Red Palm Weevil: Understanding the fungal disease mechanism and host defense

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Synthetic pesticides remained the mainstay of Red Palm Weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) control over 50 years. However, insecticide resistance, pest resurgence and concerns over human health and environmental pollution by insecticides have encouraged researchers for the development of environmentally benign strategies for pest control including the use of entomopathogenic fungi. Entomopathogenic fungi form the largest single group of insect pathogens. Such insect killing fungi are fast growing microorganisms to be recognized as disease causing agents in insects. Recent developments have revealed that successful invasion of pathogens to cause infection among insect populations relied on many fitness factors. Their failure or attenuation led to the development of disease resistance. The main purpose of this chapter is to highlight the interaction between virulence factors responsible for pathogen invasion and host defense mechanism to eradicate pathogen.

Keywords Fungal pathogens; Red palm weevil; host defense; virulence factors; Host–pathogen interaction

1. Historic perspective of Red Palm Weevil (RPW)

Red Palm Weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae), native to Indian sub-continent was unintentionally introduced to other parts of the world probably due to the import of infested palms. This carelessness allows the red palm weevils to flourish and now RPW has been reported to become a major pest in Far East (Cambodia, China, Hong Kong, Indonesia, Japan, Laos, Malaysia, Myanmar, Philippines, Singapore, Taiwan, Thailand, Vietnam), South Asia (Pakistan, India, Bangladesh, Sri Lanka), Arabian Peninsula (Bahrain, Cyprus, Egypt, Iran, Iraq, Israel, Jordan, Kingdom of Saudi Arabia, Kuwait, Lebanon, Palestine, Qatar, Sultanate of Oman, Syria, Turkey, United Arab Emirates, Yemen), Europe (Albania, France, Greece, Italy, Malta, Monaco, Netherlands Antilles, Portugal, Slovenia, Spain), Oceania (Australia, Papua New Guinea, Samoa and Solomon Islands), and United States of America [1].

2. Damage

RPW is among the most highly destructive pest of palms. It has been reported to infest $\geq$ 29 different palm species belonging to Agavaceae and Arecaceae [2]. The susceptibility of different palm species towards RPW varies with the geographical area. In Peoples Republic of China and India, RPW has been reported as primary pest against coconut palm (*Cocos nucifera*). In Spain, Canary Palm (*Phoenix canariensis*) is being reported as the most susceptible palm species. However, the infestation of RPW in the Arabian Peninsula is mainly responsible for the destruction of date palm plantations [3]. Their creamy white color larvae (grubs) are the most destructive stage. These legless larvae feed on the succulent plant tissues that create feeding galleries and move towards the center of the infested palms. Such feeding pattern disrupts the vascular system of the infested palm resulting toppling, collapse and death of the infested palm under severe attack [1].

3. Management of Red Palm Weevil

In the past, the control of RPW relies mainly on several approaches. Different strategies have been adopted against different life stages of RPW. The previous investigations have reported the control of adult RPW by adopting different tactics such as the use of Sterile Insect Technique (SIT), insect pheromones and insecticidal applications to prevent the adult entry into the tree trunk.

The use of SIT to control RPW was considered for the first time during 1970s. The investigations carried out by Rahalkar et al., [4] suggested that the 1-2 d exposure of X-rays to the newly emerged male populations of RPW at a dose of 1.5 Krad greatly (~90) induced the sterility. In the meanwhile, when the exposed male RPWs were allowed to sex with unexposed females, they produced fertile eggs that might because of resistance in sperms against radiations. In another study, field trials were conducted to investigate the effect of radiations on the growth of RPWs and the viability of eggs laid by the females. They reported that the sterile *R. ferrugineus* males remained live till 100 days post-exposure. However, they observed significant difference in the viability of the eggs between trapped females from

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experimental areas (58.9%) and wild females (72.9%) [5]. Recently, in the Kingdom of Saudi Arabia a study was conducted in order to standardize the dose of gamma radiations for sterilization among the male RPWs. They successfully optimized the dose in the laboratory for the sterilization of male RPWs [6]. Despite successful dose optimization and eggs viability reduction under controlled conditions, the use of SIT could not be practiced successfully under natural field conditions because RPWs mate within the date palm tree (concealed environment). In addition, the previous investigations further reported that the females prefer to mate with normal males that might create hurdle not to become a sole management strategy for the control of RPWs in the field.

The incorporation of pheromone usage into the management strategy of RPW started with the identification of aggregation pheromones (ferrugineol {4-Methyl-5-nonanol} and ferrugineone {4-methyl-5-nonanone} during 1993 [7]. Later on, the work on the use of pheromones to enhance their trapping potential started in different parts of the world. In Sri Lanka, different alcohols including *n*-propanol, *n*-butanol, *n*-pentanol, *n*-hexanol and *n*-nonanol were incorporated solely and in combination with ferrugineol to enhance the trapping potential of RPW populations in coconut plam plantations. Their results revealed that *n*-pentanol in combination with ferrugineol significantly enhance the trapping potential compared with all the treatments [8]. In Saudi Arabia, enhanced trapping potential (65 % increase) of the aggregation pheromone was reported during 1996. They obtained fruitful results by combining little fractions of 4-methyl-5-nonanone with 4-methyl-5-nonanol [9]. In another study, field trials were conducted at Qatif, Saudi Arabia by using 2252 pheromone traps. Their results revealed the significant reduction in RPW infestation [10]. In Egypt, it has been reported that ethyl-acetate greatly enhance the trapping potential of the aggregation pheromone [11]. In the Sultanate of Oman, field trials were conducted in the date palm plantations. Their main objective was to explore the trapping potential of food bait (fermented dates), pheromones lure (ferrugineol) and kairomone (ethyl-acetate). Their results reported interesting findings such as 1) among all the treatments, the combined effect of lure, kairomone and bait trapped the maximum number of RPWs, 2) among the trapped RPWs, majority of them were females, 3) colour of the trap might play an important role for trapping RPWs [12]. More recently, trials were conducted in order to observe the longevity of the pheromones. Their findings suggested that the pheromones used for RPWs rapidly declined in summer compared to winter [13]. Despite aggregation pheromones have multiple advantages including, easy handling, environmentally friendly, cheap, safe to humans and mammals, till now, the use of pheromones could not become a sole strategy to control the populations of RPWs. The failure might be because of high temperature prevailing especially in the Gulf. Much research needs to be done on different aspects in order to implement a successful Integrated RPW management strategy.

Insecticides are being applied to control RPW populations in different ways including spraying, dipping the offshoots with insecticidal solutions, wound dressing, frond axil filling, trunk injection, fumigation and crown drenching. Historically, insecticidal application to control RPW populations started with the use of most hazardous insecticides. For instance during 1950s, benzene hexachloride (BHC) or Chlordane dust remained the major control measure by filling the frond axil [14]. Subsequently, laboratory bioassays calimed Endrin™ as the most potent insecticide [15]. In the meanwhile, awareness of the public health hazard concerns regarding the use of insecticides came on the scene. At that advent, voices are being heard to use alternate control methods to protect environment, humans and wildlife. As a result of that most of the insecticides belonged to cyclodienes were banned because of their ability to persist within the environment. The withdrawal of banned insecticides closed this chapter and led scientists to search for alternate insecticides. In the meanwhile, the infestation of RPW in different countries was reported for the first time during 1980s and 1990s. Therefore, the search for safe, environmentally less hazardous insecticides started in different parts of the world. In its native range India, RPW was successfully controlled by monocrotophos and dichlorvos, solely and in combination through trunk injection [16]. In UAE, carbosulfan, pirimiphos-ethyl and Rogodial™ were used to explore their insecticidal potential against different larval instars of RPW. Their promising laboratory results enabled them to evaluate their potential under field conditions. They reported that the injection of these insecticides into the tree trunk successfully control RPWs [17]. Laboratory experiments conducted at Saudi Arabia reported the use of chlorpyriphos, endosulfan and pirimphos-methyl as successful preventive measure to control the attack of RPWs [18]. In another study, mixture of piperonyl butoxide and carbaryl was investigated against RPWs. They reported that the mixture is more toxic when incorporated into the diet [19]. More recently, insecticides belonging to different groups were evaluated against different life stages of RPWs. Their findings claimed pyrethroids as the most potent compared to other insecticides [20]. In Spain, different larval instars of RPWs were investigated by using imidacloprid and oxamyl. Among all the laboratory bioassays, imidacloprid effectively controlled the RPWs compared to oxamyl [21]. More recently, efforts are being done to reduce the dose of a previously effective insecticide, chlorpyriphos by introducing a micro-encapsulation formulation. This newly introduced formulation was found to be effective under laboratory and semi-field conditions [22]. Until now, multiple preventive and curative measures have been adopted to control the populations of RPWs. Because of concealed nature of grubs, the insecticides are being utilized to target adults that require frequent application. This drawback raises the concern over human health and environmental pollution that provides the impetus to look for alternative methods of RPW management.

4. Potential of bio-control agents

Naturally occurring bio-control agents are alternative to reverse the use of hazardous synthetic insecticides. Among these microorganisms, the use of entomopathogenic fungi was found to be promising alternate for insects control. According to an estimate, more than 700 species of fungi belonging to different genera are known to infect insects. In the past, the potential of entomopathogenic fungi especially *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria fumosorosea* have been evaluated against different pests including *Aphis craccivora* [23], *Aedes aegypti* [24], *Bemisia argentifolii* [25], *Coptotermes formosanus* Shiraki [26], *Melanoplus sanguinipes* [27], *Ocinara varians* Walker [28], *Odontotermes obesus* [29], *Periplaneta americana* [30], *Rhynchophorus ferrugineus* [31], *Scolytus scolytus* [32], *Thrips tabaci* [33]. The success of these naturally occurring microorganisms mainly depends on the host pathogen interaction. The most important pathogen characteristics and host events that led to the success and failure of any fungal pathogen attack are explained below.

4.1. Pathogenicity related characteristics of Entomopathogenic fungi

The access of entomopathogenic fungi to invade the host is through the cuticle that involves complex biochemical interactions between the host and the pathogen (fungus) before germination, penetration, growth, and reproduction of the fungus. Prior to host invasion, there are certain characteristics of fungi that designate them virulent or avirulent strains.

4.1.1. Conidial attachment to the host cuticle

Conidial attachment of entomopathogenic fungi (EPF) corresponds to be the first step for the establishment of mycosis as shown in Figure 1. Generally, the conidia of the EPF applied on the host through 1) direct application on the substrate, 2) dipping the target host in conidial suspensions, 3) conidial dispersion from different parts of the host. After inoculation, the success and failure of fungal infection depends on the host pathogen characteristics. In case of compatible reaction, the application of conidia could lead to a successful infection that greatly depends on the adhesion of fungal spores to the host cuticle. The previous investigations have clearly elaborated that the adhesion of conidia on the host cuticle is mainly because of some mechanisms including non-specific (hydrophobic or electrostatic) or specific (glycoprotein) [34-36].

The outer wall of the conidia that mainly determines the conidial adhesion to the host cuticle varies with the type of conidia. Approximately four decades before, adhesion process was proposed [37-38]. This model illustrated that the infection could only proceed after a successful penetration has been achieved. Subsequently, Fargues proposed that the conidial adhesion involves three steps, 1) adsorption of the fungal propagules to the cuticular surface; 2) adhesion or consolidation of the interface between pre-germinant propagules and the epicuticle; 3) fungal germination and development at the insect cuticular surface, until appresorium is developed to start the penetration stage [39]. In addition, carbohydrate binding glycoproteins such as lectins, have also been detected on the conidial surface that involved in binding between spores and the insect cuticle [40]. On the whole, the successful attachment among susceptible hosts is due to the rodlet layer of the spores that facilitates contact with the host's epicuticle, and the topography and chemical properties of the epicuticle that enhance adhesion of the spores and help to germinate the spores on the cuticle. However, non-compatible reaction might lead to the failure of infection. The fungal strains with high adhesion ability, an important trait related to pathogenicity should be considered for the development of bio-control agents.

4.1.2. Directly penetrating structures

The conidial invasion through the host depends on the penetration pattern of the conidia. Previously, it has been reported that virulence is directly related with the penetration. The conidia with early penetration are more virulent compared with late penetrating conidia [41]. Recent investigations greatly enhanced our understandings. The results obtained from these findings showed that each strain has its own penetrating potential. The strains having high potential to produce directly penetrating structures are advantageous to invade the host. Furthermore, they suggested that directly penetrating conidia greatly help to penetrate into the host's cuticle. After penetration, conidia firmly attach with the host's cuticle [42].

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Fig. 1 Sporulation of *Beauveria bassiana* on the cadaver of *Rhynchophorus ferrugineus* (Olivier) (Source: Abid Hussain).

4.1.3. Cuticle degrading enzymes

Conidial germination under terrestrial conditions lead with the formation of penetrating structures including germ tubes or appressoria [35, 37-38, 43]. These penetrating structures breach the cuticle of the host through mechanical or enzymatic means [37-38]. Among enzymes, a number of different extra-cellular enzymes including chitinases, esterases, lipases and proteases have been discovered in various fungi that synergistically degrade the cuticle of the host. However, a major virulent determinant spore bound protease Pr1 was found to play a pivotal role in host penetration. Previously, a number of investigations have been done to enhance the Pr1 expression that ultimately enhance the virulence of the EPF.

There are many reasons that might reduce the expression of this particular enzyme. Among them, continuous sub-culturing on the artificial nutrient growth medium lead to the reduction of Pr1. Recently, it has been reported that high levels of spore bound protease Pr1 in spores resulted in faster infection. The *in vivo* produced entomopathogenic spores from a lepidopterous host (*Ocinara varians* Walker) greatly enhanced the activity of Pr1 compared to the spores grown on artificial growth medium. Furthermore, they suggested that enhanced Pr1 activity along with high virulence showed that the host clearly provides the nutrition to the invading pathogenic fungal spores [44].

4.2. Host defense to combat invading pathogens

Pathogenesis among the infected insects is an important interaction between the host and invading pathogenic fungal conidia. Generally, most of the insects are resistant to microbial infections. Because they actively combat invading pathogenic fungi through complex immune reactions. The innate immune reactions responsible for the rapid removal of invading pathogens involved cellular and humoral immune reactions.

The innate immune system of insects comprised of cellular and humoral immune reactions to combat microbial infections [45]. The cellular reactions cover the series of events such as phagocytosis, nodule formation and encapsulation. These events vary with the size of the invading pathogen. Small invading pathogens are eliminated by phagocytosis. In case of larger invaders in terms of size or quantity, the invading pathogens are ingested through the process of encapsulation. However, nodule formation represents the aggregation of hemocytes that are entrapped in sticky extra-cellular materials. This process is facilitated by phenoloxidase that lead to the melanization [46-47]. On the other hand, humoral immune response is regulated in a different way by producing specific antimicrobial peptides to destroy the invading pathogens through 1) identification of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), 2) signal modulation, 3) signal transduction pathways, 4) production of antimicrobial peptides (AMPs) as shown in Figure 2.

4.2.1. Pathogen Recognition

Immune mechanism starts with the recognition of conserved pathogen-associated molecular patterns (PAMPs). The examples of such recognition molecules are lipopolysaccharides (LPS), peptidoglycan (PGN) and β-1,3-glucan from Gram-negative, Gram-positive bacteria and fungi, respectively [48-50]. These cell wall components are recognized by different pattern recognition receptors (PRRs) belonged to different classes. Among them, the most important PRRs include β-1,3-glucan recognition proteins (βGRPs), C-type lectins (CTL), down syndrome cell adhesion molecules (DSCAM), fibrinogen-like domain immunolectins (FBNs), galectins (GALE), gram negative binding proteins (GNBPs), hemolin, peptidoglycan recognition proteins (PGRPs), multidomain scavenger receptors (SCRs), nimrods, thioester containing proteins (TEPs) [51-52]. The exact mechanism responsible for the detection of invading pathogens by Red palm weevil is largely unknown. In the near future, studies must be designed to identify the recognition receptors that might help to explore the important components of immune mechanism among red palm weevils.

4.2.2. Signal Modulation

After the recognition of invading pathogen, signal modulation molecules are serially activated that either enhance the signals related to danger or dampen false alarms [53]. Furthermore, they regulate haemolymph coagulation, melanization and AMPs synthesis [54]. Among them, serine proteases (SPs) are the most important modulating molecules that are identified by their N-terminal CLIP domain (CLIPs). Various serine proteases (SPs) as immune modulators have been identified in many insect species. Recently, a genome-wide analysis in *Bombyx mori* has been performed for SPs and SPHs (serine proteinase homologs). The analysis revealed the identification of 51 SPs and 92 SPHs genes from the genome of *Bombyx mori* [55]. The genome of other insect species such as *Apis melifera*, *Anopheles gambiae* and *Drosophila melanogaster* has shown 5, 17 and 30 serpin genes, respectively [56]. Our recent findings identified 16 different types of SPs from the expressed sequence tags (ESTs) of immunized *C. Formosanus* Shiraki workers. Furthermore, the expression profiles of *C. formosanus* CLIPs have been shown to be affected by infection, indicating their possible role in the modulation of immune reactions in this species. In addition, serpin-like proteins have also been found to act as conserved suicide substrates. These proteins not only present in eukaryotes but also in viruses. Till now, more than 23 families of serpin-like proteins have been identified. Most of them were identified from insects. Among the identified serpins, the most important includes Kazal, Kunitz, alpha-macroglobulin, and serpin families [57]. As long as red palm weevil is concerned, in the past no effort has been done to explore the genes involved in modulation.

4.2.3. Signal Transduction

The immune system of insects is regulated by various types of signaling pathways including immune deficiency pathway (IMD), Toll pathway, c-jun N-terminal kinase (JNK pathway), janus kinase/signal transduction and activator of transcription (JAK/STAT pathway) [58]. The activation of these signals lead to the synthesis of AMPs. However, nothing is known about the signaling pathways that regulate the immune mechanism in red palm weevils.

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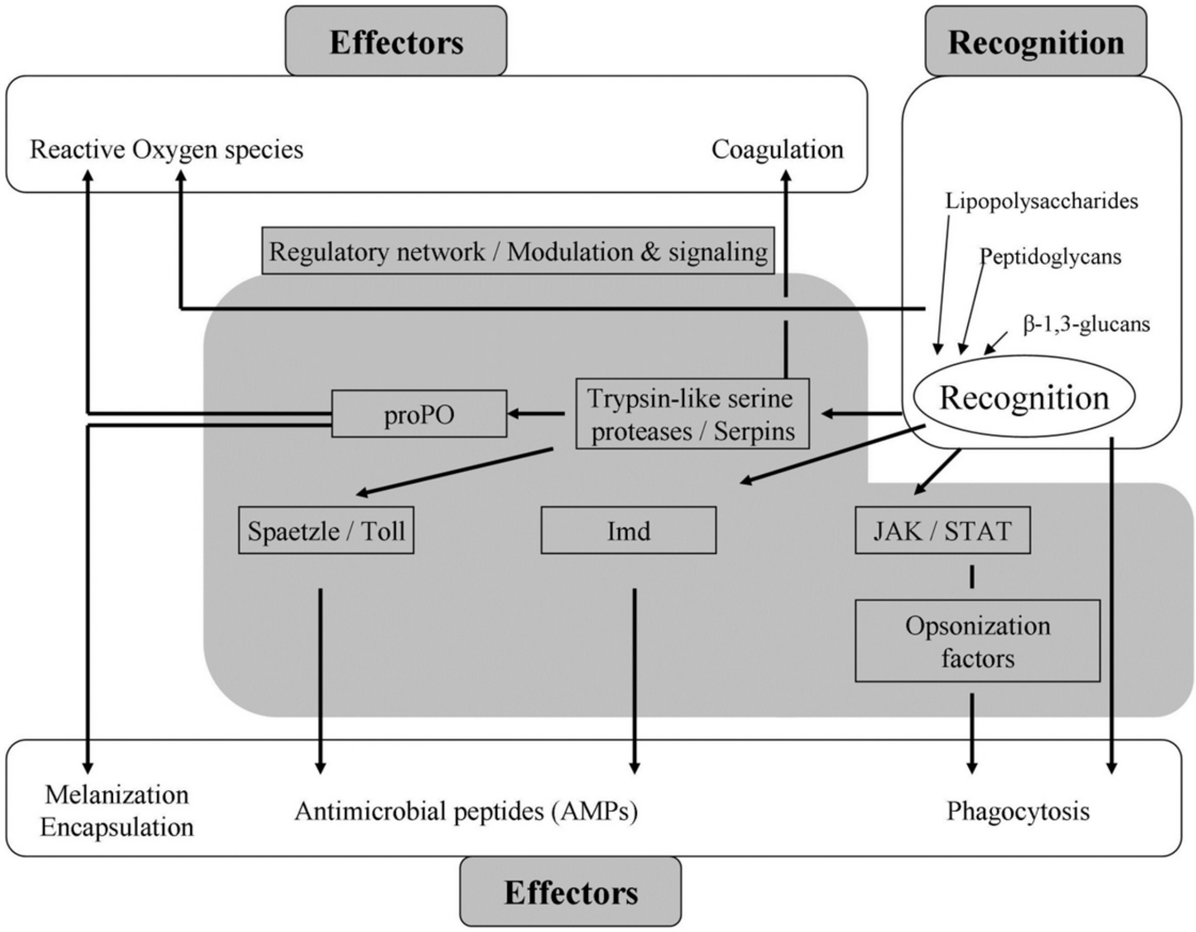

Fig. 2 Schematic overview of insect immune response modified from Schmid-Hempel, 2005 [59].

The Toll-like pathway plays a pivotal role to eradicate the gram-positive and fungal infection. This pathway induces the cleavage of Spaetzle through proteolysis. The cell wall components of these invading pathogens are sensed through their cell wall. After binding, these components activate the toll receptors [60]. In insects, one TIR domain (Toll, IL-IR) is present. This domain right after activation recruits some important death domain proteins including MyD88, Pelle and Tube. The transcription of AMPs is activated when the cactus is dissociated through Dorsal and Dif. Both of these transcription factors play crucial role in immune response.

The reduced expression of AMPs by random mutations resulted to the identification of immune deficiency (IMD) pathway [61]. This pathway is activated right after the recognition of the cell wall component of gram-negative bacteria through peptidoglycan recognition protein (PGRP-LC and PGRP-LE). The over-expression of IMD pathway is prevented by suppressors. The most important IMD suppressors includes PGRP-LB, PGRP-LF and PGRP-SC. Moreover, IMD pathway further help to activate JNK pathway [62]. Despite well-characterized components involved in these pathways, the knowledge regarding these pathways is still infancy.

The JAK/STAT pathway is controlled by kinases and transcription factors. Among insects, *Drosophilla* remained the major organism to explore this pathway. The previous findings have reported its important role in immune response against bacteria in the gut [63-64]. Moreover, its association has also been reported with antiviral immunity in different insects [65-66].

4.2.4. Antimicrobial Peptides (AMPs)

The synthesis of AMPs is considered the final step of inducible immune responses. They are systematically produced by the fat body of the host. Following AMPs synthesis, they are secreted into the haemolymph, where the large concentrations of AMPs are accumulated [67]. All the major activities of these secreted peptides against fungi and bacteria are performed in the haemolymph. The exact mode of action of these secreted peptides is largely unknown. However, in some cases these peptides have been reported to destroy the invading pathogen by disrupting cell wall of the pathogen. This cell wall disruption leads to the ultimate cell lysis [68]. In addition, these AMPs are also known to attack the intra-cellular target sites without disrupting the membrane [69].

These evolutionary conserved peptides are selectively toxic against invading pathogens. Till now, > 800 AMPs have been discovered from different organisms belonging to vertebrates, invertebrates, plants, protozoans and microbes. Because of the diversity of AMPs, it is very difficult to classify them except on the basis of secondary structure. The four major classes of AMPs based on their structure include *β*-sheet, amphipathic *α*-helical peptides, loop and extended peptides [70].

5. Conclusion and Future perspective

In summary, the successful control of red palm weevils mainly depends on the host pathogen interactions. So, there is a constant struggle between host and pathogen that ultimately lead to the success or failure of pathogens. In case of compatible interaction, the pathogen must have high number of conidia with strong adhesion that ultimately penetrate into the host through directly penetrating structures. Moreover, the invading pathogen must have the capacity to bypass or overcome the host immune system by producing toxins. In future, experiments must be conducted to explore in detail the immune mechanism of red palm weevil that might help to find out major genes involved in host defense. These findings might help to develop new products for the control of invasive populations of red palm weevils through gene silencing.

6. References

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