Effect of *Aspergillus flavus* filtrate on different stages of *Callosobruchus maculatus* (Fabricius.) (Chrysomelidae: Coleoptera) on Broad beans

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Abstract:

An experiment was conducted to use *Aspergillus flavus* filtrate in treating different stages of *Callosobruchus maculatus* (eggs, larvae, pupae) after diluting it with sterile distilled water at concentrations of 30%, 50 and %, 70% directly to calculate the mortality rates after (1, 3, 5) days After treatment, the best results of egg inhibition were in the 5th day, with rates reaching 36.9, 53.7 and 83.87%; respectively, and the best results of larvae mortality were in the 3rd, 5th, and 5th day; respectively, with rates reaching 50.33, 80.47and 93.93%, respectively. While the best results of pupae mortality were on the 5th, 5th, and 5th day; respectively, with rates that reached 63.50, 86.93and 96.97%; respectively, and the best results of mortality for adults were on the 3rd, 5th, and5th days; respectively, with rates that reached 43.73, 90.83, and 93.93% respectively. The best results of mortality were in the 1th, 3th, and 5th day respectively on adults of *C. maculatus* via the indirect method, as the highest mortality rates were at the concentration of 70% and reached 56.83, 60.36 and 67.1%, respectively. The best results were on the amount of laying eggs from an adult *C. maculatus* and by direct and indirect spraying on females. The results showed that the lowest rate of laying eggs for one female after three days of mating was zero eggs/female with a concentration of 70% compared to the indirect method which reached to 24.13 eggs/female.

Keywords: *C. maculatus*, *Aspergillus flavus*, filtrate effect, direct spray, indirect spray

Introduction

Broad beans *Vicia faba* L. are exposed to many pests, the most common of which is a group belonging to the family of legume beetles Chrysomelidae of the order Coleoptera, due to their ability to reproduce on seeds in the field and on dry seeds inside stores (11). The most important of these is *Callosobruchus maculatus* Fab, which causes damage to seed pods and annual weight losses ranging from 30-50% or 62% in quantity and quality, and sometimes more than 90% (2 and 9), and infection with this beetle leads to secondary infections in several types of aerobic bacteria such as *Staphylococcus aureus*, *S. epidermidis*, *Pseudomonas aeruginosa* and fungi such as *Aspergillus*, *Rhizopus* and *Allternaria*, and some research has shown that *Aspergillus* spp. has the ability to produce aflatoxin, which affects the general health of the consumer, especially the respiratory system (18. 4 and 20), aflatoxins are produced from the fungi *Aspergillus flavus*, *A. parasitica* and *A. Nominius* (8 and 5). Studies have shown that the use of these toxins at a concentration of 1000ppm led to mortality rate that aflatoxin at aflatoxins when present in DNA works to cause an error in the reproduction process when presented to insects orally (5). Sahayarai *et al.* (16) recorded the fungal pathogen that infects the predator, *Rhynocoris marginatus*, where the fungal tissues from the predator were cultured and microscopic examination revealed the presence of the pathogenic fungus. *Aspergillus flavus* 1 x 10^6 spores/ml were prepared from PDA slant media and sprayed on the healthy adult predator, and mortality rates were recorded, if found, for seven days. It was noted that 20% were killed, and Five days later, it became a mummy, this fungus can colonize and kill insects and produce spores from their decomposing body, and the pathogenicity test showed that an average of 96.6% of the mortality was recorded in the treated adult predatory insects, and the effect of aflatoxins was studied on the insects *Aedes aegypti*, *Musca domestica*, *Drosophila melanogaster* and in all species caused aflatoxin to reduce the production and hatching rates of eggs (15), Al-Radi (6) explain that the reason for the recurrence of *Aspergillus* fungi in the fungal isolation of species that appear on insects and in the forefront of other species is due to its being a widespread fungus. The genus *Aspergillus* appeared in all tested samples 95.43%, and the genus *Aspergillus* was the most diverse, as eight species were isolated compared to the rest of the genera, which had the lowest incidence and least diversity, (14) explained from a study of the effect of *A. flavus* fungal extract on the antioxidant and immune cellular defense of *Spodoptera lituralis*, where important information regarding stress was provided. The oxidative stress that causes the tolerant and immunosuppressive nature of *A. flavus* against *S. litura* and its non-toxicity to mammals (rats), it is environmentally friendly for pest control, where the toxicity of concentrations of Ethyl acetate extract (125, 250, 500, 1000, 2000) µg/ml extracted from *A. flavus* mixed with the artificial food provided to the insect *Spodoptera littoralis*. The mortality rates were proportional to the concentration of the extract and the concentration was 1340.84 µg/ml (LC50), i.e. the lethal concentration of the fungus at intervals of (24, 48, 72, 96) hours, and the negative effect appeared on blood cells that have a major role in the cellular immune defense of the insect as different abnormalities were observed in different

blood cells, and the toxicity was evaluated on mice and did not significantly affect all tissues.

Materials and Methods:

Insect breeding:

Southern cowpea beetle collected from grain and fodder stores in Najaf governorate, from infected Broad beans *Vicia faba* seeds in September of the year 2020, and the insects were reared after being diagnosed by the Natural Museum / University of Baghdad on Broad beans, and were sterilized by keeping them at a temperature of -20C^0 for a 20 days to get rid of any infestation of warehouse pests as a preventive procedure (1) before the biological tests, and the seeds were placed in 1 liter plastic containers at a rate of 250 g per container and 10 individuals for each male and female were placed in a plastic container for mating, after laying the eggs, The adults were removed to ensure obtaining uniform stages, and the plastic containers were covered with a Muslin and tied with a rubber band, and then transferred to an incubator at a temperature of 28 ± 2C^0 and a relative humidity of 60 ± 10% (10), and the stages (internal infection) were treated on the basis of the age period of the egg stage after laying (in days), where the stage of eggs is represented by the period (1-2) days after laying eggs on the broad bean seeds, the fourth stage larvae after 14 days and the pupal stage after 21 days of egg laying after making sure that the incubation room is configured under seed coat and the age stages were verified by dissecting the seeds with an interval of 3 days (3 and 7).

Isolation of the fungus *Aspergillus flavus*:

The fungus *A. flavus* was isolated from dead pupa and adults, where the fungus grew on it inside the seeds. These pupa and adults were collected and sterilized with 1% sodium hypochlorite for two minutes, then washed with sterile distilled water several times and placed on filter paper to get rid of the free water. These stages were transferred by sterile forceps to Petri dishes containing PDA media, and were placed in the incubator at 25 ± 2C^0 for 5 days. The developing fungus was isolated and purified and the fungus was identified by Dr. Hurria Abd Alhussain\ College of Agriculture Agricultural Engineering Sciences\ University of Baghdad. The pathogenicity was tested on the stages of the southern cowpea beetle, and the fungus was preserved on Potato dextrose broth (PDB) in test tubes in the refrigerator at a temperature of 4°C for subsequent experiments.

Preparation of *Aspergillus flavus* filtrate:

The filtrate of *A. flavus* was prepared by inoculating the bottles containing potato dextrose broth (PDB) with a 0.5 cm drop disc for each vial with a disc of *A. flavus* growing on PDA media at the age of 5 days. The inoculated bottles were incubated with the fungus for 28 days at a temperature of 25±2C^0 taking care to shake the bottle every 3-4 days. The extract was filtered using filter paper and the process was repeated twice to get rid of impurities then passed the filtrate through the Millipore filter with openings of 0.22 microns to obtain a spore-free fungus filter and to sterilize the secondary metabolites of potentially contaminating bacteria (19).

Use of *Aspergillus flavus* filtrate concentrations**:**

The filtrate was used in the treatment of the different stages of the southern cowpea beetle after diluting it with sterile distilled water, where a concentration of 30, 50, and 70% was chosen to treat the different stages of the insect in three replications, where ten individuals (eggs, larva, pupa) were chosen to spray them directly to calculate the mortality rates. After (1, 3, 5) days after treatment, the adult stage (5 females and 5 males) was also sprayed using the direct spraying method to calculate the mortality rates and its effect on the egg-laying rate for each female, hatching rates, and the resulting egg inhibition rates for each iteration three days after mating.

The healthy seeds were also sprayed with the above concentrations, and 10 individuals (5 males and 5 females) were introduced to the insect after drying the seeds to calculate the mortality rate for adults, the egg-laying rate for each female, the hatching rate and the inhibition of egg hatching resulting for each repetition after three days of The statistical program Statistical Analysis System -SAS (17) was used in data analysis to study the effect of different factors on the studied traits according to a completely randomized design (CRD), and the significant differences between the means were compared with the Least Significant Difference-LSD test.

The death percentages were corrected according to the equation (Abbott, 1925).

Results and Discussion

Effect of *Aspergillus flavus* on inhibiting *C. maculatus* F. eggs.

The results of Table (1) showed the effect of *Aspergillus flavus* infiltrate concentrations 30, 50 and 70% respectively, and the time period (1, 3, and 5) days; respectively, in inhibiting the hatching of eggs of the southern cowpea beetle. The results showed that the highest inhibition rates for eggs, it was at the concentration of 70% and it reached 50.0, 70.23, and 83.87% respectively, and the lowest inhibition rates at the concentration were 30%, and it reached 10.0, 36.73, and 36.9%, respectively. The results also showed the effect of the time period on the rates of mortality, which was the highest results at the time period of 5 days, amounted to 36.9, 53.7, and 83.87%; respectively, and the lowest results were at the time period of 1 day, and it amounted to 10.0, 26.67, and 50%, respectively. The statistical analysis of the results of the table indicated that the differences in the different concentrations of the filter were also significant. The time periods 1 and 3 days, the analysis also indicated a significant overlap between the treatments.

Table 1: Effect of *A. flavus* infiltrate on inhibiting *C.maculatus* egg hatching

Concentration%	Time/ day 1	3	5	Mean
	Hatching inhibition ratios%			
30	10.0	36.73	36.9	27.88
50	26.67	50.17	53.7	43.5
70	50.0	70.23	83.87	68.03

Mean	28.89	52.37	58.17	---

LSD values 5%: Concentration: 6.985 *, Time: 6.985 * Interference: 11.268 *.
*Based on LSD test at 5% probability level

Effect of fungal filtrate of *Aspergillus flavus* on *C. maculatus* F. larvae**.**

Table (2) shows the effect of *A. flavus* filtrate concentrations of 30, 50, 70% and the time period (1, 3, 5) days on *C. maculatus* larvae mortality, where the results showed that the highest rates of larval mortality were at concentration 70% It reached 83.87, 93.93, 93.93%, respectively, and the lowest rates of mortality at the concentration were 30%, and it amounted to 36.86, 46.96, 50.33%; respectively. It reached 50.33, 80.47, 93.93and %; respectively, and the lowest rates of mortality at the time period were 1 day, and it was 36.86, 50.3, 83.87and %, respectively. The statistical analysis indicated a significant overlap between all treatments.

Table 2: Effect of *A.flavus* filtrate on *C.maculatus* larvae mortality.

Concentration%	Time/ day 1	3	5	Mean
		mortality ratios%		
30	36.86	46.96	50.33	44.73
50	50.3	70.43	80.47	67.1
70	83.87	93.93	93.93	90.57
Mean	57.0	70.43	74.9	-----

LSD values 5%: Concentration: 6.265 *, Time: 6.265 *, Interference: 10.946 *
*Based on LSD test at 5% probability level.

Effect of *Aspergillus flavus* filtrate on *C.maculatus* F. pupae mortality**.**

Table (3) shows the effect of *A.flavus* fungal filtrate concentrations of 30, 50, 70%; respectively, and the time period (1, 3, 5) days on *C. maculatus* pupae mortality, where the table showed that the highest mortality rates of pupae It was at the concentration of 70% and it reached 76.67, 90.3, 96.97%; respectively, and the lowest rates of mortality at the concentration were 30%, and it amounted to 43.33, 46.8, 63.5%, respectively. The time period was 5 days, and it amounted to 63.5, 86.93, 96.97%; respectively, and the lowest rates of mortality were at the time period of 1 day, which amounted to 43.33, 53.33, 76.67%, respectively. The statistical analysis showed the significant differences between the treatments for the concentration of the fungal filtrate as well as for the exposure periods, as indicated Statistical analysis indicates the significant interaction differences between the three filtrate concentration coefficients and the three exposure periods in days.

Table 3: Effect of *A.flavus* filtrate on *C.maculatus* pupae mortality

Concentration%	Time/ day 1	3	5	Mean
		mortality ratios%		
30	43.33	46.8	63.5	51.2

50	53.33	66.87	86.93	69.03
70	76.67	90.3	96.97	87.97
Mean	57.77	68	82.47	---

LSD 5% values: Concentration: 7.505 *, Time: 7.505 * Interference: 12.869 *.
*Based on LSD test at 5% probability level

Effect of *Aspergillus flavus* filtrate on *C. maculatus* F. adult's mortality by direct spraying method

Table (4) shows the effect of the concentrations of the fungal filtrate of *A. flavus* at 30, 50, 70%; respect and very, and for the time period (1, 3, 5) days, respectively, on adults of *C. maculatus* by the direct spraying method on the insect. The highest mortality rates for adults were at a concentration of 70%, which of amounted to 86.9, 93.93, 90.83%; and respectively, and the lowest rates of kill were at a concentration 30%, reaching 23.4, 43.63, and 43.73%, respectively. The results also showed the effect of the time period on the rates of mortality, where the highest rates of mortality were at the time period of 5 days and amounted to 43.73, 90.83, and 90.83%; respectively, and the lowest percentages of mortality were at the time period of 1 day, amounting to 23.4, 56.87, 86.9%, respectively. The statistical analysis of the results of the table indicated significant differences between the treatments for different fungal filtrate concentrations and significant differences for the time period (1 and 3) days. The statistical analysis also indicated a significant interaction between the concentration of 30 and the time period of 3 days.

Table 4: Effect of *A.flavus* filtrate on *C.maculatus* adults mortality by direct method

Concentration%	Time/ day 1	3	5	Mean
		mortality ratios%		
30	23.4	43.63	43.73	36.92
50	56.87	83.9	90.83	77.2
70	86.9	93.93	90.83	90.55
Mean	55.73	73.83	75.13	---

LSD5% values: Concentration: 7.913 *, Time: 7.913 * Interference: 13.562 *.
*Based on LSD test at 5% probability level.

Effect of *A. flavus* filtrate on adult *C. maculatus* mortality by indirect spraying method**.**

Table (5) shows the effect of *A. flavus* fungal filtrate concentrations amounting to 30, 50, 70% and the time period (1, 3, 5) days, respectively, on adults of *C. maculatus* by indirect method, as the highest mortality rates were at The concentration was 70% and it reached 56.83, 60.36, 67.1%; respectively, and the lowest rates of mortality were at the concentration 30% and it reached 13.36, 13.4, 23.5%, respectively. The time period was 5 days, and it amounted to 23.5, 43.6, and 67.1%, respectively. The statistical analysis of Table (5) indicated the

significant statistical differences of the rates of the three concentrations used, as well as the significant differences in the treatments of the exposure time period of (1 and 5) days, as the statistical analysis indicated to the significant Overlap between exposure parameters and exposure time.

Table 5: Effect of *A. flavus* filtrate on the indirect mortality of adult *C. maculatus*

Concentration%	Time/ day 1	3	5	Mean
		mortality ratios%		
30	13.36	13.4	23.5	16.75
50	30.06	36.9	43.6	36.85
70	56.83	60.36	67.1	61.43
Mean	33.43	36.9	44.73	---
LSD 5% values: Concentration: 6.239 *, Time: 6.239 * Interference: 11.185 *.				
*Based on LSD test at 5% probability level.				

Effect of direct and indirect methods of *A. flavus* filtrate on the quantity of *C. maculatus* eggs.

The results of Table (6) showed the effect of *A. flavus* filtrate by the treatment method and with concentrations 30, 50, 70%,;respectively, on the laying of eggs from an adult *C. maculatus* and by the direct and indirect spraying method on the insect, where the results showed that the lowest rate of laying The eggs for one female after three days of mating were (0 eggs/female) at the concentration of 70% compared to the indirect method, which had results of (24.13 eggs/female) at the same concentration and the highest rate of laying eggs was (24.266 eggs/female) at the 30% concentration. Compared to the indirect method (31 eggs / female), the highest percentage of hatching inhibition was 80% at a concentration of 70% compared to the indirect method, which reached 28.34% and at the same concentration,

Table 6: Effect of direct and indirect treatment of *A. flavus* on the quantity of *C. maculatus* eggs.

Method	Concentration %	Number of eggs/female	Hatchability	Inhibition ratio
direct method	30	24.266	69.23	10.77
	50	2.6	20.51	59.49
	70	0	0	80
Mean		8.955	29.91	50.085
LSD		*4.62	*7.55	*11.47
indirect method	30	31	76.99	3.01
	50	28.2	65.96	14.04
	70	24.13	51.66	28.34
Mean		27.78	64.87	15.39
LSD		*4.39	*17.52	*5.84
*Based on LSD test at 5% probability level.				

and the lowest percentage of hatching inhibition reached 10.77% at a concentration of 30% compared to the indirect method, which reached 3.01% for the same concentration. From these results, it is clear that the rate of laying eggs is inversely proportional to the increase in the concentration of the fungal filtrate and that the percentage of inhibition is directly proportional to the increase in the concentration of the fungal filtrate, and the statistical analysis of the table indicated significant differences Statistical methods, both direct and indirect Concentrations, number of eggs per female, hatching rates and inhibition rates.

Discussions

Direct effect of *Aspergillus flavus* filtrate

The results of Tables 1, 2, 3, 4, 5 should wed that the direct effect of *Aspergillus flavus* filtrate on the different stages of the southern cowpea beetle *C. maculatus* was ranging from the most sensitive to the least sensitive as follows (pupae, adults, larvae, eggs). Pupa is the most sensitive and eggs are the most resistant and according to results, the results also showed that the results of the direct spraying treatment are better than the indirect spraying method for the adult stage, as it was proven (12) that the fungus *A. flavus* disrupts the antioxidant enzyme system of the host, which plays an important role in the elimination of oxidative toxins produced during infection with fungi. The antioxidant enzymes such as catalase, peroxidase and, phenoloxidase decreased, and infection was achieved within 48 hours. The effect of infection with the entomopathogenic fungus *Aspergillus flavus* on the antioxidant defense system was studied (13). In *Spodoptera liturliteralisme* exposure to *A. flavus* modified the levels of antioxidant enzymes, in addition to a significant decrease in the level of phenoloxidase and total blood cell count within 48 hours after exposure. a significant increase in detoxification enzymes was observed. All of these results indicate that *A. flavus* infects *S. lituralis* through a direct effect on the immune system, which leads to a decrease in immune function. The results of the bioassay showed that *A. flavus* affects the third and fourth instar larvae of *S. lituralis*, the results indicate the bioassay further indicates that *A. flavus* can kill *S. lituralis* larvae within 48 hours of exposure.

Conflict of Interest

The authors have no conflict of interest.

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