Research Article

Evaluating the Efficacy of the Fungus *Beauveria bassiana* and Indigenous Nematodes *Heterorhabditis bacteriophora* in Controlling Cucurbit Fly *Dacus ciliatus*

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Abstract | The cucurbit fly, a pest responsible for significant economic losses in cucurbit crops, lays its eggs in the fruits and feeds on them. Consequently, extensive studies and experiments have been undertaken to identify optimal methods for its control. Due to the adverse effects of chemical pesticides on consumer health, particularly human health, the pursuit of alternative safe measures has been paramount. Among the prominent alternatives, biological control has garnered attention, wherein environmentally safe organisms such as nematodes and fungi are employed. This study examines their efficacy in controlling the insect larvae and pupae. The integration of these indigenous biological control agents yielded notable mortality rates for the targeted larvae and pupae. The highest rates were achieved when using a higher concentration of fungi, specifically 20×10^6 spore/mL, in combination with 100 infective juveniles/mL of nematodes (resulting in an 83.33% mortality rate for larvae and 80% mortality rate for pupae). Remarkably, the mortality rate of the treated larvae surpassed that of the treated pupae at identical concentrations of fungi and nematodes, implying that larvae exhibit greater sensitivity and responsiveness to biological control measures compared to pupae, which benefit from partial protection due to their cocoon enclosure, mitigating the impact of external influences.

Keywords | Fungus, Biocontrol, *Dacus ciliatus*, *Heterorhabditis bacteriophora*, *Beauveria bassiana*.

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INTRODUCTION

Cucurbitaceae family crops, which include various vegetable crops, are economically significant crops worldwide (Kadio EAAB1 et al., 2011). The agricultural pest *Dacus ciliatus* Loew is among the significant pests affecting a wide range of crops belonging to the Cucurbitaceae family in Africa, Asia, and the Middle East (Hancock et al., 2010). Additionally, this insect is considered a critical and perilous pest in the Kingdom of Saudi Arabia (Fischer-Colbrie and Peteersen, 1989). In Iraq, it was first recorded in 1988 and was considered one of the most dangerous pests belonging to the order Diptera and the family Tephritidae. It causes significant economic losses to numerous cucurbit plant (Moanas and Abdul–Rassoul. M. S., 1989). Furthermore, (Mahmoudvand et al., 2011) indicated that the insecticides deltamethrin, dimethoate, trichlorfon, and malathion are effective insecticides against this pest. The frequent use of such pesticides causes significant side effects on humans, animals, and beneficial insects. They also lead to water and air pollution and are considered carcinogenic substances. Additionally, they stimulate the emergence of resistant strains. According to reports from the World Health Organization, over 1,000,000 individuals have experienced acute poisoning due to exposure to such pesticides, as reported by (Hassaan and El Nemr, 2020). Therefore, most

current studies have focused on utilizing safe alternative methods that are effective against pests while being harmless to humans and animals. One of these methods is the utilization of entomopathogenic fungi (EPFs), which are fungi that cause diseases in insects. Additionally, other biological approaches are being explored to combat such pests, as highlighted by (Quesada-Moraga et al., 2006). The utilization of entomopathogenic fungi as biological control agents is one of the commonly employed and established methods nowadays. This approach is favored due to its cost-effectiveness, non-hazardous nature to humans and the environment, specialized targeting capabilities, and absence of resistance development, as highlighted by (Coral-lo et al., 2021; Lacey and Kaya, 2007; Mascarin and Jaronski, 2016). *Metarhizium anisopliae, Beauveria bassiana,* and *Isaria fumosoroseus* are among the frequently utilized fungi as biological control agents against the pest *Dacus frontalis*. These fungi affect adults, larvae, and pupae, with adults being the most susceptible stage to fungal pathogens, as indicated by (Esam Elghadi and Gordon Port, 2019). Entomopathogenic nematodes, specifically those belonging to the families Heterorhabditidae and Steinernematidae, are effective biological control agents against insects. Additionally, symbiotic bacteria associated with these nematodes play a significant role in enhancing their efficacy against insects. The genus *Xenorhabdus* is associated with Steinernematid nematodes, while the genus *Photorhabdus* is associated with Heterorhabditid nematodes, as mentioned by (Progar et al., 2014; Sirjani et al., 2009; Toubarro et al., 2009).

MATERIALS AND METHODS

This experiment was conducted in the laboratories of the College of Science - Wasit University, Department of Biology, in collaboration with the laboratories of the Ministry of Science and Technology - Department of Agricultural Research - Biological Control Department.

Samples

Four different types of samples were prepared in order to perform the necessary tests and laboratory examinations, and careful steps were taken to ensure their proper preparation. Essential measures included providing an appropriate environment and selecting a suitable nutrient medium. The samples are described as follows:

1-Cucurbit fly larvae, *Dacus ciliatus*.

2-Cucurbit fly pupae, *Dacus ciliatus*.

3-Infective stages of the nematode, *H. bacteriophora*, see Figure 1.

4-The spores of the fungus *Beauveria bassiana* see Figure 2.

The morphological and molecular characterization of the local isolation of *Heterorhabditis bacteriophora* nematodes was conducted by (Al-Zaidawi et al., 2019). While the local isolation of the fungus was morphologically and molecularly characterized by (Diwan H. M. 2003).

Figure 1: *Heterorhabditis bacteriophora* under microscope.

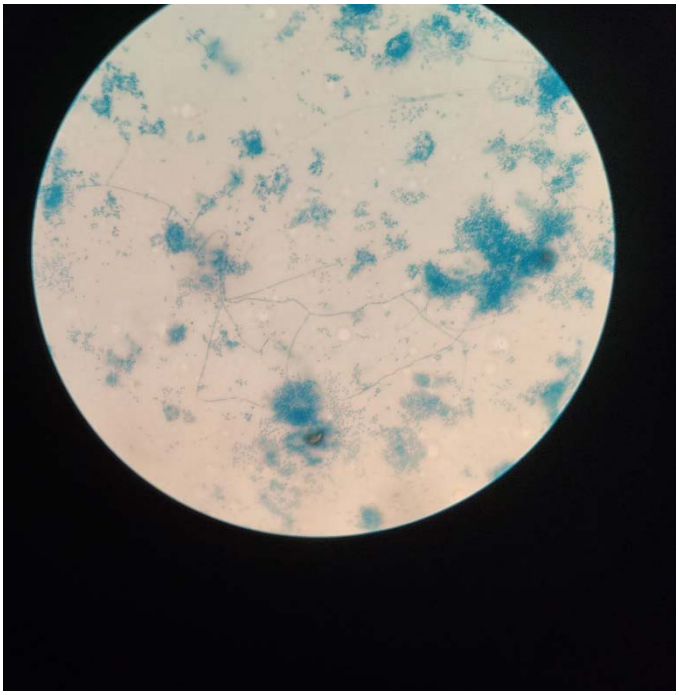

Figure 2: *Beauveria bassiana* under microscope.

Insect source

The cucurbit fruit fly was obtained by collecting the infected plants (cucurbit plants) from farms and local vegetable markets. The infected plants were diagnosed based on the presence of moist yellow lesions on the plant surface.

Growing of the cucurbit fruit fly in the laboratory

The infected plants were housed within plastic containers containing a layer of sterilized soil, 5 cm thick at the bottom of each container, to provide an optimal environment for the transformation of insect larvae into pupae. The pupae were subsequently collected and transferred to containers equipped with a sugar solution mixed with yeast extract as a nourishment medium to support the adult insects upon emergence. The pupal-to-adult metamorphosis process was completed within a span of 5 days.

Following adult emergence, a mating process was initiated after three days. Subsequently, the females began oviposition inside the cucurbit plants three days later.

The reproductive process and egg production took place under favorable environmental conditions, including a constant temperature of 25 degrees Celsius, 60% humidity, appropriate ventilation, and alternating light/dark periods (14 hours light/10 hours dark). These environmental parameters significantly contributed to expediting the reproductive process, with the eggs hatching and larvae emerging in just three days.

Collection of larvae and pupae of the cucurbit fruit fly

The larvae were collected after five days of emergence, at the pre-pupal stage, by cutting the infected plants. Due to their active and rapid movement, the larvae were collected in sealed containers to preserve them until they were exposed to biological control agents (*B. bassiana* and *H. bacteriophora*). This collection process was carried out within a short period, typically not exceeding one hour, to capture the larvae before they transformed into pupae.

As for the pupae, they were collected from the soil at the bottom of the plastic rearing boxes for the cucurbit fruit fly. Similarly, they were placed in containers until they were exposed to the biological control agent.

The cultivation of the fungus and laboratory production of the fungal suspension

A local isolate of the biocontrol agent *Beauveria bassiana* was obtained from the laboratories of the Department of Agricultural Research/Iraq. Subsequently, it was cultivated on Petri dishes containing a culture medium conducive to fungal growth, specifically Potato Dextrose Agar (PDA). The PDA medium was prepared beforehand by dissolving 40 grams of PDA in 1000 milliliters of sterilized distilled water. The medium was then subjected to sterilization using an autoclave at 121 degrees Celsius and 103.4 kilopascals of pressure for 20 minutes. Once prepared, the medium was poured into Petri dishes and allowed to solidify. The dishes were then inoculated with a streak of *B. bassiana* fungal growth at the center and incubated at a controlled temperature of 25 ± 2 degrees Celsius and humidity of 70 ± 2% for 7 to 9 days. Following confirmation of fungal growth, the Petri dishes were refrigerated at 4 degrees Celsius for future use.

After activating the biocontrol agent *B. bassiana* and allowing 14 days to elapse, a fungal broth was prepared. This involved adding 5 milliliters of sterilized distilled water to the dish containing fungal spores. The spores on the dish's surface were gently scraped off using an L-shaped loop, collected, and transferred to a 100-milliliter glass flask. The solution's volume was adjusted to 100 milliliters using sterilized distilled water mixed with 500 microliters of Tween 80. The broth was thoroughly mixed to ensure the separation of spores from fungal hyphae, resulting in the formation of the spore suspension.

A hemocytometer slide was utilized to calculate the spore concentration in 1 milliliter of the spore suspension (Lacey J., 1997) .The following equation was applied:
spore count per milliliter = spore count per square × 2.5 × 10^5
After microscopic counting and the application of the aforementioned equation, it was determined that 1 milliliter of the basic spore suspension contained 20 × 10^6 spores.

Preparation of dilutions for the spore suspension

The initial dilution of the spore suspension was achieved by transferring 50 milliliters of the original spore suspension into a glass flask and subsequently adjusting the volume to 100 milliliters through the addition of 50 milliliters of sterilized distilled water. This dilution is denoted as the first dilution.

Subsequently, the second dilution was prepared by transferring 50 milliliters of the first dilution into another glass flask, and once again, the volume was brought to 100 milliliters using sterilized distilled water.

After preparing the three dilutions, they were employed in the laboratory experiments for the biological control of the cucurbit fruit fly, *Dacus ciliatus*.

Preparation of locally isolated *Heterorhabditis bacteriophora* nematodes

The process of isolating local *H. bacteriophora* nematodes involved several steps. First, the nematodes were multiplied on last instar *Galleria mellonella* larvae in controlled conditions. This included maintaining a temperature range of 25 to 30 degrees Celsius and a relative humidity of up to 90%. The methodology used for this process was described by (Kaya H. K. and Stock S. P., 1997). After one week of multiplication, the infective juveniles (IJs) of the nematodes were collected. This was done by adding 5 mL of sterile distilled water to the Petri dish containing the nematodes and then transferring the mixture to a 20 mL sterile distilled water vial using a micropipette. In total, 25 mL of nematode suspension was obtained. To determine the concentration of infective juveniles in the suspension, a small sample of 100 µL was taken and examined under a microscope. The count in this sample was found to be 92 nematode infective juveniles (IJs) per 100 µL. The re-

searchers used the equation

c1/v1 = c2/v2

to calculate the total number of infective juveniles in the entire 25 mL suspension based on the count from the 100 µL sample. This information about the nematode concentration in the suspension is crucial for preparing the spray solution with a specific desired number of nematode infective juveniles. By accurately measuring the volumes, they were able to dilute the nematode suspension with sterile distilled water to obtain a final volume of 1 mL with 100 infective juveniles (IJs) per milliliter.

Evaluation of the compatibility between the local fungus and the local nematode.

In this research, we sought to assess the compatibility of a mixed biological control approach involving both fungi and local nematodes. The primary objective was to ensure that neither agent negatively affected the activity or effectiveness of the other.

To achieve this, we selected a concentration of 100 infective juveniles per milliliter (IJs/mL) for the local nematodes. These nematode concentrations were then combined with the highest concentrations of the fungus available. The preparation process involved taking 1 milliliter of nematode suspension at a concentration of 100 IJs/mL and 1 milliliter of fungal suspension at a concentration of 20 $\times 10^6$ spores/mL.

Next, we placed this mixture in a Petri dish lined with filter paper, which helped maintain the necessary moisture for the biological control agents. In total, three dishes were prepared with this mixture. These dishes were then carefully placed in an incubator, which maintained a constant temperature of 25±2 degrees Celsius and a humidity level of 90%.

Throughout the experiment, we closely monitored the dishes and recorded any observable impact one biological control agent had on the other. This meticulous observation and data recording allowed us to evaluate the compatibility and potential interactions between the fungus and local nematodes, ensuring the effectiveness of this mixed biological control approach.

Assessment of the compatibility of the fungus with the nematode a biological control agent against cucurbit fly

After determining the compatibility between the two biological control agents and the extent of their impact on each other's activity and effectiveness, the developmental stages of the insect, including larvae and pupae, were exposed to the combined action of the biological control agents to assess their efficacy in eliminating the insect pest when used together. This study involved using the highest concentration of the nematode suspension (100 IJs/ml) and combining it with three different concentrations of the fungal suspension (20x10^6 spore/ml, 10 x 10^6 spore/ml, and 5 x 10^6 spore/ml) as indicated in Table 1. The mixed biological control agents were then placed in Petri dishes lined with filter paper, which were sprayed with a solution containing the fungal and nematode suspensions. Subsequently, 10 larvae or pupae were placed in each dish, with three replicates for each treatment. The dishes were tightly sealed using parafilm and placed in an incubator at a temperature of 25±2 degrees Celsius and a humidity of 90%. The dishes were monitored daily, and the results were recorded after 14 days (Omar M. M. 2000).

Statistical Analysis

The collected data was meticulously recorded into columns and rows using Microsoft Excel 2019 software, ensuring its readiness for subsequent statistical analyses. GenStat version 3 software was employed to conduct the rigorous statistical analysis of all laboratory experiment results. The experiments were diligently executed following the Completely Randomized Design (CRD) approach, providing a robust experimental framework.

To account for variations arising from different concentrations, the kill rate data underwent correction utilizing Abbott's correction method (Abbott, 1925). Moreover, the corrected kill rates underwent thorough statistical scrutiny using the One-Way Analysis of Variance (ANOVA) technique, and mean kill rates were subsequently compared using the Less Significant Difference (LSD) test.

For the assessment of compatibility and interactions among various concentrations of fungi and nematodes, a Multifactor Analysis of Variance (ANOVA) design was employed. The summation of means was compared using the Fisher Less Significant Difference (LSD) test, ensuring a stringent evaluation at a 95% confidence level ($P \leq 0.05$) (GenStat 2008).

RESULTS AND DISCUSSION

The effect of the fungus *B. bassiana* on the nematode *Heterorhabditis bacteriophora*.

After mixing 1 milliliter of the fungal and local nematode cultures and distributing them across three Petri dishes, regular monitoring and examination using a microscope were conducted thrice daily. Observations encompassing fungal growth, nematode movement, and activity were meticulously recorded. By the third day, slight fungal growth became evident in the dishes under microscopic examination, remaining imperceptible to the naked eye. Nematode

Table 1: The concentrations and volumes of nematodes and fungi that were used in the experiments.

Sample type	Conc. of ijs, ijs/ ml	ijs susp. volume, ml	Conc. of Fungi spores' spore/ml	Fungi spores' volume ml
Control	-	-	-	-
Larvae	100	1	20 x x^6	1
Pupae	100	1	20 x 10^6	1
Larvae	100	1	10 x 10^6	1
Pupae	100	1	10 x 10^6	1
Larvae	100	1	5 x 10^6	1
Pupae	100	1	5 x 10^6	1

Table 2: The effect of combining the local fungus with the local nematode on larvae and pupae of the insect.

The symbols	Fungi +Nematode (100 Ijs/mL)	Mortality rate %		Average
		larvae	pupae	
A	Fungi 20×10^6 spore/mL+ Nematode 100 Ijs/ mL	83.33	80	81.66
B	Fungi 10×10^6 spore/mL+ Nematode 100 Ijs/ mL	53.33	43.33	48.33
C	Fungi 5×10^6 spore/mL+ Nematode 100 Ijs/ mL	40	26.66	33.33
D	Control	0	0	0
Average		58.88	49.99	
LSD (0.05)		0.942	0.931	
		2.191		

movement and activity exhibited normal levels during this period.

Subsequently, as the experiment progressed, fungal growth gradually intensified, becoming macroscopically visible on the fifth day. Remarkably, nematode movement and activity continued to manifest as normal under microscopic examination, unaffected by the burgeoning fungal growth. The density of fungal growth continued to increase steadily, reaching a significant level on the fifteenth day of the experiment.

In tandem, the nematodes underwent metamorphosis and experienced considerable growth, as evident from microscopic examinations on the fifteenth day of the trial

The effect of the combination between the local fungus and the local nematode on the cucurbit fly larvae and pupae

The synergistic impact of the combined biocontrol agents, the indigenous fungus, and local nematode, was investigated on the larvae and pupae of the cucurbit fly insect. The outcomes, obtained after a ten-day evaluation, as presented in Table 2 and Figure 3, unveiled that treating the larvae with the fungus at a concentration of 5×10^6 spore/mL, in conjunction with the local nematode at a concentration of 100 IJs/mL, led to a mortality rate of 40% for the larvae (12 larvae out of 30 were eradicated following treatment) and a mortality rate of 26.66% for the pupae (8 pupae out of 30 succumbed at the mentioned concentrations). The overall kill rate recorded was 33.33%.

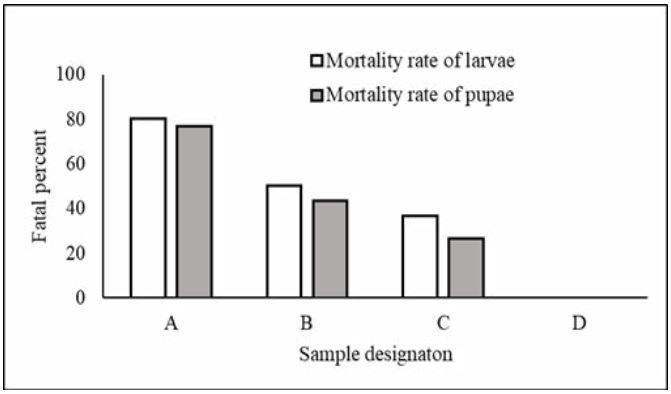

Figure 3: Mortality rate of larvae and pupae killed by the treatment with the local fungus and local nematode.

Regarding the treatment of larvae and pupae of the cucurbit fly insect with the fungus at a concentration of 10×10^6 spore/mL, combined with the local nematode at a concentration of 100 IJs/mL, the mortality rate observed for the larvae was 53.33%, resulting in the demise of 16 larvae upon the application of the combined biocontrol agents. As for the pupae, 13 out of 30 pupae distributed among three Petri dishes perished at the same concentrations of the fungus and nematode, resulting in a mortality rate of 43.33%. The overall kill rate for larvae and pupae collectively reached 48.33%.

Treating the larvae and pupae of the cucurbit fly insect with the fungus at a concentration of 20 ×10^6 spore/mL

and the local nematode at a concentration of 100 IJs/mL resulted in the death of 25 larvae out of 30 distributed evenly among three Petri dishes, with a mortality rate of 83.33%. As for the pupae, the mortality rate was 80%, indicating that 24 out of 30 adults were killed in the three Petri dishes, See Figure 4. The overall kill rate for larvae and pupae in these two concentrations was 81.66%.

Figure 4: *Dacus ciliatus* pupae infected by *Beauveria bassiana* and *Heterorhabditis bacteriophora.*

The statistical analysis indicated the presence of differences in mortality rates among the larvae and pupae that were treated with combinations of local fungus and the local nematode. There was a significant increase in mortality rates for larvae compared to pupae when treated with biological control agents.

When used on larvae ($F_{2,8}=66.5$, $p<0.01$) and on pupae regarding their pathogenicity($F_{2,8}=100.5$, $p<0.01$) However, there were no significant differences observed when comparing the effect of the same concentrations on larvae and pupae ($F_{1,17}=0.74$, $p=0.42$).

In general, it can be asserted that the efficacy of biological control agents can be optimized through the integration of multiple control factors against insect pests. The combination of two or more biological control agents targeted at a specific pest can produce comparable outcomes to individual agent actions, or even yield a slight increase in effectiveness, termed as an additive effect. Alternatively, such agent combinations may lead to a synergistic effect, resulting in a greater impact than the additive effect. Conversely, the combined effect may exhibit antagonism, resulting in an impact lower than the additive effect. In cases of synergism, one biological control agent enhances the pest's susceptibility to the other. Conversely, antagonism arises when one control agent adversely affects the other, either through competition or detrimental effects (Cavassin et al., 2011; Jaques and Morris, 1981).

As reported by (Ansari et al., 2004), the synergistic effect between fungi and nematodes on the target pest is demonstrated through the fungus infecting the pest, priming it for nematode infection. Furthermore, the fungus weakens nutrient absorption and the pest's defenses. Additionally, this study observed that after the emergence of adults that completed their life cycle without being affected by nematodes and fungi, a portion of these adults was collected and dissected. It was observed that they were mildly infected by the fungus, although the infection did not significantly impact their survival or life cycle completion. However, the remaining adults that were returned to rearing boxes exhibited a decreased growth rate and a faster mortality rate compared to the adults that were not treated with the fungal biological control agent, both in terms of their larvae and pupae.

Numerous other studies have also addressed the integration of entomopathogenic fungi with nematodes for various pests, yielding promising results (El Khoury et al., 2020; Shapiro-Ilan et al., 2004; Tarasco et al., 2011).

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

NOVELTY STATEMENT

This study demonstrates the successful integration of local biological control agents, including nematodes and fungi, for effective control of cucurbit fly larvae and pupae. The findings highlight the differential susceptibility of larvae and pupae to biological control, shedding light on targeted pest management strategies in cucurbit plants.

AUTHORS CONTRIBUTION

These authors each contributed equally.

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