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The Proteolytic and Lipolytic Activities of Entomopathogenic Fungi Isolates and Their Effectiveness in Controlling the Mealybug *Dysmicoccus neobrevipes*

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Abstract

Entomopathogenic fungi **(**EPF) are highly effective in infecting insects and are commonly used for insect pest control due to their environmental safety and bio-persistence. This study focused on selecting effective EPF isolates obtained from Nakhon Ratchasima province for controlling mealybugs and investigating their relationship with protease and lipase activity. During this study EPF isolates were collected from soil samples in Nakhon Ratchasima province using the baiting technique with yellow mealworms (*Tenebrio molitor* L.). A total of 12 isolates of *Metarhizium* spp. were obtained. When cultured on potato dextrose agar (PDA) medium for 21 days, the NMMet_BAL7/1 isolate had the largest colony diameter (71.00 mm), but this was not statistically different ($P > 0.05$) from NMMet_NS4/1, NMMet_TPR10/2, and NMMet_SD9/1 isolates. The enzyme activity tests showed that all isolates exhibited both protease and lipase, with significant differences among them ($P < 0.05$). Protease activity ranged from 0.12 to 0.33, while lipase activity varied from 0.16 to 0.54. The NMMet_SS2/2 isolate showed the highest protease (0.33) and lipase (0.54). The ability of all 12 EPF isolates to control 3rd instar nymph mealybugs was tested in a laboratory environment by spraying the mealybugs with an EPF spore suspension at a concentration of 1x10^8 spores/mL. The NMMet_SS2/2 isolate resulted in the highest mortality and infection rate of mealybugs (96.67%), which was statistically different ($P < 0.05$) from other EPF isolates. NMMet_BAL7/1 showed the second highest mortality and infection rate (68.33%). This study suggests that enzyme activity is correlated with EPF virurence, underscoring NMMet_SS2/2 as a promising candidate for field testing.

Keywords: Mealybug; Entomopathogenic fungi; Extracellular enzyme activity; Biocontrol

Introduction

The custard apple mealybug, also known as the gray pineapple mealybug (*Dysmicoccus neobrevipes* Beardsley), is a major pest that causes severe damage to custard apple trees. It also attacks various plants, including pineapple, coffee, cotton, sunflower, mulberry, banana, and citrus species (Curry 2022). Both nymphs and adults feed on the sap of custard apple fruits, preferentially targeting the vulnerable thin tissues in areolar grooves. This infestation begins at fruit set (March) and continues throughout the 110-120 day development period until harvest (June-October). If they infest a fruit during its development, it may shrivel and drop prematurely. Economic analyses indicate that mealybug infestations in commercial custard apple production systems result in average yield losses ranging from 30% to 60% during epidemic outbreaks (Bhadani and Siddhapara 2018). Furthermore, mealybugs efficiently transmit various plant virus, including numerous Ampeloviruses (Dey *et al*. 2018), and some Closteroviruses (Sether and Hu 2002). They also excrete honeydew, a thick, sticky, and sweet liquid. This honeydew leads to the growth of sooty mold, which covers parts of the plant's leaves, reducing photosynthesis. It also attracts ants, which help spread the mealybugs. Moreover, the presence of sooty mold negatively impacts the export quality of the affected produce.

Mealybugs are characterized by a white, waxy powder that covers their bodies. This wax is hydrophobic, meaning

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it does not dissolve in water, and it prevents water loss from their bodies in dry conditions. As a result, mealybugs can thrive in both rainy and dry seasons (Franco *et al*. 2009). Additionally, this waxy coating hinders the effectiveness of chemical treatments. Their dense populations and overlapping generations further complicate control and eradication efforts, leading to the development of insecticide resistance (Blumberg and Driesche 2001; Venkatesan *et al*. 2016).

Chemicals in the organothiophosphate group, such as chlorpyrifos, malathion, and dichlorvos, as well as neonicotinoid chemicals like imidacloprid, are commonly used to prevent and control mealybug infestations (Tanwar *et al*. 2007). Similarly, insecticides in the sulfoxaflor, abamectin, thiamethoxam, and spirotetramat groups have demonstrated efficacy in controlling the cotton mealybug, *Phenacoccus solenopsis* (Tinsley) (Rezk *et al*. 2019). In addition to developing resistance, mealybugs contribute to pesticide residues in agricultural produce, posing risks to consumers. Additionally, pesticides have a significant effect on reducing the activities and populations of soil bacteria, fungi, and actinomycetes. This effect varies depending on the type and dosage of pesticide applied, as well as the duration of the incubation period (Al-Ani *et al*. 2019).

The utilization of EPF such as *Beauveria bassiana*, *Cordyceps fumosorosea* (formerly *Isaria fumosorosea*), *Akanthomyces muscarius* (formerly *Lecanicillium muscarium*), *Purpureocillium lilacinum* and the *Metarhizium anisopliae* species complex (including *M. robertsii* and *M. brunneum*) is on the rise for managing over 750 species of crop insect pests (Aw and Hue 2017; Hu *et al*. 2021). EPF are deemed superior to synthetic insecticides due to their human safety, environmental sustainability, and target specificity. EPF are eco-friendly, exhibit longevity, and their spores can persist in the soil for extended periods (Al-Hedad *et al*. 2017; Yang *et al*. 2019). Farmers can easily produce and scale up their quantities at low costs. Furthermore, when insects become infected and the fungus produces spores, these spores can disperse to other insects or remain in the soil. Upon contact with other insects under suitable conditions, the fungus can effectively combat pest insects (Kaya and Vega 2012; Sirimungkararat 2014).

EPF cause insect mortality by initiating infection through spore contact with the host insect's cuticle. When environmental conditions are suitable (humidity and temperature), EPF germinates on the insect's cuticle, penetrates it, grows into the inner layers, and produces secondary conidia, which degrade the internal organs (Vega *et al*. 2012; Shin *et al*. 2020). Following this, the fungal mycelium advances through the insect's outer covering into the hemolymph, where it forms blastospores. Upon successfully invading and killing the host insect, the fungus reverts to its mycelial form and re-enters the insect's cuticle (Sharma *et al*. 2023).

The pathogenicity of EPF depends on their ability to produce toxic secondary metabolites and degradation enzymes to overcome host cuticles and induce infection, particularly enzymes that degrade insect integuments and other cellular components (Leger 1995; Butt *et al*. 2016; Pedrini 2018). Lipases are the initial enzymes secreted by these fungi, followed by proteases, phospholipases, and chitinases. Lipases function to hydrolyze the ester bonds of lipoproteins, fats, and waxes within the interior of the insect integument (Moharram *et al*. 2021). Phospholipases serve as enzymes tasked with breaking down phospholipids within the insect's cuticle, while fungal chitinases collaborate with proteases to break down the insect's outer covering. Following the compromise of the insect exoskeleton, the fungus generates substantial amounts of proteases, which continue breaking down the protein-rich material in the epicuticle. As a result, the solubilized proteins are broken down, releasing amino acids that become nutrients for the EPF (Wang *et al*. 2002).

The present study investigated the correlation between protease and lipase activity in entomopathogenic fungi isolated from Nakhon Ratchasima Province and their effectiveness in controlling mealybugs.

Materials and Methods

Collection and purification of EPF isolates

Soil samples were collected in the Nakhon Ratchasima province area and subjected to the isolation of EPF using the baiting technique. Yellow mealworms (*Tenebrio molitor* L.) at the 3rd to 5th nymph stage were used as bait. The mealworms were disinfected by immersing them in a 0.5% sodium hypochlorite solution for 5 min, followed by rinsing with sterile water. The disinfected mealworms were placed in a moist chamber until the fungi produced spores (Bidochka *et al*. 1998). Spores were then isolated to obtain pure fungal cultures. Morphological characteristics (conidia, conidiophores and spore structures) were examined to confirm that they belonged to entomopathogenic fungi, following criteria outlined in previous studies (Samson *et al*. 1998; Humber 2005).

Study of the growth and morphology of EPF

The EPF isolates, all of which had been purified, were cultured on Potato Dextrose Agar (PDA) for a duration of 14 days. Using a cork borer (Ø 0.7 mm), holes were made at the tips of the fungal hyphae, after which agar dishes were transferred to the center of PDA medium and incubated in darkness at 28 ± 2°C. The colony diameter of the fungi was measured after incubation for 7, 14 and 21 days. Colony diameter was measured using standardized metric rulers by recording both horizontal and vertical cross-sectional measurements. The mean diameter was then calculated by averaging these two perpendicular measurements to obtain a representative colony size. The experimental design followed a Completely Randomized Design

(CRD), with each treatment (isolates) replicated three times, utilizing five plates per replication. Statistical analyses were conducted using the Statistical Analysis System (SAS) software (Version 9.00; SAS Institute Inc. 2006). An analysis of variance (ANOVA) was performed to assess differences among treatments, followed by Tukey's HSD test (Tukey's test) to compare mean differences between each treatment group.

Extracellular enzyme activity assay

Protease activity assay: The EPF isolates were screened for proteolytic activity in accordance with Mohanasrinivasan *et al.* (2012). EPF mycelium from each isolate was cultivated on 14-day-old PDA and sampled using a cork borer with a diameter of 0.7 cm at the outer edge of the colony. The agar plugs were then transferred in triplicate to Skim Milk Agar (SMA) composed of: KH_2PO_4 (1 g), KCl (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.4 g), $CaCl_2 \cdot 2H_2O$ (0.1 g), powdered skim milk (25 ml of 15%), glucose (10 g), agar (12 g) and distilled H_2O (1000 mL). The plates were incubated in darkness at 28 ± 2°C and examined for the formation of clear halo zones surrounding fungal colonies. Clear zone diameters were measured using a calibrated ruler by determining the distance from the colony edge to the outer boundary of the clear zone at 7 days after incubation.

Lipase activity assay: EPF isolates underwent screening for lipase activity as outlined by Deb and Dutta (2021). This involved utilizing a substrate consisting of peptone (10 g), sodium chloride (5 g), calcium chloride dihydrate (0.1 g), agar (20 g) in 1000 mL distilled water, adjusted to pH 6.0, within Tween-80 hydrolysis media. EPF mycelium from each isolate, cultivated on 14-day-old PDA, was sampled using a cork borer with a 0.7 cm diameter at the colony's outer edge. These agar plugs were then introduced into the center of the Tween-80 hydrolysis media and allowed to incubate at 28 ± 2°C under dark conditions. Positive lipolytic activity appeared as visible precipitates or clearing of precipitates around the colony. After 7 days of incubation, precipitate diameters surrounding fungal colonies were measured on each plate using standardized metric rulers, quantifying the distance from colony margins to the precipitate peripheries. Both enzyme activity assays were conducted following a CRD, with each isolate replicated 3 times and 5 plates used per replication. Data were analyzed using SAS 9.00 (SAS Institute Inc. 2006). Treatment differences were evaluated through ANOVA with post-hoc Tukey's test for mean comparisons.

The efficacy of EPF in controlling mealybug

Mealybug preparation: Mealybugs were obtained from custard apple fruit and classified using the methods outlined by Sirisena *et al.* (2013) and Curry (2022). A pumpkin was then washed, dried, and disinfected with 70% ethyl alcohol. The mealybug eggs were divided into 10 groups and placed on the pumpkin. Subsequently, the pumpkin was transferred to insect rearing boxes (20 x 50 x 30 cm) and kept at room temperature. Mealybugs hatched from eggs to form the first-generation population, progressing through nymphal stages (first, second and third instar), followed by pupal, and adult stages, completing their life cycle in 55-68 days at 26–32°C and 60-75% RH. The adults subsequently laid eggs to initiate the next generation. The eggs were continuously grouped on newly disinfected pumpkins until they hatched into 3rd nymphs. The third-instar mealybugs were then transferred to pumpkin pieces (5 x 5 x 2 cm) in insect rearing boxes (11 x 11 x 31 cm) one day prior to experimentation to facilitate adaptation. Each box contained 20 individuals and was lined with sterile moist cotton to maintain humidity.

Entomopathogenic fungi preparation: The total isolates of EPF were cultured on PDA. The culture was maintained in darkness at a temperature of 28-30°C for 21 days. Subsequently, the spores were harvested using a surfactant (Tween 20®, 0.05%). The fungal culture was diluted in a petri dish, and a sterilized loop was employed to scrape the mycelium and spores. The spore (conidia) suspension was then quantified using a Hemocytometer under a microscope at 40X magnification. The concentration of the spore suspension was adjusted to a density of 10^8 spores/mL.

Mealybug assay: EPF spore suspensions, 5 mL each at a concentration of 1 x 10^8 spores/mL, were sprayed onto insect rearing box containing mealybug for each treatment. After spraying, the boxes were transferred to an incubator set at a temperature of 28-30°C with a 12:12 dark/light cycle. Control methods included no spraying of any substances**,** spraying with surfactant (Tween 20®, 0.05%)**,** and insecticide (Chlorpyrifos 40 w/v EC) at a rate of 50 mL per 20 L of water. The experimental design was a CRD consisting of 5 replicates per treatment. Each replicate involved the use of 20 mealybugs.

Measurement and data analysis: After 24 h of inoculation, dead mealybugs were found. The insects' skin surface was treated with a 0.5% sodium hypochlorite solution for 3-5 min, followed by rinsing with sterile water for 3-5 min (repeated twice). The mealybugs were then transferred to a moist chamber and incubated in the dark at a temperature of 28-30°C and 60-80% relative humidity (R.H.). Daily observations were recorded for mortality and fungal infections. To correct for mortality in the control treatment, Abbott's formula (Abbott 1925) was used.

$$\frac{[Test\ mortalty - Control\ mortality]\ x\ 100}{100 - Control\ mortality}$$

Data analysis

Statistical analysis was performed to identify variations and the differences in the mean values of each treatment compared using Tukey's test with the SAS statistical analysis program.

Results

Entomopathogenic fungi (EPF) isolates

The 12 isolates of EPF consisted of *Metarhizium* spp., fungi isolated from soil collected in various districts: Ban Lueam (NMMet_BAL7/1), Non Sung (NMMet_NS4/1), Thepharak (NMMet_TPR10/2 and NMMet_TPR3/3), Chaloem Phra Kiat (NMMet_CLPK4/1), Nong Bun Mak (NMMet_NBM9/1), Khon Buri (NMMet_KBR5/1), Sikhio (NMMet_SK5/1), Kham Thale So (NMMet_KTLS9/3), Pak Chong (NMMet_PC2/1), Sida (NMMet_SD9/1) and Soeng Sang (NMMet_SS2/2) (Table 1).

The colony characteristics of the fungi varied by isolate. The hyphae grew smoothly on the surface of the medium. Young fungi had hyphae that were white to yellow in color. As the hyphae aged, they produced spores at their tips, which were green to dark green or dark brown to black. The conidia were cylindrical with rounded ends, slightly constricted in the middle, and green in color. The conidia were similar in size, ranging from 2.50-2.58 x 5.00-5.17 µm. NMMet_BAL7/1 had the largest colony diameter (71.00 mm), which was not statistically different ($P > 0.05$) from NMMet_NS4/1, NMMet_TPR10/2, and NMMet_SD9/1, with colony diameters of 70.70, 68.00 and 66.00 mm, respectively. In contrast, the NMMet_NBM9/1 isolate had the slowest growth (43.70 mm) (Fig. 1).

Extracellular enzyme activity

NMMet_SS2/2 isolate exhibited the highest proteolytic activity (0.33), but it was not statistically different ($P > 0.05$) from NMMet_BAL7/1(0.26), NMMet_SK5/1 (0.23), and NMMet_KTLS9/3 (0.20). However, it was statistically different ($P < 0.05$) from the other isolates. On the other hand, NMMet_PC2/1 had the lowest proteolytic activity at 0.12. Similarly, NMMet_SS2/2 had the highest lipolytic activity (0.54), which was not statistically different ($P > 0.05$) from the NMMet_TPR3/3 and NMMet_NBM9/1 isolates. NMMet_KBR5/1 had the lowest lipolytic activity (0.16) (Table 2).

The efficacy of EPF in controlling mealybug

The use of the Chlorpyrifos insecticide resulted in the highest mealybug mortality (100%), but it was not statistically different ($P > 0.05$) from NMMet_SS2/2, which had a mortality rate of 96.67%, higher than the other isolates, followed by NMMet_BAL7/1 (68.33%). The NMMet_KBR5/1 isolate had the lowest mortality (21.67%). Regarding infection, NMMet_SS2/2 also had the highest infection rate (96.67%), followed by NMMet_BAL7/1 (68.33%) and NMMet_KTLS9/3 (66.67%), respectively. The NMMet_KBR5/1 isolate had the lowest infection rate (21.67%). Neither the untreated control nor Tween 20 treatments resulted in mealybug mortality or fungal infection.

Table 1: The mycelium growth of entomopathogenic fungi isolate on potato dextrose agar culture media

EPF Isolates	Colony diameter (mm. ± SD[1/])			Conidia
	7 days	14 days	21 days	size (µm)
NMMet_BAL7/1	27.70 ± 0.60 a	51.70 ± 3.10a	71.00 ± 2.60a	2.50x5.00
NMMet_NS4/1	25.00 ± 0.05 b	50.70 ± 0.60a	70.70 ± 1.20ab	2.50x5.00
NMMet_TPR10/2	28.00 ± 0.05 a	50.00 ± 0.04a	68.00 ± 0.07abc	2.50x5.00
NMMet_TPR3/3	23.70 ± 0.60b-d	42.00 ± 0.05c	63.30 ± 0.60cd	2.50x5.00
NMMet_CLPK4/1	23.30 ± 0.60cd	34.00 ± 0.06d	46.00 ± 3.60f	2.50x5.00
NMMet_NBM9/1	20.30 ± 0.60e	32.70 ± 0.60d	43.70 ± 1.20f	2.58x5.17
NMMet_KBR5/1	22.00 ± 0.05de	41.00 ± 0.07c	56.00 ± 2.00e	2.50x5.08
NMMet_SK5/1	25.30 ± 0.60b	48.30 ± 4.60ab	65.00 ± 2.60bc	2.50x5.00
NMMet_KTLS9/3	22.30 ± 2.90c-e	41.00 ± 2.60c	58.70 ± 1.50de	2.50x5.00
NMMet_PC2/1	24.30 ± 0.60bc	43.70 ± 0.23bc	59.00 ± 1.20de	2.50x5.00
NMMet_SD9/1	29.00 ± 2.00a	51.30 ± 0.60a	66.00 ± 0.04a-c	2.50x5.00
NMMet_SS2/2	23.70 ± 0.60b-d	42.00 ± 2.60c	62.70 ± 4.20cd	2.50x5.00
P Value	*< 0.0001*	*< 0.0001*	*< 0.0001*	-

[1/]Means ± SD in the column followed by the same common letter were not significantly different ($P > 0.05$) according to Tukey's test; SD = standard deviation

Table 2: The extracellular enzyme activity of entomopathogenic fungi isolate

Isolates	Enzyme activity[1/] (± SD)	
	Protease	Lipase
NMMet_BAL7/1	0.26 ± 0.04ab	0.41 ±0.05b
NMMet_NS4/1	0.19 ± 0.02bc	0.29 ± 0.03c
NMMet_TPR10/2	0.14 ± 0.03bc	0.38 ± 0.03bc
NMMet_TPR3/3	0.15 ± 0.04bc	0.44 ± 0.02ab
NMMet_CLPK4/1	0.18 ± 0.07bc	0.38 ± 0.02bc
NMMet_NBM9/1	0.15 ± 0.06bc	0.43 ± 0.02ab
NMMet_KBR5/1	0.15 ± 0.04bc	0.16 ± 0.04d
NMMet_SK5/1	0.23 ± 0.05a-c	0.40 ± 0.03bc
NMMet_KTLS9/3	0.20 ± 0.05a-c	0.41 ± 0.05b
NMMet_PC2/1	0.12 ± 0.03c	0.34 ± 0.01bc
NMMet_SD9/1	0.16 ± 0.03bc	0.42 ± 0.05b
NMMet_SS2/2	0.33 ± 0.02a	0.54 ± 0.05a
P Value	0.0003	< 0.0001

[1/]Means ± SD in the column followed by the same common letter were not significantly different ($P > 0.05$) according to Tukey's test; SD = standard deviation

Mealybugs treated with EPF exhibited initial mortality on day 2 post-inoculation, with significant peak mortality (10.78%) occurring on days 4-5 ($P < 0.05$). Mortality rates subsequently decreased after day 6. The progression of fungal infection showed detectable mycosis by day 3 (1.22%), peaking at 10.22% on day 6 ($P < 0.05$), followed by a gradual decline in infection rates after day 7. The use of the Chlorpyrifos insecticide resulted in mealybugs drying out, turning brown-black, with no EPF hyphae observed. In contrast, mealybugs infected by EPF showed fungal hyphae growth, green to dark green spores, resembling mummies (Table 3-4).

Table 3: The mortality percentage of mealybug inoculation by entomopathogenic fungi under laboratory conditions

Treatments	%Mortality after inoculation1/ (Days) (n = 20)										
	1	2	3	4	5	6	7	8	9	10	Accumulative mortality (% ± SD) [1/]
Non-Spraying (control)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 ± 0.00e
Tween 20®; 0.05% (control)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 ± 0.00e
Chlorpyrifos 40 w/v EC	100.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	100.00 ±0.00a
NMMet_BAL7/1	0.00	10.00	16.67	23.33	15.00	3.33	0.00	0.00	0.00	0.00	68.33 ± 14.43b
NMMet_NS4/1	0.00	0.00	3.33	16.67	1.67	1.67	0.00	0.00	0.00	0.00	23.33 ± 7.64d
NMMet_TPR10/2	0.00	0.00	20.00	23.33	16.67	5.00	0.00	0.00	0.00	0.00	65.00 ± 8.66b
NMMet_TPR3/3	0.00	10.00	15.00	15.00	18.33	0.00	0.00	0.00	0.00	0.00	58.33 ± 11.55bc
NMMet_CLPK4/1	0.00	0.00	6.67	11.67	11.67	10.00	0.00	0.00	0.00	0.00	40.00 ± 15.28cd
NMMet_NBM9/1	0.00	0.00	0.00	6.67	11.67	10.00	5.00	0.00	0.00	0.00	33.33 ± 10.41d
NMMet_KBR5/1	0.00	0.00	3.33	6.67	1.67	3.33	5.00	1.67	0.00	0.00	21.67 ± 7.64de
NMMet_SK5/1	0.00	0.00	0.00	5.00	8.33	10.00	6.67	1.67	0.00	0.00	31.67 ± 7.64d
NMMet_KTLS9/3	0.00	0.00	8.33	25.00	26.67	6.67	0.00	0.00	0.00	0.00	66.67 ± 7.64b
NMMet_PC2/1	0.00	0.00	10.00	11.67	15.00	11.67	0.00	5.00	5.00	0.00	58.33 ± 7.64bc
NMMet_SD9/1	0.00	0.00	0.00	0.00	3.33	8.33	10.00	8.33	0.00	0.00	30.00 ± 5.00cd
NMMet_SS2/2	0.00	10.00	35.00	23.33	23.33	5.00	0.00	0.00	0.00	0.00	96.67± 2.89a
Average mortality (%)[2/]	6.67B	1.33C	7.44B	10.78A	10.78A	5.78B	2.00C	1.11C	0.33C	0.00C	*< 0.0001*
P value											*< 0.0001*

[1/]Means ± SD in the column followed by the same common letter were not significantly different (*P > 0.05*) according to Tukey's test; [2/]Means ± SD in the low followed by the same common letter were not significantly different (*P > 0.05*) according to Tukey's test; SD = standard deviation

Table 4: The infection percentage of mealybug inoculation by entomopathogenic fungi under laboratory conditions

Treatments	%Infection after inoculation1/ (Days) (n = 20)										
	1	2	3	4	5	6	7	8	9	10	Accumulative Infection (% ± SD)[1/]
Non-Spraying (control)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 ± 0.00e
Tween 20®; 0.05% (control)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 ± 0.00e
Chlorpyrifos 40 w/v EC	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 ± 0.00e
NMMet_BAL7/1	0.00	0.00	6.67	21.67	23.33	16.67	0.00	0.00	0.00	0.00	68.33 ± 14.43b
NMMet_NS4/1	0.00	0.00	0.00	3.33	6.67	8.33	3.33	1.67	0.00	0.00	23.33 ± 7.64de
NMMet_TPR10/2	0.00	0.00	0.00	10.00	23.33	13.33	8.33	8.33	0.00	0.00	63.33 ± 10.41b
NMMet_TPR3/3	0.00	0.00	11.67	11.67	13.33	13.33	5.00	1.67	0.00	0.00	56.67 ± 10.41bc
NMMet_CLPK4/1	0.00	0.00	0.00	5.00	8.33	11.67	11.67	3.33	0.00	0.00	40.00 ± 15.28cd
NMMet_NBM9/1	0.00	0.00	0.00	0.00	3.33	11.67	11.67	5.00	1.67	0.00	33.33 ± 10.41d
NMMet_KBR5/1	0.00	0.00	0.00	1.67	5.00	3.33	3.33	3.33	3.33	0.00	20.00 ± 5.00de
NMMet_SK5/1	0.00	0.00	0.00	0.00	3.33	8.33	10.00	6.67	3.33	0.00	31.67 ± 7.64d
NMMet_KTLS9/3	0.00	0.00	0.00	0.00	21.67	18.33	15.00	8.33	3.33	0.00	66.67 ± 7.64b
NMMet_PC2/1	0.00	0.00	0.00	8.33	8.33	16.67	13.33	5.00	3.33	1.67	56.67 ± 10.41bc
NMMet_SD9/1	0.00	0.00	0.00	0.00	0.00	1.67	6.67	8.33	8.33	3.33	28.33 ± 2.89d
NMMet_SS2/2	0.00	0.00	6.67	10.00	30.00	23.33	18.33	6.67	1.67	0.00	96.67 ± 2.89a
Average mortality (%)[2/]	0.00C	0.00C	1.22BC	3.78B	9.67A	10.22A	8.22A	3.89B	1.67C	0.33C	*< 0.0001*
P value											*< 0.0001*

[1/]Means ± SD in the column followed by the same common letter were not significantly different (*P > 0.05*) according to Tukey's test; [2/]Means ± SD in the low followed by the same common letter were not significantly different (*P > 0.05*) according to Tukey's test; SD = standard deviation

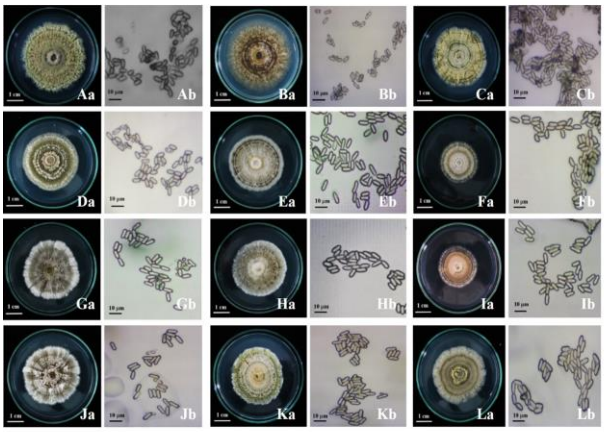

Fig 1: Colony (a) and conidia (b) characteristics of entomopathogenic fungi isolates on PDA culture media, (A) NMMet_BAL7/1, (B) NMMet_NS4/1, (C) NMMet_TPR10/2, (D) NMMet_TPR3/3, (E) NMMet_CLPK4/1, (F) NMMet_NBM9/1, (G) NMMet_KBR5/1, (H) NMMet_SK5/1, (I) NMMet_KTLS9/3, (J) NMMet_PC2/1, (K) NMMet_SD9/1 and (L) NMMet_SS2/2

Discussion

Twelve EPF fungi isolated from soil samples were collected in Nakhon Ratchasima Province, each exhibiting distinct colony characteristics and growth patterns. The conidia were densely clustered, cylindrical, blunt-ended, and slightly constricted in the middle, which are typical characteristics of *Metarhizium* spp. (Bidochka *et al.* 1998; Sun and Lui 2008; Sánchez-Peña *et al.* 2011). This finding is consistent with Thaochan and Sausa-Ard (2017) who reported that *M. anisopliae* fungi isolated from soil in the southern region showed varying colony characteristics and growth patterns when cultured on SDAY (Sabouraud Dextrose Agar + Yeast Extract).

The EPF isolates exhibited varying enzyme activities with NMMet_SS2/2 showed the highest proteolytic and lipolytic activities. This is consistent with Cortez-Madrigal *et al*. (2014), who reported that EPF isolates differed in their chitinase, lipase, and protease enzyme activities due to both intraspecific and interspecific differences. Furthermore, Gandarilla-Pacheco *et al.* (2015) found that enzyme activity varied among 30 EPF isolated from soils in different citrus-growing regions of Mexico. Of the total EPF, 51% exhibited all three activities (protease, lipase, and chitinase), 46% exhibited two activities, and 3% exhibited only one activity (chitinase). However, enzyme production is also influenced by other factors. Dhar and Kaur (2008) found that chitinase activity varied among isolates depending on the media used. The highest enzymatic activity was observed in the media containing an additional nitrogen source (yeast extract), followed by the media with colloidal chitin. Similarly, lipase production can be affected by the type and concentration of carbon and nitrogen sources (Mondal *et al.* 2016). The mechanisms regulating enzyme production and biosynthesis differ significantly among various microorganisms (Sharma *et al*. 2001).

The efficiency of EPF in destroying mealybugs is correlated with its enzyme activity. For instance, NMMet_SS2/2 exhibited higher levels of proteolytic and lipolytic activities compared to other isolates, enabling it to effectively infect mealybugs. This is consistent with Gebremariam *et al.* (2022), who reported that *B. bassiana* AAUMB-29, AAUMFB-77, and AAUEB-59 displayed the highest chitinase, lipase, and protease activities, respectively. They were most virulent against nymphs of *Bemisia tabaci* and *Trialeurodes vaporariorum*, respectively. Similarly, Elhakim *et al*. (2020) found a correlation between the virulence and proteolytic activity of certain entomopathogenic fungi isolates, such as *M. anisopliae, B. bassiana*, *V. lecanii*, and *Trichoderma harzianum.* The isolates with the highest protease activity exhibited the highest virulence against the two-spotted spider mite (*Tetranychus urticae*). Additionally, Islam *et al*. (2022) reported that each isolate of the *P. lilacinum* exhibited different levels of proteolytic activity. Isolates with high proteolytic activity were also effective in controlling *Ceratovacuna lanigera.* Similarly, *T. longibrachiatum* strain 18ASMA026, which had higher levels of protease and chitinase enzymes compared to other EPF strains, also exhibited the highest capability to destroy *Myzus Persicae* (Anwar *et al.* 2023). Meanwhile, Pelizza *et al.* (2017) reported that the fungus *B. bassiana* could produce different enzymes. Some isolates produced only lipase, while several isolates produced protease, chitinase, and lipase simultaneously. Isolates that produced multiple enzymes were more effective in controlling *Schistocerca cancellate*.

Extracellular enzymes play a dual role in degrading the insect cuticle, both thinning or weakening its structure, and, upon reaching the larval intestine, causing significant damage to the peritrophic membrane. This would result in the larvae being unable to feed themselves, ultimately leading to their demise (Binod *et al.* 2007).

Enzyme activities are vital for insect destruction, but fungal efficacy depends on factors such as fungal type, aggressiveness, density, target insect species, contact duration, and environmental conditions. Among these factors, humidity is the most important in promoting fungal growth inside insects and the emergence of fungal hyphae outside the insect body (Watson *et al.* 1996; Bugti *et al.* 2020). This study was conducted under controlled laboratory conditions regarding temperature and humidity. Results showed that the mortality of mealybugs two days after the application of fungal spore suspension, with spores covering the bodies of mealybugs on the third day. Notably, NMMet_BAL7/1, NMMet_TPR3/3, and NMMet_SS2/2 had hyphae covering the mealybug bodies faster than other isolates. This is consistent with Amutha and Banu (2017), who reported that the time required for *M. anisopliae* to destroy mealybugs (*Paracoccus marginatus*) and subsequently penetrate their bodies to form hyphae and produce spores takes approximately 96-120 h. The rate of insect destruction depends on the fungal aggressiveness in penetrating hyphae into the insect body (Leger *et al.* 1991, 1996). Moreover, EPF strains exhibit specificity towards different insect species. Some fungi produce toxins that quickly kill insects, while others simply invade to compete for essential nutrients to survive inside the insect (Sirimungkararat 2014).

This study concludes that the 12 isolates of entomopathogenic fungi from Nakhon Ratchasima soil produced protease and lipase enzymes, that effectively controlled mealybugs. Among the 12 EPF isolates tested, NMMet_SS2/2 exhibited the highest enzyme activity, leading to increased mealybug mortality. These findings suggest its potential as a biocontrol agent for field applications. Further field studies are needed to assess environmental factors influencing mealybug populations and their developmental stages.

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Author Contributions

DW planned the experiments, carried out the isolation and pathogenicity tests of entomopathogenic fungi, prepared the manuscript and approved the final version. PB carried out the cultivation of EPF and analyzed their enzyme activity. OUK handled the rearing of mealybugs for the pathogenicity test. BS conducted data analysis and interpretation, formatted the manuscript and approved the final version.

Conflict of Interest

All authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable to this paper.

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