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RESEARCH

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Identification and evaluation of isolated entomopathogenic fungus from Egyptian soil against the black cutworm larvae of *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae)

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Abstract

Background: The black cutworm, *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae), is considered as one of the most destructive pests of many field crops in Egypt. Entomopathogenic fungi (EPF) have long been accepted as relatively safe alternatives to chemical insecticides. In the past decades, many researchers have compared the effective local isolates of EPF to the formulated product of the same fungus and reported that local isolates were more efficient than formulations when used against target insects.

Results: The present study discussed the pathogenic activity of local isolate after scientific characterization. Two insect species [*Galleria mellonella* L. and *Agrotis ipsilon* (Hufn.)] were used in this evaluation. The fungus was isolated from soil samples by trapping method, and fourth-instar larvae of *Galleria* were used to evaluate the pathogenicity of the isolate. The isolate was identified as *Beauveria bassiana.* Different levels of the fungus development depending on the test temperatures (20, 25, 30, 35 and 40 °C) were tested. Optimal growth of the isolate fungus was recorded at 25 °C. The corrected mortality rate for fourth-instar larvae of *G. mellonella* treated with the isolate spores at 6.4×10^5 spores/ml was (96%) after 10 days, while the mortality of last instar larvae of *A. ipsilon* was (48 and 100%) at the concentrations of 9.2×10^4 and 2.9×10^6 spores/ml, respectively, after 14 days compared to (24%) in the control. Both pupation and the sex ratio in adults were affected at different levels according to the concentration. The effect was also very clear in the increased rates of malformation in adults.

Conclusion: The EPF, *B. bassiana,* isolated from the soil, was highly effective against *G. mellonella* larvae in storage and *A. ipsilon* larvae in soil; therefore, it could be recommended as an alternative control agent for chemical pesticides.

Keywords: Entomopathogenic fungi, Isolate, *Beauveria bassiana*, *Galleria mellonella*, *Agrotis ipsilon*, Temperature

Background

Soil is the main reservoir of entomopathogenic fungi (EPFs), which have accumulated from many insect cadavers (Butt et al. 1994). Many insect pests of field crops, especially soil insects, are naturally infected with EPFs under suitable conditions of fungal activity (Alfiky 2022). Adaptation of local isolates to environmental conditions was the main reason for their use in an IPM program more successfully than many commercial products from the same fungus (Zayed 2003). Two different methods have been accepted to isolate EPF from soil, insect bait or selective media, the first being more acceptable with regard to the higher sensitivity of the host (Zimmermann1986). The larger wax moth, *Galleria mellonella* L., is the most commonly used as larval bait in EPFs isolation technique from the soil

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(Meyling and Eilenberg 2006). Naturally, more than 700 species of EPFs are known as a bioagent has been observed only in very limited species (Rabindra and Ramanujam 2007). In various locations of Egyptian soil, according to several previous studies on EPFs isolates, *Beauveria bassiana* was the most widespread fungus, having a wide range of harmful insect hosts. Its mode of action is based on the penetration of germ tubes through the layers of insect cuticle by enzymatic influence and access to the hemocoel (Anderson et al. 1995). Alfiky (2022) collected several soil samples, at a depth of 5–10 cm from soil surface of 4 regions in 3 Egyptian governorates and reported that EMPF was found in all samples. Clifton et al. (2015) tested the abundance of EPFs in organic and conventional fields and the effect of specific cropping practices and soil properties on their abundance. They found that organic fields were more favorable environment for EPFs, and abiotic factors and crop practices such as tillage may have greater effects on EPFs abundance.

The black cutworm, *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae), is one of the major polyphagous insect pests. The larvae attack many field crops, especially in the seedling stage causing damage about 100% in some cases (Wang et al. 2021). The present study aimed to identify local isolates from different soil locations and their developmental activity under different temperatures. The pathogenicity of the isolate was evaluated against *G. mellonella* as a model insect and *A. ipsilon* as a serious ground pest.

Methods

Soil samples

A number of samples were collected from different locations of Egyptian soils (Giza, Beheira, Qalyubia and Menoufia governorates). Soil samples were collected from fields planted with wheat, Egyptian alfalfa, tomatoes, okra and zucchini in Giza Governorate, Alsaf city. In the Beheira governorate, Noubaria city, samples were collected from soil planted with wheat, tomatoes, strawberries and orange trees. Samples were also collected from Qanater City, Qalyubia governorate, from soil planted with wheat, Egyptian alfalfa, onions and okra while in Ashmoun village, Menoufia governorate, 2 samples from the field crops; wheat and Egyptian alfalfa were collected. Before collecting the samples, the surface layer of soil was removed and the soil samples were taken to a depth of 20 cm. Five samples of each crop (500 g/sample) were kept separately in plastic bags and carefully mixed in the laboratory. Samples were stored at 4 °C before starting the isolation steps (Sevim et al. 2010).

Isolation and Identification of fungi

The insect bait method technique was applied using the larvae of *G. mellonella*, to isolate EPFs from soil samples (Alfiky 2022). A number of plastic cups (120 ml volume) were filled with soil (100 g/cup), and 10 larvae (fourth instar) were placed in each cup (5 cups/sample). Cups were incubated at 25 °C and examined daily for collection of dead larvae which were carefully washed with distilled water, and placed for 30 s. in sterile 1% sodium hypochlorite solution (Aguilera Sammaritano et al. 2016). Cadavers were placed on wet filter paper in Petri dishes (10 cm diameter) after being rinsed twice with distilled water and kept under special conditions (dark and 25 °C). Fungi obtained from the surface of larvae were transferred to potato dextrose agar media (PDA) (Mwamburi 2016). The isolated fungus was identified at Fungi Research Laboratory, Department of Plant Pathology, National Research Centre, according to the methods described by Goettel and Inglis (1997). On the other hand, the definition of isolated fungus was confirmed by specialists at the Plant Diseases Institute, Agricultural Research Center, Giza, Egypt.

Effect of different temperatures on growth of entomopathogenic fungus

Amount of sterilized PDA medium (20 ml) was poured into each sterile Petri dish (9 cm in diameter) and left to be solid. Petri dishes were selected from the stock culture of isolated fungus, and each dish was divided under sterilized conditions to disks (5 mm diam. for each). One disk from fungus culture was inoculated in center of each Petri dish. Tested temperatures were 20, 25, 30, 35 and 40 °C, and 4 replicates of inoculated dishes were used for each temperature. The fungus was incubated at these temperatures for 4 days in darkness.

Insects rearing

Greater wax moth, Galleria mellonella L.

The insect was reared on artificial diet contained: wheat flour 350 g, corn flour 200 g, milk powder 130 g, yeast powder 70 g, honey 100 ml and glycerin 150 ml, for one kilogram according to Metwally et al. (2012). Adults were reared in Jars (2 l) supplied with small papers for laying eggs. The eggs were transferred to smaller jars (1 l) contained artificial diet for feeding of hatched larvae. Insect rearing conditions were 25 °C ± 2, RH 65 ± 5% and full-time darkness.

Black cut worm Agrotis ipsilon (Hufn.)

Eggs of *A. ipsilon* were obtained from the stock culture in the Department of Cutworm and Mole Cricket, Plant Protection Research Institute, Agriculture Research

Center, Giza, Egypt, for rearing. The hatched larvae were kept in glass jars (1 l) supplied with fresh castor oil leaves (*Ricinus communis* L.) for feeding. To avoid larval cannibalism, up third-instar larvae were reared individually in small cups supplied with saw dust to reduce moisture content until pupation. For adults, big jar (2 l) containing 5 pairs and a piece of cotton wet with honey solution (10%) for feeding was used. Insect rearing was performed in an incubator at 25 ± 2 °C and 65 ± 5% RH (Ahmed et al. 2013).

Preparation of the conidial suspension (conidial stock)

Conidia were harvested from the surface of stock culture dishes in the sterile condition and transferred to sterile flask (100 ml) containing 100 ml sterile distilled water and 0.05% Tween 80 for homogenization of fungal spores. The conidial suspension was stirred for 15 min and filtered through sterile gauze to separate the hyphae. The number of conidia in suspension was counted using Hemacytometer (Marienfeld, Germany) (Thungrabeab and Tongma 2007).

Evaluation of the isolate against G. mellonella larvae

The tested concentrations prepared from the stock conidial suspension were 6.4×10^5, 3.2×10^5, 1.6×10^5, 8.0×10^4 and 4.0×10^4 spores/ml. Newly molted fourth-instar larvae were collected from the stock culture and placed in 9-cm Petri dishes (5 larvae/ Petri dish). Larvae were treated topically with a specific sprayer. Control larvae were sprayed with sterile water containing 0.05%Tween 80. Five replicates (one Petri dish/rep.) were used for each concentration and control. Each Petri dish was provided with a piece of sterile wet filter paper and cotton. Treated and untreated larvae were incubated at 25 ± 2 °C and 60–70% RH for 10 days with daily checking. The number of dead larvae was counted to calculate the mortality percentage.

Evaluation of the isolate against Agrotis larvae

The fungus was tested at the concentrations 2.9×10^6, 1.47×10^6, 7.36×10^5, 3.68×10^5, 1.84×10^5 and 9.2×10^4 spores/ml, which were prepared from conidial suspension stock. The larvae used in this experiment were 2 days old in the sixth-instar larvae which placed individually in plastic cups (3 cm diameter × 3 cm depth). Each cup contained 10 g sterilized soil using an autoclave at 121 °C for 15 min, 1 ml suspension and a piece of castor oil leaves for feeding. Larvae and castor leaves were sterilized before using by dipping in 1% formaldehyde for 30 s. and left to dry on filter paper under laboratory conditions. Five replicates (5 cups/ rep.) were used for each concentration and the same number of replicates was used as a control, which was supplied with 1 ml distilled water containing 0.05% Tween 80. All cups were covered by a perforated plastic cover and incubated at 25 ± 2 °C and 60–70% RH. The cups were checked daily until adults' emergence. Number of dead larvae and pupae were counted, and morality percentages were calculated. The percentages of pupation, emergence of adults, sex ratio and adult malformation were also calculated.

Statistical analysis

The mortality percentages were corrected by Abbott's formula (Abbott 1925). Using ANOVA and Duncan F-test, analyses were performed by SPSS 11 computer program (Snedecor and Cochran 1967).

Results

The positive isolated EPF was recorded only in Giza governorate samples and identified as *B. bassiana* as shown in Table 1. Table 2 and Fig. 1 show that the EPF *B. bassiana* was sensitive to temperature at 35 °C and higher. The optimum temperature for fungus development was 25 °C, which resulted 90 cm of diameter growth in the Petri dish; the fungal development in Petri dishes incubated at 30 °C was almost the same. Corrected mortality of *Galleria* larvae exposed to prepared concentrations of the identified fungus ranged between 96.0% at 6.4×10^5 spores/ml and 56.0% at 4.0×10^4 spores/ml. Nonsignificant differences were found among the 3 highest concentrations. Also, between the 2 lowest concentrations, nonsignificant difference was recorded, while a significant difference was observed between the lowest concentration and the 3 highest concentrations as shown in Table 3. The tested concentrations of isolated fungus (*B. bassiana*) on the last instar larvae of *A. ipsilon* showed different levels of mortality (Table 4). Complete mortality was observed at the highest tested concentration (2.9×10^6 spores/ml). There was nonsignificant difference between the mortality percentages of larvae treated with concentrations of 2.9×10^6, 1.47×10^6 and 7.36×10^5 spores/ml. The lowest tested concentration of 9.2×10^4 spores/ml showed (48.0%) larval mortality compared to (24.0%) at the control, which was significantly lower than that obtained at all tested concentrations. The pupation of treated larvae was affected at different levels according to the concentrations. The concentration of 1.47×10^6 spores/ml recorded (8.0%) pupation increased to (52.0%) at the concentration of 9.2×10^4 spores/ml, while it was nonsignificant at the control (76.0% pupation). Mortality percentage of pupae formed from treated larvae was (100%) at the concentration of 1.47×10^6 spores/ml, while it was (73.3%) at the concentration of 1.84×10^5 and (60.4%) at 9.2×10^4 spores/ml. The lowest percentage

Table 1 Isolation and identification of entomopathoginic fungi from soil samples of different Egyptian governorates

No	Governorates and locations	Crop	Soil type	Larval mortality of *Galleria mellonila* (%)	The presence of fungi (+/−)	Identification of isolated fungi
1	Giza (El-Saff city)	Wheat	Clay	44	+	*Beuvaria bassiana*
		Egyptian clover		28	+	*B. bassiana*
		Tomato		24	+	*B. bassiana*
		Okra		24	+	*B. bassiana*
2	Behara (El Nubaria city)	Wheat	Sandy	0	−	−
		Tomato		0	−	−
		strawberry		0	−	−
		Orange tree		0	−	−
3	El-Qulupeia (Qanater City)	Wheat	Sandy	0	−	−
		Egyptian clover		0	−	−
		Onion		0	−	−
		Okra		0	−	−
4	Menoufia (Ashmoun village)	Wheat	Clay	10	−	−
		Egyptian clover		2	−	−

Table 2 Susceptibility of *Beauveria bassiana* to different temperatures after 4 days

Temperature °C	*Beauveria bassiana* growth (mm) Mean ± S.E
20	20.25 ± 0.25^{b}
25	90.0 ± 0.0^{a}
30	81.25 ± 5.15^{a}
35	18.25 ± 3.94^{b}
40	0.0 ± 0.0^{c}
F. value (df)	196.909 (4)
P. value	0.000

Means with different letters are significantly different at $P < 0.05$

of adult emergence was (26.7%) at the concentration of 1.84×10^5 spores/ml, while the highest percentage of adult emergence was (39.6%) at the concentration of 9.2×10^4 spores/ml, which was nonsignificant in adult emergence in the control (78.3%). Most of adults' emerged were females at all the tested concentrations, except at 7.36×10^5 spores/ml and at the control, which gave the ratio of 1:1 (males: females). Malformation of emerged adults was (100%) at the concentrations of 1.47×10^6 and 7.36×10^5 spores/ml, while the lowest adult malformation was (15.3%), recorded at the control (Table 4, Fig. 2).

Discussion

To avoid environmental pollution, economical pest management using EPFs was accepted as effective alternative agents to chemical control (Bamisile et al. 2021). EPFs isolated from the soil of certain region are more efficient in controlling local pests in the same area (Liu et al. 2021). Meyling (2007) accepted the insect bait technique which used in this study by *G. mellonella* larvae as the most successful method for isolating

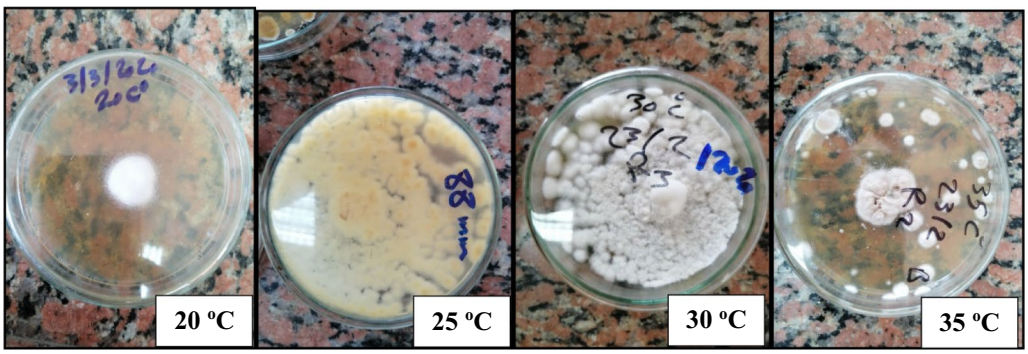

Fig. 1 Effect of temperatures on the development of isolated fungus (*Beuvaria bassiana*)

Table 3 Effect of isolated strain of *Beauveria bassiana* on *Galleria mellonila* fourth-instar larvae

Concentrations Spores/ml	Corrected larval mortality (%)
6.4×10^5	96.0[a]
3.2×10^5	92.0[a]
1.6×10^5	84.0[ab]
8.0×10^4	68.0[bc]
4.0×10^4	56.0[c]
F. value (df)	35.333 (4)
P value	0.000

Means with different letters are significantly different at $P < 0.05$

EPF from soil. *B. bassiana* was only found in clay soil samples in Giza governorate, which is consistent with the findings of Agamy (2002) who isolated the same species from cultivated clay soil samples at El-Badrashin, Giza. Vänninen et al. (2000) tested the persistence of EPF using 4 types of soil (clay, peat and 2 types of sand) and found that clay was the most suitable soil for persistence. Variations in the occurrence of EPFs from different sample sites could be due to the influence of biotic and abiotic factors including availability of insect hosts, temperature, humidity, UV radiation, soil type and organic matter in addition to application of chemical pesticides (Gebremariam et al. 2021).

Identification of the fungi isolated in the present study was based on morphological characteristics as described

Table 4 Effect of isolated strain of *Beauveria bassiana* on *Agrotis ipsilon* last instar larvae

Concentrations Spores/ml	Larval mortality (%)	Pupation (%)	Pupal mortality (%)	Adult emergence (%)	Sex ratio of emerged adults (%)		Malformation (%)
					♂	♀	
2.9×10^6	100.0[a]	–	–	–	–	–	–
1.47×10^6	92.0[ab]	8.0[d]	100.0[a]	–	–	–	–
7.36×10^5	88.0[ab]	12[cd]	66.7[a]	33.3[ab]	0	100	100
3.6×10^5	72.0[bc]	28[bcd]	62.5[ab]	37.5[ab]	50	50	100
1.84×10^5	64.0[cd]	36[bc]	73.3[a]	26.7[b]	0	100	50
9.2×10^4	48.0[d]	52[ab]	60.4[ab]	39.6[ab]	16.6	83.3	40
Control	24.0[e]	76[a]	21.7[b]	78.3[a]	50	50	15.3
F. value (df)	12.431 (6)	9.639 (5)	3.272 (5)	1.760 (4)			–
P value	0.000	0.000	0.022	0.177			–

Means with different letters are significantly different at $P < 0.05$.

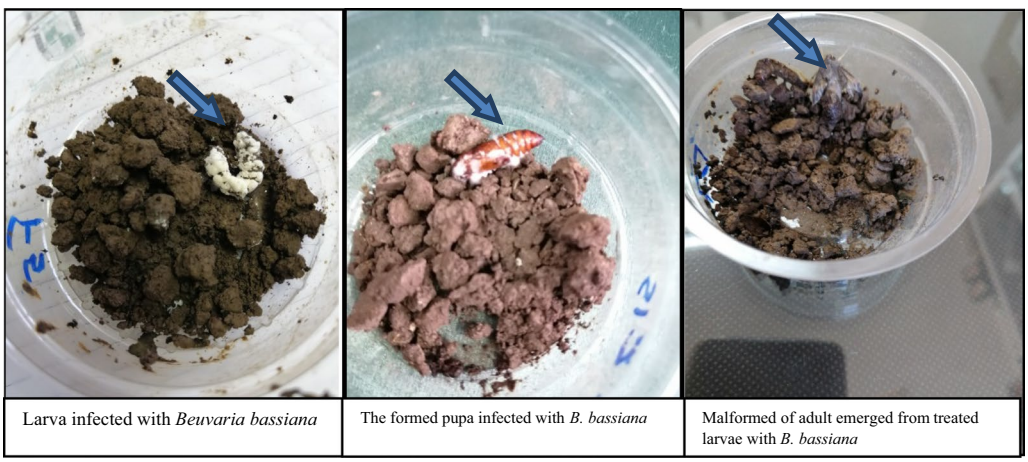

Fig. 2 Effect of entomopathoginic fungus isolated from the soil on different stages of *Agrotis ipsilon*

by Safavi (2010). Temperature plays a great role in the growth rates of *B. bassiana*. In general, the optimal temperature for the growth mycelium of *B. bassiana* is 25 °C depending on the fungal strain (Goettel and Inglis 1997). Ekesi et al. (1999) stated that the best growth rate of *B. bassiana* ranged at 25–28 °C in many isolates but few ones grew better at 30 °C. Shimazu (2004) studied the effect of temperature on the growth rate of *B. bassiana* and observed that the germination rate reached nearly (100%) at 25–30 °C while no germination was found at 34 °C or above. It was also found that the fungus does not die at high temperatures, while it recovers its growth when transferred to suitable temperatures. Wongwanich et al (2017) tested 3 isolates of *B. bassiana* at high temperatures (33 and 35 °C) and observed that no isolates grew at 35 °C. All previous results in the relation between temperature and the fungus development studies are acceptable with the present results. Using of *Galleria* larvae as an insect model for testing the activity of isolated fungus in this study was accepted by several researchers. Mortality of *G. mellonella* larvae may be due to the characteristic of the fast-growing opportunistic fungus, which may infect, injured or weaken the insect (Hajek 1997). The applied different concentrations of *B. bassiana* achieved variable results on larval mortality of *G. mellonella*. Then from this assay *B. bassiana* was the most promising agent upon achieving the highest corrected mortality percentage (96%) at concentration of 6.4×10^5 spores/ml after 10 days. In similar studies by Fergani and Yehia (2020), it was found that *B. bassiana* at concentration of 1×10^7 spores/ml against the same insect caused a very high mortality rate (98.33%) after 120-h exposure period. In another study by Abd El-Ghany et al. (2012) using different concentrations of isolated *B. bassiana* against *G. mellonella* larvae, the mortality rate increased according to spore suspension concentration reaching the maximum level (100%) at 40×10^6 spores/ml after 10 days. Several studies have discussed the effect of *B. bassiana* isolation on many lepidopterous pests such as *S. frugiperda, Helicoverpa zea, Plutella xylostella* L., *Ostrinia nubilalis* and *A. ipsilon* (Ibrahim and Gabr 2020). In the present results, the fungus toxicity reached (100%) mortality for treated last instar larvae of *A. ipsilon* at a concentration of 2.9×10^6 spores/ml after 14 days while the study of Ibrahim and Gabr (2020), the same fungus species in a formulation form prepared by Insect Pathogen Production Unit, Plant Protection Research Institute, Agricultural Research Center in Egypt, recorded (96.67%) mortality rate for third-instar larvae of *A. ipsilon* treated with 1×10^7 spores/ml for 7 days. Long time to get the positive result in our study may be due to using the isolate fungus without any additives or the relatively high tolerant of the old instar of the target insect. The last instar larvae of lepidopteran pests were more tolerant to fungus spores penetration according to the hardening of cuticle layers as stated by Wraight et al. (2010). Kaur et al. (2011) found that fourth-instar larvae of *S. litura* treated with *B. bassiana* isolate at 2.03×10^8 spores/ml, using the dipping technique recorded < 50% mortality. The variation between mortality in this study and the present study may be due to the application technique or the differences between treated insects. The mortality rate of treated larvae with *B. bassiana* was not only the observed result but both sex ratio of emerged adults and malformations were also recorded. As obtained, the imbalance in the sex ratio of emerged adults at low concentrations was very clear in female percentages to males when compared to control. This note was in agreement with that obtained by Shehzad et al. (2021) when tested other isolate of the same fungi species against another lepidopterous insect (*P. xylostella*). Other study carried out by De Souza et al. (2020) recorded that the sex ratio of emerged adults from treated larvae of *H. armigera* (Family: Noctuidae) was also affected by *B. bassiana* treatment. The imbalance in the sex ratio of emerged adults from treated larvae with the isolated fungus as shown in our findings, and results of other researchers may be due to sensitivity variations between different species to the fungus spores and some other unknown factors. A significant decrease in pupal formation and high malformation of emerged adults were also found in present results. The same results were observed by Abd EL-Wahed (2011) who evaluated *B. bassiana* isolate against second-instar larvae of *A. ipsilon.* Kaur et al. (2011) studied the effect of *B. bassiana* on development of *S. litura*, and it was found that low concentrations of *B. bassiana* led to a significant increase in the number of malformed adults as well as halving of adult emergence. On the other hand, pupal formation of *S. litura* was significantly affected by *B. bassiana* at a concentration of 1×10^8 spores/ml, while it had no effect on adult emergence as recorded by Ullah et al. (2019). Fergani and Refaei (2021) recorded morphogenetic abnormalities of *S. littoralis* adults after treatment of larvae with 1×10^8 and 1×10^9 spores ml^{-1} of *B. bassiana*. The observed malformation of emerged adults resulted from lepidopterous larvae treated with different isolates of *B. bassiana* as stated in the present and several other studies may be due to the findings obtained in Kaur et al. (2011) study. They recorded that the sublethal effects on development of the insect were very clear at low concentrations, the fungus has deformed the cuticle of larvae or pupae during molting that depend on nutrients to form the new cuticle and any imbalance of

hemolymphatic nutrient by infection with the fungus will affect any steps of molting processing.

Conclusions

The EPF *B. bassiana* is known as a promising bioagent against many insect pests. Isolation and identification of fungi were the best way to increase efficiency, especially against local pests. The effective isolate was found in clay soil. The fungus reproduced optimally at a temperature of 25–30 °C. *G. mellonella* larvae were used in the study. A high larval mortality, a significant decrease in pupation and an increase in malformed of emerged adults in treated larvae were recorded as a result of treatment of the last instar larvae of *A. ipsilon* with the isolate. Use of the isolate and re-application of *B. bassiana* for the control of many insect pests, especially those remain throughout their life cycle or some of their stages in the soil, can be recommended.

Abbreviations

EPFs: Entomopathogenic fungi; PDA: Potato dextrose agar.

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Author contributions

AAAI was a major contributor in writing the manuscript and he is the owner of the idea, KSSH did all experiments, analyzed and interpreted the data, and SAF was responsible for the identification of the fungus. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

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