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Isolation, Characterization, and Formulation of Indigenous *Beauveria bassiana* Fungus Against Colorado Potato Beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae)

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Abstract

In this study, a total of 12 different fungal isolates were obtained from two different Colorado potato beetle (CPB) populations to identify a new effective agent to control *L. decemlineata*, which has developed resistance to many chemicals. The isolates were defined as *Beauveria bassiana* based on the *ITS1-5.8S-ITS2*, *EF1-α*, *RPB1*, and *Bloc* gene sequences. Under laboratory conditions, all isolates were pathogenic, but their virulence was different on larvae and adults. LdA-1 was the most virulent isolate with a mortality of 80% in larvae and 50% in adults. The LC_{50} value of this isolate was determined to be 0.2×10^6 and 0.17×10^8 conidia/ml for larvae and adults, respectively. Based on these results, LdA-1 isolates were used for mycoinsecticide development. Conidia were produced by solid-state fermentation using rice as a substrate. The conidia were formulated as oil-in-water emulsions and their efficacy was evaluated. The efficacy of the oil-based formulation against CPB larvae and adults was tested on eggplant in pot experiments. The new oil-based product caused 100% and 97% mortality on larvae and adults, respectively, at 1×10^8 conidia/ml. The LC_{50} value of our formulation for larvae and adults was calculated to be 1.2×10^6 and 0.2×10^7 conidia/ml, respectively. These results highlight that this formulation could be a suitable product for CPB control instead of conventional synthetic insecticides.

Keywords Biological control · Colorado potato beetle · Entomopathogenic fungi · Mycoinsecticide · Oil-based formulation

Introduction

Potatoes are one of the most important staple foods in the world after wheat and rice and the third largest crop in the world in terms of human consumption (FAOSTAT 2019). In Turkey, the potato is the second most widely grown crop

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after the tomato (TUIK 2020). A wide range of insects can damage potatoes directly by feeding on the tubers and spoiling the crop or indirectly by feeding on leaves or stems. Tuber yield losses due to insects are estimated at 75% without crop protection (James 2011).

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), is a serious pest of potato (*Solanum tuberosum* L.) in the USA and Europe, as well as in Asia (Jolivet 1991; Weber 2003). The first serious damage to the potato was reported in Colorado, USA (Riley 1875). The first CPB population in Europe was discovered in Germany only a few years after its spread in the USA. By the end of the twentieth century, the pest had spread throughout Europe and Asia and continued to expand its geographic range to new regions of the world (Wang et al. 2017). In Turkey, the pest was first discovered in 1963 in Edirne (in the Turkish region of Thrace), which borders Bulgaria and Greece, and spread to the interior regions of the country (Atak 1973).

Both larvae and adults of CPB damage the leaves of potato plants. However, the main damage is caused by larval feeding. One beetle can consume 40 cm^2 of potato leaves in the larval stage. Once the foliage is eaten away, adult beetles feed on stems and exposed tubers (Ferro et al. 1985). If CPB is not effectively controlled, potato yield can be completely damaged. Chemical insecticides have been used against CPB for many years because of their rapid action. Although insecticide use initially resulted in successful control of CPB populations, resistance to the active ingredients has been reported over time. CPB is known to have developed resistance to 56 different active substances (Balasko et al. 2020). In Turkey, control of this pest is usually based on the use of synthetic insecticides. Some populations of the beetle have already developed resistance to many groups of insecticides (Keskin and Yorulmaz-Salman 2020). In addition to resistance development, chemical insecticides have triggered the elimination of natural enemies. They also pose acute and chronic risks to human health (Jepson 2020).

Challenges and concerns about chemical insecticides have led researchers to seek safer and more effective control agents for sustainable management programmes. Entomopathogenic microorganisms are an effective and environmentally safe alternative to chemical insecticides. As in the whole world, many microorganisms have been isolated from agricultural and forest pests in our country until today (Seçil et al. 2012; Sevim et. al. 2012). The most detailed descriptions of these microorganisms were made, and their insecticidal effects both on their hosts and other pests were determined (Demir et al. 2013; Eski et al. 2018; Bayramoglu et al. 2018). Biopesticides were developed from local microorganisms that were determined to be very effective, and the effectiveness of these preparations under natural conditions was determined by semi-field trials (Eski et al. 2019). Entomopathogenic fungi, which provide suppression of harmful populations in natural conditions, are one of the most common natural enemies of pests (Biryol et al. 2021; Sonmez et al. 2022). Their populations are quite high, especially in humid and hot geographical zones (Sonmez et al. 2016; Kocacevik et al. 2015; Yucel et al. 2018). Entomopathogenic fungi (EPF) play an important role in the biological control of pests. Unlike bacterial and viral pathogens of insects, they do not need to be ingested. Once they come into contact with pests, they penetrate through the cuticle.

The widely distributed entomopathogenic hyphomycete *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycota: Hyphomycetes) has been studied for microbial control of CPB (Lipa 1985; Wraight and Ramos 2017a; Ropek and Kolodziejczyk 2019; Eski et al. 2022). Some previous studies have demonstrated the potential efficacy of *B. bassiana* against the insecticide-resistant CPB strains (Roberts et al. 1981; Campbell et al. 1985). *B. bassiana* shows efficient control against adult and all larval stages of CPB and provides a very high level of control during the potato growing season because it can continue to reproduce after application. However, its pathogenicity is greatly affected by abiotic factors such as moisture, sunlight, and ultraviolet rays (Fernandes et al. 2015; Acheampong et al. 2020). Formulations improve the field performance of EPF under adverse environmental conditions because they are critical for producing large numbers of conidia for success in the field. The durability of conidia and resistance to adverse environmental conditions during field application are critical to mycoinsecticide efficacy (Herzfeld et al. 2011; Quesada-Moraga et al. 2023). Oil-based formulations provide long shelf life for fungal products and increase efficacy in the field, even in low humidity and high temperatures. Oils also facilitate the adhesion of conidia to the waxy cuticle of CPB, support germination, and aid penetration (Hong et al. 2005; Ummidi and Vadlamani 20142014; Kaiser et al. 2020).

In this study, fungi causing natural infection in different CPB populations were isolated and identified, and their insecticidal activities on larval and adult stages of CPB were determined. Furthermore, the oil-in-water emulsion formulation of the promising fungal isolate was developed, and its success was demonstrated with pot experiments.

Materials and Methods

Collection of Insects

Larvae and adult stages of the Colorado potato beetle were collected from two different potato fields in Turkey, Trabzon, and Ankara regions, in the summer of 2019. Dead and infected insects were placed individually in sterile tubes for fungal isolation. Healthy adults and larvae were placed separately in large, well-ventilated plastic containers (20 × 30 cm). Insects brought to the laboratory were fed fresh potato leaves until the bioassay was performed. Insects were maintained under controlled conditions at 25 ± 2 °C and 70 ± 5% relative humidity with a 16/8-h light/dark photoperiod.

Isolation of Fungi

Insect cadavers were surface sterilized by washing in 5% bleach for 60 s and then rinsing in sterile distilled water for 60 s. They were then incubated in moistened Petri dishes at 25 °C until fungi developed on the cadavers. Conidia on the cadavers were inoculated onto Sabouraud dextrose agar (SDA) supplemented with ampicillin

and chloramphenicol (40 µg/ml) and incubated for 2 weeks at 28 °C, RH > 60% in continuous darkness. The cultured fungi were subcultured in several rounds on SDA plates, and then the pure fungal cultures were stored in 20% glycerol at −80 °C.

Morphological Identification

For morphological identification, 2-week-old cultures grown on SDA medium were used. First, the macroscopic characteristics of the colonies were determined, including the growth pattern, colour, shape, surface texture, and height of the colony. Then microscopic observations such as the shape and colour of the hyphae and conidia were determined using the slide culture technique (Humber 2012). The morphological characteristics of the isolates were evaluated according to the identification key described by Humber (2012).

Molecular Identification

Fungal genomic DNA was extracted from the mycelium of cultures grown on an SDA medium for 10 days using the Quick-DNA Fungal/Bacterial MiniPrep Kit (Zymo Research, USA) according to the manufacturer's instructions. After DNA extraction quality and quantity were checked by NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, USA) and stored at −20 °C for further analysis. Molecular identification of the fungal strains was performed by amplification and sequencing of the genes *ITS1-5.8S-ITS2*, *EF1-α* (translation elongation factor 1-α), *RPB1* (RNA polymerase II largest subunit), and *Bloc* genes using the primer pairs listed in Table 1. The PCR mix (50 µl) consisted of 50 ng DNA, 0.2 µM of each primer, 10× standard Taq reaction buffer, 2 mM $MgCl_2$, 0.2 µM dNTP mix, and 1U Taq DNA polymerase (New England Biolabs, USA). Amplification was performed in the T100 thermal cycler (Bio-Rad, UK) according to the following protocol: initial denaturation at 95 °C for 30 s, followed by 35 cycles of 30 s at 95 °C, 30 s at 55–61 °C, 1 min at 68 °C, and a final 5-min extension at 68 °C. Amplicons were electrophoresed on 1.0% ethidium bromide agarose gels along with a size ladder and visualized in a gel documentation system. Fragments were purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Germany) and quantified using the NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, USA). PCR fragments were transformed into the *Escherichia coli* JM101 strain after cloning into the pGEM-T Easy vector (Promega Co., USA). After selection of the transformed colonies containing recombinant plasmids by 'blue/white' screening and isolation with a NucleoSpin plasmid kit (Macherey-Nagel, Germany), they were sent to Macrogen (Macrogen Europe, Amsterdam, Netherlands) for sequencing.

Sequences were reviewed, analysed, and aligned using BioEdit software (Hall 1999). Raw sequences were reviewed and cleared of pGEM-T contaminants using the VecScreen tool provided by NCBI. The sequences obtained were assembled and compared to those in the GenBank databases using BLAST (Benson et al. 2005). Multiple sequence alignment was performed using ClustalW2 in BioEdit v.7.0.5 software. Phylogenetic trees were constructed using the neighbour joining (NJ)

Table 1 Primers used in this study and their sequences

Primer	Primers name	Primer sequence	Annealing temperature	Product size (bp)	Reference
ITS	ITS5	5′-GGAAGTAAAAGTCGTAACAAGG-3′	61°C	600	White et al. (1990)
	ITS4	5′-TCCTCCGCTTATTGATATGC-3′			
Tef	EF1T	5′-ATGGGTAAGGARGACAAGAC-3′	58°C	1150	Rehner and Buckley (2005)
	1567R	5′-ACHGTRCCRATACCACCSATCTT-3′			
RPB1	RPB1Af	5′-GARTGYCCDGGDCAYTTYGG-3′	55°C	800	Stiller and Hall (1997)
	RPB1C	5′-CCNGCDATNTCRTTRTCCATRTA-3′			
Bloc	B5.1F	5′-CGACCCGGCCAACTACTTTGA-3′	56°C	1500	Rehner et al. (2006)
	B3.1R	5′-GTCTTCCAGTACCACTACGCC-3′			

algorithm with MEGA X software (Saitou and Nei 1987; Kumar et al. 2018). Bootstrap analyses were performed to evaluate the robustness of the phylogenies with 1000 replicates.

Preparation of Conidial Suspension

Conidia were harvested from 2-week-old fungal cultures. The fungal surface was scraped with a sterile loop with 10 ml of sterile 0.1% Tween 80 as a wetting agent. The suspensions were then filtered into sterile plastic tubes using sterile cotton gauze to remove fungal debris. The suspensions containing conidia were vortexed for 5 min to homogenize the preparations. To determine conidial viability, the suspensions were spread on a PDA medium, and germination was assessed after 24 h of incubation at 25 °C in the dark. Cultures with conidia viability greater than 90% were used for virulence assays. The spore suspension was counted using a Neubauer haemocytometer, and the concentration was adjusted to 1×10^7 conidia/ml for use in virulence assays (Biryol et al. 2022).

Screening and Dose-Mortality Experiments

The efficacy of the fungal isolates was screened on the larvae (3rd instar) and adults of CPB. For each fungus, ten insects were placed in disinfected Plexiglas cages (15 × 15 cm) containing potato leaves, and 1 ml of the fungal suspensions (1×10^7 conidia/ml) was applied with a hand-operated mini-sprayer. The negative control group was sprayed with sterile 0.1% aqueous Tween 80. The experiments were repeated three times on different days. Boxes were maintained at 25 ± 2 °C and ≥ 70% RH with a photoperiod of 16:8 h L:D. Mortality was checked daily and recorded for 7 days after infection. The cadavers were surface sterilized with 1% sodium hypochlorite. Dead insects were transferred to Petri dishes lined with moist filter paper to promote fungal emergence and sporulation on the cadavers.

The concentration-mortality test was performed with five different concentrations of the fungus that were highly effective in both larval and adult CBP. The five different concentrations (1×10^4–1×10^8 conidia/ml) of the fungus were prepared by serial dilution, and the tests were performed as previously described.

Development of Oil-Based Formulation

The isolate LdA-1 was selected for the development of the mycoinsecticide, which showed high efficacy on both larvae and adults. Conidia were produced by biphasic fermentation. LdA-1 was first grown in a liquid medium. The suspension containing 10^6 conidia per ml was inoculated into a 150-ml liquid medium (2% dextrose, 1% peptone, 0.25% yeast extract, 0.02% chloramphenicol) in a 500-ml flask to produce blastospores and incubated at 28 °C and 180 rpm for 3 days (Loera-Corral et al. 2016). After incubation, a liquid culture that produced blastopores was centrifuged at 10000 rpm for 1 min, and the collected spores were dissolved in sterile distilled water. Second, LdA-1 was grown on a solid substrate using soaked rice.

Semi-cooked rice was distributed in growth bags with air holes (100 g per bag) and sterilized at 121 °C and 1.1 atm pressure for 20 min (Loera-Corral et al. 2016). The growth bags were inoculated with 15 ml of blastospores from liquid fermentation and cooled to room temperature. The bags were gently shaken for homogeneous distribution of conidia and incubated at 25 °C for 20 days. To ensure uniform and stable fungal growth during incubation, the growth bags were gently shaken every 3 days. Spores were then collected by sieving the substrate through a sieve (45 mesh) and reducing the moisture content to below 5% using a vacuum desiccator. Finally, the spores were individually packed in 100-ml glass bottles and stored in the refrigerator at +4 °C until use in oil formulations.

An oil-based formulation of the fungus was developed to extend shelf life and protect physical and biological properties in nature. The oil-based formulation was prepared as described by Ummidi and Vadlamani (2014) with minor modifications. The oil-based formulation consisted of 1% vegetable oil, 1% triton X-100 (nonionic surfactant), 1% silicon (antifoaming agent), 1% sodium carbonate (stabilizer), and 10% conidia. The mixture was gently blended for 60 min at 10 °C until homogeneous. The spore concentration of the mycoinsecticide was determined using a Neubauer haemocytometer.

Pot Experiments

Pot experiments were conducted on healthy eggplant (*Solanum melongena* L.), which is one of the main hosts of CPB. Eggplant seedlings were purchased from a local seedling supplier and grown individually in 20-cm-diameter plastic pots. When the plants were 6 weeks old with four to five true leaves, ten larvae were released on each plant. Then, five concentrations of our formulation (1×10^4–1×10^8 conidia/ml) were applied to both leaf surfaces with a hand-operated sprayer until they ran off. Fungus-free formulation and sterile distilled water were used as control groups. Each experiment was repeated three times and the entire experiment was performed three times. The experiments were also performed with adult CPB. The pots were kept in a climate room (25 ± 2°C, 70% RH, L16:D8). The number of live and dead insects was recorded 7 days after application, and the dead insects were examined for fungal infection using a humidity circle.

Data Analysis

Mortality rates were adjusted for control mortality using Abbott's formula (Abbott 1925). Data were then subjected to one-way analysis (ANOVA), and means were separated using the least significant difference (LSD) test at a 5% significance level. The data from the dose-mortality experiment were subjected to probit regression analysis to determine the median lethal concentration (LC_{50}). Survival analysis was also performed using the Kaplan-Meier method, and pairwise comparisons were made using a log-rank chi-square test. All statistical analyses were performed using SPSS version 20 software (SPSS Inc. Chicago, IL, USA).

Results

Morphological Identification

Twelve fungal isolates were obtained from CPB cadavers. All isolates showed white to yellowish-white colonies with irregular margins and powdery appearance on SDA medium. Observation under the light microscope showed that the isolates have globose to sub-globose conidia with hyaline hyphae and zigzag extension of the rachis. The macroscopic and microscopic features made it possible to identify all colonies as *Beauveria* (Humber 2012).

Molecular Identification

For molecular characterization, *ITS1-5.8S-ITS2*, *EF1-α*, *RPB1*, and *Bloc* gene regions of the genomic DNA were amplified by PCR with specific primers, and DNA fragments with an expected size of 600, 1150, 800, and 1500 bp, respectively, were observed on the agarose gel. ITS sequences of the isolates were compared with those stored in the NCBI GenBank database. This showed that the isolates identified morphologically as *Beauveria* had a sequence similarity of 99% or more with *B. bassiana*. Accession numbers of the sequences were deposited in the NCBI GenBank database. The phylogenetic tree generated using the sequences from ITS showed that all isolates clustered with the *B. bassiana* reference strains reported by Rehner et al. (2011) (Fig. 1). In addition, the phylogenetic tree based on the concatenated sequences showed that all isolates belonged to the *Beauveria bassiana* species clade (Fig. 2).

Screening and Dose-Mortality Experiments

All isolates were found to be virulent against both larval and adult pests compared to control treatments (F=91.32, df=11, $P < 0.05$). However, mortality caused by fungal infection ranged from 10 to 80% at the larval stage and from 3 to 50% on the adult stage (Fig. 3). Strain LdA-1 exhibited the highest pathogenicity in both CPB larvae and adults, with an average mortality of 80% and 50%, respectively. Mortality caused by fungal infection was first observed in all fungal isolates 3 days after treatment.

The different concentrations of LdA-1 were applied separately to larvae and adults, and survival analysis showed that fewer beetles survived when exposed to the high concentration of conidia than to the low concentration or control (Log-rank, Chi-square= 875.28; df= 2; $P < 0.0001$) (Fig. 4). The LC_{50} value was determined to be 0.2×10^6 and 0.17×10^8 conidia/ml for larvae and adults, respectively (Table 2).

Development of Oil-Based Formulation and Pot Experiments

The oil-in-water emulsion formulation with 10^9 conidia per ml was developed from the LdA-1 isolate. Subsequently, the efficacy of the oil-based formulation against

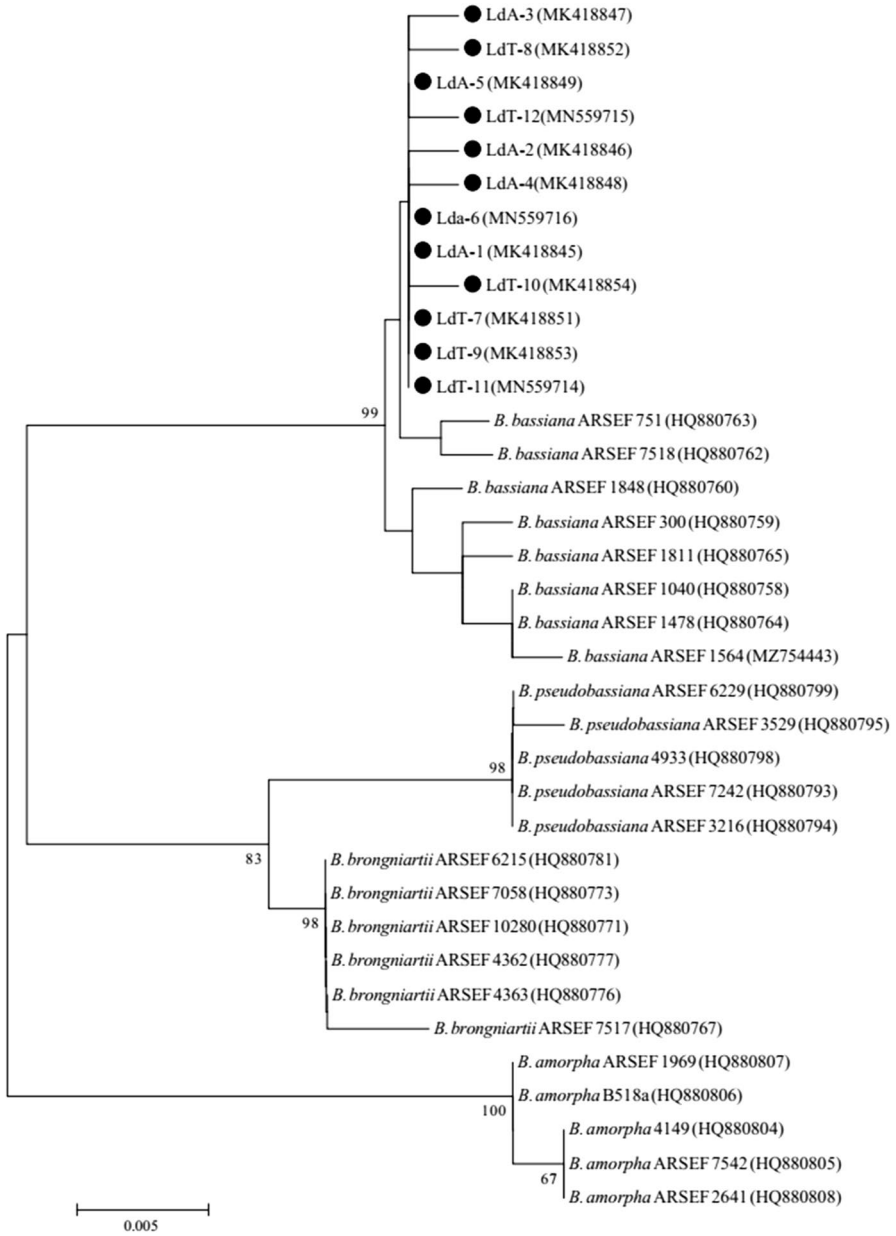


Fig. 1 Neighbour joining tree of entomopathogenic fungi associated with *Leptinotarsa decemlineata* and closely related fungal species based on the sequence of the ITS gene. Fungal isolates from *L. decemlineata* larvae are indicated with a black dot. GenBank accession numbers are shown in parentheses. The numbers at the nodes are bootstrap percentages based on 1000 replicates; only nodes supported by the bootstrap values of 70% or over are shown

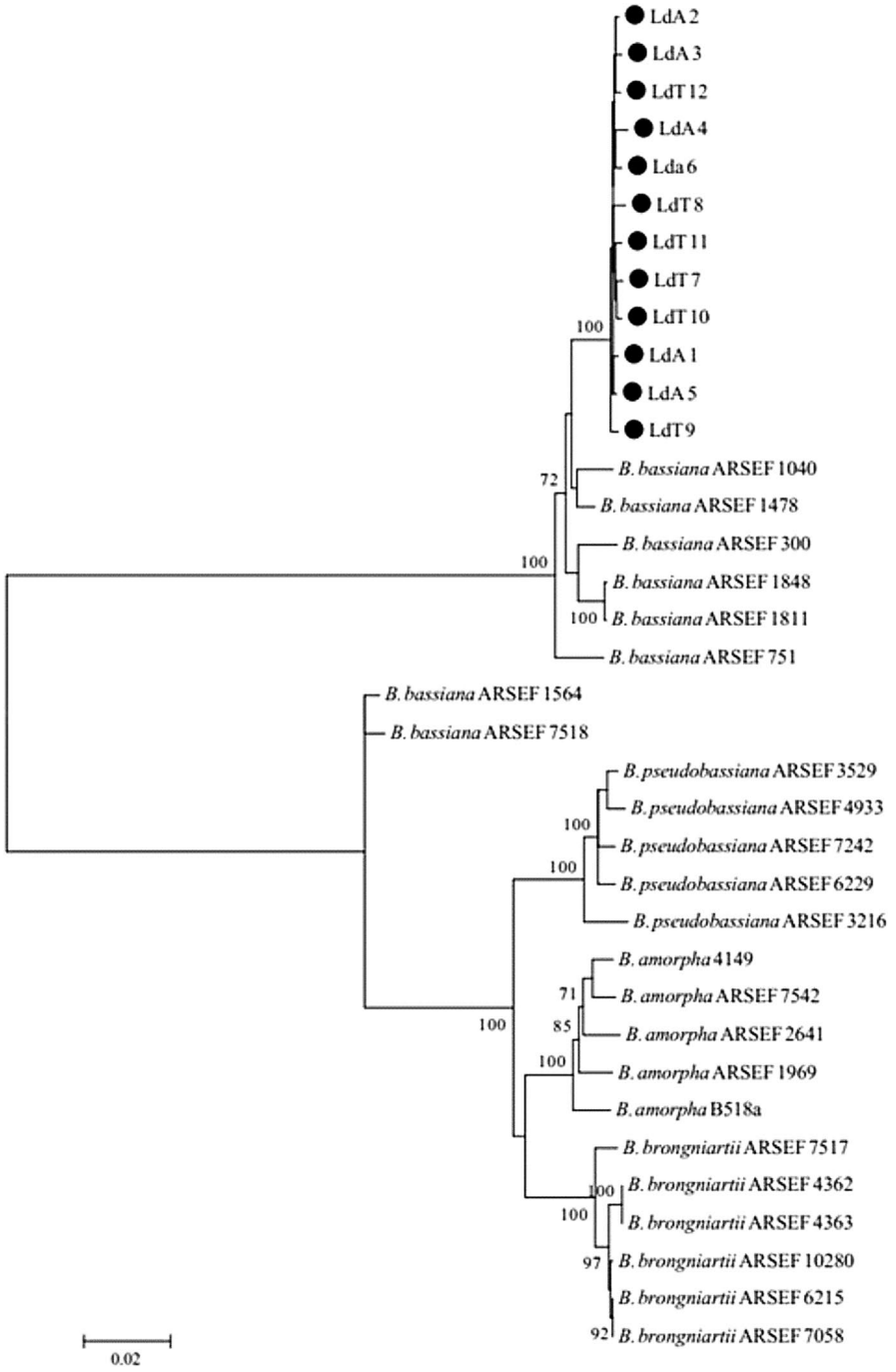
Fig. 2 Maximum likelihood phylogenetic tree of entomopathogenic fungi associated with *Leptinotarsa decemlineata* and closely related fungal species based on the concatenated sequences of ITS, TEF-1, RPB1, and Bloc genes. Fungal isolates from *L. decemlineata* larvae are indicated with a black dot. The numbers at the nodes are bootstrap percentages based on 1000 replicates; only nodes supported by the bootstrap values of 70% or over are shown ▶

CBP larvae and adults was tested on eggplant in pot experiments. The new oil-based product caused 100% and 97% mortality on larvae and adults, respectively, at 1×10^8 conidia/ml. Nostalgist-BL, on the other hand, caused 27% mortality in larvae and 3% mortality in adults at 1×10^8 conidia/ml (Fig. 5). Moreover, no mortality was observed in the fungus-free formulation and the negative control group. Seven days after infection, the LC_{50} value of our formulation was calculated to be 1.2×10^6 and 0.2×10^7 conidia/ml for larvae and adults, respectively (Table 2).

Discussion

Exploring biological approaches to control major potato pests is an environmentally friendly method instead of conventional insecticides. Entomopathogenic fungi are considered important ecological factors in suppressing pest populations in the field. Therefore, to find new indigenous fungal entomopathogens, we isolated 12 fungi from CPB cadavers by conventional morphological identification methods. The macro- and micromorphological characteristics of the present fungi are consistent with previously described characteristics of *B. bassiana* (Humber 2012). All isolates showed white colonies on SDA plates with smooth, powdery to cottony texture and round shape. Microscopic observations of the isolate showed reproductive structures and conidia with typical morphology, size, and colour of *B. bassiana*. These results are consistent with previously published studies reporting the morphological identification of *B. bassiana* (Liu et al. 2003; Sevim et al. 2010; Gençer et al. 2023). However, these characters are largely concordant among *Beauveria* species, making it difficult to distinguish them morphologically alone. Phylogenetic analysis using the ITS-rDNA nucleotide sequences of our isolates and the representative sequences published in Rehner et al. (2011) placed the high bootstrap fungi in the *B. bassiana* clade (Fig. 1). On the other hand, the ITS-rDNA sequence alone may not be sufficient for phylogenetic analysis because *Beauveria* species include cryptic species. Therefore, multilocus-based phylogenies are important for the accurate assignment of isolates to specific clades (Robène-Soustrade et al. 2015; Wang et al. 2020). In our study, phylogenetic analysis using concatenated nucleotide sequences (*ITS*, *TEF-1*, *RPB1*, and *Bloc* gene regions) confirmed that the isolates are closely related to *B. bassiana* species (Fig. 2).

The isolates showed pathogenicity against CPB larvae and adults with different corrected mortality rates ranging from 10 to 80% and 3 to 50%, respectively ($P<0.05$). Previous studies have investigated the susceptibility of different life stages of CPB to various *B. bassiana* isolates. Todorova et al. (2000) tested ten isolates of *B. bassiana* for adult CPB at a concentration of 10^7 conidia/ml and found that six isolates were highly virulent and mortality ranged from 86 to 100% 6 days after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LdT 9
B. bassiana ARSEF 1040
B. bassiana ARSEF 1478
B. bassiana ARSEF 300
B. bassiana ARSEF 1848
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B. pseudobassiana ARSEF 6229
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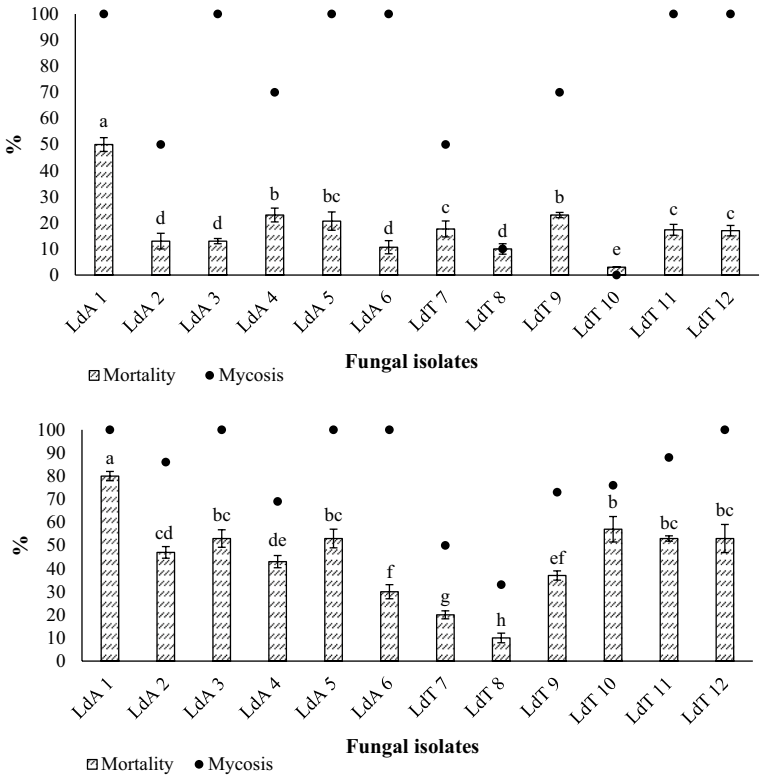

Fig. 3 Corrected mortality and mycosis of *Leptinotarsa decemlineata* larvae (up) and adult (down) stage exposed to indigenous entomopathogenic fungi at 107 conidia/ml concentration 10 days after treatment. Corrected mortality capped with different letters are significantly different (LSD comparison test: $P<0.05$)

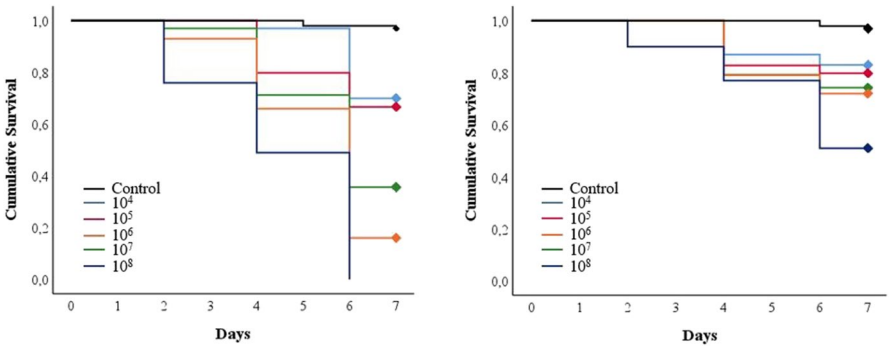

Fig. 4 Kaplan-Meier survival diagram for larval (left) and adult (right) stages of *Leptinotarsa decemlineata* exposed to isolate *B. bassiana* LdA-1 at five different concentrations 7 days after treatment

Table 2 Lethal concentrations (LC50 and LC95) of non-formulated and oil-based formulated conidia of LdA-1 isolate on larvae and adults

	Life stage	LC_{50} (conidia/ml) (*FL*, 95%)	Slope ± SE	LC_{95} (conidia/ml)	*df*	X^2	*P*-value
Non-formulated conidia	Larvae	2×10^5 (1.4×10^4 – 1.2×10^6)	0.66 ±0.05	8.9×10^7	3	14.7	<0.01
	Adult	1.3×10^9 (1.3×10^8 – 2×10^{11})	0.25±0.02	1.1×10^{17}	3	4.19	<0.01
Oil-based formulation	Larvae	1×10^6 (3.5×10^4 – 2.2×10^7)	0.75 ±0.07	5.6×10^7	3	34.8	<0.01
	Adult	4.1×10^6 (ND – ND)	1.00±0.05	1.2×10^9	3	52.2	<0.01

df, degree of freedom; *FL*, fiducial limit; *ND*, not determined; *SE*, standard error; X^2, chi square

treatment. In another study, twelve *B. bassiana* strains isolated from cadavers of CPB were tested on adults at a concentration of 1×10^7 conidia/ml, and isolated Bb8 resulted in complete mortality with an LT_{50} of 7 days (Zemek et al. 2021). On the other hand, Shafighi et al. (2012) found that two *B. bassiana* isolates showed 60% mortality within 15 days at a concentration of 10^9 conidia per ml against second-stage larvae. Akbarian et al. (2012) also reported that ABK, an Iranian *B. bassiana* isolate obtained from CPB, caused 78% mortality in second instar larvae at a concentration of 1×10^9 conidia/ml. In our study, the *B. bassiana* LdA-1 isolate showed corrected mortality of 80% with an LT_{50} of 5.09 days when larvae were treated with a concentration of 10^7 conidia/ml. However, the LdA-1 isolate also caused mortality in adults but at a lower rate than in larvae. Similarly, Baki et al. (2021) examined 14 isolates of *B. bassiana* from different sources at a concentration of 1×10^7 conidia/ml on larvae and adults of CPB and found that four isolates were more virulent than others and younger larvae were more susceptible to fungal isolates. On the other hand, Eski et al. (2022) evaluated the efficacy of two indigenous isolates of *B. bassiana* obtained from different insects on CPB and reported that isolate Gg1 was more virulent in larvae than in adults, and isolate Mm1 was more virulent in adults. Host specificity and virulence may vary among *B. bassiana* isolates, which could be related to the ability to overcome the insect immune response, as well as molecular and physiological mechanisms such as the excretion of extracellular enzymes due to wide genetic variation (Uma Devi et al. 2008; Rohrlich et al. 2018). In addition, the isolates that are most virulent to an insect host are those isolated from the same or a related host species (Goettel et al. 1990; Lacey 1997). On the other hand, the differences in susceptibility between larvae and adults of CPB can be explained by the fact that adults have a thick and highly sclerotized cuticle, whereas larvae have a softer, more flexible cuticle. Furthermore, when adults are exposed to pathogenic microorganisms, the activity of an antioxidant enzyme, GST, is upregulated and the toxic compounds of the microorganisms are eliminated. These results highlight the importance of selecting appropriate isolates for pest control.

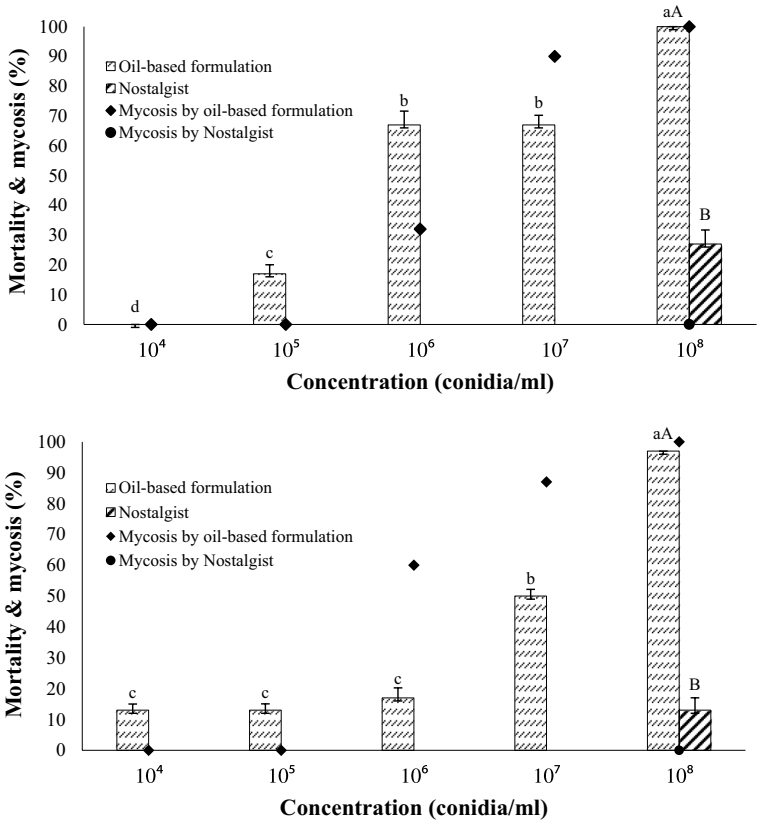

Fig. 5 Efficacy of formulations against larvae and adults of *Leptinotarsa decemlineata* in pot experiments. The oil-based formulation was used at five different concentrations. The commercial product, Nostalgist, used a concentration of only 10^8 conidia/ml. Different lowercase letters represent statistically significant differences between the different concentrations of the oil-based formulation. Different capital letters represent statistically significant differences between the oil-based formulation and Nostalgist at a concentration of 10^8 conidia/ml. (LSD multiple comparison test, $P<0.05$)

While the unformulated conidia of isolate LdA-1 appear to be a promising biological control agent with effective mortality against larval and adult CPB under laboratory conditions, their efficacy under field conditions is limited due to radiation, high temperature, and low humidity. To overcome these limitations, the conidia of LdA-1 were formulated in an oil-in-water emulsion. In addition to protecting conidia from the deleterious effects of abiotic environmental factors, oil-based formulations increase efficacy under field conditions by facilitating conidial dispersal and adhesion to the host hydrophobic cuticle. Lopes and Faria (2019) found that conidia of isolate *B. bassiana* CG425 in oil formulation exhibited 20% higher mortality on adult coffee berry borers than non-formulated conidia. Similarly, the formulation of *B. bassiana* ART2587 with canola oil showed a strong synergistic effect on pollen beetle and caused higher mortality than the non-formulated fungus

(Kaiser et al. 2020). In our study, oil-based formulated LdA-1 conidia applied in pot experiments were more effective than non-formulated conidia applied in laboratory experiments. In our case, the application of corn oil formulated LdA-1 showed a significantly lower LC_{50} value than non-formulated conidia on adult CPB. However, the LC_{50} value of the larval formulation was higher than that of the aqueous suspension of LdA-1 (Table 2). Although many studies have been conducted on the use of *B. bassiana* (unformulated conidia) for CPB control, there are few studies on the use of oil-based *B. bassiana* formulations. An oil-based formulation of *B. bassiana* GHA produced by Wraight and Ramos (2017a) caused 82.4% mortality to larvae of CPB under greenhouse conditions. Mycotrol, an oil-based formulation of *B. bassiana*, also showed mortality of 57.3% and 31.5% under unheated greenhouse and field conditions, respectively (Wraight and Ramos 2017b).

Conclusion

The introduction of new formulations to control Colorado potato beetle, which has developed resistance to many insecticides used, is very important to reduce economic losses. It has been shown that LdA-1 isolated from naturally infected insects is an important pathogen that can be used for this purpose. Subsequently, a novel oil-based mycoinsecticide was developed using this isolate, and its high efficacy was demonstrated in laboratory and pot experiments. Further studies should be conducted to validate these results under field conditions.

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Declarations

Conflict of Interest The authors declare no competing interests.

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