

Isolation, Molecular Identification and under Lab Evaluation of the Entomopathogenic Fungi *M. anisopliae* and *B. bassiana* against the Red Palm Weevil *R. ferrugineus* in Gaza Strip

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Abstract

Plant diseases generate challenging problems in commercial, agriculture and pose real economic threats to both conventional and organic farming systems. The red palm weevil (*Rhynchophorus ferrugineus*) (RPW) is one of the most destructive pests of palms in the world. Nowadays, control methods revolve around treatments based on chemicals, biotechnological systems using semi-chemicals or the development of the sterile insect technique (hardly sustainable at this time) and biological control. Biological control as the use of natural microorganisms, extracted products from microorganisms or genetically improved to resist or eliminate of pathogens. Our aim was to evaluate the entomopathogenicity of indigenous *Beauveria bassiana* and *Metarhizium anisopliae* obtained in Gaza strip against larvae and adults of *R. ferrugineus* in order to identify indigenous strains potentially suitable for Red Palm Weevil biological control. *B. bassiana* & *M. anisopliae* were isolated from larvae and adult dead of RPW from different position of Gaza strip. Morphological analysis of the isolated fungi and molecular identification was determined using PCR technique. Also, the efficiency of the isolated fungi were evaluated under lab conditions and optimized as a biological agent. On the another hand, the ability of treated RPW male to infect females is examined and calculated using Abbott's formula. Our results showed that the *B. bassiana* and *M. anisopliae* exhibited a good biological control agent against larvae and adults of RPW. The pathogenicity of the two most virulent isolates and the toxicity assay on larvae showed a highest mortality percentage nearly to 100% by 6 days after spraying the larvae with 3.4×10^8 spores/ml of *B. bassiana*. The mortality percentage reaches to 90% after spraying the larvae with 3.6×10^8 spores/ml of *M. anisopliae*. The mortality for the adults treated with pesticide arrives to 50% and the control group 10% at the same time. The results revealed that the infection of the adult males by Entomopathogenic fungi (EPF) can be disseminated into the healthy population, after RPW treatment with *B. bassiana* and *M. anisopliae*.

Our research concludes that *B. bassiana* and *M. anisopliae* locally isolated can be used as biological very effective.

Keywords

B. bassiana, *M. anisopliae*, Red Palm Weevil, Molecular Identification, Biocontrol

1. Introduction

Date palm (*Phoenix dactylifera* L.) is attacked by a large number of pests, including fungi, insects and nematodes (Carpenter and Elmer, 1978) [1]. Some of these pests are serious and difficult to control such as red palm weevil (*Rhynchophorus ferrugineus* Oliv, Coleoptera: *Curculionidae*) (El-Sufty, *et al.* 2007; Arab, 2012) [2] [3].

The red palm weevil *R. ferrugineus* is one of the most destructive pests of palms in the world. This weevil affects more than 20 palm species (Barranco, *et al.* 2000) [4] including the date palm. *R. ferrugineus* was introduced in Spain mainland in 1995 (Barranco, *et al.* 1996) [5], and then spread to all palm growing areas in the Mediterranean and recently also to the Canary Islands. The pest has caused large economic losses in date palms worldwide for the last 30 years (Murphy and Briscoe, 1999; Faleiro, 2006; Güerri-Agulló, 2010) [6] [7] [8].

Nowadays, control methods revolve around treatments based on chemicals, biotechnological systems using semi-chemicals or the development of the sterile insect technique (hardly sustainable at this time) (e.g. Paoli, *et al.* 2014) [9] and biological control (Murphy and Briscoe, 1999; Faleiro, 2006; Paoli, *et al.* 2014) [6] [7] [9].

Biological control as the use of natural microorganisms, extracted products from microorganisms or genetically improved to resist or eliminate of pathogens. It is performed by using microorganisms from the environment itself directly or makes some changes in their properties, to increase their effectiveness or use one of their products.

Advantages of using of this method are to reduce the costs of pest control. Additionally, it preserves human health and environment from pollution, of chemical pesticides usage. Many researches and studies concern only on the use of pathogens such as entomopathogenic nematodes, bacteria and EPF in controlling RPW. Naturally occurring bio-control agents are alternative tools to the use of hazardous synthetic insecticides.

The present study aim was to evaluate the entomopathogenicity of indigenous *Beauveria bassiana* and *Metarhizium anisopliae* obtained in Gaza strip against larvae and adults of *R. ferrugineus* in order to identify indigenous strains potentially suitable for Red Palm Weevil biological control.

2. Materials and Methods

2.1. Isolation of *B. bassiana* & *M. anisopliae* Fungi

B. bassiana was isolated from dead larvae of RPW from South of Gaza strip. The small larval segment were externally sterilized in 100% ethanol for about one minute and allowed to air dry for another minute. Sterilized surface segments were put into PDA medium in Petri-dishes (Abuagla & El-Deeb, 2012) [10]. *M. anisopliae* was isolated

from soil. Soil sample was also collected from Gaza strip. The sample was placed into plastic bags and stored at 4°C - 8°C (NouriAiin, *et al.* 2014) [11].

2.2. Purification of Fungi by Using Selective Medium

Selective medium is generally required for isolation of *B. bassiana* and *M. aneosiplaia* from soil. Sterilized DOC2 medium of *B. bassiana* and Oatmael agar medium (OMA) of *M. aneosiplaia* poured into 15 cm petri dishes (Liu, *et al.* 2015) [12]. The quantity of *B. bassiana* and *M. aneosiplaia* was significantly increased using Potato dextrose agar (PDA) and incubated at 25°C in the total darkness.

2.3. Spore Suspension

Liquid medium (PDB) was used for production of spores required for experiments. Liquid mediums were autoclaved and inoculated with fungal spores propagated on PDA.

Spores were harvested from 2 - 3 weeks old surface cultures by scraping and used to inoculate the liquid medium in flasks and then flasks were held on a shaker (110 rpm) for 5 days at 25°C. The suspensions were stirred and filtered through a single layer of linen to remove culture debris and mycelia. After this time, the blast spore concentrations were determined using a haemocytometer and were calibrated to 3.4×10^8 spores/ml for *B. bassiana* and 3.6×10^8 spores/ml of *M. aneosiplaia* respectively. These suspensions represented the primary stock suspensions to making the spore product (Gindin, *et al.* 2006) [13].

2.4. Morphological Identification of Fungal Isolates

When fungal colonies sporulated on PDA, small plaques from the edge and the center of each growing colony were transferred onto glass slides, and then were examined using a compound light microscope, for characteristics of their vegetative and reproductive structures such as hyphal color and structures, shape and size of conidia and conidiophores (Yu, 2010) [14].

2.5. Molecular Identification of Fungi

2.5.1. DNA Extraction

Fungal genomic DNA was extracted from the hyphae using a partially modified chemical lysis method, Approximately 50 mg of crushed mycelium was used for DNA extraction, and the rest of the sample was stored at -20°C until needed. DNA extraction was done using the DNeasy Plant Mini Kit (QIAGEN, American) and the NucleoSpin Plant Kit (Clontech) according to the manufacturers' recommendations. The extracted DNA was stored at -20°C until use as a template for PCR (Shin, *et al.* 2010; Sevim & Demirba, 2012) [15] [16].

2.5.2. Specific DNA Detection of *M. anisopliae* and *B. bassiana* by PCR

The nuclear rDNA region spanning the ITS1, ITS2, 5.8S rRNA gene for isolated fungi additionally the SCAR fragment for *B. bassiana*. All genes were amplified by polymerase chain reaction (PCR) from tow strain and all primers were presented in **Table 1**.

Table 1. The primers sequences used in ITS, β -tubulin and SCAR analysis.

| Primer Name | Sequences 5' ... 3' | Amplified Region | References |
|-------------|---------------------------------|--------------------|---------------------------------|
| ITS1 | 5'-TCCGTAGGTGAACCTGCGG-3' | ITS1 | (White, <i>et al.</i> 1990) |
| ITS2 | 5'-GCTGCGTTCCTTCATCGATGC-3' | | |
| ITS3 | 5'-GCATCGATGAAGAACGCAGC-3' | ITS2 | |
| ITS4 | 5'-TCCTCCGCTTATTGATATGC-3' | | |
| ITS5 | 5'-GGAAGTAAAAGTCGTAACAAGG-3' | ITS1 + 5.8S + ITS2 | |
| P3 | 5'-GCCGCTTCACTCGCCGTTAC-3' | | (Kusaba & Tsuge, 1995) |
| Bt2a | 5'-GGTAACCAAATCGGTGCTGCTTTC-3' | β -tubulin | (Glass and Donaldson, 1995) |
| Bt2b | 5'-ACCCTCAGTGTAGTGACCCTTGCC-3' | | |
| GHTqF1 | 5'-TTTTTCATCGAAAAGGTTGTTCTCG-3' | SCAR | (Castrillo, <i>et al.</i> 2008) |
| GHTqR1 | 5'-CTGTGCTGGGTACTGACGTG-3' | | |

In each amplification reaction, the final volume of 25 μ l consisted of 3 μ l of total genomic DNA, 0.5 μ l of each primer (forward and reverse), and 21 μ l of ultra-pure distilled water (Biological Industries). Then, all components were added to AccuPower[®] PCR PreMix tube (Bioneer Corporation-Hylabs). For each isolate, PCR amplification of ITS1, ITS2 and the whole region of ITS (ITS1 + 5.8S + ITS2) were performed in a thermocycler (Biometra, Germany) with the following conditions (Hirata and Takamatsu, 1996) [17]: an initial denaturing step at 95°C for 2 min; thermocycling for 30 cycles, where each cycle consisted of 30 s at 95°C followed by 30 s at 52°C for annealing, and 30 s at 72°C for extension, and a final extension cycle of 7 min at 72°C. For each isolate, PCR amplification of β -tubulin gene was performed in a thermocycler with the following conditions successfully used by (Devi, *et al.* 2006) [18]: an initial denaturing step at 94°C for 3 min; thermocycling for 35 cycles, where each cycle consisted of 1 min at 94°C followed by 1 min at 57°C for annealing, and 2 min at 72°C for extension and a final extension period of 5 min at 72°C. For each isolate, PCR amplification of SCAR gene was performed in a thermocycler with the following conditions successfully used by (Castrillo, *et al.* 2008) [19]: for initial denaturation at 94°C for 4 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min; and extension at 72°C for 1 min.

2.6. Bioassay (Contact Application of Fungi)

Evaluation the efficacy of the fungi isolate under *in vitro* conditions and optimize it as biological control agent after divided all insects into 4 group, (control sample, insects treated with chemical pesticide such as 1.5 cm/ml O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate, and 2.5 cm/ml N-{1-[(6-Chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl} nitramide and the third group of insects were treated with biological control agent from *B. bassiana*, and the last group of insects were treated with biological control agent from *M. anisopliae*) and data examined by (Abbott's Formula) (ABBOTT, 1925) [20], after 28 days for adults and 6 days for larvae from incubation. On the other hand, treatment the male adults to evaluate if the male infect the female by fungi or not, after divided all insects into 4 groups as mentioned above.

2.7. Development of the *B. bassiana* and *M. aneosiplaia* Based Bio-Fungicide as a Stable Liquid Formulation

The most effective *B. bassiana* and *M. aneosiplaia* isolate will be maintained in appropriate conditions and heavily tested to give a stable liquid formulation of product based bio-fungicide.

2.8. Data Collection and Statistical Analysis

The data was subjected to statistical analysis, virulence was expressed by cumulative mortality (%), treatment efficacy (Abbott's Formula) (ABBOTT, 1925) [20], within 28 days after treatment. The bar chart tested by using SPSS Statistics 17.0 (SPSS Inc., 2009).

3. Results

3.1. Isolation of *B. bassiana* & *M. aneosiplaia* Fungi

B. bassiana and *M. aneosiplaia* was isolated from dead larvae and adult of RPW, which presented in **Figure 1**.

3.2. Cultural Characterization & Microscopic Examination for *B. bassiana* & *M. aneosiplaia* Fungi

According to the macroscopic examination for *M. aneosiplaia*, we found one distinct strain based on the differences in colony morphology. After 10 days of incubation, the culture produces a white mycelial margin with clumps of more or less vermiculate branching conidiophores. The colors vary from olivaceous buff to cream color to dark green (**Figure 2**). This is akin to the observations of (Bridge, *et al.* 1993) [21]. However, there were founded the conidial shape and size of the two kinds of isolates: cylindrical with obtuse ends, slightly narrowing in the center, and the conidial width (1.5 to 3 μm) and length (4 to 8 μm), which was presented in **Figure 3**.

Generally, in culture, *B. bassiana* grows as a white mould. It produces many dry, powdery conidia in distinctive white spore balls. Each spore ball is composed of a cluster of conidiogenous cells. This result supported by (Elkichaoui, *et al.* 2016) [22].

Microscopic observation results showed that hyphae size about 1 - 2 μm which grouped on conidiogene cells with 3 - 6 μm in size. Hyphae then branched and formed conidiogene cells with bottle like form, small neck, and branch long were up to more than 20 μm and 1 μm wide, which illustrated in **Figure 4**.

Fertile hyphae was found on branch, circular and normally thicken or swollen. While



Figure 1. *B. bassiana* & *M. aneosiplaia* covered adult and larvae of RPW.



Figure 2. Culture of *M. anisopliae* on OMA selective medium and PDA medium.

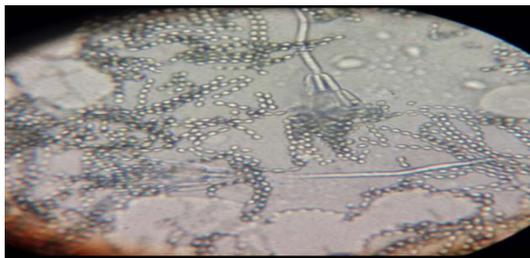


Figure 3. Microscopic examinations for *M. anisopliae* 100×.

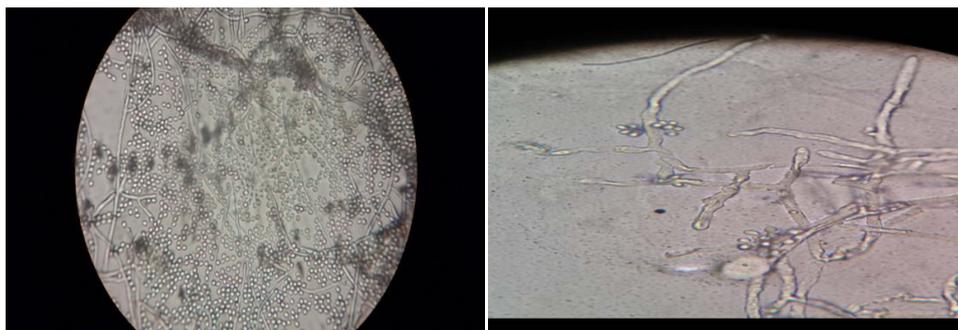


Figure 4. Microscopic examinations for *B. bassiana* 40× & 100×.



Figure 5. Culture of *B. bassiana* on DOC2 selective medium and PDA medium.

mycelium which is hyphae aggregate of *B. bassiana* was white and insulated (**Figure 5**). This result agrees with that estimated by (Elkichaoui, *et al.* 2016) [22].

3.3. Enrichment for Tow Fungi and Spore Suspension

Liquid medium Potato-dextrose-broth (PDB) was used for production of spores required for experiments. Spores were harvested from 2 - 3 weeks old surface cultures by

scraping and used to inoculate the liquid medium in flasks. After this time, the blastospore is the lethal concentration for RPW. Based on previous study for killing the RPW such as (Malik, *et al.* 2016) [23], the concentrations were determined using a haemocytometer and were calibrated to 3.4×10^8 and 3.6×10^8 spores/ml for *B. bassiana* and *M. anisopliae* respectively. These suspensions represented the primary stock suspensions of blastospore.

3.4. Molecular Characterization for *B. bassiana* & *M. anisopliae* Fungi

3.4.1. PCR Amplification of ITS

Molecular techniques are accurate and widely used for identifying species and varieties. The PCR techniques have been used in the current study. The ITS1, ITS2 as well as the whole ITS region (ITS1 + 5.8S + ITS2) were successfully amplified for two fungal species. There was a difference in fragment size of ITS1, ITS2 and (ITS1 + 5.8S + ITS2) between two fungal species. For example, the length of the ITS1 region in *B. bassiana* was larger than that of the *M. anisopliae* (230 bp and 215 bp, respectively). Whereas, the ITS2 fragment in *M. anisopliae* was greater than that of the *B. bassiana* (375 bp compared to 360 bp). These our results were confirming by (Al_qadi, 2011) [24]. The fragment sizes of all ITS regions, as obtained by gel electrophoresis. Cruz, *et al.* 2006 [25] indicated that the fragment size of ITS1 of *B. bassiana* was 570 bp. In another study, the fragment size of ITS1-5.8S-ITS2 region was 481 bp for *B. bassiana* and 540 bp for *M. anisopliae*, are shown in **Figures 6(a)-(d)**.

3.4.2. PCR Amplification of β -Tubulin

Part of β -tubulin gene was amplified successfully for tow fungal species. The size of this part in *B. bassiana* isolate was found to be greater than the corresponding one in *M. anisopliae* isolates (500 bp and 380 bp), respectively, **Figure 6(e)**. β -tubulin was developed for sequencing purposes as described by (Bischoff, *et al.* 2006) [26].

3.4.3. PCR Amplification of SCAR Region for *B. bassiana*

Standard PCR examines utilizing primers GHTqF1 and GHTqR1 against strain of *B. bassiana* created a 96 bp, recouped from tainted RPW grown-ups taking after shower application. The PCR item produced was of the anticipated length in view of the SCAR part whereupon the preliminaries were based. The fragment sizes of SCAR regions, as obtained by gel electrophoresis, were presented in **Figure 6**.

3.5. Bioassay (Contact Application of Fungi)

3.5.1. Pathogenicity of Entomopathogenic Fungi to *R. ferrugineus* Eggs

The pathogenicity of the two most virulent isolates of *M. anisopliae* and *B. bassiana*, selected in the initial screening on adult and larvae, was tested against *R. ferrugineus* eggs. Both isolates killed the eggs quickly during 3 days, without preliminary colonization on the egg surface. The characteristic symptoms, which appeared on treated eggs, e.g. loss of, tumefy lethargy and darkening of the eggs, appeared 2 - 3 days after treatment; subsequently the eggs were destroyed and disappeared in the substrate.

3.5.2. Pathogenicity of Entomopathogenic Fungi to *R. ferrugineus* Larvae

The larval mortality was measured by Bottle equation. Significant difference in growth

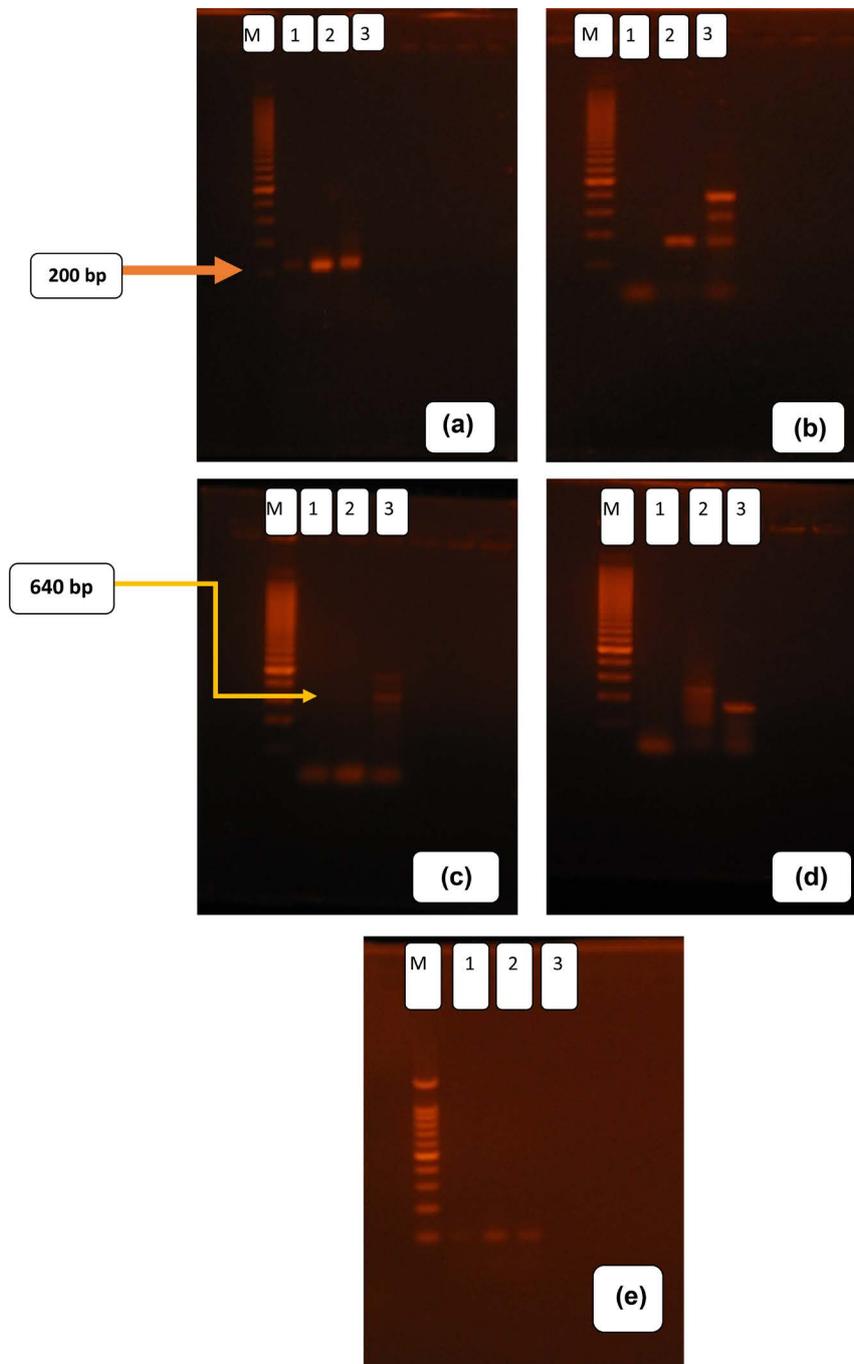


Figure 6. Molecular identification of isolated fungi for ITS regions, Bt regions and SCAR regions. (a) Fragment sizes of the PCR-amplified ITS1 regions as obtained by gel electrophoresis. In the peripheral of the photograph, bands from a DNA ladder 200 bp scale (M) are shown. L1: negative control, L2: ITS1 gene for *M. anisopliae* 215 bp & L3: ITS1 gene for *B. bassiana* 230 bp. (b) Fragment sizes of the PCR-amplified ITS2 regions as obtained by gel electrophoresis. In the peripheral of the photograph, bands from a DNA ladder 200 bp scale (M) are shown. L1: negative control, L2: ITS2 gene for *B. bassiana* 380 bp & L3: ITS2 gene for *M. anisopliae* ranging between 360 bp to 1000 bp. (c) Fragment sizes of the PCR-amplified of whole ITS regions as obtained by gel electrophoresis. In the peripheral of the photograph, bands from a DNA ladder 200 bp scale (M) are shown. L1: negative control, L2: ITS regions gene for *B. bassiana* 640 bp & L3: ITS regions for *M. anisopliae* ranging between 630 bp. (d) Fragment sizes of the PCR-amplified of whole Bt regions as obtained by gel electrophoresis. In the peripheral of the photograph, bands from a DNA ladder 200 bp scale (M) are shown. L1: negative control, L2: Bt gene for *B. bassiana* 500 bp & L3: Bt gene for *M. anisopliae* ranging between 380 bp. (e) Fragment sizes of the PCR-amplified of SCAR region as obtained by gel electrophoresis. In the peripheral of the photograph, bands from a DNA ladder 100 bp scale (M) are shown. L1: negative control, L2 & L3: SCAR gene for *B. bassiana* 96 bp.

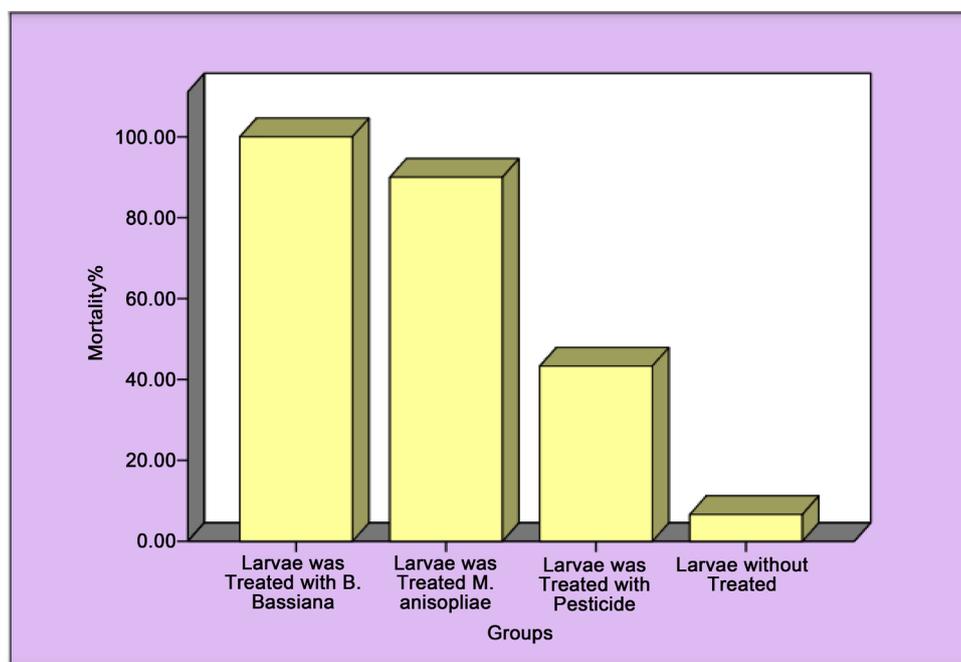


Figure 7. Compare between the mortalities percentage for groups of the larvae of RPW after treated with 3.6×10^8 spores/ml of *M. anisopliae*, 3.4×10^8 spores/ml of *B. bassiana*, chemical pesticide & negative control.

were recorded between treated and untreated larvae, the toxicity assay on larvae were treated with the *M. anisopliae* & *B. bassiana* isolate, which proved to be the most virulent to the larvae. The mortality of larvae was recorded for 6 days after contact with spraying with spore suspension.

Larvae showed a higher susceptibility than adults in terms of both mortality and speed of infection. The highest percentage mortality of the larvae reached 100% by 6 days after spraying with *B. bassiana*, but 90% after spraying with *M. anisopliae* at the same time (Figure 7).

3.5.3. Pathogenicity of Entomopathogenic Fungi to *R. ferrugineus* Adult

Results of the first experiment indicated that the mortality of *R. ferrugineus* adults differed according to the fungus application method. The mortality of adult weevils was recorded for 28 days after contact with spraying with spore suspension, which presented the adult were treated by 3.4×10^8 spores/ml of *B. bassiana* and 3.6×10^8 spores/ml of *M. anisopliae*.

The maximum mortality of weevils reached 95% by 28 days after spraying with *B. bassiana*, but 86.6% after spraying with *M. anisopliae* at the same time and was supported by recent study for Francardi, 2012 [27], when studying the effect of *M. anisopliae* was isolated from Italy, was appeared also influenced by the type of infecting substratum as resulted higher on larvae (100% mortality) and adults (90% mortality). Mortality in control groups (aqueous D.W) was 0% and 10%, but the maximum mortality of RPW reached 50% by 28 day after treated with chemical pesticide (Figure 8).

3.5.4. Pathogenicity of Entomopathogenic Fungi to *R. ferrugineus* Adult Male

The results shown in Figure 9 demonstrated that all entomopathogenic fungal strains

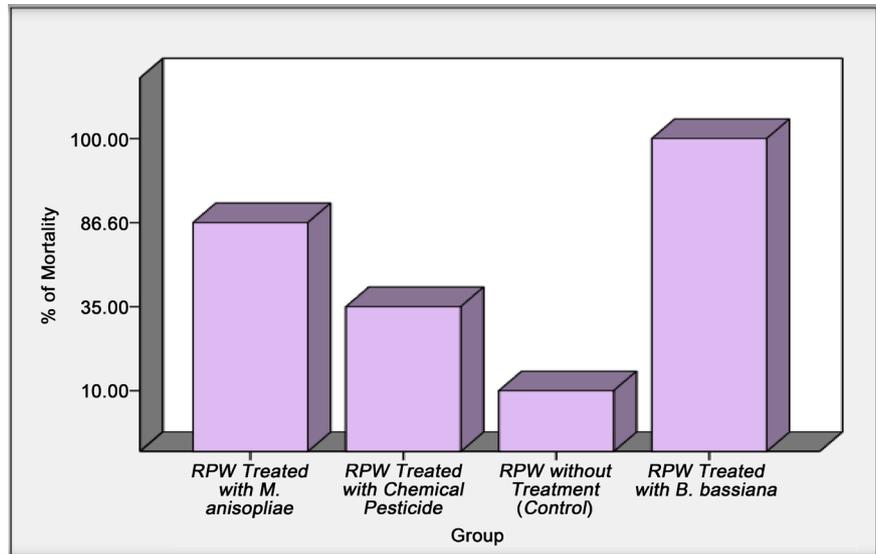


Figure 8. Compare between the mortalities percentage for groups of the adult of RPW after treated with 3.6×10^8 spores/ml of *M. anisopliae*, 3.4×10^8 spores/ml of *B. bassiana*, chemical pesticide & negative control.

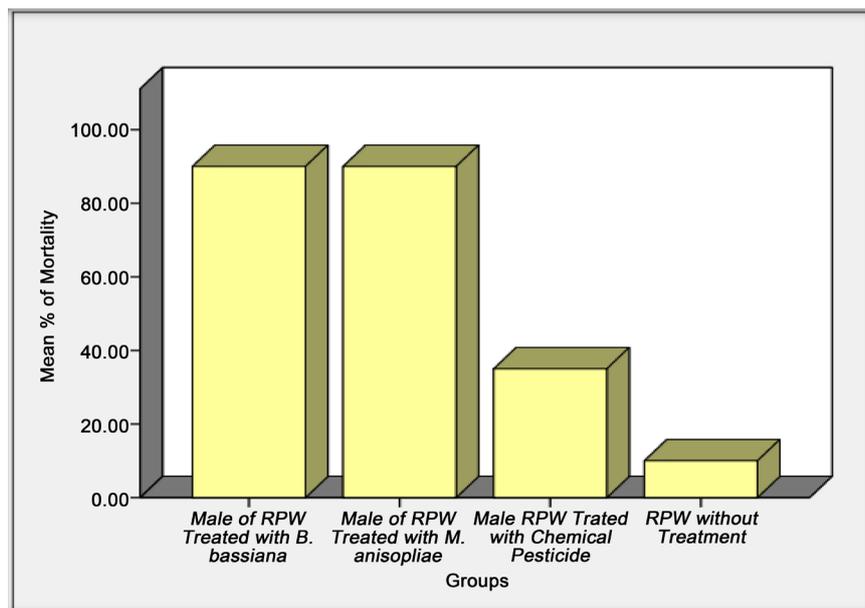


Figure 9. Mortalities percentage for *R. ferrugineus*. After treatment the Male adult of RPW with 3.6×10^8 spores/ml of *M. anisopliae*, 3.4×10^8 spores/ml of *B. bassiana* & chemical pesticide.

caused significantly increased mortality, which was investigate in the laboratory for male of RPW contaminated with entomopathogenic fungal conidia can transfer the in-oculum to female during copulation. The results appeared the male of RPW, which contaminated by *B. bassiana* & *M. anisopliae* as a vector of indirectly infected into female, which were death after 28 days.

4. Discussion

This study provides general information about the isolation and genetic diversity of

entomopathogenic fungi *B. bassiana* and *M. anisopliae* strains in the Gaza strip of Palestine. Many of molecular markers were used as a modern technique to discuss the genetic variability and to identify distinct isolates of *M. anisopliae* and *B. bassiana*. Genetic materials based technique may allow distinguishing between isolates that are very similar in morphology.

The evolution and using of PCR amplification from different rDNA regions has greatly facilitated the fungi classification studies. Alignments and molecular analyses confirmed the *B. bassiana* and *M. anisopliae* strains taxonomic identity. However, since some conserved sites were found in the ITS regions, the conserved sites of the ITS regions and the 5.8S rRNA gene were used for current analysis. Investigation of ITS-rDNA sequences had been applied to determine the genetic diversity of *M. anisopliae* and *B. bassiana* (Entz, *et al.* 2005; Becerra, *et al.* 2007; Freed, *et al.* 2011) [28] [29] [30]. Thus, (Bautista-Galvez, *et al.* 2012), made the genetic characterization of *M. anisopliae* strains obtaining fragments of 600 to 800 bp by PCR amplification from the ITS1-ITS4 rDNA regions. Our ITS1 – 5.8S – ITS2 sequencing data showed variations which allowed us to design specific primers which could not only detect and identify *M. anisopliae* but also to differentiate between *M. anisopliae* and *B. bassiana*. The ITS regions and 5.8S rDNA of *Metarhizium* were amplified using the ITS1 and ITS2 primers that was a unique fragment of approximately 630 bp for Gaza isolate. (Destéfano, *et al.* 2004) [31], analyzed at the same region with 540 bp fragment for *M. anisopliae* var. *anisopliae* strain E9, B/Vi and C isolated in Brazil and 600 bp for *M. anisopliae* strain 14 isolates in Australia. The ITS and BT markers have the different level of informativeness in discriminating *Beauveria* & *Metarhizium* isolates. While ITS regions was more informative than BT in discriminating as ITS marker distinguished.

PCR assays of *B. bassiana* which isolated from adults of RPW with SCAR primers resulted in DNA fragments of the same size as the *B. bassiana* GHA amplicons which done by Castrillo, *et al.* 2008 [19] as expected based on primer design. For field studies, the accuracy of detection from any samples may be improved by increasing the number of subsamples taken and the number of PCR assays for different genes per DNA extraction (Dionisi, *et al.* 2003) [32].

Based on the result obtained, the egg takes about 2 - 5 days to hatch while the larva takes about 80 - 90 days before molted to pupa. The pupal stage takes about 3 weeks for the emergence of the adult weevils. These results agree with that estimated by (Kaakeh, *et al.* 2001; Sharaby and Al-Dhafar, 2013) [33] [34].

Adults killed by the fungus did not change color, whereas dead adults in the control treatment darkened. After incubation of cadavers under moist conditions, fungi emerged on the dorsal and ventral surfaces of the weevil and formed conidiophores with conidia.

The current examination showed that the tested *M. anisopliae* and *B. bassiana* isolates infect the adult and larvae are fully completed their life cycles by forming conidiophores with conidia on RPW. Fungal virulence is determined by different intrinsic characteristics in the strains and their manifestation is related with biotic and abiotic variations (Hall & Papierok, 1982) [35].

The arrival of entomopathogenic fungi to infest the host is through the cuticle is

considered successfully control of RPW, which that involves complex biochemical interactions between the host and the pathogen (fungus) such as *B. bassiana* & *M. anisopliae* before germination, penetration, growth, and reproduction of the fungus. Before to host invasion, there are certain characteristics of fungi that designate them virulent or avirulent strains. So, there is a constant struggle between host and pathogen that ultimately lead to the success or failure of pathogens.

In case of compatible interaction, the pathogen must have high number of conidia with strong adhesion that ultimately penetrate into the host through directly penetrating structures. Moreover, the invading pathogen must have the capacity to bypass or overcome the host immune system by producing toxins (Hussain, 2013) [36].

The mating behavior of the RPW resulted in transferring the spores from infected male insects to the uninfected female insects. The pathogenic efficacy of *B. bassiana* & *M. anisopliae* indirectly infect females. The efficacy of indirect infection was high. The period required to obtain the maximum mortality of 90% was extended to compare the directly infected females or males, but the maximum mortality of 20% for male adult was treated by chemical pesticide. However, the time to death of indirectly infected females from mating with the infected male insects ranged from 3 to 6 day after mating. These results supported by Hajjar, *et al.* 2015 [37]. The pathogenic efficacy of the indirect infection was high, as all the indirectly infected insects of both sexes were killed after 3 d of mating. The early deaths of the directly infected insects could be due to the high load of *B. bassiana* spores on the body as compared with indirectly infected red palm.

Recently (Llacer, *et al.* 2012) [38], advanced the possibility of using sterile irradiated males as a vector of *B. bassiana* for microbiological control of *R. ferrugineus*. In laboratory bioassays, the transmission system successfully attracted, infected and released weevil adults after they contacted cereal substrata inoculated with indigenous strains of *B. bassiana* and *M. anisopliae* (Francardi, 2012) [27].

Results indicate that the EPF against *R. ferrugineus* can also provide an excellent alternative to chemical control. The EPF of *B. bassiana* and *M. anisopliae* strains obtained from different sources. All of the screened EPF strains exhibited pathogenicity to larvae stages of RPW, causing up to 90% - 100% mortality of larvae weevils under laboratory conditions. The fungal spores had a significantly reduced survival in comparison with larvae, which emerged in the control treatment.

The effects of *B. bassiana* treatments on activity and survival of larvae correlate well with previous studies conducted outside of hidden environments (Fargues, *et al.* 1994; Gindin, *et al.* 2006; Nussenbaum and Lecuona, *et al.* 2012) [13] [39] [40].

In other side the bioassays, significantly higher mortalities of RPW adult were observed at use of *B. bassiana* as biocontrol agent against the *R. ferrugineus*. After spraying RPW adult with spore suspensions (3.4×10^8 ml⁻¹) of *B. bassiana* isolates from dead adult, but in the second term the *M. anisopliae* caused the death for RPW and the mortality percentage 86.6% after treatment the *R. ferrugineus* adult with spore suspensions (3.6×10^8 ml⁻¹). While the lower mortalities of RPW adult were observed at use of chemical pesticide at the same time.

In the current bioassays, maximum number of sporulation and mycosis in cadavers

of *R. ferrugineus* was recorded at the highest and sole concentration of *B. bassiana* & *M. anisopliae* as compared to all other treatments, after treatment of adult RPW males with EPF. Similar trend in mycosis and sporulation were reported by Riasat, *et al.* 2001 [41], in (Ramakrishnan, 1999) [42].

Efficacies up to 90% were obtained compare with chemical pesticide 35%, and these results are indicative that contact infection of adults actually occurred and confirm the potential of this strain as a biological control agent against *R. ferrugineus*. Consequently, adults should be considered as the targets of any treatment involving this entomopathogenic fungus because they are actually the only free-living stage.

Overall, we can see a clear upward trend to use the EPF, which aimed at attracting and infecting adult weevils could prove the most effective way to spread the disease, and this is one of the works that our group is developing at this moments.

5. Conclusion

This work as a part of general project that aims to solve some of environmental and health problems by reducing the use of chemical fertilizers, pesticides and drugs and replace them by natural material or organisms. *B. bassiana* & *M. anisopliae* isolates from the adult RPW used in laboratory bioassays caused high mortality in larvae and adult. Natural infections by *B. bassiana* and other entomopathogenic fungi found were very high, considering the instar. For these reasons, the use of entomopathogenic fungi can be considered to be useful as a preventive tool in Gaza strip palm protection. Moreover, the high mortality of treated adults suggests that their use as vectors of *B. bassiana* & *M. anisopliae* can represent a potential tool for reducing *R. ferrugineus* populations in Gaza strip.

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