



**EFFECTIVENESS OF *Bacillus thuringiensis* MPOB Bt1-FLUBENDIAMIDE
ON *Metisa plana* WALKER (LEPIDOPTERA: PSYCHIDAE) USING GENE-
AND GENOME-CENTRIC APPROACH**

By

MOHD SHAWAL THAKIB BIN MAIDIN

**Thesis Submitted to the School of Graduate Studies,
Universiti Putra Malaysia, in Fulfilment of the Requirements for the
Degree of Doctor of Philosophy**

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December 2023

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Faculty : Biotechnology and Biomolecular Sciences

Malaysia is the second largest producer of palm oil in the world. However, palm oil production is often affected by bagworm infestation. The overuse of chemical insecticides to control the oil palm pest can lead to more persistent problems such as pest resistance to treatment and an abundance of harmful chemical residues in the environment. Therefore, this study aims to analyse the genome of *Bacillus thuringiensis* MPOB Bt1 (MPOB Bt1) and the synergistic effect of the combination treatment of MPOB Bt1 and flubendiamide (Fbd), the microbial community shift and the functional responses of metagenome-assembled genomes (MAGs) to pesticide exposure for improved control of *Metisa plana* in oil palm plantations. Whole genome sequencing of MPOB Bt1 was performed using Illumina HiSeq and PacBio platforms. Comparative genomics and analysis of insecticidal genes with other closely related genomes of *B. thuringiensis* used in commercial biopesticide products were also performed. The laboratory test and synergistic response of MPOB Bt1 and Fbd against *M. plana* larvae were then optimised using different combination ratios. The details of the changes in the microbial community of *M. plana* towards the pesticide treatments at day zero (DAT0), day one post- treatment (DAT1) and day three post- treatment (DAT3) were analysed using a gene-centric metagenomic approach. The genome-centric approach was performed for an advanced high-resolution analysis of the functional responses of the *M. plana* microbiome to the pesticide treatments. The MPOB Bt1 genome size is approximately 6.9 Mbp with the highest average nucleotide value (ANI) of 0.9765 in *B. thuringiensis* subsp. aizawai strain ABTS-1857. The bioassay results showed a synergistic ratio of more than 1 for 48 and 72 hours after the treatments, confirming the presence of synergistic interactions between MPOB Bt1 and Fbd. Community succession analysis of larval microbiome showed an increase in richness (p-value: 0.012537) and rapid growth in the combined MPOB Bt1-Fbd treatment compared to the individual Fbd treatment. Pearson correlation coefficient

analysis showed that the combined MPOB Bt1-Fbd treatment affected the growth of the microbiome that can coexist with *Bacillus*, based on the positive correlation between *Bacillus* and the genera *Enterobacter* and *Pseudomonas* in the combined MPOB Bt1-Fbd treatment. A total of 11 MAGs were successfully binned from the co-assembled shotgun metagenomics data, with genome completeness ranging from 70.42% to 98.59%, and the major genera were *Pantoea*, *Bacillus*, *Acinetobacter*, *Pseudomonas*, *Stenotrophomonas*, and *Enterococcus*. Time interval studies of MAGs and functional Kyoto Encyclopedia of Genes and Genomes (KEGG) modules in response to MPOB Bt1-Fbd treatment showed that the community and function of MAGs that initiate the degradation of xenobiotics shifts to a more resilient MAGs community that can produce the nutrient itself by synthesising terpenoids and polyketides in a state of dysbiosis. The results demonstrate for the first time how the combined MPOB Bt1-Fbd synergistically kills *M. plana* larvae and is linked to microbiome responses. This suggests that the resilience and interactions of taxonomic groups and functional shifts during dysbiosis may be responsible for community selection and the rapid rise of new taxonomic groups to survive. The study highlights the potential of the MPOB Bt1-Fbd combination for the development of effective *M. plana* control and reduces dependence on harmful chemical pesticides.

Keywords: *Bacillus thuringiensis* MPOB Bt1, Bioassay, Flubendiamide, *Metisa plana*, Microbiome Response, Metagenomics

SDG: GOAL 12: Responsible Consumption and Production, GOAL 15: Life on Land

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra
Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**MENYINGKAP KEBERKESANAN *Bacillus thuringiensis* MPOB Bt1 -
FLUBENDIAMIDE KE ATAS *Metisa plana* WALKER (LEPIDOPTERA:
PSYCHIDAE) MELALUI KAEDAH MEMUSAT GEN DAN GENOM**

Oleh

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Malaysia adalah pengeluar kedua terbesar minyak kelapa sawit di dunia. Walau bagaimanapun, pengeluaran minyak kelapa sawit sering terjejas oleh serangan ulat bungkus. Penggunaan berlebihan racun serangga kimia untuk mengawal perosak kelapa sawit boleh menyebabkan masalah yang berterusan, seperti kerintangan perosak terhadap pestisid dan kelimpahan residu kimia yang berbahaya kepada alam sekitar. Oleh itu, kajian ini bertujuan untuk menganalisis genom *Bacillus thuringiensis* MPOB Bt1 (MPOB Bt1) dan kesan sinergi gabungan MPOB Bt1 dan flubendiamida (Fbd), perubahan komuniti mikrob, dan tindak balas genom-terhimpun-metagenom (MAGs) terhadap pendedahan kepada pestisida untuk kawalan yang lebih baik terhadap *Metisa plana* di ladang kelapa sawit. Penjujukan genom penuh MPOB Bt1 dijalankan menggunakan pelantar Illumina HiSeq dan PacBio. Analisis perbandingan genom dan gen insektisid antara MPOB Bt1 dan *B. thuringiensis* berkait rapat yang digunakan di dalam produk biopestisid komersil juga telah dijalankan. Ujian makmal dan tindak balas sinergi MPOB Bt1 dan Fbd terhadap larva *M. plana* juga telah dijalankan dengan nisbah kombinasi yang dioptimumkan. Perubahan komuniti mikrob *M. plana* terhadap kawalan pestisida selepas dedahkan terus (DAT0), hari pertama selepas kawalan (DAT1), dan hari ketiga selepas kawalan (DAT3) dianalisis menggunakan kaedah metagenom berpusat gen, sementara kaedah berpusat genom dijalankan untuk analisis resolusi tinggi terhadap tindak balas fungsi mikrobiom *M. plana* terhadap kawalan pestisida. Saiz genom MPOB Bt1 adalah kira-kira 6.9 Mbp dengan nilai identiti nukleotida purata tertinggi (ANI) sebanyak 0.9765 dalam *B. thuringiensis* subsp. *aizawai* ABTS-1857. Hasil bioasai menunjukkan nisbah sinergi lebih dari 1 pada 48 dan 72 jam selepas kawalan, mengesahkan interaksi sinergi antara MPOB Bt1 dan Fbd. Analisis kejayaan komuniti bagi mikrobiom larva menunjukkan peningkatan kekayaan (nilai p : 0.012537) dan pertumbuhan cepat dalam rawatan gabungan MPOB-Bt1-Fbd berbanding dengan hanya Fbd. Analisis koefisien korelasi

Pearson menunjukkan bahawa kawalan gabungan MPOB-Bt1-Fbd mempengaruhi pertumbuhan mikrobiom yang boleh hidup bersama *Bacillus*, berdasarkan korelasi positif antara *Bacillus* dan genera *Enterobacter* dan *Pseudomonas*. Sejumlah 11 MAG berjaya digabungkan daripada data terhimpun metagenom tembak, dengan kelengkapan genomnya berkisar antara 70.42% hingga 98.59%, dan genera utama adalah *Pantoea*, *Bacillus*, *Acinetobacter*, *Pseudomonas*, *Stenotrophomonas*, dan *Enterococcus*. Kajian selang waktu MAG dan modul fungsi Kyoto Encyclopedia of Genes and Genomes (KEGG) dalam tindak balas terhadap rawatan MPOB-Bt1-Fbd menunjukkan bahawa komuniti dan fungsi MAG yang memulakan degradasi xenobiotik berubah kepada komuniti MAG yang lebih tahan terhadap ketidakseimbangan. Hasil kajian ini menunjukkan untuk kali pertama bagaimana gabungan MPOB-Bt1-Fbd secara sinergi membunuh larva *M. plana* dan berkaitan dengan tindak balas mikrobiom. Kajian ini menyoroti potensi gabungan MPOB-Bt1-Fbd untuk pembangunan kawalan yang berkesan terhadap *M. plana* dan berpotensi mengurangkan kebergantungan kepada racun serangga kimia yang merbahaya.

Kata Kunci: *Bacillus thuringiensis* MPOB Bt1, Biocerakanin, Flubendiamide, *Metisa plana*, Respons Mikrobiom, Metagenomik

SDG: MATLAMAT 12: Penggunaan dan Pengeluaran Bertanggungjawab, MATLAMAT 15: Kehidupan di Darat

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LIST OF ABBREVIATIONS

ANI	average nucleotide identity
Bt	<i>Bacillus thuringiensis</i> MPOB Bt1
Bt-F	<i>Bacillus thuringiensis</i> MPOB Bt1 – flubendiamide combination
c	Ambient laboratory conditions
DNA	deoxyribonucleic acid
F	flubendiamide
lab	Controlled environment room
°C	Degree celcius
EC	Emulsifiable Concentrate
h	hour/s
L	litre
LC	Lethal Concentration
LD	Lethal Dosage
MAG	Metagenome-Assembled Genome
NGS	Next Generation Sequencing
Mbp	Megabase pair
%	Percent
±	Plus-minus
RR	Resistance ratio
SD	Synergistic differences
SR	Synergistic Ratio
TI	Trunk injection

CHAPTER 1

INTRODUCTION

Infestations and outbreaks of bagworms have persisted in oil palm plantations in Malaysia for over five decades (Cheong and Tey, 2012; Syarif *et al.*, 2023). Despite effective control measures, bagworms have caused yield losses of about 33%-40% (Basri, 1993; Sidi *et al.*, 2023). To seriously address this issue, the bagworm was declared a dangerous pest on November 15, 2013 under the Malaysia Act 167, Plant Quarantine Act 1976. The ongoing outbreak can cause serious economic problems if not properly controlled.

Among the bagworm species, *Metisa plana* Walker (Lepidoptera: Psychidae) is the most invasive, followed by *Pteroma pendula* Joannis and *Mahasena corbetti* Tams (Basri *et al.* 1988, Norman *et al.*, 1994; Norman and Basri, 2007). Therefore, to reduce the population of *M. plana* below the threshold level, chemical control is the most commonly used strategy (Kok *et al.*, 2012; Noorhazwani *et al.*, 2017; Yap, 2000; Zulkefli *et al.*, 2021; Fuat *et al.*, 2022; Mazmira *et al.*, 2022; Nurulhidayah *et al.*, 2023). However, the frequent use of chemical pesticides to control this agricultural pest has led to other, more persistent problems, such as the disruption of beneficial insect populations, the development of pest resistance, and the spread of harmful chemical residues in the environment (Thaer *et al.*, 2021).

Out of concern for these problems, an integrated pest management (IPM) program was introduced as an environmentally friendly and more sustainable *M. plana* mitigation strategy (Wood and Norman, 2019; Sulaiman and Talib, 2021). The IPM program for *M. plana* consists of biological control agents such as *Bacillus thuringiensis*, beneficial insects and pheromone traps (Adams *et al.*, 1999; Wood and Norman, 2019; Sulaiman and Talip, 2021). The implementation of an IPM program requires a precise use of IPM components depending on the growth stages (Norman *et al.*, 2019). However, the success of the IPM program is also affected by the presence of multistage larvae, the development of a pesticide-tolerant population, and the disruption of the ecosystem of *M. plana* predators due to the long-term use of chemical pesticides (Sulaiman and Talip, 2021). *B. thuringiensis* has been shown to be an effective biological control agent against *M. plana* (Ramlah Ali and Mohd Basri, 1997; Mazmira *et al.*, 2011; Noorhazwani *et al.*, 2017; Shawal *et al.*, 2023). Therefore, enhancement of IPM components by *B. thuringiensis* is an option that can be explored. The effect of *B. thuringiensis* in combination with other class IV pesticide such as flubendiamide (fbd) and the exploration of unknown genomic features harboring the virulence genes of *M. plana* are also worth investigating (Asib and Musli, 2020). A thorough understanding of the genomic features of *B. thuringiensis* and the enhancement of mortality effects against *M. plana* larvae could lead to better control of *M. plana*.

Another area that needs to be explored is the diversity of the gut microbiome of *M. plana*. As a leaf defoliator, the microbiome of *M. plana* larvae is involved in the digestion of oil palm leaves (Paniagua Voirol *et al.*, 2018; Ting *et al.*, 2023) and the long-term application of pesticides sprayed on the leaf affects the taxonomic relationships of the insect gut microbiome community that specifically responds to pesticide treatment (Chen *et al.*, 2022). A previous metagenomic study showed differences in the composition of the *M. plana* microbiome at the taxonomic level (Ting *et al.*, 2023). However, the responses of the *M. plana* microbiome specifically to pesticide treatment are still unexplored. The reason for this is the potential identification of an *M. plana* microbiome network that can positively react with *B. thuringiensis* to kill the host. The microbiome networks are critical for the maintenance of microbial communities during pathogenesis (Hernandez *et al.*, 2021), and the changes in interactions between bacteria and bacterial networks in response to pesticide application may serve as a guide for a more effective strategy to control *M. plana* that targets the stability of microbiome networks in the gut.

Further exploration of the responses of the gut microbiome to pesticides can also be done by analyzing the taxonomically identified microbiome at a functional level. The insect microbiome has been reported to play a central role in the insect ecosystem by regulating ecological balance, insect health and pesticide detoxification (Antonelli *et al.*, 2022; Jaffar *et al.*, 2022). It is therefore hypothesized that the microbiome of *M. plana* is responsible for the host insect's resilience and susceptibility to disturbance. The reason for this is the microbiome community, which can increase or decrease depending on the combination and concentration of pesticide treatment (Wu *et al.*, 2022). A comprehensive understanding of the functional responses of the microbiome during pesticide treatment is crucial for the control of *M. plana*.

To address the challenges of IPM program and chemical pesticide use against *M. plana* in Malaysia, this study aims to investigate the genomic and insecticidal activity of *B. thuringiensis* MPOB Bt1 (MPOB Bt1) and Fbd, microbial community shifts and functional responses of metagenome-assembled genomes (MAGs) to pesticides in the control of *M. plana*. The whole genome of Bt was sequenced, and the genome and its insecticidal crystalline delta-endotoxin (Cry) genes were compared with the genomes and Cry genes in closely related *B. thuringiensis* genomes in the National Center for Biotechnology Information (NCBI) databases. Subsequently, the synergistic effect of MPOB Bt1 with flubendiamide on the fourth-instar larvae of *M. plana* will be tested. In addition, the study uses gene-centric shotgun metagenomics with the co-assembly method and genome-centric approaches to analyze the effects of pesticide treatments on broader and more detailed microbiome responses. Figure 1.1 shows the flowchart summarizing the entire work.

The importance of this study is to guide future potential strategies to increase toxicity in the control of *M. plana* by suggesting the synergistic use of pesticides and biological control measures in pest management programs. The changes in the microbial community in response to each pesticide treatment and the major

bacterial genera within the microbiome of *M. plana*, under both healthy and stressed conditions, will provide valuable insight into community succession and the function of these genera in facilitating resistance to pesticide treatments and improving efficacy.

Problem Statements:

- i. The impact of chemical pesticides on the environment, which contributed to the emergence of multistage larvae, and the development of pesticide tolerance hindered the IPM program.
- ii. The persistent use of chemical pesticides led to changes in the microbiome of *M. plana* and the microbiome network that responds positively or negatively to pesticide use is still unexplored.
- iii. The functional response of the *M. plana* microbiome involved in host resistance and susceptibility to pesticide treatments is also still unknown.

Hypotheses:

- i. The whole genome sequence (WGS) of MPOB Bt1 will reveal the insecticidal genes and virulence factors and the combined treatment of MPOB Bt1 and Fbd will enhance insecticidal activity against *M. plana* larvae.
- ii. Gene-centric metagenomic profiling of the bacterial community of *M. plana* after combined treatment with MPOB Bt1 and Fbd will reveal significant shifts in community composition through increased richness and rapidly increased abundance of the microbiome.
- iii. Genome-centric metagenome profiling of the bacterial community of *M. plana* will reveal MAGs involved in pathogenicity and resilience in response to pesticide treatments.

Aim:

To determine the effectiveness of pest control strategies (combination of MPOB Bt1 and Fbd treatment), microbial community shifts and MAGs' functional responses to pesticides exposure for guiding improved *M. plana* control in oil palm plantations.

The specific objectives of this research are as follows:

- i. To sequence and analyze the whole genome sequence of MPOB Bt1 and
- ii. To evaluate MPOB Bt1 synergy with Fbd for control of *M. plana* larvae.

- iii. To determine the community succession of *M. plana* larval microbiome after the combination of MPOB Bt1 and Fbd compared to individual treatment using gene-centric shotgun metagenomics.
- iv. To characterize the functional aspects of the metagenome-assembled genomes community in relation to the combination of MPOB Bt1 and Fbd compared to individual treatment.



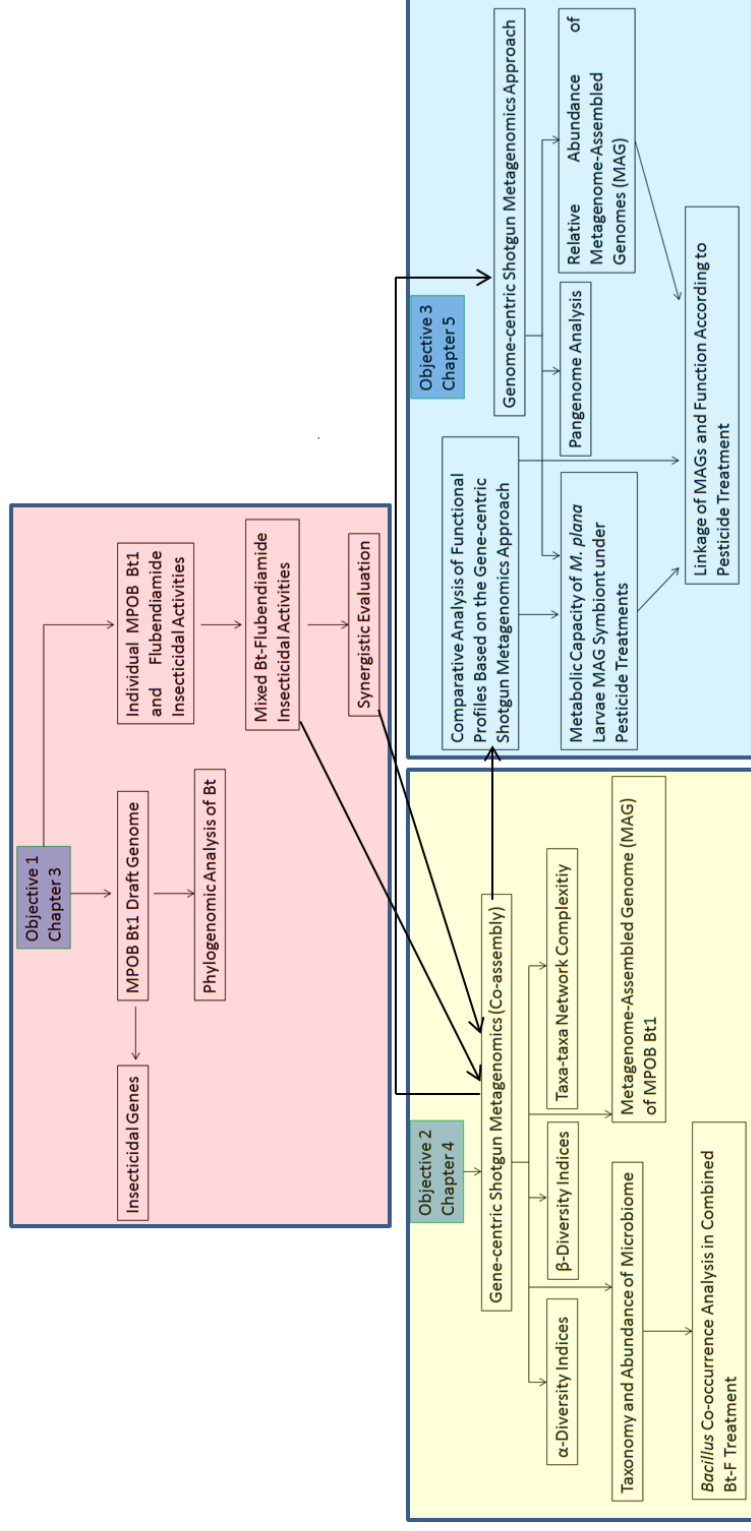


Figure 1.1 : Flowchart Summarizing the Experimental Chapters Covering Objective One through Objective Three. The red-shaded box is objective one (chapter 3), the yellow-shaded box is objective two (chapter 4), and the blue-shaded box is objective 3 (chapter 5).

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