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ISOLATION AND IDENTIFICATION OF THE MOST IMPORTANT OF ENTOMOPATHOGENIC FUNGI AND EVALUATION OF THEIR EFFICIENCY IN *MUSCA DOMESTICA* L.

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ABSTRACT

Background/Objective: Fungi that infect insects constitute a very large and diverse group, with approximately 1600 diagnosed and known species belonging to 90 genera, and The fungi have developed their capabilities and ability to infect insects and in different strains, the house fly *Musca domestica* L. (Diptera: Muscidae), has great medical importance due to its ability to transmit thousands of pathogenic microorganisms such as parasites, fungi, bacteria and viruses.

Methods/Statistical analysis: This study was conducted in the insect laboratory at the Faculty of Education for Girls / Anbar University with the aim of isolating and characterizing the most important Entomopathogenic Fungi and evaluating their efficiency on the house fly *Musca domestica* L. (Diptera: Muscidae). House fly adults were collected and raised in the laboratory, approximately 50 adult house fly insects were collected and treated at three concentrations for seven days in two ways: mixing with food and direct spraying on insects from the suspension [spores] of the fungi *Metarhizium anisopliae* and *Trichoderma harzianum* that were isolated from the Zira'a area of Ramadi city.

Findings: the results of isolation and diagnosis of fungi showed the registration of the two fungi *M. anisopliae* and *Trichoderma harzianum* and their diagnosis was confirmed. (ID: PP035895 and ID: PP065519). The results also showed that there were significant differences between the concentrations used in the experiment, and the percentage of mortality in insects was directly proportional to the increase in the suspension of fungal spores, as the concentration of 1 X 10^7 spores/ml showed the highest percentage of mortality in the house fly in both methods [direct spraying method and mixing with food], as the percentage of mortality in the direct spraying method from the fungus *M. Anisopliae* and after a period of 7 days, the percentage of mortality was 85.7% and the percentage of mortality of the same organism by mixing with food was 66.6%, while the same concentration (1 x 10^7 spores/mL) with the spore suspension of *T. harzianum* by mixing with food was mortality rate of 85.7% and gave 100% mortality with the direct spraying method after 7 days. The results also revealed that *T. harzianum* spore suspension at all concentrations used in the experiment showed a higher percentage of lethality than *M. anisopliae*.

Conclusion: The study found that molecular diagnosis is indispensable in the diagnosis of microorganisms, especially fungi because they share many phenotypic and even microscopic characteristics, and the study showed the sensitivity of house fly adults to the spore suspension of the studied fungi, and the study showed the superiority of the fungus *T. harzianum* in increasing the percentage of mortality compared to M. anisopliae.

Key word: *Musca domestica*, Entomopathogenic Fungus *Trichoderma harzianum, Metarhizium anisopliae*, PCR Technic, *Musca domestica* damage.

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INTRODUCTION

Insects represent approximately 67% of known organisms, with at least one million species of insects (Stork, 2018). Insects are a large and integral part of ecosystems (Crespo-Pérez *et al*., 2020). Although most insects depend on the nutrients found in plants, whether their leaves, fruits, stems, and even their roots, many of them depend on eating meat and are considered carnivores, and these are found in the environment in very large numbers (Cole, (2012). House flies are one of the largest health pests in all countries of the world, as they have a close relationship and association with humans as well as with his livestock or animals that he raises, as these insects transmit many different diseases to humans and his animals (Gupta *et al.* 2012). It is believed that the origin of the *Musca* house fly came from the Savannah region [in Asia] but then spread to all over the world (Hussein, and John, 2014; Ommi *et al.,* 2015). The house fly has succeeded in spreading due to its ability to reproduce rapidly due to its high fertility (Iqbal *et al.*, 2014). The house fly *Musca domestica* L. has great medical importance due to its ability to transmit thousands of pathogenic microorganisms such as parasites, fungi, bacteria and viruses (Nassiri *et al*., 2015; Tsagaan *et al*., 2015). Pathogens are transmitted to humans through direct contact with *M. domestica* to humans, food and drink, as well as to animals raised and fed (Malik *et al*., 2007). Several methods have been used to eliminate insects, including biological, plant extracts and others (Hermize *et al*., 2016).

Although insects are an important primary food source for many of their predators, including reptiles, amphibians, birds, mammals, and others, 60% of insect mortality in the ecosystem is due to fungal infections (Cole, 2012). Fungi that infect insects constitute a very large and diverse group, with approximately 1600 diagnosed and known species belonging to 90 genera, and fungi have evolved their capabilities and ability to infect insects and in different strains, several genera and fungal species have been found to cause insect diseases, many of them belonging to oomycetes, cystic fungi, basidiomycetes, myxomycetes and Blastocldiomycota (Al-Salman *et al*., 2012). Entomopathogenic fungi infect more than 18 orders and from all stages of insect development from egg to adult (Araújo and Hughes, 2016). Most insect pathogenic fungi, including *Metarhizium spp*. and *Beauveria spp*. Appressoria may be produced depending on the fungal species in order to penetrate the insects' epidermis, and penetration may take place without producing them (Ortiz-Urquiza and Keyhani, 2013; Hamad *et al*., 2015; Chethana *et al*., 2021). After penetrating the insect's epidermis, free yeast-like structures and cells are produced in order to avoid the insect's immune system, these cells are called Hyphal bodies or Blastospores inside the insect's body (Chethana *et al*., 2021). In order to minimize the danger caused by insects, especially house fly *Musca domestica*, this research was to find a biological, safe and environmentally friendly way to control the house fly, as well as to detect fungi that have a role in controlling insects.

MATERIAL AND METHODS

1. Colony rearing of *Musca domestica* L.

Approximately (50) adults of house flies were collected by means of an air net from the poultry field located inside the Faculty of Agriculture/Anbar University, during April 2023, and transferred to the insect laboratory at the Faculty of Education for Girls/Anbar University, and the adults were placed in cages with a cubic wooden structure with dimensions (30 × 30 × 30 cm) wrapped with cloth tulle, in order to multiply them, and the cages were preparing with a petri dish containing about 50 g of powdered sugar and powdered milk (1:1) (w:w) and another petri dish containing cotton soaked with

water for the purpose of feeding the adult insects in order to multiply them, and the insect was raised for four generations before conducting experiments on them (Wang *et al*., 2023).

The diagnosis of the insects at the Iraqi Natural History Museum in Baghdad was confirmed as *Musca domestica* L.

2. Isolation of fungi from soil

Fungus samples were taken from the soil of the agriculture area in the city of Ramadi / Anbar governorate after removing the surface layer of the soil, and taking samples from a depth of (10 - 15 cm) with three random samples, with a weight of approximately (500 g) soil per sample, and for the purpose of getting rid of pebbles and suspended parts of the soil, soil samples were sieved with an alcohol-sterilized sieve, and the samples were placed in polyethylene bags and the location and date of sample collection were recorded, then transferred to the laboratory for isolation and diagnosis (Mohammed *et al*., 2014; Sabr, 2018; Jaloud, and Hassan, 2018).

3. Isolation by dilution method

The fungi were isolated from the soil using the decimal dilution method, then 1 ml of the last dilution was taken and poured into Petri dishes containing Potato Dextrose Agar (PDA) medium supplemented with the antibiotic chloramphenicol (100 ppm) in three replicates per treatment, and incubated at 25°C for (4 days), and the fungi were diagnosed according to the approved taxonomic keys (Jaloud and Hassan, 2018). The diagnosis was confirmed molecularly by PCR Polymerase Chain Reaction (Al-qaysi, and Alwan, 2016).

4. Preparation of Fungal Suspension Concentrations

Three concentrations of the suspension of isolates 10^5, 10^6 and 10^7 spores/ml were prepared using sterile distilled water supplemented with Tween 20 (0.01%) for the purpose of studying the effect of these concentrations on house fly adults, as they were exposed to the above concentrations of both fungal isolates by the direct spray method, as the method involved transferring 10 larvae from the rearing colony to a plastic container with a base diameter of (4.5) cm and a height of 3.5 cm. The larvae were sprayed with about 2 ml of *M. anisopliae* suspension from a distance of 10-15 cm with a sterile hand sprayer and the larvae were then transferred to another container containing ground dried sheep manure added to sterilized dried baking powder in a ratio of 1:2 g. The mixture was moistened with distilled and sterilized water. The containers were covered with perforated lids to let air in and prevent the larvae from escaping, with three replicates for each concentration. The control treatment was done by spraying the larvae with 0.01% Tween 20 in distilled water. (Noshee, 2015; Raja and William, 2017).

5. Effect of fungal suspension concentrations in house fly adults

In order to study the effect of spore concentrations of *M. anisopliae* and *T. harzianum* The cage containing the adults was placed in the freezer for two minutes for the purpose of reducing their movement, then (10) adults were transferred to a (200) ml plastic container lined with Whatman No.1 filter paper and sprayed with (5) ml of 1×10^5 spore/ml concentration, the container was covered with tulle cloth and tied with a rubber band and a cotton swab saturated with water and 5% sugar for the purpose of feeding them. The treatment was repeated (3) times for each of the three concentrations and for the two fungi separately. As for the control treatment, the filter papers were treated with distilled water supplemented with Tween 20 at a concentration of 0.01% and the replicates were incubated at a temperature of (27±2) m, relative humidity (80±5%) and light for (12) hours. Adult mortality was monitored for 24 hours to

calculate the cumulative mortality rate and for 7 days (Noshee, 2015). The method of treating the mixture with food at the above-mentioned concentrations was by transferring 10 adults to a 120 ml Plastic container, covered with a tulle cloth and tied with a rubber band. 0.5 g of sugar was added to 5 ml of 1×10^5 spore suspension and mixed well. A sterile cotton swab was placed in the mixture and after saturating it, it was placed on top of the tulle cloth covering the container containing the adults to feed them with the sugar solution containing the fungal spore suspension. Three replicates were made for each concentration in addition to the control treatment which was done by adding 0.01% Tween 20 with sterile distilled water and the same experiment was repeated with *T. Harzianum* and then the experimental and control pots were transferred to an electric incubator under the same conditions as above (Raja and William, 2017; Al-Kaissy, 2019).

6. Statistical Analysis

A fully randomized design (C.R.D.) technique was used for all laboratory trials, and prior to statistical analysis, the mortality percentages were converted to angular transformation values. At a probability threshold of 0.05, the averages were compared using the least significant difference test (L.S.D.) and Mortality percentages were corrected based on the Schneider-Orelli [1947] formula.

RESULTS

1. Molecular diagnostics

The results of laboratory isolation and diagnosis of fungi according to fungal taxonomic keys showed the diagnosis of two fungi, *Metarhizium anisopliae* and *Trichoderma harzianum*, and this diagnosis was confirmed by genetic analysis, using the polymerase chain reaction (PCR)technique and DNA Sequencing of these fungi, and these two fungal species under study were registered in the NCBI (National Center for Biotechnology Information) gene bank in the name of the researchers and under the accession numbers: ID: PP035895 and ID: PP065519, respectively. Figure (1 and 2) shows the genetic relationship between the two species under study and the globally registered species.

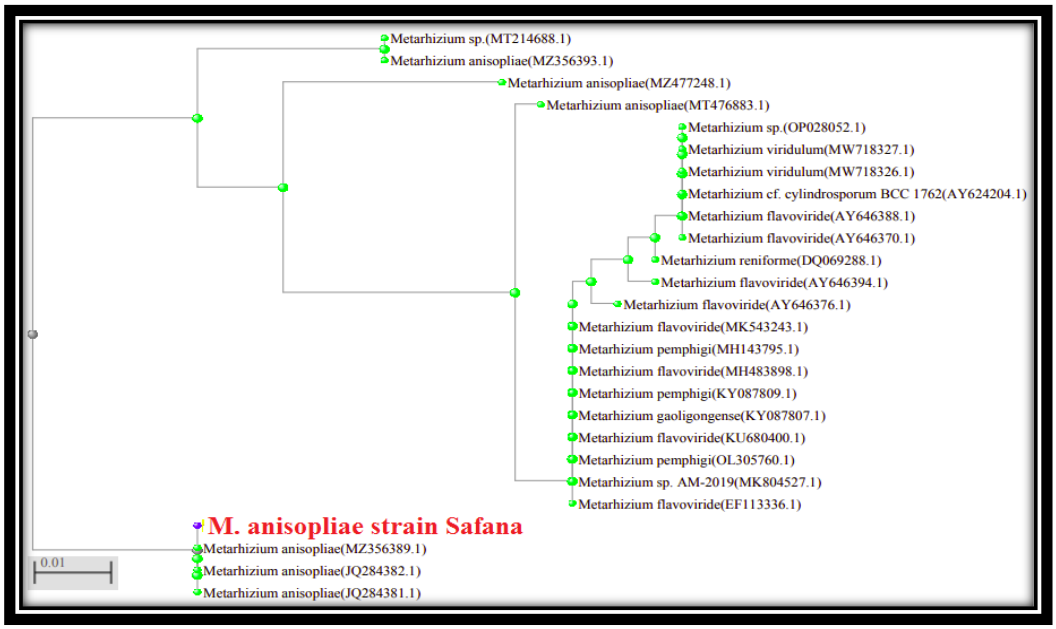

Figure 1: Phylogenetic tree of *M. anisopliae* marked in red with globally recorded species.

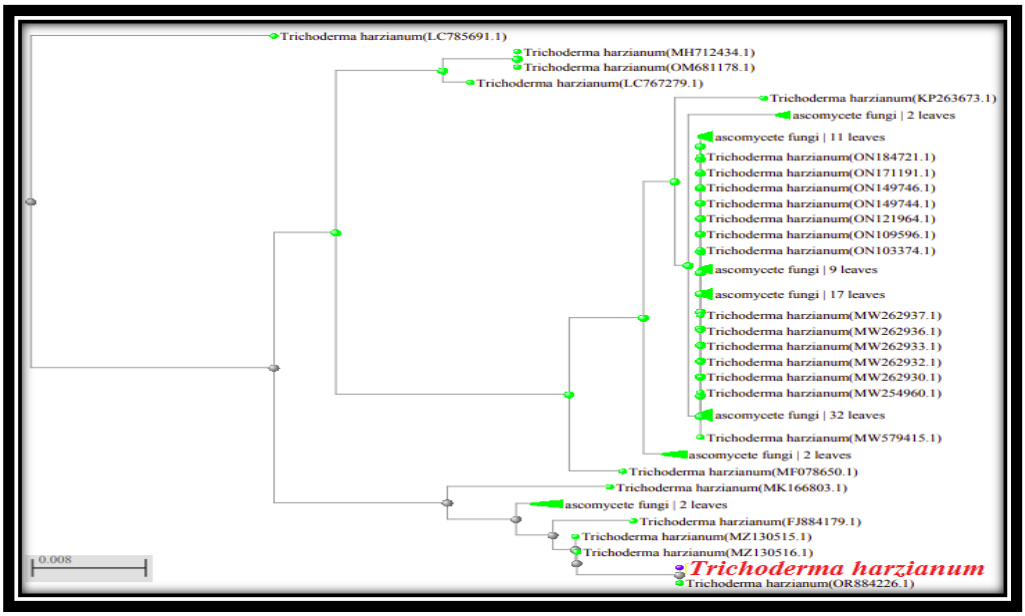

Figure 2: Phylogenetic tree of *T. harzianum* marked in red with globally recorded species.

2. Effect of *M. anisopliae* on house fly adults by the food-mixing method

The results shown in Table (9) and Figure (3) revealed the effect of three concentrations of *M. anisopliae* on house fly adults by the feeding method and the presence of significant differences between the concentrations used, the results also showed that there was no mortality on the first and second day and for all concentrations used in the experiment and the results showed that the percentage of mortality increased with increasing the concentration used as the highest percentage of mortality with 1 X 10^5 spores/mL. on the seventh day reached 38. 4%, while the highest percentage of mortality on the seventh day was 66.6% with the concentration of 1 X 10^7 spores/mL.

Table 1: Shows the effect of three concentrations of *M. anisopliae* on the mortality of house fly adults by feeding method

NO	Conce Spores\ml	Destruction rate / Day %							Transaction impact rate%
		1Day	2 Day	3 Day	4 Day	5 Day	6 Day	7Day	
1	1 X 10^5	0.0 %	0.0 %	6.6%	17.8%	17.3%	31.5%	38.4%	15.9%
2	1 X 10^6	0.0 %	0.0 %	13.3%	19.2%	28.5%	46.6%	50.0%	22.5%
3	1 X 10^7	0.0%	0.0 %	16.6%	20.0%	35.0%	53.8%	66.6%	27.4%
4	Control	0.0 %	0.0 %	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
L.S.D <0.05					**1.92**				

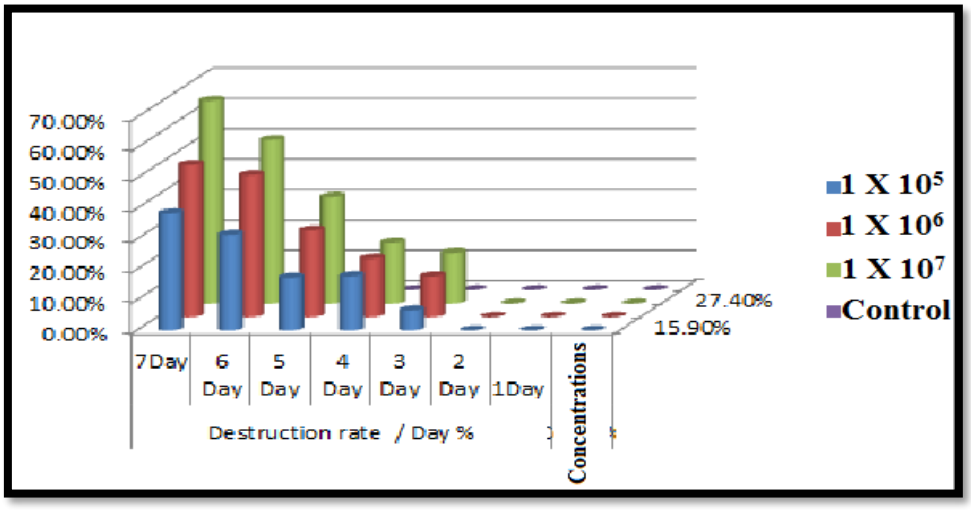

Figure 3: Shows the effect of three concentrations of *M. anisopliae* on the mortality rate of house fly adults by feeding method.

3. Effect of *M. anisopliae* on house fly adults by spraying method

The results shown in Table (2) on the effect of three concentrations of *M. anisopliae* on house fly adults by direct spraying method showed that there were significant differences between the used concentrations. The results also showed that there was no mortality on the first day only and for all concentrations used in the experiment. The results showed that the mortality rate increased with increasing the concentration used, as the highest mortality rate with 1 x 10^7 spores/ml on the seventh day reached 85. 7%, while the highest percentage of mortality on the seventh day was 20.0% with 1 X 10^5 spores/mL.

Table 2: Shows the effect of three concentrations of *M. anisopliae* on the mortality of house fly adults by direct spraying method

NO	Conce Spores\ml	Destruction rate / Day %							Transaction impact rate%
		1Day	2 Day	3 Day	4 Day	5 Day	6 Day	7Day	
1	1 X 10^5	0.0 %	٦.٦%	١٠.٧%	١٦.٠%	19.0%	41.1%	20.0%	16.2%
2	1 X 10^6	0.0 %	3.3 %	17.2%	12.5%	23.8%	25.0%	41.6%	17.6%
3	1 X 10^7	0.0%	6.6 %	7.1%	19.2%	38.0%	38.4%	85.7%	27.8%
4	Control	0.0 %	0.0 %	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
L.S.D <0.05						**1.43**			

4. Effect of *T. harzianum* on house fly adults by the food-mixing method

The results of Table (3) showed the evaluation of the effect of three different concentrations of *T. harzianum* on house fly adults and the feeding method [mixing with food]. The results revealed that there were significant differences between the concentrations used in the experiment. The results also revealed no mortality on the first day and for all concentrations, and the highest percentage of mortality on the sixth day with the concentration of 1 x 10^7 spores/ml, which amounted to 85.7%.

Table 3: Shows the effect of three concentrations of *T. harzianum* on the percentage of mortality of house fly adults by feeding method

NO	Conce Spores\ml	Destruction rate / Day %							Transaction impact rate%
		1Day	2 Day	3 Day	4 Day	5 Day	6 Day	7Day	
1	1 X 10^5	0.0 %	0.0%	13.3%	11.5%	21.7%	33.3%	41.6%	17.3%
2	1 X 10^6	0.0 %	3.3 %	17.2%	20.8%	26.3%	42.8%	62.5%	24.7%
3	1 X 10^7	0.0%	10.0 %	18.5%	31.8%	53.3%	85.7%	0.0%	66.4%
4	Control	0.0 %	0.0 %	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
L.S.D <0.05					**1.43**				

5. Effect of *T. harzianum* on house fly adults by spraying method

The results of Table (4) and Figure (4) showed the evaluation of the effect of three different concentrations of *T. harzianum* The results revealed that there were significant differences between the concentrations used in the experiment. The results also revealed that there was no mortality on the first day and for all concentrations, and the highest mortality rate on the seventh day with 1 x 10^7 spores\ml concentration was 100%, while the mortality rate with 1 x 10^5 spores\ml concentration on the seventh day was 40%.

Table 4: Shows the effect of three concentrations of *T. harzianum* on the mortality of house fly adults by direct spraying method

NO	Conce Spores\ml	Destruction rate / Day %							Transaction impact rate%
		1Day	2 Day	3 Day	4 Day	5 Day	6 Day	7Day	
1	1 X 10^5	0.0 %	0.0%	13.3%	15.3%	27.2%	37.5%	40.0%	19.0%
2	1 X 10^6	0.0 %	3.3 %	6.8%	25.9%	30.0%	57.1%	66.6%	63.2%
3	1 X 10^7	0.0%	6.6 %	17.8%	30.4%	50.0%	87.5%	100.%	97.4%
4	Control	0.0 %	0.0 %	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
L.S.D <0.05					**1.43**				

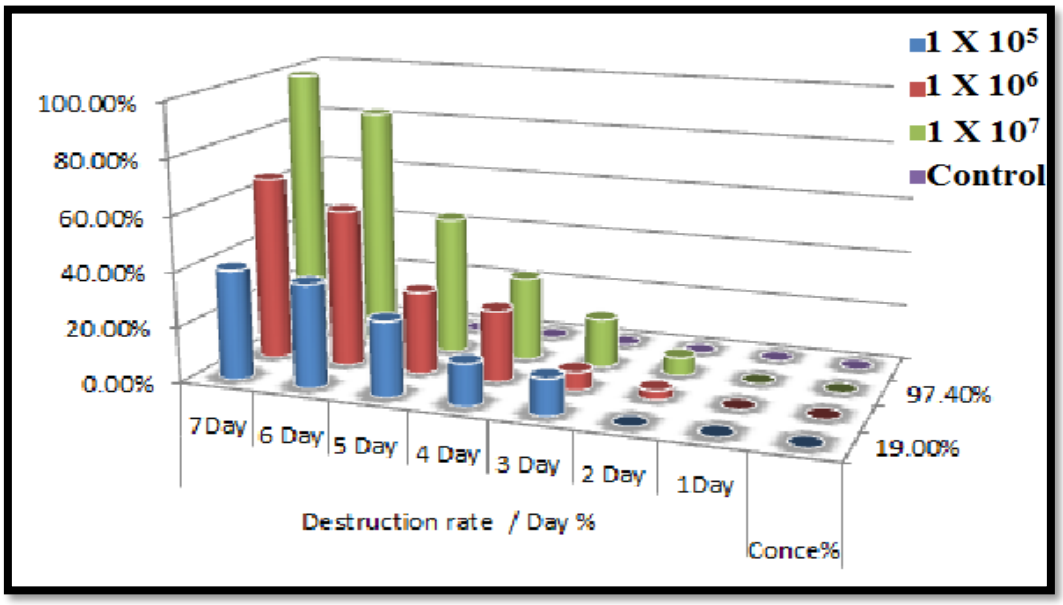

Figure 4: Effect of three concentrations of *T. harzianum* on the mortality of house fly adults by direct spraying method.

DISCUSSION

From the above results, the molecular diagnostic results are comparable to those found by (Shanker *et al*., 2023) and (Salah *et al*., 2020), who were able to diagnose the same fungal species using specific regions of DNA known as the ITS gene. Non-culture-based methods, such as polymerase chain reaction (PCR), allow rapid detection and identification of fungi, offering the possibility of prescribing effective pathogen-specific therapy and identifying genetic markers associated with antifungal resistance (Dewan *et al*., 2010; Saied Hamied, 2017). These results are consistent with Shanker *et al*. (2023), who found that the ITS gene is a unique gene for the diagnosis of the fungus *Metarhizium anisopliae*. Francis and Manchegowda (2023) were also able to diagnose two species of *Metarhizium*, *M. guizhouense* and *M. pingshaense*, using the ITS gene, and showed that molecular diagnosis is necessary and important. It has a role in distinguishing between species and strains very accurately. Mazrou *et al.* (2020) were able to diagnose *Trichoderma harzianum* using PCR and the ITS1 and ITS4 primers, finding that the percentage of identical sequences of the tested isolates is 98%. Stummer *et al.* (2020, also identified three species of *Trichoderma*: *T. harzianum*, *T. afroharzianum*, and *T. gamsii* by amplifying the ITS (internal transcribed spacer) gene by PCR. Mahmoud *et al*. (2023) were able to molecularly characterize 9 isolates of *Trichoderma harzianum* using PCR technology. In a study by Gezgin *et al*. (2023), they characterized *Trichoderma* fungi using two different ITS gene regions and a translation elongation factor for molecular identification of *Trichoderma* isolates and amplified these genes by PCR. Jang *et al*. (2012) stated that the ITS region has several advantages: multiple copies of the ribosomal gene are present in all organisms, enabling sensitive detection by PCR. The ITS region contains both a highly conserved and a variable region, making it optimal for target identification and PCR primer development. The ITS region is a highly polymorphic non-coding region with sufficient taxonomic units, allowing sequences to be separated to the species level and giving results with 450 to 750 base pairs (Op De Beeck *et al.*,2014). The ITS region is one of the most widely used markers in the phylogenetic study of most fungi. This region consists of the highly conserved 5.8S region as well as ITS1 and ITS2, the variable region that enables the distinction between different fungal species. The ITS region has been used to examine evolutionary relationships within and between species to understand the fungal community (Tamura *et al.*, 2004). This region contains many tandem repeats of ribosomal RNA, making it very useful in species identification and has been proposed as a standard marker in DNA barcoding for fungal species (Xu, 2016).

Additionally, it was shown that using *T. harzianum* with housefly insects resulted in a higher mortality rate than the use of *M. anisopliae* suspension in both methods (mixing with food and direct spraying). This result does not agree with Dewan *et al*., (Dewan *et al*., 2010), who found that the *Trichoderma* fungus had the lowest mortality rate in the development and death of housefly adults. The lethality in houseflies can be attributed to the ability of this fungus to secrete many enzymes and metabolic substances toxic to insects, such as mycotoxins, toxic acids, and other influencing substances (Alkafaji and Alzubaidi, 2014; Kumar *et al*., 2012; Kathiar *et al*., 2022). The cause of the deaths can also be attributed to the fungus's effect on and invasion of the insect's body by restricting and paralyzing its movement, especially its wings, with fungal threads and mycelium. This is confirmed by studies that show fungal threads infecting insects work to restrict their movement, preventing them from

reaching food, thus leading to their death (Obaid, 2013).

A study also found that the fungal filaments of *M. anisopliae*, when they enter the insect's body, cause the development of fungal cells, disrupting the defense and immune system in insects (Kannan *et al*., 2008). In an experiment using the spores of *M. anisopliae* on different stages of the housefly, the rotting of these insects was observed, with some turning green and others white. This indicates that the fungal mold completely surrounds the insect, leading to its death. Some insects had disrupted wings and an inability to move their heads due to the fungal filaments that infected and immobilized them (Quesada-Moraga *et al*., 2024; Obaid, 2013).

Through this study, the role of using fungi as a biological control factor in eliminating insects becomes clear. Some studies have stated that fungal control of insects (the use of fungi as biological agents) is an important and alternative means to chemicals and pesticides (Kareem and Al-Araji, 2017; Hawar, 2022).

CONCLUSION

The study found that molecular diagnosis is indispensable in the diagnosis of microorganisms, especially fungi because they share many phenotypic and even microscopic characteristics, and the study showed the sensitivity of house fly adults to the spore suspension of the studied fungi, and the study showed the superiority of the fungus *T. harzianum* in increasing the percentage of mortality compared to *M. anisopliae***.**

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عزل وتشخيص أهم الفطريات الممرضة للحشرات وتقييم كفاءتها على حشرة الذبابة المنزلية *Musca domestica* L.

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اجريت هذه الدراسة في مختبر الحشرات في كلية التربية للبنات/ جامعة الأنبار بهدف عزل وتشخيص أهم الفطريات الممرضة للحشرات وتقييم كفاءتها على حشرة الذبابة المنزلية *Musca domestica* L. إذ تم جمع يرقات الذباب المنزلي وتربيتها في المختبر وعوملت بثلاثة تراكيز لمدة سبع ايام وبطريقتين هما الخلط مع الغذاء وطريقة الرش المباشر على الحشرات من عالق (أبواغ) الفطريات *Metarhizium anisopliae* و*Trichoderma harzianum* التي تم عزلها من منطقة الزراعة في مدينة الرمادي، بينت نتائج عزل وتشخيص الفطريات تسجيل الفطرين *M.anisopliae* و *T. harzianum* وتم تأكيد تشخيصهما جزيئيا وسجلا في بنك الجينات الأمريكي تحت أرقام انضمام منحت عالميا (ID: PP035895 و ID: PP065519) ، كما بينت النتائج وجود فروق معنوية بين التراكيز المستعملة في التجربة وكانت نسبة الهلاكات في الحشرات تتناسب طرديًا مع زيادة عالق أبواغ الفطريات إذ كان التركيز 1×10^7 بوغ/ مل أظهر أعلى نسبة وفيات في حشرة الذبابة المنزلية وفي كلا الطريقتين (طريقة الرش المباشر والخلط مع الغذاء) إذ كانت نسبة الهلاكات بطريقة الرش المباشر من عالق الفطر *M.anisopliae* وبعد مدة ٧ أيام بلغت ٨٥,٧ % ونسبة الهلاكات لنفس العالق بطريقة الخلط مع الغذاء بلغت ٦٦,٦ % ، في حين أظهر نفس التركيز (1×10^7 بوغ/ مل) مع عالق أبواغ الفطر *T. harzianum* بطريقة الخلط مع الغذاء نسبة هلاكات بلغت ٨٥,٧ % واعطى نسبة هلاكات مع طريقة الرش المباشر بنسبة ١٠٠ % بعد مرور ٧ أيام ،كما كشفت النتائج أن عالق أبواغ الفطر *T. harzianum* وفي جميع التراكيز المستعملة في التجربة أظهرت نسبة هلاكات أعلى من عالق أبواغ الفطر *M.anisopliae* .

الكلمات المفتاحية: الحشرات، الذبابة المنزلية، اضرار الحشرات، الفطريات الممرضة للحشرات.