Effect of Media on Virulence and Enzyme Activity of *Pandora neoaphidis* Isolate Pd105 from Thailand to *Lipaphis erysimi* and *Myzus persicae*

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***Abstract*—The effect of artificial and mass production media on the virulence and enzyme activity of the entomopathogenic fungus, *Pandora neoaphidis* Remaudière and Hennebert (Entomophthorales: Entomophthoraceae) isolate Pd105, was investigated. Mortality of adult apterous cabbage (*Lipaphis erysimi*) and tobacco aphids (*Myzus persicae*) (Hemiptera: Aphididae) applied with 1×10^{10} spore/ml of *P. neoaphidis* cultured on various artificial media was determined and results differed significantly ($P<0.01$). Among the artificial media, the target insects infected with spore suspension cultured in SDAY showed the highest mortality in *L. eysimi* and *M. persicae* at 97.00 and 95.00%, respectively. Spore suspension obtained from mass production media including, cooked rice, rice grain and sorghum showed significant differences ($p=0.01$) of pathogenicity to target insects. Sorghum supported the growth of the fungus with the highest mycelial growth and sporulation. Fungal spores obtained from sorghum showed highest mortality to the target insects at 96.60% for *L. eysimi* and 94.80% *M. persicae*. The enzymatic activity (chitinase, lipase, galactosidase, esterase, and leucine arylamidase) of the fungus grown in different artificial and mass production media differed.**

***Index Terms*—mass production media, sustainable insect control, biological control, entomopathogenic fungus**

I. Introduction

Insecticides are commonly used to control aphid infestations in Thailand and abroad. Pest control using chemical insecticides often results in increasing pesticide resistance of many insect pest species including aphids [1]-[4]. The use of local biological control agents by small farmers in Thailand to control pests is a good aspect to reduce production costs as well as the pollution risks of using chemical insecticides. *Pandora neoaphidis* Remaudière and Hennebert (Entomophthorales: Entomophthoraceae) is an entomopathogenic fungus, originally called *Erynia neoaphidis* Remaudière & Hennebert. This fungus is naturally occurring in the agroecosystem and has high efficiency in controlling populations of aphid pest species, this fungus can be used to control and destroy global population outbreaks of aphids [5]-[9]. In Thailand, this fungus has been reported to control aphids but it has not been studied comprehensively or used in commercial scale [10]. The fungus was highly effective in controlling cotton aphids and could significantly reduce large fluctuations in aphid populations [11]-[14]. In addition, the application of this fungus to control aphids reduces the use of chemical pesticides [15]-[19].

Crops production system can be expanded without the need to increase importation of chemical insecticides for pest control. Accordingly, selection of potential artificial media for culture and mass propagation of the fungus, encouraging quality as well as quantity of the fungal isolate could provide basic information necessary for further mass propagation scale and application. By the other hand, more or less success, the propagation and application of *P. neoaphidis* have been reported. The entomopathogen was produced in vitro based on conidia or mycelium to control cereal aphids [20]. The study that evaluated production factors involved in the formulation of *E. neoaphidis* as alginate granules has been conducted and indicated the possibility for the field application [21]-[24]. The quality study, [25] cited that the efficacy of insect control through an entomopathogenic fungus is associated with the production of the enzyme.

The first investigation involved natural occurring, field collection and isolation technique for *P. neoaphidis* was reported in 2013-2014 by the reference [26]. Result from the study indicated highly potential of *P. neoaphidis* isolate Pd105 which caused disease to aphids, including *L. eysimi* and *M. persicae* as a promised biological control agent against aphids. Therefore, further investigation

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should evaluate the factors effect growth and efficacy of the biological control agent to contribute to its application as microbial insecticide. The use of biological control agent is therefore encouraging the capacities of Thai farmers to meet further safety and sustainable agricultural practices. Thus the objective of this study was to investigate the effect of artificial media to virulence and enzyme activity involved pathogenicity of the fungi, indicating potential artificial media for further mass production and application. The findings from this investigation could provide basic information necessary for further investigation in the use of local entomopathogenic fungi as potential microbial insecticides, substitute of expensive chemical insecticides for aphid pests of economic importance control in Thailand.

II. Materials and Methods

A. Mass Rearing of Target Insect Pests

The tobacco (*Myzus persicae*) and cabbage (*Lipaphis erysimi*) aphids were cultured separately following the method [27]-[30]. Aphids at different ages were collected from filed. Twenty Apterous and alate at 1:1 ratio were transferred to 60 days after transplanting chili of kale plants located in ventilated breeding cages in a small greenhouse with supplementary lighting. The aphids were allowed to complete its life cycle for 1-2 generations (10-15 days per generation) in conditions at 25-32°C and 40-60% RH (Fig. 1).

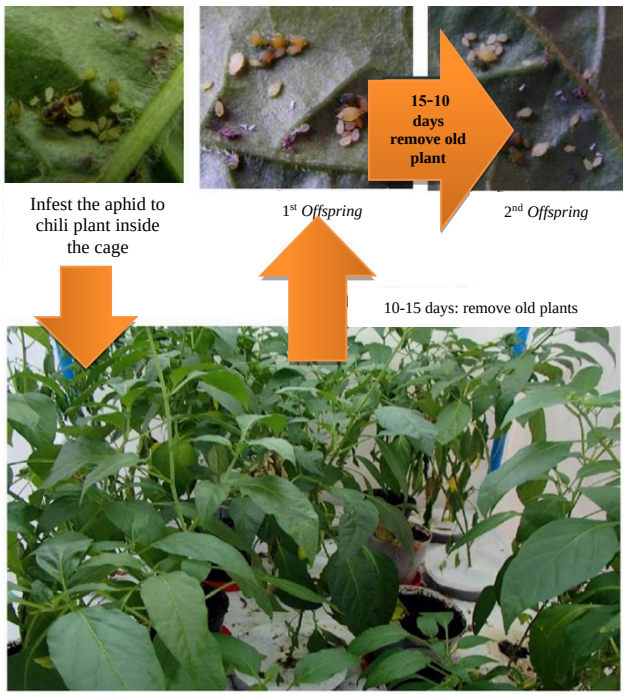

Figure 1. Mass rearing of *Myzus persicae* subjected to pathogenicity.

B. Preparation of Artificial Media and Pathogenicity to Aphid Species

Hyphae of 10 days old *P. neoaphidis* isolate Pd105 (derived from single aphid cadavers infested cabbage that the species cannot be identified, collected in 2016 in Chiang Mai, Thailand) grew on Sabouraud Dextrose Agar (SDA) was subculture to different artificial media on different media in Petri dishes separately, including Potato Dextrose Agar (PDA), SDA, SDA Supplemented with 1.5 percent Yeast Extract (SDAY), Malt Extract Agar (MEA), and Nutrient Agar (NA) and incubated under laboratory conditions at 20-22°C and 30-40% RH at dark for 20 days following [31], [15].

Spore suspension of the fungus was prepared from each 20 days old cultured by scraping the fungal materials (spore and hypha) from the surface of the agar and suspended in 10 ml sterile distilled water containing nonionic surfactant, 0.02% Tween 80. Spores were counted using hemocytometer and concentration of the spores was adjusted to 10^{10} spores/ml according to [32]-[34] using sterile distilled water containing nonionic surfactant, 0.1% solvent Tween 80 concentration [35]-[36].

The spore suspension from each media was spray to 7 days old of 30 apterous aphid infested chilly or kale leaf plated on moistening Whatman filter paper no 1, in sterilized petri dishes before incubated at the laboratory conditions, temperature 20±2°C and 80% RH at dark. Dead and alive aphids were recorded every 24 hours up to 120 hours to calculate the mortality. Data were adjusted for mortality accuracy (corrected control mortality) by a using Abbott's Correction Formula [37]-[40] as follows:

$$\text{corrected \%} = \left(\frac{\text{1-}n \text{ in } T \text{ after treatment}}{n \text{ in } Co \text{ after treatment}}\right)\times 100$$

where: n = insect population, T = treated, Co = control

C. Enzyme Activity

The enzyme activity of the fungus grew on different artificial media was determined. The sterilized media, including Potato Dextrose Broth (PDB), Sabouraud Dextrose Broth (SDB), SDB Supplemented with 1.5 percent Yeast Extract (SDBY), Malt Extract Broth (MEB), and Nutrient Broth (NB) at 50 ml in 250 ml Erlenmeyer flask were prepared by the reference [14], [41]-[43]. The medium was inoculated with one ml of 10^6 spores/ml of *P. neoaphidis* isolate Pd105 growth on SDAY (20 days of age) and the flasks then were incubated for 96 h in an orbital shaker operating at 150 rpm and 20°C. The enzyme activity, protease was analyzed as describes in reference [13] incubating at 35°C in a shaker at 150rpm for 3 days. The liquid was then filtered to obtain the culture and centrifuged at 10,000 rpm for 15 min. Enzymes were evaluated based on molecular weight acrylamide gel electrophoresis method using 12.5% SDS-PAGE. Molecular weight of the protein was measured by standard marker. Initial result showed that the enzyme extracted by such method was ineffective in extracting the fungal enzyme protease from *P. neoaphidis*. Therefore, we modified the method by measuring enzyme activity using API ZYM®-Semiquantitation. Color was measured based on the test kit system.

D. Statistical Analysis

Standard univariate ANOVA was performed on data to evaluate the pathogenicity growth and enzymatic activity according to respective PCM, growth rate and protease production, Completely randomized design (CRD) with 4 replications and Duncan's New Multiple Range Test (DMRT) were applied.

III. Results

The dead aphids were called cadavers that characterized by changing their body's color to be yellow then turned to oranges when filled with conidia. The mortality of aphid species due to exposure to 10^{10} spores/ml of *P. neoaphidis* cultured in different artificial media were significantly different (Table I).

TABLE I. Percent Corrected Mortality (PCM) of Cabbage Aphids (*Lipaphis erysimi*) and Tobacco Aphids *Myzus persicae*) after 5 Days Exposure to *Pandora neoaphidis* Isolate Pd105 Cultured on Different Artificial Media

Artificial medium	Percent Corrected Mortality (PCM) (%)	
	Cabbage aphids	Tobacco aphids
Potato Dextrose Agar	67.00 ± 9.21a	69.60 ± 3.28a
Sabouraud Dextrose Agar	76.00 ± 3.39a	76.60 ± 2.07b
Sabouraud Dextrose Agar with Yeast Extract	97.00 ± 4.53b	95.00 ± 5.52c
Malt Extract Agar	44.80 ± 6.05c	43.40 ± 3.50d
Nutrient Agar	54.20 ± 8.58c	54.40 ± 3.97e

Means and Standard deviations of PCM compared, means with the same letter are not significantly different from each other (P>0.05 ANOVA followed by Duncan's multiple range test)

The mortality for *L. erysimi* was between 37 and 100 with overall average of 59.60 to 75.19% (not shown in table). Among the artificial media, SDAY gave the highest mortality value of 97.00±4.53%, while MEA had the lowest mortality (44.80±6.05%). The pathogenicity was separated into three groups as i) low, including *P. neoaphidis* cultured on MEA and NA, ii) medium, fungus cultured on SDA and PDA, and iii) high levels, fungus cultured on SDAY. Infection in tobacco aphids showed 39 to 100% in laboratory conditions with overall average at 60.06 to 75.13% (not shown in table). Fungus cultured on SDAY caused highest disease for tobacco aphids at 95.00±5.52% while *P. neoaphidis* cultured on MEA gave the lowest pathogenesis with 43.40±3.50%.

TABLE II. Development of *Pandora neoaphidis* Isolate Pd105 according to Mycelial Growth Rate and Sporulation on Different Propagation Materials

Mass propagation materials	Development	
	Mycelial growth rate (mm/day)	Spore concentration (spore/ml)
Cooked rice	0.40 ± 0.03a	14.04 ± 1.67a
Rice grain	0.38 ± 0.07a	12.20 ± 5.11a
Sorghum	0.65 ± 0.08b	16.20 ± 5.34b

Means and Standard deviations of growth rate of mycelium and spore concentration compared, means with the same letter are not significantly different from each other (P>0.05 ANOVA followed by Duncan's multiple range test)

Development of the fungus *P. neoaphidis* indicated as mycelial growth and sporulation growth were equally well on both cooked rice and rice grain. Growth rate of mycelia were 0.40±0.03 and 0.38±0.07 mm/day, respectively (Table II).

However, the fungus grew best in sorghum grain at a rate of 0.65±0.08 mm/day. Sporulation quantity showed significant differences that the fungus growth on sorghum grain produced highest spore number averaging 16.20 spores/ml. Infected with *P. neoaphidis* culture in different growth material, the mortality of cabbage aphids was divided into two groups: fungus cultured on cooked rice and rice grain (68.20±5.35 and 56.60±10.73%), and fungus cultivated on sorghum grain which caused disease on cabbage aphids at the highest PCM of 96.60% (Table III). *P. neoaphidis* cultured on diverse media caused significantly different disease severity on tobacco aphids (p=0.01). Pathogenicity trends to tobacco aphids were similar to cabbage aphids, the result showed that the PCM were split into two groups: cooked rice and rice grain and a second sorghum group. PCM values of the first group were 76.80±7.39 and 62.40±11.71%, respectively, while *P. neoaphidis* cultured on sorghum grain gave maximum PCM at 94.80 ± 6.90%.

TABLE III. Percent Corrected Mortality (PCM) of Cabbage Aphids (*Lipaphis erysimi*) and Tobacco Aphids (*Myzus persicae*) after 5 Days Exposure to *Pandora neoaphidis* Isolate Pd105 Grown in Various Mass Propagation Material

Mass production media	Percent Corrected Mortality (PCM) (%)	
	Cabbage aphids	Tobacco aphids
Cooked rice	68.20 ± 5.35a	76.80 ± 7.39a
Rice grain	56.60 ± 10.73b	62.40 ± 11.71b
Sorghum	96.60 ± 4.97c	94.80 ± 6.90c

Means and Standard deviations of PCM compared, means with the same letter are not significantly different from each other (P>0.05 ANOVA followed by Duncan's multiple range test)

A. Enzyme Activity

The detection of protease enzyme activity following the method adapted from the reference [8] was ineffective in enzyme extraction. Thus, we modified and improved this step to determine enzyme activity using the test kit API ZYM®-Semiquantitation of enzymatic activities. Results showed that the fungus cultured on different artificial media types produced many enzymes including chitinase, lipase, galactosidase, esterase, and leucine arylamidase at diverse activity levels (Fig. 2). Maximum activity of the enzymes chitinase and lipase was shown on MEB. On SDBY, this fungus produced esterase and leucine arylamidase, with similar culture results to SDB. When cultured on PDB, the fungus did not produce any of the enzymes mentioned above but instead produced galactosidase. When cultured on NB, the fungus produced only two types of enzyme (esterase and leucine arylamidase) at low activity levels.

Enzyme activity of the fungus grown in mass production media was also determined (Fig. 3). The enzyme activity of chitinase, lipase, and galactosidase for this fungus was found to be highest when cultured on sorghum, and highest esterase and leucine arylamidase were active on cooked rice and rice grain, respectively.

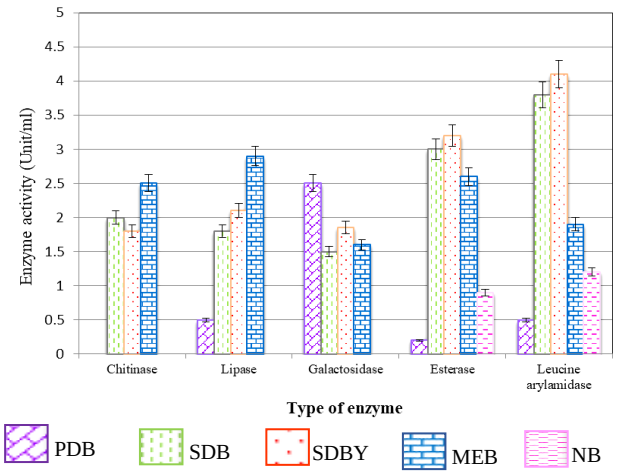

Figure 2. Enzyme activity of *Pandora neoaphidis* isolate Pd105 cultured on various artificial media.

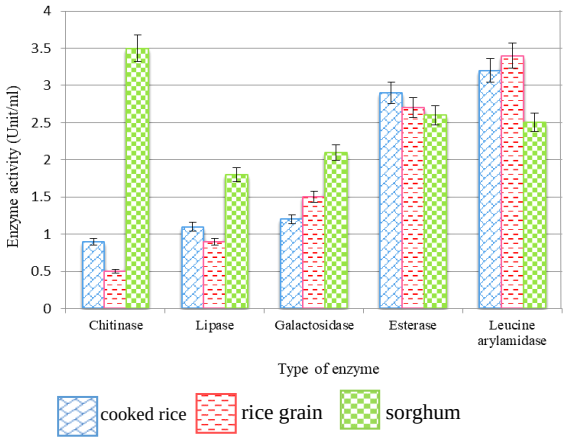

Figure 3. Enzyme activity of *Pandora neoaphidis* isolate Pd105 cultured on mass production media.

IV. Discussion

Results showed that the fungus *P neoaphidis* isolate Pd105 cultured on diverse artificial media caused significantly different pathogenicity levels in both cabbage and tobacco aphid. In the same way, this fungus grew on different mass production media also generated diverse pathogenicity levels to target aphids. Studies concerning the diverse effects of artificial media to control fungal pathogenicity levels represent a new and exciting cutting-edge research area which should be expanded to include other compounds in culture material to stimulate pathogenicity. Our results supported previous findings that different culture materials can be used to increase fungal quantities or qualities which may eventually lead to large-scale production of *P. neoaphidis*. Findings indicated that sorghum is a material suitable for cultivation and increasing the amount of fungus, consistent with the reference [18] who claimed that animal sorghum which is sold in bird food (broomcorn millet, *Panicum miliaceaum*) is a cereal grain that could be used to culture the fungus *Pandora* spp. In Thailand, detailed research should be undertaken to study these aspects more thoroughly. A simple method for increasing the amounts of fungus produced by local isolates of *P. neoaphidis* could be adapted for use by small-scale farmers in the future.

Moreover, the ability to adhere to the host, resistance to environmental conditions such as light, temperature and humidity, and the condition of the food or material culture are all important factors affecting the pathogenicity of entomopathogenic fungi [44]. The ability to produce enzymes which include protease, chitinase and lipase is also another important factor affecting the level of pathogenicity of entomopathogenic fungi which can digest cell walls of the host insect to promote the germination of fungal mycelia which then penetrate and kill the insects

In this study, the effects of artificial and natural material media on enzyme activity of the fungus *P. neoaphidis* were investigated. Results of the first part of the study on pathogenicity to target aphids determined different activity levels. The method used in this study proved to be ineffective in extracting the enzyme protease, and relationships between quantity and this enzyme activity could not be analyzed. However, by modifying the enzyme activity using the test kit API ZYM®-Semiquantitation of enzymatic activities, the fungus *P. neoaphidis* was shown to have different activities for enzyme production of chitinase, lipase, galactosidase, esterase and leucine arylamidase using diverse artificial and mass production media. These results were consistent with the reference [45] who determined a relationship between pathogenic level and enzyme activity including chitinase, protease and lipase. [45] also confirmed the influence of enzymes on pathogenesis.

Provide discussion why culturing the fungus in SDAY and sorghum contribute to the virulence and enzymatic activity of the fungus.

V. Conclusion

Several artificial media including Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SDA), Sabouraud Dextrose Agar Supplemented with Yeast Extract (SDAY), Malt Extract Agar (MEA), Nutrient Agar (NA) and mass production media of various kinds of rice and sorghum were used to grown the pathogenic fungus, Pd105. Pathogenicity was tested in vegetables aphids (*Lipaphis erysimi*) and tobacco aphids (*Mizus persicae*) (Hemiptera: Aphididae) at 20±2°C and 80% relative humidity under dark conditions. The Percent Cumulative Mortality (PCM) was determined. It was found that the PCM of each aphid species infected with Pd105 spore suspensions cultured on SDAY and on various other artificial media had significantly different ($p=0.01$) PCM values. The highest PCM values for vegetable and tobacco aphids were 97.00±4.53 and 95.00±5.52%, respectively. It was also determined that the culture media significantly affected mycelial growth and sporulation ($p=0.01$) of this fungus. Pd105 could grow on sorghum at maximal rate of 0.65±0.08 mm/day and showed average sporulation of 16.20±5.34 spores per ml. The PCM of vegetable and tobacco aphids that had been treated by suspensions of fungus spores varied with the culture media. Various strains have different

significance (p=0.01) when treated using the 18 fungal spores/ml concentration. The fungus grown on sorghum was pathogenic to vegetable and tobacco aphids at maximum PCM values of 96.60±4.97 and 94.80±6.90%, respectively. Protease production on artificial and mass production media by the Pd105 suggested that it was not an enzyme producer. In a further test of enzyme activity using a test kit (API ZYM®-Semiquantitation), it was found that the Pd105 cultured on various artificial and mass production media had measurable and different levels of chitinase, lipase, galactosidase, esterase and leucine arylamidase activities.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Rungkiat Kawpet contributed to conceptualization, methodology, data analysis, graphical analysis and major manuscript preparation. Samaporn Saengyot (corresponding author) contributed to data collection, initial manuscript writing and revision of the final manuscript.

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