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PATOGENISITAS *Beauveria bassiana* STRAIN STGD $7(14)_2$ DAN STGD $5(14)_2$ TERHADAP WERENG COKLAT (*Nilaparvata lugens* STÅL)

PATHOGENICITY OF Beavueria basiiana STRAIN STGD $7(14)_2$ *AND* STGD $5(14)_2$ *AGAINST BROWN PLANTHOPPER* (*Nilaparvata lugens* STAL)

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Abstrak

Beauveria spp. merupakan salah satu jamur entomopatogen yang dapat digunakan sebagai agen biokontrol terhadap berbagai serangga hama. Tujuan penelitian ini adalah untuk menguji patogenitas *Beauveria* spp. terhadap serangga wereng coklat (*Nilaparvata lugens* Stål). Isolat-isolat *Beauveria* spp. diisolasi dari serangga walang sangit dari Situ Gede, Bogor, Jawa Barat. Identifikasi jamur dilakukan berdasarkan analisis data sekuen dari daerah *Internal Transcribed Spacer* (ITS) rDNA. Hasil uji menunjukkan bahwa patogenisitas isolat Stgd $5(14)_2$ dan Stgd $7(14)_2$ menghasilkan tingkat mortalitas 100% terhadap *N. lugens*. Isolat Stgd $5(14)_2$ dan Stgd $7(14)_2$ memiliki nilai LT_{50} yang rendah, dan menyebabkan kematian yang cepat terhadap wereng coklat. Hasil analisis filogenetik menunjukkan bahwa sekuen Stgd $5(14)_2$ dan Stgd $7(14)_2$ termasuk ke dalam spesies *Beauveria bassiana s.str*. Hasil studi ini merupakan kajian awal terhadap patogenisitas jamur entomopatogen *B. bassiana* terhadap wereng coklat, dan distribusinya di pertanaman padi Situ Gede, Jawa Barat.

Kata kunci: *Beauveria;* ITS rDNA*; N. lugens;* Patogenisitas

Abstract

Beauveria spp. *is one of the entomopathogenic fungi that can be used as biocontrol agents against various insect pests, including brown planthopper (Nilaparvata lugens Stal). This study aimed to test the pathogenicity of Beauveria spp. against N. lugens. Beauveria spp. were isolated from the rice stink bug insects, collected from Situ Gede, Bogor, West Java. Fungal identification was carried out based on the internal transcribed spacer (ITS) rDNA analysis. The pathogenicity assay revealed that Stgd* $5(14)_1$ *and Stgd* $7(14)_2$ *isolates were virulent against N. lugens, with a mortality of 100%. The* LT_{50} *(median lethal time) determination, indicated that Stgd* $5(14)_2$ *and Stgd* $7(14)_2$ *isolates had low value indicating a faster mortality in brown planthopper insects. Phylogenetic analysis showed that the sequences of Stgd* $5(14)_2$ *and Stgd* $7(14)_2$ belong to *Beauveria bassiana s.str. This is the preliminary pathogenicity trial of entomopathogenic fungi B. bassiana against brown planthopper and their distribution in rice-growing, Situ Gede area in West Java.*

Keywords*: Beauveria; ITS rDNA; N. lugens; Pathogenicity*

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INTRODUCTION

Brown planthopper (BPH) pest (*Nilaparvata lugens* STAL) damaged more than 130,000 hectares of rice fields (Ditlintan, 2010). A high increase of BPH population led to a decline in rice production, which may have impacts on economic aspects. Pest control of BPH using pesticides has been considered as threats for the environment and human health in the long-term use. The intensive use of chemicals can cause resistance and resurgence of the target-pests, hence it may increase the population level, which is higher than the initial population (Bhisnu *et al.*, 2008). Furthermore, the use of broad spectrum pesticide can kill non-target pests. Therefore, the integrated pest management program to control insect pests, particularly BPH, is necessary (Bednarek *et al.*, 2004; Laba, 2010).

One alternative method of pest control, that is effective for insects and environmentally friendly, is biological control using the formulation of specific microbes, such as fungi, bacteria, and viruses (Bednarek *et al.*, 2004). The search of selective biopesticide against certain insects is increasing, especially the use of natural enemies as a key component of the integrated pest management. The use of biopesticides can inhibit the development of larvae and insect pests, without causing environmental damage (Thungrabeab & Tongma, 2007).

The use of entomopathogenic agent: (i) could substitute synthetic pesticides, (ii) is easy to formulate and safe, and (iii) does not produce residues. Among entomopathogenic fungi, members of *Beauveria* spp. can be used as pest control agents (Townsend *et al.*, 2003; Bednarek *et al.*, 2004; Konstantopoulou & Mazomenos, 2005; Rondelli *et al.*, 2012; Mudrončeková, *et al.*, 2013), including insects of the order *Lepidoptera*, *Coleoptera*, *Hemiptera*, *Diptera*, and *Hymenoptera* (Tanada & Kaya 1993). *Beauveria* spp. showed high chitinase activity bond to hydrolyze β-1,4-acetomido-2-deoxy-D-glycosides on chitin and chitin oligomers of insect pests (Bielka *et al.*, 1984; Suryadi *et al.*, 2013). *Beauveria* spp. has been used for controlling *N. lugens* on rice and other pests insect in Indonesia and some other countries (Townsend *et al*., 1995; Sheeba *et al.*, 2001; Safavi *et al.*, 2010; Trizelia & Nurdin, 2010; Khashaveh *et al.*, 2011; Budi *et al.*, 2013; Salbiah *et al*., 2013; Thalib *et al.*, 2013; Zibaee *et al.*, 2013).

Until now, various efforts to develop BPH control in Indonesia has still been in progress, since no commercial biological agent that is considered really effective against BPH. Therefore, an effort to utilize the potential of *Beauveria* spp. isolates as potential biocontrol agents against BPH in mass productions scale, is necessary.

The objective of this study is: (i) to determine fungal pathogenicity (LT50) of *Beauveria* spp., isolated from rice stink bug insects, from Situ Gede (Bogor West Java), against BPH, and (ii) to identify the entomopathogenic fungi using molecular method.

MATERIAL AND METHOD

Pathogenicity Assay

A total of 13 fungal isolates obtained from rice stink bug cadavers Stgd 0113, Stgd 0213, Stgd $2(14)_1$, Stgd $2(14)_2$, Stgd $2(14)_3$, Stgd $4(14)_1$, Stgd $5(14)_1$, Stgd $5(14)_2$, Stgd $6(14)_1$, Stgd $6(14)_2$, Stgd $7(14)_2$, Stgd $8(4)_2$, and Stgd $8(14)_{2]}$ was used in this study. Previously, these isolates were tested for their pathogenicity against rice stink bug insects (Wartono *et al*., 2016).

Fungal of 15 day colonies on PDA medium were harvested by adding 10 mL of sterile distilled water and 0.1% Triton X100 to the Petri dishes. The conidia then were scrapped from the solid medium with a soft brush (Kim *et al.*, 2014). The calculation of conidial density was done using hemocytometer (Bessey, 1979).

A total of 15 BPH was infested on rice plants and then covered using mylar plastic cylinder. This assay was repeated twice. The inoculation method of fungal spore suspension was carried out using the spray method following the procedure, described by Mahmoud (2009). The distilled water with 0.1% Triton X100 was used as a control treatment. A percentage of BPH mortality due to *Beauveria* spp. infestation was observed every 24 hours after application.

The experimental data were processed and analyzed by using the Sirichai program v 6.0 at 5% significance level. The determination of the median lethal time (LT_{50}) was done using probit analysis (Chi, 1997). The percentage of mortality was calculated using the following formulation:

$$P = \frac{X}{Y} \times 100\%$$

P : the percentage of mortality
X : the number of BPH mortality
Y : the total number of BPH

If the mortality occurred on control ≤25%, the percentage of BPH mortality was then calculated using Abbot formula (Busvine, 1971), as follows:

$$AI = \frac{A - B}{100 - B} \times 100\%$$

AI : percentage of mortality after correction
A : percentage of mortality of BPH on treatment
B : percentage of BPH deaths on control

Morphological Examination of Stgd 5(14)2 and Stgd 7(14)2 Isolates

The fungal isolates were rejuvenated by culturing 1–2 mycelium disks onto a potato dextrose agar (PDA). The cultures were then incubated for 2–7 days at room temperature (±25 °C). The observation of macroscopic fungal colonies includes colour, colonies form, colonies texture, and shape of the edge of colonies. Microscopic characters of spores, conidia, and hyphae were observed by using compound microscope at 40–1000x magnification (Samson *et al.*, 1988; Glare & Inwood, 1998).

DNA Extraction and PCR Amplification

Genomic DNA of fungal isolates was harvested from 4-day-old isolates on 100 mL of Sabouraud dextrose broth (SDB) medium in Erlenmeyer flask (1 L: 20 g of dextrose, 10 g peptone, and 2 g of yeast extract), and dried in an oven (50 °C) overnight (Trizelia *et al.*, 2012). The dried mycelial fungus was ground in liquid nitrogen using a mortar and pestle (Rogers & Benedich, 1994). The mycelium powder was transferred into an Eppendorf tube containing 700 mL of extraction buffer 1% (2% CTAB: 20 mM EDTA; 0.1 M Tris-HCl; 1.4 M NaCl pH 8.2; 2-mercaptoethanol) and incubated in a shaker water bath for 5 min at 65 °C, then it was vortexed and incubated again for 30 min at 65 °C. About 400 mL solution of chloroform: isoamyl alcohol (24:1v/v) was added and vortexed until the water and the organic layer portion is mixed. The mixture was centrifuged for 10 min at 12,000 rpm (Beckman Coulter Microfuge 22R), and 500 mL the top layer was transferred in new Eppendorf tube and added to 400 mL of chloroform: isoamyl alcohol (24:1v/v). The mixture was centrifuged for 10 min at 10,000 rpm, and the upper layer was moved into the new Eppendorf tube and added with 500 mL cold isopropanol, shaken until mixed, and incubated for 1 h at 20 °C. The mixture was centrifuged for 2 min at 10,000 rpm. The top layer (a mixture of water and alcohol) formed were removed. The bottom layer was air dried for 1 hour. The air-dried pellets were resuspended in 600 mL of TE buffer and stored at 4 °C. DNA was examined by electrophoresis using a 1% agarose gel, which was stained with ethidium bromide (EtBr) (Sambrook & Rusell, 2001; Khosravinia *et al.*, 2007), and the DNA was visualized using ChemiDoc.

The PCR mixture of the current study composed of 1 mL of genomic DNA (20–50 ng/mL), 1 mL of each primer (forward and reverse), 7.5 mL Kappa 2G Fast Ready Mix and 3.5 mL ddH2O. The primer pair of internal transcribed spacer (ITS) 1 (5'-TCC GCGGA GTA GGT GAA CCT-3') and ITS 4 (5'-TCC TCC GCT TAT TGA TATGC-3') (De Beeck *et al.*, 2014) was used in the amplification. PCR reactions were performed using the Esco® PCR machine with the following programs: initial denaturation at 95 °C for 5 min, followed by 30 cycles of 1 min denaturation at 95 °C; 35 s annealing at a temperature of 55 °C; and 30 s elongation at 72 °C. The DNA amplification product was visualized by electrophoresis on a 1.5% agarose gel (White *et al.*, 1990).

DNA Sequencing of Stgd 5(14)1 and Stgd 7(14)2 Isolates

The amplicon of PCR product of Stgd 5(14)1 and Stgd 7(14)2 isolates were sent to Bioneer Corporation (Korea) for sequencing analysis. Amplified ITS sequences were

compared with the GenBank Nucleotide Database (http://www.ncbi.nlm.nih.gov) using BLAST (Altschul *et al*., 1997). The entire sequences were aligned using the Clustal analysis and were grouped with other *Beauveria* sequences deposited in the GenBank. A phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap resampling (Saitou & Nei, 1987) in Mega 6 Software (Tamura *et al*., 2011).

RESULT

Pathogenicity Assay

Spore suspension density of 15-day-old fungal isolates is showed in Table 1. The highest and the lowest OD was found in Stgd 0213 (OD= 4.44×10^8) and Stgd $7(14)_2$ (OD= 1.12×10^8), respectively. The mean percentage of BPH mortality indicated that Stgd $7(14)_2$ and Stgd $8(4)_2$ isolates were highly virulent isolates, with an average mortality rate of 100% at the sixth day after the conidial suspension application (Table 1). Isolate Stgd 0213 is a low virulent isolate that showed the lowest percentage of mortality (67.50%). In this study, Stgd $5(14)_2$ and Stgd $7(14)_2$ isolates showed the ability to reduce BPH population by 50% in a shorter time. The data also showed that an average time required to kill the 50% population (LT_{50}) was after the 3rd day, while the time required for 90% (LT_{90}) was 5–6 days (Figure 1).

Table 1. Mean of BPH mortality (LT_{50})

No.	Fungal code	Mean mortality of BPH (%)	Mean of corrected mortality (%)	LT_{50} (days)
1	Stgd $7(14)_2$	100.00	100.00 a*	2.87
2	Stgd $8(4)_2$	100.00	100.00 a	3.00
3	Stgd $2(14)_1$	96.66	95.00 a	3.01
4	Stgd $6(14)_1$	93.33	90.50 a	3.08
5	Stgd 0113	93.33	90.50 a	3.09
6	Stgd $5(14)_2$	93.33	90.50 a	2.77
7	Stgd $8(14)_2$	90.00	86.00 a	2.97
8	Stgd $2(14)_2$	90.00	85.00 a	3.05
9	Stgd 0213	76.66	67.50 a	3.56
10	Control	26.67	0.00 b	-

Note: *The Means indicated with the same letters are not significantly different with the DMRT test (P= 0.05). N= 30 BPH insects

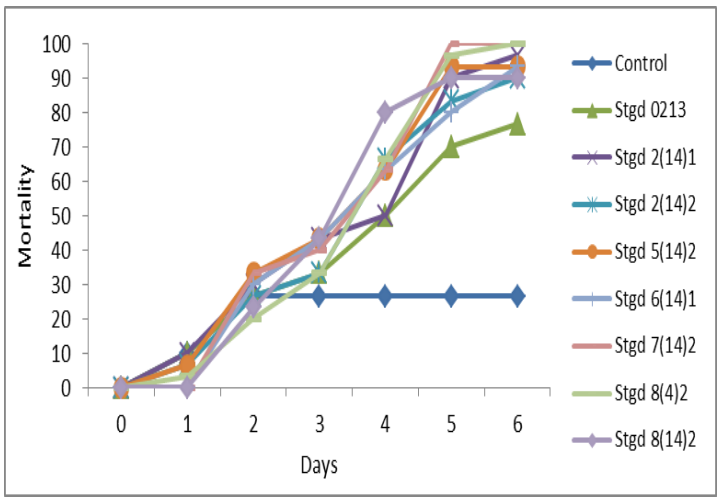

Figure 1. The Effect of fungal spore suspension of *Beauveria bassiana* on brown planthopper (BPH) mortality (LT_{90})

Morphological Examination

The observation of macroscopic and microscopic fungal colonies was presented in Figure 2. It showed that macro and microscopic characters of the fungi belong to genus *Beauveria* (due to their colony

morphology are characterised by: (i) white mycelium with white powdery appearance; (ii) conidiophores single; (iii) irregularly grouped or in verticillate clusters tapering to a slender fertile portion that appears zigzag (sympodial) after several conidia are formed on elongating conidiogenous cells.

The selected strain, Stgd 5(14)1and Stgd 7(14)2, was cultured in PDB at 25 ºC to determine its phylogenetic classification and to investigate its biochemical features. The mycelium is white, the conidial shape is cylindrical, the spore size is 2–5 μm, and the appressorium is almost cylindrical (Figure 2).

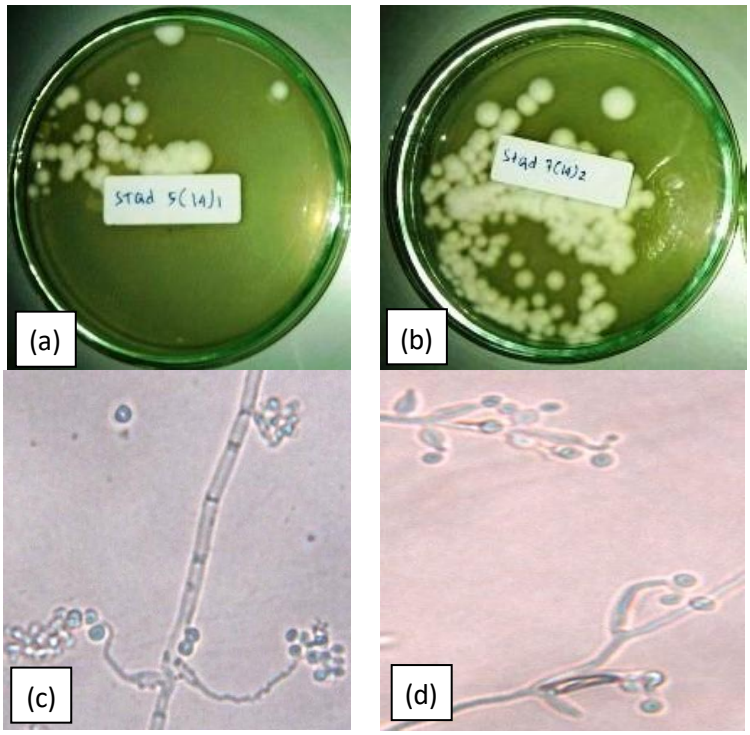

Figure 2. Morphological characteristic of the *Beauveria* spp. isolates with colony growing conidia of (a) *Beauveria bassiana* isolate Stgd $5(14)_1$ and (b) *Beauveria bassiana* Stgd $7(14)_2$, with the typical hyphae (c) Stgd 5 $(14)_1$; (d) Stgd 7 $(14)_2$

In the identification of Stgd $5(14)_1$ and $7(14)_2$, the BLAST analysis showed that both Stgd $5(14)_1$ and $7(14)_2$ sequences have 100% similarity with the *Beauveria* spp. sequences. It is obvious that Stgd $5(14)_1$ and $7(14)_2$ belong to genus *Beauveria*. In addition, the phylogenetic tree based on the ITS rDNA sequence showed that both isolates also nested in the same clade of *B. bassiana* sequences. Sequences of Stgd $5(14)_1$ and $7(14)_2$ nested in the same clade with *B. bassiana* isolates 7042 (AY 532041), *B. bassina* isolates 6722 (AY 532035), *B. basiana* isolates 6721 (AY 532034), and *B. bassiana* isolates 6723 (AY 532036) with a strong bootstrap support (89%). Therefore, Stgd $5(14)_1$ and $7(14)_2$ were identified as *B. bassiana*. (Figure 3).

DISCUSSION

This study showed that there is no variations in pathogenicity of *B. bassiana* strain Stgd $7(14)_2$ and Stgd $5(14)_1$ against *N. lugens*. Both strains were highly virulent because they cause 100% death rate at sixth day after a conidial suspension application. The degree of virulence varies among *Beauveria* spp. as a consequence of their specific virulence characteristics (Cruz *et al*., 2006; Carneiro *et al.*, 2008; Safavi *et al*., 2010). A mortality value of >90–100% indicates that both Stgd 5(14)1 and Stgd 7 (14)2 isolates are potential as biological agents against BPH.

Field observations indicated that the early symptoms of *N. lugens* mortality was shown by a rigid body and a pale colour body. The mortality of *N. lugens* can be observed from the first day until the sixth day after the conidial suspension application conducted. Indriyati (2009) reported that the mortality of insects could take place in a short period of time (the tird day to the fifth day. In another

study, mortality of *N. lugens* was obvious at the 6th or the7th day after the conidial suspension application (Wahyono, 2013). In this study, a spore germination could possibly be occurred within 24h after inoculation (Lecuona *et al*., 1997). According to Koswanudin & Wahyono (2014), at the 7th day after the application, the effectiveness of pathogenicity increased by 80-90%. This study also showed that the 10^8 conidial suspension per mL was sufficient for *Beauveria* spp. pathogenicity assay, under laboratory and greenhouse condition. A toxin released by *Beauveria* spp. may further cause insect-tissue damage and the fungal mycelia will grow throughout the body of the insect (Padmini & Padmaja, 2013). In a similar study, Shophiya *et al.* (2014) reported that the 10^4–10^6 conidial suspension/mL was effective for the pathogenicity evaluation to *Lepidoptera ricini*. Soetopo & Indrayani (2007) reported that *B. bassiana* isolates Bb4a and BbEd10 were effective against cotton bollworm (*H. armigera*). The LT_{50} and LT_{90} of both isolates were 8.96–9.62 and 19.69–22.27 days, respectively. The fastest LT_{50} fungal conidia of *B. bassiana* with 100% mortality against tea mites larvae (*Polyphagotarsonemus latus)* was determined at 10^7 conidia/mL after 31.44 hour (Yusran *et al.*, 2014).

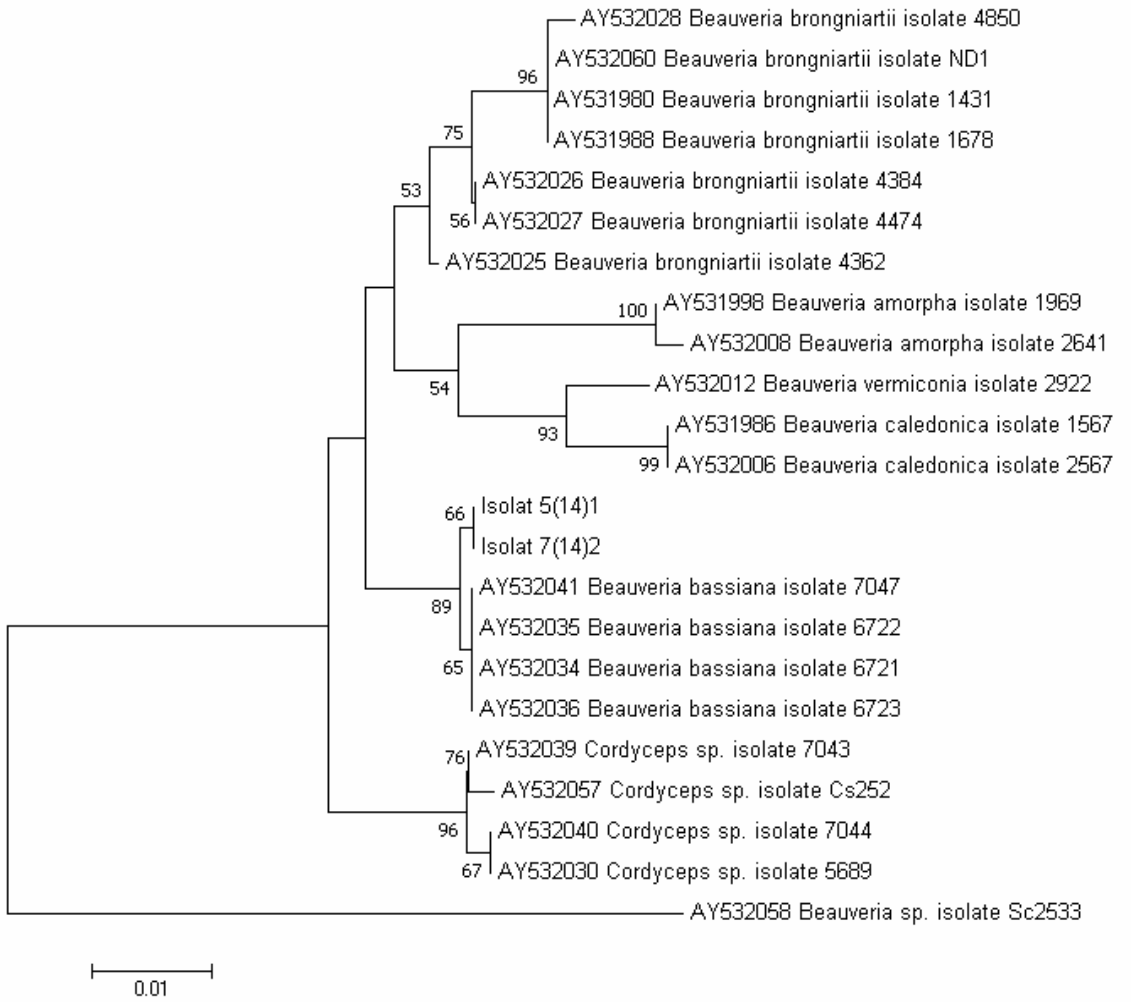

Figure 3. Phylogenetic tree generated from ITS rDNA sequence showed that Stgd $5(14)_1$ and Stgd $7(14)_2$ sequences belong to *Beauveria bassiana* with a strong bootstrap support. The phylo tree was reconstructed using NJ method with 1000 bootstrap replicates

The ability of fungal pathogen to infect an insect host is determined by several factors, such as pathogenicity, host insect fitness, and the environment (the availability of water, nutrients, oxygen, pH, and temperature) (Sandhu, 1995; Inglis *et al.*, 2001). In general, the *Beauveria* infection mechanism on insect may involve a production of beauvericin and other bioactive compounds that can damage the hemolymph function and insect host cell

nucleus (Latge *et al.*, 1988; Sandhu *et al*., 2012). The attached conidia will germinate in 1–2 days and mycelia will grow inside the host body. The infected insect will stop eating so that immunity will decrease, and within a period of 3–5 days, the insects will die with the visible fungal growth of the conidial integument (Deciyanto & Indrayani, 2008).

Beauveria bassiana has been known as the most common causative agent of disease associated with dead and moribund insects in nature (Rehner & Buckley, 2005; Rehner *et al*., 2011). The close relationship between *B. bassiana* strain Stgd $5(14)_1$ and *B. bassiana* strain Stgd $7(14)_2$ is probably due to the origin of strains. The genetic diversity of *Beauveria* spp. can be influenced by host range and ecological conditions, as well as climate conditions (Stephen & Rehner, 2005; Kaur & Padmaja, 2008; Trizelia *et al.*, 2012). *B. bassiana* is the asexual morph of *Cordyceps bassiana* (Li *et al*., 2001; Rehner *et al*., 2011).

CONCLUSION

Beuveria bassiana strain Stgd $5(14)_1$ and Stgd $7(14)_2$ isolated from rice stink bug insects (*Leptocorysa acuta*) were virulent against *N. lugens* pest with the mortality rate of 100%. Phylogenetic analysis based on the ITS rDNA sequence confirmed their identities. Additional research is needed: (i) to determine the effectiveness of both fungal strains to other rice insect pests, and (ii) to conduct further study, by using various different conidial suspension concentration and environmental condition.

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