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Effectiveness of Entomopathogenic Fungi Against Adult Red Flour Beetles (Tribolium castaneum) (Coleoptera: Tenebrionidae)

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Abstract. The current study aimed to isolate some insect pathogenic fungi from the soil of wheat crops in different areas and test their effects on the adult stages of the red flour beetle Tribolium castaneum. Five species of fungi naturally infecting the flour beetle were recorded, and these genera were Beauveria bassiana and Metarhizium anisopliae, identified morphologically. Five local strains of insect pathogenic fungi were isolated, based on their macroscopic and microscopic characteristics. The concentrations of fungal spore suspensions affected the adults of the studied insect, with Beauveria bassiana being the most effective. The mortality rate of adults reached 93.33% when the highest concentration of the fungal suspension (17x108) was used after 10 days of treatment, while the mortality rate was 86.67% when using the suspension of Metarhizium anisopliae at the same concentration. One strain matched the species B. bassiana and four strains matched M. anisopliae. The identity of the strains was confirmed by amplifying and sequencing the ITS5-ITS4 region, and comparing the sequences to molecular databases and phylogenetic analyses. 99% identity values were recorded with B. bassiana and M. anisopliae strains in molecular databases. Phylogenetic analyses confirmed that the sequences extracted from these strains fall within the group containing the reference sequences of B. bassiana and M. anisopliae, respectively, in the database. These results contribute significantly to the understanding of entomopathogenic fungi, which will aid in the development of biotechnological products in the field of biological control

Highlights:

1. Soil fungi Beauveria bassiana and Metarhizium anisopliae infect red flour beetles.
2. Fungal spore suspensions showed high mortality rates in beetle adults.
3. Molecular analyses confirmed strains' identity, supporting biological control development.

Keywords: Tribolium castaneum, entomopathogenic fungi, Beauveria bassiana. Metarhizium anisopliae. identification, contact toxicity.

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Introduction

The red flour beetle, scientifically known as Tribolium castaneum (Herbst) (Coleoptera: Tenebrionidae)(1), is a major pest that causes damage to grains and flour. This beetle is also widely used in research related to insect behavior and food safety(2). However, the use of insect-pathogenic fungi to control T. castaneum has faced limitations due to concerns about environmental sustainability(3).

With the spread of stored grain pests as a result of human activities and seed transport, these pests have evolved to adapt to diverse food sources. The red flour beetle T. castaneum, which is brown in color, is one of the most common and destructive insects of stored products(4). Due to its high appetite, it can consume a wide range of foods, including those stored in soil, warehouses, grocery stores, and homes(5), as well as grain products such as wheat and flour(6, 7). Approximately 10 to 40% of stored grains worldwide are damaged annually due to insect pests, especially in tropical and subtropical regions of developing countries (8, 9). When present in large numbers, these beetles release a chemical mixture that can negatively affect the quality of the product, as it contains carcinogenic quinones. Insects such as beetles, mites, and weevils are major factors in the destruction of stored food, imparting a pungent odor to the affected materials due to volatile compounds, such as benzoquinones(10). Grains and their products become contaminated by these secretions, affecting their market value(11, 12).

Certain types of fungi have been used as biological control agents against harmful insects(13, 14). These substances can deter these organisms from feeding or laying eggs on a resource. They have also been used to protect stored food products(15). This has led to the need for the development of non-toxic and safe alternatives for humans and animals, one of which is biological control. This involves reducing the pest population of a harmful species to a level that does not cause significant harm to humans, animals, or their activities through the use of other biological agents such as insects, nematodes, bacteria, fungi, or others. Insect pathogenic fungi are considered significant agents due to their widespread presence in nature, low cost, and high specificity in targeting particular pests(3, 16).

On the other hand, insect pathogenic fungi (EPF) used in biological control can face challenges in field environments due to environmental conditions that may affect their ability to infect and control pests. Factors such as unsuitable temperature

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and humidity can reduce their effectiveness, making them less successful compared to traditional chemical insecticides(17, 18). Other control methods, including synthetic chemicals and natural compounds, may interact with biological control agents and affect their efficacy (19, 20). These interactions may have either positive or negative effects on the growth and performance of EPFs in pest control.

Identifying fungi based on their morphological characteristics and molecular structure is a crucial step in selecting biological control agents(21, 22). Therefore, the main objective of the research was to isolate and identify certain fungal strains for their potential use and integration into control programs against the adult stages of the T. castaneum

Methods

1. Collection of Samples and Isolation of Insect-Pathogenic Fungi

Adult insect samples were collected from a laboratory colony established from infected flour samples in Al-Diwaniyah City, Iraq, between October 2023 and September 2024. The insects were reared at a temperature of 30°C and relative humidity of 75% in glass containers on wheat flour mixed with a small quantity of regular baker's yeast in a 10:1 (W: W) ratio. The insects were identified according to the taxonomic keys (23), and the adults were surface-sterilized using 70% ethanol, followed by a 5% sodium hypochlorite solution, and rinsed three times in sterilized water.

At the same time, soil samples were collected from wheat fields in Al-Diwaniyah (Shamiya area) following the methodologies outlined by (24) and (25). Five areas were randomly selected, from which samples were collected from the first 10 cm of soil depth, with a total weight of 200 g. Three serial dilutions were made using sterile water (10-1, 10-2, and 10-3). Then, 0.1 ml of the 10-2 and 10-3 dilutions were inoculated onto sterile culture plates containing growth medium. These dilutions were evenly distributed over the surface of the medium using a Drygalski spatula. Two different types of sterile culture media were used:

The medium (semi-selective growth) proposed by (26), with modifications (glucose 40 g/L, peptone 10 g/L, thiabendazole 0.004 g/L, chloramphenicol 0.5 g/L, violet 0.01 g/L, agar 15 g/L, distilled water 1 L, pH 6).A medium for insect-pathogenic fungi suggested by(27), with modifications (oat flakes 20 g/L,

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hexadecyltrimethylammonium bromide 0.6 g/L, chloramphenicol 0.5 g/L, agar 15 g/L, distilled water 1 L, pH 6). In all cases, plates were incubated at 28 ± 2 °C for 5 to 14 days. All inoculation procedures for spore suspension and dilution were performed in triplicate. Fungal growth was periodically observed on the plates. (28, 29).

2. Morphological Diagnosis of Insect-Pathogenic Fungi

To identify fungal strains morphologically, we began by observing the visible characteristics of the colonies. For each colony, characteristics such as color on the upper and lower surfaces of the plates, surface texture, shape, edges, and height were examined.(29-32). Microscopic observations were made to examine the size and arrangement of fungal hyphae, sporophytes, and spores, with the aim of determining the morphology of the entomopathogenic fungal genera of interest.(29, 32) Observations and measurements for the reproductive structures at 400x and 1000x using an ocular micrometer. Micrographs by using a Carl Zeiss optical microscope (model 467065-9902-18VA).

3. Molecular Diagnosis of Insect-Pathogenic Fungi

Each fungal sample was inoculated into a liquid medium containing barley extract (12.7 g/L, pH 6, Oxide) and incubated at 28 ± 2°C in the dark for 7 days to allow for biomass growth. DNA was isolated using the HiPurA™ Fungal Genomic DNA Purification Kit (Canvax, Spain) according to the manufacturer's protocol. The DNA concentrations of the isolated samples were determined on an agar gel by comparison with standard concentrations of Lambda DNA (Thermo Fisher Scientific Inc., USA) and using the Qubit Fluorometric Quantification Method (Qubit).

Molecular identification of fungal strains was performed by sequencing and amplifying the ITS4-ITS5 region of ribosomal DNA. ITS primers (as shown in Table 1) were used, and PCR reactions were performed in a final volume of 20 µl, which contained DNase-free water, 1X buffer, 2.5 mM MgCl , 200 µmol of each dNTP, 10 pmol of each primer, 0.5 units of Taq polymerase, and 1 µg of extracted DNA. A thermal cycler from Hangzhou Bioer Technology CO. (GenePro Thermal Cycler, Model TC-E-48D, B-48D) was used. The amplification program included an initial DNA denaturation step at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 40 s, and extension at 72°C for 40 s, with a final extension step at 72°C for 10 min. The resulting amplicons were evaluated by electrophoresis on a 2% (w/v) agarose gel stained

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with Red Gel Solution (Biotium, 10000X). The amplicons were sequenced by MACROGEN sequencing service, Korea.

Table 1: Primers that were used in this study.

Fungi name	ITS Primers Code	Sequence Product Size	Product Size	Annealing temperature	Authors
B. bassiana	ITS4	3-TCCTCCGCTTATTGATATGC-5'	620bp	55◦C	(33, 34)
	ITS5	'5-GGAAGTAAAAGTCGTAACAAG3'-G			
M. anisopliae	ITS4	3'- TCCGTAGGTGAACCTTGCGG-5'	500 bp	55◦C	(34)
	ITS5	'5-TCCTCCGCTTATTGATATGC-3'			

Sequences were analyzed and consensus sequences were generated using Geneious version 9.1.5.. These sequences were then compared to those available in the NCBI (http://www.ncbi.nlm.nih.gov/BLAST) and FungalBarcoding (http://www.fungalbarcoding.org) databases. Phylogenetic trees were constructed using ITS region sequences selected from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) corresponding to the main species within the Beauveria and Metrarhizium genera. ITS region sequences were used to determine tree roots. Sequence alignments for each fungal group were performed using MEGA 11 with the Clustal Omega, where gap penalties were applied for both gap opening and gap extension.

4. Pathogenicity Test of Fungal Species on the Insect in Laboratory Conditions

Pathogenicity tests were conducted to study the effect of graded concentrations of study fungal on the insect under laboratory conditions, where the temperature ranged from 27°C to 30°C and the relative humidity was maintained at 53%-40%. Fifteen adult insects, randomly collected, were placed inside a sterilized plastic container.

A spore suspension of both B. bassiana and M. anisopliae was prepared at a concentration of 17×10^6 spores per cubic centimeter by diluting the spores in sterile distilled water from each pure colony, which had been previously cultured on SDA medium for 7 days at 25°C ± 2°C. The suspension was filtered through two layers of gauze, and the number of spores was determined using a hemocytometer. Insects were treated with the spore suspension using the baiting method (35, 36).

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The fungal spore suspension was placed on the beetle food substrate with 5 ml of spore suspension at concentrations of 17 × 10^6 spores/cm3, 17 × 10^7 spores/cm3, and 17 × 10^8 spores/cm3 (concentrations were calculated using the formula (cm3) taken from the main suspension = desired concentration / concentration of the original suspension and then multiplied by the volume of the suspension to be prepared). These baits were used after ten days to allow the accumulation of metabolic products or enzymes from the fungus in each plastic container. After that, fifteen adults were placed in each container (37). For the control treatment, sterile distilled water was added to the food baits at a 1:1 ratio. Three replicates were performed for each treatment. The percentage of mortality was calculated using the Abbott's formula.

$$\text{The percentage of mortality} = \frac{\text{The percentage of mortality in the treatment} - \text{the percentage of mortality in the control.}}{100 - \text{The percentage of mortality in the control.}}$$

The percentage of mortality in the adult insects was calculated starting from the fifth day after treatment with the spore suspension. This was done by counting the number of dead insects, examining them under a microscope, and culturing them on PDA medium to confirm the pathogenic cause.

Statistical Analysis

All experiments were conducted using a completely randomized design with factorial experiments. The mortality percentages were calculated and analyzed statistically using Abbott's formula (38). Estimates were made for the LC50, LC90, and slope values (39). Additionally, the effectiveness of the several entomopathogens that were tested was assessed and contrasted with the most successful one (40)..

Result and Discussion

Morphological Identification of Pathogenic Fungi for insect

Five fungal species exhibiting typical characteristics of insect pathogenic fungi were isolated. Among these, one strain was identified as Beauveria bassiana labeled EB1, and four strains were identified as M. anisopliae labeled EM1, EM2, EM3, and EM4.

In this case of the fungi from the Beauveria sp. , the strain formed creamy to white colonies with irregular and a powdery appearance (Figure 1). Microscopically, conidia and reproductive structures exhibited typical, size, and color for B. bassiana. The strains showed septate hyphae, with conidial cell sizes ranging from 5.4 to 8.7

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micrometers (standard deviation 0.5 to 1.0 micrometers) × 2.0 to 2.8 micrometers (standard deviation 0.1 to 0.7 micrometers), with a broad base and a narrow apex from which multiple conidia were released in a spiraled arrangement. The conidia were glassy, smooth, and had a spherical to sub-spherical shape. The average diameter of the conidia ranged from 1.7 to 2.3 micrometers (standard deviation 0.5 to 0.6 micrometers).

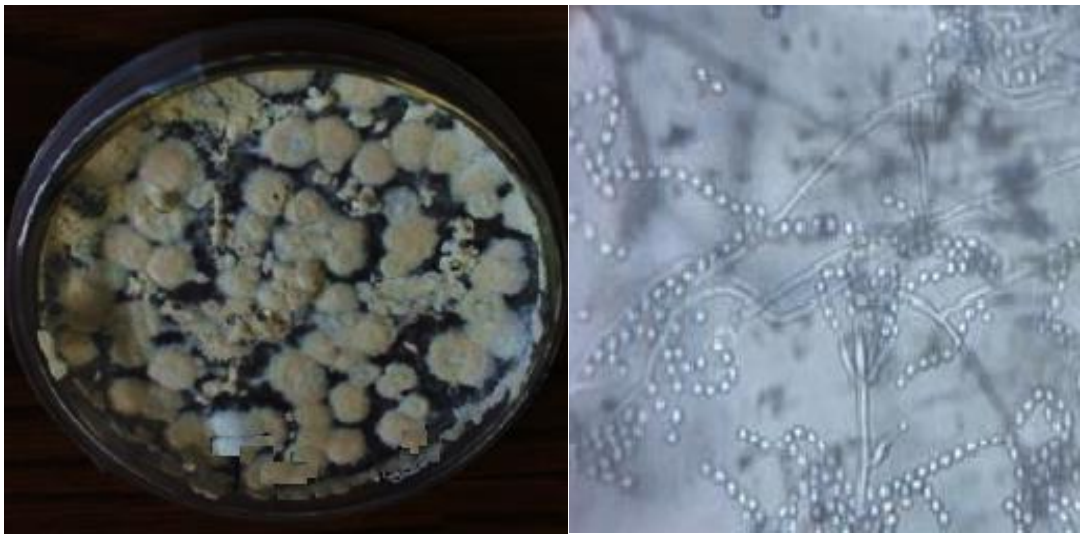

Figure (1): Photographs of B. bassiana strains on SDA culture, and a microscopic image stained with lactophenol blue.

After three weeks of incubation, Strains exhibited white colonies that developed, spore-bearing structures in tightly clustered groups, which are characteristic features of Metarhizium fungi (Figure 2). The strains showed reproductive structures and spores with typical morphology, size and color of M. anisopliae. The fungi were present as segmented hyphae and branched, candle-like sporangia, each septum containing two or three densely intertwined branches. The spore cells were cylindrical, with a slightly conical apex. Both strains showed green, cylindrical spores that formed chains clustered in cylindrical columns. The mean length of the cylindrical spores ranged from 4.5 to 5.1 µm (SD 0.7 µm), while their width ranged from 2.1 to 2.6 µm (SD 0.3 µm).

Identification based on macroscopic and microscopic information led to the classification of isolates according to genus and species levels. Fungal isolates of both B. bassiana and M. anisopliae showed spore shapes, sizes and arrangements consistent with the descriptions given in (25, 29, 31, 32).

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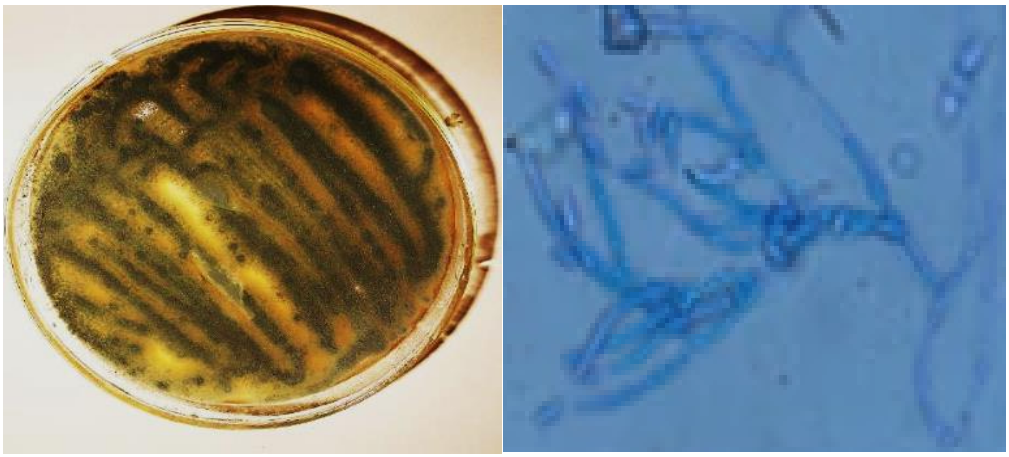

Figure (2) Photographs of M. anisopliae strains on SDA culture, and a microscopic image stained with lactophenol blue.

All the fungal strains from the phylum Ascomycota, class Sordariomycetes, and families Cordycipitaceae for Beauveria, and Clavicipitaceae for Metarhizium.

Molecular Identification of Insect Pathogenic Fungi

The results showed that the DNA purity ranged from 1.66 to 1.88. The DNA concentrations for all the tested isolates ranged from 110 to 420 ng/µL. PCR products of the ITS regions from the rRNA gene isolated from insect pathogenic fungi produced 620 and 500 base pairs according to the primers shown in Table (1).

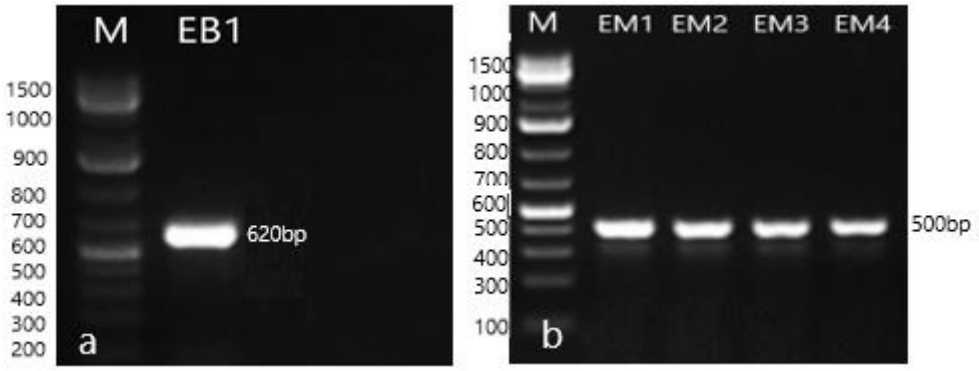

Figure 3: Molecular characterization of the isolates a: (EB1) of B. bassiana and b: (EM1, EM2, EM3, EM4) of M. anisopliae, obtained using the primers ITS5/ITS4.M: Ladder

Results of the sequence identity analysis for the EB strain, which was morphologically identified as B. bassiana, confirmed its classification as B. bassiana

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with identity index of 99%. On the other hand, for the four fungal strains EM1-4, which were morphologically identified as M. anisopliae, the sequence identity indices were also 99% with the deposited sequences of M. anisopliae. Thus, the identity or similarity at the DNA sequence of the five isolated strains of pathogenic fungi for insects was successfully verified.

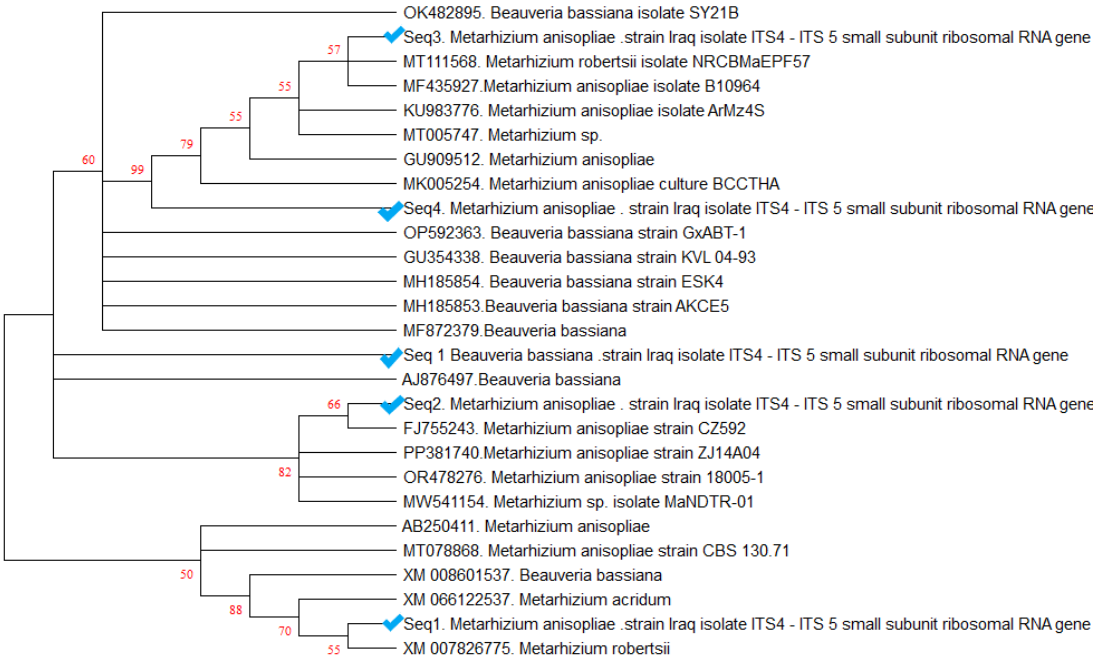

Figure 4: A phylogenetic tree showing the evolutionary relationship between B. bassiana (EB1) and M. anisopliae (EM1, EM2, EM3, EM4) isolates, which were found to exhibit high virulence in the current study, and other B. bassiana isolates from GenBank, based on the ITS region sequence.

Virulence of the Tested Microorganisms Against T. castaneum

The efficacy of the five best fungal isolates, B. bassiana and M. anisopliae, against T. castaneum adults was evaluated. The data presented in Table (2) indicate that the mortality rate of T. castaneum adults was directly related to the time elapsed after treatment. Although there was a delayed effect of the tested fungal isolates, they showed great potential against adults starting from the fifth day after treatment, as shown in Fig. 5.

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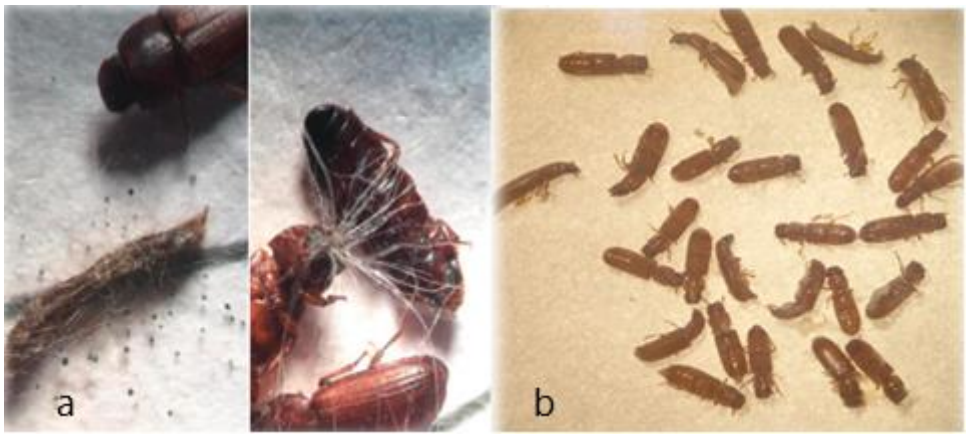

Figure 5: T. castaneum infected with B. bassiana, showing conidia covering the entire body surface (a) and (b).

This slow action was due to the fungal nature, which relies on specific time periods for tissue invasion, nutrient depletion, and the accumulation of toxins within the insect cadavers. B. bassiana was the most effective, followed by M. anisopliae, with LC50 values of 24.2×10^4 and 6.4×10^7, respectively. The concentrations of 10×10^6 spores/cm^3 and 10×10^7 spores/cm^3 gave the highest mortality rates, which were 69.89%, 78.64%, and 93.33%, respectively, when the spore suspension was used in the bait method.

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Table (2): Effect of different concentrations of B. bassiana and M. anisopliae spore suspensions on adult insect mortality.

Treatment	Conc. (cell/ ml^{-3})	Mortality % at indicated day after treatment.			LC50 (cell/ml) and confidence limits at 95%		LC90 (cell/ml) and confidence limits at 95%		Slope ± SE	X^2	Toxicity index
		5 day	7 day	10 day							
B. bassiana	$17x10^6$	30.69	55.08	69.89	$24.2x10^4$		$6.4x10^7$		0.514 ± 0.541	0.122	76.82
	$17x10^7$	38.62	65.03	78.64							
	$17x10^8$	58.33	73.33	93.33	$19.8x10^3$	$65.6x10^4$	$16.2x10^6$	$22.0x10^8$			
M. anisopliae	$17x10^6$	20.00	36.67	56.67	$5.3x10^5$		$29.5x10^7$		0.466 ± 0.114	0.144	28.90
	$17x10^7$	30.00	46.67	70.00							
	$17x10^8$	46.67	66.67	86.67	$6.7x10^4$	$17.4x10^5$	$9.6x10^7$	$35.1x10^9$			

Discussion:

Fungal identification using morphological observations (both microscopic and macroscopic) is fundamental for traditional identification(30). The results of the morphological observations for the EB isolate from SDA medium align with the characterization of B. bassiana, based on the identification by (33)and(41), as shown in Figure 1. Similarly, the four isolates of M. anisopliae formed circular, milky colonies with a cottony texture. The conidia were cylindrical, glassy, with rounded edges and an olive-green color, with an average diameter of 4.5 micrometers(42), as shown in Figure (2). In previous studies, these isolates were already recorded as natural microbial agents infecting whiteflies in tomato fields (43, 44).

Morphological similarity must be supported by molecular identification to determine and describe the genetic variation of insect pathogenic fungi. The most accurate way to identify diversity and genetic structure within a species is through molecular markers(45). The ITS region of ribosomal DNA (rDNA) is considered the official barcode for fungal DNA, due to its highly variable sequences used for identification (46,

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47). In this study, the molecular taxonomic identity of the five fungal isolates was confirmed by sequence analysis of the ITS regions of ribosomal DNA. Alignment and phylogenetic analysis also demonstrated the taxonomic identity of the studied strains. Renner (2005) compared the ITS1-5.8- ITS2 sequences of Beauveria strains with those in the NCBI database, confirming their classification as B. bassiana (48).

These results are similar to current study, where the three strains analyzed were taxonomically classified as B. bassiana, with high bootstrap. (49), who amplified the ITS1-5.8S-ITS2 region for Metarhizium strains, observed sufficient genetic variation in these markers to distinguish between different species groups within the Metarhizium genus. In the current study, genetic variation in the sequenced region allowed us to classify the indigenous Metarhizium strains as belonging to M. anisopliae var. anisopliae, with similar (50).

Morphological and molecular identification of biological control agents is ideal, as accurate identification of the fungal isolates used is essential to evaluate the effectiveness of the biological control system.(51).

The effect of different concentrations of B. bassiana and M. anisopliae spore suspensions on the mortality of adult red flour beetles, T. castaneum. The fungal suspension of B. bassiana showed significant differences in efficacy compared to M. anisopliae when the highest concentration of the fungal suspension was used, resulting in a mortality rate of 76.82% after 5 days of treatment. Mortality rates increased for all adults as the exposure period lengthened, with a direct proportional relationship between the spore suspension concentration and both mortality rate and exposure time. The reason for the increased mortality at higher concentrations is due to the greater number of spores, leading to an increased number of growing spores attacking the host, as well as weakening the insect's immune system. The immune system can defend the body only when the concentration is low, but its efficiency decreases at higher concentrations(52). The results were consistent with those reported by(53), who described the relationship between spore concentration and mortality rate as directly proportional. As the spore suspension concentration increased, the mortality rate also increased. This could be attributed to the higher number of spores (the basic units of fungal infection).

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Both B. bassiana and M. anisopliae are not harmful to natural predators and soil insects. Fungi used in pest control show high selectivity against the host and affect non-target organisms. Additionally, they are easy and inexpensive to prepare(54).

These results are consistent with (55), who reported that exposure of Ochlerotatus sierrensis adults to T. cylindrosporum spore suspension at a concentration of 5×10^6 spores/ml resulted in 50% mortality after five days and 100% mortality after nine days. (56) reported that when using M. anisopliae against Cx. quinquefasciatus and An. stephensi adults, male mosquitoes were more affected than females. Mortality rates for males were 93.33% and 100% at a concentration of 2×10^5 spores/ml after 168 hours, while mortality rates for females of both species were 90% and 96%, respectively, at the same concentration and exposure period.

Conclusion

The current study provides valuable information about insect pathogenic fungi associated with the red flour beetle (T. castaneum) in the city of Diwaniya. The fungi Beauveria and Metarhizium were isolated locally from wheat fields in Diwaniya and are recognized as insect pathogens. Based on their macroscopic and microscopic characteristics, one isolate was identified as B. bassiana, while four isolates were identified as M. anisopliae. These local isolates were further confirmed through the amplification and sequencing of the ITS5-ITS4 region, and their taxonomic identity was validated through comparison with molecular databases and phylogenetic analyses.

The high pathogenicity of these isolates suggests that they can be developed as effective biocontrol agents, reducing dependence on chemical products and promoting sustainable wheat production. Moreover, the survival curve highlights the variation in virulence among the isolates, emphasizing the importance of careful selection of the most promising isolates for practical application

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