Faculty of Resource Science and Technology

Isolation and Characterization of Sago Worm Entomopathogenic Fungi

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Isolation and Characterization of Sago Worm Entomopathogenic Fungi

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SUPERVISOR: Dr. Ngieng Ngui Sing

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Isolation and Characterization of Sago Worm Entomopathogenic Fungi

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ABSTRACT

Entomopathogenic fungi (EPF) found in the soil are also known as the natural enemies of insects have the ability to cause infection and eventually kills the arthropods. Apart from that, due to the pathogenicity of EPF, studies also showed that this fungi act as a potential insect pest control agent against the RPW. The larvae of red palm weevil (*R. ferrugineus*) known as the sago worm has been one of the most invasive species causing severe damage in the palm series where feeding of the soft tissues by the larvae of red palm weevil might leads to the death of the palm plantations. It is postulated that the natural antagonist agent such as the entomopathogenic fungi has been adapted to the harsh environment would be effective to combat the spreading of the red palm weevil pest. Hence, the objectives of this project are to isolate entomopathogenic fungi of sago worm by using the baiting method and to identify and characterize the isolated entomopathogenic fungi. EPF were isolated from three different types of soil includes the Coconut soil, Nipah soil 1 and Nipah soil 2 by using sago worm as the bait. The obtained fungal isolates were sub cultured on the Potato Dextrose Agar (PDA) in order to obtain a pure culture of the potential EPF capable of infecting the sago worm. Further molecular identification approach was done to confirm on the identity of those isolated fungi. Out of the 29 fungal EPF isolates, 5 potential entomopathogenic fungi were successfully isolated and identified namely *Talaromyces apiculatus, Penicillium ochrochloron, Fusarium sp., Aspergillus terreus* and *Aspergillus nomius* These isolates could potentially be used as biological control agent against the red palm weevil.

Key words: Entomopathogenic fungi, red palm weevil larvae, sago worm, isolation

ABSTRAK

*Kulat entomopatogenik (KEP) juga dikenali sebagai musuh semula jadi serangga dan dapat dijumpai pada serangga yang mati dalam tanah. Selain itu, disebabkan oleh daya kebolehjangkitan kulat tersebut, kajian di seluruh dunia mendapati penggunaan kulat ini berpotensi sebagai agen kawalan perosak serangga. Larva kumbang sawit merah (*R. ferrugineus*) yang dikenali sebagai cacing sagu telah menjadi salah satu spesies yang paling invasif menyebabkan kerosakan teruk dalam siri sawit di mana pemakanan tisu lembut oleh larva kumbang sawit merah mungkin akan menyebabkan kematian kelapa sawit. Kulat Entomopatogen yang terdapat di dalam tanah juga dikenali sebagai musuh semula jadi serangga yang mempunyai keupayaan untuk menyebabkan jangkitan kulat dan membunuh artropod. Selain itu, kajian di seluruh dunia telah mendapati penggunaan kulat ini berpotensi sebagai agen kawalan serangga perosak disebabkan oleh patogen kulat tersebut. Larva Kumbang Red Palm Weevil (RPW) yang dikenali sebagai ulat sagu telah menjadi salah satu spesies paling invasif yang boleh menyebabkan ancaman teruk dalam tanaman sawit di mana pemakanan tisu lembut oleh larva RPW mungkin menyebabkan kemusnahan ladang sawit. Agen antagonis semula jadi seperti kulat entomopatogen telah disesuaikan dengan persekitaran yang buruk akan berkesan untuk memerangi penyebaran perosak RPW. Oleh itu, objektif projek ini adalah untuk mengasingkan kulat entomopatogen dan ulat sagu dengan menggunakan kaedah pengumpanan dan untuk mengenal pasti serta mencirikan kulat entomopatogen. Kulat entomopatogen diasingkan daripada tiga jenis tanah yang berbeza termasuk tanah kelapa, tanah nipah 1 dan tanah nipah 2 dengan menggunakan ulat sagu sebagai umpan. Pengasingan kulat yang diperolehi adalah subkultur daripada agar dekstrosa kentang (PDA) untuk mendapatkan kultur tulen kulat entomopatogen yang mampu menjangkiti ulat sagu. Pendekatan pengenalpastian molekul selanjutnya dilakukan untuk mengesahkan identiti kulat tersebut. Daripada 29 kulat yang diasingkan, 5 kulat* *entomopatogen yang berpotensi berjaya diasingkan dan dikenal pasti iaitu* Talaromyces apiculatus, Penicillium ochrochloron, Fusarium sp., Aspergillus terreus *dan* Aspergillus nomius. *Pengasingan ini berpotensi digunakan sebagai agen kawalan biologi terhadap kumbang sawit merah.*

Kata kunci*: Kulat Entomopatogen, Larva kumbang sawit merah, ulat sagu, pengasingan*

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LIST OF ABBREVIATIONS

EPF	Entomopathogenic fungi
IPM	Integrated Pest Management
FAO	Food and Agriculture Organization
USD	US Dollar
RPW	Red Palm Weevil
w/w	Weight per weight
DOA	Department of Agriculture
FELDA	Federal Land Development Authority
EPPO	European and Mediterranean Plant Protection Organization
NaoCl	Sodium hypochlorite
PDA	Potato Dextrose Agar
PCR	Polymerase Chain Reaction

CHAPTER 1

INTRODUCTION

1.1 Study Background

Entomopathogenic fungi (EPF) - the cosmopolitan components of the soil have the ability to cause infection and eventually kills the arthropods. The entomopathogenic fungi acts as a vital niche in the microbial control of insect pests. In fact, most of the insect orders are at risk of being infected by fungal diseases (Sharma et al., 2020). These entomopathogenic fungi infects the insect's entire life cycle by penetrating directly through their cuticle. The infection process starts with fungal spores breaching the insect cuticle through mechanical pressure and degradation of enzyme to gain entry into the insect's body under ideal conditions of moderate temperatures. Litwin et al. (2020) stated that the fungi eventually start to grow and multiply once they are inside the arthropod's body. In addition, some EPF may have the ability to produce blastospores that will enter the host's hemolymph producing secondary hyphae that invade the tissues of the host. Because of their mode of infection, EPF are gaining the interest of numerous researchers as a potential source of biological control agent for the control of insect pest in the agriculture field. Some of the commonly studied entomopathogen includes the *Beauveria bassiana* and *Metarhizium anisopliae* (Fong et al., 2018).

To date, little studies have been done on the entomopathogenic fungi capable of infecting *Rhynchophorus ferrugineus*. *R. ferrugineus* or commonly known as the "sago worm" by the locals is characterized under the family of Curculionidae. Native to Southeast Asia, sago worms are also known to be a kind of exotic delicacies to some of the local especially among most Sarawak tribes such as the Dayak and Melanau due to its high nutrient content such as the essential amino acids and fatty acids that is necessary for human health (Ogbuagu & Emodi, 2014). The life cycle of the palm weevil begins when the adult female weevils laid around 200 eggs into the holes of the trunk that were chewed using their long rostrum. The plump yellowish larvae with dark brown hard heads which hatched out of the eggs after several days started to feed on nutrients by burrowing into the interior part of the palm and thus causing major damage to the soft tissues surrounding the apical meristems (Dembilio & Jacas, 2011). Because the larvae of the red palm weevil burrows and eats voraciously into the heart of sago palm, this will then lead to mortality of the trees. RPW was reported as a major invasive insect pest for a large portion of palm species around the globe (Azmi et al., 2017). The severe damage of the palm cultivations might lead to a major reduction in yield of the palms, as well as the increasingly cost of pest management.

According to Kassem et al. (2020), the red palm weevil causes around 40 palms series belonging to 23 different genera to be damaged in more than 60 countries. In countries like Saudi Arabia, the red palm weevil was found to be responsible for the loss of 80,000 palm trees with an estimated economic loss that reaches USD 8.69 million for its destruction and management. Even in Malaysia, along the East coast to be precise, there were multiple reports of red palm weevil infecting coconut palms which caused severe damage to a total of 465 hectares of coconut palm plantations in all seven Terengganu districts that leads to a drastic loss in Malaysia's economy (Lin et al., 2017).

Nowadays, one of the commonly used approaches in pest control and management mostly relies on the use of insecticide. Based on Lin et al. (2017), chemical insecticides such as Cypermethrin are widely utilized in Malaysia to control pest infestations. However, insecticide application is unfeasible as a long-term solution because they often show high properties of persistence and toxicity in the environment that causes negative impact to the ecosystem and human health (Yan et al., 2021). Thus, this called for a rapid search for the entomopathogenic fungi that is potentially useful as a biological agent due to their environmentally friendliness which also leads us to the objective of this study.

1.2 Objectives

The objective of the study is to isolate and identify potential sago worm entomopathogenic fungi. More specifically, the objectives of this study include:

i. To isolate entomopathogenic fungi of sago worm by using the baiting method

ii. To identify and characterize the isolated entomopathogenic fungi

The potential entomopathogenic fungi capable of infecting sago worm were baited using baiting method in order to obtain the potential EPF. The fungal isolates were further identified via the molecular approach to confirm on the identity of the isolated fungi. Recent studies reported that entomopathogenic fungi plays a significant role as a pest biocontrol. However, entomopathogenic fungi living in association with larvae have not been fully characterized and identified.

CHAPTER 2

LITERATURE REVIEW

2.1 Basic Taxonomy of Sago Worm, *Rhynchophorus ferrugineus*

The red palm weevil *(Rhynchophorus ferrugineus),* also known as the Asian palm weevil or sago palm weevil is considered to be one of the most exotic pest species on palm plantations. The sago grub (sago worm) is the larvae stage of the sago palm weevil that consumes sago palm during its infancy. The red palm weevil is a native species of Southeast Asia, and this palm borer has been expanding towards the Middle East, Mediterranean Basin and the Caribbean since mid-1980s due to several accidental anthropogenic introductions and the cryptically infested planting material (Hazzouri et al., 2020). The red palm weevil (RPW) was classified under the Phylum Arthropod, Class Insecta, Order Coleoptera, Family Curculionidae and the genus Rhynchophorus.

The Coleoptera order is known as the largest among insects having over 400,000 species that accounts for more than one fifth of metazoans including forest and agriculture pest. Based on Hazzouri et al. (2020), *R. ferrugineus* is categorized as a Coleopteron pest under the Cruculionidae family whose larvae are a threat palm species around the globe. Besides, as stated by Giblin-Davis et al. (2013), the weevil borers of palms are members of the seven natural lineages with the ‘Curculionidae’ sensu lato, with the subfamily Dryophthorinae, being the largest threat to the palm species.

According to Soroker and Colazza (2017), *R. ferrugineus* is one of the weevil species belonging to the Dryophthorinae subfamily, which is the most economically important insect group. In addition, Dryophthorinae subgroup is a ubiquitous monophyletic group of pantropical weevils that includes 1,200 species in 153 genera (Chamorro et al., 2021). The Dryophthorinae family has four tribes which are well-known from palms such as the

Rhynchophorini that includes the genus Dynamics and Rhyncophorus; the Diocalandrini that includes the genera Diocalandra; the Sphenophorini that includes Rhabdoscelus, Rhabdoscelus and Temnoschoita; and the Orthognathini which includes the genus Mesocorylus and Rhinostomus (Giblin-Davis et al., 2013). Dryophthorines are commonly used as a model for the study of weevil development. Studies had shown that the Dryophthorine red palm weevil are known to be the first weevil to have a genome published due to their widespread economic importance, and the fact that a study has never been conducted on this well- known weevil subfamily is surprising (Hazzouri et al., 2020).

Based on Giblin-Davis et al. (2013), the Rhynchophorus species are commonly known as ‘palm weevils’ and have relatively large sizes, with the adult having a length up to 5 cm in the body and 3 cm wide, whereas the size of the larvae is approximately 5 cm long and 2 cm wide. The coloration in the Rhynchophorus species varies greatly, ranging from entirely black to reddish brown. In addition, the Rhynchophorus species such as, *R. ferrugineus, R. richteri, R. quadrangulus, R. bilineatus, R. distinctus, R. lobatus and R. vulneratus* are known to be polyphagous and have life histories that is similar, which is the behaviours and characteristic activity periods that each species in the genus exhibit (Mazza et al., 2014). For instance, the female palm weevil that are commonly attracted by the volatiles palm and lay eggs at the damaged part of the palm that might results in plant death when the eggs successfully hatch into larvae developing within the trunks of the palm (Mazza et al., 2014). Interestingly, Murphy and Briscoe (1999) stated that the red palm weevil (*R. ferrugineus*) is the most widely distributed species among Rhynchophorus species as stated above.

2.2 Background of Sago worm

2.2.1 The life cycle of sago worm

Sago worm is known as the larvae of sago palm weevil (*R. ferrugineus*) that is classified under the order of Coleoptera and family of Curculionide. According to Engsontia and Satasook (2021), this weevil completes almost entire of its life cycle depending on the host plant Arecaceae or palms that includes the date palm, sago palm, oil palm and coconut palm. *R. ferrugineus* commonly takes approximately four to five months to complete its life cycle that comprises four developmental phases including egg, larva, pupa and adult (Azmi et al., 2017).

According to Leatemia et al. (2021), the life cycle begins with the female weevil chewing a hole that has a depth of approximately 3-8 mm penetrating the palm tissue, which in most cases is the petiole base of the palm leaves, by using their long beak or snout. Eggs were then laid by the female weevil into these holes or the damaged surfaces (after Oryctes attack) in the trunks of the sago palm, the eggs are observed to be whitish yellow in color, cylindrical in shape which is around 2-2.75 mm in length and 0.75-1 mm in width (Leatemia et al., 2021). Studies had shown that the gravid female weevil could lay up to 530 eggs within 120 days during their life span, with the mean number of eggs laid being around 250 eggs (Mahmud et al., 2015).

After 3 to 5 days, the eggs will hatch into plump creamy white colored legless larvae with dark brown hard heads, which is known as the apodous first instar larvae (Leatemia et al., 2021). The newly emerged larvae that have a segmented body texture feed on the surrounding succulent palm tissue by tunneling deep towards the center of the palm. The feeding galleries formed were subsequently contained with a distinctive odor frass mixed with the plant exudates that resulted in the fermentation of palm tissue. This palm tissue fermentation process favored the development of the larvae stage as it produces heat up to 40 ℃ which results in fragile palms. The larvae grow up to 5 cm in length, this stage is known to be harmful and lasts between 36-78 days (Mahmud et al., 2015). A full larvae development comprehends 6 instars with an increasing potential host damage and size in which the duration is based on the nutrition available in the host, humidity as well as temperature (Giblin-Davis et al., 2013).

The larva migrates periphery at maturity forming a dark brown hard cocoon with the plant fiber. Pupation occurs in the spiral and oval-shaped cocoon that is about 60 mm in length and 30 mm in width (Bozbuga & Hazir, 2008). This stage takes up roughly 4-17 days and form an imago, which is the adult stage of the weevil. The adult weevil has an average life span of approximately 2-3 months. The soft strands of hairs on the snout of male weevils can be used to distinguish them from female weevils (Hussain et al., 2013). The life cycle of the sago palm weevil can be described as a monoxenous parasitic cycles as all their life cycle stages occur on the same tree (Mahmud et al., 2015).

2.2.2 Nutritional composition and utilization of sago grub

The community has been grappling with the issue of food security due to the increasing demands of consumers caused by the growing global population. According to Tuhumury (2021), the world population approached 7.8 billion people in 2020 and is projected to exceed 9 billion people by 2050. Nutritional needs and sustainability are considered vital to the concept of food security in providing an environmentally food system. Among animal resources, it was recommended that the edible insects are considered as a potential nutritional alternative, and also a role in sustainable diets (Solomon et al., 2020). However, the nutritional aspects of edible insect changes depending on their living condition and feed composition as well as the preparation and processing before consumption (Oonincx & Finke, 2021).

According to Chinarak et al. (2020), the larva of red palm weevil (*Rhynchophorus ferrugineus*) is commonly known as the “sago grub” or the “sago worm” since they are produced from the sago palm. It is among the popular edible insects that are rich in both macro (sodium, potassium, calcium, magnesium and phosphorus) and micro (zinc, iron, copper and manganese) elements (Chinarak et al., 2020). It can also act as secondary protein source and other nutrients supplement in both humans' and animals' diet, the mineral analysis of the RPW larva is as shown in Table 1.1 (Abdel-Moniem et al., 2017). Studies from Cito et al. (2017), have shown that the red palm weevil larva has a fat content percentage of 59.71% (w/w) consisting of saturated fatty acids (palmitic and stearic acid), monounsaturated fatty acids (oleic and palmitoleic acid) and polyunsaturated acids (a-Linolenic acid and a-Linoleic acid). The Linolenic acid which is also known as the omega 3 acid while Linoleic acid by means of the omega 6 acid. Both the Omega-3 and Omega-6 are the important human nutrition that cannot be produced by the human body and had to be taken from sources of food intake. Thus, sago grubs are said to be a potential source of protein for human and livestock (Leatemia et al., 2021).

Table 1.1: Mineral composition (mg/kg) of the red palm weevil larva (*R.ferrugineus*) (Abdel-Moniem et al., 2017)

Minerals	**Mg/100kg**
Sodium (Na)	460.0
Potassium (K)	45.0
Calcium (Ca)	22.0
Magnesium (Mg)	210.0
Phosphor (P)	43.0
Zinc (Zn)	11.0
Iron (Fe)	99.0
Copper (Cu)	ND
Manganese (Ma)	4.0

Commonly, these larvae of sago palm weevil can be consumed raw after removing their head that gives a creamy tasting or either being cooked (boiled, fried or sauteed) (Ogbuagu & Emodi, 2014). It is regarded as a delicacy in Papua New Guinea, where the sago worms were roasted on spit for the celebration of special occasions. In South Asian countries like Malaysia, “Fried Sago Worms” are considered as one of the specialities by the Melanau ‘river people’ in Sarawak and the Kadazandusun tribespeople in Sabah (Sageng et al., 2020).

2.2.3 Signs and symptoms of the RPW infestation

Edible as it may be, it is undeniable that the RPW is a kind of mortal insects, causing the most devastating damage to the palm. In China, several studies had been conducted to study the RPW’s feeding pattern along with their growth and development on different host plants, the findings show that the grubs that spending their early stages of life inside the tree trunk boring through soft tissues will often result in the disruption of the palm’s vascular system (Hussain et al., 2013). The signs of an infested palm are visible only after the palm trees are damaged, thus it is challenging to identify any infested palm plantations at the early stage of infestation without specialized equipment, the trees will be destroyed beyond remediation before the presence of the pest is detected (Hazzouri et al., 2020).

According to Alshammari et al. (2021), the host infection by red palm weevil begins with an injury such as a cut in tender areas like the apical zones and armpit palm, or the digging tunnel. Dembilio & Jacas (2015) stated that the detection of the Canary Island palm (*Phoenix canariensis*) and date palm infestation based on the visual symptoms was done recently that it relies on the staff’s experience and their detection skills in the field. The signs that show the presence of the RPW is the perforations found in the trunk or crown of the palm tree and the ejections of fibers that are chewed-up from the crown of an infested date palm as shown in Figure 2.1. The wilting of the developed fronds and frond loss (Figure 2.2) will hinder symmetrical growth of the crown and might cause it to later collapse (El-Shafie & Faleiro, 2020). As stated by Witt et al. (2020), the apodous last instar larvae with a brown-red head capsule and a curved body had caused a serious damage to the date palm on the Socotra Island located between Hadiboh and Hawlaf as shown in Figure 2.3.

Figure 2.1: Example of an ejected fibers that are chewed-up from the infested date palm's crown (El-Shafie & Faleiro, 2020)

Figure 2.2: Example of the visible frond loss in the infested date palms (Dembilio & Jacas., 2015).

Figure 2.3: Example of the date palm trees infected by the red palm weevil on Socotra Island (Yemen) (Witt et al., 2020).

Based on Fui (2013), the infestation of RPW was first reported on coconut palm where the damage started mainly with the larvae moving inside the palm from the top of the palm making tunnel. Azmi et al. (2013) stated that the infection of the coconut palm (*Cocos nucifera*) by red palm weevil occurs in three different ways. The first way is through the shoot and to the cabbage of the coconut trees while the second way is through the trunk by chewing holes on the trunk with their snout. The third way is through soil digging and tunnelling of the adults red palm weevil into the coconut palm root system.

The signs and symptoms on the infected coconut palms that are commonly observed are the holes and tunnels found in the petioles (Figure 2.4) which are dark in color that oozed out thick brownish fluid (Azmi et al., 2017). The most destructive stage in the damage of petiole is the appearance of chewed up plant tissue, the soft tissue that were eaten by the larvae will later turn into dark and brownish color. Thus, with the minimal hard tissue left, the trunk will become hollow at the end stage of the infestation (Azmi et al., 2017). The shoots of the infected coconut tree will turn brown and become dried entering the advanced stage of the infestation which is the drooping of the fronds and the umbrella-shaped of the infested coconut trees (Fui, 2013). Figure 2.5 shows an example of the yellowing and drooping of the fronds. At this point, the increasing feeding of the larvae causes the tree to become weak and might lead to the break off of the stem or the complete death of the coconut palm trees as shown in Figure 2.6 (Nangai & Martin, 2019).