

# Newly Isolated *Paecilomyces lilacinus* and *Paecilomyces javanicus* as Novel Biocontrol Agents for *Plutella xylostella* and *Spodoptera litura*

Hoang Chinh NGUYEN<sup>1</sup>, Thi Van Anh TRAN<sup>1</sup>, Quoc Linh NGUYEN<sup>2</sup>,  
Nhu Nhut NGUYEN<sup>2</sup>, Minh Khiem NGUYEN<sup>1</sup>,  
Ngoc Thanh Tam NGUYEN<sup>1</sup>, Chia-Hung SU<sup>3\*\*</sup>, Kuan-Hung LIN<sup>4\*</sup>

<sup>1</sup>Ton Duc Thang University, Faculty of Applied Sciences, 700000 Ho Chi Minh City, Vietnam; [nguyenhoangchinh@tdt.edu.vn](mailto:nguyenhoangchinh@tdt.edu.vn);  
[bsy@staff.pccu.edu.tw](mailto:bsy@staff.pccu.edu.tw); [minhkhkiem2710@gmail.com](mailto:minhkhkiem2710@gmail.com); [theresapi@yahoo.com.tw](mailto:theresapi@yahoo.com.tw)

<sup>2</sup>Gia Tuong Company Ltd., Binh Duong, Vietnam; [nguyenquoclinh@giatuong.vn](mailto:nguyenquoclinh@giatuong.vn); [nhunhutnguyen@yahoo.com.uk](mailto:nhunhutnguyen@yahoo.com.uk)

<sup>3</sup>Ming-Chi University of Technology, Graduate School of Biochemical Engineering, 24301 Taipei, Taiwan; [chsui@mail.mcut.edu.tw](mailto:chsui@mail.mcut.edu.tw) (\*\*co-corresponding author)

<sup>4</sup>Chinese Culture University, Graduate Institute of Biotechnology, 11114 Taipei, Taiwan; [rlin@faculty.pccu.edu.tw](mailto:rlin@faculty.pccu.edu.tw) (\*corresponding author)

## Abstract

Biocontrol offers an attractive alternative to the use of chemical pesticides in agricultural pest management. The development of high levels of resistance to chemical pesticides have forced researchers to find more alternative biological control agents. The aims of this study were to isolate *Paecilomyces* spp. with high virulence against diamondback moth (*Plutella xylostella*) and Oriental leafworm moth (*Spodoptera litura*), and to investigate suitable agro-industrial residues as a substrate used for solid state fermentation for sporulation of isolates. In this study, *Paecilomyces* spp. were isolated from soil and insects and identified by morphological and sequencing analyses. The pathogenicity of these isolates was evaluated on *Pl. xylostella* and *S. litura* to identify strains with the highest virulence. In addition, agro-industrial residues were used as a cheap substrate for investigating a suitable medium for sporulation on an industrial scale. Six strains of *Paecilomyces* spp. were isolated including one strain of *P. lilacinus* and five strain of *P. javanicus*. *P. lilacinus* PL01 showed the highest virulence against both *Pl. xylostella* and *S. litura* with respective LT<sub>50</sub> values of 2.51 and 7.09 days. The five isolated *P. javanicus* strains also strongly infected *Pl. xylostella* with LT<sub>50</sub> values of 2.52~6.59 days. For sporulation, brown rice alone or brown rice mixed with rice husks and wheat bran or rice bran was suitable for cultivating these isolates. Two newly isolated species of *Paecilomyces*, *P. lilacinus* and *P. javanicus*, can be used as biological control agents for controlling *Pl. xylostella* and *S. litura*.

**Keywords:** agro-industrial residues, biocontrol, bio-pesticides, entomopathogenic fungi, solid-state fermentation, sporulation

## Introduction

The diamondback moth (*Plutella xylostella*) and Oriental leafworm moth (*Spodoptera litura*) are major pests of cabbage, canola, broccoli, and other crucifers. To control these pests, farmers all over the world spend more than \$1 billion on chemical insecticides each year (Talekar and Shelton, 1993). However, many populations of these pests have become resistant to chemical pesticides and were detected in Asia (Syed, 1992), Central America (Perez and Shelton, 1997), the continental United States (Shelton and Wyman, 1992), and Hawaii (Tabashnik *et al.*, 1990). Biocontrol offers an attractive alternative or supplement to the use of chemical pesticides in agricultural pest management to protect crops (Lopez *et al.*, 2014; Kepenekci *et al.*, 2015). The use of fungal agents for biological control of pests is an important strategy to minimize synthetic chemical pesticides which often have adverse effects

on humans, animals, and the environment (Mar *et al.*, 2012). However, it is hard to develop specific fungi as potential bio-pesticides because their sporulation on culture media usually depends on the species and the components used in the artificial media. There are several ways to manipulate fungi becoming a biocontrol agent, but fungi must be available in large quantities. In general, the success of microbial bio-pesticides depends on obtaining the pathogen at competitive prices to permit its mass-production and commercialization (Gao *et al.*, 2007). Production processes for fungal bio-pesticides must be low-cost, and yield high concentrations of viable, virulent, persistent spores that can be stabilized to provide a product shelf-life of 12~18 months (Cliquet and Jackson, 2005).

The nutritional composition of the production medium significantly impacts the biocontrol efficacy, tolerance, desiccation, and persistence (Lane *et al.*, 1991). Utilization of

industrial residues or agricultural products is a great alternative to achieve a competitive price, resulting in the utilization of agro-industrial residues with added value (Soccol and Vandenberghe, 2003). Sorghum, white rice, wheat, coffee husks, rye, barley, sugarcane, cassava bagasse, refused potatoes, corn, beans, soy, glucose syrup, and grapes have been used as materials for spore production (Buzzini and Martini, 1999; Pandey et al., 2000; Kamp and Bidochka, 2002; Santa et al., 2005; Robl et al., 2009; Mishra and Thawani, 2016). Solid-state fermentation of these crop residues is advantageous because it is easy to carry out, and raw materials are cheap (Mishra and Thawani, 2016). In general, product costs and storage stability have driven the development of production and formulation processes (Ying and Feng, 2006).

The development of high levels of resistance to most chemical pesticides and the side effects of pesticides on natural enemies of the pest have forced researchers to find alternative biological control agents (Lacey et al., 1996; Lopez et al., 2014; Kepenekci et al., 2015). The genera *Beauveria* and *Paecilomyces* have been recognized as important biocontrol agents of aleyrodid pests of field and greenhouse crops (Wraight et al., 1998; Kepenekci et al., 2015; Ibrahim et al., 2016). *Paecilomyces* contains members which are often thermophilic, a perfect state for placement in the ascomycetous genera, *Talaromyces* and *Thermoascus*. *Isarioidea* contains mesophiles, including several well-known entomopathogenic or nematophagous species, such as *Paecilomyces lilacinus* and *P. javanicus* (Samson, 1974; Kepenekci et al., 2015). *Paecilomyces lilacinus* is a soil fungus and has been used as an efficient and common nematocide (Kiewnick and Sikora, 2006; Siddiqui and Futai, 2009; Kepenekci et al., 2015), and also as a controller of greenhouse insects and mite pests (Fiedler and Sosnowska, 2007; Wraight et al., 2000; Zawadneak et al., 2015). To date, no comparison of the efficacy of different *P. lilacinus* and *P. javanicus* isolates against diamondback moth (*Pl. xylostella*) and Oriental leafworm moth (*S. litura*) has been reported. In this paper, we aimed to isolate new *Paecilomyces* spp. with high virulence against *Pl. xylostella* and *S. litura*, and analyze the spore production of isolates in agro-industrial residues. The objectives of this study were to isolate and identify *Paecilomyces* spp. as biocontrol agents against diamondback moth (*Plutella xylostella*) and Oriental leafworm moth (*Spodoptera litura*) under laboratory conditions, and to investigate suitable agro-industrial residues as a substrate used for solid state fermentation for sporulation of isolates.

## Materials and Methods

### Materials

All the chemicals and reagents were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). The cultural media were from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Primers were synthesized by Nam Khoa Biotek Comapany (Ho Chi Minh, Vietnam).

### Collection, isolation, and identification of *Paecilomyces* spp.

Soils and naturally infected insects were collected from cultivated fields and greenhouse areas in Binh Duong Province, Vietnam. Dead insects with external signs of mycosis were collected and promptly transported to the laboratory. The fungi

were isolated directly from dead insects by transferring external conidia from dead insects onto potato dextrose agar (PDA) medium amended with 0.01% (w/w) chloramphenicol and 3% (w/w) sodium chloride plate (PDA+) and incubated at 28 °C for 7 days (Pau et al., 2012). Two grams of soil were added to a flask containing 20 ml sterilized water. The soil suspension was diluted into concentrations of  $10^{-1}$ ,  $10^{-2}$ , and  $10^{-3}$  after shaking for 10 min, subsequently spread onto PDA+ plates, and incubated at 28 °C for 7 days (Pau et al., 2012). For pure culture isolation, the hyphal tip of a small fungal colony was cut and transferred to PDA plates. Preliminary identification of the collected entomopathogenic fungi was conducted by examining macro- and microscopic features of the colonies after culturing fungi on PDA plates at 28 °C. The morpho-taxonomic characteristics of conidia-forming mycelia and conidia structures were identified based on Samson (1974), Humber (1997), and Tasanathai et al. (2010).

Ribosomal RNA genes (rDNA) typically exist as a tandem repeat that includes coding regions, which are conserved to varying degrees, as well as highly divergent spacer regions. These spacer regions, or internal transcribed spacer (ITS) sequences, are widely used in fungal systematics (Driver et al., 2000). In the case of the genus *Paecilomyces*, analysis of sequences of the large and small subunit rRNA genes indicates the polyphylogeny of the genus (Obornik et al., 2001; Inglis and Tigano, 2006). Therefore, rDNA-ITS sequencing was used to identify *Paecilomyces* isolates held in our culture collections. A single pair of primers 18S-ITS1-5.8S-ITS2-28S rDNA was custom-synthesized by Nam Khoa BioTek Company (Ho Chi Minh City, Vietnam). The polymerase chain reaction (PCR) products were checked using agarose gel electrophoresis. DNA sequences were compared to sequences deposited in the National Center of Biotechnology Information (NCBI) server (<http://www.ncbi.nlm.nih.gov>) with Entrez and BLAST.

### Preparation of spore suspensions

The sterilized substrate (a mixture of 25 g rice and 25 mL distilled water, at pH 6.5) filled in 250-mL conical flasks was inoculated with  $10^8$  conidia of fungal isolates. The inoculated medium was incubated in the dark at 28 °C for 14 days. The fermented substrate (5 g) was collected in a sterile test tube, supplemented with 10 mL of 0.05% Tween 80, and vortexed for 2 min to dislodge and suspend the spores before being filtered through a double-layered sterile cheese cloth. The spore concentration was determined using a hemocytometer (Marienfeld GmbH, Marienfeld, Germany) and standardized to  $10^8$  spores.mL<sup>-1</sup> for further uses.

### *In vitro* pathogenicity of *Paecilomyces* to *Pl. xylostella* and *S. litura*

Third instar larvae of *Pl. xylostella* and *S. litura* were obtained from a laboratory colony maintained at 15 °C and 15:9 h light:dark and reared on wheat germ diet until used in this study (Altret et al., 1999). Cultures of isolates were maintained for 25 days at 28 °C and used for the bioassay. Petri dishes were prepared by placing filter paper, lightly moistened with sterile distilled water at the bottom of the dish. Third instar of *Pl. xylostella* and *S. litura* larvae were dipped for 5 seconds in a spore suspension of each isolate ( $10^8$  spores.mL<sup>-1</sup>) and placed on moistened sterile Whatman no. 4 filter paper (Whatman International Ltd., Maidstone, UK) in a Petri dish. A control

larvae were immersed in sterile aqueous 0.05% Tween-80 solutions. For each treatment, ten larvae were kept in dishes with cabbage (*Brassica chinensis* L var. *graciosa*) as food, and mortality was recorded daily. A corrected mortality and the lethal time causing 50% mortality ( $LT_{50}$ ) were calculated using the formula recommended by the Food and Agriculture Organization (FAO) of the United Nations (Abbott, 1925; FAO, 2004; Sun *et al.*, 2011). The assay was repeated three times. Corrected mortality = [treated mortality % - control mortality %] / [100 - control mortality %].

#### *Effect of the solid substrate on spore production of Paecilomyces spp.*

Different concentrations of solid substrates of brown rice (*Oryza sativa* L.), rough rice (*Oryza sativa* L.), bran, and rice husks, were used for testing spore production. Each 250-mL conical flask was filled with 25 g of substrate, mixed with 25 mL of distilled water, and sterilized in an autoclave at 121 °C for 20 min. The sterilized substrate was inoculated with  $10^8$  spores of fungal isolates and incubated in the dark at 28 °C for 10 days. Five grams of the fermented substrate was collected in a sterile test tube, supplemented with 10 mL of 0.05% Tween 80, and vortexed for 2 min. The spores were suspended before being filtered through double-layered sterile cheesecloth. Spore concentrations were detected using a hemocytometer (Marienfeld GmbH, Marienfeld, Germany), and the number of spores was analyzed to determine the most suitable medium for spore production of *Paecilomyces*.

#### *Statistical analysis*

Data shown in Tables 1 and 2 and Fig. 2A and 2B are the means of at least three independent sets of experiments with similar results. Measurements of  $LT_{50}$  values, % mortality, and the number of spores were analyzed by an analysis of variance (ANOVA) with a completely randomized design. For significant values, means were separated by the least significant difference (LSD) test at  $p \leq 0.05$  using PC SAS 8.2 (SAS Institute, Cary, NC, USA).

## Results

#### *Isolation and identification of Paecilomyces spp.*

Six strains of *Paecilomyces* isolates, designed *P. lilacinus* (PL) 01, *P. javanicus* (PJ) 01, PJ02, PJ03, PJ04, and PJ05 were isolated from the original infected insects. The growth of colonies of PL01 on PDA plates formed a basal felt with floccose aerial mycelium giving rise to conidiophores. The diameter of the colony reached 3.2 cm within 15 days of incubation at 28 °C. Conidial heads appeared white but gradually became light brownish when sporulated (Fig. 1A). This color change is consistent with species of *P. lilacinus* reported by Samson (1974). The polyphylogeny of the genus *Paecilomyces* was previously observed by analysis of rRNA gene sequences (Luangsa-ard *et al.*, 2004), and the ITS region was found to be useful in *Paecilomyces* taxonomy and classification of our isolates. Sequence analysis of the PL01 clone showed 100% similarity to the *P. lilacinus* sequence in the GenBank database (accession no. AB 1033801.1) and confirmed that the PL01 isolate was the species *P. lilacinus*.

For other isolates, PJ01~PJ05, colonies grew slowly on PDA with diameters of colonies ranging 2.6~3.1 cm within 10 days of incubation at 28 °C. Colonies were round, flat, short floccose, and white, then became cream-colored with age (Fig. 1B). Conidiophores are erect, arising from the aerial mycelium bearing branches with phialides, consisting of a cylindrical basal part, tapering into a thin neck. Conidia are fusiform, sometimes cylindrical, hyaline, and smooth-walled, which is consistent with species of *P. javanicus* reported by Samson (1974). Sequence analysis of the PJ01, PJ02, PJ03, PJ04, and PJ05 clones also showed 100% similarity to the *P. javanicus* sequence in the GenBank database (accession no. AB 263744.1) and confirmed that PJ01, PJ02, PJ03, PJ04, and PJ05 isolates were the species *P. javanicus*.

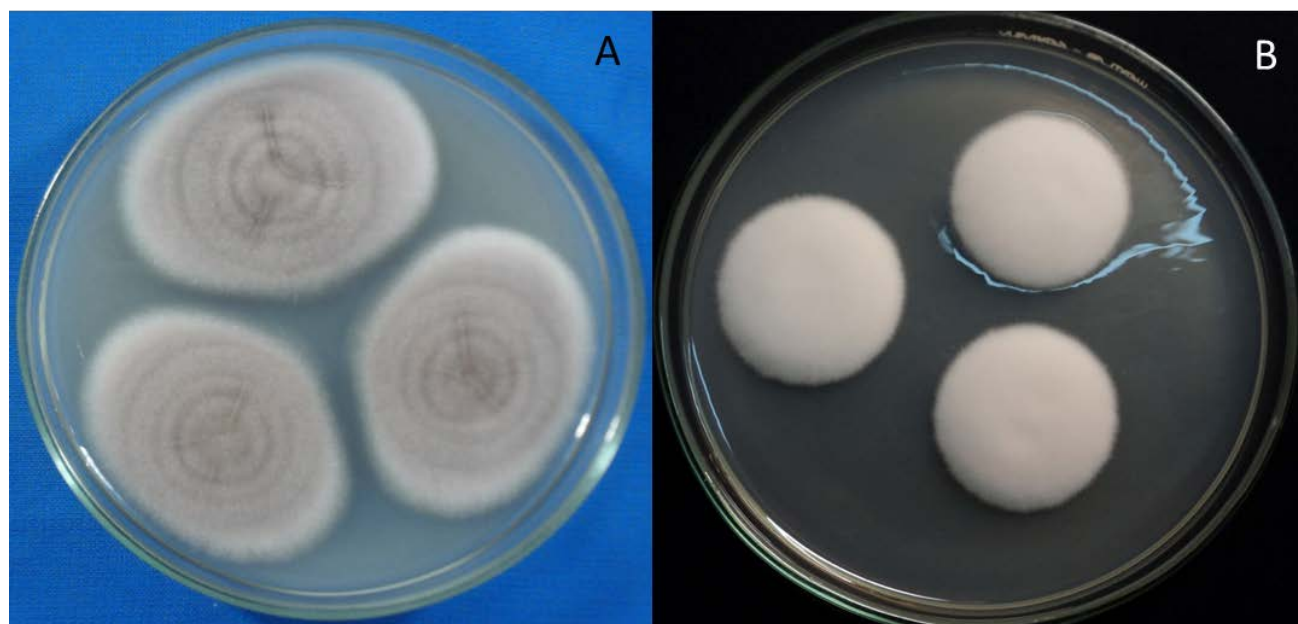


Fig. 1. Colony characteristic of isolated *Paecilomyces lilacinus* (A) and *P. javanicus* (B) on PDA medium

*In vitro* pathogenicity of *Paecilomyces* to *Pl. xylostella* and *S. litura*

To investigate the pathogenicity of isolates to insects, percentages of corrected mortality for *P. xylostella* third instar larvae exposed to six isolates were calculated. Virulence of the six isolates against *Pl. xylostella* and *S. litura* significantly differed ( $p < 0.5$ ) (Table 1). In the pathogenicity tests, all fungal isolates were pathogenic to *Pl. xylostella* and *S. litura*. The mortality of *Pl. xylostella* was 100% with the PL01, PJ02, and PJ04 isolates after 6 days of treatment (Fig. 2A). The *P. lilacinus* isolate strain PL01 showed the highest pathogenicity to *Pl. xylostella* and *S. litura* with respective  $LT_{50}$  values of 2.51 and 7.09 days. Two *P. javanicus* isolates, strains PJ04 and PJ02, also strongly infected *Pl. xylostella* with respective  $LT_{50}$  values of 2.52 and 2.55 days, while isolates PJ01 and PJ03 demonstrated the lowest pathogenicity to *Pl. xylostella* (6.56 days) and *S. litura* (17.26 days). Furthermore, six isolates showed higher virulence against *Pl. xylostella* than *S. litura*. More than 90% of *Pl. xylostella* was infected by four isolates (PL01, PJ02, PJ03, and PJ04) after 6 days of treatment, while all six isolates weakly infected *S. litura* with mortalities below 72% after 10 days of treatment (Fig. 2B).

*Effect of solid substrates on spore production by Paecilomyces spp.*

Agro-industrial residues such as brown rice, rough rice, bran, and rice husks, were used as substrates, and spore production of two potential isolates, PL01 and PJ04, was compared. The number of spores varied between the two isolates and among various media (Table 2). The isolated *P. lilacinus* strain, PL01, showed the highest number of spores from brown rice alone as a substrate ( $32.83 \times 10^8$  spores.g<sup>-1</sup>) followed by brown rice substrate mixed with rice husks, and wheat bran ( $13.36 \times 10^8$  spores.g<sup>-1</sup>) or rice bran ( $15.67 \times 10^8$  spores.g<sup>-1</sup>). The lowest number of spores was observed from the

corn bran substrate when the PL01 isolate was grown in M5 medium. For the PJ04 isolate, brown rice mixed with wheat bran and rice husks was the most suitable medium for sporulation of the isolated *P. javanicus* with a number of spores of  $9.67 \times 10^8$  spores.g<sup>-1</sup>, while the rough rice substrate alone was unsuitable for cultivating PJ04 to produce spores. Generally, brown rice, wheat bran, and rice bran were suitable for sporulation of both PL01 and PJ04 isolates.

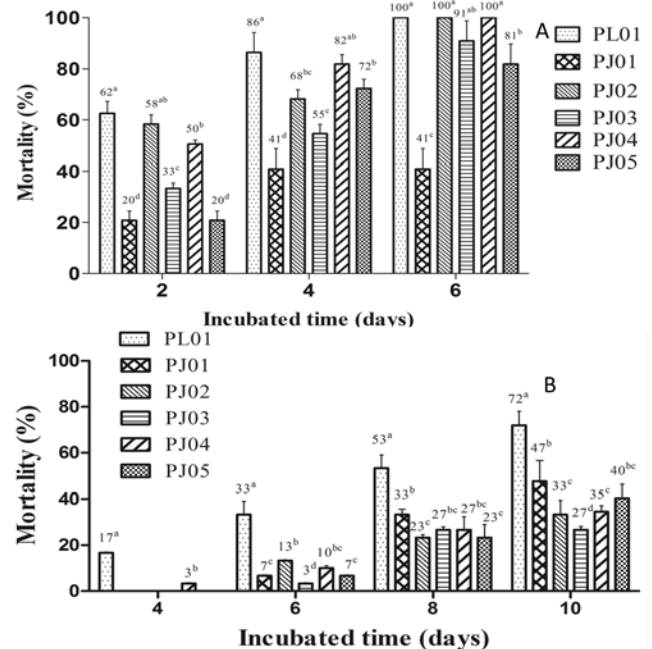


Fig. 2. Corrected mortality of third instar larvae of *Plutella xylostella* (A) and *Spodoptera litura* (B) with incubation times (day) when exposed to six isolates

Table 1. Lethal time (days) for 50% mortality ( $LT_{50}$ ) of *Plutella xylostella* and *Spodoptera litura* third instar larvae exposed to six strains of *Paecilomyces* spp. isolates

Isolate	Fungal species	$LT_{50}$ value (days)	
		<i>Plutella xylostella</i>	<i>Spodoptera litura</i>
PL01	<i>P. lilacinus</i>	2.50 <sup>c</sup>	7.09 <sup>c</sup>
PJ01	<i>P. javanicus</i>	6.56 <sup>a</sup>	10.10 <sup>d</sup>
PJ02	<i>P. javanicus</i>	2.55 <sup>c</sup>	11.31 <sup>bc</sup>
PJ03	<i>P. javanicus</i>	3.36 <sup>b</sup>	17.26 <sup>a</sup>
PJ04	<i>P. javanicus</i>	2.52 <sup>c</sup>	10.53 <sup>cd</sup>
PJ05	<i>P. javanicus</i>	3.61 <sup>b</sup>	11.91 <sup>b</sup>

Values in the same column with different letters significantly differ ( $p < 0.05$ ).

Table 2. The number of spores of isolated *Paecilomyces lilacinus* strain PL01 and *P. javanicus* strain PJ04 on different substrates

Media	Composition (%)						Number of spores ( $\times 10^8$ spores.g <sup>-1</sup> )	
	Brown rice	Rough rice	Wheat bran	Rice husks	Rice bran	Corn bran	PL01	PJ04
M1	100	0	0	0	0	0	32.83 <sup>a</sup>	4.09 <sup>b</sup>
M2	0	100	0	0	0	0	6.93 <sup>c</sup>	0.55 <sup>c</sup>
M3	60	0	30	10	0	0	13.36 <sup>bc</sup>	9.67 <sup>a</sup>
M4	60	0	0	10	30	0	14.67 <sup>b</sup>	4.75 <sup>b</sup>
M5	60	0	0	10	0	30	1.78 <sup>f</sup>	2.02 <sup>c</sup>
M6	0	70	30	0	0	0	8.42 <sup>d</sup>	1.17 <sup>d</sup>
M7	0	70	0	0	30	0	10.50 <sup>cd</sup>	4.54 <sup>b</sup>
M8	0	70	0	0	0	30	5.09 <sup>e</sup>	1.00 <sup>d</sup>

Values in the same column with different letters significantly differ ( $p < 0.05$ ).



## Discussion

Entomopathogenic fungi are currently considered to be the important factors controlling insect population, and are well suited for being developed as ecofriendly biopesticide (Kumar et al., 2015). Among them, *Paecilomyces* are important natural control agents and sources of mycopesticides for pests management worldwide (Han et al., 2014; Jamali and Ghasemi, 2016; Sanjaya et al., 2016). To isolate entomopathogenic fungi, susceptible insect host and selective media have been utilizing to the isolation of entomopathogenic fungi from soil. Use of infected insect is a very sensitive detection method where entomopathogenic fungi can be selectively isolated (Ibrahim et al., 2016). In this study, six strains of *Paecilomyces* were isolated from infected insects and identified as *P. lilacinus* and *P. javanicus* based on morphological and molecule studies. The macro- and micro-morphological features of these fungi grown on PDA agreed with the features reported by Samson (1974) for *P. lilacinus* and *P. javanicus*. The further studies based on sequence analysis using ITS region confirmed that the PL01 isolate was the species *P. lilacinus* (accession no. AB 1033801.1) and PJ01, PJ02, PJ03, PJ04, and PJ05 isolates were the species *P. javanicus* (accession no. AB 263744.1 in the GenBank database).

Several studies have examined the potential use of *Paecilomyces* as biological control agents. Kepenekci et al. (2015) reported a potential infectivity of *P. lilacinus* isolated in Turkey against the last larvae instar of the potato tuber moth [(*Phthorimaea operculella* (Zeller)] and the Colorado potato beetle [(*Leptinotarsa decemlineata* (Say))] (43.3% and 33.2% mortality, respectively) on the 10<sup>th</sup> day of treatment with the fungal concentration of 10<sup>8</sup> cfu.mL<sup>-1</sup>. Another study by Lopez et al. (2014) revealed that *P. lilacinus* illustrated the potential pathogenicity against cotton aphids and herbivores under greenhouse and field conditions. In this study, all six isolates showed a strong virulence against the diamondback moth (*Pl. xylostella*), with LT<sub>50</sub> values ranging 2.5~6.5 days. The pathogenicity of the six isolates to *Pl. xylostella* was much higher than to *S. litura*. Infection with entomopathogenic fungi also differs depending on the ability of the fungal strains to directly penetrate the insect through the cuticle. It seems that it was more difficult to infect *S. litura* than *Pl. xylostella* by these entomopathogenic fungi. Among the six isolates, *P. lilacinus* showed the highest infectivity against both *S. litura* and *Pl. xylostella*. In addition, *P. lilacinus* has been used as an efficient nematicide against nematodes (*Meloidogyne* spp.) (Sharma et al., 2014; Wang et al., 2010; Sharma et al., 2014). Moreover, *P. fumosoroseus*-infected *Pl. xylostella* exhibited the highest mortality of 79% (Altre et al., 1999), and yet *P. tenuipes* infected *Pl. xylostella* third instar larvae with an LT<sub>50</sub> of 2.33 days at a spore concentration of 10<sup>8</sup> spores.mL<sup>-1</sup> (Baksh and Khan, 2012). In our study, the newly isolated *P. lilacinus* PL01 and *P. javanicus* PJ04 strains were potential biological control agents and can be used for crop protection against *Pl. xylostella* and *S. litura*.

*Paecilomyces* isolates were firstly selected for diamondback moth and Oriental leafworm moth control due to the high mortality against the target organism. They are the only pathogens that have been developed for the control of insects

with piercing and sucking mouthparts (Eslamizadeh et al., 2015). Entomopathogenic fungi usually infect insect hosts by penetrating the cuticle after their conidia attach and germinate on the insect host cuticle (Han et al., 2014). The synthesis of extracellular enzymes is crucial for the infection process. These fungi secrete extracellular enzymes proteases, chitinases and lipases to degrade the major constituents of the cuticle (protein, chitin and lipids) and allow hyphapenetration (Moorthi et al., 2014; Ibrahim et al., 2016).

Entomopathogenic fungi are an important option in integrated pest management programs so large scale production of the ecofriendly biopesticide is a primary objective in the biocontrol programs for increasing agricultural output. A high spore number is one of the main criteria for choosing a fungal pathogen for biological control of pests in the field. The success of microbial control often depends on producing the pathogen at competitive prices. To achieve low-costs and high yields of viable fungal spores and make good use of agricultural wastes, the biotechnological potential of these agroindustrial refuse can be employed in byproduct development and establishing improved biocontrol programs. Several studies were performed on utilizing agro-industrial residues as materials for spore production. The highest number of spores in *Metarhizium flavoviride* was 102.80 x 10<sup>9</sup> spores.g<sup>-1</sup> sorghum, whereas white-rice yielded the greatest amount of spores for *Beauveria bassiana* (141.0 x 10<sup>9</sup> spores.g<sup>-1</sup>) after 60 days of incubation (Mar et al., 2012). Robl et al. (2009) reported that refused potatoes yielded the highest amount of spores for *P. lilacinus* (1.75 x 10<sup>8</sup> spores.g<sup>-1</sup>) after 14 days of incubation followed by 80% cassava bagasse supplemented with 20% coffee husks (1.56 x 10<sup>8</sup> spores.g<sup>-1</sup>), wheat (1.18 x 10<sup>8</sup> spores.g<sup>-1</sup>), rye (1.08 x 10<sup>8</sup> spores.g<sup>-1</sup>), barley (0.80 x 10<sup>8</sup> spores.g<sup>-1</sup>), cassava bagasse (0.69 x 10<sup>8</sup> spores.g<sup>-1</sup>), and soy (0.67 x 10<sup>8</sup> spores.g<sup>-1</sup>). Kamp and Bidochka (2002) demonstrated that spore production by *Beauveria bassiana*, *Metarhizium anisopliae*, and *Verticillium lecanii* was affected by different solid substrate culture conditions. In our study, mixed substrate fermentation was carried out to make the substrate less expensive and also to test whether they had any beneficial effects on spore production. Methods for commercial production of spores were evaluated on solid substrates consisting of brown rice, rough rice, rice husks, rice bran, wheat bran, and corn bran, and spore production of six isolates was affected by the different solid substrate culture conditions. Different nutrient types in the media resulted in variability in the number of spores produced after the 10-day growth period. Among all the natural media assayed, brown rice alone as a substrate or brown rice mixed with rice husks and wheat bran or rice bran was suitable for the sporulation of both isolated species: *P. lilacinus* and *P. javanicus*. It may because the grains are carbon sources in the form of starch and the utilization of starch of these isolates depends upon its hydrolysis by the action of the enzyme amylase (Mishra and Thawani, 2016). In addition, it was reported that amount of moistening and other agents also played very important role for the growth of fungus (Mishra and Thawani, 2016). High amount of moistening agent caused clumping of the substrate particles, which hindered the optimal utilization of substrate whereas less amount of moistening agent caused dried and non soft condition of the grain and thus fungus could not grow and sporulate. Furthermore, a nutrient-rich medium might not stimulate sporulation, while a nutrient-poor medium would

not offer extensive mycelial growth. In agreement with study reported by Mishra and Thawani (2016), due to maximal spore production efficiency, rice was suggested as a good solid substrate for the mass production of spores. These tested substrates in our study can be applied by industry to culture two isolates of entomopathogenic fungi for spore production at low costs and with a high concentration of fungal spores.

## Conclusions

The strongly virulent *P. lilacinus* and *P. javanicus* against *Pl. xylostella* and *S. litura* were isolated. Among six isolates, *P. lilacinus* strain PL01 showed the highest pathogenicity to both *Pl. xylostella* and *S. litura* with respective LT<sub>50</sub> values of 2.51 and 7.09 days. Five isolated *P. javanicus* strains also strongly infected *Pl. xylostella* with LT<sub>50</sub> values of 2.52~6.56 days. For sporulation of these isolates, brown rice alone or mixed with rice husks, wheat bran, or rice bran was suitable for cultivation with low costs and high yields of spores. The newly isolated *P. lilacinus* and *P. javanicus* strains can be used as biological control agents for controlling *Pl. xylostella* and *S. litura*.

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