

ORIGINAL ARTICLE

## Development of thermotolerant isolates of *Beauveria bassiana* (Bals.-Criv.) Vuill. with ethyl methanesulfonate

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### Abstract

*Beauveria bassiana* is an entomopathogenic fungus that is widely used in Thailand to control pest insects. However, the increasing temperature has influenced the insect control efficiency of the fungus. Therefore, determination of thermotolerant isolates of *B. bassiana* that can grow and remain pathogenic at higher temperatures than its current optimum temperature may be a better way to control pest insects in a high temperature environment. Three isolates of *B. bassiana* obtained from the Rice Department, Thailand were selected for mutagenesis using ethyl methanesulfonate (EMS) with subsequent screening at high temperatures (33 and 35°C). In addition, the recovery of fungal growth after exposure to a high temperature for a period of time (5–15 days) and then transferring to 25°C was evaluated. No isolates were found that grew at 35°C but one mutant isolate (BCNT002MT) produced larger diameter colonies and more spores than the corresponding wild type (WT) at 33°C. Growth and spore production of the BCNT002MT isolate were greater than its WT when incubated at 25°C for 14 days following exposure to 33°C for 7 days. In addition, the spore germination level (%) of BCNT002MT was significantly higher than its WT during culture at 25°C after prior exposure to 33°C for 5, 10 and 15 days. The pathogenicity against the brown planthopper, *Nilaparvata lugens* (Stål), of this mutant isolate was also prominent.

**Key words:** *Beauveria bassiana*, brown planthopper, ethyl methanesulfonate (EMS), mutation, thermotolerance

## Introduction

The entomopathogenic fungus *Beauveria bassiana* is used as a biological agent for controlling insect pests. The fungus has a reported optimum temperature of around 25°C for hyphal growth (Hallsworth and Magan 1999; Tefera and Pringle 2003), while the highest pathogenicity against *Diatraea saccharalis* was reported to be at 26°C (Svedese *et al.* 2013). Temperature, humidity and solar radiation are all important factors for fungal growth, germination and viability (Zimmermann 2007). Climate change has a serious impact on the environment, including drought and higher temperatures, which can affect the fungal pathogenicity by reducing activities of the fungus before contacting its host or by reducing fungal growth within the host (Zimmermann 2007). Moreover, prolonged exposure

to the sun's rays reduces the fungal viability and its sporulation. For example, the pathogenicity and spore germination level of *B. bassiana* grown at an optimum temperature or in a shady environment was higher than those that grew at a higher temperature or exposed to the sun, even when they were the same isolate (Inglis *et al.* 1997).

The mean annual temperature of Thailand during the last 35 years (1981–2015) has increased from 26.7 to 27.5°C (The Thai Meteorological Department 2017). However, Thailand has experienced a long period of warm weather during the hottest time of the year (March to May), when temperatures usually reach up to 40°C. The efficiency of *B. bassiana* to control the brown planthopper (BPH), *Nilaparvata lugens*,

during the rice growing season has decreased with the increasing temperature. In central Thailand, the average temperatures in irrigated areas (December to June) during the last three years (2014–2016) were 28.0, 28.6 and 29.4°C, respectively, while during the rainy season (July to November) they were 28.4, 28.8 and 28.6°C, respectively. Krutmuang (2011) studied two strains of *B. bassiana* (BCC6241 and BCC2637) for killing BPH in Thailand (28±2°C, 70–80% relative humidity) and found only a 60–67% induced mortality in BPH, representing a low control efficiency. One way to increase the efficiency of *B. bassiana* pathogenicity is to improve the fungal ability to grow and control insect development at high temperatures. Moist heat treatment and UV irradiation have been used to induce mutagenesis in *B. bassiana*, where the derived mutant *B. bassiana* produced more blastospores than its respective wild type (WT) at 35°C (Avanti *et al.* 2014). However, moist heat could degrade the spore germinating protein (Wang *et al.* 2012), which may then expose the germinating spores to DNA changes or germinating protein degradation. The UV induced mutagenesis of *Saccharomyces cerevisiae* showed a low percentage of mutation (Lawrence and Christensen 1976), which may result from the ability of self DNA repair.

A chemical mutagen that is widely used and has a good efficiency to induce mutagenesis in fungi is ethyl methanesulfonate (EMS). Typically, EMS induces base-pair substitution, changing GC to AT and AT to CG, but sometimes EMS induces base-pair insertion or base-pair deletion (Sega 1984). Reports of EMS inducing mutagenesis in fungi include induced oxalic acid production in *Metarhizium anisopliae* (Leger 1999) and temperature-sensitive mutants in *S. cerevisiae* (Momose and Gregory 1998). In this study, EMS was used to induce thermotolerance of *B. bassiana*. The mutant isolates were screened up to 35°C. Growth, sporulation, spore germination and pathogenicity of the selected mutant isolate were then evaluated.

## Materials and Methods

### Fungal isolates and molecular identification

Three isolates of *B. bassiana* (BCNT001 to BCNT003) were obtained from the Rice Department, Thailand (RDT). The identification of each isolate was confirmed using morphological (Humber 2005; Rehner *et al.* 2011) and molecular characteristics. Molecular identification of *B. bassiana* isolates was performed by PCR amplification of a specific region in the translation elongation factor 1 alpha (EF1- $\alpha$ ) gene using the species-specific primer pair EFFO (5'-TCGACCTCGACGAGCAATACATACTG-3')

and EFRO (5'-GATACGACGAAAAAAATTTGC GCAG-3') as previously reported (Johny and Kyei-Poku 2014) (Bio Basic Canada Inc., Canada). Three other isolates of *B. bassiana*, one each from the Thailand Institute of Scientific and Technological Research (TISTR3617), National Science and Technology Development (BCC22355) and Department of Agricultural Extension (BDOAE001), Thailand, were used as positive controls, while three isolates of *Metarhizium* spp. (MNBKK037, MNBKK039 and MNBKK040) and *Xanthomonas oryzae* (BB2014-288 and BB2014-292 for pv. *oryzae* and BLS2014-5 for pv. *oryzicola*) were obtained from the RDT, and used as negative controls.

The mycelial preparation and DNA extraction were adapted and performed as previously described (Carneiro *et al.* 2008; Safavi 2010). All fungal isolates were grown in Sabouraud's Dextrose Broth (SDB) medium at 27°C for 4–7 days with agitation at 120 rpm. Fungal mycelia were then collected using No. 1 Whatman filter paper and ground to a fine powder in liquid nitrogen. The powdered fungal mycelia were transferred to 1.5-ml microtubes for DNA extraction in 450  $\mu$ l of TES [1.4 M NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 M Tris-HCl, pH 8.0], 50  $\mu$ l of 20% (w/v) cetyltrimethylammonium bromide and 2  $\mu$ l of  $\beta$ -mercaptoethanol, mixing and incubating at 65°C for 90 min. After that the tubes were centrifuged (2,236  $\times$  g, 4°C for 5 min), the supernatant was harvested and extracted with an equal volume of 25 : 24 : 1 (v/v/v) phenol : chloroform : isoamyl alcohol. After phase separation by centrifugation (2,236  $\times$  g, 4°C for 5 min) the aqueous phase was harvested and extracted in the same manner with the exception that 24 : 1 (v/v) chloroform : isoamyl alcohol was used. The DNA was precipitated from the aqueous layer by adding an equal volume of absolute isopropanol, centrifuged (12,879  $\times$  g, 4°C for 5 min) and the pellet washed twice with 70% (v/v) ethanol. The DNA pellet was then air dried and dissolved in 50  $\mu$ l TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). The quantity and quality of extracted DNA was measured using a Nanodrop spectrophotometer (ND-1000 Spectrophotometer, NanoDrop, USA).

The total volume of the PCR reaction (Johny and Kyei-Poku 2014) was 25  $\mu$ l containing 10 pM of each primer, 1X PCR Master mix (Promega Corporation, USA) and 20 ng genomic DNA. The PCR was thermocycled at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, and then a final step at 72°C for 10 min. The products were resolved alongside low molecular weight size markers by electrophoresis in 1.5% (w/v) agarose gels and visualized by Gel Doc 2000, Bio-Rad, USA. The PCR products were purified and then direct sequen-

ced commercially (Bio Basic Inc., Canada). Consensus sequences were checked for homologues in the NCBI GenBank data base using the BLASTn algorithm, and aligned and analyzed using ClustalW software.

### Pathogenicity test

Four isolates of *B. bassiana* (BCNT001 to BCNT003 and BDOAE001) were screened for their pathogenicity against BPH. Fungal spore suspensions of each isolate were prepared at  $10^9$  spores  $\cdot$  ml<sup>-1</sup> in 0.05% (w/v) Tween 80 in distilled water. Fungal spores were sprayed onto 60 fourth to fifth instar nymphs of BPH as described by Li *et al.* (2014), and then the sprayed insects were released into cages that contained 15-days old TN1 rice cultivars. Control samples were sprayed with 0.05% (v/v) Tween 80. Dead BPH were collected and recorded daily for 14 days, from which the pathogenic efficiency of each *B. bassiana* isolate was calculated. Sample means were analyzed with Duncan's Multiple Range test (DMRT) at 95% confidence interval by SPSS Statistics (ver. 17.0, Chicago, IL).

### Induced thermotolerance of *Beauveria bassiana* using EMS mutagenesis

The three *B. bassiana* isolates (BCNT001, BCNT002 and BCNT003) were determined for inducing mutagenesis by using EMS, according to a method adapted from Ho and Ho (2015). These isolates were cultured on potato dextrose agar (PDA) at 25°C for 14 days, and then a spore suspension of each isolate was prepared at  $10^6$  spores  $\cdot$  ml<sup>-1</sup>. Next, 100  $\mu$ l of 0.5 or 1.0% (v/v) EMS was added (0.5 and 1.0% (v/v) final concentration) into each 900  $\mu$ l spore suspension of each isolate in a microcentrifuge tube, or sodium phosphate buffer pH 7 for the control, and then incubated at 30°C for 60 min. The EMS-treated spore suspension and control treatments were spread on PDA and incubated at 25°C for 7 days. For each treatment, fungal colonies that grew on PDA were then sub-cultured on PDA at 31, 33 and 35°C for 7 days. After that, colony diameters of the mutant and its respective WT isolates were measured and compared. The mean colony diameter was analyzed with a T-test at 95% confidence interval using the SPSS Statistics ver. 17.0 (Chicago, IL) software. Those EMS-treated colonies that generated a larger colony (diameter) than their respective WT at the highest temperature were selected for further analysis.

### DNA polymorphism test

The colonies selected from the EMS mutagenesis and their respective WT isolates were screened for DNA polymorphism using the Random Amplified Poly-

morphic DNA (RAPD) technique with four primers: OPA02 (5'-TGCCGAGCTG-3'), OPA03 (5'-AGT CAGCCAG-3'), OPA09 (5'-GGGTAACGCC-3') and OPA13 (5'-CAGCACCCAC-3'). The PCR reaction was performed as reported by Carneiro *et al.* (2008) in a total volume of 25  $\mu$ l containing 0.4  $\mu$ M of each primer, 1X PCR Master mix (Promega Corporation, USA) and 25 ng genomic DNA, and thermo-cycled at 94°C for 15 s, followed by 40 cycles at 94°C for 15 s, 36°C for 30 s and 72°C for 1 min, and then a final 72°C for 7 min. The products were resolved and visualized as above. The DNA band patterns obtained with each primer pair for each isolate was recorded.

### Characterization of thermotolerant *Beauveria bassiana*

The EMS-treated *B. bassiana* [0.5 and 1.0% (v/v) final concentration] that generated a colony diameter larger than their respective WT at 33 or 35°C were examined for their thermotolerant characteristics. In addition, a recovery test was performed to evaluate the growth characteristics of the mutant isolate during the reported optimum temperature after encountering a high temperature. To this end, the mutant and its respective WT were cultured on PDA at 33°C (the highest tolerant temperature) for 7 days and then incubated at 25°C for 14 days, whereupon the colony diameter and spore numbers of all the isolates were measured.

The level of fungal spore germination (%) is an important determinant in the efficiency of killing the host insect by pathogenic fungi. Thus, the percentage spore germination was studied during growth at the optimum temperature (25°C) after exposure to a high temperature (33°C) for various periods of time (5, 10 and 15 days). The number of germinated spores was counted. Then, the potato dextrose broth (PDB) flasks containing fungal spores were transferred from 33 to 25°C and the number of germinated spores was counted daily for 4 days. Spore germination percentages were calculated. Sample means were analyzed with T-test at 95% confidence interval by SPSS Statistics (ver. 17.0, Chicago, IL).

### Pathogenicity of the EMS mutant isolate

Comparison of pathogenicity between the mutant and respective WT isolates was performed according to Li *et al.* (2014) with minor adaptations, as in the pathogenicity test section above, with the exception of using spore suspensions at  $10^8$  and  $10^9$  spores  $\cdot$  ml<sup>-1</sup>. Dead BPH were collected and recorded daily for 14 days, from which the pathogenic efficiency of each isolate of *B. bassiana* was calculated. Sample means were analyzed with DMRT at 95% confidence interval by SPSS Statistics (ver. 17.0, Chicago, IL).

## Results

### Molecular identification

Verification of *B. bassiana* morphological identification was performed by PCR amplification with the species-specific EFFO and EFRO primer pair. The species-specific 307 bp band amplified from the EF-1 $\alpha$  gene for *B. bassiana* (Johny and Kyei-Poku 2014) was present in all isolates of *B. bassiana* that were used in this study. No PCR product appeared in the three isolates of either *Metarhizium* spp. or *Xanthomonas oryzae* (Fig. 1). DNA sequence analysis of all three *B. bassiana* isolates from the RDT (BCNT001 to 003) showed 100% nucleotide identity to *B. bassiana* (GenBank accession code KJ500423). Thus, the molecular identification result supported their morphological identification as *B. bassiana*.

### Pathogenicity test

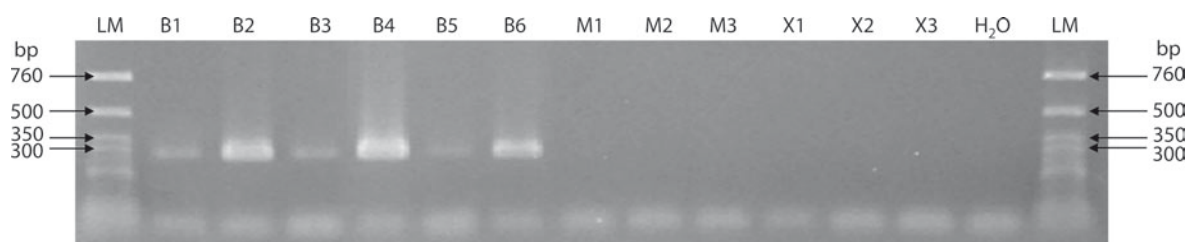
The pathogenicity of the three *B. bassiana* isolates from the RDT against BPH was evaluated in August 2016 (31.5 $\pm$ 1.2 $^{\circ}$ C, 76.8% relative humidity). Two isolates (BCNT002 and BCNT003) showed a greater efficiency to kill BPH than the isolate from the Department of Agricultural Extension (BDOAE001) that is promoted for controlling pest insects in Thailand's rice fields (Table 1). Then, three isolates, BCNT001, BCNT002 and BCNT003, were chosen for inducing mutagenesis by EMS. However, several dead BPH were found in control samples without any mycelial growth detected on the insects.

### Induced thermotolerance in *Beauveria bassiana* using EMS mutagenesis

Induced thermotolerance by EMS mutagenesis was evaluated in the three most pathogenic *B. bassiana* isolates (Table 2). Fungal growth after treatment with both 0.5% and 1% (v/v) EMS was decreased by 25% compared to the control at 25 and 31 $^{\circ}$ C. No colonies were found at 35 $^{\circ}$ C and only one EMS-treated colony [from BCNT002 treated with 0.5% (v/v) EMS] was found to survive and generate a larger diameter colony than its respective WT at 33 $^{\circ}$ C. Even at 31 $^{\circ}$ C, although there were many surviving EMS-treated colonies, their respective WT isolates could grow equally well at this temperature (data not shown).

### DNA polymorphism test

The Random Amplified Polymorphic DNA analysis of the selected EMS-treated isolate (BCNT002MT) and its respective WT isolate (BCNT002WT) presented different DNA band patterns (Fig. 2). DNA bands present in the WT but not in the EMS-treated isolate were 120 bp from primers OPA02  $\times$  OPA03 and 180 and 1,300 bp from primers OPA02  $\times$  OPA09. On the other hand, DNA bands present in the mutant but not present in the WT isolate were 1,000 and 1,500 bp from primers OPA02  $\times$  OPA09, 600 and 1,000 bp from primers OPA03  $\times$  OPA09 and 160 bp from primers OPA03  $\times$  OPA13. Thus, detectable changes in the DNA of *B. bassiana* occurred after the EMS treatment.



**Fig. 1.** *Beauveria bassiana* identification by PCR with the EF-1 $\alpha$  species-specific primers EFFO and EFRO, showing the resolved PCR amplicon of 307 bp for *B. bassiana*. Lanes: LM – low molecular weight DNA ladder; B1–B6 – *B. bassiana*: B1 – BCNT001, B2 – BCNT002, B3 – BCNT003, B4 – TISTR3617, B5 – BCC22355 and B6 – BDOAE001; M1–M3 – *Metarhizium* spp.: M1 – MNBKK039, M2 – MNBKK040 and M3 – MNBKK037; X1–X2 – *Xanthomonas oryzae* pv. *oryzae*: X1 – BB2014-288 and X2 – BB2014-292; X3 – *Xanthomonas oryzae* pv. *oryzicola*: BLS2014-5 and H<sub>2</sub>O. The gel shown is representative of three such amplifications

**Table 1.** Mortality of the brown planthopper (BPH) by *Beauveria bassiana* infection at 4 to 14 days after inoculation

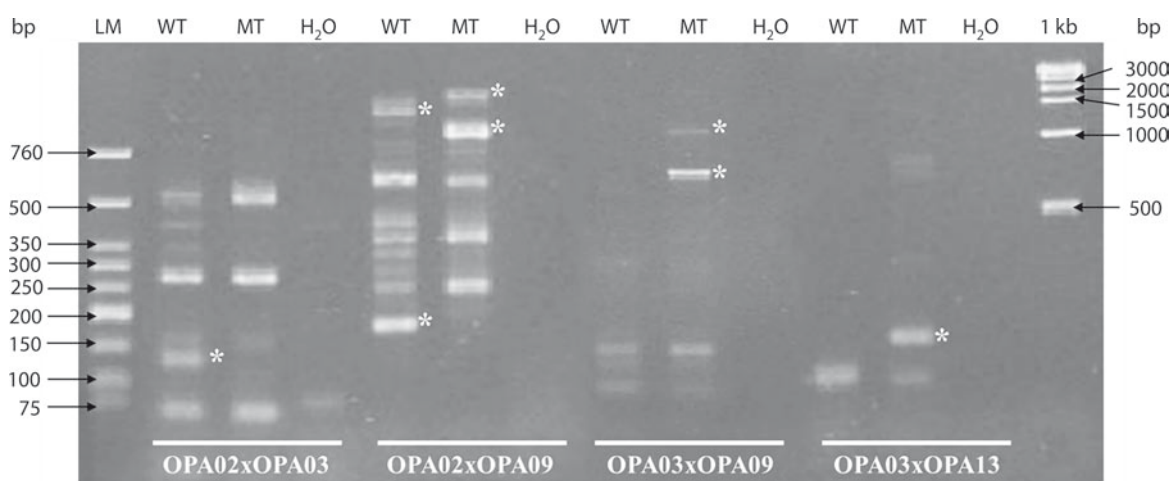
Mortality of BPH [%]	Treatment				
	BCNT001	BCNT002	BCNT003	BDOAE001	Tween 80
	20 $\pm$ 3 b	37 $\pm$ 4 c	36 $\pm$ 1 c	22 $\pm$ 5 b	0 $\pm$ 0 a

Data are shown as the mean  $\pm$  1SD, derived from 3 replications. Means followed by different letters were significantly different according to the DMRT ( $p < 0.05$ )

**Table 2.** Colony growth (% of the control cells) of *Beauveria bassiana* after ethyl methanesulfonate (EMS) treatment and then incubated at 25, 31, 33 and 35°C for 7 days

Isolate	Control				0.5% (v/v) EMS				1.0% (v/v) EMS			
	25°C	31°C	33°C	35°C	25°C	31°C	33°C	35°C	25°C	31°C	33°C	35°C
BCNT001	100	100	0	0	29	23	0	0	27	17	0	0
BCNT002	100	100	0	0	31	22	1	0	22	13	0	0
BCNT003	100	83	0	0	24	12	0	0	22	14	0	0

Data are shown as the mean, derived from 3 replications for control while only one replication for 0.5 and 1.0% EMS mutations due to slow mycelial growth



**Fig. 2.** The Random Amplified Polymorphic DNA (RAPD) results of the *Beauveria bassiana* isolates: BCNT002WT (WT), BCNT002MT (MT), and H<sub>2</sub>O (negative control). Lanes: (1) low molecular weight DNA ladder (LM), (2–4) OPA02 × OPA03, (5–7) OPA02 × OPA09, (8–10) OPA03 × OPA09, (11–13) OPA03 × OPA13 and (14) 1 kb ladder. DNA bands with asterisk (\*) are of a different size between the wild type (WT) and mutant type (MT) isolates. Results shown are representative of three independent PCR RAPD assays

### Thermotolerant characteristics of *Beauveria bassiana*

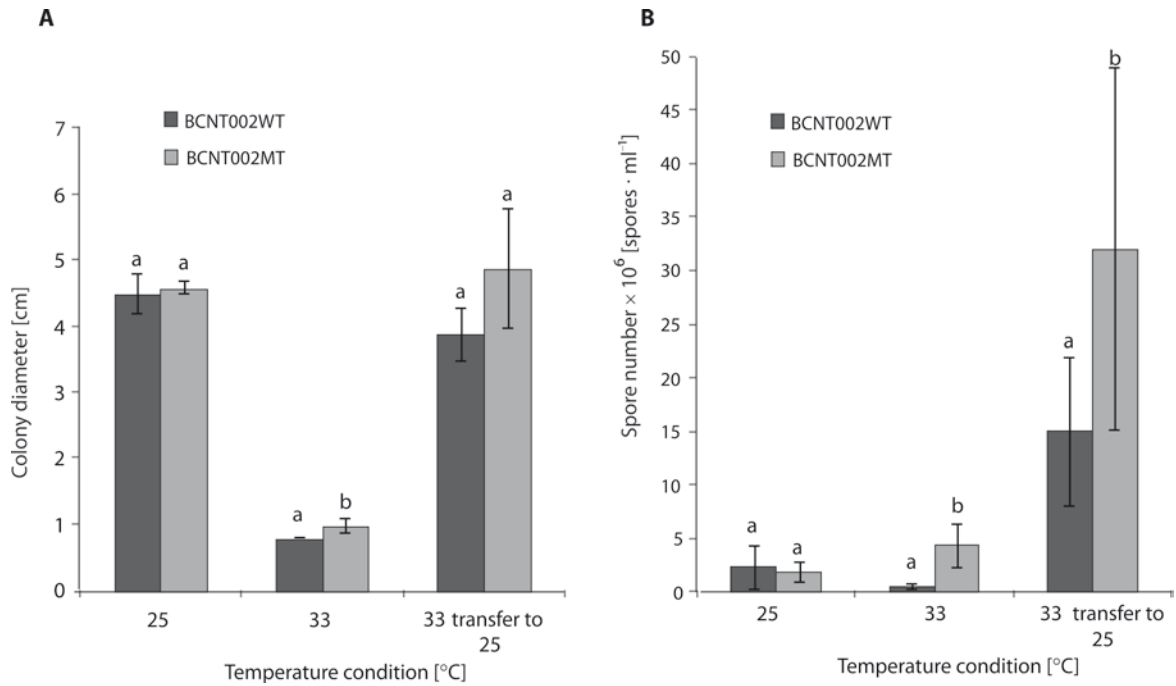
The thermotolerant *B. bassiana* isolate (BCNT002MT) grew significantly faster than the WT isolate when evaluated at 33°C (Fig. 3A). However, the colony growth rate of BCNT002MT (0.07 cm · d<sup>-1</sup>) at 33°C was very slow compared to being cultured at 25°C (0.33 cm · d<sup>-1</sup>). Therefore, a recovery test was performed by culturing the fungal mycelia at a high temperature (33°C) for 7 days and then transferring them to the optimum growth temperature (25°C). The mutant (BCNT002MT) recovered and grew better than the WT (BCNT002WT), where the colony diameter of the mutant isolate in the recovery test (4.9±0.9 cm) was close to that when cultured at 25°C (4.6±0.1 cm), whereas the WT had smaller colonies in the recovery (3.9±0.4 cm) than at 25°C (4.5±0.3 cm). In addition, a greater sporulation level (more spore formation) was evident for the mutant BCNT002MT after recovery from high temperature than its respective WT (Fig. 3B).

Spore culture at 33°C was conducted to assess the germination efficiency. However, no spore germination was found in both the mutant (BCNT002MT) and its WT (BCNT002WT) isolates when cultured at 33°C for

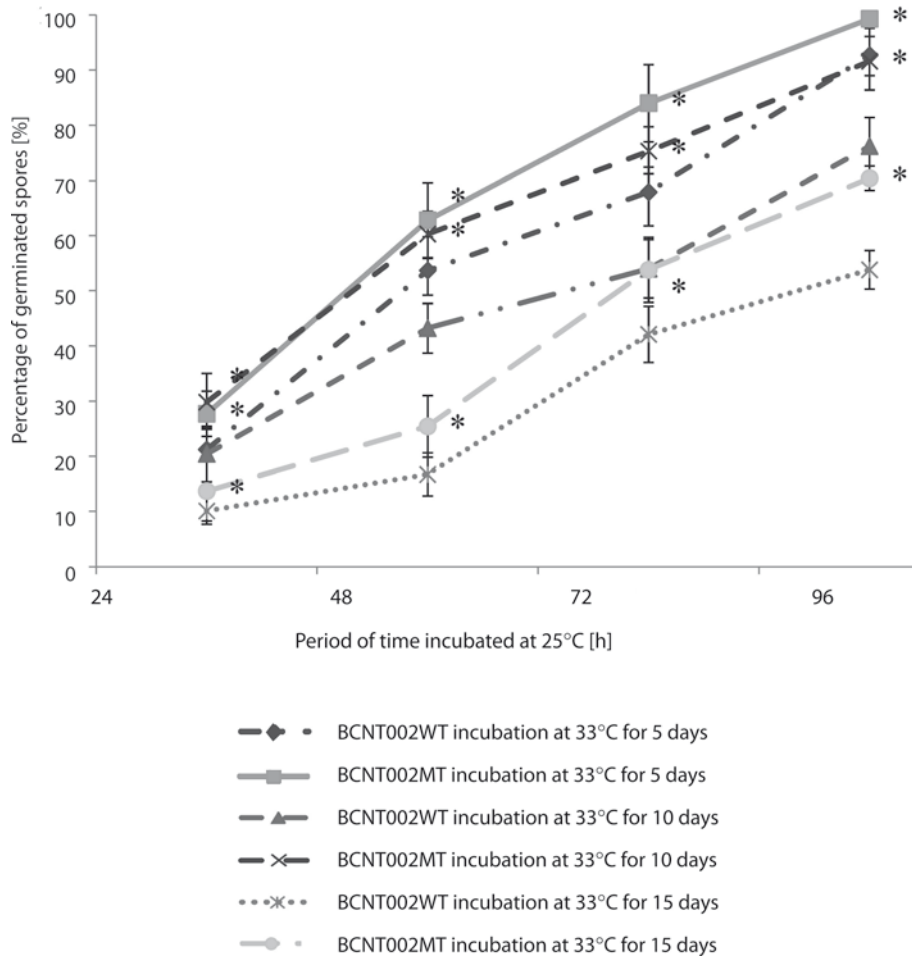
5, 10 or 15 days (data not shown). Within the recovery test, spore germination at 25°C decreased with longer prior exposure times to the high temperature (33°C). Spores cultured at 33°C for 5 days and then transferred to 25°C had a higher germination percentage than those incubated at 33°C for 10 or, especially, 15 days in both the mutant and WT isolates (Fig. 4). However, the spore germination percentage after transfer to 25°C was significantly greater in the mutant isolate than in the WT for all germination periods (Fig. 4).

### Pathogenicity test of the thermotolerant BCNT002MT isolate compared to its parental WT (BCNT002WT) isolate

The bioassay to assess the pathogenicity of the thermotolerant mutant (BCNT002MT) isolate against BPH in comparison to its WT (BCNT002WT) isolate was performed in March 2017 (35.0±2.5°C, 63.8% relative humidity). The only significant difference between mutant and wild type isolates was the concentration 10<sup>9</sup> spores · ml<sup>-1</sup>. However, the BPH control efficiency was greater for the thermotolerant BCNT002MT isolate than the WT (BCNT002WT) at inoculation levels of both 10<sup>8</sup> and 10<sup>9</sup> spores · ml<sup>-1</sup> (Table 3).



**Fig. 3.** The average (A) colony diameter and (B) number of spores for *Beauveria bassiana* isolates: BCNT002WT and BCNT002MT after incubation at 25°C and 33°C for 14 days, and at 33°C for 7 days and then transferred to 25°C for 14 days. Data are shown as the mean ± 1SD, derived from three repeats. Means with a different letter above them are significantly different ( $p < 0.05$ )



**Fig. 4.** The percentage of germinated spores when incubated at 25°C following prior incubation at 33°C for 5, 10 and 15 days. Data are shown as the mean ± 1SD, derived from three repeats. Means with asterisk (\*) indicate a significant difference between the WT and mutant ( $p < 0.05$ )

**Table 3.** Mortality (%) of the brown planthopper (BPH) (after 14 days) induced by *Beauveria bassiana* infection inoculated with various spore concentrations

Spore concentration [spores · ml <sup>-1</sup> ]	Treatment		
	BCNT002WT	BCNT002MT	Tween 80
10 <sup>8</sup>	22 ± 9 b	30 ± 12 b	0 ± 0 a
10 <sup>9</sup>	18 ± 3 B	46 ± 8 C	0 ± 0 A

Data are shown as the mean ± 1SD, derived from three replications. Means followed by different letters were significantly different according to the DMRT ( $p < 0.05$ )

## Discussion

To support the morphological identification of the three isolates of *B. bassiana* from the RDT, PCR amplification of a 307 bp fragment of the EF-1 $\alpha$  gene was performed using the species-specific primers. The appropriate amplicon was detected in all three *B. bassiana* isolates from the RDT, plus the three control *B. bassiana* isolates, but not in the other six isolates from two other species, which agrees with a previous report (Johny and Kyei-Poku 2014). Moreover, DNA sequencing of the 307 bp fragment of the EF-1 $\alpha$  gene (GenBank accession codes JQ043235) supported the PCR result that three isolates from the RDT were *B. bassiana*. Therefore, the identification of these isolates as *B. bassiana* was accepted.

Two isolates (BCNT002 and BCNT003) revealed a higher pathogenicity under these conditions against BPH than the promoted isolate from the Department of Agricultural Extension (BDOAE001). These two isolates were recently isolated from infected BPH in rice fields, and so should have retained a high level of their virulence compared to repeatedly subcultured isolates (Ansari and Butt 2011). Improvement of these pathogenic isolates of *B. bassiana*, in terms of increased thermotolerance, was attempted by EMS mutagenesis with screening at the elevated culture temperatures of 33 and 35°C. No colonies were found at 35°C, but at 33°C one 0.5% (v/v) EMS-treated colony (BCNT002MT) was found that could survive and generate a larger colony diameter than its respective WT (BCNT002WT). DNA polymorphism comparison between the BCNT002MT and BCNT002WT isolates showed different band patterns indicating changes in the DNA sequences. Normally EMS-induced mutations are evidenced by base-pair substitution (Sega 1984; Shiwa *et al.* 2012). However, whether the DNA band patterns presented in BCNT002MT but not in BCNT002WT (or vice versa) might be involved (causal rather than incidental) with thermotolerant genes in *B. bassiana* is unknown, but of course it does not have to be the case. Certainly, while whole genome analysis of EMS-treated *Lotus japonicus* revealed high single-nucleotide polymorphisms that were causal mutations for phenotypic changes (Mohd-Yusoff *et al.* 2015), we sampled only a small random fraction of the genome by this RAPD analysis.

The mutant isolate BCNT002MT produced a larger colony diameter and greater spore production than its corresponding WT isolate at 33°C. Comparing the colony diameter and sporulation between the 25 and 33°C cultured conditions, both the mutant and WT isolates grew better at 25°C (optimum temperature) than at 33°C, which is consistent with previous studies where high temperatures retarded or inhibited spore germination, hypha growth and sporulation of *B. bassiana* and also affected the fungal virulence (Shimazu 2004; Bugeme *et al.* 2008). Other studies have reported higher temperature tolerant isolates of *B. bassiana* up to 35°C (Shimazu 2004; Bugeme *et al.* 2008; Avanti *et al.* 2014). However, the optimal growth temperature of entomopathogenic fungi is likely to vary within a species depending on the geographic origin (Orduño-Cruz *et al.* 2015) due to adaptation to local selection pressures, including the thermotolerant character.

In this study, the effect of fluctuating temperatures was tested. Exposure to high temperatures (33°C) for a relatively short duration (5 to 15 days) had a lower inhibitory effect on growth as it was followed by recovery with culturing at the optimum temperature (25°C). After treating the mutant isolate and its WT at the high temperature (33°C) and transferring to the optimum temperature (25°C), the mutant isolate revealed better growth and sporulation levels than its corresponding parental WT. The ability of *B. bassiana* to recover and grow after exposure to a high temperature has been reported before (Shimazu 2004).

No spore germination was found in all isolates at 33°C but after transferring to 25°C, some spore germination was seen. Fungal spores typically need a specific temperature range for germination, and for *B. bassiana* the optimum temperature for germination was reported to be about 25°C, depending on the fungal isolate. Thus, Qazzaz *et al.* (2015) reported that the optimum temperature range was 20–25°C, whereas Bugeme *et al.* (2008) presented it as 25–30°C, but at the non-optimal temperature the level of spore germination was low. Sivasankaran *et al.* (1998) reported that *B. bassiana* isolates require high levels of water activity for germination. This experiment showed that the higher temperature of 33°C significantly affected spore germination when culturing spores in a liquid media

(and so a high water activity). In addition, the high temperature tolerance of *B. bassiana* depended on the fungal stage, with spores being more tolerant to the high temperature than hypha. In accordance, Shimazu (2004) reported that *B. bassiana* spores survived at high temperatures for a longer time than the hypha, which reflects that spores are better adapted to survive in high stress environments (Shimazu 2004; Liu *et al.* 2015). Interestingly, the mutant isolate of this study (BCNT002MT) produced a higher number of spores than the WT isolate, which could potentially promote spore survival. Moreover, non-optimal temperatures induced exogenous dormancy in asexual fungal spores as a response to the unfavorable conditions (Feofilova *et al.* 2012).

The level of spore germination (%) of the BCNT002MT isolate was significantly higher than the WT when transferred from 33°C and incubated at 25°C. High temperatures normally decrease the viability of fungal spores (Zimmermann 2007), which explains why fewer spores germinated after incubating the fungal spores at 33°C for 15 days, than at 10 or 5 days, before being transferred to 25°C. This is consistent with the report that exposure to a high temperature for a longer time caused a lower germination rate, and that at over 36°C no spores germinated (Shimazu 2004). Overall, the fungal radial growth, sporulation and spore germination level of the BCNT002MT mutant isolate were greater than the WT, suggesting that the mutant isolate is better adapted under these experimental conditions to fluctuating temperatures than the WT. However, the trade-offs and stability of this/ these trait(s) remain unknown.

The thermotolerant BCNT002MT mutant and its respective WT isolate were tested for their pathogenicity against nymphal stages of BPH, where the mutant isolate caused higher insect mortality than the WT isolate at a spore inoculum of both  $10^8$  and  $10^9$  spores  $\cdot$  ml<sup>-1</sup>. Bugeme *et al.* (2008) reported that fungal virulence on insects was associated with the fungal isolate and temperature, where different isolates of *B. bassiana* resulted in a different mortality of tomato spider mites at different temperatures. For example, isolate ICIP278 caused a higher mortality at 25°C than ICIP279, but this was vice versa at 30°C, where the mutant isolate adjusted itself better than the WT isolate under stable greenhouse conditions (35.0±2.5°C). The efficiency of fungal pathogenicity is known to involve many factors, including spore germination efficiency, hyphal growth and sporulation (Zimmermann 2007).

The improved hyphal growth, sporulation and germination after confronting a high temperature of the mutant BCNT002MT isolate makes it a candidate for further study as a potential agent for Thai agriculturists to use for controlling insect pests in Thailand.

However, the trade-offs of such genetic changes and their stability under heterogeneous environmental conditions are unknown and require evaluation prior to mass rearing and release.

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