RESEARCH ARTICLE Legume Research- An International Journal, Volume 45 Issue 12: 1572-1579 (December 2022)

Efficacy of Three Entomopathogenic Fungi *Beauveria bassiana, Metarhizium anisopliae* and *Lecanicillium lecanii* Isolates against Black Bean Aphid, *Aphis fabae* (Scop.) (Hemiptera: Aphididae) on Faba bean (*Vicia faba* L.)

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10.18805/LRF-706

ABSTRACT

Background: A laboratory bioassay study was conducted to evaluate the *in vitro* pathogenicity of different isolates of *B. bassiana, M. anisopliae* and *L. lecanii,* against the adults of black bean aphid.

Methods: The PCR-based method was used to identify the different isolates molecularly using sequence information from the ITS region. The total genomic DNA of the 19 fungal isolates was recovered from aphid cadavers using CTAB. The amplified DNA using QRT-PCR showed no significant differences in the ANOVA that tested mean cycle threshold (CT) values from the control. Post-molecular identification of the isolated entomopathogen was approved. The single discriminative concentration bioassay was carried out to determine LT_{50} values for each of twelve isolates to determine the most virulent for further studies.

Result: LT_{50} values for *B. bassiana, M. anisopliae* and *V. lecanii* isolates varied from 110-113, 71-75 and 64-77 h, respectively. *B. bassiana* isolate BBK2, *M. anisopliae* isolate MAA2 and *V. lecanii* isolates VLJ2 were selected for further experiments based on their discriminating concentration values. LC_{50} of BBA post-exposure to isolates of *V. lecanii, M. anisopliae* and *B. bassiana* was 46, 269 and 251 ppm, respectively. A significant difference in cumulative mortality was recorded between the three EPF. *M. anisopliae* showed a higher significant cumulative mortality during the first and second days post-application. Then *V. lecanii* recorded higher significant cumulative mortality from the third until the seventh-day post-application. *V. lecanii* showed higher virulence among the other entomopathogenic isolates.

Key words: Cumulative mortality, Entomopathogenic fungi, Median lethal concentration, Median lethal time.

INTRODUCTION

Faba bean (*Vicia faba* L.) is an important winter legume crop that originated in West Asia and is considered as a cheap and primary protein source for the population in the Mediterranean region (Rahate *et al*., 2021). They play an essential role in fixing atmospheric nitrogen through the symbiotic relationship with Rhizobium bacteria and improving the soil's nitrogen status (Köpke *et al*., 2010). Aphids are a large group of phloem-feeding insects that cause severe damage to major crops worldwide (Maketon *et al*., 2013). Black bean aphid (BBA), *Aphis fabae* (Scop.) (Hemiptera: Aphididae) is a significant insect pest of *V. faba* and *Beta vulgaris* L. crops (Almogdad and Semaškienė, 2021). BBA adults and nymphs feed directly on the plant sap and transmit many plant viruses such as bean yellow mosaic (BYMV) and pea leaf roll (PLRV) virus and other mosaic viruses (Almogdad and Semaškienė, 2021). Entomopathogenic fungi (EPF) gained a significant role in insect pest management programs (Reddy *et al*., 2021). More than 700 species belonging to more than 90 genera are classified as entomopathogens. EPF strains, such as *Lecanicillium lecanii* (Zimm.) Viegas, *Beauveria bassiana* (Bals.) Vuill., *Isaria fumosorosea* (Wize) and *Metarhizium anisopliae* (Metsch.) Sorokin. were used worldwide as a natural enemy for controlling a wide range of insects species, including *Helicoverpa armigera*, *Alphitobius diaperinus*, *Plutella xylostella*, *Laniifera cyclades*, *Prostephanus truncatus*, *Nilaparvata lugens*, *Polyphagotarsonemus latus* and *Bemisia tabaci* (Atta *et al*., 2020; Ojha *et al*., 2018; Singh and Joshi, 2020).

Although many EPF strains are commercially mass-produced and available as bioagents, the native isolates

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How to cite this article: Qubbaj, T. and Samara, R. (2022). Efficacy of Three Entomopathogenic Fungi *Beauveria bassiana, Metarhizium anisopliae* and *Lecanicillium lecanii* Isolates against Black Bean Aphid, *Aphis fabae* (Scop.) (Hemiptera: Aphididae) on Faba bean (*Vicia faba* L.). Legume Research. 45(12): 1572-1579. DOI: 10.18805/LRF-706.

Submitted: 24-06-2022 **Accepted:** 01-09-2022 **Online:** 14-09-2022

may still be adapted to the dominant environmental conditions. Therefore, the present study aims to evaluate the bio-efficacy and effectiveness of three Palestinian EPF isolates, *B. bassiana*, *M. anisopliae* and *V. lecanii*, as potential biological control agents against *A. fabae* in faba bean (*V. faba*). Furthermore, we also aimed to investigate fast and reliable molecular identification tools for the three Palestinian EPF isolates. These results could help to establish an effective integrated pest management method that is eco-friendly and cost-effective to mass-produce locally, reducing BBA population below economic thresholds while minimizing the use of synthetic chemical insecticides .

MATERIALS AND METHODS

Sources and preparation of fungal isolates

The eleven virulent isolates of the EPF *B. bassiana, M. anisopliae* and *V. lecanii* were collected and maintained in the PTUK laboratories during 2021-22. EPF isolates were sub-cultured and incubated by a single spore method (Sufyan *et al*., 2019) for 7-10 days at 28±2°C on PDA media (Fig 1 A-C). A pathogenicity test was carried out after the 6-7 subcultures to maintain the virulance of entomopathogen isolates. The spore concentration was prepared following the method of Samara (2016). Under the microscope, the spore concentration was then determined using a hemocytometer. All the cultures were adjusted to 1×10^{10} spores ml^{-1}, from which the lower concentrations were prepared by serial dilution technique for bioassay studies.

Insect culture

BBA adults were collected from a broad bean field at Najah National University Farm, An-Nassarya field station. Then reared and maintained on the young broad bean plants in the PTUK glasshouses at 25±5°C, 65±5% RH and 16:8h L:D. The mature aphids were kept on plants for 24 h, resulting in neonate nymphs with an age of 0-24 h that were used throughout the bioassay experiments.

Molecular identification of the EPF

Genomic DNA of the fungal isolates was extracted from the growing fungal mycelium from insect cadavers using modified cetyl-trimethyl-ammonium bromide (CTAB) protocol (Reineke *et al*., 1998). A positive source of the EPF used in this experiment was obtained from the culture maintained at PTUK lab. The PCR-based method was amplified using sequence information from the ITS region from all fungal isolates. Preparation of the qPCR reaction mixture of the final volume of 20 µl; each PCR reaction contains 1 µl of DNA isolate, SsoAdvanced™ SYBR® Green Supermix (Bio-Rad Lab. Inc.) and 0.3 µM of the forward specific primers and 0.3 µM of the reverse specific primers. For *B. bassiana* (Hegedus and Khachatourians, 1996), for *M. anisopliae* (Destéfano *et al*., 2004) and *V. lecanii* were used (Nam *et al*., 2020). The 96 well plates were loaded using two technical replicates for each isolate: positive, negative and water control (Husien, 2019). Plates were then placed in CFX Connect Real-Time PCR Detection System (CFX Connect®; Bio-Rad, Hercules, CA, USA). Data analysis was carried out using CFX Manager™ Software (Bio-Rad) with auto-calculated baseline and fixed threshold fluorescence units (RFU) settings. Once the identity was confirmed, isolated samples were used in the next bio-assays.

Median lethal time (LT_{50}) assessment

V. faba seedlings were grown in plastic pots in the greenhouse at Najah Farm, An-Nassarya. Two-week-old seedlings were used in this bioassay study. A single discriminative concentration bioassay was carried out to determine LT_{50} values for each of the 12 isolates to select the most virulent for further studies (Samara 2016). For each, an aqueous suspension containing 1×10^{10} spores conidia ml^{-1}

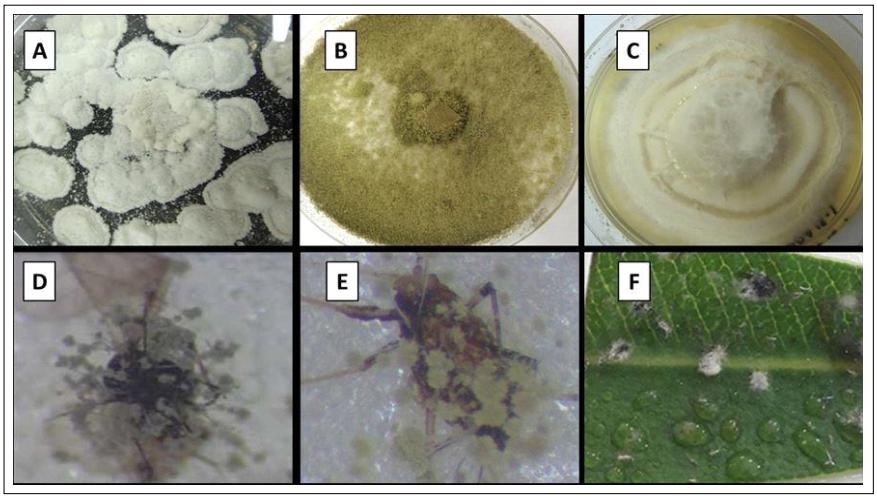

Fig 1: Three colony growth of the EPF *B. bassiana* (A) *M. anisopliae* (B) and *V. lecanii* (C) were maintained in PTUK on PDA at 28±2°C. Growth of the entomopathogen on aphid cadavers during bioassay assessment *B. bassiana* (D) *M. anisopliae* (E) and *V. lecanii* (F).

was prepared in Tween 80 (0.05% v/v). Sub-samples were plated onto Sabouraud dextrose agar (SDA) and the germination percentage was counted after 24 h. LT_{50} was assessed at 24 h intervals for a 7-day mentoring period (Fig 1 D-F). Each date was replicated three times. Ten aphids were transferred to a broad bean seedling using a camel hairbrush. A Total of 210 aphids were used per EPF isolates per bioassay. Every plant infected with aphids was sprayed with an aqueous suspension containing 1×10^{10} spores conidia ml^{-1} per EPF isolates by a hand atomize. Dead aphids were collected daily and transferred to a moist filter paper in a self-sealed Petri dish; once the dead aphid produced mycelial growth, they were considered for the mortality count (Pegu *et al*., 2017). The mortality data were then corrected using Abbott's formula (Abbott 1925).

Median lethal concentration (LC_{50}) assessment

B. bassiana isolate BBK2, *M. anisopliae* isolate MAA2 and *V. lecanii* isolates VLJ2 were selected for the further experiments based on their discriminating dose bioassay carried out in the previous experiment. Seven serial dilutions (50, 100, 200, 350, 500, 750, 1000 ppm) were prepared as described above and each dilution was replicated three times. Ten aphids were transferred to a broad bean seedling using a camel hairbrush. A total of 30 aphids were used per concentration and a total of 210 aphids were used per bioassay. Every three plants infected with aphids were sprayed with one of the seven dilutions of the fungal spore suspensions by a hand atomize. The mortality of aphids was counted every 24 h up to seven days (Fig 1 D-F). Dead aphids were collected daily and transferred to a moist filter paper in a self-sealed Petri dish; once the dead aphid produced mycelial growth, they were considered for the mortality count (Parveen *et al*., 2021). The mortality data were then corrected using Abbott's formula (Abbott 1925). Data were analysed by Probit analysis and LC_{50} and LT_{50} values and their 95% confidence limits (CL 95%) were calculated from Probit regressions using SAS software.

Cumulative mortality

Same EPF isolates were used to assess the cumulative mortality of *B. bassiana* isolate BBK2, *M. anisopliae* isolate MAA2 and *V. lecanii* isolates VLJ2 on BBA. Ten aphids were transferred to a self-sealed petri dish with wetted filter paper using a camel hairbrush after spraying an aqueous suspension containing 1×10^{10} spores conidia ml^{-1} per EPF isolates by a hand atomize. A total of 100 aphids were used per EPF isolates per bioassay (Samara 2016). Dead aphids were assessed as described above. The mortality data were then corrected using Abbott's formula (Abbott 1925).

Statistical analysis

All the data were analysed by Probit analysis and LC_{50} and the LT_{50} values and their 95% confidence limits (CL 95%) were calculated from Probit regressions using SAS software. Each fungus's per cent corrected cumulative mortality was analyzed using ANOVA as a general linear model (PROC GLM) procedure. The significance level was determined by applying the Student-Newman-Keuls test at P = 0.05.

RESULTS AND DISCUSSION

Molecular identification of the EPF

Amplification of the isolated DNA sample using QRT-PCR results is presented in Fig 4. The statistical analysis of seven EPF isolates with qPCR amplification experiments showed no significant differences in the ANOVA that tested mean

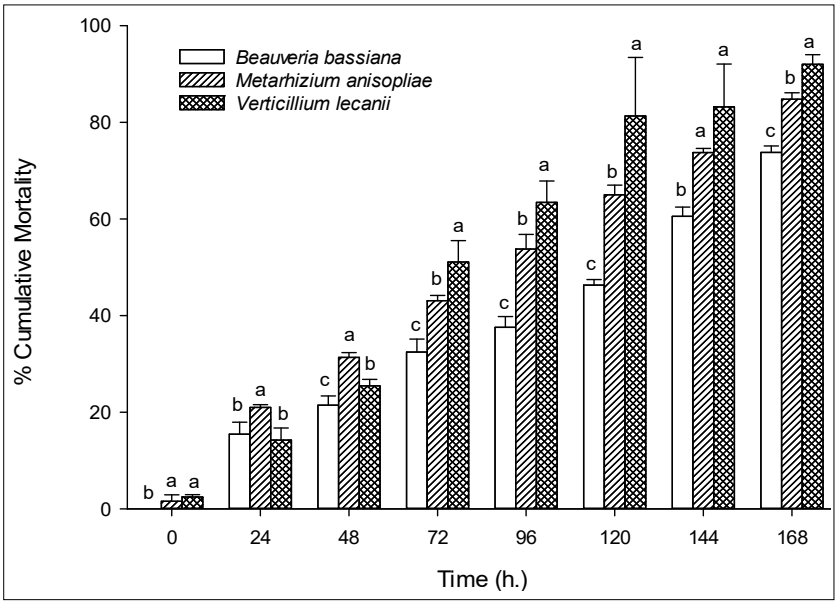

Fig 2: Mean percent ± S.D. Cumulative mortality of BBA exposed to EPF *B. bassiana* isolate BBK2, *M. anisopliae* isolate MAA2 and *V. lecanii* isolate VLJ2. The bars indicated standard errors; Different letters represent significant differences between fungal isolates treatment according to the Student-Newman-Keuls (SNK), ($p \leq 0.05$).

cycle threshold (CT) values from the positive (Fig 4). The Real-time amplification of the PCR product generated by specific primers confirmed that the three EPF isolates of *B. bassiana, M. anisopliae* and *V. lecanii* isolates under 20 cycles. Using this protocol, amplifying the control samples began around 20 cycles in a real-time experiment, whereas the target DNA (*i.e.*, *B. bassiana, M. anisopliae* and *V. lecanii* isolates) was detected later to 21 cycles. Three isolated samples, *B. bassiana* (isolate BBK2), *M. anisopliae* (isolate MAA2) and *V. lecanii* (isolate VLJ2), were used in the next bio-assays.

Identifying entomopathogenic bio-agents is one of the main challenges in using indigenous microbial pesticides (Samada and Tambunan, 2020). Morphological, developmental and physiological characteristics were the sole methods used for identification, but they required taxonomical experiences and took a long time (Hetjens *et al*., 2021). Recently DNA and RNA-based molecular techniques have been used for taxonomical hierarchy and phonological classification (Goettel and Glare, 2005). The QRT-PCR protocols with specific primers have proven to be very sensitive in detecting and identifying the EPF isolates (Sabbahi *et al*., 2009). They are also considered a standard approach for accurately and rapidly identifying microorganisms. The modified specific primers for PCR have been used effectively to detect and differentiate plant pathogenic fungi in the current study. The genomic DNA of the fungal isolates was isolated to obtain pure DNA from cadavers using CTAB.

LT_{50} assessment

BBA LT_{50} and LT_{90} with the corresponding 95% confidence limits after exposure to twelve isolates of *B. bassiana*. *M. anisopliae* and *V. lecanii* are presented in Table 1, along with the value of Pearson Chi-square, degree of freedom (df) and regression equations. Mortality in the control treatments was consistently below 10%. LT_{50} values for *B. bassiana* isolates varied from 110.39-113.74 h, while LT_{50} values for *M. anisopliae* varied from 71.88-75.27 h and LT_{50} values for *V. lecanii* isolates varied from 64.86-77.17 h.

LT_{50} of BBA post-exposure to VLJ2 isolates of *V. lecanii*, was the shortest 64.86 h, while the longest for VLK2 isolates 77.17 h. There were no significant differences between the four isolates of *V. lecanii* based on the non-overlapping of the fiducial limits 95%. LT_{50} for MAA2 isolate of *M. anisopliae*, was the shortest 71.88 h, while LT_{50} for MAJ2 isolate of *M. anisopliae*, was the longest 75.27 h. No significant differences between the four isolates of *M. anisopliae*. As for *B. bassiana* BBK2 isolates LT_{50} was the shortest 110.39 h, while BBA1 isolates LT_{50} was the longest 113.74 h. No significant differences between the four isolates of *B. bassiana*. Similar results were found with LT_{90} of BBA post-exposure to isolates of *V. lecanii, M. anisopliae* and *B. bassiana*. Based on the discriminating dose bioassay, *B. bassiana* isolate BBK2, *M. anisopliae* isolate MAA2 and *V. lecanii* isolates VLJ2 were selected for further experiments.

LC_{50} assessment

The probit and logit analysis graph for BBA (Fig 3) and LC_{50} and LC_{90} with the corresponding 95% confidence limits after exposure to isolates of *B. bassiana. M. anisopliae* and *V. lecanii* are presented in Table 2, along with the value of Pearson Chi-square, degree of freedom (df) and regression equations. Mortality in the control treatments was consistently below 10%. LC_{50} of BBA post-exposure to

Table 1: LT_{50} and LT_{90} values (with corresponding 95% confidence limits) for BBA adults after exposure to broad bean leaf sprayed with different concentrations of the EPF *B. bassiana. M. anisopliae* and *V. lecanii.*

Bio-insecticide		Aphid mortality							
		NO*	Regression equations[1]	X^2 (df)	Slope ± SE	LT_{50}** (h.)	(95 % CL)[2]	LT_{90}** (h)	(95% CL)
B. bassiana	BBK1	240	y = 0.075x+0.7033	12.39 (19)	-3.88±0.41	112.43	99.46-129.72	540.07	381.75-931.51
	BBK2	240	y = 0.078x+0.6385	27.84 (19)	-3.76±0.72	110.39	88.71-147.28	477.04	296.27-1338
	BBA1	240	y = 0.079x+0.6405	24.22 (19)	-6.32±1.29	113.74	91.3-153.8	589.09	333.15-2222
	BBJ2	240	y = 0.069x+0.8151	32.15 (19)	-4.21±0.74	113.13	93.00-145.9	564.44	324.05-2022
M. anisopliae	MAK1	240	y = 0.070x+0.6103	8.69 (19)	-3.89 ±0.39	74.36	65.74-83.41	307.56	242.54-431.83
	MAK2	240	y = 0.0707x+0.600	28.23 (19)	-6.41±1.21	74.49	58.66-91.17	326.63	220.17-716.04
	MAA2	240	y=0.0637x+ 0.7105	31.90 (19)	-7.06±1.25	71.88	57.50-86.46	272.24	194.78-509.47
	MAJ1	240	y = 0.0719x+0.5817	27.63 (19)	-6.31±1.20	75.27	59.15-92.44	338.86	225.66-768.45
V. lecanii	VLK1	240	y = 0.047x+0.9800	18.64 (19)	-5.48±0.44	67.37	61.26-73.46	180.19	158.04-213.15
	VLK2	240	y = 0.0526x+0.9406	38.45 (19)	-8.68±1.39	77.17	64.27-90.37	231.86	177.31-370.88
	VLA1	240	y = 0.0397x+1.1067	47.18 (19)	-11.09±1.62	66.30	56.25-75.81	167.52	136.78-212.07
	VLJ2	240	y = 0.0454x+0.9953	43.60 (19)	-9.66±1.46	64.86	54.03-75.13	152.12	135.93-204.39

Mortality in all control treatments was consistently below 10%. The results presented as LT_{50} and LT_{90} with corresponding 95% confidence limits (CL), Pearson Chi-squareresults, degree of freedom (df) and regression equations.

*Number of BBA used in the bioassay.

**LT_{50} and LT_{90} values in having different letters are significantly different (95% CL did not overlap).

[1]Regression equations estimated by probit regression.

[2](95%) Confidence limits for LT_{50} and LT_{90}.

isolates of *V. lecanii* was 46.47 ppm, *M. anisopliae* 269.53 ppm and *B. bassiana* was 251.48 ppm. LC_{90} of BBA post-exposure to isolates of *V. lecanii* was 215 ppm, *M. anisopliae* 1311 ppm and *B. bassiana* was 1274 ppm.

Cumulative mortality

BBA cumulative post-treatments mortality with the isolates of the EPF *B. bassiana, M. anisopliae* and *V. lecanii* is presented in Fig 2. A significant difference in cumulative mortality was recorded between the three EPF. *M. anisopliae* showed a higher significant cumulative mortality during the first and second days post-application. Then *V. lecanii* recorded higher significant cumulative mortality starting from the third unit the seventh-day post-application. The 24 h monitoring intervals showed that *V. lecanii, M. anisopliae* and *B. bassiana* caused 50% cumulative death by the 3rd,

Table 2: LC_{50} and LC_{90} values (with corresponding 95% confidence limits) for BBA adults after exposure to broad bean leaf sprayed with different concentrations of the EPF *B. bassiana* (BBK2), *M. anisopliae* (MAA2) and *V. lecanii* (VLJ2).

Bio-insecticide	Aphid mortality							
	NO*	Regression equations[1]	X^2 (df)	Slope± SE	LC_{50}** (ppm)	(95 % CL)[2]	LC_{90}** (ppm)	(95% CL)
B. bassiana BBK2	210	y = 0.0731x+1.0848	86.78 (19)	-4.36±0.74	251.48	177.02-348.09	1274	786.91-3141
M. anisopliae MAA2	210	y = 0.078x+1.0412	70.65 (19)	-4.53±0.69	269.53	199.93-359.34	1311	846.39-2807
V. lecanii VLJ2	210	y = 0.0672x+0.4793	4.88 (19)	3.21±0.42	46.47	33.67-58.86	215.19	178.82-269.86

Mortality in all control treatments was consistently below 10%. The results presented as LC_{50} and LC_{90} with corresponding 95% confidence limits(CL), Pearson Chi-square results, degree of freedom (df) and regression equations.

*Number of BBA used in the bioassay.

**LC_{50} and LC_{90} values in having different letters are significantly different (95% CL did not overlap).

[1]Regression equations estimated by probit regression.

[2](95%) Confidence limits for LC_{50} and LC_{90}.

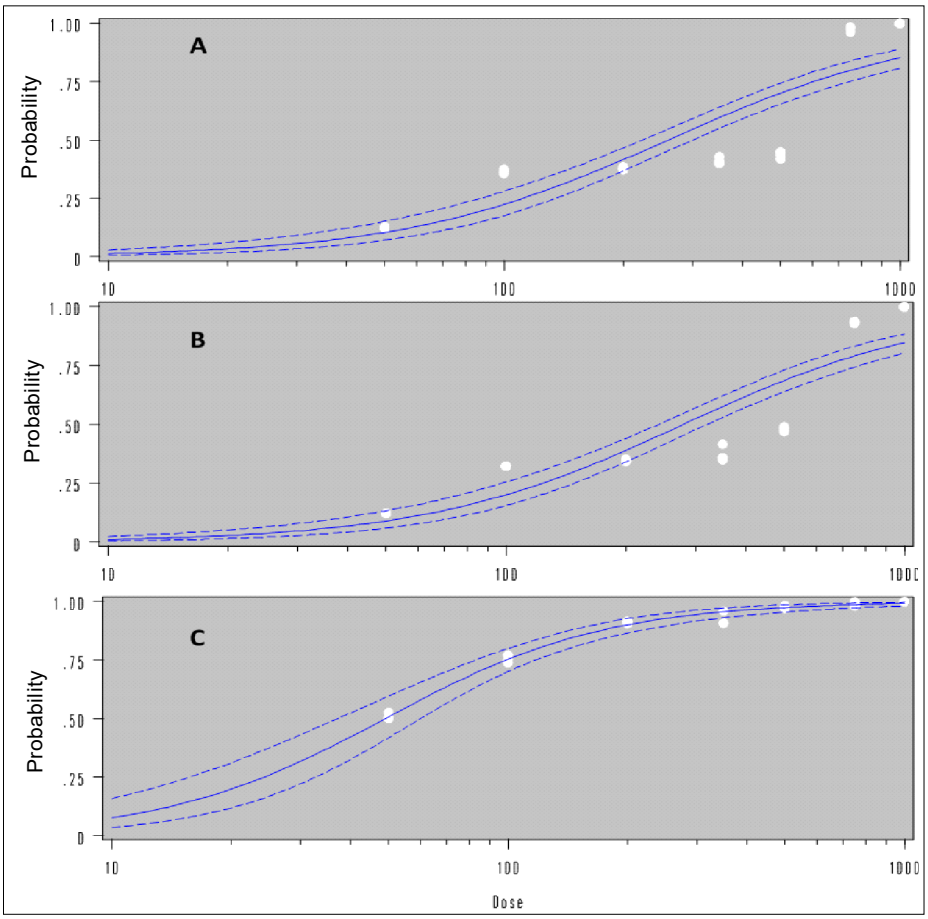

Fig 3: The output of the probit graph for the probability of BBA mortality at different doses of the EPF *B. bassiana* isolate BBK2 (A), *M. anisopliae* isolate MAA2 (B) and *V. lecanii* isolate VLJ2 (C). Graphs were created in SAS software using PROC PROBIT. Circles represent average BBA mortality treated with serial concentrations of 50, 100, 200, 350, 500, 750 and 1000 ppm.

4th and 5th day, respectively. *V. lecanii, M. anisopliae* and *B. bassiana* cumulative mortality of BBA on the 7th day were 92, 84.8 and 73.8%, respectively.

The current study evaluates the efficacy of EPF in controlling back bean aphids under laboratory environmental conditions. In most soil systems, *B. bassiana, M. anisopliae* and *V. lecanii* are naturally occurring EPF. These entomopathogens infect insects when their spores penetrate insect cuticles, produce toxins and cause the death of their host insect (Islam *et al*., 2021). Identifying the virulence between the different entomopathogen species is one of the critical tools before further genetic, biochemical and environmental risk assessment investigation is carried out (Plantey *et al*., 2019). On the other hand, these EPF were described to affect their host insects by starving them (Mannino *et al*., 2019), deteriorating insect tissue (Altinok *et al*., 2019) and discharging toxic substances (Bamisile *et al*., 2021). The EPF fungi produce chitinase, protease and lipase enzymes that degrade the insect cuticle (Singh and Joshi, 2020). Once the fungal germ tube penetrates the insect cuticle, they start releasing more mycotoxins in the hemocoel that destroy insect cells and cause their death (Mahankuda and Bhatt, 2019).

Screening bioassays of EPF isolates under laboratory conditions is a crucial step toward identifying the most virulence strains prior to field assessments. Due to the constraint status of pesticide registration legislation and regulations in West Bank, the limitations on agrochemical

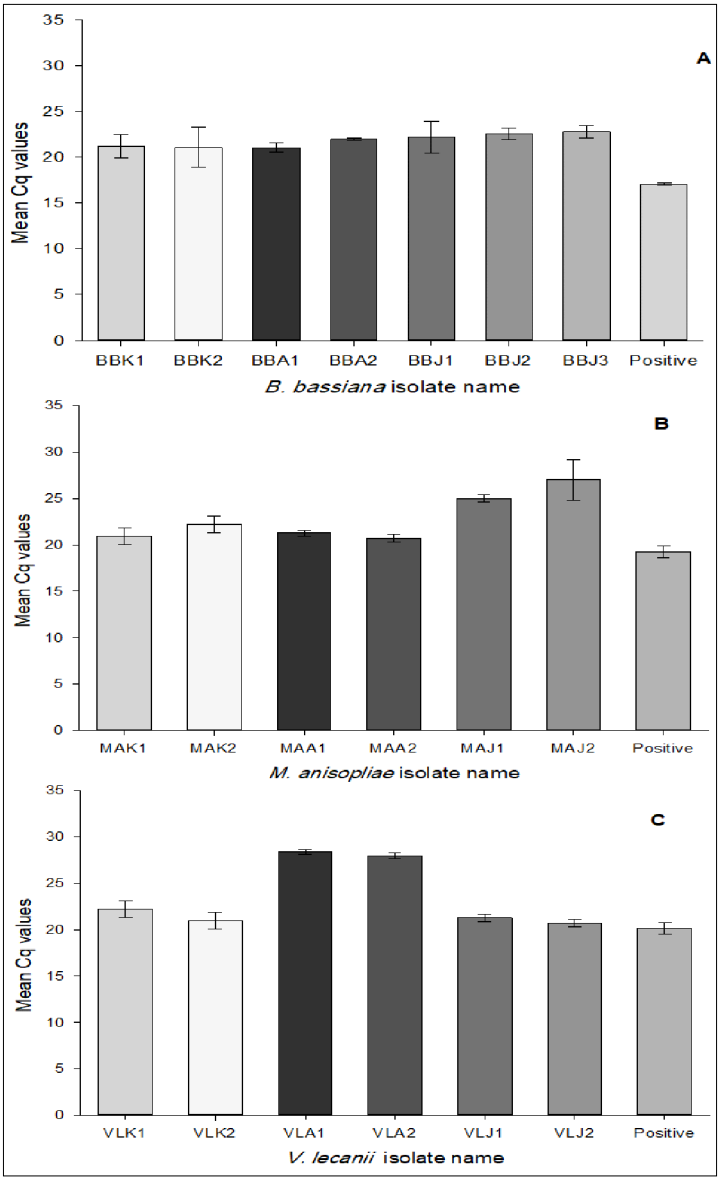

Fig 4: Mean Cq values in RT-qPCR study measured using Bio-Rad CFX Maestro software®. Bars and error bars represent the mean value ± standard deviation for the biological samples and technical replicates of Cq values. (A) *B. bassiana* isolates; (B) *M. anisopliae* isolates, (C) *V. lecanii* isolates.

importation and the high market prices of most pesticides. Many local growers showed an increased interest in developing an indigenous EPF over exotic isolates due to political and ecological boundaries.

The current study investigated the toxicity and virulence of three EPF isolates. *B. bassiana*. *M. anisopliae* and *V. lecanii* have been used to control many insects of economic importance, *Cylas formicarius* (Reddy *et al*., 2014); *Aphis craccivora* (Maketon *et al*., 2013); *Leptinotarsa decemlineata* (Anderson and Roberts, 1983); *Rhynchophorus ferrugineus* (Gindin *et al*., 2006); *Agrotis ipsilon* (Gabarty *et al*., 2014); and *Spodoptera littoralis* (Amer *et al*., 2008). Similar results were found in several studies; Saranya *et al*., (2010) reported a 100% mortality of *A.craccivora* post-application of *V. lecanii* followed by *B.bassiana, M. anisopliae*. LC_{50} value was the highest virulence for *V. lecanii* compared to *B. bassiana*, *M. anisopliae*. Similar to our results, LT_{50} was the highest for *V. lecanii*, then *M. anisopliae* and *B. bassiana*. These three EPFs have been used wildly against aphids and other insect pests worldwide. They are cheap for mass production, have a broad host range and can tolerate a wide range of temperatures and humid conditions (Milner, 1997). *B. bassiana* and *M. anisopliae* are one of the most abundantly and commercially available and used EPF (Peng *et al*., 2021), but *V. lecanii* is the only hyphomycete fungi that attack aphids in greenhouses because they need very high humidity (Goettel and Glare, 2005). Javed *et al*., (2019) reported that *V. lecanii* have a higher virulence and mortality rate due to their ability to germinate under a broad range of temperatures and humidity, increasing their virulence.

LT_{50} value of *V. lecanii* against *Macrosiphoniella sanborni* aphid was three days (Jackson *et al*., 1985) and they caused higher mortality to *Myzus persicae* (Sulzer) aphid than *B. bassiana* (Javed *et al*., 2019), while *M. anisopliae* was more efficient than *B. bassiana* against brown plant hopper (Atta *et al*., 2020), which is similar to our findings.

CONCLUSION

EPF for insect pests is one approach to non-chemical crop protection. Studying the bio-efficacy and virulence of the potential EPF is a prerequisite for optimizing the application strategy for controlling insect pests in biological control. *V. lecanii, M. anisopliae* and *B. bassiana* were found to be the promising EPF against BBA. They can be used as potential biocontrol agents to manage BBA by further testing their field efficacy as a good alternative insect pest control of aphids for faba bean cultivation.

ACKNOWLEDGEMENT

The authors acknowledge An-Najah National University and Palestine Technical University-Kadoorie for the logistical and facility support.

Conflict of interest: None.

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