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Pathogenicity of the entomopathogenic fungus, *Beauveria bassiana* (Bals.) Vuill. endophytic and a soil isolate against the squash beetle, *Epilachna chrysomelina* (F.) (Coleoptera: Coccinellidae)

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Abstract

Laboratory and field bioassays were conducted to evaluate the pathogenicity of an endophytic and a soil isolate of the entomopathogenic fungus, *Beauveria bassiana* (Bals.) Vuill. against different stages of squash beetle, *Epilachna chrysomelina* (F.) (Coleoptera: Coccinellidae). Both isolates were identified by ITS rDNA sequence analysis. Both isolates were pathogenic to the squash beetle; however, their potential was different according to the conidia concentration and the exposure period. Three days post treatment, (100%) mortality rate was obtained, when the first and second larval instars were treated by the *B. bassiana* ES (soil isolate) compared to 83.67 and 72.60%, respectively when treated with the endophytic isolate. A percentage of 17.67% malformation occurred among the adults that emerged from treated pupae. The highest mortality percentage under field conditions were 28.67 and 22.33% for larvae and adults, respectively.

Keywords: Pathogenicity, *Beauveria bassiana*, Endophyte, *Epilachna chrysomelina*, Soil, Iraq

Background

The squash beetle, *Epilachna chrysomelina* (F.) (Coleoptera: Coccinellidae), is a common pest of cucurbit plants in Iraq. It causes a severe damage to most of the plants of family Cucurbitaceae (Al-Iraqi 1978). Both larvae and adults feed on the epidermal tissues; the larvae confine their attack to the lowest surface of the leaves. Adults usually feed on the upper surface of leaves (Khan et al. 2000). The damage to the leaves reduces the vegetative production of the host plant and consequently affects the plant growth and yield (Awadalla et al. 2011). Aldigail et al. (2013) in Saudi Arabia reported that *E. chrysomelina* as one of the phytophagous insects of cucurbit plants and is considered an economic pest. In addition, the severe damage of *E. chrysomelina* is capable of transmitting the squash mosaic virus (SQMV) (Cohen and Nitzany 1963; Campbell 1971 and Lockhart et al. 1982). Smith et al. (2017) recorded *Epilachna varivestis* as an efficient vector of several soybean- infecting viruses, including Bean pod mottle virus (BPMV) in the USA. Today the entomopathogenic fungus, *Beauveria bassiana*, is used for the control of many insects in greenhouses and fields; however, field data on the impact of the fungus attacking coccinellids is limited but suggests that natural infection levels are less than 20% (Ceryngier 2000; Beyene et al. 2007). Ghosh and Chakraborty (2012) reported that microbial pesticides such as *B. bassiana* provided only 39.56% suppression of *Epilachna* beetle population.

In Iraq, no biological agents have been reported on *E. chrysomelina*, so this study aimed to evaluate the pathogenicity of two isolates of *B. bassiana* (soil and endophytic isolate) against various stages of *E. chrysomelina* under laboratory and field conditions to develop application strategies suitable for future use in biological control.

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Fig. 1 Cages used for field experiments

Materials and methods

Preparation of the entomopathogenic fungus

Two isolates of *B. bassiana* were used; *B. bassiana* ES isolated from soil samples, collected beneath fallen litter under plants that are regarded as most suitable hibernation sites for sunn pest (The most important insect attacks wheat plants in Iraq) from Gara Mountain (N 37 1.51″ E 43 23 34″, 2066 m above sea level) and *B. bassiana* EE isolated from cucumber leaves collected from Amadia district (N 37.0917°, E 43.4877°, 1122 m above sea level) Duhok city, Kurdistan region, Iraq. The extraction of isolate DNA was done according to a commercial animal and fungi DNA preparation kit protocol (Jena Bioscience, Germany). Genomic DNA was used as a template for PCR amplification of ITS region, using universal primers ITS5 and ITS4 (White et al. 1990). The sequencing was performed at Macrogen Company, Korea, and submitted to GenBank (GenBank accession MH374537 and MH374538, respectively). Dried and living cultures were deposited at the mycology bank, Department of Plant Protection, College of Agriculture, Duhok University, BEG22 and BEG23, respectively.

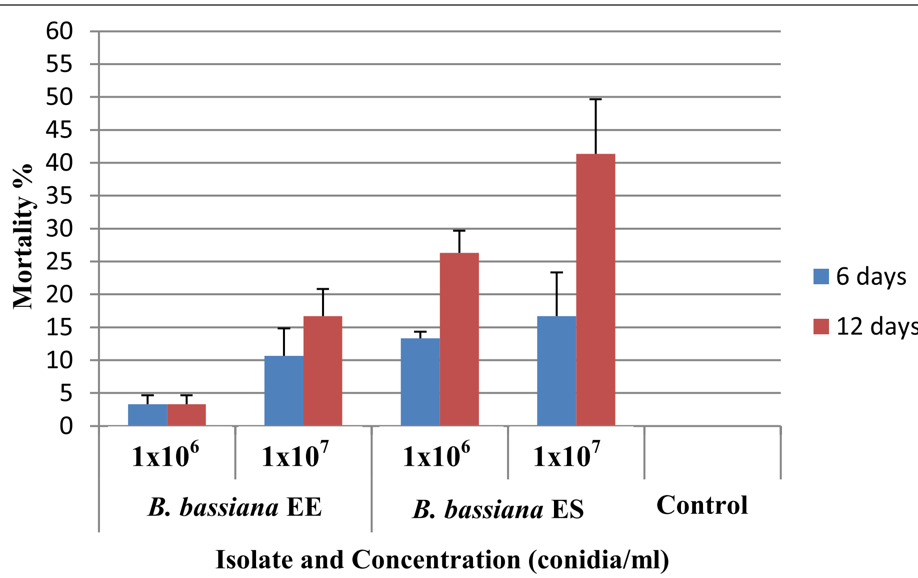

Fig. 2 Pathogenicity of both *Beauveria bassiana* isolates to squash beetle *Epilachna chrysomelina* adults under laboratory conditions

Table 1 Pathogenicity of *Beauveria bassiana* isolates to squash beetle *Epilachna chrysomelina* larval instars, 3 days post treatment

Isolate	Concentration Conidia /ml	Corrected mortality %/instar larvae			
		First instar larvae	Second instar larvae	Third instar larvae	Fourth instar larvae
B. bassiana EE	10^6	61.33 ± 5.77 c	45.20 ± 5.77 c	39.67 ± 6.67 c	24.99 ± 4.17 b
	10^7	83.67 ± 8.29 b	72.60 ± 8.29 b	42.86 ± 6.67 c	32.14 ± 6.67 b
B. bassiana ES	10^6	89.73 ± 10.7ab	89.36 ± 8.77ab	64.29 ± 8.12 b	37.71 ± 8.12 b
	10^7	100.00 ± 0.00a	100.00 ± 0.00a	82.14 ± 10.22a	53.57 ± 10.33 a
Control		16.70 ± 3.33 d	16.70 ± 3.33 d	3.33 ± 1.01 d	6.67 ± 2.33 c

Means followed by a common letter within the same column are insignificantly different at 5% level of probability (Duncan's multiple-range test)

Culture of the squash beetle *Epilachna chrysomelina* for laboratory and field experiments

Adults of *E. chrysomelina* (females and males) were collected from infested melon fields at Tilakru village (37° 03′ 45″ latitude, 42° 51′ 35″ longitude and 637 m above sea level), located at northwest of Duhok by early July 2016. Adults were placed in wooden cages measured (75 × 75 × 75) cm, with one face of glass, while the other sides were covered by sieves under growth chamber laboratory conditions (26 ± 2 °C and 14:10 L:D) in Plant Protection Department/College of Agriculture/Duhok University. The cages were supplied daily by pumpkin leaves, fixed inside a jar, and filled with water daily to keep the leaves fresh (Hassan 2003). The cages were also supplied with pieces of pumpkin fruit to enhance mating and ovipositional sites to obtain *E. chrysomelina* different stages for laboratory and field experiments.

Pathogenicity of both isolates under laboratory conditions

Under laboratory conditions, two concentrations (10^6 and 10^7 conidia/1 ml water) were used to evaluate the pathogenicity of both *B. bassiana* isolates to the adults which were sprayed directly by 3 ml of spore suspension/ isolate/ concentrations/ replicate. Tween 80 at a conc. of 0.02% was added to the suspension. Four replicates were determined (10 adults/replicate) in a small plastic container (20 × 10 × 10 cm) lined with moistened filter paper, supplied with fresh and clean pieces of pumpkin leaves and fruit when required. For control treatment, the replicates were sprayed with 3 ml of distilled water by a new parfan sprayer (50 ml capacity). The application was repeated twice. The mortality percentage was recorded daily for 12 days after treatment. Cumulative mortality counts obtained from experiments were corrected for natural mortality, using Abbott's formula (Abbott 1925).

For the 4 larval instars, each 10 larvae/instar/isolate/concentration/replicate were spread out individually in a Petri dish of 9 cm diameter, containing moistened filter paper in the bottom and then sprayed with isolate suspension (3 ml/ replicate) in two concentrations (10^6 and 10^7 conidia/ml), while the control treatment was sprayed by distilled water, using a new parfan sprayer (50 ml capacity). Fresh leaves were provided daily to the larvae and monitored to record the mortality percentage for 3 days. The same procedure was used for the pupal stage; however, the mortality rate was recorded up to the seventh day posttreatment. Data of lab experiments were statistically analyzed by SAS program using a complete randomized design (CRD) with four replicates and the means were compared, using Duncan's multiple-range tests at $P \leq 0.05$.

Pathogenicity of both isolates under field conditions

A randomized complete block design (RCBD), with four replicates, was designed in a pumpkin field at the College of Agriculture. A branch of pumpkin's plant with three to four leaves was determined for each replicate. The plant branch was surrounded by a steel frame and then covered with a muslin cloth bag provided with a zipper (Fig. 1). Ten adults

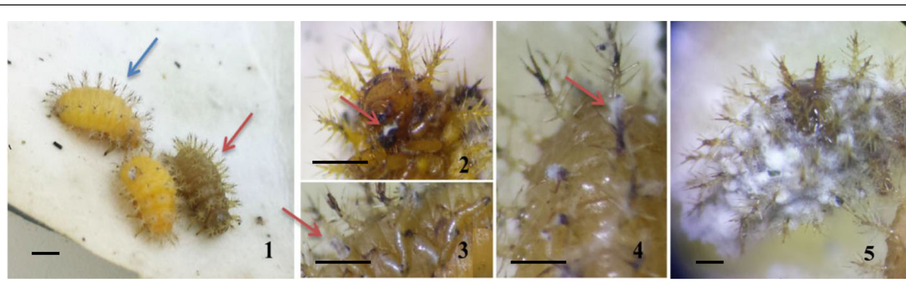

Fig. 3 *Epilachna chrysomelina* fourth instar larvae infected with *Beauveria bassiana*. 1—(Red arrow) Infected larva (color changed to brown) and (blue arrow) healthy larva (shiny yellow) scale bar = 2 mm. 2—Mycelium emerged from mouth parts. 3—Mycelium emerged from leg parts. 4—Mycelium emerged from setae. 5—larvae covered with mycelium 2–5 scale bar = 1 mm

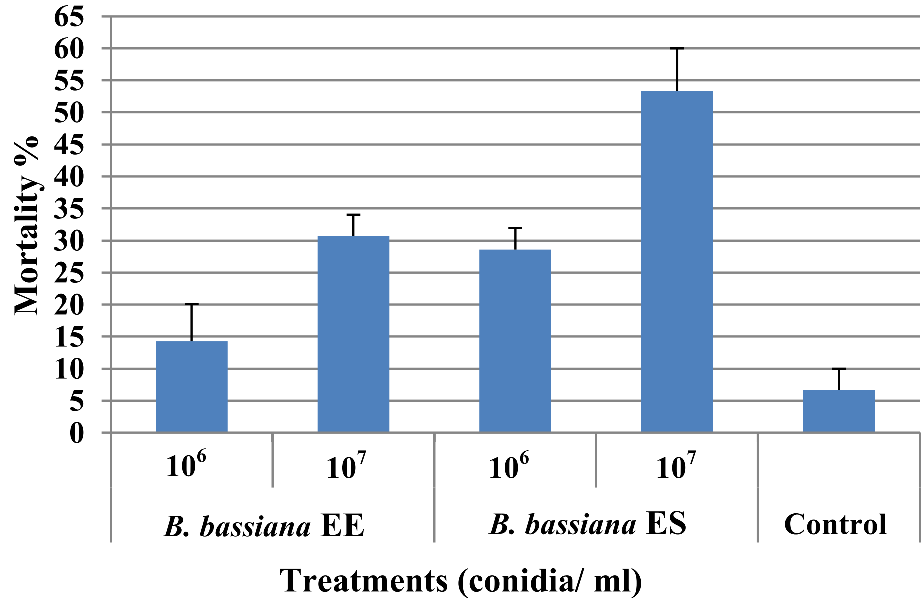

Fig. 4 Pathogenicity of *Beauveria bassiana* isolates to squash beetle *Epilachna chrysomelina* pupae, 7 days post treatment

were added to each replicate and then the adults at the plant were sprayed by 15 ml of each fungus isolate conidial suspension (1×10^7 conidia/ml) of water with 0.02% Tween 80. For the control treatment, the plant was sprayed by 15 ml distilled water. The mortality percentage of adults was recorded after 4, 8, and 12 days of treatment. For larvae, at each replicate, 10 larvae (fourth instar) were added and then the larvae with plants were sprayed by 15 ml of each fungus isolate conidial suspension (1×10^7 conidia/ml) of water with 0.02% Tween 80. The mortality percentage of the fourth instar larvae was recorded after 1, 2, and 3 days post treatment. The data of the field experiments were statistically analyzed by SAS program, using a randomized complete block design (RCBD) with four replicates, and the means were compared, using Duncan's multiple-range tests at $P \leq 0.05$.

Results and discussion

Pathogenicity under laboratory conditions

Corrected mortality percentages of *E. chrysomelina* adults treated with the two concentrations (10^6 and 10^7 conidia/ml) of endophytic isolate *B. bassiana* EE and soil isolate *B. bassiana* ES, respectively, under laboratory conditions are illustrated in Fig. 2. Both isolates showed pathogenicity to adults. The highest mortality percentage (16.67%) was recorded by the adults treated with *B. bassiana* ES isolate at (1×10^7 conidia/ml) 6 days post treatment. No significant differences of mortality rates were recorded by the endophytic isolate at the same concentration as 10.67%. The final cumulative mortality percentage of adults, 12 days post treatment, were 41.33 and 26.33%, when the adults were treated by *B. bassiana* ES isolate at 10^7 and 10^6, respectively. Mortality of adults treated with the endophytic isolate *B. bassiana* EE was lower than 20% compared to 0% in the control treatment.

The data in Table 1 showed that both isolates of *B. bassiana* were found to be pathogenic to the four instars of *E. chrysomelina* larvae under laboratory conditions. The mortality percentages ranged between 61.33 and 100%) for the first instar larvae than 16.70% in the check. The highest mortality percentage was reached (100%) for the second instar larvae treated with *B. bassiana* ES isolate (1×10^7 conidia/ml) than 72.60%, when treated with endophytic isolate *B. bassiana* EE at the same concentration. The mortality percentage decreased as larvae developed in age. The highest mortality percentages (82.14 and 53.57%) were for third and fourth larval instars treated with *B. bassiana* ES isolate, respectively, while it was almost equal to duplicate when the third and fourth larval instars were treated by the same concentrations of the endophytic *B. bassiana* EE exhibiting 42.86 and 32.14% mortality, respectively.

Obtained results agree with the previous studies of Olson and Oetting (1999), Parker et al. (2003), and Assaf (2007) who reported that the mortality percentage was low at low concentrations and duplicated by increasing the spore concentration and period of exposure. Assaf et al. (2011) recorded 53.90% in the third instar larvae of poplar leaf beetle *Melasoma populi* L. after 6 days of treatment with *B. bassiana* at 1×10^7 conidia/ml. Ramirez-Rodriguez and Sánchez-Peña (2016) mentioned that 98.3% of the fall armyworm, *Spodoptera frugiperda* third instar larvae inoculated with *B. bassiana* isolated from soil died by day 14, while the same strain as an endophyte from maize plant killed (75%). Both differed significantly from each other than the control.

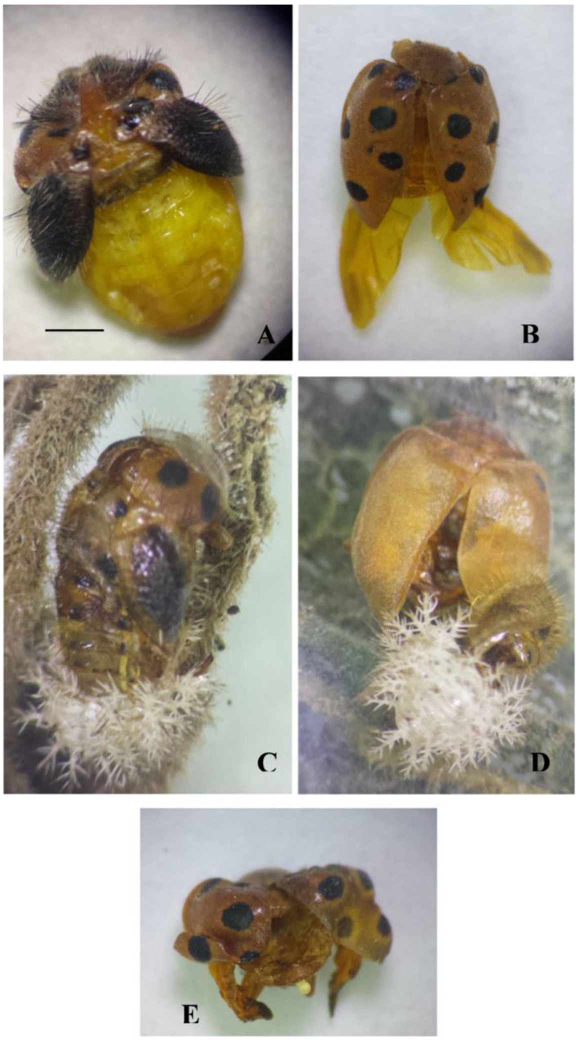

Fig. 5 Malformation in adults emerged from treated pupae with *Beauveria bassiana* ES (1×10^7) **a-e** scale bar = 2 mm

Mohamed (2016) reported that different isolates of the same fungal species were affected by many factors, as insect species, experiment conditions, the materials used in the bioassays, and the pathogenicity effect of isolates.

Larvae affected by *B. bassiana* showed characterized symptoms as feedless, motionless, and color changes from the shine yellow to brown as a result of mycelium growth and fruiting structures emerging from the cadaver and produce infectious spores (Fig. 3). Mirza (2014) reported that the tomato moth, *Tuta absoluta*, fourth instar larvae infected with *B. bassiana* changed their color from pink green to brown within the larvae mines.

For pupae (Fig. 4), *B. bassiana* ES (1×10^7 conidia/ml) significantly scored the highest mortality percentage reaching 53.33% compared to other treatments, followed by 30.71% caused by *B. bassiana* EE isolate at the same concentration, which was similar to that caused by *B. bassiana* ES at 1×10^6 conidia/ml as 28.59%. The lowest percentage of mortality (14.28%) was recorded at *B. bassiana* EE (1×10^6 conidia/ml) but insignificantly with control treatment (6.67%).

A percentage of 17.67% malformation occurred with adults emerged from pupae treated with *B. bassiana* ES at higher concentration as compared to 0% in other treatments and control. Deformation at wings, legs, and abdomen was recorded. The malformed adult was shorter in length with crumpled wings (Fig. 5b) or wings reduced highly in length (Fig. 5a). The abdomen was reduced in length and deformed wings did not reach the ovipositor (Fig. 4e). The deformed adult was unable to extricate from the old cuticle (Fig. 5c, d).

Malarvannan et al. (2010) recorded 75% malformation in *Spodoptera litura* pupae treated with *B. bassiana* at 2.4×10^7 conidia/ml, and attributed that to the decrease in the juvenile hormone titre and its associated disturbances in oogenesis, larval-pupal, and pupal-adult moults. Decrease in juvenile hormone influences the storage proteins and fat which are highly essential for metamorphosis, moulting, and reproduction (Koul and Isman 1991).

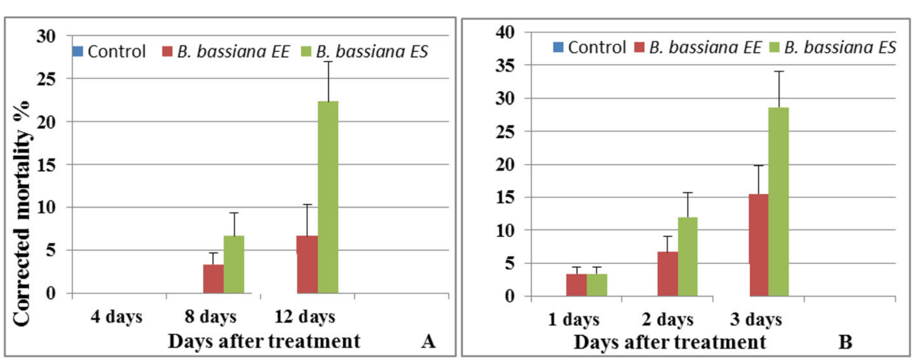

Fig. 6 Pathogenicity of *Beauveria bassiana* EE and *Beauveria bassiana* ES on squash beetle *Epilachna chrysomelina* larvae and adults under field conditions. **a** = adults, **b** = larvae

Pathogenicity under field conditions

As shown in Fig. 6, the mortality percentages of *E. chrysomelina* adults and larvae caused by the endophytic isolate (EE) and soil isolate (ES) of *B. bassiana* at 1×10^7 conidia/ml. The results showed that no mortality was recorded, 4 days post adults' treatment by the suspension of both isolates of *B. bassiana*. The highest mortality percentage (6.67%) was recorded, 8 days post treatment, at the adults treated with *B. bassiana* ES isolate, followed by (3.33%) for adults treated with *B. bassiana* EE isolate. It was zero % in the control treatment.

The results also showed significant differences between both isolates in their effect on adult's mortality after 12 days of treatment, the adults treated with *B. bassiana* ES isolate mortality percentage reached 22.33% and 6.67% for adults treated with *B. bassiana* EE isolate. Riddick et al. (2009) stated that coccinellid mortality resulting from *B. bassiana* infection occurs naturally but has not clearly been shown to regulate populations. For larvae, the mortality percentage, 1 day post treatment was 3.33% recorded with the larvae treated with both isolates compared to zero % in control treatment. The mortality percentage increased to reach 12.00 and 28.67% with larvae treated with *B. bassiana* ES after 2 and 3 days of treatment, respectively, and significantly differed from that recorded from larvae treated with *B. bassiana* EE isolate (6.67 and 15.5%) after 2 and 3 days of treatment, respectively.

Conclusion

Pathogenicity evaluation indicated that both isolates of *B. bassiana* were pathogenic to the squash beetle *E. chrysomelina* larvae and adults under laboratory and field conditions. The results showed a decrease in mortality rates as larvae developed in age and increased with increasing the concentration and exposure period. Malformation occurred with adults emerged from pupae treated with *B. bassiana*. The study suggests that the entomopathogenic fungi can be a tool to control *E. chrysomelina*, the serious pest of cucurbits in Iraq.

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Authors' contributions

FH analyzed the data and have written the manuscript. SA identified the fungus isolates. LA prepared the fungus isolates and squash beetle cultures for bioassays. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors read and approved the final manuscript and gave consent to this publication.

Competing interests

The authors declare that they have no competing interests.

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