Arab Universities Journal of Agricultural Sciences
(2024) 32 (1) 139-148
Website: http://ajs.journals.ekb.eg

Isolation and Characterization of Native Entomopathogenic Fungi and Their Pathogenicity Against Cowpea aphid

Alyaa M Elsawy[1]*, Mohamed S El-Zemaity[1], Yosra Ahmed[2], Walaa El-Sayed[1]
1- Plant Protection Dept, Fac of Agric, Ain Shams Univ, P.O. Box 68, Hadayek Shoubra 11241, Cairo, Egypt
2- Plant Quarantine Pathogens Laboratory, Mycology Research & Disease Survey Dept, Plant Pathology Research Institute, Agricultural Research Centre, Giza, Egypt

*Corresponding author: alyaa1@agr.asu.edu.eg

https://doi.org/10.21608/AJS.2024.267259.1554

Received 4 February 2024; Accepted 8 May 2024

Keywords:

Aphis craccivora, *Beauveria bassiana*, Biocontrol, *Metarhizium anisopliae*, Molecular identification

Abstract: The research aimed to isolate and characterize local entomopathogenic fungi from different agricultural ecosystems and assess their pathogenicity against cowpea aphid. Forty soil samples were collected from different ecosystems. In total, 38 fungal isolates were collected from the cadavers' exteriors of *Galleria mellonella* larvae. Microscopic observation of these fungal isolates revealed that they belonged to nine fungal genera among them *Metarhizium* sp. (3 isolates) and *Beauveria* sp. (5 isolates). Primary experiments showed different bioactivities of the isolates of the two selected genera against the target insect. Only *Metarhizium* isolates (M3 & M5) and *Beauveria* isolates (B1 & B5) were selected for further investigations. Identification of fungal cultures by morphological characters, molecular characterization, ITS sequencing, and NCBI Blast showed that two isolates were confirmed as *M. anisopliae* and two isolates as *B. bassiana*. The bioassay evaluation revealed that *M. anisopliae* (M3 and M5) and *B. bassiana* (B1) exhibited significant virulence against *A. craccivora*, as evidenced by their high LT50 and LC50 values and substantial sporulation on the cadavers. Therefore, they could be used for the formulation of pesticides targeting this pest on cowpea and other vegetable crops.

1 Introduction

The cowpea aphid, *Aphis craccivora*, is a significant plant pest and a highly polyphagous that attacks a diverse range of leguminous crops globally (Juliya 2020) as well as plants from various botanical families (Blackman and Eastop 2017). This species is a prominent insect pest of faba bean, *Vicia faba*, responsible for significant yield loss (Abdel-Raheem et al 2021). Moreover, the cowpea aphid is a vector for the dissemination of the cowpea mosaic virus (CPMV) which can result in substantial reductions in crop productivity and yield losses (Ramanujam et al 2017). Aphid ravenously feeds on soft, rapidly growing plant tissues and induces symptoms of chlorosis, stunting, and delaying the onset of flowering. This reduces crop productivity by depleting plant sap and interfering with photosynthetic processes (Mweke et al 2019). Since many chemical pesticides are registered for use in the management of aphids, farmers heavily rely on their use to control *A. craccivora* on faba bean in order to increase crop profitability and minimize yield losses. Despite the susceptibility of aphids to pesticides, their high fecundity and ability to develop resistance to some chemicals make them difficult to control.

In addition, chemical pesticides pose health risks, raise concerns about environmental pollution, have negative impacts on nontarget species and kill natural enemies of *A. craccivora* (Mweke et al 2020). Scientists are, therefore, adopting alternative non-chemical strategies to control insect pests (Ahmad et al 2023, Hamza et al 2023). Entomopathogenic fungi exhibit significant pathogenicity towards certain plant pests including aphids and are responsible for regulating plant populations in natural ecosystems (Kavallieratos et al 2013). The use of commercial biopesticides derived from entomopathogenic fungi has proven to be effective in the control of various aphid species, leading to a decreased reliance on chemical control (Mohammed et al 2018, Juliya 2020). Among these entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana* were the most widely species used commercially (Imoulan et al 2017, Batta 2018). However, the large-scale application of entomopathogenic-based biopesticides is hampered by their low efficacy and persistence under field conditions. Hence, integrating entomopathogenic-based biopesticides with other pest management strategies, like cultural control, can enhance their efficacy in controlling aphids (Mweke et al 2020). In addition to the morphological identification of fungi, recent molecular phylogeny techniques are utilized to detect and identify promising fungi in controlling such pests (Bich et al 2021). The present research aimed to i) isolate indigenous soil-borne entomopathogenic fungi; ii) characterize the selected isolates using morphological and molecular analysis and iii) assess their pathogenicity against the cowpea aphid under laboratory conditions.

2 Materials and Methods

2.1 Sampling sites and soil collection

Forty soil samples were collected from various ecosystems at El Behera and Giza governorates, Egypt. Five samples of non-rhizospheric soil were collected from each site during the growing of faba bean, tomatoes, lime, date palm, wheat, onion, and banana. Each soil sample (250 g each) was taken from a depth of 0–10 cm at different field sites using a trowel after removing litter and weeds. Samples were separately put in plastic bags and maintained at -20°C in the laboratory until analysis and fungal isolation.

2.2 Isolation and purification of entomopathogenic fungi

2.2.1 Rearing of the greater wax moth, *Galleria mellonella*

Cultures of *G. mellonella* larvae were obtained from the Bio-Insecticide Production Unit, Plant Protection Research Institute (PPRI), Agricultural Research Centre (ARC), Giza, Egypt. The insect culture of *G. mellonella* was reared on an artificial diet in plastic containers filled with 200 g of the mixture (350 g of wheat flour, 200 g of corn flour, 130 g of milk powder, 70 g of backing yeast powder, 100 ml of honey and 150 ml-glycerine). Insects were reared at 25 ± 2°C, relative humidity of 75 ± 5,% and a photoperiod of 12 h light:12 h dark cycle. The eggs hatched within 3-4 days and larvae were fed on the mentioned diet. Last instar larvae were collected to be used for entomopathogenic fungi isolation using insect bait.

2.2.2 Insect baiting method

The entomopathogenic fungi were isolated from soil samples using the Galleria bait method (Zimmermann 1986). The 3rd or 4th instar larvae (about 30 days post-hatching) were utilized as bait. The wax moth larvae were submerged in water heated to 55°C for 25-30 s in order to hinder their capacity to generate silk webbing within the soil (Meyling and Eilenberg 2006). Containers (50 mL each) were filled with soil up to the lid. A spray of water was added to add moisture to the soil. In each container, 5 larvae were emerged into the soil then covered by the lid and incubated at 25±2°C for 2 weeks considering turning around the containers from time to time. The larvae were checked on days 7 and 14 days after incubation. The dead larvae were collected and surface sterilized for 3 min using a 2% solution of sodium hypochlorite and then rinsed twice with sterile distilled water. All larval specimens exhibiting symptoms of fungal infection were transferred into a sterile petri dish containing moist filter paper. After incubating the plates for 7–14 days at 25 ± 2°C, fungal isolates were collected from the larvae displaying external mycelial growth, and then cultured on Sabouraud dextrose agar medium (SDA) and incubated at 26± 2°C for 10-15 days. The developing fungal colonies were checked under the light microscope (100x and 400x) and were transferred to new SDA plates. The cultures were purified by single spore technique and kept on Potato Dextrose Agar (PDA) slants at 5°C for further experiments.

2.3 Morphological identification of entomopathogenic fungi

Preliminary identification was performed by analysing morphological characteristics including colony morphologies, asexual structures, conidiophores shape and length, conidia shape and size, and conidiogenous cells according to the identification key of Barnett and Hunter (1986).

2.4 Molecular identification of entomopathogenic fungi

2.4.1 DNA extraction

The identification of four selected isolates was confirmed by the molecular characterization of the internal transcribed spacer (ITS) region. The genomic DNA (gDNA) was extracted according to Dellaporta et al (1983).

2.4.2 Amplification of PCR and sequencing

The PCR amplification of the ITS region was carried out using the ITS4 and IT5 primer pair according to White et al (1990). The PCR amplification was conducted using a thermocycler (Applied Biosystems, Foster City, CA); the PCR amplicons were purified and then sequenced in two directions using the ITS4 and ITS5 primers by Macrogen company in Korea.

2.4.3 Sequence comparisons and Phylogenetic analysis

Using Blastn, the consensus sequences of the four isolates were compared with the partial sequences of the ITS region available in the National Centre for Biotechnology Information NCBI GenBank. The comparison of pair-wise nucleotide sequences was done by the Muscle algorithm incorporated in the species demarcation tool (SDT**)** (Muhire et al 2014). The phylogenetic relationship of *Metarhizium* and *Beauveria* species was inferred by the Maximum Parsimony method (MP). The MP tree was constructed by the Subtree-Pruning-Regrafting (SPR) method at search level 1 (Nei and Kumar 2000), the branches indicate the proportion of duplicate trees in which the corresponding taxa formed clusters during the bootstrap test, which consisted of 1000 repetitions. The first 10 trees were generated by randomly adding sequences together. A total of 21 nucleotide sequences from each of *Beauveria* and *Metarhizium* species were used in this study. First, second, third, and non-coding positions were all accounted for MEGA11 (Tamura et al 2021). The phylogenetic trees of *Metarhizium* and *Beauveria* were visualized using MEGA11. The source and place origin and NCBI GenBank accession numbers of selected internal transcribed spacer ITS sequences used in this study are given in **Table 1**.

2.5 Screening and bioassays of entomopathogenic fungal isolates against cowpea aphid

Colonies of cowpea aphid, *A. craccivora* originally obtained from Syngenta Lab. (Qalyubia government, Egypt), were established on faba bean plants, grown in plastic Potts (11 cm diam.). The plants were kept in a growth chamber with a temperature of 23 ± 2 °C, a relative humidity of 65±5% and a photoperiod of 16h of light and 8h of dark cycle. For bioassay experiments, two isolates of *M. anisopliae* and two isolates of *B. bassiana* were initially screened for their pathogenicity against the adults of cowpea aphids using one concentration of 1×10^8 conidia/mL. The spore suspension was prepared from 12-to 14-day-old cultures grown on SDA plates at 24±2°C. The colony surface was scraped with a sterile glass slide and harvested into 20 mL of sterile distilled water. The suspensions were filtered using a double-layer muslin cloth and transferred to a conical flask containing distilled water with a drop of tween-80 to keep the conidia dispersed uniformly and then shaken thoroughly for 10 min. The conidia were counted in the suspension using a hemocytometer and the required concentrations were prepared by diluting the stock inoculum with sterile distilled water. The bioassay experiment was repeated using 2 fungal isolates of *M. anisopliae* (M3 and M5) and only one fungal isolate of *B. bassiana* (B1) using 1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia/mL as serial conidial concentrations. Faba bean plants, 10-15 days old, were cut, the stem was wrapped with a piece of wet cotton, covered with a piece of tin foil to prevent rapid wilting, and then placed in perforated plastic containers. Apterous adults of aphids were carefully transferred onto fab bean plants using a soft brush and thereafter left to settle on the leaves before undergoing any treatment (Mweke et al 2018). Every plastic container containing the insects and the plants was sprayed with 10 mL of each concentration of the spore suspension. Each treatment was replicated four times, with each replicate containing 50 insects. The control insects were sprayed with sterilized distilled water with 0.05% Tween-80. The containers containing tested insects were allowed to dry for 5 min to eliminate any remaining moisture and then covered with perforated lids to let air circulate. All the treated insects were kept at lab temperature (26 ± 2 °C). The

data on mortality were collected daily for 7 days after treatment. The deceased insects were placed in Petri dishes that were lined with moist tissue paper to promote fungal growth and induce sporulation. Fungi-induced mortality was verified by microscopic examination of fungal growth, the presence of hyphae, and spores on the deceased insect's surface (Ekesi et al 2000).

2.6 Statistical Analyses

The data of mortality were calculated using Abbott's formula (Abbot 1925). Median lethal concentration values (LC_{50}), the median Lethal Time (LT_{50}), and the toxicity lines were statistically measured according to Finney (1971) using software computer programs Ldp Line and SigmaPlot.

3 Results and Discussions

3.1 Isolation and Morphological Identification

The examination of the soil sample showed that the bait larvae of *G. mellonella* had some indications and symptoms of fungal infection. A total of 38 fungal isolates were pre-described as entomopathogens when mycelial growth was observed outside the cadavers of *G. mellonella* larvae. Microscopic observation of these fungal isolates showed morphological and cultural characteristics similar to 9 fungal genera including *Aspergillus* sp. (10 isolates), *Alternaria* sp. (7 isolates), *Beauveria* sp. (5 isolates), *Cladosporium* sp. (4 isolates), *Metarhizium* sp. (3 isolates), *Paecilomyces* sp. (1 isolate) *Penicillium* sp. (5 isolates), *Stemphylium* sp. (2 isolates), and *Verticillium* sp. (1 isolate). The isolates of *Metarhizium* spp. were initially identified using morphological and colony characteristics. After two weeks of incubation, isolates developed white colonies with compact green clusters. Microscopic observations of the isolates revealed the presence of septate mycelium and conidiophores arranged in the shape of a candelabrum. Each septum included two or three branches that were tightly entangled. The conidia exhibited a green colour and cylindrical shape as well as forming chains that aggregated into cylindrical columns. *Beauveria* species colonies were distinguished by their smooth, rounded shape, powdery to fluffy appearance, white colour on the surface, yellowish reverse side, and dense and dispersed growth. The microscopic observation revealed globose and round conidia; in addition, the conidia-producing cells formed a globular or bottle-like morphology, which was mostly irregular in shape. In this study, *Metarhizium* and *Beauveria* were the main abundant entomopathogenic fungal genera collected from the soil samples**.** Several species within the Phylum Ascomycota, including *Metarhizium anisopliae* and *Beauveria bassiana*, develop their life cycle in the soil, particularly when they are not relying on insect hosts. Gürlek et al (2018) emphasized that soil serves as a significant reservoir for entomopathogenic fungi.

The use of the Galleria bait method for isolating entomopathogenic organisms has been demonstrated to be a more efficient technique for isolating native species compared to the conventional plating on media approach. Zimmermann (1986) proposed the use of *Galleria mellonella* larvae, which are highly susceptible, as the standard method for isolating entomopathogenic fungi. However, other studies have demonstrated that entomopathogenic strains with higher virulence are more commonly isolated from both larval and adult stages of the target insect, rather than from *G. mellonella* (Klingen and Haukeland 2006). On the other hand, the identification of such fungi based on their morphological properties is usually confirmed by molecular characterization (Gürlek et al 2018, Bich et al 2021).

3.2 Molecular characterization and phylogenetic analysis

Molecular characterization confirmed the results of the morphological identification. The selected four isolates were molecularly characterized by partial sequencing of the ITS region using ITS 4 and ITS 5 primers. The *Metarhizium* sp. and *Beauveria* sp. isolates gave clean sequence results. Sequences of the two isolates of *Metarhizium* sp. (M3, and M5) were 99% homologous to other *M. anisopliae* sequences in GenBank. The sequences were deposited in NCBI with the accession numbers OR826406 (*M. anisopliae* M5) and OR826407 (*M. anisopliae* M3) (**Fig 1**).

Beauveria isolates, when sequenced and blasted on GenBank, were identified as *B. bassiana* (**Table 1**) with 100% homologies (**Fig 2**). The nucleotide sequences were deposited in GenBank under the accession numbers OR826409 (*B. bassiana* B1) and OR826410 (*B. bassiana* B5).

From the NCBI database, 21 isolates from each of *Metarhizium* and *Beauveria* species were retrieved and used for the sequence alignment, calculation of pairwise identity using species demarcation tool (SDT), and MP analysis (**Fig 1 and 2**). MP analysis of the ITS sequence data of *M. anisopliae* (OR826406) and *M. anisopliae* M3 (OR826407) made a cluster within the

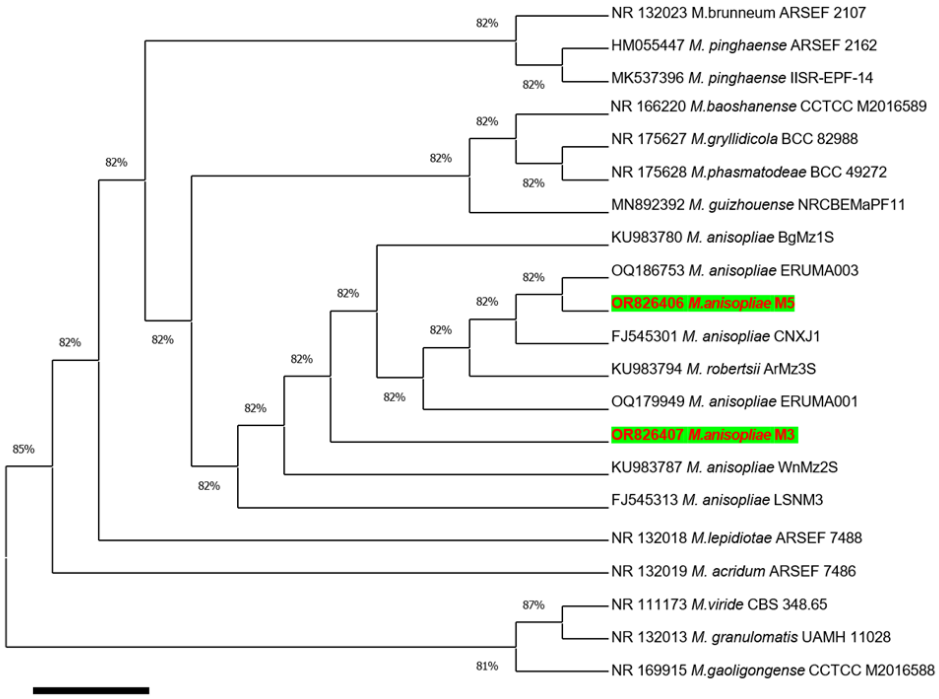

Fig 1. A phylogenetic tree constructed using MP analysis of ITS region for nucleotide sequences of *Metarhizium* spp. The nodes displayed bootstrap values obtained from 1000 replicates. The phylogenetic scale shown at the top of the phylogram represents the level of dissimilarity.

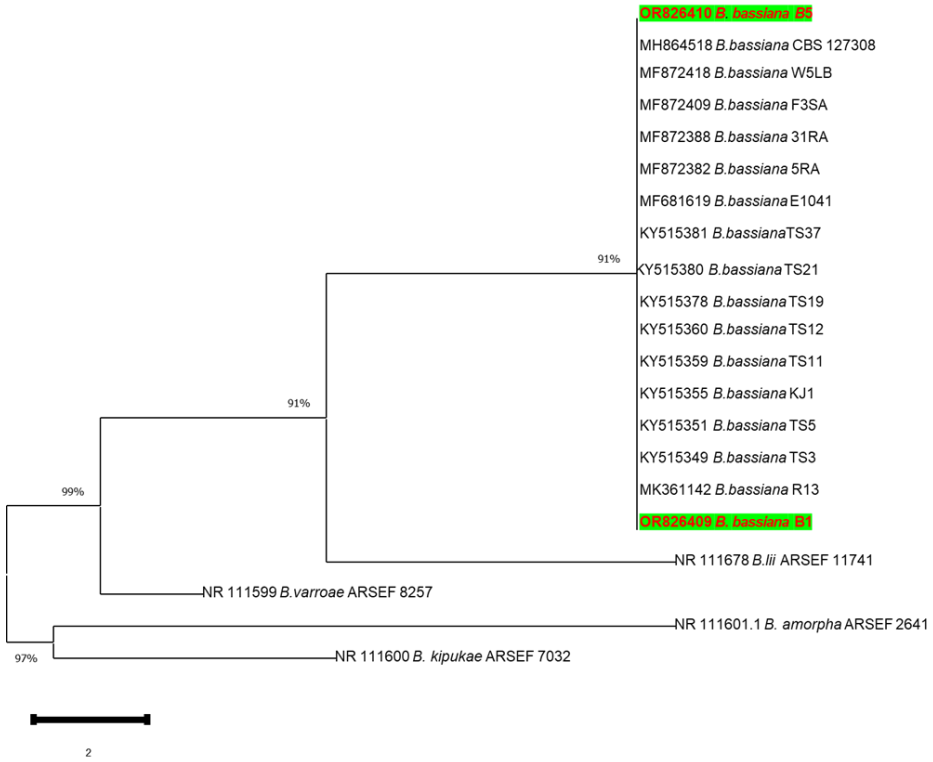

Fig 2. A phylogenetic tree constructed using MP analysis of ITS region for nucleotide sequences of *Beauveria* spp. The nodes displayed bootstrap values obtained from 1000 replicates. The phylogenetic scale shown at the top of the phylogram represents the level of dissimilarity.

Table 1. GenBank accession numbers of selected ITS sequences.

Fungus	**Isolate**	**Source**	**Region**	**NCBI accession No. ITS sequence**
Beauveria bassiana	B1	Soil/ Tomato	Giza, Egypt	OR826409
B. bassiana	B5	Soil/Onion	Giza, Egypt	OR826410
B. bassiana	R13	Soil of halophilic plants	Algeria	MK361142
B. bassiana	TS3	Soil	Iran	KY515349
B. bassiana	TS5	Soil	Iran	KY515351
B. bassiana	KJ1	Soil	Iran	KY515355
B. bassiana	TS11	Soil	Iran	KY515359
B. bassiana	TS12	Soil	Iran	KY515360
B. bassiana	TS19	Soil	Iran	KY515378
B. bassiana	TS21	Soil	Iran	KY515380
B. bassiana	TS37	Soil	Iran	KY515381
B. bassiana	E1041	Soil	USA,	MF681619
B. bassiana	5RA	Soil	USA	MF872382
B. bassiana	31RA	Soil	USA	MF872388
B. bassiana	F3SA	Soil	USA	MF872409
B. bassiana	W5LB	Soil	USA	MF872418
B. bassiana	CBS127308	Soil	USA,	MH864518
B. amorpha	NR 111601	fire ants/ *Solenopsis* sp	Brazil	ARSEF 2641
B. kipukae	NR 111600	NA	USA	ARSEF 7032
B.lii	NR 111678	NA	China	ARSEF 11741
B.varroae	NR 111599	Mite/ *Varroa destructor*	France,	ARSEF 8257
Metarhizium anisopliae	M5	Soil/Broad bean	Behera, Egypt	OR826406
M. anisopliae	M3	Soil/ Common wheat	Giza, Egypt	OR826407
M. anisopliae	BgMz1S	Forest soil	India	KU983780
M. anisopliae	ERUMA003	*Dysdercus cingulatus*	India	OQ186753
M. anisopliae	CNXJ1	Soil/ cotton field	China	FJ545301
M. anisopliae	ERUMA001	*Dysdercus cingulatus*	India	OQ179949
M. anisopliae	WnMz2S	Forest soil	India	KU983787
M. anisopliae	LSNM3	Forest soil	Laos	FJ545313
M.brunneum	*ARSEF 2107*	NA	USA	NR 132023
M. robertsii	*ArMz3S*	Forest soil	India	KU983794
M.lepidiotae	ARSEF 7488	NA	Switzerland	NR 132018
M. acridum	ARSEF 7486	NA	Switzerland	NR132019
M. viride	CBS 348.65	NA	Thailand	NR111173
M. granulomatis	UAMH 11028	NA	Canada	NR132013
M. gaoligongense	CCTCC M2016588	Soil	China	NR169915
M. pinghaense	ARSEF 2162	NA	China	HM055447
M. pinghaense	IISR-EPF-14	Lepidoptera	India	MK537396
M. baoshanense	CCTCC M2016589	Soil	China	NR166220
M. gryllidicola	BCC 82988	NA	Thailand	NR175627
M. phasmatodeae	BCC 49272	NA	Thailand	NR175628
M. guizhouense	NRCBEMaPF11	Insect/*Odoiporus Longicollis Olivier*	India	MN892392

M. anisopliae sequences (**Fig 1**). The phylogenetic tree of *B. bassiana* isolates revealed the presence of a single well-supported clade together with 17 fungal strains of *B. bassiana* with Bootstrap values of 91% and different from the clades of the other *Beauveria* sp. (*B. lii, B. amorpha, B. kipukae* and *B. varroae* (**Fig 2**). The results of phylogenetic analyses confirmed the morphological characterization data and fungal identity as *M. anisopliae* and *B. bassiana* (Gürlek et al 2018, Bich et al 2021).

3.3 Bioassays of entomopathogenic fungi against the cowpea aphid

The initial screening of the 4 isolates using 1×10^8 conidia/mL concentration against the adults of cowpea aphids showed notable variations in their pathogenicity. Isolates of *M. anisopliae* (M3, M5) and *B. bassiana* (B1) had the highest % of mortality, while *B. bassiana* isolate B5 showed weak pathogenicity and was excluded. Data in **Table 2** showed that the % mortality of *A. craccivora* adults was 73.3% after 3rd day and reached 97.7% after 6 days when tested at the concentration of 1x 10^8 conidia/mL of *M. anisopliae* (M5). While *M. anisopliae* isolate M3 showed lower pathogenicity where the % mortality recorded 28.9, 53.6, 71.0 and 83.5 % after the 3rd, 4th, 5th, and 6th day respectively when tested with the highest concentration of 1x 10^8 conidia/mL. *B. bassiana* isolate B1 at the concentration of 1x 10^8 conidia/mL was also found to be pathogenic to *A. craccivora* and caused 86.3% mortality 6 days after the infection. Results presented in **Table 2** and illustrated in (**Fig 3**) revealed that *M. anisopliae* isolate M5 gave the most effect against *A. craccivora*, the LC_{50} value was 1.2×10^5 conidia/mL, while data showed greater LC_{50} values for M3 and B1 which were 4.8×10^6 and 4.8×10^6 conidia/mL respectively. **Table 3** shows the LT_{50} values at different concentrations of *M. anisopliae* and *B. bassiana* against *A. craccivora* adults. The LT_{50} values decreased with the increasing conidial concentrations. LT_{50} values at 1×10^8 conidia/mL were 1.475, 3.8594, and 3.1413 days for isolate M5, M3, and B1 respectively.

The local entomopathogenic fungi isolates have many advantages over exotic isolates, including ecological compatibility with pest species and adaptability to their natural habitat and soil conditions; they also exhibit a reduced risk of non-target organisms (Gürlek et al 2018). Different published articles showed the activity of entomopathogenic fungi (EPFs), including *B. bassiana* and *M. anisopliae*, against many insect pests (Zimmermann et al 2013). Ekesi et al (2000) evaluated the entomopathogenicity of *B. bassiana* and *M. anisopliae* against the cowpea aphid and obtained mortality of between 58-91%. Vu et al (2007) recorded 100% mortality of *Myzus persicae* and *Aphis gossypii* treated with *Lecanicillium lecanii*. In addition, Gürlek et al (2018) stated that *Beauveria* and *Metarhizium* spp. are the most common EPFs and could potentially be valuable in the biological control of *Cydia pomonella*.

Table 2. Mortality % and LC_{50} values of *A. craccivora* adults treated with series concentrations of *M. anisopliae* (isolate M3& M5) and *B. bassiana* (isolate B1) spores.

Entomopathogenic fungal species	Concentration (spore/ml)	Corrected mortality % after different days					LC50 (Spores/ml.)
		2nd	3rd	4th	5th	6th	
M. anisopliae (isolate M5)	1×10^5	0.0	6.3	26.3	37.5	42.5	1.2×10^5
	1×10^6	0.0	12.3	51.9	71.6	81.5	
	1×10^7	0.0	69.5	84	90.3	92.4	
	1×10^8	0.0	73.3	86.9	93	97.7	
M. anisopliae (isolate M3)	1×10^5	0.0	11	19	21	21.8	4.8×10^6
	1×10^6	0.0	22.3	28.5	33	37	
	1×10^7	0.0	28	32	41	43.6	
	1×10^8	0.0	28.9	53.6	71.0	83.5	
B. bassiana (isolate B1)	1×10^5	0.0	4.7	11.6	17.9	20	4.8×10^6
	1×10^6	0.0	2.8	18.6	26.2	29.7	
	1×10^7	0.0	21.9	32.3	40.6	45.8	
	1×10^8	0.0	44.7	69.4	83	86.3	

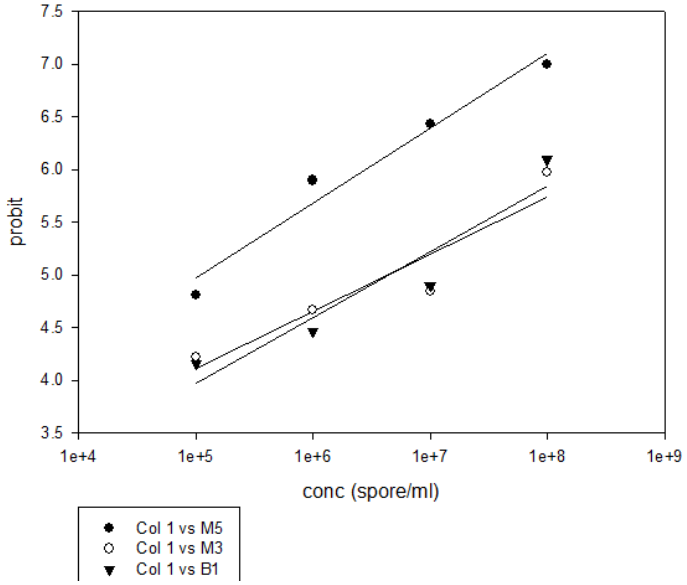

Fig 3. Lethal concentration lines of *A. craccivora* adults treated with series concentrations of *M. anisopliae* (isolate M3& M5) and *B. bassiana* (isolate B1) spores

Table 3. Time mortality response of *M. anisopliae* and *B. bassiana* against *A. craccivora* adults

Entomopathogenic Fungi	**Median lethal time LT_{50} of different concentration (spores/ml.) in days**			
	1×10^5	**1×10^6**	**1×10^7**	**1×10^8**
M. anisopliae (isolate M5)	6.2662	3.986	2.0358	1.475
M. *anisopliae* (isolate M3)	19.5853	10.2069	7.5589	3.8594
B. bassiana (isolate B1)	11.6874	7.6877	6.534	3.1413

4 Conclusions

Thirty-eight fungal isolates were collected from various soil samples and morphologically characterized. Among entomopathogenic fungi, two isolates of *M. anisopliae* (M3 and M5) and two isolates of *B. bassiana* (B1 and B5) were molecularly characterized. The accurate characterization of these EPFs requires a combination of techniques, including macroscopic and microscopic examination, sequencing, and phylogenetic analysis. Obtained results of bioassays showed that only three isolates (M3, M5, and B1) caused a highly pathogenic effect against cowpea aphid, *Aphis craccivora*. These results encourage to development of suitable formulations based on such isolates for application in pest management programs.

Abbreviations

EPFs Entomopathogenic Fungi
ITS Internal Transcribed Spacer
LC_{50} Median lethal concentration values
LT_{50} Median Lethal Time
MP Maximum Parsimony method
nt Nucleotide
PCR Polymerase Chain Reaction
PDA Potato Dextrose Agar
SDA Sabouraud Dextrose Agar
SDS Sodium Dodecyl Sulfate
SDT Species Demarcation Tool
SPR Subtree-Pruning-Regrafting method

Acknowledgment

The authors are grateful to Dr. Sahar S. Ali for her technical assistance in bioassay experiments and Dr. Sherif Gianny for his assistance in the phylogenetic analysis.

References

Abbott WS (1925) A method of computing the effectiveness of an insecticide. *Journal Economic Entomology* 18, 265–267. https://doi.org/10.1093/jee/18.2.265a

Abdel-Raheem M A, Saad A F, Abdel-Rahman I E (2021) Entomopathogenic Fungi on Fabae bean Aphid, *Aphis craccivora* (Koch) (Hemiptera: Aphididae). *Romanian Biotechnological Letters* 26, 2862–2868. https://doi.org/10.25083/rbl/26.4/2862-2868

Ahmad S, Shoaib M, Iqbal M, et al (2023) Influence of foliar application of micronutrients on population dynamics of aphids on wheat. *Journal Plantarum* 5, 87–94. https://plantarum.pk/ojs/index.php/1/article/view/48

Barnett H L, Hunter B B (1986) Illustrated Genera of Imperfect Fungi, 4th Edition. Macmillan Publishing Co., New York. 218 Pp.

Batta YA (2018) Efficacy of two species of entomopathogenic fungi against the stored-grain pest, *Sitophilus granarius* L. (Curculionidae: Coleoptera), via oral ingestion. *Egyptian Journal of Biological Pest Control* 28, 44. https://doi.org/10.1186/s41938-018-0048-x

Bich GA, Castrillo ML, Kramer FL, et al (2021) Morphological and molecular identification of entomopathogenic fungi from agricultural and forestry crops. *Floresta e Ambiente* 28, e20180086. https://doi.org/10.1590/2179-8087-FLORAM-2018-0086

Blackman RL, Eastop VF (2017) Taxonomic Issues. In: van Emden HF, Harrington R (Eds). Aphids as Crop Pests. CABI Publishing, Wallingford United Kingdom, pp 1–36. https://doi.org/10.1079/9781780647098.0001

Dellaporta SL, Wood J, Hicks J B (1983) A plant DNA minipreparation: Version II. *Plant Molecular Biology Reporter* 1, 19–21. https://doi.org/10.1007/BF02712670

Ekesi S, Akpa AD, Onu I, et al (2000) Entomopathogenicity of *Beauveria bassiana* and *Metarhizium anisopliae* to the cowpea aphid, *Aphis Craccivora* koch (Homoptera: Aphididae). *Archives of Phytopathology and Plant Protection* 33, 171–180. https://doi.org/10.1080/03235400009383341

Finney D J (1971) Probit Analysis. Cambridge University Press. (3rd ed), UK.. 333 Pp. https://rb.gy/616oeg

Gürlek S, Sevim A, Sezgin FM, et al (2018) Isolation and characterization of *Beauveria* and *Metarhizium* spp. from walnut fields and their pathogenicity against the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae). *Egyptian Journal of Biological Pest Control* 28, 50. https://doi.org/10.1186/s41938-018-0055-y

Hamza A, Manzoor M, Anees M, et al (2023) Bioefficacy of some botanical extracts against brinjal fruit and shoot borer [*Leucinodes orbonalis* (Guenee); Lepidoptera, Pyrallidae]. *Plant Protection* 7, 263–272. https://doi.org/10.33804/pp.007.02.4728

Imoulan A, Hussain M, Kirk PM, et al (2017) Entomopathogenic fungus Beauveria: Host specificity, ecology and significance of morpho-molecular characterization in accurate taxonomic classification. *Journal of Asia-Pacific Entomology* 20, 1204–1212. https://doi.org/10.1016/j.aspen.2017.08.015

Juliya RF (2020) Phylogeny, chitinase activity, and pathogenicity of *Beauveria*, *Metarhizium* and *Lecanicillium* species against cowpea aphid, *Aphis craccivora* Koch. *International Journal of Tropical Insect Science* 40, 309–314. https://doi.org/10.1007/s42690-019-00082-x

Kavallieratos NG, Athanassiou CG, Aountala MM, et al (2013) Evaluation of the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria fumosorosea* for control of *Sitophilus oryzae*. *Journal of Food Protection* 77, 87–93. https://doi.org/10.4315/0362-028X.JFP-13-196

Klingen I, Haukeland S (2006) The Soil as a Reservoir for Natural Enemies of Pest Insects and Mites With Emphasis on Fungi and Nematodes. In: Eilenberg J, Hokkanen HMT (Eds), An Ecological and Societal Approach to Biological Control. Progress in Biological Control, vol 2. Springer, Dordrecht, pp 145–211. https://doi.org/10.1007/978-1-4020-4401-4_9

Meyling NV, Eilenberg J (2006) Occurrence and distribution of soil borne entomopathogenic fungi within a single organic agroecosystem. *Agriculture Ecosystems and Environment* 113, 336–341. https://doi.org/10.1016/j.agee.2005.10.011

Mohammed AA, Kadhim JH, Kamaluddin ZNA (2018) Selection of highly virulent entomopathogenic fungal isolates to control the greenhouse aphid species in Iraq. *Egyptian Journal of Biological Pest Control* 28, 71. https://doi.org/10.1186/s41938-018-0079-3

Muhire BM, Varsani A, Martin DP (2014) SDT: A virus classification tool based on pairwise sequence alignment and identity calculation. *PLoS One* 9, e108277. https://doi.org/10.1371/journal.pone.0108277

Mweke A, Ulrichs C, Nana P, et al (2018) Evaluation of the entomopathogenic fungi *Metarhizium anisopliae*, *Beauveria bassiana* and *Isaria* sp. for the management of *Aphis craccivora* (Hemiptera: Aphididdae). *Journal of Economic Entomology* 111, 1587–1594. https://doi.org/10.1093/jee/toy135

Mweke A, Akutse KS, Ulrichs C, et al (2019) Efficacy of aqueous and oil formulations of a specific *Metarhizium anisopliae* isolate against *Aphis craccivora* Koch, 1854 (Hemiptera: Aphididae) under field conditions. *Journal of Applied Entomology* 143, 1182–1192. https://doi.org/10.1111/jen.12705

Mweke A, Akutse KS, Ulrichs C, et al (2020) Integrated management of *Aphis craccivora* in cowpea using intercropping and entomopathogenic fungi under field conditions. *Journal of Fungi* 6, 60. https://doi.org/10.3390/jof6020060

Nei M, Kumar S (2000) Molecular Evolution and Phylogenetics. Oxford University Press, New York. 352 Pp. https://rb.gy/czak11

Ramanujam B, Poornesha B, Dileep RC, et al (2017) Field evaluation of entomofungal pathogens against cowpea aphid, *Aphis craccivora* Koch and their effect on two coccinellid predators. *International Journal of Pest Management* 63, 101–104. https://doi.org/10.1080/09670874.2016.1227881

Tamura K, Stecher G, Kumar S (2021) MEGA 11: Molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution* 38, 3022-3027. https://doi.org/10.1093/molbev/msab120

Vu VH, Hong S I, Kim K (2007). Selection of entomopathogenic fungi for aphid control. *Journal of Bioscience and Bioengineering* 104, 498–505. https://doi.org/10.1263/jbb.104.498

White TJ, Bruns T, Lee S, et al (1990) Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (Eds), PCR Protocols: A Guide to Methods and Applications, Academic Press, New York, pp 315–322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Zimmermann G, Huger AM, Kleespies RG (2013) Occurrence and prevalence of insect pathogens in populations of the codling moth, *Cydia pomonella* L.: A long-term diagnostic survey. *Insects* 4, 425–446. https://doi.org/10.3390/insects4030425

Zimmermann G (1986) The 'Galleria bait method' for detection of entomopathogenic fungi in soil. *Journal of Applied Entomology* 102, 213–215. https://doi.org/10.1111/j.1439-0418.1986.tb00912.x