Malaysian Applied Biology (2024) 53(5): 123-139
https://doi.org/10.55230/mabjournal.v53i5.3178

Research

Genomic Analysis And Synergistic Biocontrol Potential of *Bacillus thuringiensis* MPOB Bt1 With Flubendiamide Against Oil Palm Bagworm, *Metisa plana* Walker (Lepidoptera: Psychidae)

Mohd Shawal Thakib Maidin[1,2], Amalia Mohd Hashim[2,3*], Ahmad Zuhairi Abdul Malek[2,3], Mohamed Mazmira Mohd. Masri[1], Nurhafizhoh Zainuddin[1], Raha Abdul Rahim[2,4], Syari Jamian[5]

1. Malaysian Palm Oil Board, 6, Persiaran Institusi, Bandar Baru Bangi, 43000 Kajang, Selangor, Malaysia
2. Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
3. Halal Products Research Institute, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
4. National Institutes of Biotechnology Malaysia, Level 2, Block A, Agro-Biotechnology Institute Malaysia (ABI) Complex, Jalan Bioteknologi, 43400 Serdang, Selangor, Malaysia
5. Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

*Corresponding authors: amalia@upm.edu.my

ABSTRACT

Bacillus thuringiensis MPOB Bt1 (MPOB Bt1) is a biological control agent used to suppress bagworm larvae in Malaysian oil palm plantations. Although MPOB Bt1 has been utilized in the field for biocontrol of oil palm bagworm larvae, its genetic basis for biocontrol capabilities and the combined effectiveness of MPOB Bt1 with flubendiamide have not been fully investigated. This study aimed to provide a genomic foundation for understanding the insecticidal properties of MPOB Bt1 by identifying specific genes that may be responsible for its biological activity. In addition, the study focused on evaluating the practical biological efficacy of MPOB Bt1, both alone and in combination with flubendiamide, against *Metisa plana*. The draft genome sequence of MPOB Bt1 was determined using Illumina HiSeq and PacBio platforms. The genome size was 6.9 Mb, with a GC content of 35.1%, and containing 5,558 coding DNA sequences, which included *Cry9Ea*, *Cry1Ab*, *Cry1Ca,* and *Cry1Da* of δ-endotoxin genes, 23 rRNAs, and 86 tRNAs. Bioassays showed that MPOB Bt1 exhibited toxicity to oil palm bagworm larvae, with an LC_{50} of 3.31 × 10^{10} spores/mL after 72 hr of treatment. The combination of MPOB Bt1 and flubendiamide showed a synergistic effect (LC_{50} of 1.19 × 10^{9} spores/mL), with a ratio of experimentally observed efficacy to predicted efficacy greater than one. This study presents the draft genome sequence of MPOB Bt1 and identifies multiple insecticidal genes that potentially exhibit inhibitory effects against *M. plana* larvae. The toxicity and synergistic effect of MPOB Bt1 and Fbd suggest a potential strategy for controlling bagworm infestation in oil palm plantations. These findings provide a promising safer alternative to chemical insecticides for sustainable *M. plana* management in oil palm plantations.

Key words: Bacterial genome, bioassay efficacy, bioinsecticide, insecticidal genes, integrated pest management

Article History
Accepted: 26 September 2024
First version online: 30 November 2024

Cite This Article:
Maidin, M.S.T., Mohd Hashim, A., Abdul Malek, A.Z., Mohd. Masri, M.M., Zainuddin, N., Abdul Rahim, R. & Jamian, S. 2024. Genomic analysis and synergistic biocontrol potential of *Bacillus thuringiensis* MPOB Bt1 with flubendiamide against oil palm bagworm, *Metisa plana* Walker (Lepidoptera: Psychidae) 2024. Malaysian Applied Biology, 53(5): 123-139. https://doi.org/10.55230/mabjournal.v53i5.3178

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INTRODUCTION

Pest management in oil palm plantations is important to ensure oil palm health and productivity, ensuring the economic viability of the industry. Recently, sustainable methods like Integrated Pest Management (IPM) have gained prominence. IPM combines biological controls, chemical pesticides, cultural practices, and mechanical practices (Wood & Kamarudin, 2019; Sulaiman & Talip, 2021). Among oil palm pests, *Metisa plana* has emerged as one of the most problematic bagworm species in Malaysia.

To address this issue, IPM strategies have been implemented for controlling the bagworm populations in Malaysian oil palm plantations (Wood & Kamarudin, 2019). The component of the IPM program consists of spraying with *Bacillus thuringiensis* bio-insecticides, planting beneficial plants, and installing pheromone traps (Sulaiman & Talip, 2021) for an effective and sustainable bagworm control strategy (MPOB, 2016).

The success of the IPM depends on precise deployment techniques and understanding the bagworm's life cycle (Kamarudin *et al*., 2019). However, challenges persist due to the indiscriminate use of chemical pesticides, which can lead to pesticide resistance and disrupt natural predator ecosystems (Zainuddin *et al*., 2023). This can potentially contribute to recurring bagworm infestations, especially with the *M. plana* species, which is the most damaging leaf defoliator among the oil palm bagworms (Samada & Tambunan, 2020; Sulaiman & Talip, 2021).

In addressing these problems, developing safer alternatives to strengthen the IPM program became the focus (Masri *et al*., 2021). *B. thuringiensis*, known for its environmental friendliness and specificity, has been widely used to control *M. plana* (Ali & Wahid, 1997; Masri *et al*., 2010; Kamarudin *et al*., 2017). Due to its high specificity and environmental friendliness, *B. thuringiensis* has become the world's most effective commercially used biological control agent (Glare *et al*., 2012; Jurat-Fuentes & Crickmore, 2017). MPOB Bt1 isolated by Ali and Wahid (1997) has been applied as bio-insecticide for aerial spraying mainly on smallholder plots to control infestations below the threshold (Ali & Wahid, 1997; Ali, 2000; Masri *et al*., 2010; Kamarudin *et al*., 2017).

Besides *B. thuringiensis*, Flubendiamide (Fbd) is another stomach poison known for its ability to target insects of the order Lepidoptera (Kato *et al*., 2009). The diamide compounds of Fbd namely the phthalic acid diamide have emerged as a new insecticide targeting insect Ryanodine Receptor (RyR) (Kato *et al*., 2009). This diamide can trigger intracellular release of Ca^{2+} from the endoplasmic or sarcoplasmic reticulum and has significant insecticidal activity against several Lepidoptera pest species (Zhang *et al*., 2021). According to Priwiratama *et al*. (2018), Fbd was effective against defoliating moths at an application rate of 150-200 mL ha^{-1}, while an application rate of 200 mL ha^{-1} was required to control bunch moths. In addition, Fbd had no adverse effects on male inflorescence activity and the development of *Elaeidobius kamerunicus* (Priwiratama *et al*., 2018). Therefore, these diamides can be considered as another potential insecticide to be utilized together with *B. thuringiensis* for the *M. plana* mitigation strategy.

Exposure of insect pests to substances in a mixture or combination, even at low concentrations, is thought to cause additive, antagonistic, and synergistic effects (Kortenkamp, 2007). To ensure continuous and consistent crop yields, pesticide mixtures with different pesticides are used against pests (Legrand *et al*., 2016; Zhao *et al*., 2018; Mesnage *et al*., 2021). As part of the IPM strategy, the approach of combining multiple pesticides has been thoroughly researched (Delnat *et al*., 2019; Stemele, 2017; Rajendran, 2020). However, there is a lack of studies on the combination of *B. thuringiensis* toxins with pesticides to control *M. plana*. The use of *B. thuringiensis* and chemical pesticides is synergistic (Liu *et al*., 2019; Shabbir *et al*., 2021; Polenogova *et al*., 2021). Therefore, the combination of *B. thuringiensis* with other pesticides can potentially lead to better control of *M. plana* due to a higher mortality rate.

Next-generation sequencing (NGS) by whole genome sequencing (WGS) has advanced the identification of insecticidal genes in *B. thuringiensis* (Naveenarani *et al*., 2022). This study aims to sequence the whole genome of MPOB Bt1 using advanced technologies (Illumina HiSeq & PacBio) and identify the specific insecticidal genes and virulence factors. To compare these genes with those of closely related *B. thuringiensis* strains available in the NCBI database. To assess the effectiveness of MPOB Bt1 and Fbd separately and in combination (MPOB Bt1-Fbd) against *M. plana* larvae. This research would provide valuable information on the molecular identification of insecticidal genes and the potential of combination pesticide application for the development of alternative pesticides that exhibit increased mortality against *M. plana* larvae.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and chemical pesticide

MPOB Bt1 was obtained from the culture collection of the Entomology and Integrated Pest Management Unit (EIPM), Malaysian Palm Oil Board (MPOB), Malaysia (Ali & Wahid, 1997). The strain was maintained at -80°C in nutrient broth (Oxoid, UK) containing 20% (v/v) glycerol. To obtain pure culture, the isolated bacterium was aseptically propagated three times at 30°C in Nutrient Broth (Sigma-Aldrich) for activation before use. The culture was incubated at 30°C with shaking at 150 rpm for 48 hr for sporulation in AgroNat-7 medium (patent no. P12011000307) (Masri *et al*., 2021; Ahmad *et al*., 2012). The number of MPOB Bt1 spores per mL of water was counted and adjusted to a standard concentration of ±1 x 10^{10} spores mL^{-1} (Ali *et al*., 2005). The commercial formulation of flubendiamide 20% w/w was obtained from Agricultural Chemicals (M) Sdn. Bhd. (ACM).

DNA extraction

The total genomic DNA of MPOB Bt1 (accession number JASVEG000000000) was isolated using the innuPREP DNA Mini Kit (Analytic Jena, Germany) according to the manufacturer's recommendations with minor modifications. DNA was quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, USA) and homogeneity was assessed by agarose gel electrophoresis (0.8% agarose). Genomic DNA was randomly fragmented by sonication, and then DNA fragments were end-polished, A-tailed, and ligated to full-length Illumina sequencing adapters, followed by further PCR amplification with P5 and indexed P7 oligos. PCR products for final library construction were purified using the AMPure XP system (Beckman Coulter, USA). Libraries were then screened for size distribution using the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) and quantified by real-time PCR to meet the 3 nanoMolar (nM) criteria and to ensure that libraries met the minimum concentration required for successful sequencing on the selected systems. Samples were sent to Apical Scientific, Malaysia, for next-generation sequencing, which was performed using Illumina (HiSeq) and PacBio Sequel systems.

Quality check and *de novo* assembly

The Illumina raw reads were first cleared of primer sequences using BBDuk from the BBTools packages (https://jgi.doe.gov/data-and-tools/bbtools/). QC Illumina and PacBio reads were assembled de novo using Spades (Prjibelski *et al*., 2020) and polished using Pilon version 1.23 (Walker *et al*., 2014) implemented in Unicycler (Wick *et al*., 2017). All reads were assembled into 28 contigs. All contigs were subjected to taxa classification using Kraken 2 (Wood *et al*., 2019).

Analysis of genome sequence

All 28 contigs were scaffolded against a reference genome of *B. thuringiensis* BT 407 (NZ_ CM000747.1) using Ragtag (https://github.com/malonge/RagTag), resulting in a single scaffold of > 6 Mb with several smaller contigs (17 contigs in total). BLASTN was used to analyze the best match of the 17 contigs in the database. Prodigal was used to predict the open reading frames (Hyatt *et al*., 2010) and annotated with Prokka 1.14.6 (Seemann, 2014). The ORF was mapped to the eggNog classification using eggNog Mapper 5.0 (Huerta-Cepas *et al*., 2019). The presence of cry/vip/cyt type genes was analyzed using the BtToxin_scanner tool (Ye *et al*., 2012). GenoVi v0.2.16 was used to generate circular representations of the genome (Cumsille *et al*., 2023). The draft genome sequence of MPOB Bt1 was finally deposited into the NCBI database with accession number JASVEG000000000.

Comparative genome analysis

A phylogenomic analysis was performed to compare MPOB Bt1 with 10 other commercially available active ingredients of the *B. thuringiensis*-based pesticide product in the market that has been used as biocontrol. For this purpose, the pangenomics pipeline of Anvi'o v7.1 was used with default parameters, which are available at https://merenlab.org/2016/11/08/pangenomics-v2/#generating-an-anvio-genomes-storage (Eren *et al*., 2015). The list of eleven (11) *B. thuringiensis* strains, including MPOB Bt1, is shown in Table 1. The *B. thuringiensis* strains were selected based on the commercially available active ingredients of the biopesticide products on the market. The Anvi'o genome repositories were generated and analyzed before the average nucleotide identity value was calculated. The average nucleotide identity based on the BLAST (ANIb) table with the complete percent identity is presented in Appendix A. The list of δ-endotoxin gene clusters in each genome was summarized as a comprehensive TAB-limited file in Appendix B. The amino acid sequences of Pfam δ-endotoxins were split from the pangenome database using Anvi'o script, anvi-split.

Bagworm collection and laboratory maintenance

An oil palm smallholder plantation in Sungai Suli, (4°02'45.2"N & 101°01'57.7"E), Perak, Malaysia was selected as the bagworm collection site. This plantation was identified to be heavily infested (above threshold level or more than 10 larvae/frond) by Metisa plana bagworm. Stage four of *M. plana* larvae (average case length of 9.5 mm) on the infested palms was examined (Kok *et al*., 2011) and were collected with three replicates for a plot size of 5 × 5 palms using a randomized complete block design (RCBD). Larvae were kept under laboratory conditions (temperature: day 27±1°C, night 24±1°C; relative humidity 35%-50%; photoperiod, light: darkness = 12:12 hr) for 24 hr before bioassay treatments.

***Bacillus thuringiensis* MPOB Bt1 – flubendiamide concentrations and viability**

The concentration and viability of MPOB Bt1 spores were determined according to Ali *et al*. (2003).

Table 1. List of eleven (11) *Bacillus thuringiensis* genomes that have been used as active ingredients in commercial biopesticides

Bt strain list	Genome Size (bp)	Description	RefSeq	Submitter
MPOB Bt1	6,912,457	Isolated from oil palm plantation soil	This study	This study
strain HER1410	6,147,475	Host bacterium for the tectiviridae Bam35	GCF_013340745.1	KU Leuven
Bt407	6,026,843	A well-studied isolate that has been cured of the plasmid that encodes the insecticidal crystalline toxin	GCF_000161495.1	Naval Medical Research Center
strain c25	5,661,190	Isolated from mulberry orchard soil in Iksan, South Korea	GCF_002222555.1	Chungbuk National University
subsp. *aizawai* strain ABTS-1857	6,694,857	Biopesticide, XenTari strain ABTS-1857	GCF_020809245.1	University of Zurich
subsp. *kurstaki* strain ABTS-351	6,834,111	Biopesticide, DiPel DF	GCF_020809105.1	University of Zurich
serovar *kurstaki* str. HD-1	6,386,251	Insecticidal bacterium	GCF_000710255.1/ GCF_000717535.1	Huazhong Agricultural University
subsp. *aizawai* strain GC-91	6,756,558	Biopesticide, Agree WP	GCF_020809205.1	University of Zurich
serovar *israelensis* strain AM65-52	6,700,047	"Commercial powder, Vectobac"	GCF_003445395.2	Micalis Infra
serovar morrisoni str. 4AA1	6,313,491	Spore-forming gram-positive bacterium which produces highly toxic compounds against *Tribolium castaneum*	GCF_022810725.1	Georg-August-University Goettingen, Genomic and Applied Microbiology, Goettingen Genomics Laboratory
serovar indiana strain HD521	6,198,448	Microbe sample from *Bacillus thuringiensis* serovar indiana	GCF_001183785.1	Rice Research Institution of SICAU

The number of MPOB Bt1 spores per mL of water was determined using spore counting to a standard concentration of ±1 × 10^{10} spores/mL (Ali *et al.*, 2005). The concentration of Fbd was standardized at 0.22 g L^{-1} according to the manufacturer's recommendations.

Dosage response bioassay

A dose-response bioassay was performed and the lethal concentration (LC_{50}) for each pesticide was recorded. For the freshly prepared MPOB Bt1, five concentrations of spore suspensions were prepared starting from the standard concentration: 10^{10} spores mL^{-1} (C1), 10^9 spores mL^{-1} (C2), 10^8 spores mL^{-1} (C3), 10^7 spores mL^{-1} (C4), and 10^6 spores mL^{-1} (C5). For the Fbd treatment sample, five concentrations of the chemical were prepared starting from the standard concentration: 2.2 × 10^{-1} g L^{-1} (C1), 2.2 × 10^{-2} g L^{-1} (C2), 2.2 × 10^{-3} g L^{-1} (C3), 2.2 × 10^{-4} g L^{-1} (C4), and 2.2 × 10^{-5} g L^{-1} (C5). A total of six replicates including negative control with a sample size of 10 larvae per dilution were used and treated with MPOB Bt1 or Fbd for 12 consecutive days. MilliQ water was used as a negative control. The bioassay was performed on leaves of oil palm with sublethal concentrations of a substance. In the bioassay, 30 mL of each MPOB Bt1 and Fbd concentration was aseptically sprayed onto the leaves, which were then placed in water containers. Larvae in each rearing container were then placed on the sprayed leaves. The rearing containers were then placed in a controlled environment (temperature: day 27±1°C, night 24±1°C; relative humidity 35%-50%; photoperiod, light: dark = 12:12 hr). Larval feeding, mortality, and leaf damage were recorded daily for seven days. After one week, all larvae were provided with leaf segments freshly sprayed with pesticide for another five days. Final records were made 12 days after treatment.

Data analysis

The efficacy of MPOB Bt1 against M. plana was analyzed using Abbott's control-adjusted mortality (Abbott, 1925) for 3, 7, and 12 days after treatment (DAT) followed by a one-way analysis of variance (ANOVA). Corrected mortality was calculated according to Abbot (1925).

Means were compared using Tukey's test at a 5% probability level. The interaction monitored was the difference in response between treatments (as a factor) that contributed to bagworm mortality. Means were separated using the least significant difference (LSD) analysis (SPSS version 11.5). Dose-response data from the bioassays were subjected to Probit Analysis to calculate LC_{50} values at the 95% cutoffs and mortality rates (regression slope) according to Finney (1971).

Optimized mixed MPOB Bt1 - flubendiamide, individual *Bacillus thuringiensis* MPOB Bt1, and individual flubendiamide dosage response assay

Different ratios of MPOB Bt1 mixed with Fbd were prepared using MPOB Bt1: Fbd (0.5:0.5), MPOB Bt1: Fbd (0.6:0.4), MPOB Bt1: Fbd (0.7:0.3), MPOB Bt1: Fbd (0.8:0.2), and MPOB Bt1: Fbd (0.9:0.1) according to Sayed and Behle (2017). Spore concentrations in each MPOB Bt1 or Fbd sample were based on LC_{50} values to standardize the efficacy of the samples. The mortality rate of fourth instar (L4) larvae was recorded and determined. Bioassays were performed in replicates of five. Ten larvae were inserted in each replicate (50 larvae per strain). Larval mortality was recorded for 10 days after treatment (DAT).

Synergism analysis

The combination index (CI) method proposed by Gisi (1996) was used to calculate the synergistic effect of the mixture of MPOB Bt1 and Fbd. Expected efficacy, represented as percent control ($\%C_{exp}$), was predicted using Abbott's formula (Abbott, 1925): $\%C_{exp} = A+B-(AB/100)$, where A and B are the control values for the single pesticide. Synergistic interactions are present in the mixture if the ratio between the experimentally observed efficacy (C_{obs}) and the predicted efficacy (C_{exp}) is larger than one.

RESULTS AND DISCUSSION

Draft genome

Table 1 shows the summary of the MPOB Bt1 genome compared to other *B. thuringiensis* genomes that have been used in commercial *B. thuringiensis* products. The genome consists of 6,912,457 base pairs (bp), with an average G+C content of 34.95%. This genome also contains 7,354 protein-coding sequences (CDS), 42 ribosomal RNA genes (*rRNA*), and 125 transfer RNA genes (*tRNA*) (Table 2). A circular representation of the MPOB Bt1 genome is shown in Figure 1.

Table 2. *Bacillus thuringiensis* MPOB Bt1 genome with a summary of sequencing, assembly, and genomic statistics

Attribute	Value
Genome size (bp)	6912457
%GC	34.95
Number of contigs	17
N50 (bp)	6311061
L50	1
Sequencing technology	Illumina and PacBio
Annotation pipeline	BLASTN and Prokka annotation pipeline
CDS	7354
rRNAs	42
tRNAs	125
tmRNAs	1
Repeat region	2
Genbank accession number	JASVEG000000000

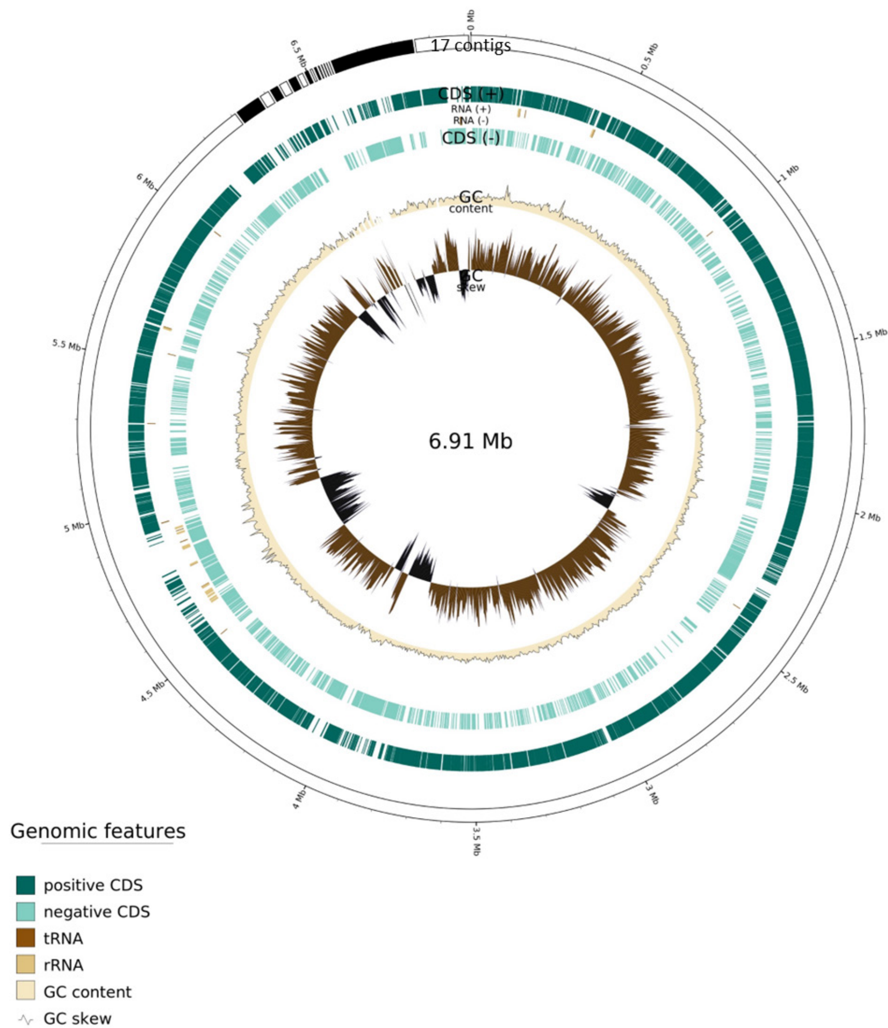

Fig. 1. Circular representation of the *Bacillus thuringiensis* MPOB Bt1 draft genome, generated with GenoVi (Cumsille *et al*., 2023). The 17 contigs are the individual bands in one circular representation. The labeling is arranged from the inside to the outside, starting with GC skew, GC content, reverse strand CDS reverse strand RNAs forward strand rRNAs, forward strand CDS, and contigs.

In this work, dual bacterial genome sequencing was conducted using Illumina HiSeq and PacBio platforms to obtain the complete genome of MPOB Bt1. Illumina reads were used to fill gaps, while PacBio long reads were used to recover end-stage bacterial genomes (Jeong *et al*., 2016). Following

the development of various freeware programs, the use of next-generation sequencing technology for whole genome analysis has proven to be an economical and advantageous strategy for searching for new insecticidal genes (Ye *et al*., 2012).

BLASTn analysis of all 17 contigs shows that many of these smaller fragments have best matches to *B. thuringiensis* plasmid DNA (Table 3). In the assembly, *B. thuringiensis* BT 407 (NZ_CM000747.1) was selected as the reference genome for homologous analysis to compare and personalize the genome sequence of MPOB Bt1 due to high sequence similarity, as revealed by 16s *rRNA* BLASTn search. Genome sequence analysis of MPOB Bt1 also revealed a total of 11 gaps of 100 bp length. These missing unassembled regions are often referred to as genomic “dark matter” (Peona *et al*., 2021; Sedlazeck *et al*., 2018; Weissensteiner *et al*., 2019). The “dark matter” is the gaps in the assembled genome sequence, which might be due to the abundance and repetitive nature of the sequence that resulted in the incomplete assembly. A total of 7108 protein-coding genes were assigned a presumed function based on the functional categories of Clusters of Orthologous Groups (COG) (Figure 2). Genes with unknown function (1526 ORFs) and not in COGs (635 ORFs) were among the most abundant COG functional categories. This was followed by replication, recombination, and repair (530 ORFs), transcription (512 ORFs), and amino acid transport and metabolism (485 ORFs).

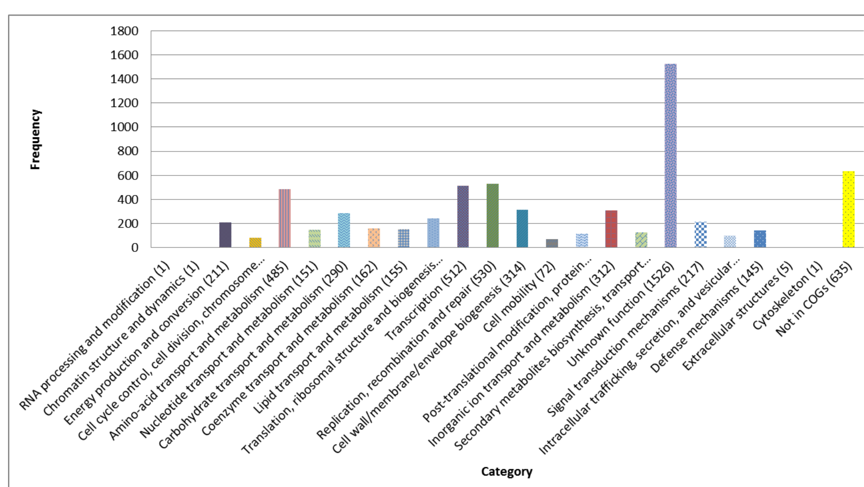

Fig. 2. Distribution of protein-coding genes in *Bacillus thuringiensis* MPOB Bt1 assigned to general COG functional categories. Genes that are not associated with any COG annotation were assigned as ‘not in COGs’.

Insecticidal genes

The BtToxin_scanner tool (Ye *et al*., 2012) was used to highlight potential genes, and the results are shown in Table 4. Four δ-endotoxin genes were identified using the BtToxin_scanner: *Cry9Ea*, *Cry1Ab*, *Cry1Ca*, and *Cry1Da*, with total sizes of 1150 aa, 1189 aa, 1189 aa, and 1165 aa, respectively. These genes have previously been reported to have insecticidal activities against Lepidoptera (Konecka *et al*., 2018). Table 4 also provides a summary of additional potential proteins that may play a role in the insecticidal activity of MPOB Bt1 against *M. plana*.

The progress of genome sequencing technology and the lack of information on the whole genome sequence of MPOB Bt1 and its insecticidal genes led to an attempt to fill this knowledge gap. The BtToxin_scanner tool (Ye *et al*., 2012) was used and was able to identify four δ-endotoxin genes, namely *Cry9Ea*, *Cry1Ab*, *Cry1Ca,* and *Cry1Da*. These genes, known for their insecticidal activities against Lepidoptera, suggest a potential multicomponent mechanism of action employed by MPOB Bt1 against the oil palm bagworm. The possible involvement of these proteins suggests synergistic interaction in insecticidal activity in a multicomponent mechanism of action against the *M. plana* larvae (Bergamasco *et al*., 2013; Mathur *et al*., 2019). The insecticidal mechanism of action of the δ-endotoxins comprises the steps of ingestion, binding to brush border membrane vesicle (bbmv), intercalation into the membrane, and cation-selective channel formation (Mathur *et al*., 2019). Ali *et al*. (2003) described one of the multicomponent mechanisms of action of *Cry1Ac*, *Cry1C,* and *Cry1Ab* against *M. plana* larvae, which involves the bbmv of the susceptible larval epithelial gut toxin receptor. The findings showed that the δ-endotoxin binds specifically to the bbmv irreversibly for the formation of gut pores, resulting in insect death. This provides a good foundation for the selection of effective toxin against *M. plana* larvae especially using MPOB Bt1.

Table 3. BLASTn of *Bacillus thuringiensis* MPOB Bt1 contigs

Organisms	Description	%GC	% Pairwise Identity	Accession
Bacillus thuringiensis	*Bacillus thuringiensis* serovar *kurstaki* strain HD 1 plasmid unnamed6, complete sequence	39.10	99.20	CP010003
Bacillus thuringiensis	*Bacillus thuringiensis* serovar *thuringiensis* str. IS5056 plasmid pIS56-68, complete sequence	32.50	100.00	CP004132
Bacillus thuringiensis	*Bacillus thuringiensis* HD-771 plasmid p06, complete sequence	34.90	98.90	CP003758
Bacillus thuringiensis	*Bacillus thuringiensis* serovar *galleriae* strain HD-29 plasmid pBMB47, complete sequence	34.40	100.00	CP010095
Bacillus thuringiensis	*Bacillus thuringiensis* strain HM-311 chromosome, complete genome	35.70	93.00	CP040782
Bacillus thuringiensis	*Bacillus thuringiensis* strain YWC2-8 plasmid pYWC2-8-5, complete sequence	36.70	100.00	CP013060
Bacillus thuringiensis	*Bacillus thuringiensis* strain YC-10 plasmid pYC10, complete sequence	43.80	98.10	CP011355
Bacillus thuringiensis	*Bacillus thuringiensis* serovar *kurstaki* str. HD73 plasmid pHT8_2, complete sequence	29.50	98.80	CP004075
Bacillus thuringiensis	*Bacillus thuringiensis* strain Bc601 plasmid pBTBC6, complete sequence	35.20	98.50	CP015156
Bacillus thuringiensis	*Bacillus thuringiensis* strain K1 plasmid pK1S1, complete sequence	33.10	99.20	EF406356
Bacillus thuringiensis	*Bacillus thuringiensis* serovar *coreanensis* strain ST7, complete genome	36.90	89.70	CP016194
Bacillus phage	*Bacillus* phage phiS58, complete genome	32.40	100.00	KT970646
Bacillus thuringiensis	*Bacillus thuringiensis* serovar *galleriae* strain HD-29 plasmid pBMB426, complete sequence	35.60	100.00	CP010090
Bacillus thuringiensis	*Bacillus thuringiensis* HD-771, complete genome	31.50	94.50	CP003752
Bacillus phage	Bacillus phage vB_BthS-TP21T, complete genome	34.40	99.30	MK843319
Bacillus thuringiensis	*Bacillus thuringiensis* serovar *kurstaki* strain HD 1 plasmid unnamed6, complete sequence	39.10	99.20	CP010003
Bacillus thuringiensis	*Bacillus thuringiensis* serovar *thuringiensis* str. IS5056 plasmid pIS56-68, complete sequence	32.50	100.00	CP004132

Beyond the previously identified genes, the presence of additional proteins in Table 4 adds complexity to the potential mechanisms underlying the insecticidal activity of MPOB Bt1 against the *M. plana* larvae. A hypothetical protein of 83 aa length closely aligns with a gene responsible for encoding a vegetative insecticidal protein, *Vip3Bc1*, with a significant identity of 71.08%. The *B. thuringiensis* Toxin Nomenclature Committee has classified Cry toxins based on their primary amino acid sequences and identified 312 Cry holotype protein genes across 78 different categories (Crickmore *et al*., 2016). The potential involvement of these proteins in the insecticidal activity against the *M. plana* larvae suggests a diverse mechanism of action employed by MPOB Bt1 (Ahmad *et al*., 2009). The findings indicate the complexity of MPOB Bt1's interactions with the target pest and warrant further investigation into the specific roles and synergies among these proteins in enhancing the efficacy of MPOB Bt1 as a biopesticide against the oil palm bagworm.

All *Cry1* genes identified in this study are known for their involvement in toxicity to Lepidoptera insects such as *M. plana* (Ali *et al*., 2005), *Spodoptera exigua* (Hernández-Martínez *et al*., 2008), *Earias insulana* (Ibargutxi *et al*., 2006), *Spodoptera littoralis* (BenFarhat-Touzri *et al*., 2013) and *Helicoverpa armigera* (Estela *et al*., 2004). Another Cry gene, *Cry9Ea*, has shown significant insecticidal activity against another Lepidoptera species, the larvae of *Cydia pomonella* with a potentially narrow spectrum of activity (Baranek *et al*., 2021). The presence of multiple *Cry* genes in this study could suggest a synergistic interaction that enhances insecticidal activity. The synergistic interaction of *Cry1* and *Vip3* genes was demonstrated in another study for *Spodoptera cosmioides*, *Spodoptera albula*, and *Spodoptera frugiperda* in toxicity tests at a protein ratio of 1:1 (Bergamasco *et al*., 2013).

Table 4. Protoxin characteristics from the predicted Coding DNA Sequences (CDS) of different *Bacillus thuringiensis* MPOB Bt1 contigs using BtToxin_scanner (Ye *et al*., 2012)

ID	Protein Description	Length (bp)	Best hit	Hit length (bp)	Coverage (%)	Identity (%)
1	Pesticidal crystal protein Cry9Ea	1150	*Cry9Ea9*	1150	100	100
2	Pesticidal crystal protein Cry1Ab	1155	*Cry1Ab9*	1189	100	100
3	Pesticidal crystal protein Cry1Ca	1189	*Cry1Ca9*	1189	100	100
4	Pesticidal crystal protein Cry1Da	1165	*Cry1Da2*	1165	100	99.91
5	Hypothetical protein	83	*Vip3Bc1*	803	10.34	71.08
6	Ornithine carbamoyltransferase, catabolic	332	*Zwa6*-other	310	100	29.07
7	Chitinase A1	674	*Chitinase C*-other	688	97.97	99.11
8	Immune inhibitor A	799	*InhA2*-other	799	100	98.37
9	Immune inhibitor A	796	*InhA1*-other	796	100	100
10	Cysteine synthase	305	*Zwa5A*-other	325	94.15	29.08
11	Ornithine carbamoyltransferase	310	*Zwa6*-other	310	100	100
12	N-(2-amino-2-carboxyethyl)-L-glutamate synthase	325	*Zwa5A*-other	325	100	99.08
13	N-((2S)-2-amino-2-carboxyethyl)-L-glutamate dehydrogenase	322	*Zwa5B*-other	322	100	99.07
14	Hypothetical protein	742	*Enhancin*-other	742	100	92.86
15	Immune inhibitor A	795	*InhA1*-other	796	100	75.25
16	Thermolysin	567	*Bmp1*-other	893	63.61	78.87
17	Hypothetical protein	893	*Bmp1*-other	893	100	96.86
18	Ornithine carbamoyltransferase	316	*Zwa6*-other	310	96.45	34.11
19	O-acetylserine-dependent cystathionine beta-synthase	307	*Zwa5A*-other	325	92.31	28.67
20	Cysteine synthase	307	*Zwa5A*-other	325	96.31	30.99
21	Thermolysin	583	*Bmp1*-other	893	66.63	31.43

Phylogenomic analysis of *Bacillus thuringiensis*

Phylogenomic comparisons between MPOB Bt1 and other *B. thuringiensis* strains were performed to show the relatedness of MPOB Bt1 to other commercial active components of pesticide products. The ANIb table, which displays the complete percentage identity (Appendix A) among 11 bacterial strains, was transformed into a heatmap for clustering analysis (Figure 3). MPOB Bt1 has a very high Average Nucleotide Identity (ANI) value with BT_1857 (0.9765), indicating that these two strains are very closely related (Figure 3). This is reflected in the heatmap as a dark blue color and in the clustering as a close grouping. *B. thuringiensis* subsp. *aizawai* strain BT-1857 has a relatively large genome size of 6,694,857 bp composed of 12 contigs. It has a moderate GC content of 34.5%, which is typical for *Bacillus* species. This strain also contains a high number of coding DNA sequences (6779) as well as 28 *rRNAs* and 104 *tRNAs*, which are important functional elements in the genome.

ANI is a measure of genomic similarity between two bacterial strains and is commonly used to determine if two strains belong to the same species (Jain *et al*., 2018). The higher the ANI value between two strains, the more closely related they are (Han *et al*., 2016). The genome features of the *B. thuringiensis* species suggest the adaptation capabilities to different ecological niches and hosts (Bonis *et al*., 2021). Furthermore, the high genome similarities of MPOB Bt1 with the commercial *B. thuringiensis* products indicate their close relationship, making them suitable as insecticides for moths and caterpillars (Plata-Rueda *et al*., 2020; Wu *et al*., 2022). To date, there are many *B. thuringiensis*-based biopesticide products in the market (Bonis *et al*., 2021). Among them, XenTari WG is a commercial biopesticide product containing *B. thuringiensis* subsp. *aizawai* strain ABTS-1857 as the active component (Ortiz and Sansinenea, 2022). Basri *et al*. (1994) also reported that *B. thuringiensis* subsp. *aizawai* was effective in controlling bagworm as compared to other subspecies such as *kurstaki*, *berliner*, and *morrisoni*. This suggests that MPOB Bt1, which is very similar to the *aizawai* subspecies, has the potential for effective biocontrol of oil palm bagworm.

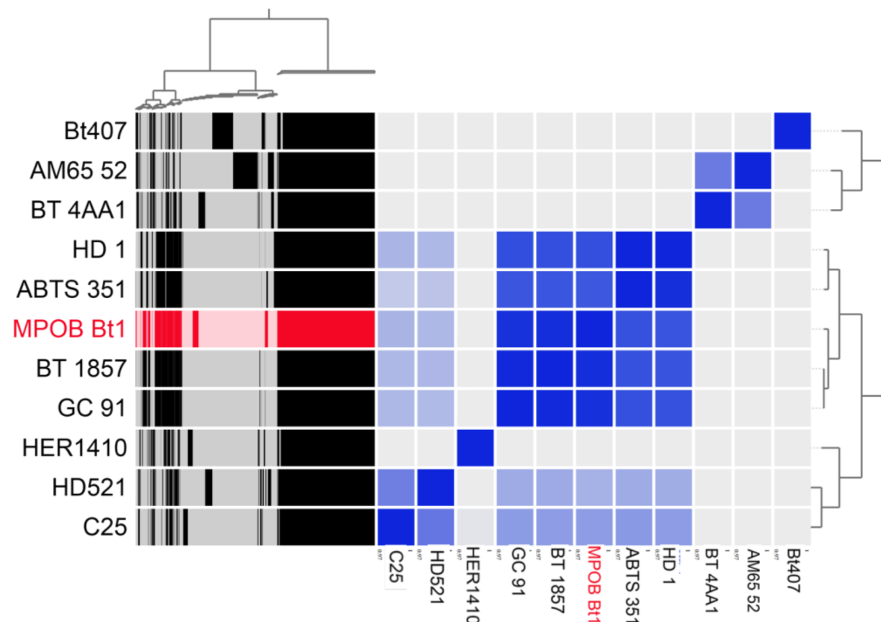

Fig. 3. Heatmap clustering of *Bacillus thuringiensis* MPOB Bt1 and other *B. thuringiensis* genomes based on ANIb percentage identity values set at 97%. The gene clusters were arranged based on the gene cluster present and absent (D: Euclidean; L: Ward). The shade of blue represents the ANIb values, whereas darker blue indicates a higher percentage of identity between the genomes.

Bacillus thuringiensis subspecies *aizawai* produces protein toxins commonly known as δ-endotoxins, which are highly specific to larvae of insects of the order Lepidoptera (Zhao *et al*., 2021). These toxins are not harmful to humans, animals, or beneficial insects, making them an ideal biopesticide for use in IPM programs (Sabbahi *et al*., 2022). In Figure 3, the δ-endotoxins MPOB Bt1 also have relatively high ANI values with GC_91 (0.974221884), an active ingredient in the commercial biopesticide Agree WP, suggesting that they are also closely related. This is reflected in the heatmap by a darker blue color compared to the other strains and they were clustered as a close group. MPOB Bt1 has lower ANI values with HD_1 (0.897522103) (DiPel BMP 123 Thuricide), ABTS_351 (0.891710198) (DiPel DF), C25 (0.832180984), HER1410 (0.793026517), HD521 (0.788192056), BT_4AA1 (0.745137161), Bt407 (0.726480198), and AM65_52 (0.662739832) (Vectobac) indicating that they are less closely related. This is reflected in the heatmap as a lighter blue color compared to the other strains and they were clustered more distantly. MPOB Bt1 is most closely related to BT_1857, followed by GC_91, and is distantly related to the other strains.

Insecticidal genes were compared by splitting the δ-endotoxins (accession number: PF00555.22) from the *B. thuringiensis* genomes in the Protein family database (Pfam) (Figure 4). As expected, the insecticidal protein of MPOB Bt1 was clustered with BT-1857, GC-91, ABTS-351, and HD-1. The close clustering of these genes (red-shaded box for MPOB Bt1 & black-shaded box for other *B. thuringiensis* genomes) in the phylogenetic tree also suggests that they may have similar modes of action or insecticidal properties-, consistent with the high degree of genomic relatedness observed between these strains (heatmap clustering of the dark red color showed high ANI value, which indicates the highest similarity) as shown in Figure 3.

Clustering analysis of *B. thuringiensis* genomes showed that two major clades with the genomes exhibiting insecticidal activity against Lepidoptera were clustered together (ABTS-1857, GC-91, ABTS-351, HD-1 & MPOB Bt1). In contrast, other genomes that are not specific to Lepidoptera were split into other clades. The AM65-52, 4AA1, and HD521 genomes were reported to have insecticidal activities against the insect order Diptera (Fayad *et al*., 2019) and Coleoptera (Al-Saeedi & Al-Jassany, 2019; Schäfer *et al*., 2023). This means that the MPOB Bt1 and reference *B. thuringiensis* genomes in this study were clustered according to the insecticidal specificity of the target insect order. The insecticidal proteins that were clustered together (Figure 4) also suggest that these proteins share a common ancestor and have a closer evolutionary relationship than other proteins in the analysis (Bonis *et al*., 2021).

Individual *Bacillus thuringiensis* MPOB Bt1 and flubendiamide insecticidal activities

The results of leaf-dip bioassays revealed that both MPOB Bt1 and Fbd were toxic to laboratory-reared *M. plana* larvae (Table 5). The LC_{50} value of MPOB Bt1 was 4.57 x 10^{10} spores mL^{-1} at 48 hr after treatment and decreased to 3.31 x 10^{10} spores mL^{-1} at 72 hr after treatment. For Fbd, the LC_{50} value increased slightly from 1.16 x 10^{-2} g mL^{-1} to 1.25 x 10^{-2} g mL^{-1} at 48 and 72 hr after treatment, respectively. The results indicate that MPOB Bt1 becomes more effective over time, as shown by the decrease in the LC_{50} value (lower concentration needed to kill 50% of the larvae at 72 hr compared to 48 hr). On the other hand, the LC_{50} value for Fbd slightly increased from 48 to 72 hr, indicating a minor decrease in its effectiveness over time.

Combining different *B. thuringiensis* toxins in a single mixture may not be more effective than using a single toxin (Roush, 1998). However, to delay the emergence of resistance, *B. thuringiensis* toxins are often used together with other pesticides (James, 2006; Carrière *et al*., 2016). Insect resistance to *B. thuringiensis*-based pesticides can be delayed by incorporating different mechanisms of action into pest management programs' efficacy (Tabashnik, 1994; Ferré & Van Rie, 2002). Fbd, a Class IV pesticide (DOA, 2024), has been shown to have no adverse effects on the oil palm pollinator, *Elaeidobius kamerunicus* (Priwiratama *et al*., 2018), making it a suitable and sustainable candidate for controlling bagworm populations in this study.

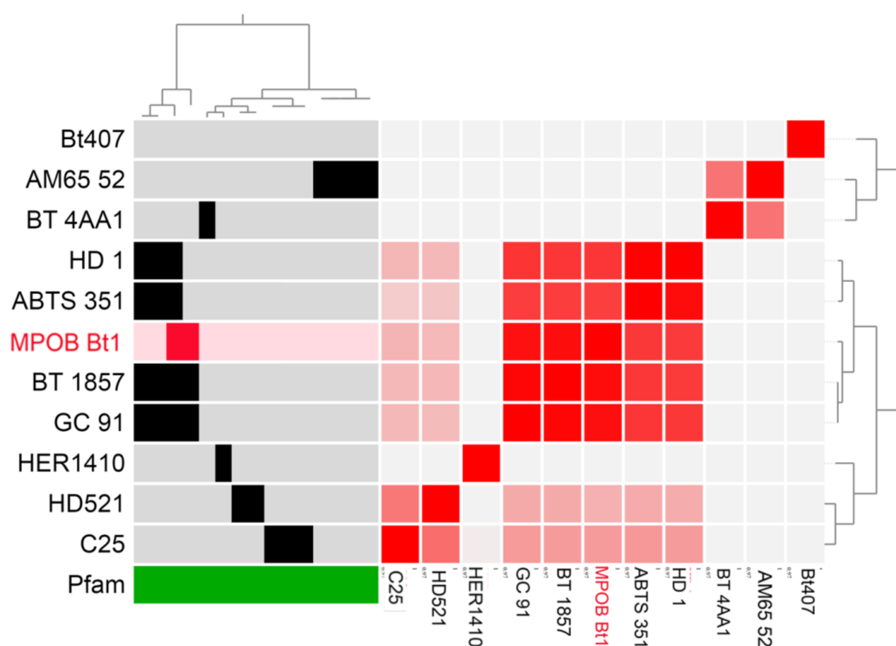

Fig. 4. Split δ endotoxin gene clusters from *Bacillus thuringiensis* genomes. The separation was conducted using anvi-split. The gene clusters were arranged based on the gene cluster present and absent (D: Euclidean; L: Ward). The shade of red represents the ANIb values, whereas darker red indicates a higher percentage of identity between the genomes. The shaded green represents the Protein family database (Pfam).

Table 5. Toxicity of individual *Bacillus thuringiensis* MPOB Bt1 and Flubendiamide, respectively against oil palm bagworm, *Metisa plana* Walker (Lepidoptera: Psychidae)

Treatment	Observation time (H)	df	LC50 (95% FL)	Slope ± SE	X2
Bacillus thuringiensis MPOB Bt1	48	4	4.57x10^{10} spore/mL (1.20-11.22)	0.565 ± 0.15	16.37
Bacillus thuringiensis MPOB Bt1	72	4	3.31x10^{10} spore/mL (1.53-9.35)	0.578 ± 0.12	9.13
Flubendiamide	48	4	1.16x10^{-02} g/mL (1.14-27.55)	0.748 ± 0.22	12.50
Flubendiamide	72	3	1.25 x 10^{-02} g/mL (1.10-6.95)	0.442 ± 0.09	2.95

Mixed *Bacillus thuringiensis* MPOB Bt1-flubendiamide insecticidal activities

To optimize the efficacy of the combined MPOB Bt1-Fbd, combinations of MPOB Bt1 and Fbd were conducted in the form of ratios between the two pesticides (Table 6). The study included five combination ratios of 0.5:0.5, 0.6:0.4, 0.7:0.3, 0.8:0.2, and 0.9:0.1, and the most effective combination

was determined based on lethal LC_{50} values at 48 and 72 hr after treatment. LC_{50} values were calculated in spores mL^{-1} to relate to MPOB Bt1, while they were calculated in g mL^{-1} to relate to Fbd. Among the five combination ratios, the 0.5:0.5 combination ratio showed the most lethal LC_{50} values at 48 hr (2.45 × 10^9 spores mL^{-1} / 2.99 × 10^{-3} g mL^{-1}) and 72 hr (1.19 × 10^9 spores mL^{-1} / 1.45 × 10^{-3} g mL^{-1}) after treatment.

Table 6. Toxicity of mixed *Bacillus thuringiensis* MPOB Bt1 and *Flubendiamide* against oil palm bagworm, *Metisa plana* Walker (Lepidoptera: Psychidae)

Treatment	Ratio	Observation time (hr)	df	LC_{50} (95% FL) (spore mL^{-1} / g mL^{-1})	Slope ± SE	X^2
MPOB Bt1 + Fbd	0.5:0.5	48	4	2.45 x 10^9 / 2.99 x 10^{-3} (1.48-14.97)	0.672 ± 0.16	9.60
MPOB Bt1 + Fbd	0.5:0.5	72	3	1.19 x 10^9 / 1.45 x 10^{-3} (1.95-6.19)	0.541 ± 0.06	3.83
MPOB Bt1 + Fbd	0.6:0.4	48	4	6.27x10^9 / 7.67 x 10^{-3} (0.76-14.77)	0.638 ± 0.24	10.62
MPOB Bt1 + Fbd	0.6:0.4	72	3	8.32x10^{10} / 1.02 x 10^{-1} (0.97-2.69)	0.209 ± 0.05	0.751
MPOB Bt1 + Fbd	0.7:0.3	48	4	5.14x10^9 / 6.29 x 10^{-3} (1.25-20.60)	0.706± 0.19	10.62
MPOB Bt1 + Fbd	0.7:0.3	72	3	8.19x10^9 / 9.97x10^{-3} (0.62-4.56)	0.227± 0.10	1.13
MPOB Bt1 + Fbd	0.8:0.2	48	4	1.78 x 10^{10} / 2.17 x 10^{-2} (1.10-11.67)	0.684 ± 0.12	10.99
MPOB Bt1 + Fbd	0.8:0.2	72	4	4.26x10^9 / 5.21x10^{-3} (1.67-22.39)	0.786± 0.18	12.78
MPOB Bt1 + Fbd	0.9:0.1	48	4	3.22x10^{10} / 3.93x10^{-2} (1.35-12.71)	0.618± 0.15	10.78
MPOB Bt1 + Fbd	0.9:0.1	72	4	4.34x10^9 / 5.31x10^{-3} (0.84-20.82)	0.622± 0.22	9.58

Synergistic evaluation

The efficacy and synergism of the MPOB Bt1-Fbd combination are shown in Table 7. Efficacy at 48 hr for MPOB Bt1 and Fbd combination (0.5:0.5 ratio) was 60%, which was higher than the individual efficacies of MPOB Bt1 (24%) and Fbd (40%). At 72 hr, the efficacy of the combination increased to 76%, compared to 28% for MPOB Bt1 and 60% for Fbd individually. The synergy ratio at 48 hr and 72 hr was observed as 1.103. and 1.067, respectively. The synergy ratio was consistently above 1 at both time points, showing a synergy effect. The expected inhibition of the mixture is expressed as a percentage, C_{exp} = A + B- AB /100 (Gisi, 1996). Synergy ratios of C_{obs} over C_{exp} above 1 indicate synergism (Gisi, 1996; Liu *et al*., 2019).

As noted in previous studies, *B. thuringiensis* and the insecticides chlorantraniliprole, tricyclohexyltin hydroxide, chlordimeform, binapacryl, and fentin hydroxide have been reported to have synergistic effects when used together to suppress *P. xylostella* strains (Hamilton & Attia, 1977; Shabbir *et al*., 2021). Similarly, this result supports a previous study by De Liguoro *et al*. (2018) in which a mixture of sulfonamides had synergistic effects on *Daphnia magna*, and reports have shown that chemical combinations have greater toxicity to the pests of interest (Zhao *et al*., 2018; Rizvi *et al*., 2018). Because they have multiple mechanisms of action, pesticide combinations are more effective than individual insecticides. Thus, combinations of chemicals may be useful in the control of Lepidoptera pests.

Table 7. Mixtures of *Bacillus thuringiensis* MPOB Bt1 and Flubendiamide efficacy towards oil palm bagworm, *Metisa plana* Walker (Lepidoptera: Psychidae)

% Efficacy					
Time after treatment (h)	Neg. Control dH2O	MPOB Bt1 (1.8 x 109 spores/mL) (A)	Fbd (0.022g/L) (B)	MPOB Bt1:Fbd (0.5:0.5) (Cobs)	Synergy Ratio
48	0	24	40	60	1.103
72	0	28	60	76	1.067

CONCLUSION

Whole genome sequencing results show that the MPOB Bt1 isolate contains the *Cry9Ea*, *Cry1Ab*, *Cry1Ca*, and *Cry1Da* genes, which have been reported to be toxic to Lepidoptera. MPOB Bt1 is also closely related to *B. thuringiensis* subsp. *aizawai* strain BT-1857 (XenTari), *B. thuringiensis* subsp. *kurstaki* strain ABTS_351 (DiPel DF), *B. thuringiensis* subsp. *aizawai* CG-91 (Agree WP), and *B. thuringiensis* subsp. *kurstaki* HD_1 (DiPel BMP 123 Thuricide). All of the individual MPOB Bt1, the individual Fbd, and the mixture of MPOB Bt1 and Fbd treatments are toxic to the oil palm bagworm *M. plana*. The combination of MPOB Bt1 and Fbd showed a synergistic effect at a ratio of 0.5:0.5,

suggesting that the combination of MPOB Bt1 and Fbd may increase toxicity to *M. plana* when used together. The future use of combined pesticides has not yet been determined. However, these synergistic combinations could be an opportunity to initiate the use of *B. thuringiensis* products and pesticides to control *M. plana*. Further studies also need to be conducted to understand the functional response of the host microbiome using high-throughput omics approaches and to develop a next-generation integrated pest management program for *M. plana*.

ACKNOWLEDGEMENTS

We thank the Director General of the MPOB and the Director of the Biology and Sustainability Research Division for approving the budget and permission to conduct this study. Additionally, we extend our appreciation to the team at Entomology and Integrated Pest Management Unit, including the head and staff, for their valuable guidance and support throughout the experiment and manuscript preparation. Special thanks are also due to Dr. Amalia's Postgraduate Data Analysis Group.

ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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