อุบัติการณ์ของเชื้อราก่อโรคในแมลง *Beauveria bassiana* และ *Metarhizium anisopliae* และแมลงศัตรูข้าว ในจังหวัดพิษณุโลก ประเทศไทย

Incidence of Entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* and Associated Rice Pest Insects in Phitsanulok Province, Thailand

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Abstract: This research investigated the incidence of *Beauveria bassiana* and *Metarhizium anisopliae*, entomopathogenic fungi (EPF), from infected rice and insect pests in paddy fields at Pitsanulok province, Thailand. The results of the survey of EPFs and insect pests (rice insect pests infected by those two EPFs) revealed that the abundance of some important Lepidoptera pests were 0.01–0.58 insects per site. Pyralid larvae, including the Yellow stem borer (*Schoenobis bipunctifer*) and Rice leaffolder (*Cnapholochrocis medinalis*) were mainly found. The dead insects that were infested with *B. bassiana* or *M. anisopliae* included the green leafhopper (*Nephotettix virescen*), and other members of Hemiptera, including plant-leafhoppers, as well as members of Coleoptera, Lepidoptera, and Diptera. Isolation and identification of *B. bassiana* revealed it had white colonies filamentous and had definite cell walls. Under the same laboratory condition, *M. anisopliae* had hyaline to greenish hyphae that formed a sporodochium where conidiogenous cells were born. The basipetally produced chains adhered in distinct cylindrical columns with rounded ends, and were green (phialospores) in color.

Keywords: *Beauveria bassiana*, biological control, *Metarhizium anisopliae*, paddy field, survey

บทคัดย่อ: งานวิจัยนี้ค้นหาการอุบัติของเชื้อราก่อโรคในแมลง (EPF) ได้แก่ *Beauveria bassiana* และ *Metarhizium anisopliae* และแมลงศัตรูพืชในนาข้าวที่จังหวัดพิษณุโลกประเทศไทย จากการสำรวจและเก็บตัวอย่าง พบว่ามีแมลงศัตรูพืชสำคัญในอันดับ Lepidoptera จำนวน 0.01–0.58 ตัวต่อพื้นที่สำรวจ ตัวอ่อนของแมลงวงศ์ Pyralid ประกอบด้วย หนอนกอสีครีม (*Schoenobis bipunctifer*) และ หนอนห่อใบข้าว (*Cnapholochrocis medinalis*) พบแมลงที่ตายจากการเข้าทำลายของ *B. bassiana* หรือ *M. anisopliae* ได้แก่

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เพลี้ยจั๊กจั่นสีเขียว (*Nephotettix virescen*) และสมาชิกอื่นในอันดับ Hemiptera ประกอบด้วย เพลี้ยจักจั่น สมาชิกในอันดับ Coleoptera Lepidoptera และ Diptera การคัดแยกและจัดจำแนกเชื้อรา *B. bassiana* พบว่ามันมีลักษณะโคโลนีสีขาว เส้นใยแบบมีหลายเซลล์ (filamentous) มีผนังเซลล์ชัดเจน ภายใต้สภาวะเดียวกันในห้องปฏิบัติการ *M. anisopliae* มีเส้นใยสีใสจนถึงสีเขียวที่เกิดจากการสร้างก้านชูสปอร์สั้นที่อัดตัวกันแบบ sporodochium รูปห่วงโซ่ที่ผลิตขึ้นจากฐานถูกยึดติดในคอลัมน์ทรงกระบอก (phialospores) มีปลายมน และมีสีเขียว

คำสำคัญ: *Beauveria bassiana*, การควบคุมทางชีวภาพ, *Metarhizium anisopliae,* นาข้าว, การสำรวจ

INTRODUCTION

Globally there are over 800 species of insects found in paddy fields, but less than 200 species are general regarded as of economic importance (pests or beneficial insects) (Barrion and Litsinger, 2010). Many rice insect pests attack sporadically, so that an important insect pest at one locality may become unimportant or disappear there at later times. These complexes in Southeast Asia are comprised of the rice stem borer complex, which consists of *Scirpophaga incertulas, Chilo suppressalis, C. polychrysa* and *Sesamia inferens*; the leafhopper and planthopper complex, which consists mainly of the green leafhopper, *Nephotettix virescens*, plus *N. nigropictus*; and the planthopper complex, consisting of the brown planthopper, *Nilaparvata lugens* and white-backed planthopper, *Sogatella furcifera*. (Rombach *et al*., 1987). Well known minor insect pests of rice are the rice leaf folder (RLF), *Cnaphalocrocis medinalis*; rice gall midge, *Orseolia oryzae*; rice hispa, *Dicladispa armigera*; and rice thrips, *Baliothrips* sp. (Wongsiri, 1991; Liong, 2015). The survey was carried out in Phitsanulok Province at various paddy fields, particularly where the rice farmers applied less pesticide. Rice production is the central component of the agricultural sector. Most paddies at the study sites are in plain regions with rich black soil. Moreover, intricate canal systems and abundant rainfall provide a suitable environment for growing rice.

As for the naturally-occurring entomopathogenic pathogens in rice pest insects, the green rice leafhoppers and brown planthoppers are infected by various entomopathogenic fungi (EPF), such as *Metarhizium anisopliae*, *M. flavoviride*, *Beauveria bassiana*, and *Hirsutella citriformis* (Rombach *et al*., 1987; DeFaria and Wraight, 2007). However, no significant attempts have been made to extensively utilize them as microbial control agents yet. In this study, we examined the incidence of EPF, found to be *B. bassiana* (Balsamo) Vuillemin and *M. anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae), and associated rice pest insects, to evaluate their potential to control rice pest insects as part of augmentative biological control using native or endemic natural enemies as well as to support organic farming.

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MATERIALS AND METHODS

A study was made during the rice planting season in Phitsanulok province, Thailand at 10 different paddy fields. The locations chosen were in the three Districts of Wat Bot, Mueang Phitsanulok, and Wang Thong (16.60–17.50° N and 100.30–100.60° E). Ten contiguous paddy fields of 4,000 m^2 each were set up and all fields were mapped. Each study site was divided into 10 subplots of 20 x 20 m^2, and in each subplot 50 samples of rice plants were sampled each month. Each sample was evaluated using the top half of the rice tiller. Dead insects (cadavers) found to be sticking to the leaves or part of the tiller or covered by cottony mycelia and/or overgrown by chalky white or green mass of conidia were collected in a sterile plastic tube and kept in a cooler box to avoid cross contamination from handling. Soil samples were also taken from each of the ten survey sites using a spoon. At each random sampling point (0.36 m^2) five subsamples of approximately 0.5–0.8 g soil samples were taken from the top 2 cm of soil because the majority of fungal inoculums are usually concentrated in this soil layer (Storey *et al.*, 1989). For each site, the five subsamples were pooled and mixed thoroughly in the laboratory for further investigation.

Isolation and Identification of the EPF Collected from the Paddy Fields

The insect cadavers that appeared to be infected by fungi were collected during the survey and isolated as detailed above. The isolation of *B. bassiana* and *M. anisopliae* was conducted using the descending conidia showering method (Papierok and Hajek, 1997). Field-collected rice pests infected with *B. bassiana* that were filled with hyphal bodies were surface sterilized by dipping them in 70% (v/v) ethanol for 15 s, 2% (w/v) sodium hypochlorite solution for 2–3 min, and then washed twice with sterile water.

Isolation of *B. bassiana* from soil samples was performed using the "Galleria bait method" (GBM) (Goettel and Inglis, 1997) with slight modifications. Silkworm larvae, *Bombyx mori* L. fed with cut mulberry leaves in a continuously reared colony maintained at 28–30 °C, were used as the bait insect. Before the baiting procedure, the *B. mori* larvae were soaked in 50 °C water for 15 s to reduce their ability to produce silk webbing in the soil, which might otherwise limit the exposure of the larvae to EPF spores in the soil. Approximately 400 mL of the moistened sampled soil (moistened with distilled water if necessary) was placed in a 500-mL clear plastic box with a perforated cover and was then baited with at least ten third to fourth instar *B. mori* larvae.

Isolation of *M. anisopliae* from the cadavers of infected field-collected rice insect pests was performed in the same manner as for *B. bassiana*, while isolation of *M. anisopliae* from soil samples used the GBM as above except as follows. After baiting on the soil sample, dead *B. mori* larvae were removed, surface-sterilized, and incubated at 25–30 °C for a few days until the white color mycelium was observed and turned green because of sporulation. The diluted suspensions were then inoculated on PDA plates using the dilution plate method and cultured at 20–25 °C for 3–5 d and examined for the growth and development of *M. anisopliae* single colonies (Ali *et al.*, 2010). The obtained EPF isolates were then sub-cultured to clonality and stored on PDA slants for further study.

Macroscopic and microscopic identification of the EPF

The *B. bassiana* and *M. anisopliae* isolates obtained from cultures grown on PDA were examined. Monosporic isolates of each fungal species were incubated in Petri dishes at 25 ºC in darkness for 72 h. Then, three single colonies from each plate were removed and transferred to three sterile Petri dishes containing malt extract agar and PDA and incubated for 14 d in darkness at 23–25 °C. Micro-morphological characteristics were studied using and mounting young colony structures in lactophenol cotton blue [0.01% (w/v)] (Poinar and Thomas, 1984). Morphological characteristics of the hyphal body and conidia, and pigmentation descriptions were made and identified according to Steinhaus (1967).

Quantity and Viability of the EPF

The conidia from 14-d-old culture were collected in 0.5 mL of sterile distilled water supplemented with 0.02% (v/v) Tween 80 (carrier) and then the concentration of suspension was determined using hemocytometer with 5 replications/isolate (Goettel and Inglis, 1997). All isolated EPF were subjected to conidia germinating test on PDA in order to determine their viability as previously reported (Migiro *et al*., 2010). The isolate from each study site that produced the highest conidial amount and viability was selected for further study, giving 10 isolates of either each of *B. bassiana* and *M. anisopliae.*

RESULTS AND DISCUSSION

The incidence of some important insect pests of rice and the two obtained EPF species, *B. bassiana* and *M. anisopliae*, were evaluated at 10 study sites in Phitsanulok province. As shown in Table 1, the important rice pests were found in the paddy fields at 0–0.14 and 0.01–0.02 insects per site, respectively, while the rice leafhopper and planthopper complex, consisting of rice green leafhoppers, Nephotettix virescens, *N. nigropictus*, zigzag leafhopper, *Recilia dorsalis*, white leafhopper, *Cofana spectra*, orangeheaded leafhopper, *Thaia oryzivora*, brown planthopper (BPH), *Nilaparvata lugens*, and the white-backed planthopper (WBPH), *Sogatella furcifera* were commonly found in paddy fields in almost all locations at 0.01 to 0.14 insects per site.

In contrast, lepidopteran pests were observed at 0.01–0.58 insects per site (Table 1). Amongst these lepidopteran species, the yellow rice stem borer, *Scirpophaga incertulas*, and the RLF, *Cnaphalocrocis medinalis*, were the main species found in the paddy fields and to a lesser extent were the rice caseworm, *Nymphula depunctalis*, rice skipper, *Pelopidas mathias*, bush hopper, *Ampillia dioscorides*, according to Wongsiri (1991), there are at least 15 lepidopteran pests recorded as rice pests.(it was similar or different to the reference) Although the yellow stem borer and RLF were the main lepidopterous pests encountered in the paddy fields at Phitsanulok province, they were apparently not serious pests in the area during the survey. Paddy fields in South East Asia play the importance of the RLF and rice stem borer also reported very few cases of their severe damage (Rubia *et al*., 1996). This may be due to the benefits of natural enemies in the paddy fields, such as the predators, parasitoids, and entomopathogens, which suppress the pest population, and so implies some sort of rough balance between these pests and their natural enemies (DeBach and Rosen, 1991; Migiro *et al*., 2010).

This study indicated that the natural occurrence of white or green muscardine diseases on insect pests in the paddy fields was scarce. In total, from both the field and laboratory screening of insects, 36 insects (39.13%) were infected with *B. bassiana*, giving an incidence of 0.08±0.08 samples per site (Table 1). The infected insects included Coleoptera, rice green leafhopper, *Nephotettix virescens*, Diptera and lepidopteran larvae. For *M. anisopliae*, a total of 12 infected insects were found (13.04%), giving an incidence of 0.04±0.05 samples per site. The insects infected with *M. anisopliae* were leafhoppers, Asian rice gall midge, *Orseolia oryzae*, lepidopteran larva and Hemipter at an average of 0.04±0.05 samples per site. The difference in the incidence of *B. bassiana* and *M. anisopliae* was not significant.

Table 1 Incidence of important rice pests infected by *B. bassiana* and *M. anisopliae* in paddy fields at Phitsanulok Province (16.60-17.50º N; 100.30-100.60º E) (n = 100 subplots)

Organism	Average number of rice pests infected (mean ± SD)
Leafhoppers (Hemiptera: Cicadellidae):	
Rice green leafhopper, *Nephotettix virescens*	0.14 ± 0.05
Rice green leafhopper, *N. nigropictus*	0.02 ± 0.02
Zigzag leafhopper, *Recilia dorsalis*	0.02 ± 0.13
White leafhopper, *Cofana spectra*	0.01 ± 0.01
Orangeheaded leafhopper, *Thaia oryzivora*	0.01 ± 0.02
Planthoppers (Hemiptera: Delphacidae):	
Brown planthopper (BPH), *Nilaparvata lugens*	0.01 ± 0.02
Whitebacked planthopper (WBPH), *Sogatella furcifera*	0.02 ± 0.03
Lepidopterous insects	
Lepidoptera: Pyralidae:	
Yellow stem borer, *Scirpophaga incertulas*	0.58 ± 0.22
Rice leaffolder, *Cnaphalocrocis medinalis*	0.44 ± 0.16
Rice caseworm, *Nymphula depunctalis*	0.01 ± 0.22
Lepidoptera: Hesperiidae:	
Rice skipper, *Pelopidas mathias*	0.10 ± 0.02
Entomopathogens: Hyphomyces:	
Beauveria bassiana	0.08 ± 0.08
Metarhizium anisopliae	0.06 ± 0.15

EPF Collected from Paddy Fields

Beauveria spp. are cosmopolitan in distribution, easily recognized and isolated, occur at a high frequency in nature, and have a wide and broad host range, including more than 700 insect species. On the the other hand, *Metarhizium* spp. are known to infect more than 200 insect species, many of which are major agricultural pests (Steinhaus, 1967). All the dead insects infected with *B. bassiana* were covered with a dense cottony white mycelium covering on their exoskeleton, and some of these mycelia had sporulated. This characteristic of the dead insects was consistent with *Beauveria vuillemin* or the white muscardine (Lacey *et al*., 1999). According to the macro- and micro-characteristics of the EPF isolates mentioned above, they were identified as *B. bassiana* and *M. anisopliae*, had been isolated and identified from different insect hosts collected from different study sites of Phitsanulok province. A total of 99 EPF isolates were obtained from the cadavers and soil samples. Of these, 52 were from *B. bassiana*-infected insects, including 19 *Nephotettix* spp., 6 *R. dorsalis*, 2 *C. spectr*a, 14 *O. oryzae*, 3 unknown coleopteran insects, 3 unknown hemipteran insects, 4 unknown lepidopteran larvae, and one dipteran larva. Another 40 were from *M. anisopliae*-infected insects, including 13 *Nephotettix* spp., 2 *R. dorsalis*, 1 *C. spectra*, 16 O. oryzae, 3 unknown coleopteran insects, 2 unknown hemipteran insects, 4 unknown lepidopteran larvae, and 1 dipteran larva. In addition, 2 isolates of *B. bassiana* and 5 isolates of *M. anisopliae* were obtained from 7 out of 10 soil samples from the 10 different study sites using the GBM (Figure 1).

Quantity and Viability of EPF spores

The susceptibility of most insects to EPF infection depends on the spore dosage, with a positive correlation between the number of infective spores and mortality by mycosis (Ferron, 1981). The EPF characteristics, spore production, and spore viability were evaluated for selecting the highest productivity isolate from each of the ten sampled locations (Figure 1).

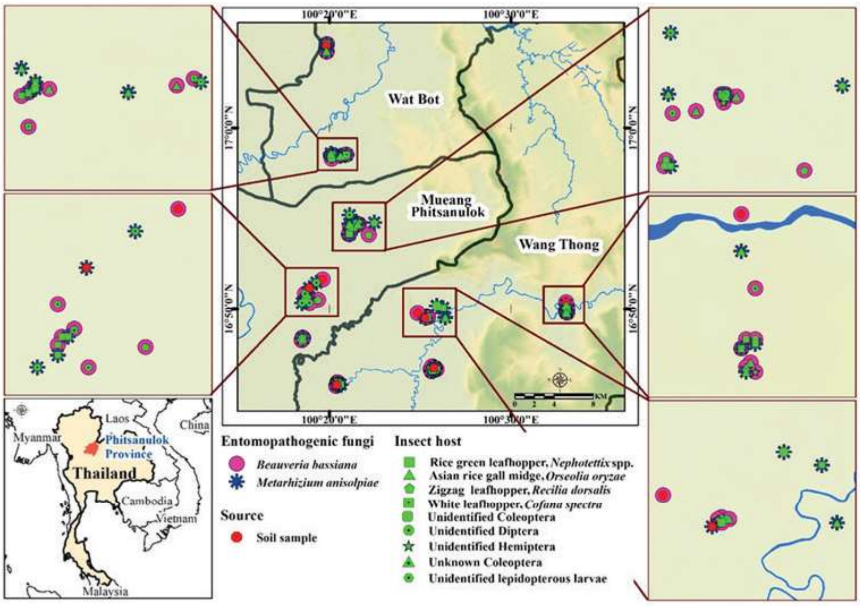

Figure 1 The location of found insect pests infected by *B. bassiana* or *M. anisopliae* and soil samples collected sites in Phitsanulok province (Source: Author's elaboration)

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The *B. bassiana* and *M. anisopliae* isolates with the highest productivity of viable conidia were selected from each of the ten locations. It shows that the selected *B. bassiana* isolates (Bb01–10) produced conidia ranging from 107–1016 spores mL^{-1}, while their spore germination rate at 23 - 28 °C for 24 h ranged from 85.0 ± 3.96% to 92.0 ± 3.53% (Table 2). Marti *et al*. (2005) reported that the *B. bassiana* isolated from 301 adults and 274 nymphs of *Triatoma infestans* (Hemiptera: Reduviidae) collected in nine localities of five provinces in Argentina had a spore viability of 100% at 24 h at 25 °C. Whereas, the germination rate of *B. bassiana* isolated from *Lygus lineolaris* (Heteroptera: Miridae) at 25 or 35 °C ranged from 88–100% at both 24 and 48 h (Leland and Behle, 2005). This difference may reflect that spore germination is very variable and depends on the spore type, host species, and oxygen level (Feng *et al*., 1994). Accordingly, the germination level observed in this study is in the acceptable range and the difference could due to the different insect host species.

Table 2 The EPF isolates selected for pathogenicity testing based on the spore quantity and viability.

Location (subdistrict)	*B. bassiana* (Bb)			*M. anisopliae* (Ma)		
	Source	Spore mL^{-1}	Germination (%)	Source	Spore mL^{-1}	Germination (%)
Tha Ngam	*Nephotettix* spp. (Bb01)	1.54×10^{10}	90.8 ± 3.4	Soil (Ma01)	1.42×10^{15}	91.6 ± 4.3
Wat Bot	*Nephotettix* spp. (Bb02)	1.15×10^{10}	85.8 ± 4.0	*Orseolia oryzae* (Ma02)	1.41×10^{13}	93.0 ± 4.4
Ban Pa	Coleopterous insect (Bb03)	1.56×10^{10}	89.2 ± 1.9	*Orseolia oryzae* (Ma03)	1.98×10^{16}	93.1 ± 3.8
Don Thong	*Recilia dorsalis* (Bb04)	1.39×10^{10}	85.6 ± 6.9	*Recilia dorsalis* (Ma04)	1.53×10^{10}	91.8 ± 1.3
Samo Khae	*Nephotettix* spp. (Bb05)	1.40×10^{10}	88.2 ± 4.7	*Nephotettix* spp. (Ma05)	1.49×10^{14}	91.8 ± 3.0
Aranyik	*Nephotettix* spp. (Bb06)	1.45×10^{14}	91.8 ± 3.0	*Nephotettix* spp. (Ma06)	1.54×10^{14}	94.2 ± 5.3
Wang Thong	Hemiptera (Bb07)	1.53×10^{16}	92.0 ± 3.6	*Nephotettix* spp. (Ma07)	1.48×10^{14}	92.2 ± 0.8
Wang NokAen	*Nephotettix* spp. (Bb08)	1.13×10^{10}	92.8 ± 2.9	Soil (Ma01)	1.81×10^{10}	93.8 ± 2.5
Din Thong	*Nephotettix* spp. (Bb09)	1.41×10^{10}	91.8 ± 2.0	*Orseolia oryzae* (Ma08)	1.56×10^{12}	90.8 ± 1.8
Wang Phikun	Diptera (Bb10)	1.19×10^{7}	91.6 ± 4.8	*Orseolia oryzae* (Ma09)	1.47×10^{10}	90.8 ± 1.3

Metarhizium anisopliae isolated from different insect hosts have varying degrees of virulence, which is linked with its spore germination (Altre *et al*., 1999). Goral (1978) reported that the virulence of *M. anisopliae* conidia against insect species was increased when the spores were produced on certain complete media. In our study, each of the ten selected *M. anisopliae* isolates produced a spore amount ranging from 10^{10} to 10^{16} spores mL^{-1}, with isolate Ma03 obtained from *Orseolia oryzae* in Subdistrict Ban Pa, District Mueang Phitsanulok having the highest spore productivity (1.98 x 10^{16} spores mL^{-1}). On the other hand, there was no difference in the spore viability (as germination) among these 10 selected isolates, which ranged from 90.80 ± 1.30% to 94.20 ± 5.31% at 23 - 28 °C for 24 h (Table 2). The germination level in this study is within the range found from 27 cultures of *M. anisopliae* isolated from different insects in the Orders Coleoptera and Hemiptera in Argentina, which varied from 85.30 ± 9.40% to 100% (Toledo *et al*., 2008).

CONCLUSIONS

The incidence of *B. bassiana* and *M. anisopliae* in natural paddy fields was found to range from 0–0.16 and 0.01–0.11 samples per site. The insect pests associated with the EPF were the rice leafhopper and planthopper complex which ranged from 0.01 to 0.14 samples per site. Moreover, changes in the population density of these organisms were dependent on the rice growing stages; all insect pests increased gradually from the tillering to milky stages, followed by the occurrence of the two EPF, *B. bassiana* and *M. anisopliae*. At the laboratory level, the EPF collected from the paddy fields were isolated and identified based on their macro- and micro-morphologies according to the current identification keys. A total of 99 EPF isolates were obtained from 92 dead insects (cadavers) and seven soil samples (following the GBM). Major insect hosts infected with *B. bassiana* and *M. anisopliae* were the rice green leafhopper, *N. virescens*, and other Hemiptera. The result indicated that the natural occurrence of *M. anisopliae* and *B. bassiana* on insect pests in paddy fields were scarce up to 0.06-0.08 samples per site. Additionally, the occurrence of *M. anisopliae* was lower in frequency when compared to *B. bassiana*, but this difference was apparently not significant.

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