Evaluation of the effectiveness of spore suspension and fungal filtrate of *Metarhizium anisopliae* fungus in controlling *Tribolium castaneum* (Coleoptera: Tenebrionidae)

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Abstract

This study evaluated the effect of spore suspension and fungal filtrate against different developmental stages of *Tribolium castaneum third and fifth larval instars and adults*. Two isolates of entomopathogenic fungus *Metarhizium anisopliae* were used (commercially (Met 52 EC), and domesticly). For the two isolates, the effectiveness of various conidial concentrations were (1×10^4 ; 1×10^6 ; 1×10^8 conidia/ml) and various concentrations of fungal filtrate (100,75,50%) were evaluated. It is observed that the fungal filtrate at a concentration of 75% and the conidial concentration of 1 x 10^8 conidia /ml for both isolates were the most effective in causing the highest mortality rates to the third and fifth instar larva and adults of *T. castaneum*. Additionally, it resulted in less eggs produced by females treated with conidial concentration and fungal filtrate compared to control treatment. The results of this study demonstrate that the fungus can be used as a biological control agent against *T. castaneum*, which infects stored products. However, further research studies under storage conditions are required.

Keywords: Insects storage pests, biological control, *Tribolium castaneum*

Introduction

T. castaneum is one of the most important and widespread stored insect pests. This insect feeds and lives, in all its different instars, on dried warehoused products such as grains and flour, causing qualitative and quantitative loss to these products, Moreover, flour unsuitability for bread and pastries, as well as the lack of protein, starch, minerals and various vitamins is also one of the most important economic damages [1]. This insect secretes chemical compounds such as ketones and aldehydes, which are carcinogenic chemicals when the infection is extremely severe [2]. Since chemical control is the fastest approach to control pests, it has been widely used to control *T.castaneum* compared to other control methods. Chemical insecticides are used through spraying, fogging, and fumigation to control warehouse insects, including this pest [3]. However, the use of chemical pesticides has resulted in the emergence of insect strains that are resistant to insecticides. In addition to the fact that insecticides are harmful to other

living organisms and environment [4]. Thus, the research's objective was to determine whether it is possible to eliminate *T.castaneum* and minimize the impact and damage caused to stored products by using alternative methods such as biological entomopathogenic fungi .

Materials and methods:

The culture medium used:

Potato Dextrose Agar (PDA) culture media

To prepare potato infusion, 200 g of boiled potato were sliced and placed in 500 ml of distilled water for 20- 30 min using a glass beaker. Then, it was filtered through cheesecloth to obtain the potato infusion. 20 g of dextrose sugar and 17 g of acre were dissolved in 500 ml of distilled water in another 500 ml glass flask . then , potato filtrate was added to it to make the entire volume , which is 1L .The medium was poured into the petri dishes according to the required experiments and some of medium was kept in the refrigerator until use. This medium was used to grow the fungi under study.The culture media was placed into glass flasks as needed and sealed with cotton plugs and autoclaved for 20 minutes at 121 °C and pressure of 15 pounds / square inch. After sterilization, the flasks were allowed to cool down, then 250 mg / 1 liter of chloramphenicol was added. Then, the media was poured into petri dishes in line with to the required experiment and part of it stored in refrigerator until use. This media is used to grow the fungi under study.

Potato Dextrose Broth (PDB) Media

This media was prepared in the same manner described above, but without the addition of agar. This media is used to grow fungi to obtain fungal secretions.

Obtaining *Metarhizium anisopliae* fungus used in experiments

The domestic isolate of this fungus is obtained from the Faculty of Agriculture, University of Babylon, and diagnosed by Prof. Dr. Jamal Hussein, while the commercial isolate is obtained, from Britain, loaded on powder , then , activated and propagated in the PDA medium, several times.

Collecting and diagnosing *T. castaneum*

A pure culture of *T. castaneum* is obtained from the entomology laboratory at the Faculty of Agriculture, University of Kufa and diagnosed as *Tribolium castaneum* by Dr. Rasha Abdul Razzaq Jawad Al-Taie , Department of Plant Protection , Faculty of Agriculture , University of Kufa . Moreover, the pure culture is reared using sterilized flour and incubated at a temperature of 2±30ºC and the culture is constantly monitored and renewed after each generation.

Preparation of conidial concentrations of the fungus *Metarhizium anisopliae* (local and commercial isolates)

The conidial concentrations of *M. anisopliae* fungus is prepared through activating and propagating it by transferring it multiple times using the P.D.A media. A petri dish containing the 7-day -old fungus is taken and washed with 5 mL of sterile distilled water containing 0.02% of Tween 20. After thoroughly stirred , 1 mL of is taken out of the petri dish and diluted in a ratio of (1:10) using sterile distilled water containing 0.02% of the tween material to the first dilution, and then the second, third and fourth dilution and so on. The commercial isolate suspension of *M. anisopliae* fungus is prepared as follows : by taking 10 g of loaded fungus and added into 1L of sterile distilled water, contains 0.02% of the tween material ,thus , the first dilution was obtained 1×10^{10} .Then, 1 mL of it was taken out and added into 9 ml of sterile distilled water, as a result , the required dilutions were obtained for the experiment, which is (1×10^{8}-1×10^{6} -1×10^{4}) conidia / mL.

Preparation of the *Metarhizium anisopliae* filtrate

In this method, 250 mL wide-bottom glass flasks were used and 150 mL of the previously prepared (Potato Dextrose Broth) PDA culture media was placed into these flasks. After cooling the medium, the glass flasks were inoculated with 0.5 cm diameter culture discs from 7-day- old *Metarhizium anisopliae* culture and grown on dishes containing PDA and by 1-3 disc / flask for all fungi isolates used in the experiment. To divide the hypha and separate the spores, the glass flasks were shaken every three days while being incubated at a temperature of 2±25°C for a total of 28 days. The fungus cultures were filtered when the incubation period was over using a filtration glass funnel and Whatman Filter Paper Grade No 1; the filtrate was then stored in glass flasks until use [5].

Preparation of different concentrations of domestic-commercial *M. anisopliae* filtrates

Different concentrations of the produced infiltrate were prepared, namely (50, 75, 100) % for each fungal isolate through taking a portion of the fungal filtrate in line with the required concentration and dissolving it into sterile distilled water. Using a 100 mL mini hand sprayer, the treatment dishes were sprayed with fungal filtrate at the required concentration for each treatment. Unlike the treatment dishes, the control dishes were sprayed with distilled water only, and all the treatments were incubated at a temperature of approximately 30ºC for 10 days.

Isolation of different larval instars of *T. castaneum*

To isolate different larval instars of *T. castaneum*, 100 pairs of adults were collected and placed in 10 cm long and 5 cm wide plastic dishes, each one contains 2 grams of sterilized flour, by 10 pairs of adults in each dish . The plastic dishes were incubated at a temperature of 30 ± 2 ºC and a relative humidity 50 ±5 % for three days for laying eggs, after that the adults were taken out and the plastic dishes containing the eggs were left in the incubator for five days for hatching. The flour containing a group of first larval instar in each replicate was placed on a relatively large white piece

of paper with a bright light (W100) applying on it, getting difficult to see them easily with the naked eye. After that, a number of them were moved using a hairbrush, by 10 larvae per replicate, to new plastic dishes having lids with small holes for ventilation , making 10 replicates. They were allowed to develop to the next larval instar based on the time required for each instar as follows : (second larval instar after 3 days from first larval instar , third larval instar after 6 days, fourth larval instar after 9 days, fifth larval instar after 12 days and the sixth larval instar after 15 days).

The effect of the spore suspension and fungal filtrate of *M. anisopliae* on adult productivity

Females and males were isolated from each other in pupal instar; the pupae were placed in petri dishes with food for five days until they emerge out of their skins, and then left another five days until they grew adult. After that, each 4 pairs (male and female) were placed in a 9 cm diameter petri dish containing filter paper. Furthermore, 1 mL of each concentration, 75% of the fungal filtrate and 1 x 10^8 conidia /mL of the spore suspension were sprayed, per isolate, using a mini hand sprayer, by four replicates per concentration. As for the control treatment, the adults were sprayed with sterile distilled water (four replicates as well). The adults of the treatment were left at laboratory temperature for 30 minutes to dry up. Then, they were transferred using hairbrushes to (3 x 3 x 3) cm3 plastic dishes, each containing 2 grams of sterilized flour, by a pair per plastic dish. The perforated plastic dishes were tightly closed to prevent the adults to escape, and they were incubated at a temperature of 30 ± 2 °C, for 5 days. After that, they were taken out of the dishes and then, the plastic dishes were left in the incubator under the same conditions referred to above for an additional 5 days, after which the number of hatched first-instar larvae was calculated in each replicate, since distinguishing eggs was not possible .

Designing experiments and statistical analysis

The laboratory experiments were conducted using completely randomized design (C.R.D) and with a single factor, the means were compared using the least significant difference (LSD), and the probability level less than 0.05. The mortality percent were corrected based upon Abbott formula [6]. The corrected percent morality is calculated as follows:

$$\text{Corrected percent mortality} = \frac{\%\ \text{test mortality} - \%\ \text{control mortality}}{100 - \%\ \text{control mortality}} \times 100$$

The mortality rates were converted into mortality percent, and the corrected morality percentage is converted into angular values to be included in the statistical analysis [7].

Results and Discussion

The effect of different concentrations of *M. anisopliae* spore suspension on mortality rates of *T. castaneum* different instars

Table (1) indicates the effect of the interference of different concentrations of spore suspension of commercial *M. anisopliae* fungus as well as the time duration in the corrected percentages of mortality of *T. castaneum* third and fifth larval instars and adults. The results showed that the highest effect rate of varied concentration factor of the spore suspension was at a concentration of 1×10^8 conidia / mL after 10 days of treatment. The corrected mortality rate for adults and the fifth and third larval instars was (40, 60, and 70) respectively.

The lowest mortality rate was at a concentration of 1×10^4 conidia / ml in all the larval instars of this insect, reaching (30, 40, and 57.5) of the adults and the fifth and third larval instars, respectively, after 10 days of treatment. With respect to the effect of time duration, it was significant after treatment with the spore suspension, as the greatest corrected mortality rate was on the tenth day in adults and the fifth and third larval instars in all the concentrations. The lowest mortality rate was on the first and third day, as the effect of the spore suspension started on the third day of treatment for both the fifth and the third larval instars, and the corrected mortality rate of the concentrations was 1×10^8 spore/ml (2.5, 10), respectively.

Table (1): The corrected mortality percentages of different larval instars treated with different concentrations of commercial fungus spore suspension (Met 52 EC) based on *M.anisopliae* strain F52

Developmental stage	Conidial concentration conidia ml^{-1}	Corrected mortality (%)				
		1 day	3 days	5 days	7 days	10 days
Adult	1×10^4	0.0	0.0	5.0	22.5	30.0
	1×10^6	0.0	0.0	15.0	27.5	37.5
	1×10^8	0.0	0.0	27.5	35.0	45.0
Fifth instar larvae	1×10^4	0.0	0.0	2.5	10.0	40.0
	1×10^6	0.0	0.0	5.0	20	47.5
	1×10^8	0.0	2.5	12.5	27.5	60.0
Third instar larvae	1×10^4	0.0	0.0	10.0	30.0	57.5
	1×10^6	0.0	0.0	20.0	40.0	60.0
	1×10^8	0.0	10.0	32.5	50.0	70.0

LSD for developmental stage = 0.23; LSD for Conidial concentration = 0.27; LSD for days = 0.25

Table (2) indicates the effect of the interference of different concentrations of spore suspension of the domestic *M. anisopliae* fungus as well as the time duration in the corrected mortality percentages of *T. castaneum* third and fifth larval instars and adults. The results showed that the highest effect rate of varied concentration factor of the spore suspension was at a concentration of 1×10^8 conidia / ml after 10 days of treatment. The corrected mortality rate for adults and the fifth and third larval instars was (40, 50, and 67.5) respectively. The lowest mortality rate was at a concentration of $10^4\times1$ conidia / ml in all larval instars of this insect, reaching (30, 40, and 55) in adults and the fifth and third larval instars, respectively, after 10 days of treatment. With

respect to the effect of time duration, it was great after treatment with the spore suspension, as the greatest corrected mortality rate was on the tenth day in adults and the fifth and third larval instars in all the concentrations. Furthermore, the lowest mortality rate was on the first and third day, as the effect of the spore suspension started on the third day of treatment for both the fifth and the third larval instars, and the corrected mortality rate of the concentrations was 1×10^8 conidia /ml (2.5, 5), respectively.

The results showed that highest mortality rate is achieved in the third larval instars in all the concentrations, followed by the fifth larval instar and then the adult instar. More importantly, the third larval instar has the highest mortality rate due to being more defenseless and sensitive due to its thin chitin cell wall, which is easily penetrated by pathogenic fungal enzymes, followed by the damage of its internal organs and death.

What [8] found that the modern stages of the rusty red flour beetle *Tribolium castaneum* had the highest morality rate reaching 97% at the concentration of 5×10^8 conidia / ml. The results also in line with [9] when studying the effect of the *Lecanicillium lecanii* fungus on larvae of *oryzaephilus surinamensis* (L.) (saw -toothed grain beetle). The results showed that morality rates in the early larval instar were high reaching 60% at a concentration of 1×10^3 conidia / ml. This is attributable to the fact that it is more sensitive since the chitin wall has fewer tanning substances than the developed instars, which makes it easier for pathogenic fungal enzymes to penetrate, leading to the damage of its internal organs and inevitable death.

The morality rates increase as the concentration of the fungal suspension increases. These findings are in line with [10], who found that *Trogoderma granarium* (Everts) morality rates increased as the concentration of the fungal suspension of *Beauveria bassiana* fungus increased.

Table (2): **The corrected mortality percentage of *T. castaneum's* different larval instars treated with different concentrations of the domestic spore suspension of *M. anisopliae.***

Developmental stage	Conidial concentration conidia ml^{-1}	Corrected mortality (%)				
		1 day	3 days	5 days	7 days	10 days
Adult	1×10^4	0.0	0.0	2.5	17.5	30.0
	1×10^6	0.0	0.0	2.5	25.0	37.5
	1×10^8	0.0	0.0	5.0	30.0	40.0
Fifth instar larvae	1×10^4	0.0	0.0	5.0	12.5	40.0
	1×10^6	0.0	0.0	5.0	20.0	45.0
	1×10^8	0.0	2.5	12.5	25.0	50.0
Third instar larvae	1×10^4	0.0	0.0	10.0	22.5	55.0
	1×10^6	0.0	2.5	15.0	40.0	60.0
	1×10^8	0.0	5.0	25.0	45.0	67.5

LSD for developmental stage = 0.22; LSD for Conidial concentration = 0.25; LSD for days = 0.19

The effect of different concentrations of *M. anisopliae* filtrate on mortality percentages of *T. castaneum'* different larval instars

The results presented in Table (3) illustrated the effect of the commercial fungus filtrate via direct spraying, as well as the time duration, on the corrected mortality percentage of adults and the fifth and third larval instars of *T. Castaneum* insect. The highest cumulative mortality rate was at the concentration of 100% for adults and the fifth and third larval instars, reaching (37.5, 47.5, 55), respectively, after 10 days of treatment. The lowest cumulative mortality rate was for adults and the fifth and third larval instars at the concentration of 50% after 10 days of treatment, the corrected mortality percentage was (17.5, 20,22.5), respectively. With respect to time effect factor, the time duration of 10 days for adults and the fifth and third larval instars had higher significant difference than the rest of the time duration 1, 3, 5, 7 in all the concentrations. Whereas the lowest mortality rate was on the third day of treatment for the adults, the fifth and the third larval instars, Therefore , the corrected mortality percentage of concentration of 100% was (5, 5, 5), respectively. It is concluded from that that the time duration had an important effect on biological control, as there is a direct relationship with the morality percentages.

Table (3): The corrected mortality percentage of *T. castaneum* different larval instars treated with different concentrations of the commercial fungus filtrate (Met 52 EC) based on *M. anisopliae* strain F52

Developmental stage	EPE filtrate concentration	Corrected mortality (%)				
		1 day	3 days	5 days	7 days	10 days
Adult	50%	0	0.0	2.5	12.5	17.5
	75%	0	0.0	12.5	22.5	27.5
	100%	0	5.0	17.5	27.5	37.5
Fifth instar larvae	50%	0	5.0	7.5	17.5	20.0
	75%	0	12.5	12.5	20.0	32.5
	100%	0	5.0	15.0	22.5	47.5
Third instar larvae	50%	0	2.5	7.5	12.5	22.5
	75%	0	2.5	15.0	27.5	37.5
	100%	0	5.0	25.0	32.5	55.0

LSD for developmental stage = 0.26; LSD for Filtrate concentration = 0.30; LSD for days = 0.21

The results presented in Table (4) illustrated the effect of the domestic fungus filtrate as well as the time duration, on the corrected mortality percentage of adults and the fifth and third larval instars of *T. castaneum* insect via direct spraying. The 100% concentration has higher significant difference than the two concentrations, 50, 70 % in the mortality rate for larval instars. The highest cumulative mortality percentage was at the concentration of 100% for adults and the fifth and third larval instars, reaching (45, 40, 37.5), respectively, after 10 days of treatment. The lowest cumulative mortality rate was for adults and the fifth and third larval instars at the concentration of 50% after 10 days of treatment, the corrected mortality percentage was (21, 22,25.5), respectively. With respect to time effect factor, the time duration of 10 days of the

adults and the fifth and third larval instars had higher significant difference than the rest of the time duration 1, 3, 5, 7 in all the concentrations. Whereas, the lowest mortality rate was on the third day of treatment for the third larval instars. Therefore, the corrected mortality percentage of concentration 100% was (10), respectively. It is concluded from that that the time duration had an important effect in biological control, as there is a direct relationship with the morality percentages.

Table (4): **The corrected mortality percentage of *T. castaneum's* different larval instars treated with different concentrations of the domestic fungus filtrate , *M. anisopliae***

Developmental stage	EPE filtrate concentration	Corrected mortality (%)				
		1 day	3 days	5 days	7 days	10 days
Adult	**50%**	**0**	**0.0**	**2.5**	**20.0**	**21.0**
	75%	**0**	**0.0**	**7.5**	**20.0**	**32.5**
	100%	**0**	**0.0**	**12.5**	**25.0**	**37.5**
Fifth instar larvae	**50%**	**0**	**0.0**	**5.0**	**17.5**	**22.0**
	75%	**0**	**0.0**	**12.5**	**17.5**	**35.0**
	100%	**0**	**0.0**	**12.5**	**25.0**	**40.0**
Third instar larvae	**50%**	**0**	**0.0**	**7.5**	**12.5**	**25.5**
	75%	**0**	**0.0**	**10.0**	**10.0**	**37.5**
	100%	**0**	**10.0**	**15.0**	**25.0**	**45.0**

LSD for developmental stage = 0.24; LSD for Filtrate concentration = 0.28; LSD for days = 0.21

The effect of the spore suspension and fungal filtrate of *M. anisopliae* on the adult productivity of *T. castaneum*

Table (5) results showed the significant effects of spore suspension and fungus infiltrate on the egg numbers of *T. castaneum* females. The egg number decreased significantly to its lowest levels reaching (17.3 a and 23.2 a) eggs per female treated at a concentration of 1×10^6 conidia /ml for the commercial and the domestic isolates, respectively. Whereas the egg numbers of *T. castaneum* females treated with fungal filtrate was (24.8 b, 27 b) eggs per female treated with a concentration of 75% for the commercial and domestic isolates, respectively, compared with (43.5 c) egg / female) in the control treatment. The low number of eggs laid by adults is due to the consumption of nutrients by the fungus inside the insect's body. This result was observed when the *M. anisopliae* fungus infected pupae of Rhynchophorus ferrugineus insect [11]. These results are also consistent with [12] who indicated that the use of the spore suspension of the pathogenic fungus, Clonostachys rosea against three types of warehouse insects *Trogoderma granarium*, *T. castaneum* and *Collosbruchus. maculatus* demonstrated high effectiveness in reducing their total fertility rate of the three treated insect pests.

Table (5): The mean of eggs laid per *T. castaneum* female treated with spore suspension and fungal filtrate of the domestic and commercial isolates of *M. anisopliae* with control treatment.

Treatment	Total fecundity mean No. of eggs per female (±SE)
M. anisoplae **(Met 52 EC)**	**17.3 a**
M. anisoplae **(Local isolate)**	**23.2 a**
Fungal filtrate (Met 52 EC)	**24.8 b**
Fungal filtrate (Local isolate)	**27 b**
Control	**43.5 c**

Means within a column followed by different lowercase letters indicate significant differences among treatments at each insect species at *P* = 0 .05 using LSD test. The lowercase letters in the Total Fertility column indicate the treatment types that T. castaneum adults treated by , using (P=0.05) LSD

Under laboratory conditions, the *M. anisopliae* fungus and its domestic and commercial isolates were effective against *T. castaneum*. Additionally, a decrease in the fertility of the treated insect was also observed. However, additional researches are required to demonstrate the *M. anisopliae's* effectiveness under commercial storage conditions. Additionally, in conjunction with various other control methods, the fungus's potential effects should be evaluated.

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