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RESEARCH

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Characterization of a new isolate of *Beauveria bassiana* in Algeria and evaluation of its pathogenicity against the cowpea aphid (*Aphis craccivora* Koch)

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Abstract

Background The cowpea aphid, *Aphis craccivora* Koch (Hemiptera: Aphidiidae) is a polyphagous aphid species that causes various damage on different crops. The conventional method of controlling this pest is the use synthetic insecticides that threaten both the environmental safety and human health. Moreover, it contributes to the emergence of insecticide-resistant generations. Hence, relying on Entomopathogenic fungi (EPF) remains one of the most safe and effective alternative solutions to control insect pests. For the mentioned reasons, the EPF, *Beauveria bassiana* was isolated and characterized; besides, its efficiency against adults' *A. craccivora* was evaluated both in the laboratory and in the greenhouse.

Results A new isolate of *B. bassiana* was isolated from collected cadavers' insects associated with the population of *A. craccivora* in a rural area in the Northwest of Algeria. This isolate was identified on the basis of its morphological and molecular characteristics and was referred to as *B. bassiana* BBAA. The enzymatic activities of this isolate revealed a high production of chitinase, protease and lipase, without any production of amylase. The use of different concentrations of *B. bassiana* BBAA conidia against *A. craccivora* led to a high mortality rate, ranging from 64 to 74% mortality on the seventh day post-treatment in vitro and 58 to 70% in greenhouse.

Conclusion Virulence and enzymatic activities produced by *B. bassiana* BBAA demonstrated the necessity to exploit entomopathogenic fungi (EPFs) in pest control.

Keywords *Beauveria bassiana*, Enzymatic activities, *Aphis craccivora*, Pathogenicity

Background

The cowpea aphid, *A. craccivora* Koch, 1854 (Hemiptera: Aphidiidae), is a highly polyphagous species which attacks 50 cultivated plant species belonging to 19 botanical families, mainly those species belonging to the Fabaceae (Blackman and Eastop 2017). It causes serious economic harms caused by the sucking of the sap associated with the injection of toxic saliva inside the plant tissues (Rakhshani et al. 2005). On the other hand, the sticky and sugar-rich honeydew droplets resulting from an excess of phloem metabolism affect the growth of sooty mold by preventing the photosynthesis and respiration of the host plant, reducing thus the commercial value of the plant product (Castro et al. 2020). Besides, this aphid is responsible for the transmission of about 30 viral diseases, such as cucumber mosaic virus (CMV) and Alfalfa mosaic virus (AMV) in the non-persistent mode (Blackman and Eastop 2017), as well as in a persistent

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mode like the lucerne enation virus (LEV) and Alfalfa leaf curl virus (ALCV) (Ryckebusch et al. 2020).

Most of the time, due to their effectiveness and low cost, pesticides are the most widely used control method for crop protection. Moreover, they represent significant risks to both the cultivators and the consumers, due to the acute toxicity of their components (Cross et al. 2008). Adding to this the fact that the overuse of chemical insecticides leads to an environmental pollution and a poisoning of the organisms threatening the ecological balance (Mahmood et al. 2016), as well as the emergence of progenies' insecticide-resistant (Foster et al. 2017). On the other hand, the disadvantages of these pesticides have forced researchers to find more sustainable alternative means of control which are safer and less harmful. Natural environments are a reservoir of microorganisms used in biological control, mainly entomopathogenic fungi (EPF). Compared to pesticides, such effective fungi are easily produced and used with no undesirable consequences (Vega 2018). Fortunately, *Beauveria bassiana* is one of the most common antagonists in terms of virulence with a host range of about 700 arthropod species (Vega 2018). Despite the role of insect cuticle in defending against microbes, it produces numerous extracellular enzymes including chitinase, protease and lipase that lead to hydrolyze components of this defensive barrier. This allows the mycelium of *B. bassiana* to penetrate and develop into the hemolymph and tissues of the host (Ramzi and Zibaee 2014), and this fungus also secretes toxic substances in the form of proteins, and secondary metabolites such as beauvericin, Oosporein, beauverolides, bassianolides, isarolides and tenellins that are toxic and lethal to insects (Rustiguel et al. 2018).

In this respect, a new isolate of *B. bassiana*, isolated from cadavers collected from insects in a rural environment in the Northwest of Algeria, was recorded. Its enzymatic activities were evaluated and its pathogenicity was tested against *A. craccivora* under laboratory and in greenhouse conditions.

Methods

Fungal isolate

Different dead insects were collected during 2020 from a rural area in Mascara (35°23′39.64 "N; 0°25′19.58 "E). The samples were examined in the laboratory of Research on Biological Systems and Geometry LRSBG (Faculty of life Sciences, Mascara University) where they were observed under a binocular magnifying glass. The cadavers were sterilized by immersion in a solution of sodium hypochlorite (1%) for 2 to 3 min and then rinsed successively with sterile distilled water, and finally they were dried with sterile filter paper (Doolotkeldieva et al. 2019). The sterile cadavers were placed in Sabouraud Dextrose Agar (SDA) and incubated for 5 days at 25 ± 2 °C and 75 ± 5% R.H. When the fungal complex appeared, it was replanted until a pure strain was obtained (Awan et al. 2021).

Morphological and Microscopic identification of *B. bassiana* isolate

Identification of the fungal isolate was based on the different morphological characteristics of its colonies such as, growth, color, shape and texture (Doolotkeldieva et al. 2019). An aliquot of the fungal culture was placed on a glass slide, and then a drop of lactophenol cotton blue stain was added and covered with a cover slip. The slide was observed using a binocular light microscope. Thanks to the taxonomic key described by Humber (1997), the fungal isolate was identified based on its microscopic characteristics regarding the shape of the conidia and their arrangement on the conidiophore.

Molecular identification of *B. bassiana* isolate in Sabouraud Dextrose Agar (SDA) medium

The isolate was cultivated on SDA nutrient medium for 5 days at 28 °C. The mycelium thus obtained was collected by filtration, and only 0.5 g of this mycelium was used for DNA extraction, performed according to the protocol of the Nucleo Spin Plant II extraction kit (Macherey–Nagel, Germany). An internal transcribed spacer region (ITS) was amplified by PCR with universal primers ITS1 (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) (Gardes and Bruns 1993), using the following conditions: initial denaturation of 1 cycle at 95 °C for 5 min, followed by 35 cycles of denaturation at 95° C for 30 s, an annealing step at 55° C for 30 s, followed by an extension at 72 °C for 45 s and final extension step at 72 °C for 7 min. Amplification products were revealed after electrophoresis on a 1.5% agarose gel and purified by kit NucleoSpin® Gel and the Macherey- Nagel's PCR Clean-up system. The amplifiers were sequenced by Sanger technique (Sanger et al. 1977) using the Applied Biosystems BigDye v3.1 kit, and PCR primers were used to amplify the fragments of interest. The sequences obtained were analyzed and cleaned by Finch TV software and then identified using BLAST program.

Alignment and phylogenetic analysis were conducted in MEGA 11 software (Tamura et al. 2021) with the Neighbor-Joining method (Saitou and Nei 1987) based on 1000 bootstrap replicates (Felsenstein 1985).

Evaluation of efficacy of *B. bassiana*—BBAA

Aphids

A colony of *A. craccivora* was collected from carob trees. Aphids were reared on beans in a laboratory at 25 ± 2 °C

and 45 ± 5% R.H at the Department of Agronomy Sciences, University of Mascara.

Preparation of conidia suspension

The fungus *B. bassiana* was incubated in SDA medium for 15 days; their conidia were scraped by a scalpel and suspended with distilled water containing 0.05% Tween 80. The suspension was homogenized by a vortex shaker and then filtered through a cloth to reduce the mycelium. The main concentration (10^8 conidia/ml) of conidia suspension was determined by the Thoma counting cell under a light microscope. Concentrations (10^6 and 10^4 conidia/ml) were also obtained by diluting the main concentration in tubes of distilled water containing 0.05% Tween 80.

Laboratory efficacy

First, the bean leaves were disinfected with sodium hypochlorite and rinsed in a beaker of sterile distilled water, after that they were dipped for 15 s in each of the three concentrations of the suspension, and finally they were placed on filter paper in Petrie dishes. Ten adults of *A. craccivora* were transferred to the treated leaves with 5 replicates. The control group was treated only with distilled water containing 0.05% Tween 80. Each treatment was repeated five times, and the mortality rate of aphid individuals was daily determined after the treatment. To confirm that the cause of aphid mortality is caused by the treatment with *Beauveria* isolate, Koch's postulate was applied. The dead individuals were sorted before sporulation to avoid horizontal transmission of the infection, and then the cadavers were replanted on SDA medium and incubated at 25 ± 2 °C and relative humidity of 75 ± 5% for five days.

Greenhouse efficacy

After the infestation of the bean plants by the cowpea aphid, they were transferred to the greenhouse located at the Department of Agronomy Sciences, University of Mascara, where the number of aphids was determined on each bean plant, before the test application. Each five plants were sprayed with one of the following concentrations of conidia suspensions of *B. bassiana*: 10^8, 10^6 and 10^4 conidia/ml, respectively. The remaining five bean plants, used as a control, were treated only with distilled water containing 0.05% Tween 80. All plants were daily checked; dead aphids were sorted and counted with a hand magnifier plus a brush.

Enzymatic activities

The amylolytic activity of *B. bassiana* isolate was tested by using plates of starch agar medium; the plates were then incubated at 28 °C for 3 days. The halo appeared around the fungal colonies in the case of amylase production (Doolotkeldieva et al. 2019). Protease activity of *B. bassiana* isolate was demonstrated using Skim milk agar. After three days of incubation at 28 °C, the clear zone around the fungal colony indicates protease production.

Lipase activity of *B. bassiana* isolate was determined using lipid medium agar that contains olive oil as a lipid substrate (Pignède et al. 2000). After 48 h of incubation at 37 °C, the diameter of the halo around the colonies was measured to assess the production of lipase.

B. bassiana isolate was tested for chitinase production on chitin-agar medium prepared with colloidal chitin as a carbon source. Disks of fungal isolate were inoculated into plates of culture medium and then incubated at 25 °C for three to five days. As the pH increases, the yellow color of the medium changes to purple. This is due to the production of chitinase by *B. bassiana* and the breakdown of chitin to N-acetyl glucosamine (Kamala and Indira 2011). Chitinase production was thus evaluated based on the color intensity and diameter of the purple zone around the colonies.

Statistical analysis

First, using the Abbott's formula (1925), mortality data were corrected for natural mortality, then transformed into arcsine square-root percentage values to meet normality criterion which allows application of the variance analysis (Sokal and Rohlf 1981). Subsequently, a two-way ANOVA with two factors, dose and time, was performed. For significant differences, Tukey's HSD test was applied to construct homogeneous groups of means. LC_{50} and LT_{50} values were estimated by probit analysis.

Results

Fungal isolate

Morphological and microscopic identification of B. bassiana isolate

Colonies of the *B. bassiana* isolate were characterized by dispersed and dense growth, a cloudy shape and a white color with a yellowish reverse side. The microscopic observation revealed globose to sub-globose conidia supported by translucent branched hyphae (Fig. 1). The same criteria were mentioned by Humber (1997) in their taxonomic key.

Molecular identification of B. bassiana isolate in Sabouraud Dextrose Agar (SDA) medium

Molecular analysis confirmed the results of the morphological identification, it indicated that the EPF preserved in the cadavers' insects associated with the population of *A. craccivora* was the *B. bassiana* isolate. The fungal isolate was characterized by the sequencing of the internal transcribed spacer (ITS) of the rDNA using primers ITS1

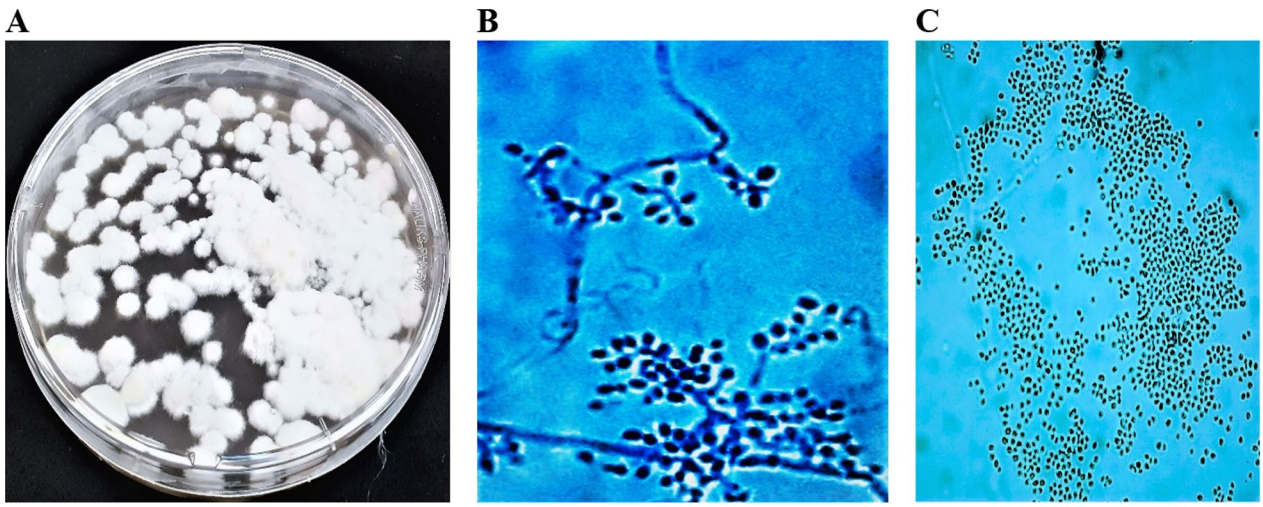

Fig. 1 Morphological characterization of *Beauveria bassiana isolation*. **A** Colony morphology of *B. bassiana*; **B** Mycelia, Conidiophore and conidia; **C** Conidia

Fig. 2 Phylogenetic tree of the isolated entomopathogenic *Beauveria bassiana* BBAA based on the ITS sequences. Tree constructed using the Neighbor-Joining method integrated in MEGA 11. The bootstrap consensus tree inferred from 1000 replicates

and ITS4. The sequences of this isolate were 99% homologous to other *B. bassiana* isolates in GenBank. The nucleotide sequences were deposited in GenBank under the accession number ON715442, https://www.ncbi.nlm.nih.gov/nuccore/ON715442.1/. This *B. bassiana* isolate was named BBAA and referenced by a black disk in the phylogenetic tree (Fig. 2).

Evaluation of efficacy of B. bassiana—BBAA

The death of *A. craccivora* started two days after treatment with three concentrations (1×10^8, 1×10^6 and 1×10^4 conidia/ml) of *B. bassiana* BBAA. Four days later, the killed aphids were covered with a cottony mycelium (Fig. 3). On the other hand, no mycosis was observed in the group of *A. craccivora* treated with distilled water. Statistical analysis showed that the concentration of the conidia suspension had a significant difference in the mortality rate in vitro ($F=93.53$, $df=3$, and $P<0.001$), likewise in the greenhouse ($F=28.55$, $df=3$, and $P<0.001$). Concerning the time, a significant difference in mortality was also recorded in the Petri dish trials ($F=98.83$, $df=1$, $P<0.001$) and in the green house ($F=93.27$, $df=1$, $P<0.001$).

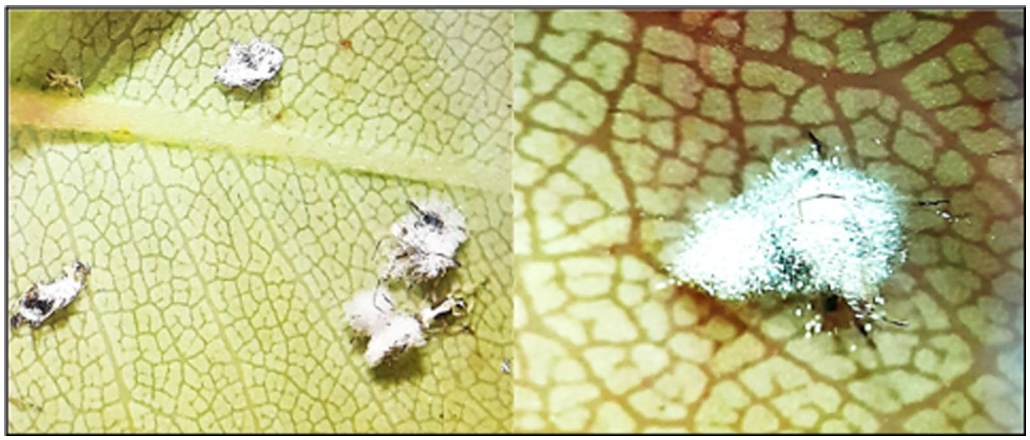

Fig. 3 Cadavers of the cowpea aphid (*Aphis craccivora*), 96 h after treatment with *Beauveria bassiana* BBAA isolate

Table 1 Efficacy of *Beauveria bassiana* BBAA isolate against adults of *Aphis craccivora* in vitro

Days after treatments	% Mortality			
	Concentrations			
	Control	1×10^4 conidia/ml	1×10^6 conidia/ml	1×10^8 conidia/ml
Day 1	0.00^c	0.00^c	0.00^c	0.00^c
Day 2	1.00^c	24.02^b	28.03^b	40.04^b
Day 3	1.40^c	40.06^b	52.07^{ab}	54.07^{ab}
Day 4	1.80^c	48.08^b	54.10^{ab}	66.12^a
Day 5	2.00^c	54.10^b	62.12^a	72.14^a
Day 6	2.40^c	56.13^b	68.16^a	72.17^a
Day 7	2.60^c	64.16^a	74.19^a	74.19^a

The different letters denote the statistical significance determined by ANOVA followed by Tukey test ($p < 0.05$)

Laboratory efficacy

After only three days of treatment, as shown in Table 1, the origin concentration 1×10^8 conidia/ml caused the death of 54% of aphids, while the concentrations 1×10^6 and 1×10^4 conidia/ml killed, respectively 52.07 and 40% of *A. craccivora* adults. The highest mortality rate recorded was 74% after the seventh day of treatment with concentrations 1×10^6 and 1×10^8 conidia/ml. At the same time, the lowest concentration eradicated 64.16% of aphids. However, the lowest mortality rate of 2.60% was recorded in the control treatment.

Greenhouse efficacy

Two days after treatment, insect death started in the greenhouse with concentrations of 1×10^6 and 1×10^8 conidia/ml. However, even after three days, mortality appeared in the group of aphids treated with the lowest dose of 1×10^4 conidia/ml, and even among those treated with distilled water. Seven days after treatment (Table 2), the origin concentration had the highest level of toxicity (70.11%). It was followed by the intermediate concentration 1×10^6 conidia/ml (60.09%) and then the lowest concentration (56.08%), while the control treatment caused the mortality rate of *A. craccivora* population (15%).

Concerning the estimation of lethal concentrations and lethal time's values, the results are presented in Table 3: the LC_{50} was 2.23×10^2 conidia/ml in Petri dish trials and 5.43×10^8 conidia/ml in greenhouse test. LT_{50} values with the concentration (1×10^8 conidia/ml) were 3.34 days in vitro and 5.62 days in vivo trials.

Finally, the results of Koch's postulate test indicated that the death plus the total coverage of aphids with white colonies was due to the treatment with the BBAA isolate. Yet, there was no growth of fungal colonies on the cadavers of the control series.

Table 2 Efficacy of *Beauveria bassiana* BBAA isolate against adults of *Aphis craccivora* in greenhouse

Days after treatments	%Mortality			
	Concentrations			
	Control	1×10^4 conidia/ml	1×10^6 conidia/ml	1×10^8 conidia/ml
Day 1	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}
Day 2	0^{c}	0^{c}	8^{c}	18^{bc}
Day 3	6^{c}	12^{c}	18.01^{bc}	30.02^{b}
Day 4	8^{c}	26.02^{b}	28.02^{b}	46.04^{b}
Day 5	10^{c}	32.03^{b}	40.04^{b}	58.06^{ab}
Day 6	12.66^{c}	46.05^{b}	50.06^{b}	62.07^{a}
Day 7	15^{c}	56.08^{ab}	60.09^{ab}	70.11^{a}

The different letters denote the statistical significance determined by ANOVA followed by Tukey test ($p < 0.05$)

Table 3 LC and LT values for *Aphis craccivora* population treated with conidia of BBAA isolate

		In vitro	In vivo
LC concentrations at 7 days)	LC_{10}	0	3.00×10^{-4}
	LC_{30}	1.9×10^{-3}	5.48×10^{3}
	LC_{50}	2.23×10^{2}	5.43×10^{8}
	LC_{90}	5.40×10^{14}	8.81×10^{20}
	LC_{99}	6.74×10^{24}	7.92×10^{30}
	Slope.SE	0.017 ± 0	0.024 ± 0
	Chi	208	103
	P	4.723	3.370
LT times (at 1×10^8 conidia/ml)	LT_{25}	1.96	3.51
	LT_{50}	3.34	5.62
	LT_{75}	5.71	8.97
	LT_{90}	9.25	13.68
	Slope.SE	0.112 ± 0	0.217 ± 0
	Chi	278	138
	P	3.463	1.684

Enzymatic activities

The evaluation of the enzymatic activities of amylase, protease, lipase and chitinase produced by *B. bassiana* isolate was carried out by measuring the diameter of the halo around the fungal colony and the color change of the agar media. The BBAA isolate was able to grow on the entire agar media that were tested in this study (Fig. 4). Halos appeared around the fungal colonies in the different agar media indicating the production of proteases, lipases, and chitinases, which hydrolyzed all of the specific substrates, casein, lipids, and chitin, respectively. But neither lysis zone nor color changes were observed surrounding the colonies of the BBAA isolate on the plates of starch medium, showing the incapacity of the BBAA isolate to produce amylase to break down the starch.

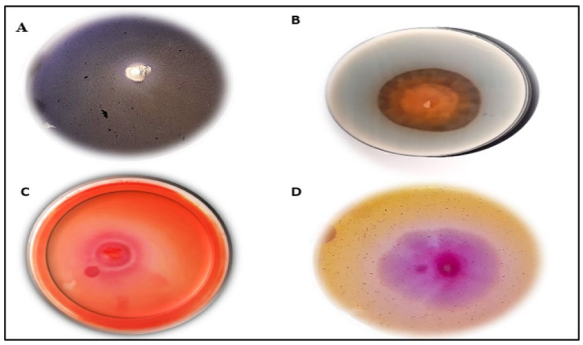

Fig. 4 Substrate hydrolysis zones for the detection of enzymatic activities. **A** Negative production of amylase; **B** positive production of protease; **C** positive lipolytic activity; **D** positive production of chitinase

Discussion

The present study revealed that the EPF isolated from *M. domestica* cadavers was *B. bassiana*. It was identified on the basis of morphological and microscopically features and then confirmed by molecular identification. This isolate was deposited in GenBank and referred to as BBAA. The concentration 1×10^8 conidia/ml was most commonly used by researchers. Toxicity rate of 40% of *A. craccivora* appeared within two days post-treatment and a rate of 74% within seven days, following the treatment in vitro. On the second day of treatment in greenhouse, the same concentration caused a death rate of 18 and 70% after seven days post-treatment. Similarly, even the diluted concentrations of, respectively, 1×10^4 and 1×10^6 conidia/ml eliminated a high number of aphids. On other hand, the LT_{50} value in Petri dishes was 3.34 days, while it was 5.62 days in greenhouse. These results revealed the rapid development and highly insecticidal potential of the *B. bassiana* BBAA isolate against adults of *A. craccivora*. Important

results of various studies on the effects of *B. bassiana* against different aphids were similar to the results of the present study. Among them, some tried three concentrations of, respectively, 1×10^4, 1×10^6 and 1×10^8 conidia/ml against different stages of the lettuce aphid *Nasonovia ribisnigri*. The mortality rate resulting from these concentrations varied between 10 and 94% after nine days of inoculation, whereas the highest concentration (1×10^8 conidia/ml) was the most effective, besides the adult stage as being the most susceptible to infection (Shrestha et al. 2015). On their part, Selvaraj and Kaushik (2014) declared that under greenhouse conditions the concentration (1×10^{10} conidia/ml) of the spore suspension of *B. bassiana* killed 85.04% of *A. craccivora* on fenugreek under greenhouse conditions, while 55.21% of mortality rate was induced by the lowest concentration (1×10^4 conidia/ml) in the seventh day post-treatment. The same researcher reported that the LC_{50} values were 1.2×10^8 conidia/ml, and the TL_{50} values were 97 h for a concentration of 1×10^8 conidia/ml and 157 h for a concentration of 1×10^4 of conidia/ml. Likewise, a study on the *Sitobion avenae* conducted by Ali et al. (2018), in laboratory, reveals that the treatment with a concentration 1×10^6 conidia/ml of the spore suspension of *B. bassiana* caused 39% mortality and a 15% reduction in fertility within 96 h of inoculation. Jandricic et al. (2014) compared the effects of 44 fungal isolates and four commercial products of *Beauveria*, *Metarhizium* and *Isaria* against larvae and adults of *M. persicae* and *A. gossypii*. Six days after application, *Beauveria* isolates were the most effective, especially *B. bassiana* 5493, which killed 61.6% of *M. persicae* and 55.6% of *A. gossypii*, while the most virulent isolates 738 of *M. anisopliae* caused 48% of mortality in *A. gossypii*. However, *I. javanica* isolates were less effective; 30% mortality only in *A. gossypii* and 23% of mortality only in *M. persicae* were recorded as an immediate result of treatment by *I. javanica* 2749. The same study reported that mortality of aphid larvae was about 35% lower than that of the adults. All the studies, mentioned above, confirm that insect mortality controlled by EPF is associated with the virulence of the strain, the conidia concentration, the time after inoculation and the life stage of aphids.

The insect cuticle is composed of many proteins, the chitin and the lipids, which act as a defensive barrier against the external environmental factors, the predators and the microbes, as well as the chemical insecticide resistance (Wang et al. 2019). The present study revealed that the enzymatic activity tests of *B. bassiana* BBAA had an effect on the decomposition of substrates used in agar media due to the production of hydrolytic enzymes, including protease, chitinase and lipase; hence, this isolate was able to break down the components of aphid cuticle. Thus, the fungus was allowed to penetrate and grow into the whole body of *A. craccivora*. Several studies have shown the role of enzymatic activities produced by EPF in their pathogenicity against insects. According to Cheong et al. (2020), due to their increased chitinase production, *B. bassiana* Bb0062 and BbK4B3 were more virulent against adult of *M. persicae*.

Conclusions

This study has shown the importance of natural environments in finding alternatives to chemical pesticides, such as the EPF *B. bassiana* that was isolated from dead insects, identified morphologically and on the basis of its microscopic characteristics and on sequencing of the ITS region. It was then deposited in NCBI GenBank under the accession number ON715442 and was coded *B. bassiana* BBAA. This isolate grew rapidly and was highly virulent against *A. craccivora*. It was characterized by notable production levels of chitinase, protease and lipase that play a crucial role in the pathogenicity of *B. bassiana* against aphids, while the secondary metabolites and the effectiveness of this isolate on insect pests will be studied in prospective research. These biological resources need to be explored and exploited to develop safer and more effective strategies for controlling pests and protecting crops.

Abbreviations

ALCV	Alfalfa Leaf Curl Virus
AMV	Alfalfa Mosaic Virus
CMV	Cucumber Mosaic Virus
EPF	Entomopathogenic fungi
ITS	Internal transcribed spacer
LC	Lethal concentration
LEV	Lucerne Enation Virus
LRSBG	Laboratory of Research on Biological Systems and Geometry
LT	Lethal time
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
RH	Relative humidity
SDA	Sabouraud Dextrose Agar

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Author contributions

AA and KR designed this study; AA performed experiments; AL analyzed data. AA wrote the paper; KR and AFR revised the paper. All authors approved this final manuscript. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

All data generated and analyzed during this study are indicated in the manuscript.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
The co-authors gave their permission for publication.

Competing interests
The authors have no conflict of interest to declare.

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