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Combining the entomopathogenic fungi *Beauveria bassiana*, and the parasitoid *Bracon hebetor* for controlling *Ephestia cautella*

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Abstract

Stored-product insects cause considerable post-harvest losses, the development of resistance to chemical insecticides, and concerns over the adverse effects of chemicals on the environment and human health, have provided the stimulus for developing biological control agents to control pests of stored product. The compatibility of *Beauveria bassiana* and the parasitoid *Bracon hebetor* in controlling *Ephestia cautella* Walker (Lepidoptera: Pyralidae), a pest of a wide range of commodities including dates was evaluated. The highest effect of fungus was at a concentration of 10^9 spores / ml. (85% mortality) and the LT50 value of *B. bassiana* at this concentration was 1.92 days, the LC50 value was 2.8 x 10^3 spores/ml. The ability of *B. hebetor* to paralyze the fig moth larvae was 24 ± 3 larvae in the first day and the cumulative rate was 48 and 63 larvae in the second and third days respectively. The combination of both agents enhanced their activity in controlling the *E. cautella* larvae; the mortality rate was 94%, while it was 70% for parasitoids and 56.7% for fungus at LC50 concentration. The highest mortality of the parasitoid due to this fungus was recorded after 7 days of treatment with a concentration of 10^9spores/ml (73.3%) and the LC50 value was 2.7 x 10^3 spores/ml. Effectiveness against a particular pest and safety of non-target organisms are key characteristics of good IPM practices. Despite the increased effectiveness of *B. bassiana* and *Bracon hebetor*, its carries the risk of the dual action of fungus on the target stored-product insects and arthropod natural enemy.

Introduction

The date palm tree, *Phoenix dactylifera* is exposing to infestation by various kinds of insects and diseases. One of the most important insect species is the *Ephestia cautella* (Walker), it was the main insect pest in Iraq that infests dates in orchards and continues to stores throughout the months of the year (Abd al-Husain, 1985; Khadir, 1998; Al-Taweel & Al-Jboory, 2007). If this infestation is left without any treatment the dates will be seriously affect and become unsuitable for human consumption (Hameed, 2002). During the storage period methyl bromide (CH3Br) is usually applied to control this pest in spite of its dangers (Ahmed, 1998), moreover *E. cautella* is capable to develop resistance to it (Ahmed, 1998) and this fumigant appeared to be the carcinogenic agent to the human being in addition to its ability to deplete the Ozone layer (Leesch *et al*., 1992; Marcot, 1993; Ross &Vail, 1993). These reasons stimulate researchers to find alternative ways against such pests

(Al-Taweel and others, 1990; Talebi et al., 2011). The biological control of this pest is one of the alternatives and most effective methods of controlling this pest, which includes the use of natural enemies such as insect parasitoids or entomopathogenic fungi. The insect parasitoid *Bracon hebetor* Say (Hymenoptera: Braconidae) is one an effective natural enemy on many insect larvae in particular the Pyralidae family, which including the target insect in this study, as well as other field pests, (Gurbuz, Aksoylar, 2006; Landge et al., 2009). Entomopathogenic fungi play a major role in the control of many insect pests. There are more than 750 species, the most important are *Beauveria*, *Metarhizium*, *Verticillium* and *Paecilomyces* (Dent, 2002). *Beauveria bassiana* strains are the most virulent on the *Ephestia kuehniella*, followed by the strains of fungus *Metarhizium anisopliae* and *Verticilium lecanii* (Draganova and Markova, 2006). Sultan (2016) demonstrated the possibility of compatibility between the insect predator *Chrysoperla carnea* (Stephens), the fungus *Beauveria bassiana* and *Metarhizium anisopliae* against the *Ephestia cautella* (Walker). According to the importance of *Ephestia cautella* on dates due to its significant damage, this study was carried out to evaluate the efficiency and the compatibility of *Beauveria bassiana*, and *Bracon hebetor* against this pest

Materials and methods

Insects

The target insect (*E.cautella*) was reared on an artificial diet 81% crushed whole wheat, 1% yeast, 6% syrup(dibis), and 12% glycerol. Stock cultures were maintained at 27±2°C and 60-70% relative humidity (RH), with 16 hours photo phase and 8 hours Scot phase (Saad and Mahdi, 2017).

The adults of ***B. hebetor***, were reared on a large scale, by using the larvae of *E. cautella*, as a host, at 25 ± 1 °C, 65 ± 5% R.H and 16 hours photo phase and 8 hours Scot phase. A number of 2 to 3 larvae of *E. cautella* of the 4th stage were placed in the glass vial, with a fertilized female of the parasitoid, *B. hebetor* (Say.). The female, was provided with a cotton swab, soaked in 20 % honey-solution, for feeding the parasitoid. After 24 hours, the females were shifted to the other vials, with a lot of the larvae of the host, and honey-soaked, cotton swab. This process was continued up to the end of female parasitoid's life. The parasitized larvae of the host were incubated, under the optimum conditions of 25 ± 1 °C of temperature and 65 ± 10 % of the relative humidity.

Preparation of Spore Suspensions

The fungal pathogen used in the present study was cultured on potato dextrose agar medium (PDA) autoclaved at 121○C (15 Psi) for 15-20 minutes and poured into sterilized Petri plates. The Petri plates containing PDA medium were incubated at 27 ± 1○C, 80 ± 5% relative humidity and a photoperiod of 12 hours. The conidia were harvested gently by scraping the surface of the 15-days old culture with an inoculation needle. The conidia were suspended in distilled water containing 0.05% Tween-80. The mixture was stirred on a magnetic shaker for 10 minutes. The hyphal debris was removed by filtering the mixture through fine-mesh sieve. The conidial concentration of the final suspension was determined by direct count using haemocytometer. Suspension concentrations (1 x 10^5, 1 x 10^7 and 1 x 10^9 conidia ml^{-1}) were prepared and used in bioassay (Vinod Kumar and Chowdhry 2004)**.**

Bioassay procedures

Immersion method

The fourth larval stage of the *E.cautella* (10 larvae per replicate) was immersed for 10 seconds in different concentrations (1 x 10^5 , 1 x 10^7 and 1 x 10^9 conidia ml^{-1} + 0.05% Tween 80) of the fungal suspension of *Beauveria bassiana* and in distilled water+ 0.05% Tween 80 for the control treatment (Jarrahi and Safavi , 2016).The excess moisture was removed from the treated larvae by placing them on filter paper and then transferred to a Petri dish containing 5 grams of diet and incubated under controlled laboratory conditions . Mortality rates were calculated and the dead insects were placed on moisturized filter paper for 7 days to determine the cause of mortality (the fungal growth) (Jarrahi and Safavi, 2016). The experiment was replicated three times.

Exposure to treated filter papers with fungal suspension

E. cautella larvae were exposed to treated filter papers with different concentrations of fungal suspension (1 x 10^5 , 1 x 10^7 and 1 x 10^9 conidia ml^{-1} + 0.05% Tween 80) of *Beauveria bassiana* in Petri dishes supplied with 5 grams of diet, and incubated at 27 ± 1 ° C , 60-70% relative humidity and photoperiod 8:16 hours (D: L). Daily mortality was recorded and dead insects were transferred to the moisturized filter paper for 7 days to determine the cause of mortality depending on the fungal growth (Jarrahi and Safavi, 2016). The experiment was replicated three times.

Efficiency of B.hebetor

Couples of newly emerging adults of *B.hebetor* were kept in test tubes 2.5 x 7.5 cm for mating (adults were fed on 10% honey solution) and then were introduced in glass bottles of 7.5 x 12 cm containing 100 larvae of fig moth for 24, 48 and 72 hours., At each time, the percentage of paralyzed larvae were calculated. The experiment was replicated ten times.

Integrated Effect of entomopathogenic fungus and parasitoids

This experiment was carried out by immersing the fifth larval stage of the *E.cautella* in the LC50 concentration of the fungus *B. bassiana* for 10 seconds. Each 100 treated larvae were placed in plastic bottles 12 x 11 cm with pair of adult parasitoids and covered with muslin, then

incubated under controlled laboratory condition. The results are recorded after 72 hours to determine the mortality rate and reasons. These results compared with result of treated larvae with fungi alone and parasitoid alone.

Effect of fungus on the adult of Bracon hebetor

The effect of the fungus on the parasitoids adults as well as the LC_{50} and the LT50 was determined by exposure the parasitoid adult (10 individuals) to treated filter papers with fungal suspension. And then placed in a glass container with a piece of cotton saturated with 10% sugar solution and incubated under controlled laboratory conditions. Mortality rates and its reasons were determined.

Exposure of parasitoids adult to treated larvae with fungal suspension.

The larvae of *E.cautella* were dipped in the fungal suspension of *B. bassiana* (20 larvae per replicate) for each fungal concentration and in distilled water as control. 10 adult parasitoids (5 males and 5 females at age of 24-48 hours) were introduced in a container of treated larvae and supplied with a piece of saturated cotton by sugar solution (10%) to feed parasitoids and incubated under controlled laboratory conditions. The mortality rate of parasitoids was calculated with determining the reason of mortality. The experiment was replicated ten times.

Experimental design and statistical analysis: The experiments were designed in a completely randomized design CRD, and data were analyzed by using SPSS program, and Duncan multiple range tests (DMRT) to compare the means in probability level of 0.05. Standard probit analysis was used to obtain LC50 and LT50 values.

Results and Discussion

The results of immersion last larval stage of *E.cautella* in different concentrations of fungal suspension of local isolates of *B. bassiana* appeared that the susceptibility depended on the concentrations , the mortality has increased with increasing of concentrations (Table 1), highest impact was at concentration of 10^9 spore / ml (85%) after four days of treatment, and the lowest impact (77 %) was at the concentration of 10^5 spore / ml. The median lethal time (LT_{50}) which determines the virulence of fungal isolate has been associated with the concentration, it was ranged from 1.92 - 2.9 days at concentrations of 10^9 and 10^5 spore \ ml respectively for *B. bassiana* and 1.82 - 3.92 days . The median lethal concentration LC50 of *B. bassiana* was 2.8×10^3 spore / ml.

Table 1: Mortality of *Ephestia cautella* larvae *treated* by immersion method with different concentrations of fungal suspensions of *B.bassiana*

Treatment	the concentrations	% of mortality	LT50	X^2	P Values	LC50	X^2	P value
B.bassiana	10^5	60.5a	2.9	14.4	0.044	2.8×10^3	11.5	0.17
	10^7	80 b	2.43	18.7	0.001			
	10^9	85 b	1.92	11.7	0.11			

The means followed by the same letter in the same column are not significantly different according to the Duncan test (0.05)

Pathogenicity is the most important indicator when measuring the effectiveness of entomopathogenic fungi against pests (RobertAnd St Leger , 2004), Fungal isolates were elected as successful biological agents according to their pathogenecity , ease of production and adaptation to the environmental conditions (Reay ,et al., 2008) . Using of pathogenic microorganisms was increased especially entomopathogenic fungi to combat warehouse insects because of low toxicity and risks to mammals and the environment .Their effect was occurred by contact with the insects and penetrate their bodies through analysis of the integument and then spread inside the body and causing mortality (Cox and Wiking, 1996). According to the mentioned reasons, the entomopathogenic fungi were tested against many stored-products insects (Kavallieratos et al, 2006; Sabbour and Abd El-Aziz, 2007a, b, 2010). One of the mechanisms that causing insect mortality by *Beauveria bassiana* is produce a number of toxic compounds (Zacharuk, 1971; Vey et al. 2001). In a local study the pathogenicity of three isolates of *B. bassiana* fungus were tested against larvae of *Ephestia cautilla* , the highest pathogencity of isolates were at the concentration 10^6 spore / ml, which achieved mortality percentage 92.7 and 91.2 and 90.3% after 18 days and these isolates achieved protection of dates in store for six months , with the percentage of injury , 1.3, 1 , and 3% for the three isolates ,while it ranged between 5 - 16.5% in the control (Jassim and Laith 2012). Using of *Beauveria bassiana* , *Metarhizium anisopliae* and *Isaria fumosorosea* against *Plodia interpunctella* , *Ephestia cautella* and *E. Kuehniella* showed that the highest efficacy was for *Beauveria bassiana* fungus and effectiveness was enhanced by addition of diatomaceous (Sabbour et al. 2013) . Dragamora and Markoa (2006) Tested Four fungal isolates of *Beauveria bassiana* and two isolates of *M.anisopiliae* and one isolate *of Verticillium lecanii* against larvae of *Ephestia kuehniella* and the percentage mortality was measured within 8 days , the virulence was determined by LT_{50}, results appeared that the

isolate Bb383 of *B.bassiana* caused the highest lethal effect of larvae (87.88%) , followed by Bb399 and Bb382 ,mortality rates was 68.84% and 60%respectively .lowest lethal effect was by two isolates of *M.anisopiliae* and one isolate of *V. Lecanii* . Bb383 isolate was the most virulent, according to the value of the median lethal time with limited confidence 5.234- 4.81 days and rate of 5.019 days. Virulence of ten fungal isolates of the fungus *Beauveria bassiana* and *Metarhizium anisopliae* under laboratory conditions against the third larval stage of *E. kuehniella* , by using the method of immersion (10 ml) for 5 seconds , percentage of the cumulative mortality was between of 11-92% for *Metarhizium anisopliae* and the LC_{50} values ranged between 5.4×10^7 to 3.4×10^8 and for *Beauveria bassiana* the mortality was ranged between 17-88% and the LC_{50} between 8.3×10^5 to 6.5×10^6 (Faraji et al. , 2013). According to Wakefield (2005) the greatest results were obtained within the Mycopest project of definition of the most entomopathogenic fungi efficacy in the control of stored product insects, work within the project appeared that the *B bassiana* had better control to insect and mites. Four of the 12 isolates of *B bassiana* were caused 100% mortality of *E.kuehniella* larvae after 10 days of treatment by concentration of 10^8 conidia / ml. According to the Lord (2005) the application of entomopathogenic fungi achieved development when combined with other materials such as *Diatomaceous earth.*

Exposing to treated filter paper with fungal suspension

The results of treatment the last larval stage of *E.cautella* to different concentrations of *B. bassiana* on treated filter paper with fungal suspension (Table 2), the mortality rate was 60 % at the concentration of 10^5 spore / ml and LT_{50} was 3 days , and at 10^9 spore / ml the mortality was 73 % and the LT_{50} was 2.9 days. The LC_{50} value was1.4×10^2**.**

Table 2: Mortality of *Ephestia cautella* larvae *treated* by different concentrations of fungal suspension of *B.bassiana* on filter paper

Treatment	the concentrations	% of mortality	LT50	X^2	P values	LC50	X^2	P values
B.bassiana	10^5	60a	3.0	6.26	0.51	1.4×10^2	4.9	0.66
	10^7	66 ab	2.96	9.5	0.22			
	10^9	73 b	2.9	12.6	0.08			

The means followed by the same letter in the same column are not significantly different according to the Duncan test (0.05)

Entomopathogenic fungi (EPF) represent one of the most promising alternatives to chemical control against the stored-products insect. *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) is the most widely tested EPF for the control of stored-grain insects (Sabbour et al. 2012; Kaur et al. 2014).

The efficiency of the parasitoid *B. hebetor* and *B.bassiana*

The results (fig. 1) appeared that the ability of *B. hebetor* on paralyze the fig moth larvae was 24 ± 3 larvae in the first day and the cumulative rate was 48 ± 9 and 63 ± 19 larvae in the second and third days respectively. Comparing the effectiveness of parasitoid alone and with the Microbial control agents (*B. bassiana*) showed The combination of biological control agents gave an enhancing to the effectiveness (fig. 2) , the percentage of larvae mortality was 94 % as the impact of parasitoid with *B. bassiana* , while it was 70%, 56.7% and 51.6% for the parasitoid alone and the mentioned fungus.

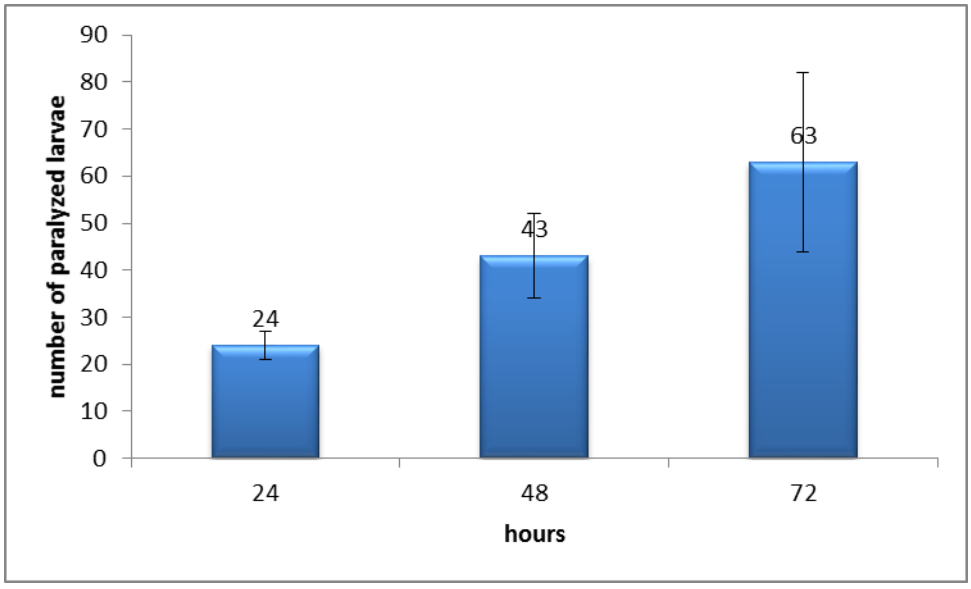

Figure 1: Cumulative parasitism efficiency of *Bracon hebetor* on larvae of *Ephestia cautella*

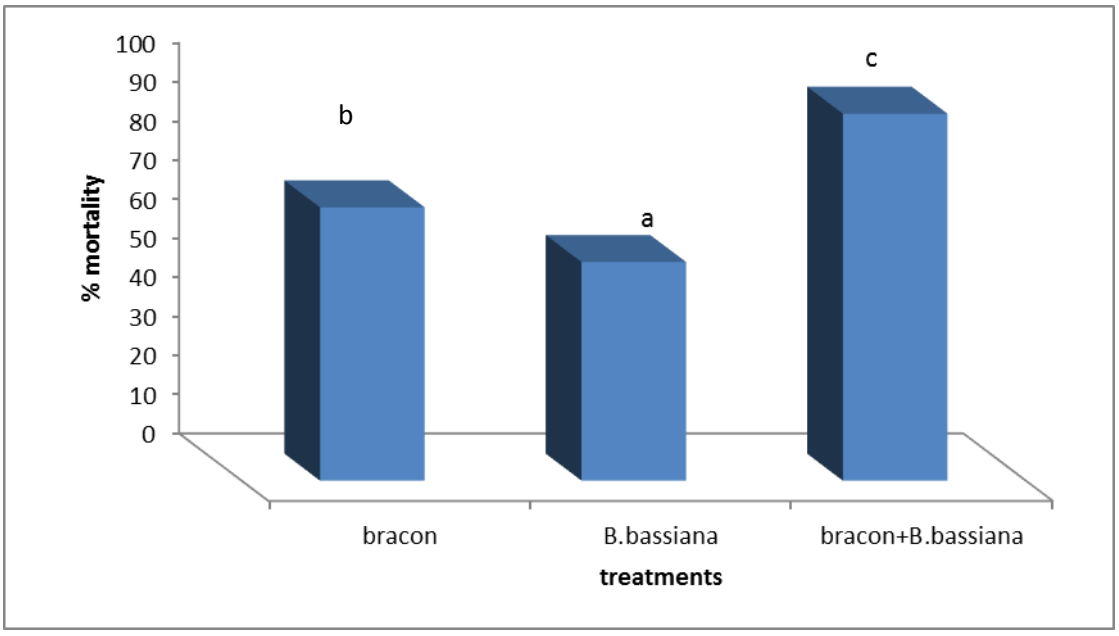

Figure 2: The effectiveness of the f biological control agents (*Bracon hebetor*, B*. bassiana*) against the larvae of *Ephestia cautella*

The organization of host population by external parasitoids mainly through injecting of females secretions toxicity in larval host that caused rapid permanently paralysis, which allows for females laying eggs and then feed of parasitoid larvae that caused inhibiting of the defensive response of host larvae (Pennacchio et al., 2014). This mechanical adopted as a measure of the effectiveness of B. *hebetor* especially toxic secretions that cause complete and permanent paralysis at the level of one part of toxin to 200 million liquid part of the blood host (Weaver et al., 2001). The increase of efficiency is due to increase the sensitivity of paralyzed larvae to fungal infection, speed and increase fungal growth as containing toxic secretions which disable the endocrine and immune system of the host (Kryukova et al., 2011). Kryukov et al. (2013) confirmed increase the effectiveness of entomopathogenic fungi in Paralyzed individuals compared with non – paralyzed ones and the LC50 value has decreased by 5,000 times after entering the toxicity secretions to the body of the larva and low dose (114 conidia per larva) was sufficient for the development of Fungal growth, this phenomenon is widespread in nature and that individuals that fall down on the surface of the soil and thus more vulnerable to infection. It was indicated to increase of Lepidoptera larvae sensitivity after injected of parasitoid, such as internal *parasitoids Micropliti scroceiper* (King and Bell, 1978) and Oomyzuss okolowskii (Dos santos et al., 2002) and *Pimplahy pochondriaca* (Dani et al., 2004). Shonouda and Nasr (1998) stated that the parasitism efficiency of the *B. hebetor* against *Ephestia Kuehniella* larvae ranged from 90 - 100 % for all instars except the

first instar (0 %) For its activity and smallness size. Reinert and King (1971) noted that the release of 250 females of parasitoid leads to 97% mortality of the Indian flour moth larvae (1800 larvae). Nickle and Hagstrum (1981) have indicated that the *B. hebetor* was high efficiency against almond moth *E. cautella* in the stores. The parastoid is more efficient when it is ready for release at an early stage of infection. The paralyzed hosts by parastoids female are ready source for their food and thus increase their survival and fertility and is a suitable place to lay eggs. According to Cline et al. (1984) that the release of 50 pairs of *B. hebetor* twice a week in the corn flour store containing an infected packs by *E. cautella*: first packed and tightly closed, and the second packed but without integrated closing, and the third is open, these led to a variation infection rates in the three packages as follows: infections was reached in the first pack to 7.5% compared with 42.5% in the control treatment group, while it was 10% compared to 75% in the control treatment for the second pack, and 50% in the third pack compared to 100% in the control treatment. Keever et al. (1985 and 1986) pointed out the possibility of reducing the infection of *E. cautella Plodia interpunctella* using *B. hebetor* and the predator X. *flavipes* in the store of Pistachio. While Brower and the Press (1990) pointed out the importance of biological control using *B. hebetor* and *Trichogramma pretiosum* against *E. cautella* and *Plodia interpunctella*, the first achieved a mortality ratio of stages of *Plodia interpunctella* 66.1%, while the second has achieved 37.3%, release both parasitoids together they achieved 84.3% mortality. Mortality rate of *E. cautella* was 96.7% in the case of the first parasitoid, while it was 97.3% when using the second parasitoids while using both parasitoid the mortality was 98%. Hagstrum and Smittle (1978) stated that when paralyse the larvae of the host by a parasitoids female, the paralyzed larvae remain moist and suitable for feeding, or to lay eggs and the growth of their offspring last enough period, the rate of deterioration of bodies does not exceed 15% per week. Female choose newly and soft paralyzed larvae usually for the purpose of eggs laying, which represents 68.7% of the total laid eggs. The researchers also found that the number of eggs laid by single female on the single larva does not double with increase the number of female to 1, 2, 5 and 10 to a single larva of the host , the number of eggs was 13.6, 14.4, 10.5 and 6.7respectively. Some researchers have pointed to the importance of Kairomones that released by some moths that inferred them by B. hebetor. Scholler et al. (1997) explain that Kairomones are material if it released the benefit the recipient alone , which includes smells or tastes attractive and exciting to attack the host or prey whether an insect or vegetable host. To reduce the crop field losses and a few risks on the environment and human is recommended to use microbial control of (Hull and Beer, 1985), but the use with parasitoids and predators carries negative effects on the life and effectiveness of those parasitodes and predators (Hajek and St Leger, 1994; Thungrabeab and Tongma, 2007).

The effect of *B. bassiana* on *Bracon hebetor*

The highest mortality rate after 7 days of treatment was 73.3% by exposed to the concentration of 10^9 spores/ml of fungal suspension of *B. bassiana* in filter paper. The shortest LT50 was 2.3 days, the LC50 was 2.7 × 10 spore / ml.

Table 3: Cumulative mortality, LC_{50} and LT_{50} of *Bracon hebetor* adult's exposed to fungal suspension of *B.bassiana* in filter paper

Treatment	concentrations	% of mortality	LT50	X^2	P Values	LC50	X^2	P Values
B.bassiana	10^5	56.7a	2.82	5.2	0.64	2.7 x 10^3	12	0.09
	10^7	66.7ab	2.33	9.32	0.23			
	10^9	73.3b	2.3	23	0.03			

The means followed by the same letter in the same column are not significantly different according to the Duncan test (0.05)

The direct effect of fungi on the parasitoid that has been exposure to fugal suspension on the host larvae bodies (table 4) showed that the highest rate of mortality after 7 days of treatment was 83% by the concentration of 10 9 spores / ml of the fungus B. bassiana and reached the shortest LT50 was 2.1 days for B. bassiana , the LC50 reached 4.2×10^5 .

Table 4: Cumulative mortality, LT50 and LC50 of adults of Bracon *hebetor* exposed to different Suspensions *B.bassiana* through the bodies of host larvae

Treatment	the concentrations	% of mortality	LT50	X^2	P Values	LC50	X^2	P Values
B.bassiana	10^5	47a	3.7	17	0.04	4.2×10^5	17.6	0.01
	10^7	57b	3.33	19	0.03			
	10^9	83 c	2.1	32	0.01			

The means followed by the same letter in the same column are not significantly different according to the Duncan test (0.05)

Studies have indicated that the pathogenic fungi effect on natural enemies under the optimum environmental conditions of fungus, for example, Act of Aphidius nigripes was obstructed by infection of aphids by *Lecanicillium lecanii,* but the successful biology evolution in a high percentage of parasitoid occurs when the exposure to the fungus was after four days of parasitism (Askary and Brodeur, 1999). In a study of the impact of of two Iranian isolates of *B. bassiana* and *M. anisopliae* using the method of immersion of an immature individuals of parasitoid , the LC 50 value of IRAN187C *B. bassiana* was 4 × 109 spore ml and the mortality rate for other isolates are very low,so the LC50 value didn't account. It confirmed the lack of impact of all isolates on the pupal stages of the parasitoids and recommended using the Isolates in the field of integrated control (Mahdavi et al., 2013). Exposing the two species of B. hebetor and *Apoangyrus lopezi* to *B. bassiana* and *M. anisopliae*. Under the development of locust and leaves hoper control program, Danfa and Vandervald in 1999 found that the laboratory application of the fungus *Metarhizium spp.* and one isolate of *B. bassiana* at a rate of 5×10^{12} spore/ml in 280 litres of water per hectare (the field rate recommendation) gave 100% mortality in *Bracon hebetor* and *Apoanagyrus lopezi.* Some parasitoids show a decrease in attempts of egging in the fungal infected hosts compared to non - infected, some parasitoids can distinguish the infected hosts. Some lay slightly eggs on the infected hosts compared to non - infected (Brobyn, 1988; Fransen and Van Lenteren, 1993).

In a study on the use of *Lariophagus distinguendus* and *Anisoptermalus calandrae* alone and in combination with the fungus *Beauveria bassiana* against *Sitophilus granarius* on grain, the results showed that the highest suppression of the pest population (99.9%) was due to L. distinguendus, and then A. *Calandrae* either when combine both of them the suppression has been 83-98% indicative of a negative impact of fungi on parasitoid (Hansen and Steenberg, 2006). Increase in mortality can occur when the effect of interference between the pathogen and the parasitoid, the fungal pathogen *Hirsutella cryptosclerotium* and *parasitoid Gyranusoi deatebygi*, both natural enemy of *Rastrococcus invadens*, the fungi has decreased the level of parasitism (Akalach et al., 1992).

Conclusion

Effectiveness against a particular pest and safety of non-target organisms are key characteristics of good IPM practices. Despite the increased combined effectiveness of *B. bassiana* and *Bracon hebetor*, its carries the risk of the dual action of fungus on the target stored-product insects and arthropod natural enemy. . Further research is needed to confirm this and to determine the damage to the parasitoids larvae.

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