**Combination of A Nano-Emulsion Formulation of An Entomopathogenic Fungus, *Metarhizium anisopliae* with A Catch-And-Release Pheromone Trap for the Management of Red Palm Weevil (RPW) in Coconut**

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ABSTRACT

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| **The red palm weevil (RPW, *Rhynchophorus ferrugineus* Olivier) (Colioptera: Curculionidae) is a damaging insect pest of coconut in Malaysia. Severe infestation can kill the infested coconut trees. RPW is currently managed by chemical measures, removal of infested coconut trees and mass trapping of adult weevils by using pheromone traps. In 2020-2021, Malaysian Agricultural Research and Development Institute (MARDI) and Universiti Malaysia Terengganu (UMT) conducted a collaborative research to develop a biological control system for RPW by using a nano-emulsion formulation of an entomopathogenic fungus, *Metarhizium anisopliae* (MET-GRA4)  combine with a new design of catch-and-release pheromone trap. The mechanism began with pheromone attracts RPW to enter the trap. Upon entering through the inlet funnel, the beetle passes through gauze saturated with the emulsion, wetting its body with the MET-GRA4 emulsion which is sprayed at the inlet funnel, particularly the gauze area, according to the pre-set intervals.** **Since the RPW cannot access the pheromone and food bait inside the trap due to the inlet hole is covered by a gauze, it will eventually exits and returns to its population. The spores of MET-GRA4 start infecting the weevil's body 48-72 hours after exposure, leading to its death. This biological control method aims to infect the entire RPW population within the area. Three RPW hotspot locations were selected as evaluation sites in Kelantan (Kota Bharu, Pasir Putih and Bachok) and Terengganu (Kuala Terengganu, Kuala Nerus and Marang), respectively. Comparison of RPW population at the sites before and after treatment with the biological control system applications showed 28.78% reduction in Kelantan and 60.27% in Terengganu. Impacts of the RPW population reduction were significant in both states. This environmental-friendly innovation is potential to be used for monitoring surveillance and control of RPW in coconut planting areas as well as in other palms.** |

***Keywords:*** *coconut, Metarhizium anisopliae, nano-emulsion, pheromone trap, RPW*

1. INTRODUCTION

Coconut (*Cocos nucifera*), often regarded as “the tree of life” is one of the economically important industrial crop in Malaysia in term of land usage after oil palm, rubber and rice. In 2023, the planting areas of coconut was reported as 79,411.95 ha which contributed to 623,662.73 metric tonne of nut production [1]. Johore, Selangor, Perak and Kelantan are major coconut producers in Malaysia. Nevertheless, The coconut productivity are affected by insect pests infestations including the red palm weevil (RPW), thus threatening the sustainability of the crop sector locally.

RPW, scientifically known as *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae) that is originally native to Southeast Asia, particularly Indonesia, Malaysia and the Philippines [2], has successfully spread worldwide where palm trees such as coconut, date, oil palm and ornamental palms are cultivated. The economic importance of RPW as a destructive pest for palm trees has been recognized since 1880s [3]. All *Rhynchophorus* species are polyphagous. Females are usually attracted by palm volatiles and lay several eggs in dying or damaged parts of palms, although undamaged palms could also be attacked, and after a few days, eggs hatch into larvae which develop within trunks of palms, frequently leading to the plant death [4]. The natural habit of RPW as a concealed tissue borer causes difficulty to detect its infestation at an early stage. Symptoms such as wilting and drooping of the dried leaves forming like an umbrella-shaped or skirting shaped leaves are the signs of a late stage of RPW infestation [5]. The larval stage of RPW is the most critical to control because the larvae generally cause serious damage to the palm tree [6]

Destructive effects on coconut trees due to RPW infestation are often resulting in removal of the affected trees, and require additional cost for treatments to overcome the pest spread. Treatment with chemical insecticides are still the most effective RPW control. In 2019, Syngenta Tree Micro injection using emamectin benzoate micro emulsifier in two formulations Revive®4% and ReviveII®9.5% with previlage to ReviveII®9.5% showed a very promising techniques to control and protect the invasion of RPW on date palm [7]. Meanwhile, penitrothion, thiamethoxam, imidacloprid and fipronil caused 100% mortality against 8th instar RPW larvae when tested under laboratory conditions [8]. In Malaysia, studies showed that carbosulfan was the most effective chemical control against RPW of which delivered via trunk injection method in coconut compared with cypermethrin and acephate [9]. Another common method of RPW control as reported elsewhere is using pheromone traps with or without food baits. The traps not only serve as monitors to indicate the presence of the pest in and around the plantation, but also play a vital role in reducing the RPW population levels in the field [10,11]. The role of entomopathogenic fungi (EPFs) as biological control agents for RPW had been revealed. *Beauveria bassiana* and *Metarhizium anisopliae* are two of the most potential EPFs for killing the weevil, thus reducing its population, particularly when used in conjuction with other means of control such as pheromone traps, insecticides and sanitation [12]. In Malaysia, management of RPW by the Department of Agriculture is via chemical measures, removal of infested coconut trees and mass trapping of adult weevils by using pheromone traps [13]. However, the efficacy of the control techniques is yet unknown as infestations have steadily increased over the past decades [14]. In 2020-2021, Malaysian Agricultural Research and Development Institute (MARDI) and Universiti Malaysia Terengganu (UMT) conducted a collaborative research to develop a biological control system for RPW by using a nano-emulsion formulation of an entomopathogenic fungus, *Metarhizium anisopliae* (MET-GRA4)  combine with a new design of catch-and-release pheromone trap.The present article describes laboratory and field evident of the new biological control system for reducing RPW population in coconut growing areas.

2. material and methods

**2.1 Chemicals and Materials**

The pure culture and spores of MET-GRA4 were prepared by UMT. The spores were freshly grown, mass-produced through solid-substrate fermentation, and subsequently dry-harvested using a sieve shaker. The drying temperature for post-fermentation solid substrate was maintained at room temperature (25 ◦C ± 3 ◦C). The spore viability was assessed before using them in experiments. Only MET-GRA4 with a high viability of over 70% were selected and used for subsquent experiments. Potato dextrose agar (PDA), Tween-20 from Sigma Aldrich (USA), ultrapure water (Direct Q3, Millipore, Billerica, MA, USA) were used throughout the study.

**2.2 Preparation of Invert Emulsion Containing MET-GRA4**

An invert emulsion consisting of MET-GRA4 was produced according to Batta, 2016 [15] with slight modifications. The oil phase and the water phase containing the Metarhizium fungus, MET-GRA4 were combined and stirred using a vortex for 5 minutes at 2000 × g to form a coarse emulsion. The fine emulsion was prepared by homogenising the coarse emulsion using a homogeniser at 3000 × g for 1.5 minutes at room temperature (27 °C). The produced invert emulsion from this process proceeded for stability test, polydispersity (PDI), size of the emulsion droplets characterisation by dynamic light scattering method using Zetasizer (Brookhaven Instruments, USA) and the ability of the invert emulsion formulation containing *M. anisopliae* to grow on Potato Dextrose Agar (PDA).

**2.3** **Pathogenicity Test of MET-GRA4 Nano-emulsion Against RPW**

For pathogenicity test, healthy larvae and adults of RPW, free of mites, and measuring 3.0-3.5 cm in length were selected and sterilised by dipping in 70% alcohol for no more than 15 s, followed by three simultaneous dips in sterile distilled water. The nano-emulsion were adjusted to a concentration of 1 x 102, 104, 106 and 108 spore mL-1 for the treatments. The disinfected RPW larvae and adults were sprayed with the prepared spore suspensions for around 60 s (~1 mL) to ensure that the spores were evenly distributed on the surface of the weevils’ bodies. The inoculated RPW were kept in containers at a temperature of 25 ± 1 °C and a photoperiod of 12 h (L)/12 h (D). The lid of each container had small holes punctured in it. Three replicates were used for each treatment, with 10 RPWs per container (560 mm in length × 270 mm in width × 360 mm in height). Control treatments involved RPW treated with distilled water.

**2.4 Fabrication of a Catch-and Release Pheromone Trap**

Trap was constructed from PVC pipe and incorporated several key components, such as an automatic sprayer, a bait container, and a compartment for pheromones. The automatic sprayer was programmed to activate at adjustable intervals of 9, 18, and 36 minutes and powered by two 1.5V alkaline batteries for ease of use and maintenance in the field. Additionally, the trap featured an inlet funnel that served as an entry and exit point for RPW beetles. Gauze was placed at the inlet to ensure that the nano-emulsion of MET-GRA4 continually wetted the entrance area, enhancing the infection rate of RPW. However, the beetles were not able to reach the bait section, aligning with the trap's catch, infect, and release design. The trap also has a cover to protect it from rain, making it suitable for field use.

**2.5 Evaluation Site, Experimental Design and Data Analysis**

Evaluation sites for validation of the new biological control system for RPW in coconut areas were conducted at three hotspots in Terengganu (Kuala Terengganu, Kuala Nerus and Marang) and Kelantan (Pasir Putih, Kota Bharu and Bachok), respectively. Each evaluation site was 1-2 ha. Experimental design was carried out in Completely Random Design (CRD) with three replications. Seven new catch-and-release pheromone traps were installed for two months at each evaluation site including one trap as a negative control (without pheromone). The RPW population before and after treatment (application of the new biological control system) at each site was determined by trapping with current pheromone trap for six months. Parameters that were measured were monthly collection of RPW adults trapped in the current pheromone traps and mean number of the population at each evaluation site in Kelantan and Terengganu before and after treatment with the biological control system. Statistical analysis was conducted by using SAS 9.4 software and GraphPad Prism. The RPW populations before and after treatment were compared and statistically analysed using Student’s T-test.

3. results

**3.1 Properties of Invert Emulsion Containing MET-GRA4 and Pathogenicity Test Against RPW**

The invert formulation was optimised by observing the stability properties and the results are shown in Table 1 and Figure 1. Based on the result, the droplet size obtained was estimated at 79.1 nm and the polydispersity index (PDI) value was estimated at 0.208, which showed a good production of polydispersity of the invert emulsion droplet. The sized droplet was found to be physically stable for up to six months at room temperature.

Table 1. Physicochemical Properties of the Invert Emulsion Containing MET-GRA4

|  |  |  |
| --- | --- | --- |
| Characteristic | Value | |
| Appearance | | Milky white |
| Droplet size | | 79.1 nm |
| PDI | | 0.208 |
| Storage stability | | No phase separation up to 6 months |

A picture containing cup, table, indoor, sitting

Description automatically generatedA picture containing indoor, plastic, milk

Description automatically generated

(A) (B)

Figure 1. Observation On A) A Stable Invert Emulsion Formulation Without Phase Separation and B) An Example of Unstable Formulation Showing Two Phases of Separated Formulation.

The efficacy of the developed formulation was determined by the growing ability of MET-GRA4 spores on PDA. Figure 2 shows that MET-GRA4 spores in the invert emulsion were successfully cultured on PDA, indicating the viability and suitable use as a biocontrol agent for RPW.

A picture containing indoor, cup, sitting, doughnut

Description automatically generated

Figure 2. Growth of MET-GRA4 on PDA.

Data showed that the pathogenicity of the nano-emulsion formulation was confirmed against RPW. The mortality of RPW larvae and adults at 10 days after treated with different concentrations of nano-emulsion of MET-GRA4 are shown in Table 2. There was no significant difference in larvae mortality between the emulsion with 106 of spore concentration and 108 concentration. Thus, 106 conidia mL-1 was selected for further application.

Table 2. Mortality of RPW Larvae and Adults Treated With Different Concentrations

of Nano-emulsion of MET-GRA4 Using Direct Dipping Method

|  |  |  |
| --- | --- | --- |
| Concentration  (conidia mL-1 ) | % mortality  Larva + S.E | % mortality  Adult + S.E |
| 108 | 76 ± 0.37a | 88 ± 0.26a |
| 106 | 86 ± 0.35a | 60 ± 0.42a |
| 104 | 46 ± 0.67b | 28 ±0.45b |
| 102 | 23 ± 0.92c | 8 ± 1.09c |
| Distilled water | 0 ± 0d | 0 ± 0d |

Same alphabet in a column is not significant at p<0.05

**3.2 Fabrication of a Catch-and Release Pheromone Trap**

A new design of catch-and-release pheromone trap is shown in Figure 3. The trap's mechanism begins with pheromones attracting the RPW to the trap. Upon entering through the inlet funnel, the beetle passes through gauze saturated with the emulsion, wetting its body and initiating infection. The MET-GRA4 emulsion is regularly sprayed at the inlet funnel, particularly the gauze area, according to the pre-set intervals. Since the RPW cannot access the pheromone and food baits inside the trap due to the inlet hole is covered by a gauze,it eventually exits and returns to its population. The spores of MET-GRA4 start infecting and attacking the weevil's body 48-72 hours after exposure, leading to its death.



Figure 3. A new design of catch-and-release pheromone trap hang on a coconut tree at 1-2m from the ground.

**3.3 Field Evaluation of New Biological Control System for RPW in Kelantan and Terengganu**

The effectiveness of the new biological control system for RPW were evaluated at hotspots of coconut growth areas in Kelantan and Terengganu. The mean populations of RPW at evaluation sites in Kelantan before and after the application of the new biological control system were 82 and 58.4, respectively. Meanwhile, the mean population of RPW at evaluation sites in Terengganu before and after the application of the new biological control system were130.25 and 51.75, respectively. Therefore, based on the mean populations, significant reduction of the RPW population were determined as 28.78% in Kelantan and 60.27% in Terengganu.

Table 3. T-test Comparison of Mean Population of RPW at Three Evaluation Sites in Kelantan and Terengganu Before and After Treatment With the New Biological Control System

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Parameter / State | Rt | N | Min ± S.E | SD | T value | df | P value | Mean difference | 95% |
| confidence interval |
|  | SB | 5 | 82 ±3.9 |  | 2.613 | 8 | 0.031 | 23.6 | -44.43 to |
| Kelantan | -2.774 |
|  | SL | 5 | 58.4±8.1 | 18.15 |  |  |  |  |  |
|  | SB | 4 | 130.25±19.9 | 39.85 | 2.995 | 6 | 0.0242 | 78.25 | -142.6 to |
| Terengganu | – 14.36 |
|  | SL | 4 | 51.75±17.0 | 34.06 |  |  |  |  |  |

Rt=Treatment, SB=Before Treatment, SL=After Treatment

4. DISCUSSION

In this work, an invert emulsion formulation based approach was developed by incorporating the Metarhizium fungus, MET-GRA4 in the system. The sized droplet was found to be physically stable for up to six months at room temperature as Tween 20 used in this formulation was effective in minimising the droplet size owing to its rapid adsorption onto the droplet surface [16]. The viability of MET-GRA4 is essential, as the preparation process may have killed the fungus by affecting its viability. The ability of MET-GRA4 spores grown on PDA proved the efficacy of the developed formulation, indicating that they were viable and suitable for use as a biocontrol agent for RPW.

When sprayed on RPW adult, the MET-GRA4 spores in the invert emulsion adhered to the cuticle with the aid of the oil in the formulation. The outer layer of MET-GRA4 spores is composed of interwoven fascicles of hydrophobic rodlets that adhere to the insect cuticle due to non-specific hydrophobic forces [17]. The spores will eventually germinate and invade the interior, infecting the cuticular membrane of the weevil. The advantage of using invert emulsion in the formulation of entomopathogenic fungi is that these emulsions contain the water necessary for the germination of fungal conidia during or after application, thereby enhancing the fungal efficacy during application against targeted insects [18]. The prepared nano emulsion showed capability in killing both RPW larvae and adults in the laboratory bioassays, thus indicating its pathogenicity against the weevil.

Application method of the entomopathogenic fungus as a biological control agent for RPW was facilitated by using a new design of catch-and-release pheromone trap. The nano-emulsion formulation of MET-GRA4 when combined with a new design of a catch-and-release pheromone trap was found effective in reducing the RPW population at evaluation sites in Kelantan and Terengganu. The obtained results are in agreement with Sewify and colleagues [19] whom applied an IPM-based approach using *Beauveria bassiana* as an entomopathogenic fungus combined with pheromone-mass trapping which was found successful in reducing the RPW population in Egypt. The same approach was also successful in suppressing *Spodoptera frugiperda* (Lepodoptera: Noctuidae) population under laboratory condition as reported by Akutse and co-workers [20].

5. Conclusion

The significant reduction of RPW populations at evaluation sites (coconut areas) in Kelantan and Terengganu has proven the function and effectiveness of the new biological control system. This IPM based approach could be used as a new strategy and offers a more environmental-friendly control methods for the RPW management in coconut and other palms.

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