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Isolation and identification of bacteria associated with red palm weevil, *Rhynchophorus ferrugineus* from Hail region, northern Saudi Arabia

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ABSTRACT

The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) is an obtrusive, ruinous pest of date palms, (*Phoenix dactylifera)* causing critical economic losses in the Middle East including the Kingdom of Saudi Arabia. However, relying on insecticides alone for controlling the RPW (*R. ferrugineus*) can have negative effects on human health and the environment. Natural enemies are considered as the fundamental part of the biological control, which is safe for controlling the RPW. Therefore, knowledge of the natural enemies against the RPW (*R. ferrugineus*) is an important to create techniques for the integrated pest management (IPM). The present study aimed to isolate and identify the bacterial species associated the RPW (*R. ferrugineus*) in Hail region during. Adults of the RPW were monthly collected from infested date palm farms in various sites in Hail region. Several bacterial species were isolated from the investigated RPW and the obtained sequences were edited in MEGA7 software and compared to available sequences in the Gen Bank database. The 16S rDNA sequencing showed that bacteria isolated from the investigated RPW were mostly Gram positive and belonged to *Proteus mirabilis* (33.3%), *Klebsiella pneumonia* (25%), *Serratia marcescens* (25%), *Staphylococcus sciuri* (8.3%) and *Providencia rettgeri* (8.3%). Overall, the results of this study can be utilized a baseline data for applying the biological control program of the RPW.

KEY WORDS: RHYNCHOPHORUS FERRUGINEUS, BIOLOGICAL CONTROL, INTEGRATED PEST MANAGEMENT, NATURAL ENEMIES, BACTERIA, CHEMICAL CONTROL

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INTRODUCTION

The red palm weevil *Rhynchophorus ferrugineus* (Olivier) (RPW) (Coleoptera: Curculionidae) is considered as a dangerous insect pest of date palm trees and makes huge losses to date palm's farmers. Indeed, the Food and Agriculture Organization (FAO) has recorded the RPW as a class -1 pest of date palms in the Middle East (Al-Shawaf *et al.*, 2013). This, subsequently, has encouraged to utilized several strategies to control the *R. ferrugineus* including chemicals, biotechnological frameworks utilizing semi chemicals or the improvement of the Sterile Insect Technique, and biological control (Mazza *et al.*, 2014). Although chemical control has been broadly connected for controlling the R. ferrugineus, there is creating stress over the negative impacts of insecticides application on human health and the environment (Mazza *et al.*, 2014; Asiry, 2015; Nicolopoulou-Stamati *et al.*, 2016). *R. ferrugineus* collected from different typologies of prothoracic spots have been found in Malta, Sicily and Pakistan (Bannu, Khyber Pakhtunkhwa) which shows a degree of diversity in RPW population around the globe (Ul Haq *et al.*, 2018).

Biological control can be characterized as an ecosystem service which advances the concealment of pests by their common natural enemies including their parasites, parasitoids, predators and pathogens (DeBach and Rosen, 1974; Bale *et al.*, 2008). Biological control offers ecological and monetary favorable circumstances as yield loss might be diminished without undesirable natural outcomes coming with application of insecticides (Bianchi *et al.*, 2006). The improvement of a biological control segment for a productive IPM requires the distinguishing proof of the common adversaries of the RPW and its defensive mechanisms against its regular natural enemies.There have been a few endeavors to separate pathogens from the RPW (Gindin *et al.*, 2006; Güerri-Agulló *et al.*, 2008; Salama and Abd-Elgawad, 2002; Salama *et al.*, 2004). These reviews prompted to the disclosure of a cytoplasmatic polyhedrosis infection, and a yeast isolated from the RPW's haemolymph. Be that as it may, none of these can be classified as potential biocontrol agents, mostly because their application in normal conditions is restricted (Banerjee and Dangar, 1995; Salama *et al.*, 2004). Cytoplasmic polyhedrosis virus infected RPW larva showed 80-100 % mortality with a viral dose of 80×106 (Mahmoud *et al.*, 2018). However, the accomplishment of biological control agents is frequently lacking and any control of the RPW is by all accounts.

Pathogenic entobacteria generally belong to the families Bacillaceae, Pseudomonadaceae, Enterobacteriaceae, Streptococcaceae and Micrococaceae (Tanada and Kaya, 1993). Although numerous bacteria can infect insects, just members of two genera of the order Eubacteriales, *Bacillus* and *Serratia*, have been enrolled for the control of insects (Tanada and Kaya, 1993). For the genus *Rhynchophorus*, bacteria have only been isolated from the RPW: Dangar and Banerjee (1993) found some pathogenic entobacteria belonging to *Bacillus* sp., *Serratia* sp. and the coryneform group in larvae and adults in India, while Alfazairy *et al.* (2003) and Alfazariy (2004) isolated *Bacillus thuringiensis* Berliner and *Bacillus sphaericus* Meyer and Neide from larvae and adults in Egypt. Alfazariy (2004) reported successful control of the RPW in laboratory conditions by infection with *B. thuringiensis* subspecies kurstaki isolated from larvae in Egypt. Conversely, different authors demonstrated an alternate weakness of the RPW to *B. thuringiensis* (Manachini *et al.*, 2008a,b, 2009). *Pseudomonas aeruginosa* (Schroeter) was isolated from infected larvae collected in Kerala, India (Banerjee and Dangar, 1995). Research facility examines that this bacterium was pathogenic for weevils when ingested through force-feeding or when insects were forced to wade through a suspension of bacterial cells. Mortality occurred eight days after inoculation and small larvae were more susceptible than larger larvae (Banerjee and Dangar, 1995), probably most likely because of absence of antimicrobial cuticular compounds (Mazza *et al.*, 2011a).The current study was carried out to characterize bacterial flora associated with the RPW in Hail region. Also to identify and screen bio-control bacterial strains. As a result, this can be set as a baseline data on the screening of natural enemies of the RPW in this region.

MATERIALS AND METHODS

Collection of red palm weevils (RPW) samples: Monthly, 120 adults of the red palm weevils (RPW) were collected from five different infested farms in Hail region, namely: Al Gayed, Jubbah, Helala, Horir and Gutha Sharagiya. The study was conducted during the period October 2017 to December 2018. The pheromone traps were used for the RPW's collection. In each area, 40 adults of the RPW were separately placed in plastic boxes. The collected RPW were kept in a freezer at –20 °C at the laboratory at the Department of Biology, Faculty of Science at University of Hail until used for investigating the bacteria.

Identification of microorganism flora: Isolation of bacteria: For bacterial isolation, the collected RPW were surface-sterilized with 70% ethanol for 5 min (Poinar and Thomas, 1978) and washed 3 times in sterile distilled water. The bodies of the investigated RPW were homogenized in nutrient broth using a glass tissue grinder, and the homogenate was filtered. Then, 10, 25, and 50 µL of

sample extracts was plated on nutrient agar and incubated at 30 °C for 2–3 days. The remaining mixtures were incubated at 30°C for 3–4 h to enrich the number of bacteria that have low concentration. From these mixtures, 10, 25, and 50 µL were also plated on nutrient agar and incubated at 30°C for 2–3 days. Finally, the incubated RPW suspensions were heated in a water bath at 80°C for 10 min to eliminate nonspore-forming bacteria (Ohba and Aizawa, 1986). After heat inactivation, 10, 25, and 50 µL of the heated suspensions were plated on nutrient agar and incubated at 30 °C for 2–3 days. The bacterial Isolates were distinguished according to their colony color and morphology. Pure cultures of bacterial colonies were prepared and stocked in 20% glycerol at –80°C in the Microbiology Laboratory, Department of Biology, and Faculty of Sciences at University of Hail. Bacterial cultures were identified according to their morphology, nutritional features, and biochemical and molecular characteristics.

Molecular identification of the bacterial isolates: Inoculation of isolates: The bacterial isolates were grown on 5 ml tubes contained 2 ml of Luria broth (LB). The tubes containing isolates were incubated horizontally at 37°C for overnight with shaking at 200 rpm in an incubator shaker (Lab-line Instruments, Inc.). To precipitate bacterial pellets for extraction of DNA, 2 ml of LB media was centrifuged at 5000 rpm for 5 min.

DNA Extraction using modified Dellaporta procedure: DNA was extracted using modified method of Dellaporta *et al*., (1983) as the following protocol: Twenty mg of fresh harvested mycelium or bacterial pellet were ground with pestles in a 1.5 ml tube with 500 µl of Dellaporta buffer (100 mM Tris pH 8. 50 mM ethylenediamine-tetraacetate EDTA, 500 mM NaCl, 10 mM beta mercaptoethanol) (BME). Thirty three µl of 20% sodium dodecyl sulfate (SDS, w/v) were added, and the mixture was vortexed and incubated for 10 min at 65°C.One hundred and sixty µl of 5 M potassium acetate KoAc (Sigma chemicals) were added and vortexed. The mixture was then centrifuged for 10 min at 10,000 rpm, and 450 µl of supernatant was transferred to a new tube. Four hundred and fifty µl phenol, chloroform and isoamyl-alcohol (PCI) were added with a ratio of 25:24:1 and vortex for 5 min and then centrifuged for 5 min at 10,000 rpm. 400 µl of the upper phase were then removed to a clean microcent. The supernatant was removed and the total nucleic acid was precipitated in the bottom of the tube. The pellet was washed with 70% ethanol and spun 5 min at 10,000 rpm. Then, the pellet was re-suspended in 100 µl of Double-distilled water (ddH2O).

Polymerase Chain Reaction (PCR): Amplification of the 16S rRNA gene from bacterial isolates was carried out using the universal primers; 27F 5'-AGA GTT TGA TCM TGG CTC AG-3' and 1492R 5'-TACGGYTACCTTGTTACGACTT (Weisburg *et al*., 1991), in a total 50 µl of PCR reaction. The main PCR steps were programmed as follows: denaturation at 94 °C for 45 s, annealing at 55 °C for 60 s, and extension 72 °C for 60 s. in 30 amplification cycles, followed by a final extension step at 72 °C for 10 min. PCR was conducted in the ESCO Swift Maxi Thermal Cycler with initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 sec, 52°C for 30 sec, and 72°C for 30 sec, and the final cycle is a polymerization cycle performed at 72°C for 10 min. PCR Products were purified using QIAquick® PCR Purification Kit (Cat. No. 28106) according to manufacturing procedures.Macrogen Inc., (Korea), sequenced the purified PCR products and sequencing of the purified isolates was performed in both directions using ITS5 and ITS4 primer pairs. Sequence alignments were edited by MEGA7 (Kumar *et al*., 2016).

RESULTS AND DISCUSSION

Rhynchophorus palm weevils are large insects belonging to the family Dryophthoridae, subfamily Rhynchophorinae, and tribe Rhynchophorini (Bouchard *et al*., 2011). All *Rhynchophorus* species are polyphagous and have a comparable life history and some of them are significant pests due to the serious economic damage they cause, specifically to several species of the family Arecaceae. In excess of 50 characteristic adversaries have been accounted to attack *Rhynchophorus* species, among the considered organisms, bacteria is a critical to be considered for incorporation in the integrated pest management programs. In the present study, obtained sequences were edited in MEGA7 and compared to available sequences in GenBank database. The 16S rDNA sequencing showed that bacteria isolated from the investigated RPW were mostly Gram positive and belonged to *Proteus mirabilis* (33.3%), *Klebsiella pneumonia* (25%), *Serratia marcescens* (25%), *Staphylococcus sciuri* (8.3%) and *Providencia rettgeri* (8.3%) as shown in Fig. 1 and 2.

The dominated bacteria *P. mirabilis* was found to be on the bodies of the tested dead red palm weevil (RPW)

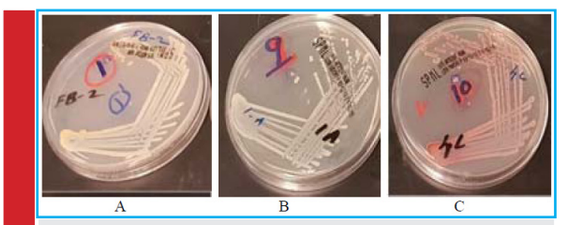

FIGURE 1. A: Proteus mirabilis: B: Klebsiella pneumonia; C: Serratia marcescens

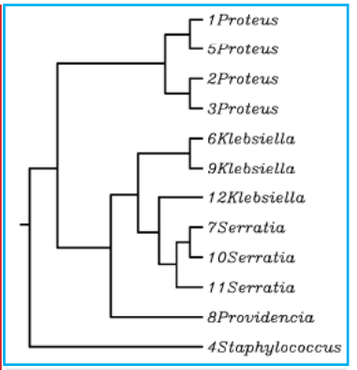

FIGURE 2. Phylogenetic analysis of 16S rDNA sequence by UPGMA software.

and it was accounted 33.33% of all bacterial isolates from RPW body. *Proteus* species are part of the Enterobacteriaceae family of gram-negative bacilli. *Proteus* organisms are embroiled as genuine causes of diseases in humans along with *Escherichia, Klebsiella, Enterobacter,* and *Serratia* species. *Proteus* species are the most regularly found in the human intestinal tract as a component of ordinary human intestinal flora, alongside *Escherichia coli* and *Klebsiella* species, of which *E. coli* is the dominating inhabitant. *Proteus* is also found in various natural environments, including long-term care facilities and medical clinics (https://emedicine.medscape.com/article/226434-overview).

The most characterizing normal for *Proteus* microscopic organisms is a swarming marvel, a multicellular differentiation procedure of short rods to extended swarmer cells. It allows population of bacteria to move on strong surface. The virulence of *Proteus* rods has been related to several factors including fimbriae, flagella, catalysts (urease - hydrolyzing urea to CO_2 and NH_3, proteases corrupting antibodies, tissue framework proteins and proteins of the supplement system), iron acqusition frameworks and poisons: hemolysins, *Proteus* poison agglutinin (Pta), as well as an endotoxin - lipopolysaccharide (LPS)

Proteus mirabilis, a Gram-negative rod-shaped bacterium most noted for its swarming motility and urease activity, frequently causes catheter-related urinary tract infections (CAUTI) that are frequently polymicrobial (Chelsie *et.al.*, 2017). *P. mirabilis* belongs to the class Gammaproteobacteria, and has long been perceived as a member of the order Enterobacteriales, family Enterobacteriaceae. However, one group recently created a reconstructed phylogenetic tree based on shared core proteins, ribosomal proteins, and four multilocus sequence analysis proteins, and has suggested that the order Enterobacteriales be renamed, putting *Proteus* within a new Morganellaceae family (Adeolu *et. al.*, 2016).

P. mirabilis can be found in a wide assortment of environments, including soil, water sources, and sewage, yet it is transcendently a commensal of the gastrointestinal tracts of humans and animals (Armbruster and Mobley, 2012). While the bacterium is fit for causing an assortment of human diseases, including those of wounds, the eye, the gastrointestinal tract, and the urinary tract, it is most noted for infections of the siphoned urinary tract, known as catheter-associated urinary tract infections (CAUTI) (Warren *et.al.* 1982; Mobley and Warren 1987; Breitenbucher 1984; Jacobsen 2008; Armbruster *et. al.*, 2016). These infections are common in long-term siphoned patients, for example, the individuals who dwell in nursing homes and chronic care facilities, and may be of particular danger to spinal cord injury patients. During infection, histological damage is brought about by cytotoxins including hemolysin and a assortment of proteases, some autotransported. The pathogenesis of infection including evaluation of individual genes or global screens for virulence or fitness factors has been evaluated in murine models of ascending UTI or CAUTI using both single-species and polymicrobial models. Global gene expression studies carried out in culture and in the murine model have revealed the remarkable metabolism of this bacterium (Chelsie *et.al.*, 2017). Vaccines, utilizing MR/P fimbria and its adhesin, MrpH, have been appeared to be strong in the murine model.

Klebsiella pneumoniae was also found in high numbers in the body of the tested RPW. It represented 25% of the total numbers of isolated bacteria. *Klebsiella* species are Gram-negative coliform bacteria that can cause mastitis, prompting noteworthy economic losses on dairy farms. *K. oxytoca* and *K. pneumoniae* are the species that are responsible for causing mastitis. Typical *K. pneumoniae* is an opportunistic pathogen, which for the most part influences those with debilitated immune systems and will in general reason for nosocomial infections. A subset of hypervirulent *K. pneumoniae* serotypes with elevated production of capsule polysaccharide can affect influence previously healthy persons and cause hazardous community acquired infections, such as pyogenic liver abscess, meningitis, necrotizing fasciitis, endo- phthalmitis and severe pneumonia. *K. pneumoniae* uses an assortment of virulence factors, particularly capsule polysaccharide, lipopolysaccharide, fimbriae, outer external membrane proteins and determinants for iron procurement and nitrogen source usage, for survival and immune avoidance during infection (Bei *et. al.*, 2014).

K. pneumoniae, a member of the family Enterobacteriaceae, is a rod-shaped, Gram-negative, lactose-fermenting *bacillus* with an unmistakable case. Normal *K.*

pneumoniae is an opportunistic pathogen that is widely found in the mouth, skin and intestines, as well as in hospital settings and medical devices. Opportunistic *K. pneumoniae* mostly influences those with compromised immune systems or who are weakened by other infections. Colonization of the GI tract by opportunistic K. pneumoniae generally occurs prior to the development of nosocomial infections, and *K. pneumoniae* colonization can be additionally found in the urinary tract, respiratory tract and blood (Podschun 1998). *K. pneumoniae* biofilms that structure on therapeutic gadgets (e.g., catheters and endotracheal tubes) provide a significant source of infection in catheterized patients (Schroll et. al., 2010). Nosocomial infections brought about by *K. pneumoniae* tends to be chronic due to the two following major reasons: *K. pneumoniae* biofilms formed in vivo protect the pathogen from attacks of the host immune responses and antibiotics (Jagnow and Clegg 2003); and nosocomial isolates of *K. pneumoniae* often display multidrug-resistance phenotypes that are commonly caused by the presence of extended-spectrum β-lactamases or carbapenemases, making it hard to choose appropriate antibiotics for treatment (Paterson et. al., 2004; Munoz-Price et. al. 2013).

Serratia marcescens was also found in high numbers in the body of tested RPW. It represented 25% of the total numbers of isolates. Analysis by the 16S rDNA sequences allotted the selected bacteria to the genus Serratia (family Enterobacteriaceae), with the most noteworthy similitude found for the species *Marcescens*. The genus *Serratia* includes, at least, 10 species (Grimont and Grimont 2006). Serratia is a bacterium found in the family Enterobacteriaceae that can cause opportunistic infections even though it is usually a weak pathogen. Analysis by the 16S rDNA sequences, classified our isolates into the species *marcescens*. The phylogenetic trees dependent on the 16S rDNA and the linked housekeeping gene sequences arranged our strains within the *S. marcescens* cluster (Figure -2).

This cluster was plainly particular from those of the other known red pigment-producing *Serratia* species (Grimont and Grimont 2006; de Araujo *et al*. 2010). *S. marcescens* has likewise been accounted for, at least for some red pigment-producing strains, to display an antimicrobial activity against some gram-positive and gram-negative bacteria (Ibrahim *et al*. 2014; Lapenda *et al*. 2015). I then verified if this was also the case for my isolates. *S. marcescens* is available as extracellular symbiont in various formative phases of the RPW. Additionally, the antimicrobial activity exhibited versus *Bacillus* spp., *Paenibacillus* spp., and *Lysinibacillus* spp., reported as insect pathogens and potential candidates for biocontrol agents, could attribute for *S. marcescens* a potential protective role (Maria Scrascia *et. al*. 2016).

Serratia marcescens is among the most widely recognized irresistible agents in infections related with *Serratia*. They cause infections with noteworthy mortality and morbidity in infants (Edmond *et. al*., 1999; Roy *et. al*., 1997; Ania et. al., 2008; Bayramoglu et. al., 2011). Moreover, *S. marcescens* is a significant irresistible agent that causes hospital-acquired respiratory and urinary tract infections in neonatal-adult intensive care unit and immunodeficient patients. The diffuse nearness of *S. marcescens* inside the infested palms highlighted the capacity of this bacterium to replicate and spread along the palm tissue.

The RPW is a singular insect with no or restricted contact between adult and developing individuals. Solitary insects, moreover to their own safeguards, can make utilize of symbionts to better protect themselves, offspring, or nutritional *assets* against pathogens, predateors, parasites, or parasitoids (Kellner 2002; Kaltenpoth *et al*. 2005; Brownlie and Johnson 2009*)*. This protection can be interceded by various mechanisms, which include the production of antimicrobials. The cooperation among microorganisms and hosts have dependably been the object of escalated studies. Specifically, studies on the mutualistic relationships among bacteria and insects have dynamically uncovered the pertinent pretended by the formers on the life cycle of their hosts. The identification of red pigment-producing *S. marcescens* as extracellular symbiont of the RPW will add to the knowledge on a mutualistic connection among bacteria and the RPW (Maria Scrascia *et. al*. 2016). It has been reported that populations of certain weevils are sometimes definitely decreased by naturally occurring pathogens, for the most part under conditions for example, delayed high humidity or dense pest populations. Banerjee and Dangar (1995) isolated the bacterium *Pseudomonas aeruginosa* from naturally infected adults of *R. ferrugineus*. The bacterium is observed to be pathogenic to adults forced to feed on a suspension of bacterial cells and mortality happened 8 days after ingestion.

Staphylococcus sciuri was identified in 8.3% of the bacterial isolates from the tested RPW body. *S. sciuri* belongs to the group of oxidase-positive, novobiocin-resistant coagulase negative *staphylococci* (CoNS) (Stepanovic *et. al.,* 2006). This bacterium is widespread in nature and can be isolated from an assortment of pets, wild and domestic animals, insects, environment (soil, sand, water, air samples, etc.), and foods (Stepanovic *et. al*., 2006, Stepanovic *et. al.* 2005).

It has additionally been recuperated from the hospital environment (Dakic *et. al*., 2005) and in spite of the fact that *S. sciuri* is just occasionally isolated from humans, it has been associated with a number of serious infections such as septicemia, endocarditis, peritonitis, pelvic inflammatory disease, urinary tract infections and

FIGURE 3. A: Staphylococcus sciuri ; B: Providencia rettger

wound infections (Severin *et. al*., 2010; Stepanovic *et. al*., 2005). However, there is a little information regarding the pathogenicity of *S. sciuri*. Members of the *S. sciuri* group are widely distributed in nature, and they can be isolated from a variety of animals and the products of animal origin (Takeuchi *et. al*., Gianneechini *et. al*., 2002; Waage *et. al*., 1999) as well as from human (Stepanovic *et. al*., 2001; Couto *et. al*., 2000), but most of them are a pathogenic to animals. It has been reported that numerous bacteria pathogens to insects are dynamic makers of secondary metabolites harmful to insects or other organisms that can be utilized as novel particles for controlling both plant pathogens and pests (Bode, 2009).

Providencia represents a genus of urease producing, gram negative bacilli which although rare, are very omnipresent in the environment. *Providencia* species intently take after *Proteus* and *Morganella* species. They are often isolated from wounds, respiratory tract and urinary tract (*P. alcalifaciens*, *P. rettgeri* and *P. stuartii*), stool of humans (*P. alcalifaciens*), poultry, faeces from reptiles (*P. rettgeri*), throat, perineum, axilla and blood of humans (O'Hara *et. al*., 2000). A report from Nepal in 2014, a cluster of surgical infections with regards to the isolation of *P. rettgeri*, demonstrated the presence and significance of this organism in the Asia-Pacific region (Tada *et al*., 2014). *P. rettgeri* has been involved in the etiology of gastrointestinal sickness in 1986, traveler's diarrhea in 2004, and ocular infection in 2006 (Muller 1986; Yoh *et. al*., 2005; Koreishi *et al*., 2006). *P. rettgeri* has additionally been involved as a causative agent of "purple bag syndrome", where the enzymatic activity gives rise to a purple tinged urine (Peters *et. al*., 2011).

There are not many records about the occurrence of natural enemies of *R. ferrugineus*, which may be ascribed to the mysterious living space of the eggs, larvae and pupae, which protect them from such common adversaries. Ordinarily, the natural enemies do not play an important part in controlling of *R. ferrugineus*. Reginald (1973) suggested that natural enemies don't have a significant impact in controlling world's worst pest of palm trees, *R. ferrugineus* (RPW) and few studies have been conducted on natural enemies of *Rhynchophorus* (Murphy and Briscoe, 1999; Faleiro, 2006a,b). In this study, bacteria associated with the red palm weevils have been investigated by considering their pitfalls and potentialities in order to pinpoint management techniques to be considered in the development and reconciliation of biological control procedures.

CONCLUSION

The study demonstrated the many pathogenic bacteria were associated with the red palm weevil, *Rhynchophorus ferrugineus* Olivier adults, however, the pathogenicity of these bacteria could be attributed to the production of secondary metabolites harmful to the RPW. The extraction and recognizable proof of secondary metabolites delivered by the entomopathogenic bacteria isolated in this study as well as the in vivo activity of bacterial cells against *R. ferrugineus* require further investigation.

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