Isolation, Identification, Pathogenicity bioassay and Mass production of Indigenous Isolate of Entomopathogenic Fungi against Red Palm Weevil *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae)

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This Thesis was Submitted in Partial Fulfillment of the Requirements for the Master's Degree in:

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July, 2019

عزل وتشخيص فطريات محلية ممرضة للحشرات وتقييمها مخبرياً ضد حشرة

سوسة النخيل الحمراء *Rhynchophorus ferrugineus* (Olivier)
(Coleoptera: Curculionidae)

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This thesis/dissertation (Isolation, identification, pathogenicity bioassay and mass production of indigenous isolate of entomopathogenic fungi against Red Palm Weevil *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae)

was successfully defended and approved on 22/July/2019.

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Dedication

I dedicate this thesis to the four pillars of my life: God, Parents, sisters and nephews, until now I do not know where the life's road will take me, but walking in your side my merciful God prove that I am in the right path.

My mom and dad, I would never be who I am today, without your unconditional love, your doa'a and faith in me.

My sisters, Ghadeer and Atheer and little lovely nephews, Moa'taz and Yousef who provide me strength through their bright eyes.

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May Allah accept this research as useful science and grant me his mercy.

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"Praise to Allah, Lord of the worlds"

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List of Abbreviation

Abbreviation	Description
Bp	Base pair
CTAB	Cetyl trimethyl ammoniun bromide
D.W	Distilled water
DNA	Deoxy ribo nucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
EPB	Entomo pathogenic bacteria
EPF	Entomo pathogenic fungi
EPN	Entomo pathogenic nematodes
EPV	Entomo pathogenic viruses
FAO	Food and agriculture organization
GLM	General linear model
Ha	Hectares
IPM	Integrated pest management
ITS2	Internal transcribed spacer
L: D	Light : dark
LT100	100% lethal time
LT50	Median lethal time
M	Molar
MEM	Mediterranean flour moth
Mg SO4	Magnesium sulfate
mL	Milliliter
mM	Millimolar
mm	Millimeter
NA	Nutrient agar
NaCl	Sodium chloride
NB	Nutrient broth
NH4OAC	Ammonium acetate
PAAC	Palestinian Agricultural Academic Cooperation Project
PCR	Polymerase chain reaction
PDA	Potato dextrose agar

PEG	Poly ethylene glycol
PTUK	Palestine Technical University - Kadoorie
rDNA	Ribosomal DNA
RH	Relative humidity
rpm	Round per minute
RPW	Red Palm Weevil
SAS	Statistical analysis system
SDS	Sodium dodecyl sulfate
SIT	Sterile insect technique
sp.	Species
Std	Standard deviation
TAE	Tris base, acetic acid and EDTA
TE	Tris- HCl and EDTA
Tris- HCl	Tris hypochlorite
USD	United states Dollar
UV	Ultra violet
x	Times

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Abstract

The high incidence rates of red palm weevil (RPW) *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) in Palestine consider as serious threat to palm industry; due to the sudden death of the whole trees. In this study, different Palestinian indigenous entomopathogens were isolated from different locations in the West Bank such as entomopathogenic fungi, entomopathogenic bacteria, entomopathogenic nematodes and entomopathogenic viruses. The entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae* were identified morphologically and molecularly, then their pathogenicity against the RPW was evaluated. Results showed that they have a significant effective against RPW. Thus they could be considered as alternative to the chemical control. Results also revealed that mass production of these indigenous entomopathogenic fungi could be carried out by using cheap and affordable agricultural by-products, that enhances the activity and provide date palm growers alternative active bio-control agents in a wide scale and for a long-term control.

Key words: *B. bassiana*, *M. anisopliae*, integrated pest management, bio pesticide, indigenous natural enemy.

1. INTRODUCTION

1.1 General introduction

Date palm tree *Phoenix dactylifera* L. (Arecales: Arecaceae) is considered one of the most important fruit tree; as it was mentioned in the Holy Quran and the Bible. The genus consists of fourteen species distributed in the tropical and sub-tropical regions (Al Antary et al., 2015). This perennial tree can live for more than 150 years, and normally starts to bear dates in their fifth year (Ajlan and Abdulsalam, 2000). Date palm fruits have been used as a nutritional food source for thousands of years (Shabani et al., 2014). They have many health benefits like antioxidant, anti-diabetic, sex hormone modulator, anti-inflammatory and anti-tumour (Rahmani et al., 2014). It contains the basic nutrients components such as proteins, fibers, minerals (selenium, copper, potassium, and magnesium) and vitamins especially B-complex and C (Al-Farsi and Lee, 2008).

The total number of date palms in the world is about 120 million trees, distributed in 30 countries and producing nearly 7.5 million tons of fruit per year (Abd Rabou and Radwan, 2017). Arab countries possess 70% of the 120 million world's date palms and produce 67% of the global date production (El-Juhany, 2010). Cultivated date palms have existed in Palestine for more than 5,000 years in Jericho and the Jordan Valley in the West Bank and in the Gaza Strip; due to their optimal climate conditions,

including high temperatures and low relative humidity. The total grown area with dates in both West Bank and Gaza was 725 ha in 2012 (Abu-Qaoud, 2015). Medjool, Barhi and Hayani cultivars are the most planted in the West Bank and Gaza, with an average production of 70 –120 kg /tree /year and with a total revenue of 140 $/ tree, which is considered as the highest crop revenue (Abu-Qaoud, 2015).

Serious problems and challenges have been facing date industry over the past decade. Productivity has declined due to insect pest attacks and disease infestations. Almost 50 species of insects and mites were recorded to attack date palm worldwide. The main pests and diseases included red palm weevil (*Rhynchophorus ferrugineus* Olivier), date palm scale (*Parlatoria blanchardi* Targ.), Rhinoceros beetle (*Oryctes rhinoceros* L.), alternaria leaf spot (*Alternaria radicina* Meier), Fusarium wilt (*Fusarium oxysporum*), (Haldhar et al., 2017), Diplodia leaf base rot, Graphiola leaf spot, Terminal bud rot, Inflorescence rot, Bending head, and fruit rot (Abu-Qaoud, 2015).

Red Palm Weevil (*Rhynchophorus ferrugineus* Olivier) (Coleoptera: Curculionidae) (RPW) is one of the most important insect pest of date palm worldwide (El-Juhany, 2010), and the most destructive insect pest in the Middle East (Bokhari and Abuzuhira, 1992). According to Alhudaib, et al., (2017), FAO has identified it as a category number one of date palm pests in the Middle East. This pest has been known with various names such as the

Red Stripe Weevil, Coconut Weevil, Asiatic Palm Weevil, Hidden Enemy, and AIDS of palm (Wakil, et al., 2017).

The RPW adult females dig holes at the base of the fronds, and lay their eggs (Abraham et al., 1998), when larvae bore and tunnel into the palm and feeding on the inner tissues. Progressively the larvae tend feed on the soft tissues surrounding and reach to the periphery of the stem destroying the palm tree (Dembilio et al., 2019; Soroker, et al. 2005). It was found around the whole year in date palm areas and their damage increased when chemical application is cease during pollination and maturing period (Al Saoud, 2007). Non-chemical method by frond pruning help to minimize the infestation (Suma, et al., 2014).

Chemical insecticides were the most commonly used method to control RPW (Jacas and Dembilio, 2013) but record of insect resistance against most insecticides used (Unruh and Willett, 2008), and the general public concerns related to environmental pollution and insecticide residues promoted other control methods (Abdel-Azim, et al.,2017). Many integrated pest management (IPM) programs considered alternative approaches to the chemical control with biological, semi-ochemical, sterile insect technique (SIT) (Lacey, et al., 1986; Abraham, et al., 1998).

Entomopathogenic natural enemies (Biopesticides) served as potential insect pest control method to reduce the negative impact of insecticides on the

public health and the environment (Ummidia and Vadlamani, 2014; Cito, et al., 2014; and Garcia et al., 2017). Thus, mass production of indigenous beneficials could increase the effectiveness of pest management programs (Latifian, 2014).

1.2 Research objectives

The aims of the current study were to:

1. Determine the pathogenicity of isolated indigenous entomopathogenic fungi.
2. Mass production of the most effective bio-agent for mass releases and management program against RPW.
3. Viability assessment of the mass produced entomopathogenic fungi.

2. LITERATURE REVIEW

Many researchers studied the nature of RPW and its rapid dispersal internally and across international borders resulted in making it a trans-boundary pest (Al-Saroj, 2017). It was first described as a dangerous date palm pest in Punjab, India (1917), and recorded for the first time in the Arabian Peninsula in the mid of 80s (Abraham et al., 1998). Ever since, RPW had spread all over the world (Cox, 1993). The first detection of the weevil infestation in the Jordan Valley, in the west bank of the Jordan River and in the northern area of the Dead Sea was in 1999 (Soroker, et al., 2005). The presence of the pest and their host during the entire year enabled RPW to spread widely (Al-Saoud and Ajlan, 2013). Adult females begin their infestation using their strong mandibles to create a very small wound or attack tree from the tender areas to lay their eggs (Ince et al., 2011; Giblin-Davis et al., 2013).

The RPW is a polyphagous insect attacking several palm species such as coconut, oil palm and sago but date palm cultivar has been favored due to their high sugar content; which helps the insect in their development (Vidyasagar, et al., 2000; Mahmud, et al., 2015 and El-Shafie et al., 2017). The RPW female lays nearly 300 creamy eggs, which hatches in 2-5 days to 50 mm long, Legless, black head with strong mouthparts larvae (Dembilio et al., 2018). The larvae stay for 1-3 months, then pupate in cocoons form for 2-3 weeks (Rochat, et al., 2017). Adult weevil (30 mm long) emerges and

live nearly 4 months depends mainly on environmental conditions. Well-developed winged adults increase the ability to fly from dead palm trees to new ones (Al-Saad and Aletby, 2018).

Larvae and adults prefer to remain and multiply in the infested trunk. It is difficult to detect early infestation because they do not show any clear symptoms (Al-Saoud and Ajlan, 2013). Symptoms of infestation were described as strong and distinctive fermented odor, insect frass (Dembilio, et al., 2010). Wilting and remarkable reduction in date production are the late infestation symptoms (Rach, et al., 2013), trunk broken due to wind or other factors, as well as heavy viscous yellow to brown fluids oozing from the boring tunnels (Soroker, et al., 2005).

Mass rearing of RPW on sugarcane and the artificial diets for laboratory studies, and their results showed that the average biomass of larvae at various stages, and biomass and percentage of emergence of adults varied greatly (Kaakeh et al., 1997 a; Kaakeh et al., 1997 b). According to Shahina et al., (2009) cotton wool with 20 % honey solution was the best artificial medium for egg deposition.

In the past decades, the entomopathogenic fungi were used increasingly as biological control agents for insect species worldwide (Hussain, et al., 2013). Mazza et al., (2014) isolated an indigenous strain of entomopathogenic viruses (EPV) (Cypovirus sp.), entomopathogenic fungi (EPF) (*Beauveria*

bassiana, Metarhizium anisopliae), entomopathogenic bacteria (EPB) (*B. thuringiensis, B. sphaericus, Pseudomonas aeruginosa, Serratia marcescens*), and entomopathogenic nematodes (EPN) (*Heterorhabditis bacteriophora, Steinernema carpocapsae*). They concluded that the natural enemies of RPW failed to stop the spread of the pest. In another study in Italy reported that, more than 90% of the larvae inside the palm trees were naturally infected with entomopathogenic fungi, *B. bassiana and M. anisopliae* (Tarasco et al. 2008). Similar results were confirmed in other studies (Gindin et al., 2006; El-Sufty et al., 2007; Yasin et al., 2017a ; Dembilio and Jaques, 2015; El Kichaoui, et al., 2017; Ricano et al., 2013; Jalinas et al., 2015).

Mass production of *B. bassiana* and *M. anisopliae*, using grain by-products, cereals and other agricultural products showed that rice, sorghum seeds and wheat were the most suitable media for the mass production of *B. bassiana* , and *M. anisopliae* (Sahayaraj and Namasivayam, 2008; Latifian et al., 2014).

Pu, et al., (2016) in their study found that the entomopathogenic bacterium *B. thuringiensis* (Bt) was the most used biological insecticide from bacteria that control Coleoptera and other insect orders. EPNs were also consider an effective control bioagent against RPW and was reported harmless to non-target vertebrates and the environment, the treatments with nematodes were 80% effective as a curative, and up to 98% in preventing RPW infestation

(Dembilio and Jaques, 2015). While, entomopathogenic viruses are obligate pathogen that cannot live or reproduce without a host. The viral infection was found infecting all the developmental stages of RPW under laboratory condition or at field, diseased and infected larvae that suspected of having the infection appear very weak, become slow in their movement, their midgut turn thinner as the infection advances until it ruptures, and black to grey liquid is released from them, adult stage resulted in malformed and deformed adults and reducing their life span (Yasin, et al., 2017b). Entomopathogenic viruses have been used in biological control in very small scale but have been improved if combined with nematodes (Garcia, et al., 2017).

3. MATERIALS AND METHODS

3.1 Collecting field samples

Larvae, cocoons and adults of RPW were collected from fallen and infested date palm trees in 6 date palm farms located in West Bank) Fig (1).

Insect were collected from Kofor-Aboosh (32°13′20″N 35°05′04″E), Attil (32°22′10″N 35°04′18″E), Baqa-Alsharqea (32°24′38″N 35°04′09″E) and Nazlit Essa (32°24′58″N 35°03′20″E) in Tulkarm district, Al-Zubaidat (32°10′24″N 35°31′47″E) and Al- Jeftlik (32°08′39″N 35°29′36″E) in Jericho district Fig (2).

Figure 1: Sudden death of the whole infested date palm tree in Jericho district

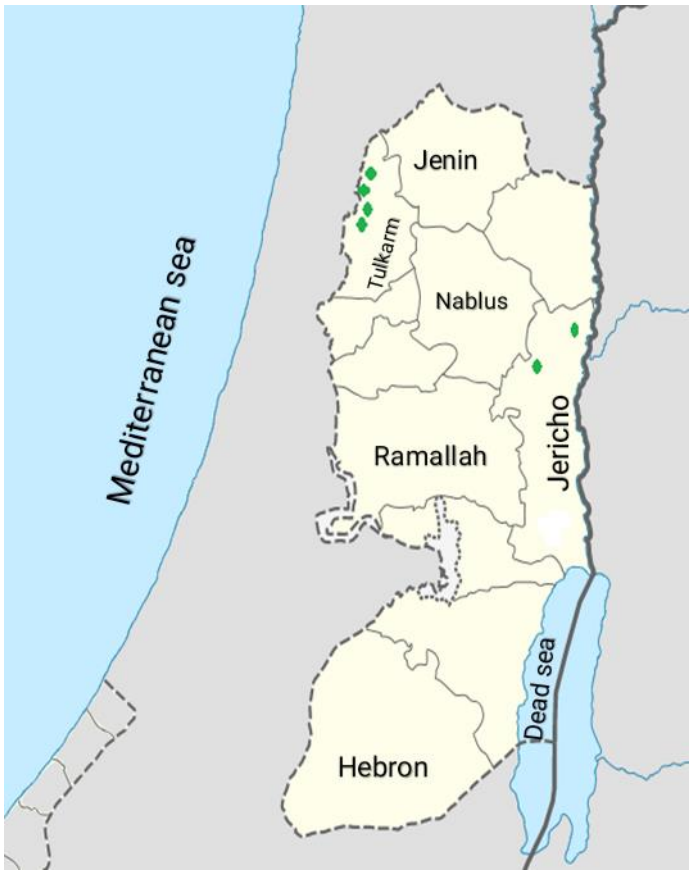

Figure 2: Field collection of RPW carried out through 6 sites in 2 governorates in the West Bank-Palestine

Each developmental stage was placed individually in covered plastic container. Larvae were separated by size, and cocoons were kept in individual covered plastic boxes until adult emergence. Adults were collected from the field, cleaned well and kept in plastic boxes with fitting lids and small pores in the middle for aeration and provided with at least two absorbent cotton pads saturated with diluted honey solution (10%) for feeding, mating and egg laying.

3.2 Rearing the RPW

All experiments were conducted in the Agricultural Sciences and Technology College's laboratory, Palestine Technical University- Kadoorie (PTUK), Tulkarm, Palestine. Various life stages of RPW were reared in sugarcane (*Saccharum officinarum* L.; Poales: Poaceae) diet in the beginning of the rearing process, the rearing room was maintained at 26 ± 2ºC and 60-70% relative humidity (RH), and the photoperiod was approximately 12:12 (L:D). Collected RPW were divided in pairs (male and female), visual inspection of the fine hair on the dorsal end of the rostrum of the male was used to differentiate between the sexes (Dembilio and Jaques, 2015). Paired insects were kept for 24 hours in (15 x10 x 15 cm) plastic containers for mating, feeding on pieces of sugarcane (5x 3 cm), shredded coconut and piece of cotton pad soaked in diluted honey. Females were daily observed for egg laying and kept at 27±3°C for 3 to 4 days. Using fine camel hairbrush, sugarcane and cotton pad were checked gently to collect fresh laid eggs. Feeding medium was changed every 2-3 days with fresh prepared diet. Eggs were transferred in 90 mm petri dishes with a wet filter paper and were monitored for egg hatching at 27±3°C and 50% RH.

New emerged larvae were transferred gently into a new container with artificial diet, incubated at 27±3 ° C, and used in the bioassay tests. Emerged adults from collected cocoons were fed on diluted honey solution.

Modified meridic artificial diet was prepared according to El-Shafi, et al., (2013) Fig (3).

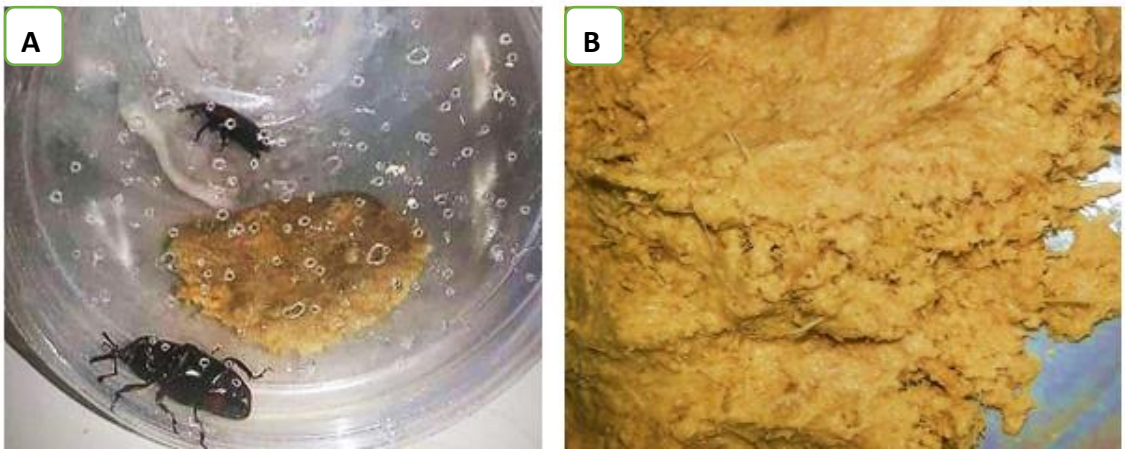

Figure 3: **A:** pair of RPW reared on artificial diet, **B:** the artificial diet

Preparation of the modified diet:

The mixture was prepared with the ingredients in Table (1), ingredients were boiled and stirred very well until complete dissolve and left to cool until 60°C. The homogenized mixture poured in to sterilized containers and kept in the refrigerator at 4±2°C for future use.

Table 1: The ingredients of the rearing diet for red palm weevil

Component	**Amount**	**Component**	**Amount**
Grinded date palm frond	**500 g**	Sodium penzoic	**1.6 g**
Corn flour	**250 g**	Sorbic acid	**1.6 g**
Wheat flour	**250 g**	Agar	**20 g**
Ascorbic acid	**2 g**	Distilled water	**2 L**

3.3 Isolation the entomopathogenic indigenous natural enemies from the *R. ferrugineus*

Cadavers of RPW adults and Larvae were placed in 90 mm petri dishes on an autoclavable filter paper saturated with D.W at 26±3°C. Enotmo-pathogenic growth was noticed after 4-7 days were sporulating structures appeared. Many natural enemies were collected and isolated from either dead adults or larvae or other insect stages (Gabarty and Salem, 2014; Sabbahi, et al., 2009).

3.3.1 Isolation of Entomopathogenic Fungi (EPFs)

Entomopathogenic fungi isolation was carried out by transferring fungal mycelium from the dead weevils to potato dextrose agar (PDA) media (Rathod, et al., 2015) and incubated for 7-10 days at 28± 2°C.

The inoculated plates were checked daily. Then sub-cultured on a new PDA media to obtain pure culture. The subculture was done every 2-3 weeks to maintain a new fresh and pure culture (Ravimannan, et al., 2014).

Morphological identification of the isolated fungi through microscopic examination was achieved by wet mount slides, spores and mycelia were examined under an inverted microscope-Optika XDS-2 Trinocular (AIPTEK international GmbH, Italy).

Conidia and spores were harvested in dry conditions by scraping the surface of agar plate (about 1g) with a sterile loop or scalpel, then suspended in 100 ml sterile D.W containing of 1 ml 0.1% Tween-20, stirred very well by vortex mixer (Scientific, Bohemia, NY) to completely homogenized for 15 minutes, then filtered through three layers of cheesecloth to obtain pure suspension without any debris, large particles or hyphae. Serial dilutions for each fungal isolate were prepared and by using a hemocytometer (ART.No.1280) (Gilchrist-Saavedra, 1997), the conidial concentration and viability in the mixture was determined and calculated.

3.3.2 Isolation of Entomopathogenic Bacteria (EPBs)

Isolation of entomopathogenic bacteria occurred by taking a swab from the infected larvae or adults then spread into nutrient agar (NA) media, then inoculated petri plates were sealed, labeled and incubated at 35±3°C for 12 -24 h. All plates were observed for bacterial growth and the number of colonies was counted. Gram staining technique was applied to differentiate whether the bacteria is Gram-positive or Gram-negative (Moyes, et al., 2009). Isolated entomopathogenic bacteria was suspended in sterile distilled water, kept at 4±2°C in the refrigerator.

3.3.3 Isolation of Entomopathogenic Nematodes (EPNs)

The isolation of EPNs was done by adding sterile distilled water on the dead cadavers of RPW adults and larvae in sterile petri dishes and check their presence under microscope (Optika XDS-2 Trinocular (AIPTEK international GmbH, Italy). Many life stages of nematodes were found, these nematodes were collected while they are under microscope by a needle and dropper, transferred to nutrient broth media (NB) tubes and kept at 4±2°C in the refrigerator.

3.3.4 Isolation of Entomopathogenic Viruses (EPVs)

RPW larvae and adults showed weakness, immobility and typical viral epizootic symptoms have been transferred individually into test tubes container. Under laboratory conditions, the infected larvae (which covered with the black liquid) were transferred into sterilized tubes and D.W was added above them, close the tube and stir, then remove the larvae and kept the resulted liquid in closed tubes in the refrigerator.

3.4 Pathogenicity Test (Bioassay)

Five fungal suspension were prepared for both pathogenicity test and mass production Fig (4). The concentration of each type was measured using hemocytometer Table (2).

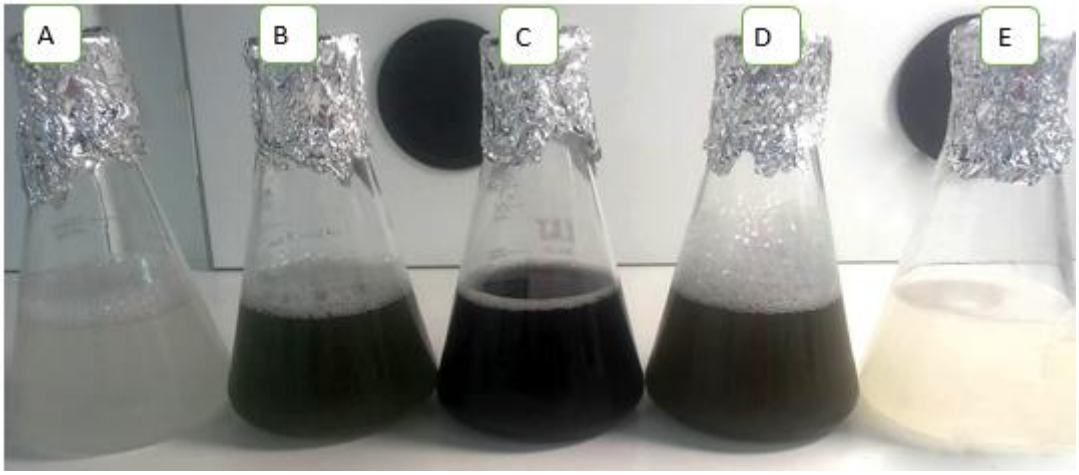

Figure 4: Five fungal suspension, **A:** *B. bassiana*, **B:** *M. anisopliae,* **C:** *Rhizopus sp*., **D:** *Aspergillus sp.* and **E:** *Verticillium sp.*

Table (2): The concentration of the entomopathogenic fungi

Fungi name	Concentration (spores/ml)
B. bassiana	4×10^{-10}
M. anisopliae	4.5×10^{-10}
Rhizopous sp.	1×10^{-10}
Aspirigullus sp.	3×10^{-11}
Verticillium sp.	4.5×10^{-7}

Pathogenicity test was carried out on RPW and the Mediterranean flour moth (MFM) *Ephestia kuehniella* (Zeller) reared on wheat flour and oat flaks in plastic containers (15cm x 10cm x 15cm). Throughout the experiment, insect culture was maintained at constant temperature 27±3ºC, 14L: 10D photoperiod and 60% RH. *E. kuehniella* 3rd larval instars were treated with

the isolated natural enemies. Five fungal suspension were used to detect their pathogenicity.

The insects were divided in to three groups:

Control group in which *E. kuehniella* were treated by water only, the other two groups: one was sprayed with *B. bassiana* and the other with *M. anisopliae*. Then each group (4 insect/group) were put in a plastic container with their nutrient media under control conditions, the insects were observed daily and their mortality were recorded.

The second part was to test the RPW larval bioassay for EPFs, a group of four RPW larvae immerse in *B. bassiana* suspension for one minute by a dipping method while trying to keep the head above using sterilized plastic needle. The same was done for *M. anisopliae* and the control group immerse in D.W only. Then each group was placed in an individual agar petri dish containing 4% agar. Each treatment was repeated four time. All petri dishes were incubated at 27±3°C in darkness for 7-14 days; the plates were observed every day to estimate the lethal time 100 (LT_{100}).

3.5 Molecular identification of the EPFs

The most effective EPFs that isolated and tested were *B. bassiana* and *M. anisopliae*. Therefore, DNA extraction and PCR (Polymerase chain reaction)

which relies on primer sequences designed to facilitate the identification at strain level of specificity were done.

3.5.1 Deoxyribonucleic acid (DNA) extraction using CTAB protocol

Following a modified cetyl-trimethyl-ammonium bromide (CTAB) protocol (Reineke, et al., 1998; Rogers and Bendich, 1994). Briefly, 50-100 mg of five to ten days grown fungi were scraped from PDA surface media using clean and sterile scalpel, then transferred into sterile 1.5 ml microfuge tube (eppendorf tube), 1 µL Protenese K (from a stock solution of 20 mg/ml) was added to each microfuge then heated in water bath for 1 hour at 50-60ºC. 28 µL NaCl (5M NaCl) and 13 µL CTAB 10% (Sigma, St. Louis, MO) were added, mixed gently and heated in water bath for 10 min at 65ºC (CTAB must be rotate on the magnetic plate). Then under fume-hood 142 µL Chloroform: isoamylalcohol (24:1) was added and incubated in ice for 30 min. Centrifuged for 20 min (12000 rpm at 4ºC), the supernatant was transferred (under fume-hood) in new microfuge about 100 µL, and added to each 45 µL NH4Ac (5 M ammonium acetate), mixed gently then put in ice for 1 hour. Centrifuged for 20 min (12000 rpm at 4ºC) using (Hettich® MIKRO 200/200R centrifuge, Z652121 SIGMA), the upper phase was transferred into new 25 µL microfuge. Then added to each 37 µL 30% poly ethylene glycol (PEG) (must be rotate of the magnetic plate) mixed gently, then put in the refrigerator at 4ºC for 1 hour or longer.

Then centrifuged for 30 min to precipitate DNA, the supernatant was poured in glass with water to discard it, and the pellet was washed twice very fast with 500 µL 70% ethanol, which is cold at 4ºC. Then allowed to dry in the drier machine or air dry for 20 min, re-suspended with 50 µL TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8) to each and let in room temperature for overnight to use in gel electrophoresis.

3.5.2 Polymerase Chain Reaction (PCR)

PCR of the internal transcribed spacer 2 region (ITS2) of the ribosomal DNA (rDNA) was performed using an automated veriti 96 well thermal cycler (applied biosystem company) device. The following mixture was done for one sample: PCR reactions were performed in 25 µL reaction mixtures containing 1 µL DNA template, 2.5 µL dNTP's (each one in a 10 mM concentration), 2 µL MgCl2 (25 mM concentration), 0.5 µL 10% BSA (Bovine Serum Albumin), 0.21 µL Taq DNA polymerase, 1 µL of each primer (forward and reverse) Table (3), (0.2 mM) in 2.5 µL (10x) PCR-buffer (TE-buffer) and 16 µL ultra-pure sterile dnase free water (Biological Industries, 1710266). The amplification reaction with the pair of primers.

Table 3: Forward and reverse primers for *B. bassiana* and *M. anisopliae*

Fungi	Forward primer	Reverse primer
B. bassiana	(5'AAGCTTCGACATGGTCTGAT3')	*(*5'-*GGAGGTGGTGAGGTTCTGTT3')*
M. anisopliae	(5'GAAACCGGGACTAGGCGC3')	(5' TCTGAATTTTTTATAAGTAT3 ')

The primers were used to amplify the ITS-2 region of rDNA. The PCR device was programmed for PCR as follows: initial denaturation at 94 ºC for 2 minutes followed by 45 cycles of 1 min. at 94 ºC, 1 min. at 53 ºC and 2 min. at 72 ºC, with 1 min. at 72 ºC after the last cycle. Then reaction products were analyzed by electrophoresis.

3.5.3 Gel electrophoresis

The gel (30ml) was prepared by heating 1% agarose in microwave for 1 minute until it become liquid by dissolve 0.3 g of agarose in 30 ml of 0.5x Tris base, acetic acid and EDTA (TAE) buffer, then 1 µL 1000 x gel red dye (Biotium GelRed, 41003- F) was added and stirred by magnetic bar on hot plate, then it was poured in the electrophoresis tray (Submarine Horizontal) type electrophoresis system (Helixx, Mupid- exU), and let the gel for 15 minutes until it becomes hard or solid. 1200 ml TAE buffer (Tris Acetate EDTA) (0.5x) was poured in the glass plate. 1 µL from bromophenol blue gel loading dye was mixed with 4 µL DNA, 5 µL of the mixture was absorbed and put in to the agarose gel cells (5 µL / well). A 100 bp (base pairs) DNA

ladder (100 µg / ml) was used as a size marker to reveal the PCR product size in the first well, and then the mixture allowed at 80 volt for 1 hour in the electrical field to rundown. After 1 hour, the buffer was poured off, and the gel was viewed under the ultra violet light (UV) device (UVITEC, Cambridge) to visualize the DNA bands, a photocopy of the gel was taken into the computer and the results were read. Negative controls which lacking DNA template were included in all experiments.

3.6 Mass production of the entomopathogenic fungi

Different agricultural products and by-products were used for mass producing, such as Rice, Wheat, Maize, Barley, Oat, Coffee, Mixture of (wheat, oat, and barley), Mixture of (Olive mill pomass, rice, wheat and barley), Hay, Perlite, and Olive mill pomass (Sahayaraj and Namasivayam, 2008). Each experiment was replicated four times. Different by-product grains were used with the five-entomopathogenic fungi suspension. 50 g of different grains were slightly crushed using mechanical mixer (Kumar and Tyagi, 2013), then treated by 50 ml of D.W in an autoclavable polypropylene bags in order to maintain suitable moisture content. Grain bags were placed in another autoclavable polypropylene bag (double bags), then autoclaved at 121 ºC for 15 min. When completely cooled, 10 ml of the fungal suspension prepared in (3.4) was used to inoculate each page of autoclaved grains, then incubated at 28 ºC in dark condition for 10 days. After 2 weeks, the fungal

conidia were observed very well and covered the grains, then the covered grains were transferred to aluminum containers with small holes and left to dry. Then grains with fungi transferred to paper bag, labeled and kept at 4±2 ºC in the refrigerator.

3.7 Viability test

The viability of mass produced bio-pesticide stored at room temperature or in the refrigerator were re-calculated and the spores were counted after one year of packaging, using the same method mentioned in (3.4)

3.8 Data Analysis of the Pathogenicity test and Mass Production

Collected spread data sheets were analyzed using Analysis of variance (ANOVA) test using general linear models procedure (PROC GLM). Level of significance were determined by applying Duncan's multiple grouping test. On means and the standard deviation (Std) of 3 replicate readings at P= 0.05. All statistical analysis was performed using Statistical Analysis System (SAS) (SAS Institute 2008). Figures and diagrams were carried out using sigma plot system.

4. RESULTS

4.1 Entomopathogenic morphological identification

Seven days post the death of the larvae and adult, mycelium growth was observed on the insect. White, green and black clusters of spores and mycelia appeared. Five species of entomopathogenic fungi were found, *Beauveria bassiana*, *Metarhizium anisopliae*, *Rhizopus stolonifer*, *Verticillium sp.* and *Aspergillus sp.* on RPW adult and larvae cadavers Fig (5).

Figure 5: Symptoms of some fungal infection on RPW, **A and B**: *Beauveria bassiana* on larvae, **C:** *Metarhizium anisopliae* on larvae, **D**: *Metarhizium anisopliae* on adults, **E**: *Beauveria bassiana* on adults and **F**: *Verticillium* sp. on larvae

Isolation and growth of the fungi on PDA media showed, mixed growth of fungi found in the same plate, sub- culturing afterward resulted in pure and non- contaminated cultures Fig (6).

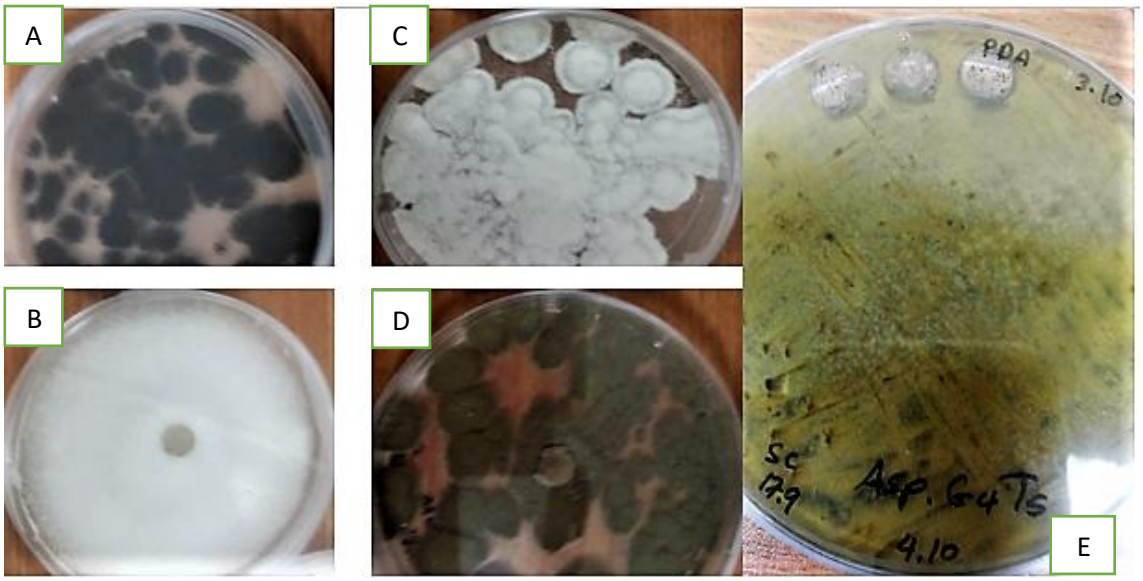

Figure 6: Entomopathogenic fungi plates, **A:** *Rhizopus sp.*, **B:** *Verticillium sp.*, **C:** *B. bassiana*, **D:** *M. anisopliae*, **E:** *Aspergillus sp.*

4.2 Pathogen microscopic identification

Microscopic and morphological identification of the entomopathogenic fungi *M. anisopliae* detected under light microscope in both the mycelium and spore stage showed, compact patches of the conidiophores, and the individual broadly branched (candel abrum-like) Fig (7).

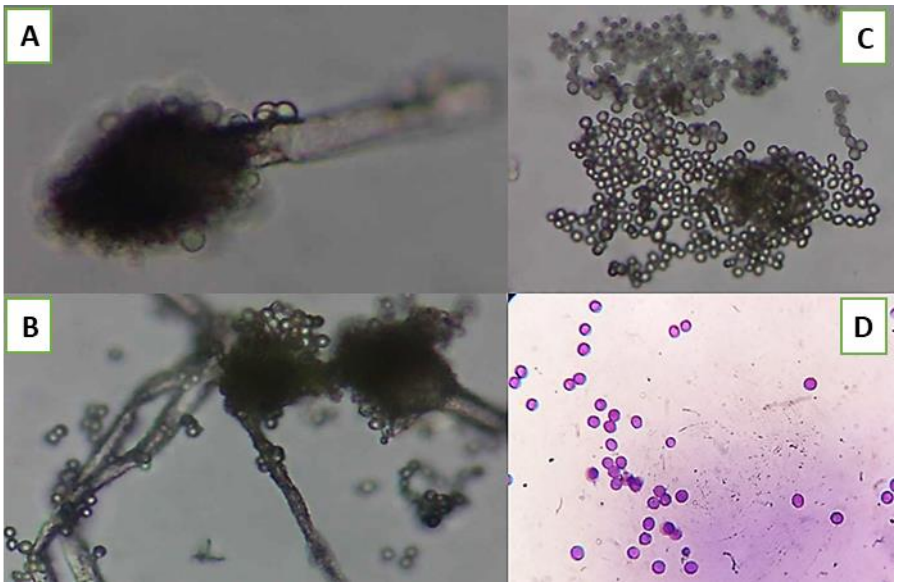

Figure 7: Microscopic image for A and B: mycelium of *Metarhizium anisopliae*, c: the spores of *Metarhizium anisopliae*, and D: is the diluted and stained spores of *Metarhizium anisopliae* fungi (1000x).

Microscopic identification of *B. bassiana* fungi was densely clustered, colorless, and short with base globose which is presented in Fig (8).

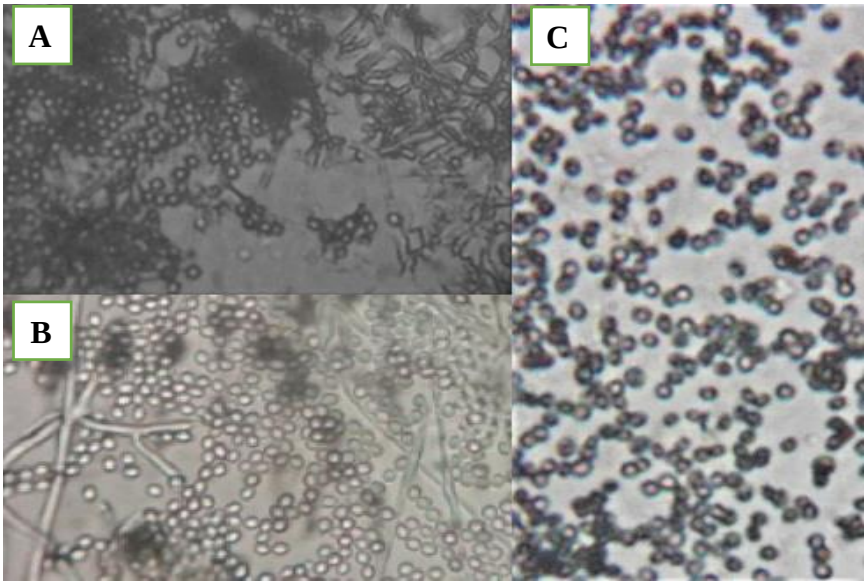

Figure 8: Microscopic image for A and B: mycelium and spore stage of *B. bassiana* C: the spores of *B. bassiana* fungi (1000x).

Isolated Bacterial colony grown on the (NA) medium plate appeared as creamy to white in color with dentate margins and this morphological margin shape to distinguish the (Bt) bacterium from others. It's gram positive one as it appeared in purple-blue color and rod shape under microscope Fig (9).

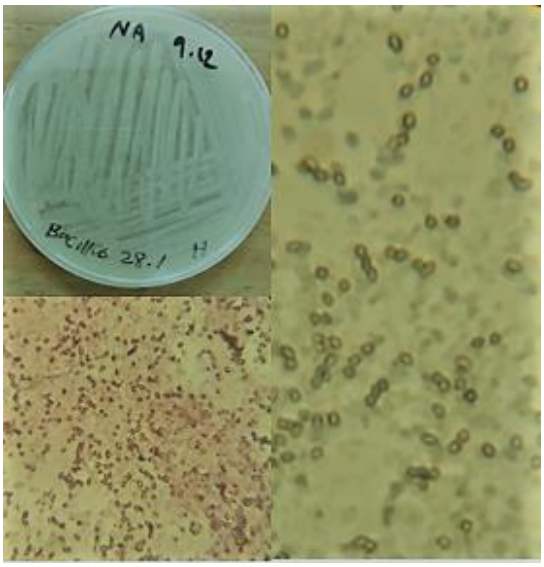

Figure 9: (Bt) bacterium colony and bacterium spores under (1000x)

4.3 The total DNA Extraction

Verification of *B. bassiana* and *M. anisopliae* isolates were confirmed by the total genome DNA extraction and Amplification of the ITS-2. An approximate size of 400 pb bands in lane 1, 2 and 3 from fungal samples, confirmed that these samples are belong to *Beauveria bassiana* Fig (10). While *Metarhizium anisopliae* showed weak PCR signals. Products in lane 2 and 3 with an approximate size of 226-bp length could be observed in the gel Fig (11).

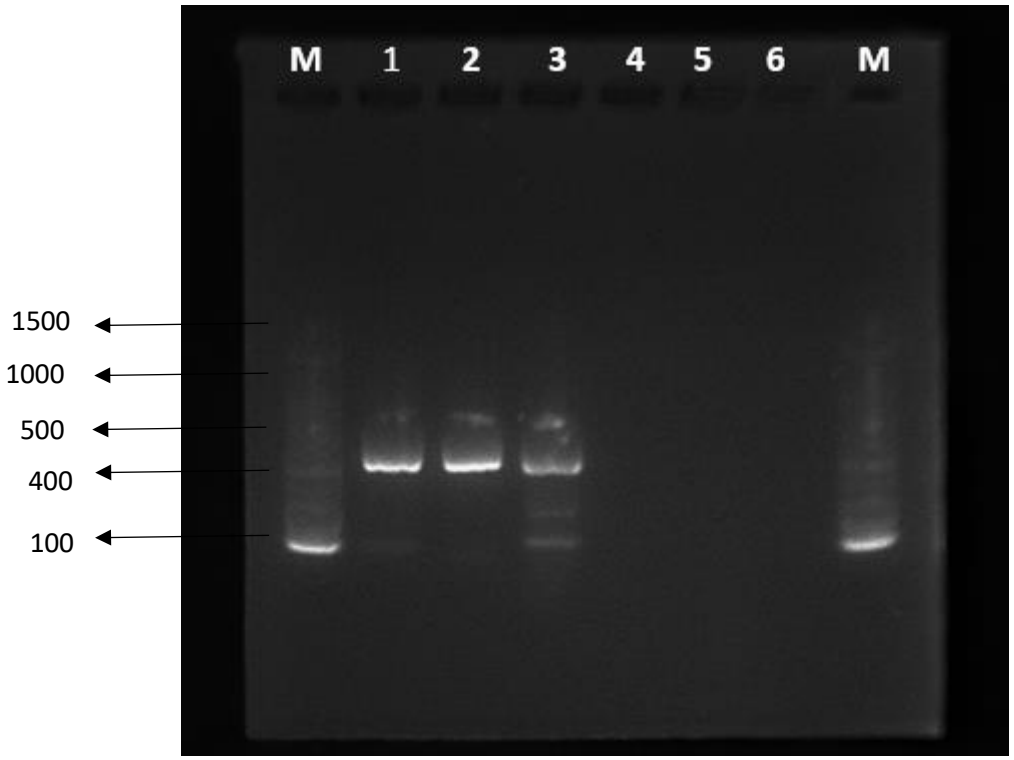

Figure 10: Agarose gel-electrophoresis of PCR products amplified from *Beauveria bassiana* DNA extract. M=100 bp DNA marker. Lane 1, 2 and 3= *Beauveria bassiana* fungi samples; Lane 4=other type of fungi as positive control, Lane 5, 6= negative control without DNA.

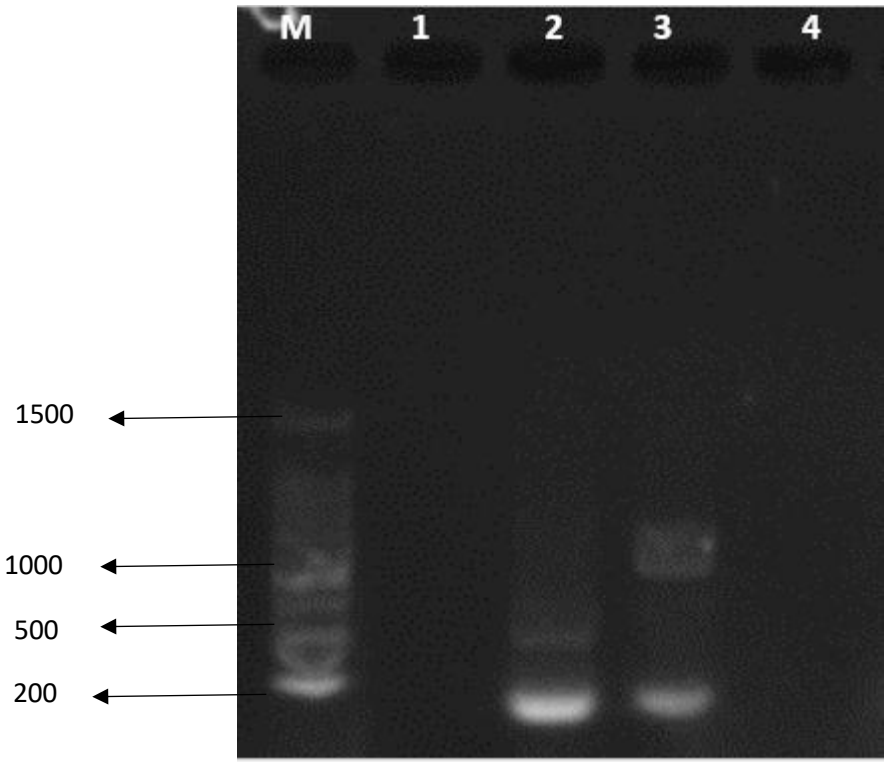

Figure 11: Agarose gel-electrophoresis of PCR products amplified from *Metarhizium anisopliae* DNA extract. M=100 bp DNA marker. Lane 1=other type of fungi as positive control, Lane 2 and 3= *Metarhizium anisopliae* fungi samples; Lane 4= negative control without DNA.

4.3.1 Pathogenicity test

Results of the pathogenicity test (lethal time 100) post *B. bassiana* and *M. anisopliae* suspension treatments against RPW showed a significant difference between both fungi compared to control where the LT100 for control is 17 while 8 for *B. bassiana* and 9.750 for *M. anisopliae* in Fig (12).

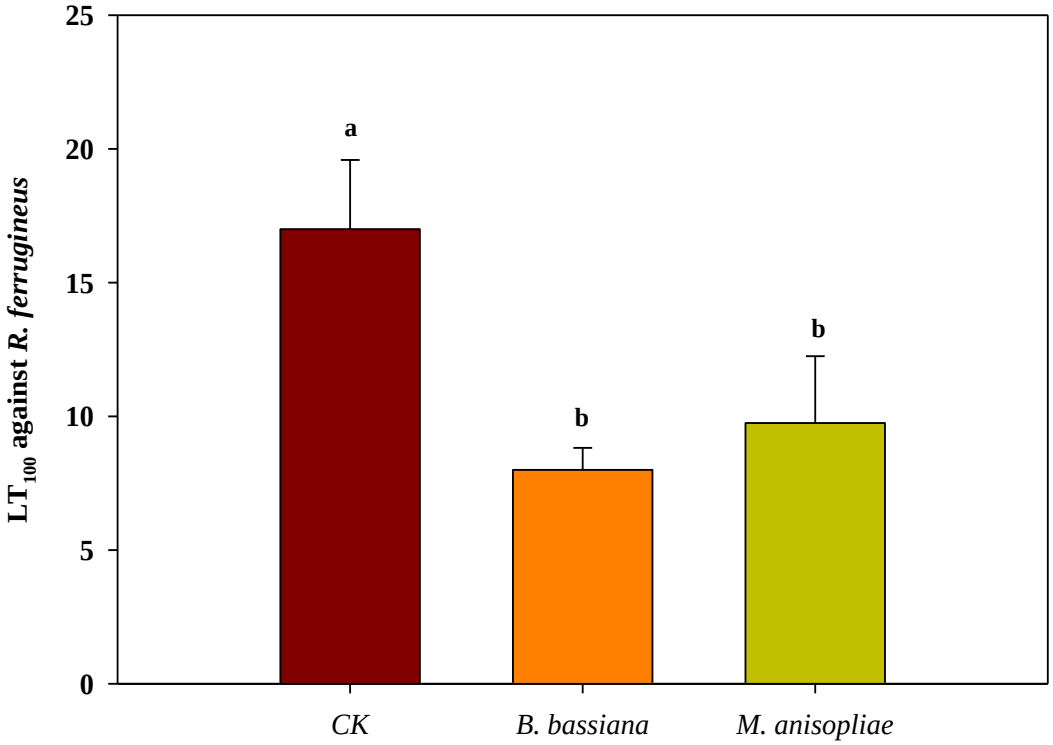

Figure 12: The lethal time 100 pathogenicity test against *R. ferrugineus*. Each column represents mean and Std at P= 0.05, F= 20.12, Pr 0.0005, Similar latter represent same impact.

Pathogenicity test (lethal time 100) post *B. bassiana* and *M. anisopliae* suspension treatments against *E. kuehniella* showed a significant differences between both fungi compared to control where the LT100 for control is 12.5 while 6.5 for *B. bassiana* and 8.5 for *M. anisopliae* Fig (13).

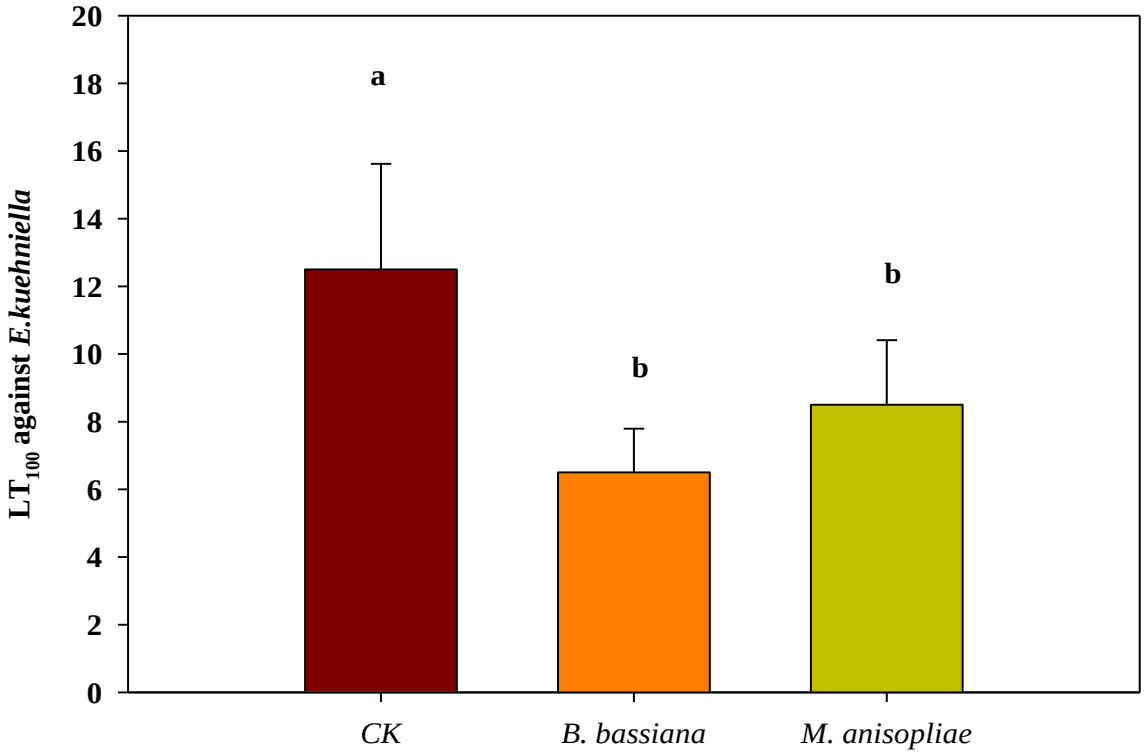

Figure 13: The lethal time 100 pathogenicity test against *E. kuehniella*. Each column represents mean and Std at P= 0.05, F= 7.47, Pr 0.0123, Similar latter represent same impact.

4.3.2 Mass production of bio control agents

Mass production of *B. bassiana* and *M. anisopliae* using four different by products resulted in significate varied results. Wheat and barley were the most efficient by-products for mass producing *B. bassiana*, compared to coffee and oat (Fig 14). *M. anisopliae* mass production recorded significant higher value for barley and oat compared to coffee and wheat Fig (15).

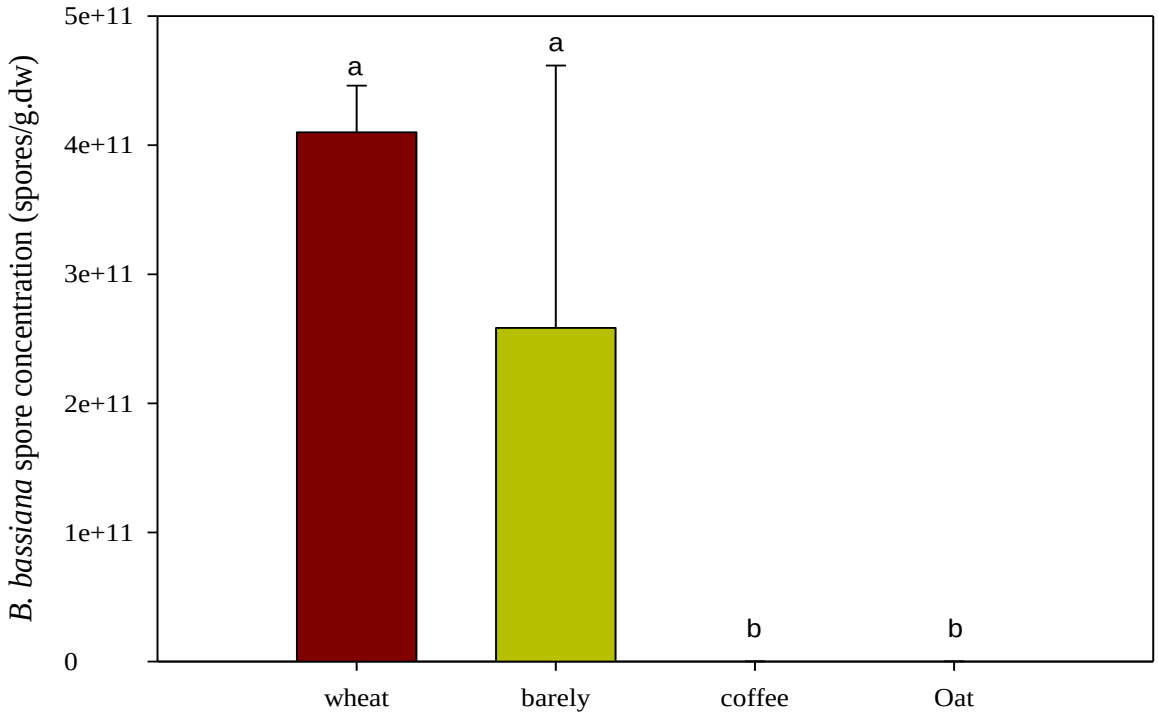

Figure 14: The mass production results of *B. bassiana* on four by-products at P= 0.05, F= 11.55, Pr 0.0028, Similar latter represent same impact. Each column represents mean and Std.

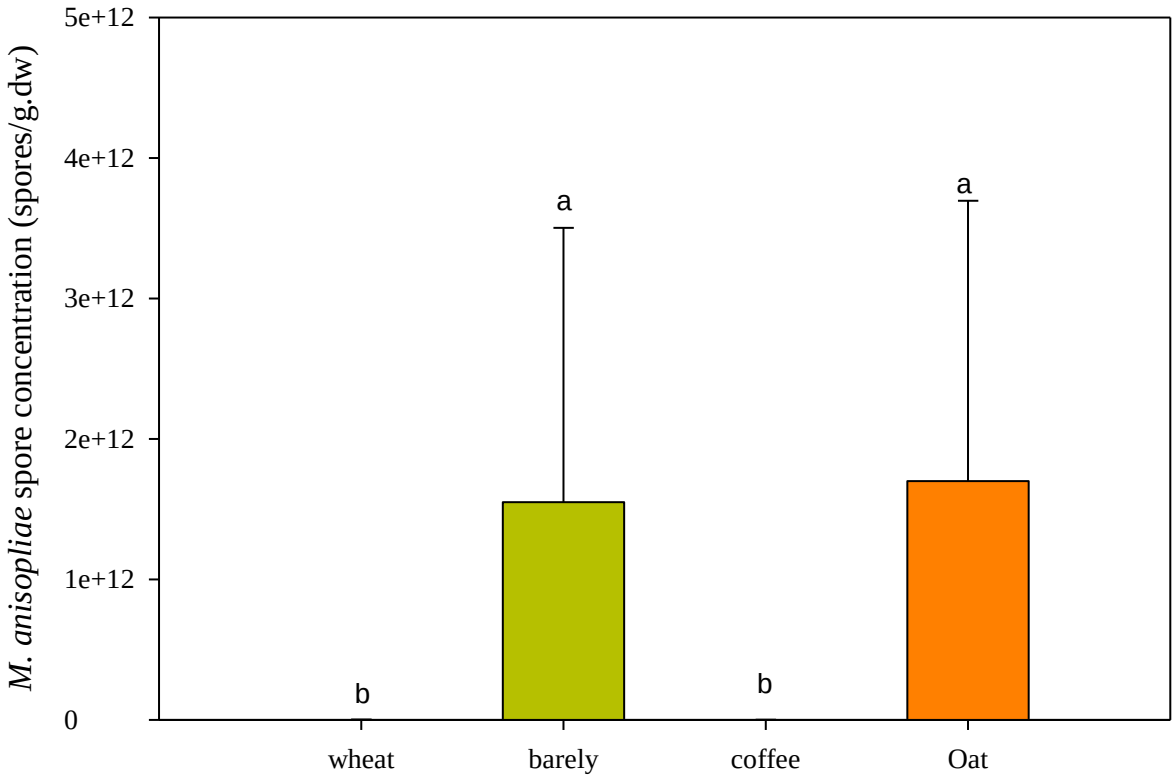

Figure 15: The mass production results of *M. anisopliae* on four by-products and, at P= 0.05, F= 1.36, Pr 0.3223, Similar latter represent same impact. Each column represents mean and Std.

Aspirigullus sp., showed significant and poor mass production impact Fig (16.A), while *Verticillium* sp., has a significant growth on barley compared with wheat, oat and coffee Fig (16.B). *Rhyzopous* sp., showed significant growth on both barley and oat in a large scale. Fig (16. C).

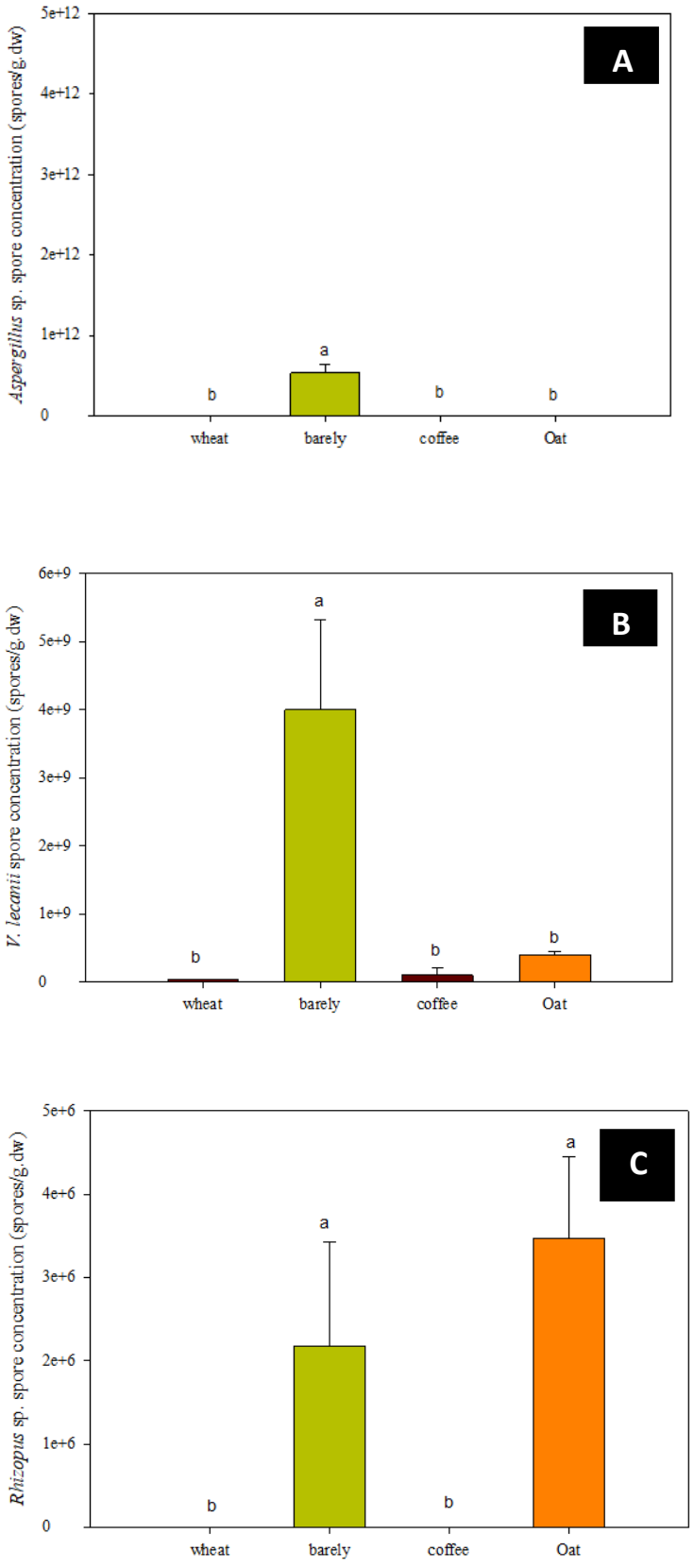

Figure 16: Mass production of **A**: *Asperigullus* sp., **B**: *Verticillium* sp., and **C**: *Rhyzopous* sp., at P= 0.05, Similar latter represent same impact. Each column represents mean and Std.

4.3.3 Viability test

The viability of the mass produced *B. bassiana* on wheat and barley showed significant higher viable spore count post 1 year storage compared with coffee and oat at P= 0.05 Fig (18). Mass produced of *M. anisopliae* on oat and barley showed significant higher viable spore count post 1 year storage compared with coffee and wheat at P= 0.05 Fig (19).

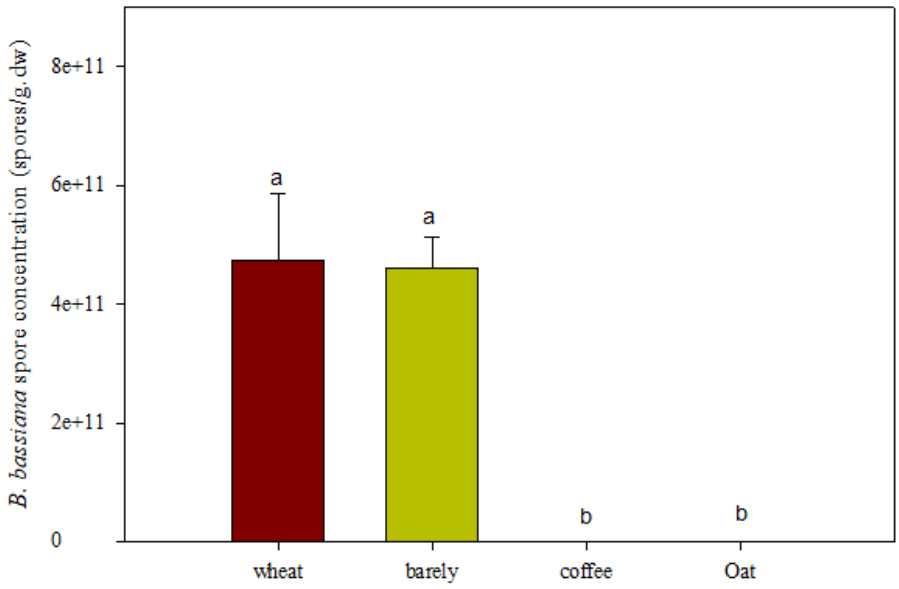

Figure 17: The viability of mass production results of *B. bassiana* after one year of packaging, at P= 0.05, Similar latter represent same impact. Each column represents mean and Std.

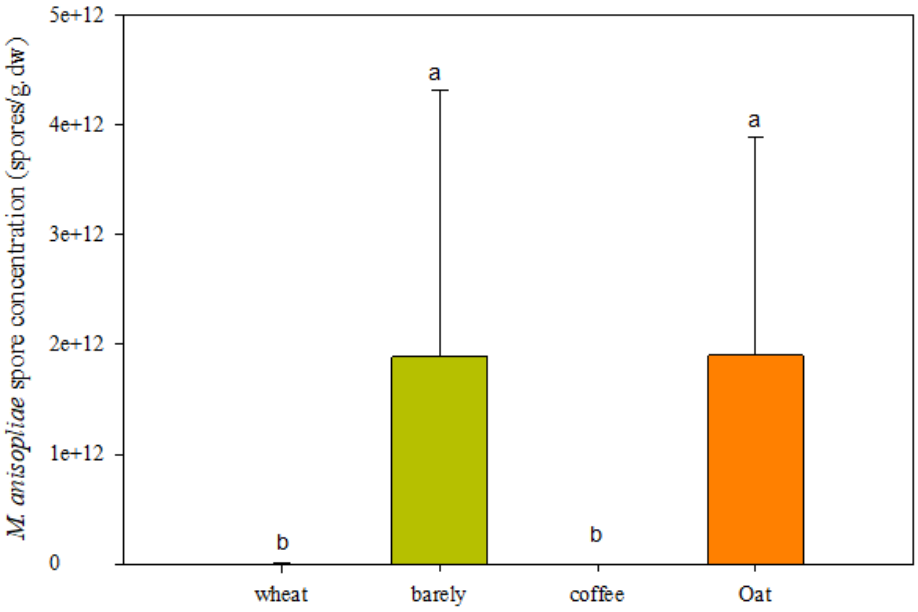

Figure 18: The viability of mass production results of *M. anisopliae* after one year of packaging, at P= 0.05, Similar latter represent same impact. Each column represents mean and Std.

5. DISCUSSION

Several indigenous bio-agents were successfully isolated from infected larvae and adult's cadavers of RPW in this study. *B. bassiana, M. anisopliae, Asperigulus sp., Verticillium sp., and Rhyzopous sp.,* entomopathogenic bacteria *B. thuringiensis,* entomopathogenic nematodes and entomopathogenic viruses. They were used increasingly as biological control agents for insect species and diseases worldwide (Mazza et al., 2014; Hussain, et al., 2013; Tarasco et al. 2008; Gindin et al., 2006; El-Sufty et al., 2007; Yasin et al., 2017a). In 2006, a study and a survey was conducted about the natural enemies of the RPW in Italy by (Tarasco et al. 2008) isolated two species of entomopathogenic fungi, *B. bassiana* and *M.anisopliae,* and that more than 90% of the larvaes inside the palm trees were found to be diseased. The same results were found in Israel (occupied Palestine) according to (Gindin et al., 2006) and in United Arab Emirates according to (El-Sufty et al., 2007). Another study by Yasin et al., (2017a), isolated the two fungi both from soil samples and from insect cadavers, and then they were tested for their virulence against RPW larvae and adults. The results confirmed that the isolates from dead cadavers of RPW gave more mortality compared to isolates from soil samples, and the isolates of *B. bassiana* caused the highest percentage of mortality. The lethal action of both fungal isolates depends on their conidial concentration. *B. bassiana* caused mortality up to 85.7%

against RPW which made it as an excellent bio control agent (Dembilio and Jaques, 2015). The pathogenicity test based on the LT_{100} of *B. bassiana* and *M. anisopliae* against *E. kuhenilla* and RPW. LT_{100} of *B. bassiana* treatment against *E. kuhenilla* was recorded on 6.5 days and *M. anisopliae* is 8.5 days compared with 12.5 days in control insects. LT_{100} of *B. bassiana* treatment against *R. ferrugineus* recorded on 8 days, and 9.75 days for *M. anisopliae* compared to 17 days in control insects.

B. bassiana and *M. anisopliae* pathogenicity against larvae and adults of RPW showed that both fungi have an effective biological control against RPW larvae and adults (El Kichaoui, et al., 2017). They also conclude that both fungi showed 100% mortality percentage after 6 days of spraying the larvae with 3.4 x 10^8 spores/ml of *B. bassiana,* and 90% after spraying the larvae with 3.6 x 10^8 spores/ml of *M. anisopliae*. Ricano et al., (2013) reported that using *B. bassiana* as dry conidia reduced RPW adult survival, because it's more efficient for host penetration and infection and it performed better than conidial suspensions at degrading RPW cuticle and developing abundant infection structures, while conidial suspensions were found less pathogenic to RPW than the same amount of dry conidia formulations, and they also found that *B. bassiana* conidia had a low germination rate in water (Jalinas et al., 2015). *B. bassiana* and *M. anisopliae* can remarkably reduce

survival and increase mortality rate of RPW. (Khalaf, et al., 2017) had the same results in their study.

The isolation, identification, characterization and pathogenicity are not enough for the success of microbial control pests; it must be also a good and effective mass production of the microbial agents to make a complete bio-control program for a successful integrated pest management program. Therefore, the mass production and multiplication of the entomopathogenic fungi was easy and cheap by using agricultural grains and by-products that cheap, easily available and act as best nutritive media for the mass multiplication of many microorganisms.

The agriculture product, by-product or solid substrates that available for use in the multiplication and mass production of fungi for biological control varies widely such as (Rice, bran, Vermiculite, Wheat, Maize, Oats, Barley, Sorghum, coffee, hay, perlite, etc...), and the substrate can be chosen depend on more than one factor like cost, availability and also isolate preference. (Jenkins, et al., 1998) in their study used the same materials and had the same results. In this study, wheat was found to be the suitable media for the mass culture of *B. bassiana* which was recorded the maximum spore production on wheat with (4.1 $x10^{11}$ spore), and barley in the second position with (2.5 $x10^{11}$), while the lowest spore production was recorded in coffee with (6.7 $x10^{4}$). *M. anisopliae* fungi was recorded the maximum spore production on

Oat with (1.7 $x10^{12}$), followed by barley with (1.5 $x10^{12}$), while the lowest spore production was recorded in coffee with (2.5 $x10^{5}$).

The viability of mass produced bio-pesticide after one year of storage is consistent and even relatively increasing due to good storage conditions from favored temperature and low light intensity (darkness) which is favorable for both fungi we had. The virulence of *B. bassiana* fungi and *M. anisopliae* against RPW was accomplished the usefulness of acoustic methods to evaluate the fungal efficacy against internally feeding pests. Therefore, *B. bassiana and M. anisopliae* as EPF considered as a new policy in promoting effective biological control against RPW.

6. CONCLUSION

This study focused on isolation of indigenous natural enemies from RPW cadavers then use them as bio-control agents against RPW on date palm trees. Isolated natural enemies were the entomopathogenic fungi, bacteria, nematodes and viruses. Results revealed that the most effective and virulence bio-agent against RPW was entomopathogenic fungi especially *B. bassiana* and *M.anisolpiae*.

Modified methods for mass production and storing of the entomopathogenic fungi for mass release and management program for the RPW naturally with affordable, available, safe and cheap products like wheat, oat and barley without any contamination and with good quality. The final product post one-year storage were found highly viable and effective on wheat and barley mass produced bio-pesticides.

7. RECOMMENDATIONS

Indigenous bio-agent isolated from local habitat are highly recommend; because they might be more effective, well adapted to local niche conditions and eco-friendly agents against RPW.

B. bassiana and *M. anisopliae* fungi showed higher potential and more efficacy against RPW.

Mass production of indigenous beneficial using available products such as wheat, oat and barley are recommended, they play a major role in enhancing the efficiency of safe and affordable pest population management programs.

ABSTRACT IN ARABIC

(الملخص)

عزل وتشخيص فطريات محلية ممرضة للحشرات وتقييمها مخبرياً ضد حشرة

سوسة النخيل الحمراء *Rhynchophorus ferrugineus* (Olivier)

(Coleoptera: Curculionidae)

الطالبة: هديل حسين

المشرفة: د. رنا سمارة

الملخص

تتمتع شجرة النخيل بمكانة كبيرة عالميا و في دول الشرق الأوسط و خاصة العالم العربي و منها فلسطين، لأنها تعتبر ذات أهمية دينية و ثقافية و صحية و كذلك قيمة اقتصادية كبيرة. تتعرض شجرة النخيل الى الإصابة بعدة أمراض و آفات حشرية ومن أخطر هذه الآفات الحشرية سوسة النخيل الحمراء *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) والتي تسمى بإيدز النخيل دلالة على خطورتها إذ لا يمكن التنبؤ بالإصابة إلا بعد وقت طويل منها الأمر الذي يؤدي إلى السقوط والموت المفاجئ للشجرة. يتم مكافحة الحشرة عادة باستخدام المكافحة الكيميائية ولكن نظراً لخطورة هذه المواد على البيئة المحيطة والصحة العامة وكذلك تطور قدرة الحشرة على مقاومة أغلب المبيدات المسجلة; فقد تم حظر الكثير منها لذلك باتت طرق المكافحة الحيوية المتكاملة هي البديل عن المكافحة الكيميائية.

استخدام الأعداء الحيوية الطبيعية المحلية هو الخيار الأفضل لمكافحة الحشرة بطرق غير ضارة بالبيئة و الصحة العامة، كما ان إنتاج هذه الأعداء الحيوية على نطاق واسع وبكميات وافرة (mass production) يعمل على زيادة فعالية برامج المكافحة المتكاملة.

في هذه الدراسة تم عزل عدة أعداء حيوية مثل الفطريات والبكتيريا و الفيروسات و النيماتود و تم دراسة تأثير بعض الفطريات مثل (*B. bassiana* and *M. anisopliae*) ضد سوسة النخيل الحمراء. أظهرت النتائج فعالية كبيرة ضد الحشرة وثم تم عمل انتاج على نطاق واسع لهذه الفطريات باستخدام حبوب زراعية و منتجات ثانوية رخيصة الثمن و متوفرة حتى تستخدم للمكافحة الحيوية كمبيد حشري حيوي محلي بديل عن المبيد الكيماوي.

كلمات افتتاحية:

آفات شجرة النخيل ,أعداء حيوية طبيعية, مكافحة متكاملة, فطريات ممرضة

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