International Journal of Tropical Insect Science
https://doi.org/10.1007/s42690-024-01230-8

ORIGINAL RESEARCH ARTICLE

A new fungal entomopathogen has potency as a biocontrol agent of longhorn beetle larva, *Osphranteria coerulescencs*

Hadi Farrokhzadeh[1] · Shervin Sharifi[2] · Gözde Büşra Eroğlu[3] · Javad Karimi[2]

Received: 12 June 2023 / Accepted: 5 March 2024

Abstract
Entomopathogenic fungi (EPFs) are a significant group of insect pathogens that are used as microbial insecticides with distinct physiological mechanisms of pathogenicity. During our survey in the middle of March 2020 to early October 2022 on natural EPF as a biological control agent of the longhorn beetle (LHB) *Osphranteria coerulescens*, new isolates of EPFs were found on LHB larvae in apple orchards of Dargaz, Razavi Khorasan province, Iran. The nBLAST results of this these indigenous isolate indicated the highest similarity to *Cladosporium* sp. (Ascomycota: Davidiellaceae) with ON307222.1 access number and for EF-1α indicated the highest similarity to *Cladosporium* sp. with MH724951.1 access number. To assess the virulence of these indigenous isolates of EPF, a series of tests were conducted on the LHB larvae stages. Conidial concentrations 10^4, 10^5, 10^6, 10^7, and 10^8 (conidia/ml^{-1}) caused (10.21 ± 2.1), (23.44 ± 2.4), (28.72 ± 2.6), (33.23 ± 3.1) and (34.02 ± 2.8) % of mortality in LHB, respectively and there was no significant difference between 10^8 and 10^7 (conidia/ml^{-1}) concentrations. The mortality results by the time showed that the process did not start until 3 days after treatment, and after that, it sped up. This research gathers new information about cryptic inhabiting larvae which shows considerable potential for this *Cladosporium* species to be implemented within the microbial control program of the LHB. This research is a new report isolating the pathogenicity of *Cladosporium* sp. on LHB. These fungal isolates have considerable potential for the microbiological control of the LHB.

Keywords Epizooty · Natural infection · Insect pathology · Microbial control · Pest management · Conservation

Introduction

The long horn beetle, *Osphranteria coerulescens* Redtenbacher (Cerambycidae: Coleoptera) is a species that extensively dispersed in Afghanistan, Iran, Iraq, Pakistan, and Turkey. These Cerambycid's larvae feed on living twigs and branches of various Rosaceae, and on occasion, when populations are excessive, they can seriously harm fruit trees (Sharifi et al. 1970). The LHB larvae spend winter within larval galleries in immature stages (Behdad 1984). Due to the cryptic behavior of the larvae, the management of this beetle is a challenging issue. One of the main methods for managing *O. coerulescens* involves mechanical management, which involves cutting and burning the infested branches. Since many different groups of natural enemies find shelter in abandoned larval galleries and numerous species of parasitoids and predators seek refuge there throughout the winter (Karimi et al. 2017), However, this is not seen as a viable environmental strategy. Due to the inaccessible habitats of the pest, cryptic behavior during the immature stages under the bark of host trees, and also asynchronously in hatching the eggs and emerging the larvae, the application of chemical pesticides against this pest poses unique management challenges (Esmaeili 1983). To control these types of cryptic inhabiting pests, numerous researchers have addressed the use of entomopathogenic fungi (Liu and Bauer 2008). Entomopathogenic fungi (EPFs) are a significant group of insect pathogens that are used as microbial

✉ Javad Karimi
Jkb@um.ac.ir

[1] Department of Entomology, Southern Piedmont Agricultural Research & Extension Center, Virginia Tech, Blackstone, VA, USA

[2] Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad, P.O. Box 91775-1163, Mashhad, Iran

[3] Departmant of Molecular Biology and Genetics, Faculty of Science, Erzurum Technical University, Erzurum, Türkiye

Published online: 03 April 2024

insecticides and have physiologically distinct mechanisms of pathogenicity. These pathogens can infect their hosts without being ingested, which is a critical factor because microbes that require ingestion to infect a host are not appropriate for use against cryptic larvae like wood borer larvae (Hajek and Bauer 2007). Since the 1960s, more than 170 entomopathogenic fungi-based biopesticides have been produced; about 75% of these are still in use or have been registered for use (Faria and Wraight 2007). These kinds of entomopathogens frequently infect a wide range of insect pests, including Coleopterans, Lepidopterans, and other groups (Lee et al. 2015; Khoury et al. 2020). While several reports are available on the application of fungi as microbial agents of wood-boring pests, in the case of LHB, due to our knowledge, no record of this type of attempt has been found. Among the natural enemies of *O. coerulescens* larvae, several species of entomopathogens, including fungi were found infecting larval populations in Iran (Mohammadyani et al. 2016).

Several studies have been conducted on the effective use of EPFs against wood-boring insect infestations. *Beauveria bassiana* (Balsamo) Vuillemin caused 75% mortality of *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) larvae (Hajek and Bauer 2007). In another works, fiber bands containing EPFs were applied to control *Anoplophora glabripennis* Motschulsky (Coleoptera: Cerambycidae) (Hajek et al. 2006) and strains of *B. bassiana*, *Metarhizium anisopliae* and *Lecanicillium muscarium* were effective against the sarta beetle larvae, *Aeolesthes sarta* (Farashiani et al., 1998). Also, the conidia of *Beauveria bassiana* were sprayed on trees infested with *Enaphalodes rufulus* Haldeman (Coleoptera: Cerambycidae) (Meyers et al. 2013). A recent study about the use of entomopathogenic fungi on Asian longhorn beetle showed that *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocerales: Clavicipitaceae) and *Metarhizium brunneum* Petch (Hypocerales: Clavicipitaceae) killed 100% of treated beetles with the shortest survival times. After that five strains of *Beauveria bassiana* ranging from 0 to 77.3% mortality within 28 d and finally two *Isaria fumosorosea* Wize (Hypocerales: Cordycipitaceae) and the *Lecanicillium muscarium* Zare & Gams (Hypocerales: Cordycipitaceae) were not pathogenic to Asian longhorn beetle adult (Clifton et al. 2020).

The first step toward the implementation of effective microbial management is to look for virulent strains of indigenous microorganisms and subsequently assessing their effectiveness against pests (Lacey and Shapiro-Ilan 2008). Therefore, in this study, we aimed to characterize a native EPF as a natural pathogen from the larvae of LHB and evaluate its pathogenicity against LHB at different concentrations and times in laboratory conditions.

Materials and methods

Insect sources

The *Ospheranteria coerulescens* larvae were collected from the LHB-infested apple shoots in orchards of Dargaz, Razavi Khorasan Province, North East Iran, (37.44° N, 59.10° E) from late March 2020 to early October 2022.

The larvae were taken out of the galleries, put into perforated plastic containers (175 mm×85 mm), and kept at 4 ± 1 °C until needed. The weight of the larvae was 0.5 ± 0.02 gr on average. In all bioassays, insects with estimated equal weights were used across treatments using the method described by Dobies et al.(2008).

Isolation of fungi

A fungal isolate was collected from *O. coerulescens* larvae found in an apple orchard, from Dargaz, Khorasan Razavi. During the survey, the larvae that seemed to be infested by EPFs were removed and translocated into the lab. The described method by Kumar et al. (2015) was used to isolate putative EPF. The individual larva was immersed in 70% ethanol (Merck, Darmstadt, Germany) for 3 s before being rinsed three times with deionized distilled water (ddH2O). The air-dried individuals were then put in the glass Petri dish (10 cm diameter), lined with wet Whatman paper (no. 1), and incubated at 25 ± 2℃ for 72 h. Cadavers that showed signs and/or symptoms of fungal infection were placed on a 1% potato dextrose agar (PDA) medium, where they were cultured for monitoring the infection [25 ± 2 ℃, 60 ± 5% RH with a photoperiod of 16:8 h (L: D)] for two weeks till sporulation.

To test Koch's ideas, healthy larvae were dipped for 5 second in suspensions of 1.0×10^4, 10^5, 10^6, 10^7, and 10^8 conidia per ml in 0.05% Tween 80 for possible confirmation of fungal pathogenicity. There were 10 larvae per treatment. The treatments were incubated for seven days while being closely observed. The insects of the control group were submerged in sterile 0.05% Tween 80 (Merck, Darmstadt, Germany) for the same time and under the same controlled situations as the treated insects. Mycosis symptoms were noted four days after the cadavers had been surface sterilized with 0.2% sodium hypochlorite solution.

Characterization of fungi isolates

Morphological identification

For morphological identification, macro- and micromorphological features of fungal colonies were examined. During the macroscopic characterization, the conidial color and

colony growth in pure fungal cultures were noted. Conidiogenous cells, conidiophores, and other characteristics were examined under a microscope, and the genes were identified using associated keys (Bensch et al. 2011; Humber 2012). Twenty samples were mounted, dyed with aniline blue, and then taken pictures using a 400x digital microscope (Olympus, Tokyo, Japan).

Molecular characterization

For molecular investigation, the isolated fungus were grown on PDA plates for 15 days in controlled conditions. Following that, the mycelial mass was removed and placed in Erlenmeyer flasks with 50 cc of sterile PDB liquid medium. (200 g potato and 10 g dextrose in 1 l of sterilized water plus 0.035 g streptomycin). The flasks were kept in a shaker at room temperature for ten days. Following collection, the mycelia were extracted with sterile distilled water, placed in a sterile 2-ml microtube, and maintained at 4 °C until required.

Using the CTAB technique, genomic DNA was extracted based on the method described by Doyle and Doyle (1987). Nitrogen-frozen samples were ground using laboratory Mortar & Pestle and placed into a 1.5 ml microcentrifuge tube. 500 µl CTAB isolation buffer (2% hexadecyltrimethylammonium bromide, 1.4 M NaCl, 0.2% β2-ME, 20 mM EDTA, 100 mM Tris-HCl, pH 8) were added into a 1.5-ml microcentrifuge tube. The sample was incubated at 65 °C for 60 min with occasional mixing, and then 500 µl chloroform/isoamyl alcohol (24:1 v/v) was added to each sample. The sample was briefly vortexed and then centrifuged in a microcentrifuge for 4 min at 13,000 rpm. To precipitate the nucleic acid, 400 µl of supernatant was transferred into a fresh 1.5 ml microcentrifuge tube, then 400 µl of ice-cold isopropanol was added to the tube, and the tube was inverted five times before being placed in the refrigerated at -23 °C for 10 min. At 13,000 rpm for 4 min, the sample was centrifuged in a microcentrifuge, and the supernatant was discarded. The pellet was suspended in 50 ml of nuclease-free water after being air-dried for two hours.

The ITS and EF-1α gene regions were subjected to Polymerase Chain Reactions (PCR) with the ITS4 and ITS5 and EF1α-F and EF1α-R primers respectively (Table 1).

Table 1 The primers used for molecular identification of EPF

Target region	Primers and sequence	Reference
ITS	ITS-F: 5′-TCCTCCGCTTATTGATATGC-3′ ITS-R: 5′-GGAAGTAAAAGTCGTAACAA-3′	(White et al. 1990)
EF-1α	EF1α-F: 5′-ATGGGTAAGGARGACAAGAC-3′ EF1α-R: 5′-GGARGTACCAGTSATCATG-3′	(Donnell et al. 2009)

PCR was performed in an Eppendorf Mastercycler gradient (Eppendorf, Hamburg, Germany) in a standard 25 µl reaction containing 5 µl *Taq* DNA Polymerase Master Mix (Ampliqon, Odense, Denmark), 1 µl of each primer, 3 µl DNA template and 15 µl of double distilled water. Temperature conditions were denaturant at 94 ℃ for 60 S, annealing at 51 ℃ for 90 S, and extension at 72 ℃ for 90 S (30 cycles, plus an initial denaturation at 94 ℃ for 1 min and a final extension at 72 ℃ for 8 min). Temperature conditions of the EF-1α amplification were similar to ITS except for annealing which was set at 53 ℃.

The PCR product was loaded onto a 1% agarose gel and visualized under UV light. For direct sequencing of double strands of DNA, the PCR products were sent to the Macrogen sequencing service facilities (Macrogen Inc., Seoul, Korea).

The raw sequence file is first checked then a consensus sequence is created and submitted. The sequences were assembled using the DNA Baser program. Using NCBI Genbank's nBLAST search tool, our consensus sequence was compared with the database sequence. Genbank sequences with the highest similarity were obtained, and the phylogenetic tree was reconstructed using valid and confirmed data from those sequences. Multiple alignments were carried out using the ClustalW software (Thompson et al. 1994). MEGA6 software was used to reconstruct phylogenetic trees using the neighbor-joining method (Tamura et al. 2013). A bootstrap analysis was performed with 1000 replicates.

Preparation and viability test for the conidia

Using a PDA method, conidial viability was evaluated and all bioassays were conducted using cultures having conidial viability of at least 90%. Three replicates were used to evaluate the germination percentage of conidia. After preparing the stock cultures of conidia with three replications, the number of conidia was counted using a Neubauer hemocytometer (Superior, Marienfeld). The stock culture was diluted to prepare other concentrations.

Virulence assays

Following the results of the preliminary testing, five concentrations of EPF isolates were chosen for the virulence assay and tested on LHB larvae using the dipping method as described by (Dubois et al. 2008). In brief, larvae were directly immersed separately for 5 seconds in conidial suspension ranges (1×10^4, 10^5, 10^6, 10^7, and 10^8 conidia/ml^{-1}), then transferred to sterile Petri dishes lined with Whatman No. 1 filter paper and maintained under controlled conditions. After treatment, the larvae were placed in plastic

containers (120 × 100 mm), along with fresh apple twigs as food, and maintained in a controlled condition [25 ± 2 ℃, 60 ± 5%RH with a photoperiod of 16:8 h (L: D)]. Four larvae per treatment were used in a four-replicates random design.

Sterilized water with 0.05% Tween 80 without fungal conidial was used as the control treatment. The treatments were examined every day until 21 days. Any dead larvae were surface sterilized, washed with sterile distilled water three times, placed in sterile Petri dishes lined with filter paper, and kept in a controlled condition [25 ± 2 ℃, 60 ± 5% RH with a photoperiod of 16:8 h (L: D)] to investigate for mycosis. All experiments were repeated twice.

Statistical analysis

For all bioassays, statistical analysis was performed using SAS software version 9.1. (SAS Institute 2002). The general linear model (PROC GLM) was used to analyze the variance of mean numbers. Data were control-corrected for mortality (Abbott 1925). The arcsine square root transformation was used to standardize percentage data as needed. Data from the larval bioassays were evaluated using a completely random design (5 concentrations 7 days). The mortality data were analyzed via Proc ANOVA and the means were separated using Tukey (HSD) test ($p = 0.05$). Using the Probit procedure, the median lethal concentration (LC_{50}) values (fiducial 95%) for the larvae exposed to EPFs were determined in IBM-SPSS statistics, version 20 (SPSS Inc. IBM Company 2010).

Results

Entomopathogenic fungus identification

During our survey we identified one isolate of an EPF belonging to *Cladosporium* sp. Link (Ascomycota: Davidiellaceae) in the population of *O. coerulescens* larvae from the apple orchards in Dardgaz, Khorasan Razavi, Iran.

The hyphae of *Cladosporium* sp. had a velvety appearance and were green to brown. Furthermore, long, branched chains of conidia (3-5.5 × 2–5 µm), most of which were small ellipsoidal, were seen in branched conidiophores (25–165 × 2.5-4 µm) (Vries 1952; Campbell and Johnson 2013).

For molecular identification, the ITS and EF-1α sequences of the fungal isolated were used to reconstruct of a phylogenetic tree. The obtained DNA sequences of the isolated species were deposited in the NCBI Genbank under accession numbers OQ430660 for ITS and OR102503 for EF-1α. For ITS gene region nBLAST search indicated the highest similarity to *Cladosporium* sp. with ON307222.1 access number and for EF-1α indicated the highest similarity to *Cladosporium* sp. with MH724951.1 with a high bootstrap value. The results of reconstruction Neighbor-joining tree based on ITS and EF-1α showed that this species sited in a single clade with *Cladosporium* sp. (ON307222.1) and (MH724951.1) respectively (Figs. 1 and 2). Consequently, morphological identification and ITS and EF-1α sequencing analyses were used to identify the genes of the fungi.

Bioassay

Bioassay results revealed that *Cladosporium* sp. caused the infection in LHB larval stages. The findings also indicated that a rise in conidial concentration was associated with a rise in mortality. All of the dead larvae, which were found to be cadavers 18 days after inoculation, confirmed Koch's hypotheses.

Virulence assays

Cladosporium sp., an entomopathogenic fungus, caused (mean ± SE) 25.92 ± 2.6% mortality in LHB (Table 2). Conidial concentrations 10^4, 10^5, 10^6, 10^7, and 10^8 (conidia/ml^{-1}) caused (10.21 ± 2.1), (23.44 ± 2.4),

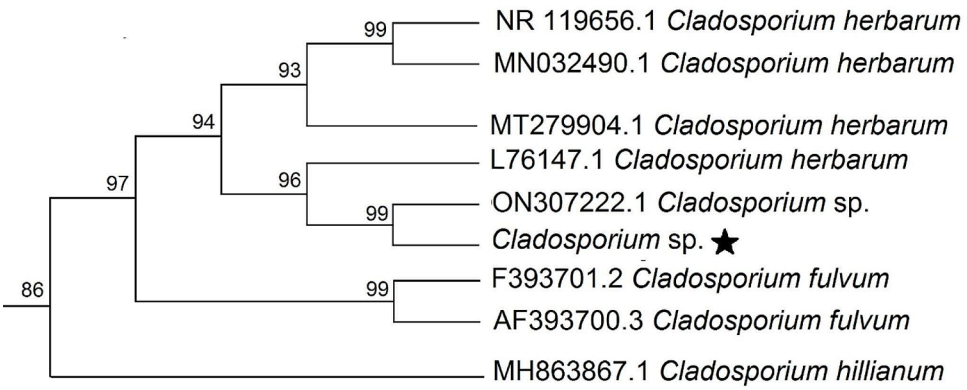

Fig. 1 Phylogenetic reconstructed based on the ITS gene using the NJ method. Numbers at nodes represent the percent bootstrap values. The samples of this study are shown with star symbols

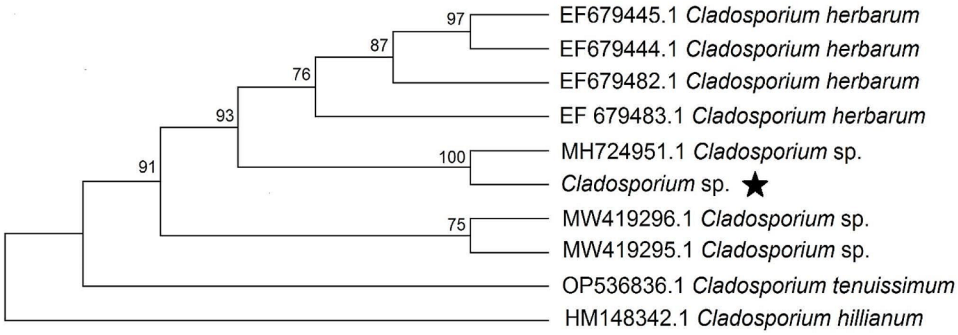

Fig. 2 Phylogenetic reconstructed based on the EF-1α gene using NJ method. Numbers at nodes represent the percent bootstrap values. The samples of this study are shown with star symbols

Table 2 ANOVA parameters for main effects and interactions caused by entomopathogenic fungi on LHB mortality

Source	D.f	MS	F	P
Concentration (C)	4	27.47	26.85	< 0.0001
Days After Treatment (DAT)	6	251.71	246	< 0.0001
C*DAT	24	5.03	4.92	< 0.0001

(28.72 ± 2.6), (33.23 ± 3.1) and (34.02 ± 2.8) % of mortality in LHB, respectively (Fig. 3).

Five days after inoculation, the equivalent LC_{50} values for EPF on the larvae were determined, and they were used to compare the virulence of the applied fungal species. The LC_{50} values also indicated that the *B. bassiana* has high virulence on 10^8 and 10^6 (conidia/ml^{-1}) against larva insects of *O. coerulescens*. No mycosis was found in the control ($n = 120$, $LC_{50} = 4.8 \times 10^5$, Slope ± SE^b = 0.35 ± 0.08, χ^2 (df) = 9.57 (22), *P* value^c 0.99).

Mortality rates by EPF increased over time. Larva mortality caused after 3,6,9,12,15 and 18 days were 11.01, 12.43, 24.28, 37.64, 45.11, and 47.32%, respectively (Fig. 4). There is no mortality at 1 day after treatment and also there is no significant difference between mortality rate after 3 and 6 days and also between 15 and 18 days after treatment (Fig. 4).

Discussion

During our survey, one native entomophatogenic fungus was isolated from *O. coerulescens* larva in an apple orchard, described, and tested in a trial series to examine its interaction with its natural host insect. Previous research by Mohammadyani et al. (2016) has shown that LHB infested by *Beauveria bassiana* and *Metarhizium anisopliae* as two native entomophatogenic fungi. Although, *Cladosporium* spp. have been identified as entomopathogens on several insect pests (Abdel-Baky 2000; Narmani et al. 2016; El-Sawy et al. 2019; Kishore et al. 2019; Mousavi et al. 2022). However, occurrence of *Cladosporium* sp. within the larvae of *O. coerulescens* is a new report for the incidence of this EPFs as a natural entomopathogen on the *O. coerulescens*.

To identify species within the *Cladosporium* genus, molecular analysis of DNA sequences such as translation elongation factor 1- alpha and ITS has been proposed (Becchimanzi et al. 2021). As a result, the fungus isolates used

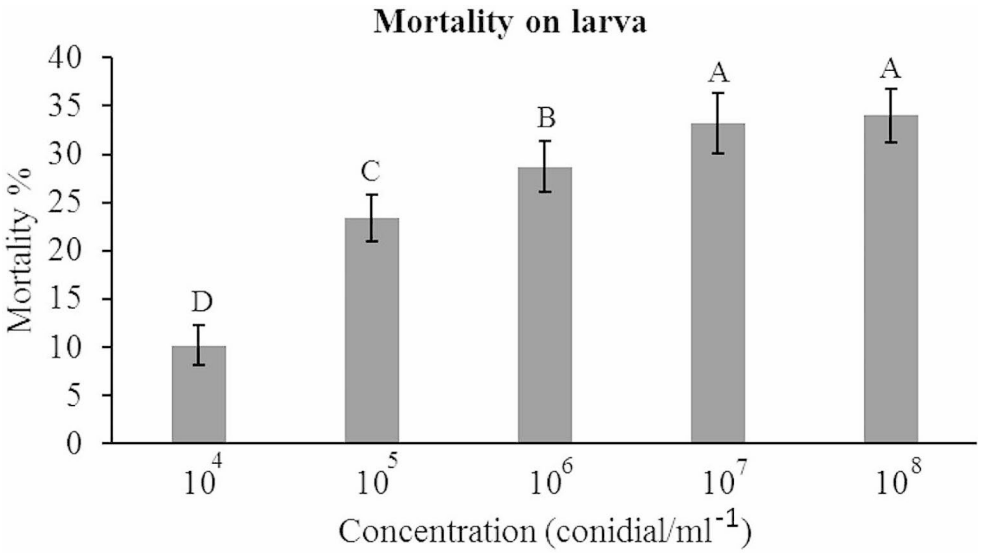

Fig. 3 Mean corrected mortality (% ±SE) of *Osphranteria coerulescens* larvae at 5 concentrations (1×10^4, 10^5, 10^6, 10^7, and 10^8 conidia/ml^{-1}). Different letters indicate a significant difference between concentration, Tukey test ($p < 0.05$)

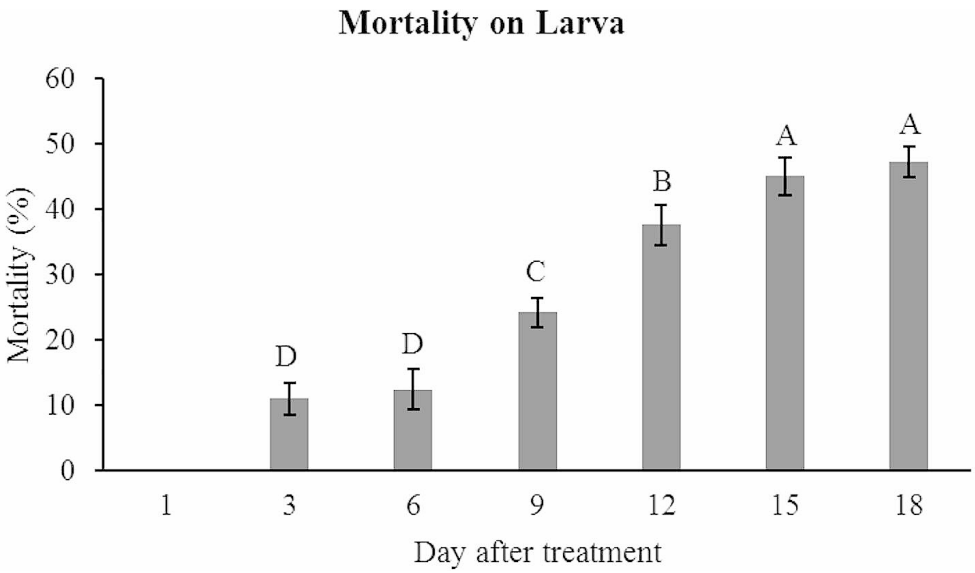

Fig. 4 Mean corrected mortality (% ±SE) of *Osphranteria coerulescens* larva, by EPF at 3–18 days after treatment. Different letters indicate a significant difference between concentration, Tukey test ($p < 0.05$)

in this study was identified up to the genus level. Although *Cladosporium* sp. was identified using morphological and molecular features, more investigation is necessary to ensure precise species-level identification of the fungus.

As other EPF, *Cladosporium* sp. conidia germinate after adhering to their host's cuticle, the hyphae penetrate the cuticle, and proliferation occurs for a few days within the host's hemocoel. Furthermore, EPF produces some mycotoxins leading to the host's death (El-Sawy et al. 2019; Elbanhawy et al. 2019; Shaker et al. 2019).

It is crucial to research the distribution, isolation, and characterization of native EPF species (Bidochka et al. 1998), as exotic species/strains of EPFs have often failed in biocontrol programs due to factors relating to host species, or poor adaptability of exotic fungal strains to local environmental conditions (Lacey et al. 2015). Native EPF isolates may be more resistant to environmental changes and compatible with the ecosystem here than exogenous ones, which is a well-known fact (Kim and Kim 2008). This study's primary objective was to determine whether local isolates of EPF species could protect against longhorn beetle larvae.

We attempt to explain the significance of the key components of each component involved by taking into account the "disease triangle" that represents the link between the insect host, pathogen, and environment (Tanada and Kaya 1993; Scholthof 2007). According to the LHB larva bioassay, maximum mortality was observed in the 10^7 and 10^8 conidia/ml^{-1}, causing 33.23 and 34.02% mortality respectively. Determining whether or not a control strategy can be successful critically depends on an insect host's susceptibility to fungal infection. Host, pathogen, and environmental interactions are influenced by host density as well as genetic and behavioral traits (associated with resistance). For instance, the wax layer's thickness (Shimazu et al. 2002) and amino acid properties of the insect cuticle (Tanada and Kaya 1993) play critical roles in preventing hyphal invasion.

The EPF characteristics are the second part of the disease triangle. Many factors, such as fungal strain, the nutritional condition of fungi, and different insect orders, have demonstrated different levels of sensitivity to *Cladosporium* sp. Bahar et al. (2011) reported that *Cladosporium* sp. treatment resulted in mortality of 54% of first instar larvae of *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) compared with 5% in the controls. This species was also efficacious against *Aphis gossypii* Glover (Hemiptera: Aphididae) and *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) with 67% and 75% mortality rates, respectively. Furthermore, it was discovered that *Cladosporium uredinicola*, when used as endophytic treatment, causes abnormalities such as form changes, extensive vacuolization, and necrosis in a significantly greater portion of the hemocytes of *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) larva (Thakur et al. 2014). Mousavi et al. (2022) evaluated the pathogenicity of *Cladosporium* sp. on the black bean aphid, *Aphis fabae* Scopoli (Hemiptera: Aphididae) under laboratory conditions and found a mortality rate of 26.4 ± 1.1% in nymphs and 24.0 ± 1.2% in adults of aphids.

The concentration of entomopathogenic fungi can indeed play a significant role in their virulence and effectiveness as biocontrol agents against insect pests. In our study we observed that the highest mortality rate was observed in a concentration of 10^8 Conidia/ml. It was also observed that there is no significant difference between mortality rate in concentrations of 10^8 and 10^7 conidia/ml. This suggests that the effectiveness of the EPF in causing mortality reached a plateau at the higher concentration, and further increasing the concentration did not significantly enhance the mortality rate. It's worth noting that the optimal concentration of EPF

can vary depending on various factors, including the target insect pest, the specific EPF strain used, and the environmental conditions. Previous studies suggested that applying higher concentrations of fungal conidia quickly causes mortality (Castrillo et al. 2008; Mnyone et al. 2009; Shanley et al. 2009). Low conidia concentrations can result in small infective doses that can be countered by insect immune responses like melanization, encapsulation, and phagocytosis of blastospores. According to Shanley et al. (2009), determining the LC_{50} values is an essential factor in figuring out how active EPFs are. The LC_{50} of *Cladosporium* sp. on the LHB larvae in our experiment was determined as 4.8×10^5 conidia/ml^{-1}.

In our research we found that the mortality started three days after treatment and then sped up suggests that there was a delayed onset of action followed by a rapid progression of the infection process. This pattern is consistent with the typical behavior of entomopathogenic fungi once they have established an infection within the insect host. Dhawan and Joshi (2017) found that the mortality of *Beauveria bassiana* at 1×10^7 conidial/ml on *Pieris brassicae* Linnaeus, 1758 (Lepidoptera, Pieridae) was 30% ten days after infestation. Overall, The infection process of entomopathogenic fungi involves several steps and depends on various factors like conidial attachment, cuticle degradation using enzymes, reaching the hemolymph using the formation of specialized infection structures such as appressoria and penetration pegs, creating the hyphal bodies, and eventually the mycosis (Mannino et al. 2019). Jiang et al. (2020) found that the cuticle penetration is an important factor for killing insect and on *Locusta migratoria* Linnaeus (Orthoptera: Acrididae) by *Metarhizium anisopliae* depends on the assistance from such related enzymes (Yu et al. 2016). Abdel-Baky et al. (2005) found that proteinase and chitinase on *Cladosporium* sp. are the most important enzymes that has key factor of cuticle degradation on *Bemisia argentifolii* and *Aphis gossypii* adult.

In conclusion, finding native EPF species/isolate that can exist and grow in the larval growth environment, isolating them, and re-examining their pathogenicity in different concentrations and times are crucial in the microbial control of LHB because the LHB larva is the most vulnerable stage to fungal pathogens and lives inside the branch. Our results are in agreement with those of previous works (Shimazu et al. 2002; Reay et al. 2007; Meyers et al. 2013; Mohammadyani et al. 2016, Ranjbar et al. 2023). These findings are consistent with earlier research and suggest that native EPFs have great potential as wood-boring insect biocontrol agents. It is necessary to conduct more research on the use of EPF suspensions in unique formulations under unique climatic conditions.

Declarations

Conflict of interest We don't have any conflict of interest.

References

Abbott W (1925) A method of computing the effectiveness of an insecticide. J Econ Entomol 18:265–267. https://doi.org/10.1093/jee/18.2.265a

Abdel-Baky N (2000) *Cladosporium* spp. an entomopathogenic fungus for controlling whiteflies and aphids in Egypt. Pakistan J Biol Sci 3:1662–1667 https://doi.org/10.3923/pjbs.2000.1662.1667

Abdel-Baky NF, Fadaly HA, EI-Nagar ME, Arafat NS, Abd-Allah RH (2005) Virlence and enzymatic activities of some entomopathogenic fungi against whiteflies and aphids. J Plant Prot Pathol 30(2):1153–1167. https://doi.org/10.21608/jppp.2005.238696

Bahar MH, Backhouse D, Gregg PC, Mensah R (2011) Efficacy of a *Cladosporium* sp. fungus against *Helicoverpa armigera* (Lepidoptera: Noctuidae), other insect pests and beneficial insects of cotton. Biocontrol Science and Technology 21(12)

Becchimanzi A, Zimowska B, Nicoletti R (2021) Cryptic diversity in *Cladosporium cladosporioides* resulting from sequence-based species delimitation analyses. *Pathogens* 10(9): 1167

Behdad E (1984) Pests of fruit trees in Iran. Isfahan, Neshat press

Bensch K, Braun U, Groenewald J, Crous P (2011) The genus *Cladosporium*. Study Mycol 72:1–401. https://doi.org/10.3114/sim0003

Bidochka M, Kasperski J, Wild G (1998) Occurrence of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria Bassiana* in soils from temperate and nearnorthern habitats. Can J Bot 76:1198–1204. https://doi.org/10.1139/b98-115

Campbell C, Johnson E (2013) Identification of pathogenic Fungi. Wiley, Oxford, UK. https://doi.org/10.1002/9781118520055

Castrillo L, Ugine T, Filotas M, Sanderson J, Vandenberg J, Wraight S (2008) Molecular characterization and comparative virulence of *Beauveria bassiana* isolates (Ascomycota: hypocreales) associated with the greenhouse shorefly, *Scatella tenuicosta* (Diptera: Ephydrididae). Biolgical Control 45:154–162. https://doi.org/10.1016/j.biocontrol.2007.10.010

Clifton EH, Jaronski ST, Hajek AE (2020) Virulence of commercialized fungal entomopathogens against Asian longhorned beetle (Coleoptera: Cerambycidae). J Insect Sci 20(2):1–6. https://doi.org/10.1093/jisesa/ieaa006

Dhawan M, Joshi N (2017) Enzymatic comparison and mortality of *Beauveria bassiana* against cabbage caterpillar *Pieris brassicae* LINN. Brazilian J Microbiol 48(3):522–529. https://doi.org/10.1016/j.bjm.2016.08.004

Donnell OK, Sutton D, Rinaldi M (2009) Novel multilocus sequence typing scheme reveals high genetic diversity of human pathogenic members of the *Fusarium incarnatum-F. Equiseti* and *F. Chlamydosporum* species complexes within the United States. J Clin Microbiol 47(12):3851–3861. https://doi.org/10.1128/JCM.01616-09

Doyle J, Doyle J (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Dubois T, Lund J, Bauer L, Hajek A (2008) Virulence of entomopathogenic hypocrealean fungi infecting *Anoplophora glabripennis*. Biocontrol 53:517–528. https://doi.org/10.1007/s10526-007-9112-2

El-Sawy M, Mostafa E, Ismail N (2019) Secondary metabolites of the entomopathogenic fungus, *Cladosporium cladosporioides* and its relation to toxicity of cotton aphid, *Aphis gossypii* (Glov). Int J Entomol Nematology 5:115–120

Elbanhawy A, Elsherbiny E, El-Mageed AA, Abdel-Fattah G (2019) Potential of fungal metabolites as a biocontrol agent against cotton aphid, *Aphis gossypii* Glover and the possible mechanisms of action. Pestic Biochem Physiol 159:34–40. https://doi.org/10.1016/j.pestbp.2019.05.013

Esmaeili M (1983) Important pests of fruit trees. Tehran, Sepehr Publishing Center

Farashiani ME, Askary H, Hoseini E (1998) Laboratory investigation on virulence of three entomopathogenic fungi against the larvae of *Aeolesthes sarta* (Col.: Cerambycidae). J Entomol Soc Iran 28:19–34

Faria MR, Wraight SP (2007) Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. Biol Control 43:237–256. https://doi.org/10.1016/j.biocontrol.2007.08.001

Hajek A, Huang B, Dubois T, Smith M, Li Z (2006) Field studies of control of *Anoplophora glabripennis* (Coleoptera: Cerambycidae) using fiber bands containing the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria Brongniartii*. Biocontrol Sci Technol 16:329–343. https://doi.org/10.1080/09583150500335962

Hajek A, Bauer L (2007) Microbial control of wood-boring insects attacking forest and shade trees. Field manual of techniques in invertebrate pathology. L. LA and K. HK, Springer, pp 505–525. Secaucus. https://doi.org/10.1007/978-1-4020-5933-9_24

Humber R (2012) Identification of entomopathogenic fungi. *Manual of techniques in invertebrate pathology*. L. LA, Amsterdam, Academic Press, pp 151–186

Jiang W, Peng Y, Ye J, Wen Y, Liu G, Xie J (2020) Effects of the entomopathogenic fungus *Metarhizium anisopliae* on the mortality and Immune Response of *Locusta Migratoria*. Insect 11(1):1–12. https://doi.org/10.3390/insects11010036

Karimi J, Darsouei R, Sharifi S (2017) A Bethylid wasp (Hymenoptera: Bethylidae) as a promising biocontrol agent of Rosaceous long horn beetle *Ospheranteria coerulescens* (Coleoptera: Cerambycidae). Entomol News 127(2):123–132. https://doi.org/10.3157/021.127.0207

Khoury YE, Noujeim E, Ravlić J, Oreste M, Addante R, Nemer N, Tarasco E (2020) The effect of entomopathogenic nematodes and fungi against four xylophagous pests. Biocontrol Sci Technol 30(9):983–995. https://doi.org/10.1080/09583157.2020.1781059

Kim J, Kim K (2008) Selection of a highly virulent isolate of *Lecanicillium attenuatum* against cotton aphid. J Asia Pac Entomol 11:1–4. https://doi.org/10.1016/j.aspen.2008.02.001

Kishore PV, Sekhar VC, Bhavani B, Upendhar S (2019) *Cladosporium cladosporioides*: a new report of parasitism on sugarcane woolly aphid, *Ceratovacuna Lanigera* Zehntner. J Entomol Zoological Stud 7:1122–1126

Kumar C, Jacob T, Devasahayam S, D'Silva S, Kumar N (2015) Isolation and characterization of a *Lecanicillium psalliotae* isolate infecting cardamom thrips (*Sciothrips cardamomi*) in India. *BioControl* 60: 363–373

Lacey L, Shapiro-Ilan D (2008) Microbial control of insect pests in temperate orchard systems: potential for incorporation into IPM. Ann Rev Entomol 53:121–144. https://doi.org/10.1146/annurev.ento.53.103106.093419

Lacey L, Grzywacz D, Shapiro-Ilan D, Frutos R, Brownbridge M, Goettel M (2015) Insect pathogens as biological control agents: back to the future. J Invertebr Pathol 132:1–41. https://doi.org/10.1016/j.jip.2015.07.009

Lee S, Yu J, Nai Y, Parker B, Skinner M, Kim J (2015) *Beauveria bassiana* sensu lato granules for management of brown planthopper, *Nilaparvata lugens* in rice. Biocontrol 60:263–270. https://doi.org/10.1007/s10526-014-9632-5

Liu H, Bauer L (2008) Microbial control of emerald ash borer, *Agrilus planipennis* (Coleoptera: Buprestidae) with *Beauveria bassiana* strain GHA: greenhouse and field trials. Biol Control 45:124–132. https://doi.org/10.1016/j.biocontrol.2007.12.008

Mannino MC, Huarte-Bonnet C, Davyt-Colo B, Pedrini N (2019) Is the insect cuticle the only entry gate for fungal infection? Insights into alternative modes of action of Entomopathogenic Fungi. J Fungi 5(2):33–43. https://doi.org/10.3390/jof5020033

Meyers J, Stephen F, Haavik L, Steinkraus D (2013) Laboratory and field bioassays on the effects of *Beauveria Bassiana* Vuillemin (Hypocreales: Cordycipitaceae) on red oak borer, *Enaphalodes rufulus* (Haldeman) (Coleoptera: Cerambycidae). Biol Control 65:258–264. https://doi.org/10.1016/j.biocontrol.2013.02.001

Mnyone LL, Kirby MJ, Lwetoijera DW, Mpingwa MW, Knols BG, Takken W, Russell TL (2009) Infection of the malaria mosquito, *Anopheles gambiae*, with two species of entomopathogenic fungi: effects of concentration, co-formulation, exposure time and persistence. Malar J 8(309).

Mohammadyani M, Karimi J, Taheri P, Sadeghi H, Zare R (2016) Entomopathogenic fungi as promising biocontrol agents for the rosaceous longhorn beetle, *Osphranteria coerulescens*. Biocontrol 61:579–590. https://doi.org/10.1007/s10526-016-9745-0

Mousavi K, Rajabpour A, Parizipour MHG, Yarahmadi F (2022) Biological and molecular characterization of *Cladosporium* sp. and *acremonium zeylanicum* as biocontrol agents of *Aphis fabae* in a tri-trophic system. Entomol Exp Appl 170(10):877–886. https://doi.org/10.1111/eea.13217

Narmani A, Arzanlou M, Kazemi F, Karimzadeh R (2016) First report of *Cladosporium cladosporioides* on Alfalfa weevil larvae (Hyperapostica) in the world. Proceedings of 22th Iranian Plant Protection Congress. Tehran, Iran: 679

Ranjbar Aghdam H, Marzban R, Mafi-Pashakolaei S, Sedighi L (2023) Study on the possibility control of box tree moth,*Cydalima Perspectalis* (Lepidoptera: Crambidae) using microbial, organic and low risk insecticides. J Entomol Soc Iran 43(1):23–29. https://doi.org/10.52547/JESI.43.1.4.

Reay S, Hachet C, Nelson T, Brownbridge M, Glare T (2007) Persistence of conidia and potential efficacy of *Beauveria bassiana* against pinhole borers in New Zealand southern beech forests. For Ecol Manag 246:232–239. https://doi.org/10.1016/j.foreco.2007.04.005

Scholthof K (2007) The disease triangle: pathogens, the environment and society. Nat Rev Microbiol 5:152–156. https://doi.org/10.1038/nrmicro1596

Shaker N, Ahmed G, Ibrahim H, El-Sawy M, Mostafa M, Ismail H (2019) Secondary metabolites of the entomopathogenic fungus, *Cladosporium cladosporioides* and its relation to toxicity of cotton aphid, *Aphis gossypii* (Glov). Int J Entomol Nematology 5:115–120

Shanley R, Keena M, Wheeler M, Leland J, Hajek A (2009) Evaluating the virulence and longevity of non-woven fiber bands impregnated with *Metarhizium anisopliae* against the Asian longhorned beetle, *Anoplophora Glabr* Ipennis (Coleoptera: Cerambycidae). Biol Control 50:94–102. https://doi.org/10.1016/j.biocontrol.2009.03.013

Sharifi S, Javadi I, Chemsak JA (1970) Biology of the Rosaceae branch borer, *Osphranteria coerulescens* (Coleoptera: Cerambycidae). Ann Entomol Soc Am 63:1515–1520. https://doi.org/10.1093/aesa/63.6.1515

Shimazu M, Zhang B, Liu Y (2002) Fungal pathogens of *Anoplophora glabripennis* (Coleoptera: cerambycidae) and their virulences. Bull Forestry for Prod Res Inst 1:123–130

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version6.0. Mol Biol Evol 30:2725–2729. https://doi.org/10.1093/molbev/mst197

Tanada Y, Kaya H (1993) Insect pathology. Academic, San Diego, CA

Thakur A, Singh V, Kaur A, Kaur S (2014) Suppression of cellular immune response in *Spodoptera litura* (Lepidoptera: Noctuidae)

larvae by endophytic fungi *Nigrospora oryzae* and *Cladosporium uredinicola*. Ann Entomol Soc Am 107(3):674–679. https://doi.org/10.1603/AN13164

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22(22):4673–4680. https://doi.org/10.1093/nar/22.22.4673

Vries dG (1952) *Contribution to the Knowledge of the Genus Cladosporium*. PhD Dissertation, Utrecht University

White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics. *PCR protocol, a guide to methods and appliaction*. M. Innis, D. Gelfand, J. Sninsky and T. White. San Diego, Academic press: 315–322

Yu Y, Cao Y, Xia Y, Liu F (2016) Wright-Giemsa staining to observe phagocytes in *Locusta migratoria* infected with *Metarhizium acridum*. J Invertebr Pathol 139:19–24. https://doi.org/10.1016/j.jip.2016.06.009