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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
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308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS

Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2021.944.952

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Research Article

Bioassay of Some Indigenous Entomopathogens for Controlling *Rhynchophorus ferrugineus*, Olivier in Saudi Arabia

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Abstract

Background and Objective: The red palm weevil is a dangerous date palm pests that cannot be controlled with chemical pesticides only. As a result of the justified concerns of the negative use of synthetic insecticides on human health and the environment. So on, candidate eco-friendly micro-organisms isolated from KSA agri-ecosystems were evaluated in controlling RPW. **Materials and Methods:** Some indigenous entomopathogenic fungi and bacteria were isolated from naturally infected RPW larvae and adults and evaluated as alternative control methods. **Results:** The infection of RPW larvae with entomopathogenic fungi and bacteria under natural conditions was higher than in adults. *Beauveria bassiana* was the most prevalent followed by *Aspergillus* sp., *Metarhizium anisopliae*, *Mucor* sp., *Cladosporium chlorocephalum*. In contrast, both *Bacillus thuringiensis* and *Bacillus popilliae* formed 73.9 and 26.1%, respectively. From the 7th day, mortalities (%) increased gradually and recorded the highest mortalities with 21st days after treatment and recorded 93.33, 66.70, 53.36, 46.69 and 60.00% when treated with *B. bassiana*, *M. anisopliae*, *C. chlorosphalum*, *Mucor* sp. and *Aspergillus* sp., respectively. **Conclusion:** Although there was evidence indicating midgut damage and feeding inhibition among larvae that survived the treatments, instead of lower activity of *B. thuringiensis* against *R. ferrugineus* immature stages may refer to that, Both species of *Bacillus* were more virulent as the days 15-21 post-treatment.

Key words: Red palm weevil, *Rhynchophorus ferrugineus*, Bio-control, biological control, entomopathogenic fungi and bacteria, eco-friendly micro-organisms

Citation: Abdel-Baky, N.F., K.E. Hamed, N.D. Al-Otaibi and M.A. Aldeghairi, 2021. Bioassay of some indigenous entomopathogens for controlling *Rhynchophorus ferrugineus*, olivier in Saudi Arabia. Pak. J. Biol. Sci., 24: 944-952.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The Red Palm Weevil (RPW) *Rhyncophorus ferrugineus* Olivier (Curculionidae: Coleoptera) is considered the most lethal insect pest of date palm since introduced to Gulf countries in the early 1980s. So, among all palm pests, it can be considered that RPW is a devastating and dangerous palm pest in the Mediterranean, Middle East and Eastern countries, meaning that the insect has a wide geographical distribution plague in temperate and tropical areas[1-3]. This insect is a highly prevalent pest worldwide, damaging more than 26 palm species belonging to 16 different genera[4] including date palm (*Phoenix dactylifera* L.)[5]. *Rhynchophorus ferrugineus* has a unique infestation process to the date palm, which caused economic losses to exceed the capacity of farmers to produce dates continuously with low economic costs in gulf country and worldwide within the last 30 years, so may lead to a disaster in date palm production[6,7]. The insect is a tissue-boring pest which most of its life stages live hidden inside date palm tree parts destroying its vascular system and eventually causing the collapse and death of the tree. Consequently, leads to rotting the internal tissue of the palm tree trunk leads to destroying, the disintegration of the trunk tissues and in most cases, trees collapse and die[8]. So, many of the control methods used till now still ineffective to reduce the RPW population to a non-economic Injury level[8,9].

There are many factors behind the success of RPW colonization in palm countries and became one of the invasive species including, agro-environmental conditions, monocultures and intensive modern agronomic practices[8]. As well as, selling date palm parts and transmission of palm trees materials among cities inside the same country and/or between centuries becomes another reason behind RPW spread worldwide[10].

Prophylactic treatments against *R. ferrugineus* generally consist of using chemical insecticides periodically which may involve problems of environmental pollution, reduction of entomophagous fauna and human health especially when carried out in urban areas. Because of the cryptic habitats of RPW, so its management with chemical insecticides is difficult. In general, the control tactics that are applied worldwide against RPW are based on chemical insecticides through the injection of chemical pesticides inside the palm trunk[11]. Microbial control agents offer alternatives to chemical pest control and can be more effective and selective than chemical pesticides[3]. Additionally, there are concerns regarding the potentially detrimental effects of chemical pesticides on non-target insects including natural enemies[12]. It is, therefore, necessary to test the compatibility and effectiveness of biological control agents within the IPM program. No effective control measures are available[9], despite, the high efficacies and persistence of imidacloprid Oil Dispersion (OD) as a drench in preventive and curative treatments against *R. ferrugineus* in young palms of *Phoenix canariensis, Washingtonia robusta, Washingtonia filifera* and *Trachycarpus fortunei* show the potential of this product for the management of *R. ferrugineus*[13].

Rhynchophorus ferrugineus has been found naturally infected by the entomopathogenic fungus *Beauveria bassiana* but its infection process in this host is unknown[3]. Fungal entomopathogens infect a wide range of economically important insect pests[12]. The fungus *Beauveria* spp. is a significant coleopteran pathogen[14]. To suppress current outbreaks of *R. ferrugineus*, entomopathogenic fungi are being proposed as biological control agents in IPM programs[8,9]. Entomopathogenic agents are considered a unique way and have well achieved in insect pest control compared with other methods. This may be due to their mode of actions which destroy insect hemolymph even though penetration the insect cuticle or swallows from the mouth. Therefore, these agents show great potential to control insects whether insects were sucking or chewing[15].

Therefore, the current study aimed to look for, (1) an expanded scientific search area to get isolates of the entomopathogenic fungi to be more effective against *R. ferrugineus* and other insect pests and that more adapted to the local environment, (2) virulence study of the fungus and determine its efficiency as a biological control agent, (3) examine the host range of insect pests as possible to be infected under laboratory conditions, (4) study the safety to the host plant and associated beneficial insects and (5) study the effect of some ecological factors on production and growth of the fungus to contribute the mass production under laboratory conditions.

MATERIALS AND METHODS

Surveying entomopathogenic organisms associated with *R. ferrugineus*: In 2017-2018, a survey study was carried out at different date palm farms infested by *R. ferrugineus* in the Qassim region. Both RPW larvae and adults were collected and involved in the study. About Thirty date palm trees were chosen from three date farms by 10 trees to each farm. RPW larvae and adults were collected and inserted in paper bags, then transferred to the laboratory for examination. Infected larvae and adults by entomopathogenic organisms (cadavers or a healthy one) were counted and a single hypha and/or spore was used to get a new colony under lab condition. This procedure was done with each farm infested with RPW in the Qassim region.

Fungal and bacterial isolations and identification

Following two procedures were used for fungi isolation: RPW cadavers (larva and/or adults) showing natural external growth of fungi were collected and maintained in Petri-dishes contain Potato Dextrose Agar (PDA) media. The inoculated Petri-dishes were kept in an incubator at 27±2 and 75±5% R. H. until further growth of the fungi. A compound microscope was used to examine spores from pure cultures. Predicted RPW (larva and/or adults) to be infected due to their abnormal movement were surface-sterilized in a 1% sodium hypochlorite solution for 30 sec and washed in distilled water. Then the insects were cultivated in Petri-dishes (25 insects/dish) on PDA media and kept in an incubator under the same regime of temperature and R. H.

Identification of isolated fungi and bacteria was done primarily by Project teamwork. Confirmation of the Fungus identification was based on the external symptoms and the morphology of the entomopathogenic organisms and then habit characters were used in consultation with Waterhouse and Brady[16], Humber[17] and Common wealth Mycology Institute, Kent, Surrey, England[18,19] to confirm the preliminary identification.

Bioassay: Each isolate of microorganisms associated with RPW individuals was used to assay its pathogenic role against RPW larvae and adults under the laboratory. Twenty-five individuals of each *R. ferrugineus* larvae or adults were used after surface-sterilized in a 1% sodium hypochlorite solution for 30 sec and washed in distilled water. Each Prti-dish contained 25 individuals were considered as one replicate. The insects were placed in a dark colour blotter moist with the fungal suspension with one concentration (10×10^6 spores mL^{-1}). A piece of sugar cane was added to each Petri dish after sterilization to be a source of food[20] techniques for the preparation of the fungal inocula were followed. Data collected daily and continued for 7 days.

Statistical analysis: Analysis of variance, correlation coefficient and stepwise regression models was used for Data analysis[21]. Percentages of entomopathogenic-infected RPW larvae and/adults were calculated by dividing the total number of infected RPW (summed over all samples) by the total recorded numbers of each species sampled then multiplying by 100. Treatment means, Standard Deviations (SDs) and significant differences were analyzed using the CoStat software[21] test program. The significance of the main effects was determined by ONE-way analysis of variance (ANOVA). The Significant differences between treatments were determined using Tukey's multiple range tests ($p\leq0.01$, 0.05). Percentage mortality was calculated according to the Abbott Eq. [21]:

$$CM = \frac{Nc - Nt}{Nt} \times 100$$

where, CM is the Corrected mortality in (%), Nc is the Live individuals in the control after the treatment and Nt is the Live individuals in the treatment after the treatment.

RESULTS

Identification of the micro-organisms: The identification methods represented two micro-organisms groups associated with RPW larvae and adults in palm farms. The 1st group includes five species of fungal pathogens, while the other group contains two species of bacterial pathogens (Table 1). From the Entomopathogenic fungi group, *Beauveria bassiana* occupied the 1st rank, followed by *Aspergillus* sp. (in the 2nd rank). Whereas *Metarhizium anisopliae*, *Cladosporium chlorosphalum* and *Mucor* sp. were in equal degree of importance in terms of their presence and occurrence in the examined samples of RPW (Table 1). In the entomopathogenic bacteria group, *Bacillus thuringiensis* and *B. popilliae* were found associated with RPW larvae but *Bacillus thuringiensis* occurrence was higher (occupied the 1st rank) comparing with *B. popilliae* (occupied the 2nd rank).

Occurrence and natural infection of micro-organisms associated with RPW under field condition: Filed observation showed that the infection of RPW larvae under natural conditions was higher in comparison with the adults. The natural infestation percent reached 72.5 and 27.5% of both

Table 1: Groups of the entomopathogenic that naturally infected RPW in the qassim region in 2016-2017

Entomopathogenic species		Occurrence rate
Entomopathogenic fungi	*B. bassiana*	***
	M. anisopliae	*
	C. chlorosphalum	*
	Mucor sp.	*
	Aspergillus sp.	**
Entomopathogenic bacteria	*B. thuringiensis*	****
	B. popilliae	**

*Occurrence rate less than 25% **occurrence rate less than 50% and ***occurrence rate less than 75% ***occurrence rate less than 90%

larvae and adults. Additionally, Entomopathogenic fungi represented 42.2% compared with 47.8% for Entomopathogenic bacteria (Fig. 1 and 2).

Among entomopathogenic fungi, *Beauveria bassiana* was the most dominant one by 41.2% followed by *Aspergillus* sp. (25.4%), *M. anisopliae* (18.8%), *Mucor* sp. (8.9%), *C. chlorocephala* (5.7%). On contrary, both *Bacillus thuringiensis* and *B. popilliae* formed 73.9 and 26.1% of the total entomopathogenic bacteria, respectively (Fig. 3 and 4).

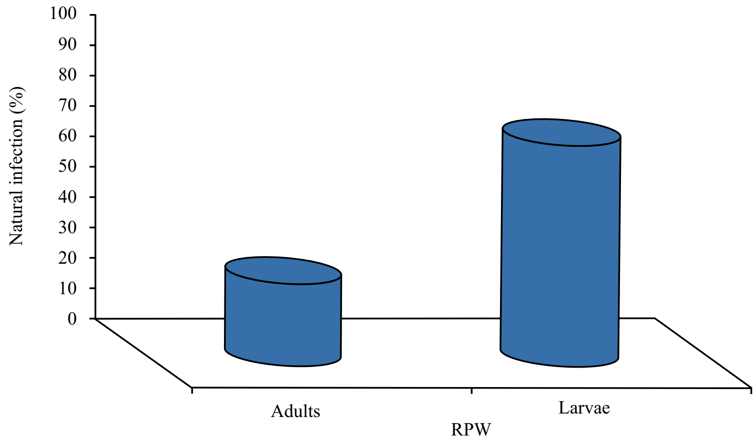

Fig. 1: Natural infection (%) among *Rhyncophorus ferrugineus* adults and larvae in qassim region, during 2016-2017

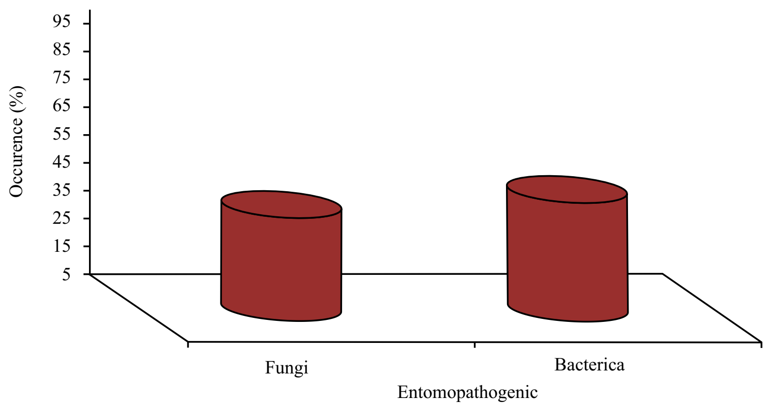

Fig. 2: Occurrence (%) of the entomopathogens infected *Rhyncophorus ferrugineus* adults and larvae in qassim region, during 2016-2017

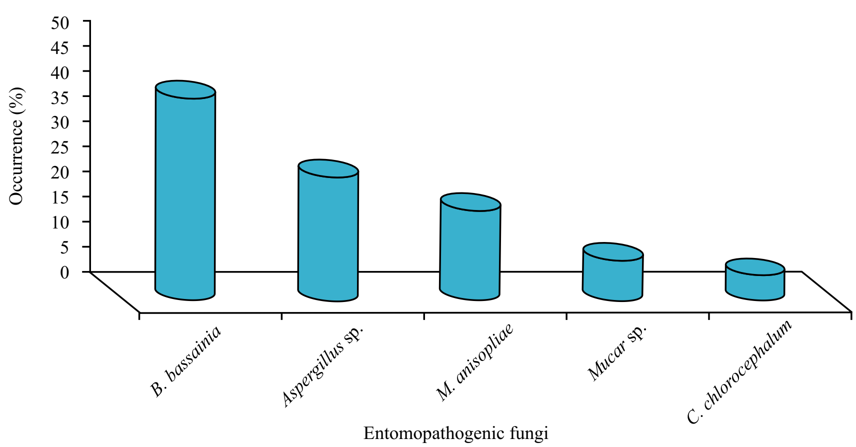

Fig. 3: Occurrence (%) of the isolated entomopathogenic fungi from *Rhyncophorus ferrugineus* adults and larvae, qassim, KSA 2016-2017

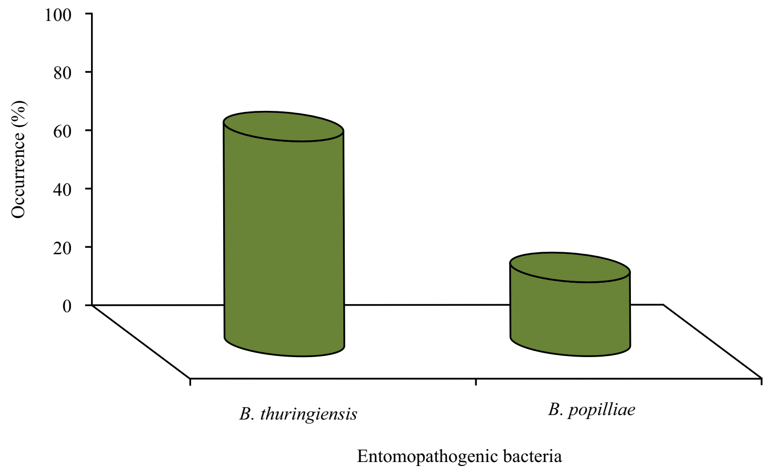

Fig. 4: Occurrence (%) of the isolated entomopathogenic Bacteria from *Rhyncophorus ferrugineus* adults and larvae, qassim, KSA 2016-2017

Table 2: Mortality percentages among *R. ferrugineus* larvae as a result of treatment by 7 entomopathogenic organisms, under lab conditions

Entomopathogenic species	Initial kill (0 time)	Mortality % (days after treatment)* 3	5	7	10	15	21	Residual activity (RA)**	Total activity (TA)**
B. bassiana	00.00^{g}	6.67^{f}	13.33^{e}	20.00^{d}	33.35^{c}	60.03^{b}	93.33^{a}	28.34^{C}	14.17^{C}
M. anisopliae	00.00^{g}	0.0^{f}	6.67^{e}	13.33^{d}	20.01^{c}	40.56^{b}	66.70^{a}	19.24^{D}	9.24^{D}
C. chlorosphalum	00.00^{g}	0.0^{f}	6.67^{e}	13.33^{d}	20.01^{c}	33.35^{b}	53.36^{a}	15.84^{DE}	7.92^{DE}
Mucor sp.	00.00^{e}	0.0^{e}	00.00^{e}	6.67^{C}	6.67^{C}	26.67^{b}	46.69^{a}	10.84^{E}	5.42^{E}
Aspergillus sp.	00.00^{e}	0.0^{e}	6.67^{e}	13.33^{d}	26.68^{c}	33.35^{b}	60.03^{a}	17.51^{D}	8.75^{D}
B. thuringiensis	00.00^{g}	6.67^{e}	26.67^{d}	46.69^{c}	60.03^{b}	100.00^{A}	100.00^{a}	42.51^{A}	21.51^{A}
B. popilliae	00.00^{g}	6.67^{f}	20.01^{e}	40.02^{d}	53.36^{c}	86.71^{b}	100.00^{a}	38.35^{B}	19.18^{B}

*Small letters meaning that the averages followed by the same letter within a row are not significantly different from each other at 5% (duncan) and **capital letters meaning that the averages followed by the same letter within a column are not significantly different from each other at 5% (duncan)

Bioassay studies

Entomopathogens associate with the larvae of RPW: Laboratory studies revealed that the five candidate entomopathogenic fungi isolated from RPW were pathogenic to *R. ferrugineus* larvae (Table 2). Despite their initial kill were zero and their activities had a higher pathogenic virulence against RPW larvae (Table 2).

Also, the current revealed that the pathogenic by five entomopathogenic fungi varied significantly in terms of the initial kill, days after treatment, residual activity and total activity. Despite this, the 1st mortality cases among RPW larvae appeared on the 3rd day of treatment with *B. bassiana* (6.67%). On the 5th day of treatment, all entomopathogenic fungi caused mortalities amongst RPW larvae except *Mucor* sp. From the 7th-day mortalities (%) increased gradually and recorded their highest mortalities with 21st days after treatment. On day 21st, mortality (%) listed 93.33, 66.70, 53.36, 46.69 and 60.00% when treated with *B. bassiana, M. anisopliae, C. chlorocephala, Mucor* sp. and *Aspergillus* sp., respectively (Table 2). From this table, we can conclude that *B. bassiana* was the most virulent fungi followed by *M. anisopliae* and *Aspergillus* sp.

The entomopathogenic bacteria were more virulent than the entomopathogenic fungi (Table 2). The 1st mortality symptoms caused by *Bacillus thuringiensis* and *B. popilliae* was observed with the 3rd day of treatment (6.67% mortality for each one). On the 7th day, the mortality rose sharply to 46.69 and 40.02 and then increased to 60.03 and 53.36 on the 10th day for *B. thuringiensis* and *B. popilliae*, respectively. Fifteen days after treatment, *B. thuringiensis* caused 100% mortality. *B. thuringiensis* and *B. popilliae*, cause 100% mortalities as cumulative mortality (Table 2).

Entomopathogen associate with the adults of RPW: The five candidate entomopathogenic fungi isolated from RPW were pathogenic to *R. ferrugineus* adults despite their lowest initial kill values and their activities had a moderately effective (Table 3).

Despite this, the 1st mortality cases among RPW adults appeared on the 7th day of treatment with *B. bassiana* (13.33%) and *Aspergillus* sp. (6.67%). Ten days post-treatment, mortalities (%) increased gradually and recorded their highest mortalities with 21st days after treatment. On day 21st, mortality (%) listed 66.67, 46.67, 40.0, 33.33 and 46.67% when treated with *B. bassiana, M. anisopliae*, *C. chlorosphalum*, *Mucor* sp. and *Aspergillus* sp., respectively (Table 3).

Effect of various constant temperatures on the linear growth of entomopathogenic fungus, *Beauveria bassiana*: This study showed that *B. bassiana* is influenced by the studied temperature (Fig. 5). The linear growth of the fungus was faster when incubated at 25 followed by 20°C than at 15 and 30°C. Furthermore, 20 and 25°C are more suitable for mass production of the fungus under lab conditions.

Influence of temperature on the entomopathogenic bacteria *Bacillus thuringiensis'* synthesis of exopolysaccharides (EPS): Temperature and bacteria growth conditions are important factors for EPS production. In this study, the production of EPS by *B. thuringiensis* varied according to the suitable temperature used (Fig. 6). Incubated *B. thuringiensis* at 28°C produced more EPS (24.77 g L^{-1}) followed by 23 and 33°C which produced 21.47 and 18.3 g L^{-1} of EPS. Meanwhile, incubated *B. thuringiensis* at 43°C was not preferred for EPS production (Fig. 6).

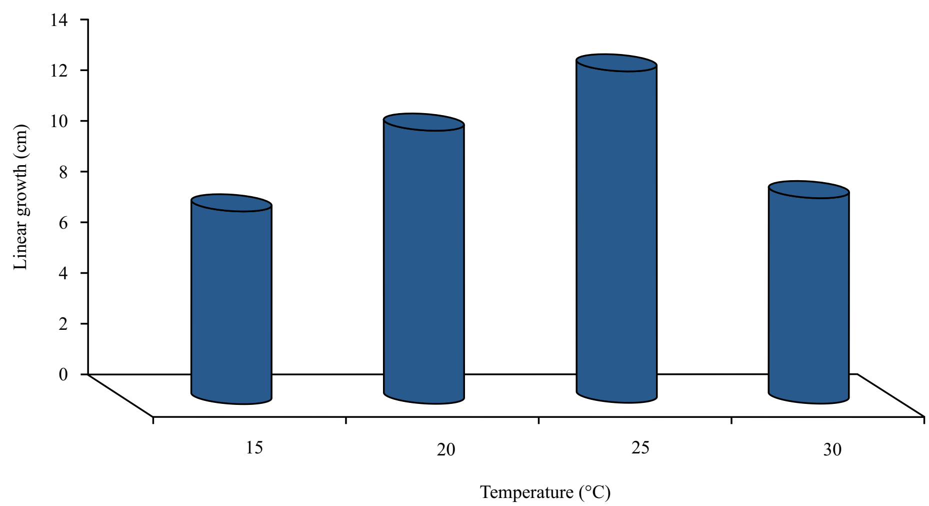

Fig. 5: Effect of four constant temperatures on *Beauveria bassiana* growth, after 14 days of inculcation

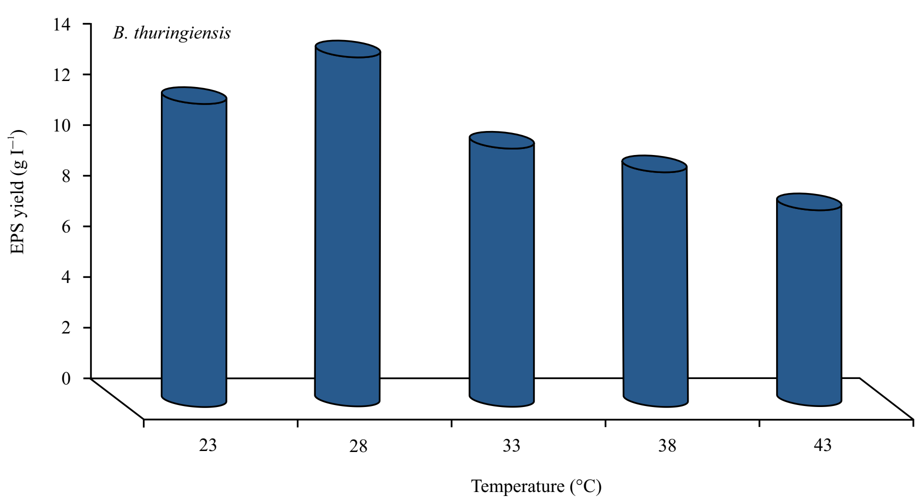

Fig. 6: Production of exopolysaccharides (EPS) by *B. thuringiensis* after exposure to various constant temperatures

Table 3: Reduction percentage in *R. ferrugineus* adults after treatment by seven entomopathogenic organisms under lab conditions

Entomopathogenic species	Initial kill (0 time)	Mortality % (days after treatment)* 3	5	7	10	15	21	Residual activity (RA)**	Total activity (TA)**
B. bassiana	00.00^{e}	00.0^{e}	00.00^{e}	13.33^{d}	20.00^{c}	46.67^{b}	66.67^{a}	20.95^{C}	10.475^{C}
M. anisopliae	00.00^{d}	00.0^{d}	00.00^{d}	00.00^{d}	6.67^{c}	20.00^{b}	46.67^{a}	10.477^{D}	5.284^{D}
C. chlorosphalum	00.00^{d}	00.0^{d}	00.00^{d}	00.0^{d}	6.67^{c}	20.0o^{b}	40.00^{a}	9.52^{DE}	4.76^{D}
Mucor sp.	00.00^{d}	00.0^{d}	00.00^{d}	00.00^{d}	6.67^{c}	20.0o^{b}	33.33^{a}	8.57^{DE}	4.285^{D}
Aspergillus sp.	00.00^{d}	00.0^{d}	00.00^{d}	6.67^{c}	6.67^{c}	20.00^{b}	46.67^{a}	11.43^{D}	5.72^{D}
B. thuringiensis	00.00^{e}	00.0^{e}	00.00^{e}	20.00^{d}	46.67^{c}	53.33^{b}	86.67^{a}	29.52^{A}	14.76^{A}
B. popilliae	00.00^{e}	00.0^{e}	00.00^{e}	13.33^{d}	40.00^{c}	53.33^{b}	73.33^{a}	25.71^{B}	12.86^{B}

*Small letters meaning that the averages followed by the same letter within a row are not significantly different from each other at 5% (duncan) and **capital letters meaning that the averages followed by the same letter within a column are not significantly different from each other at 5% (duncan)

DISCUSSION

The current study provides that the entomopathogenic fungi and bacteria can also provide an excellent alternative mean to control RPW instead of chemical control. The host can be infected both by direct treatment and by horizontal transmission from infected insects or cadavers to healthy insects. Subsequently, the infection can occur via the new generation of spores[22,23]. These unique characters make the entomopathogenic fungi and bacteria especially important for the control of concealed insects such as *R. ferrugineus*. Different strains of *M. anisopliae* and *B. bassiana* were tested against *R. ferrugineus*[24]. The former proved more virulent than *B. bassiana*. More recently, in preliminary studies,[25] successfully reduced the incidence of *R. ferrugineus* under field conditions using a native strain of *B. bassiana* isolated from *R. ferrugineus* cadaver. Consequently, adults should be considered as the targets of any treatment involving this entomopathogenic fungus because they are the only free-living stage. In some cases, these endophytic fungi have resulted in complete control of the target pest[23]. However, the use of fungal species and strains with endophytic behaviour is still a poorly explored tool for the systemic protection of palms against *R. ferrugineus*. As a result, it is critical to conduct sound open-field tests using *B. bassiana.* Therefore, it is urgent to perform sound open-field experiments with *B. bassiana* strains to explore their biocontrol potential.

The efficacy of the entomopathogenic bacteria *B. thuringiensis* to control *R. ferrugineus* has been tested in laboratory conditions[26,27]. Although there was evidence indicating midgut damage and feeding inhibition among larvae that survived the treatments, results showed that the activity of *B. thuringiensis* against *R. ferrugineus* immature stages was low. But our current study referred that the two species of Bacillus were more virulent which caused 100% mortality with 15-21 days of treatment.

CONCLUSION

The Pathogenicity of the isolated entomopathogenic fungi and bacteria was higher in RPW larvae than adults under the natural field conditions. The natural infestation percentage reached 72.5 and 27.5% on larvae and adults respectively. Entomopathogenic fungi represented 42.2% compared with 47.8% for Entomopathogenic bacteria. There was evidence indicating midgut damage and feeding inhibition among larvae that survived the treatments, instead of lower activity of *B. thuringiensis* against *R. ferrugineus* immature stages may refer to that, both species of Bacillus were more virulent as the days 15-21 progressed from the start of the treatment.

SIGNIFICANCE STATEMENT

In this study, we found the indigenous entomopathogenic fungi and bacteria in the Qassim region, KSA provides some highly safe effective non-chemical control procedures against RPW, which considered a serious pest in date-palm fields. The results confirmed certain candidate entomopathogenic fungi and Bacteria could provide eco-friendly control procedures against RPW in the field and lab. The effectiveness of these entomopathogenic fungi and bacteria could be reasonably improved through combination with other biotic agents or by using suitable facilities and techniques or other control measures and/or through integration with other IPM components.

ACKNOWLEDGMENT

We acknowledge the team of the Red Palm Weevil Control Program at the Ministry of Environment, Water and Agriculture, Qassim Branch, for the technical support and assistance in collecting samples and conducting the experiments.

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