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Screening and evaluation of entomopathogenic fungi, *Metarhizium anisopliae*, against rice leaf folder, *Cnaphalocrocis medinalis* (Guenée)

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Abstract
The rice leaf folder, *Cnaphalocrocis medinalis* (Guenée), is a destructive insect pest of rice in Asia. Screening for the promising fungal strains of *M. anisopliae* from the *C. medinalis* cadavers collected from the rice paddy field in lower northern Thailand for controlling *C. medinalis* was carried out. A total of 27 *C. medinalis* cadavers infected by *M. anisopliae* were obtained from 11 survey locations in 5 provinces of lower northern Thailand. The pathogenicity screening found that three isolates of *M. anisopliae* (MPC03, MST01 and MPL02) were highly efficient in controlling *C. medinalis* at 100% mortality 6 days after application with LT_{50} at 3.01-3.23 days. The efficacy test revealed that those three isolates caused 100% mortality on *C. medinalis* 7 days after application.

Keywords: Rice leaf folder, *Metarhizium anisopliae*, entomopathogenic fungi, screening, isolate

Introduction
The rice leaf folder, *Cnaphalocrocis medinalis* (Guenée), is an economically important rice insect pest in Asia [1, 2]. The larvae folds up the rice leaf from the rim, lives and scraps the leaf inside, leaving only the wax layer of rice leaf, thereby, causing white stripes on the damaged leaf and resulting in the reduction of photosynthetic ability and rice disease infection [3]. The yield of infested rice is decreased positively relating to the percentage of damaged leaves [4]. The current control method for *C. medinalis* is through the use of broad-spectrum insecticides such as chlorpyrifos, carbosulfan and cypermethrin because they are very easy to use, fast action, involves less work and cheap. However, the misuse of insecticides has caused serious problems such as a reduction in the natural enemies of *C. medinalis* and beneficial insects on the field, insect pest resistance, pest resurgence, environmental pollution and potential toxicity to humans and animals [5-8]. The concern over pesticide efficacy and safety to humans and the environment has led to an intensive search for alternative control methods. Biological controls with microorganisms are considered to be an effective alternative or substitute to chemical pesticides [9]. Entomopathogenic fungi such as *Metarhizium anisopliae* has long been studied and developed to control wide range of pests [10]. *M. anisopliae* is a naturally occurring entomopathogenic fungi, spore and conidia forming, soil borne with a high potency to infect and kill wide range of insect pest species. Many isolates of *M. anisopliae* have been developed for commercial products as biological control agents for controlling various pests in agriculture [11]. However, the efficiency of *M. anisopliae* in both commercial products and newly explored isolates on *C. medinalis* has been scarcely studied. Therefore, the aim of this study was to use standard laboratory procedures to screen and select virulent isolates of *M. anisopliae* from the cadavers of *C. medinalis* found in a rice paddy field and to also determine their efficacy in controlling *C. medinalis* compared to commercial products and commonly used insecticide.

Materials and methods
Investigations were carried out on the natural occurrence of fungal pathogens on *C. medinalis* and its susceptibility to entomopathogenic fungi, *M. anisopliae*, between 2016-2017 at the Department of Agricultural Sciences, Faculty of agriculture natural resources and environment, Naresuan University and National Biological Control Research Center lower northern regional center, Phitsanulok. The experiments were composed of 4 parts as follows.

Laboratory mass rearing of *C. medinalis*

Adults of *C. medinalis* were collected from the paddy fields in and around Muang district Phitsanulok province, Thailand. They were screened and only the healthy insects were collected for rearing. A total of 20 male and female each of *C. medinalis* was reared in 0.5x0.5x1.0 m^3 insect case in a greenhouse at 30 ± 2 °C and 85% relative humidity. The five pots of rice variety, Tai Chung Native 1 (TN1), at 60 days old were planted inside and 10% honey solution pots were placed to feed the moths. The third instar larva of *C. medinalis* were collected and used in the experiments. The insect culture was reared continuously as a way of maintaining the amount of insects that were readily available for use in the experiments.

Survey and collection of infected *C. medinalis*

Surveys were carried out on the irrigated rice paddy fields in Phitsanulok, Nakhon Sawan, Phichit, Tak, Sukothai and Uttaraditt from July 2016 to February 2017 for natural occurrence of fungal pathogens on *C. medinalis*. The naturally dead *C. medinalis* larva were collected from the leaf of rice using forceps, put separately in plastic bags and brought back to the laboratory. The infected *C. medinalis* were washed once in a solution of 1% sodium hypochlorite for 3 minutes, twice in sterile water, stored in test tubes, covered with muslin cloth, coded by collecting place and number and kept at 27 ± 2 °C with 85% relative humidity for at least 5 days. After fungal conidia were presented on the larva, standard procedures for isolation of fungus from cadavers were performed following the procedure of Lomer and Lomer (1996) [12], subcultured and purified by hyphal tip method [13], and identified based on conidia structures. The colonies of *M. anisopliae* in each cadaver were screened, collected and preserved in PDA as a stock for further use.

Preparation of *M. anisopleae*

The fungal isolates were streaked on Potato Dextrose Agar (PDA) and incubated at 27 ± 2 °C with 85% relative humidity for 2 weeks or when the plate was full of the conidia. The conidia from culture surface were harvested by adding 0.01% Tween 80 at 20 ml into the plate, and gently mixed using a sterilized glass rod. The conidia suspension was filtered through a double-layered muslin cloth. The conidia concentration was determined and adjusted by haemacytometer to obtain 1×10^8 conidia per ml for pathogenicity screening.

Pathogenicity screening

Pathogenicity screening were performed based on a completely randomized design with 4 replications. A total of 10 *C. medinalis* at the third instar larvae was used in each experimental unit. Each *M. anisopleae* isolate at the concentrations of $1x10^8$ conidia/ml was applied with an applicator on the leaves of TN1 rice variety planted in pots and placed in the insect rearing case (0.3x0.3x1.0 m^3), while distilled water was used as a control. The third larval instars were starved for 4 hours prior to their release on the leaves. The mortality and morphological changes in larvae were observed and recorded daily for 15 days. The data were analyzed and probit analysis [14] for the median lethal time (LT_{50}) was calculated. The effective isolates were screened and selected based on the mortality rate and lethal time.

Efficacy test compared to *Bacillus thuringiensis* (BT), commercial *M. anisopliae* and insecticide

The experiment was carried out in a greenhouse based on a completely randomized design with 5 replications. A total of 10 *C. medinalis* at third instar larvae was used in each experimental unit. They were reared on 30 days old of TN1 rice variety in the insect rearing case (0.3x0.3x1.0 m^3). The treatments, all effective *M. anisopleae* selected from screening test at the concentrations of $1x10^8$ conidia/ml were applied, compared to the commercial products, *Metarhizium anisopleae* (METAZAN®, Appliedchem (Thailand) Co., Ltd.), *Bacillus thuringiensis* (BT) (BACTOSPIN® Thep Wattana Co., Ltd., Thailand) and chlorpyrifos (LORSBAN®, Sotus International Co., Ltd., Thailand) at the concentrations on the recommendation of product, and the control was distilled water. The third larval instars were starved for 4 hours prior to release on the leaves. The mortality and morphological changes of larvae were observed and recorded at 1, 3, 5, and 7 days after application.

Statistical analysis

The corrected percent mortality was obtained using Abbott's formula [15]. The data were analyzed using analysis of variance and Duncan's multiple range test [16].

Results

Survey and collection of infected *C. medinalis*

A total of 27 *C. medinalis* cadavers infected by *M. anisopliae* were obtained from 11 survey locations in 5 provinces of lower northern Thailand. All *M. anisopliae* from infected *C. medinalis* were isolated, coded and numbered. (Table 1)

Table 1: Survey of *C. medinalis* cadavers infected by *M. anisopliae* at different locations in lower northern Thailand

provinces	places	locations	numbers of infected cadavers	code number
Phitsanulok	Tha Nang Ngam, Bang Rakam District	16°50'56.2"N 100°05'47.6"E	3	MPL01-03
	Wong Khong Phrom Phiram District	17°07'37.1"N 100°05'47.1"E	2	MPL04-05
Phichit	Huai Kaeo, Bueng Na Rang District	16°13'54.0"N 100°07'23.0"E	4	MPC01-04
	Wang Samrong, Taphan Hin	16°13'55.9"N 100°20'38.7"E	2	MPC05-06
	Phai Tha Pho, Pho Prathap Chang District	16°15'42.9"N 100°14'39.5"E	3	MPC07-09
Kampaeng Pet	Khlong Khlung District	16°12'55.2"N 99°42'52.6"E	4	MKP01-04
	Song Tham, Mueang district	*16°30'19.3"N 99°27'51.3"E*	3	MKP05-07
Nakhon Sawan	Ban Makluea, Mueang district	15°47'31.9"N 100°06'53.3"E	1	MNW01
	Nam Song, Phayuha Khiri District	5°25'26.5"N 100°07'04.2"E	2	MNW02-03
Sukothai	Kong, Kong Krailat District	16°54'09.1"N 99°56'54.6"E	2	MST01-02
	Yan Yao, Sawankhalok district	17°16'25.1"N 99°51'28.4"E	1	MST03

Pathogenicity screening

The accumulated mortality rates recorded during 1-15 days showed that the significantly different mortality rates (18 and 100%) were found from all 27 isolates. The mortality rate below 50% in 15 days after contacting fungus was found in 14 isolates. These isolates were classified as nonpathogenic against the *C. medinalis* and it is not appropriate to calculate LT_{50}. The mortality rate between 50-100% in 7-15 days after contacting fungus and LT_{50} ranging between 6.61-10.21 days was found in 10 isolates. The mortality rate at 100% in 6 days after contacting fungus and LT_{50} ranging between 3.01-3.23 days was found in 3 isolates, MPC03, MST01 and MPL02. Among the determined *M. anisopliae* isolates, only 3 isolates, MPC03, MST01 and MPL02 showed the highest mortality rate at 100% with the shortest lethal times at 6 days and LT_{50} at 3.01-3.23 days on *C. medinalis*. Therefore, they were selected for further study. *(*Table 2*).*

Table 2: Percentage mortality with the number of observed days in parenthesis and median lethal time *(*LT_{50}*)* of the third instar larva of *C. medinalis* infected by *M. anisopliae* isolates

isolates	% mortality(days)	LT_{50}	CI
MPL01	83(15)	10.21	9.16-11.43
MPL02	100(6)	3.23	2.70-3.77
MPL03	28(15)	na	
MPL04	35(15)	na	
MPL05	45(15)	na	
MPC01	98(15)	8.22	7.42-9.05
MPC02	33(15)	na	
MPC03	100(6)	3.01	2.48-3.54
MPC04	48(15)	na	
MPC05	85(15)	7.42	6.52-8.39
MPC06	100(13)	6.89	6.06-7.73
MPC07	100(12)	6.61	5.85-7.37
MPC08	28(15)	na	
MPC09	93(15)	8.74	7.85-9.67
MKP01	45(15)	na	
MKP02	38(15)	na	
MKP03	100(15)	7.79	6.99-8.60
MKP04	45(15)	na	
MKP05	100(14)	7.24	6.47-8.00
MKP06	43(15)	na	
MKP07	33(15)	na	
MNW01	48(15)	na	
MNW02	28(15)	na	
MNW03	100(14)	7.2	6.43-7.96
MST01	100(6)	3.04	2.48-3.60
MST02	93(15)	8.46	7.54-9.38
MST03	18(15)	na	

Efficacy test with BT, commercial *M. anisopliae* and insecticide

The results obtained from the study clearly revealed that all treatments of *M. anisopliae*, BT and chlorpyrifos were effective against *C. medinalis* at 3, 5, and 7 days after application. On the first day after application, none of the entomopathogenic organism, *M. anisopliae* and BT affected *C. medinalis*. The highest percentage mortality (30%) was found only in chlorpyrifos. On day three after application, the mortality of *C. medinalis* was found in *M. anisopliae* isolates, (MPC03, MST01 and MPL02) at the rate of 23, 28 and 18%, respectively and chlorpyrifos at the rate of 98%. Meanwhile, no mortality was found in commercial *M. anisopliae* and BT. On day five after application, again chlorpyrifos showed superior efficacy by recording 100% mortality of *C. medinalis*, followed by *M. anisopliae* isolates, (MPC03, MST01 and MPL02) (70-83% mortalty) which was at the same level with BT (76% mortality) and the least efficacy was observed in commercial *M. anisopliae* (35%mortality). On day seven after application, the 100% mortality of *C. medinalis* in *M. anisopliae* isolates (MPC03, MST01 and MPL02) was recorded at the same level with BT and chlorpyrifos, but was significantly different from commercial *M. anisopliae* which was the least effective (75% mortality). (Table 3).

Table 3: Accumulated percent mortality of *C. medinalis* after contact with selected *M. anisopliae* at 1, 3, 5 and 7 days compared to commercial Metarhizium, chlorpyrifos and BT

treatments	percent mortality of *C. medinalis (*days*)*			
	1	3	5	7
MPL02	0^b	18^b	70^b	100^a
MPC03	0^b	23^b	78^b	100^a
MST01	0^b	28^b	83^b	100^a
BT	0^b	0^c	76^b	100^a
chlorpyrifos	30^a	98^a	100^a	100^a
Metarhizium®	0^b	0^c	35^c	75^b

*Mean values followed by different lowercase superscript letters in the same column are significantly different at the 5% level by Duncan's multiple range test

Discussion

The entomopathogenic fungi, *M. anisopliae* have high potential as a bio-pesticide to control rice insect pests similar to the previous finding of Jin *et al*. [17], Hong *et al*. [18] and Aker and Tuncer [19]. However, when recovered from the natural environment, the isolates of entomopathogenic fungi were commonly varied in their efficiency and lethal time [20-21]. Thus, screening of the significant isolates is needed.

In this study, a total of 27 cadavers of *C. medinalis* infected by *M. anisopliae* was found and isolated from the rice paddy field during the survey in lower northern region. A primary screening of *M. anisopliae* isolates at the concentration of $1x10^8$ conidia/ml [22-24] against the *C. medinalis* showed that all isolates apparently could infect the third instar larva of *C. medinalis* but their virulence were obviously varied. The mortality rates and lethal time were found to range from 18 to 100% and 6-15 days. A total of 13 isolates showed mortality rate more than 50% and only 3 of the isolates (MPC03, MST01 and MPL02) had a highly effective control at 100% mortality in the shortest lethal times at 6 days (LT_{50}=3.01-3.23). This high variation in virulence may be caused by the varying abilities of cuticle enzymes and toxic substance that *M. anisopliae* produced while infesting the host [25-27].

The process of killing entomopathogenic microorganisms is a time consuming process that is longer than using insecticides because they need time for hyphae to invade the integument, body cavity, destroy hemolymph, various tissues, and produce large number of conidia in the body of the insect host before causing the death of host [28-29]. Meanwhile, insecticides act directly with the nerve system resulting in the faster death of host. In this study, chlorpyrifos showed significant mortality occurred on day 3 and 100% complete mortality occurred on day 5, whereas, all entomopathogenic microorganisms showed only 35-83% mortality. Among the entomopathogenic microorganisms, our selected isolates had the mortality rate on the third instar of *C. medinalis* in the same trend which was equal to BT, and reaching 100% in 7

days where the mortality rate of commercial Metarhizium was only 75%.

The entomopathogenic fungi is usually of high virulence when infecting the species of insect host on which the fungus was originally isolated [30]. Here, all three selected *M. anisopliae* isolates were obtained from cadaver of infected *C. medinalis* and re-infested to the same host species. Therefore, their efficiency was better than the commercial fungi product which was less specific to *C. medinalis*. When compared to the commercial Metarhizium and BT, this result confirmed that these three *M. anisopliae* isolates had very high potential for being biocontrol agents similar to other research works on *C. medinalis* [31, 32] and others insect pests [33-36].

Conclusion

In this work, the *M. anisopliae* isolates, (MPC03, MST01 and MPL02) screened and selected from the *C. medinalis* cadaver collected from rice paddy field in lower northern Thailand caused the mortality rate of *C. medinalis* to be above 50% in 3.01-3.23 days and reached 100% in 6 days after application under laboratory condition. The efficiency of MPC03, MST01 and MPL02 isolates on the third instar larva of *C. medinalis* increased and was equal to that of BT at 7 days after application. This study strongly confirmed that all these three *M. anisopliae* isolates can be used as the biocontrol agents to control *C. medinalis*.

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