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Efficacy of indigenous isolates of entomopathogenic fungi, *Beauveria bassiana* against the box tree moth, *Cydalima perspectalis*, invasive pest in Iranian forests

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Abstract

Cydalima perspectalis (Walker), box tree moth (BTM) is a major invasive pest of *Buxus hyrcana* Pojark in northern Iran that has spread across Asia and Europe. To reduce the use of insecticides in the management of forest pests, developing integrated pest management (IPM) programs using biocontrol agents can be an effective strategy. During our studies on native entomopathogenic fungi (EPF) in northern Iran, *Beauveria bassiana* (B2, F4, D2, B1) were naturally isolated from BTM larvae in Hyrcanian forests. Under laboratory conditions, the potential of the naturally occurring EPFs as a biological control method against the third-instar larvae of BTM was evaluated. The mean lethal concentration values (LC_{50}) of isolates B2, F4, D2 and B1 were found to be 1.2×10^7, 2.0×10^7, 2.8×10^7 and 1.0×10^8 conidia ml^{-1}, respectively on third-instar BTM larvae in the laboratory tests 96 hours after the treatment at 26 ± 1 °C, 75 ± 5% relative humidity. Under field conditions, the foliar applications of isolate B2 formulations containing linseed oil, corn oil, soybean oil, and wettable powder resulted in 13.2-82.2% mortality against BTM in Maskupa, Mazandaran and 8.8-70.4% mortality in Sisangan Forest Park, Mazandaran (after 14 days at a concentration of 10^8 conidia ml^{-1}), respectively. This investigation suggests that BTM larvae in Hyrcanian forests contain pathogenic *B. bassiana* isolates and highlights for the first time their potential as a biological control agent against BTM.

Key words: *Buxus hyrcana*, biocontrol agents, Hyrcanian forests, oil formulation.

Introduction

The Hyrcanian boxwood *Buxus hyrcana* Pojark is one of the endemic and endangered species of Hyrcanian forests in northern Iran (Jalili and Jamzad, 1999). The box-tree moth, *Cydalima perspectalis* (Walker) (Lepidoptera Crambidae) (BTM) has caused extensive damage to *B. hyrcana* habitats in Hyrcanian forests in recent years (Ahangaran, 2016). BTM is a major invasive pest native to Eastern Asia that was introduced to Europe in 2007 and spread rapidly throughout Europe, Asia, Canada and USA (Wan *et al*., 2014; Bras *et al*., 2019; Coyle *et al*., 2022). In Iran, it was first reported from Banafsheh Park in Chalus (Mazandaran province) in June 2016 (Ahangaran, 2016). BTM completes three to five generations per year in the area of origin (She and Feng, 2006; Wang *et al*., 2008; Sun *et al*., 2009) and two to three generations per year occur in the European continent (Leuthardt *et al*., 2010; Brua, 2014). Depending on the climate conditions, BTM has six to seven larval instars per year (Matsiakh *et al*., 2018; Coyle *et al*., 2022) and overwinters as a 3rd-4th instar larva, protected in a cocoon spun between *Buxus* leaves (Matošević, 2013).

Sex pheromone traps will be a useful method for integrated pest management (IPM) to assess the spread of BTM into new areas and to determine the best time for control treatments (Santi *et al*., 2015). The results of Kazerani *et al*. (2019) confirmed that the delta traps baited with sex pheromone are very efficient in capturing BTM males in order to estimate flight trends and developing management strategies. Several essential oils derived from plants in the families Asteraceae, Apiacaeae, and Lamiaceae have also shown promise as potential controls of BTM larvae (Gokturk *et al*., 2021).

Microbial biopesticides offer a unique alternative to broad-spectrum chemical insecticides against BTM (Zemek *et al*., 2020). Several studies investigated the usage of entomopathogenic bacteria (Harizanova *et al*., 2018), fungi (Kenis *et al*., 2013; Rose *et al*., 2013; Tabone *et al*., 2015), and nematodes (Göttig and Herz, 2018; Gholami Ghavamabad *et al*., 2021a; 2021b) to control BTM as an invasive forest pest. The ability of *Beauveria bassiana* (Balsamo) (Hypocreales Cordycipitaceae) to manage a variety of insect pests has been extensively studied and it appears to be a potential alternative to conventional insecticides (Alves *et al*., 1998). Biological control of BTM with entomopathogenic fungus, *B. bassiana* GY1-17 in Yangsan, south Korea was investigated, it was not affected at the rate of 2.0×10^7 to 10^4 conidia ml^{-1} (Lee *et al*., 1997). According to Bereś (2020), *Bacillus thuringiensis* was the most effective against BTM larvae (efficacy up to 90%) followed by Spinosad (85%) and *B. bassiana* (65%). In field experiments, *B. thuringiensis* subsp. *kurstaki* Lepidocid CKM, DiPel® and *B. bassiana*-MB-103 against BTM larvae were used and mortality were observed 60.6%, 88.6%, 60% respectively (Burjanadze *et al*., 2019). In another study, the 1×10^8 CFU/ml of bacterial strains: *Bacillus cereus* Frankland et Frankland FD-63, *Brevibacillus brevis* (Migula) FD-1 and *Vibrio hollisae* (Hickman) FD-70; also, 1×10^8 conidia ml^{-1} concentration of ET 10 fungal isolate of *B. bassiana* were able to cause mortality rate of 100% against BTM larvae under laboratory conditions (Tozlu *et al*., 2022). The fall webworm, *Hyphantria cunea* (Drury) (Lepidoptera Erebidae) was successfully controlled by *B. bassiana* strains Bb10331 (LC50 4.72×10^6 conidia·ml^{-1} after 120 hours post treatments) and Bb7725 (LC50 3.28×10^6 conidia·ml^{-1} after 120 hours post treatments) (Hu *et al*., 2021).

Characteristics of *B. bassiana* are perfectly matched to the environment and when optimized lead to excess production, making the formulations readily available (Singh *et al*., 2015). Spraying of entomopathogenic fungi formulated as wettable powders and suspension concentrates based on oils or water has been used to control pests (Kim *et al*., 2014). Most mycoinsecticides require a carrier of natural or synthetic oils to be effective when sprayed on forest-defoliating caterpillars such as the eastern spruce budworm, *Choristoneura fumiferana* (Clemens) (Lepidoptera Tortricidae) (Hicks, 2016). Virulence potential of the entomopathogenic fungi vary depending on different biological stages of the target pest, the type of isolate and conidia concentration. The susceptibility of the insect to entomopathogenic fungi decreased with the advancement in age of larvae of the insect (Asi *et al*., 2013).

Natural stands of *B. hyrcana* have been severely harmed by pests and diseases such as boxwood blight disease and the box-tree moth. BTM larvae infected with *B. bassiana* fungal mycelium were previously isolated and identified from some parts of BTM distribution zones in Iran (Zamani *et al*., 2017). The goal of this study is to find the most effective concentration and treatment dose of *B. bassiana* conidial suspension as well as to compare the efficacy of different fungus formulations in laboratory and field.

Material and methods

Fungal isolation

Fungi were isolated from BTM larvae with superficial hyphal development collected directly from the Hyrcanian boxwood *B. hyrcana*, in different regions of Hyrcanian forests. Infected insects were surface-sterilized and incubated in a humid chamber until the appearance of an actively growing mycelium. The mycelium was transferred to Sabouraud's dextrose agar supplemented with 1% yeast extract (SDYA medium) and then fungal isolates were subcultured on the selective media containing 40 g glucose, 10 g proteose peptone, 15 g agar, 0.01 g crystal violet, 0.25 g cyclohexamide, 0.5 g chloramphenicol and 1 litre distilled water. Pure cultures from all isolates were obtained by single spore isolation (monosporic cultures). The isolates were identified morphologically as *B. bassiana* by a microscope Olympus BX51 (Olympus, Japan) with 400× magnification and the morphological key (Humber, 1998). Four *B. bassiana* isolates were preserved in the microorganisms and they were deposited in the fungal culture collection of the Plant Pathology Laboratory of Research Institute of Forests and Rangelands, Tehran, Iran.

Spores preparation for *in vitro* entomopathogenicity assays

The obtained isolates were cultivated for 14 days on SDYA medium plates at 24 ± 1 °C. Fresh conidia were extracted from the Petri dish by mixing 10 ml of sterile distilled water with 0.05% v/v adhesive spreader (Tween® 80), filtered through a sterilized filter paper (Whatman n. 01) and finally transferred to a sterile falcon 50 ml conical centrifuge tube. Conidial concentrations were determined using a Neubauer chamber under optical microscopy and the initial suspensions were adjusted to 10^8 conidia ml^{-1}. The viability of the spores was confirmed by inoculating the center of a 9 cm Petri dishes containing SDYA medium with 2 µl of obtained spores suspensions. The germination percentage was measured 12 hours after inoculation by counting 100 conidia per Petri dish. Then the remaining concentrations of *in vitro* entomopathogenicity tests; 10^5, 10^6, 10^7 and 10^8 conidia ml^{-1}, as well as the control composed of sterilized distilled water and the adhesive spreader at 0.05% v/v, were all adjusted from the initial solution.

Insect collection and rearing

During May-July 2021, BTM eggs were collected from infested *B. hyrcana* in Mazandaran province, northern Iran (36°34'N 51°47'E). The larvae were kept in cages (20 × 30 × 30 cm) with holes covered by fine mesh in growth chambers (26 ± 1 °C, 75 ± 5% RH. and 16:8 L:D) to get third instar larvae under laboratory conditions. Fresh *B. hyrcana* leaves were provided daily to the larvae. The larval instars were determined by the presence of newly moulted exuviae and head capsules.

In vitro entomopathogenicity assays

In the laboratory, the susceptibility of BTM third instar larvae to the four *B. bassiana* isolates (B2, F4, D2, B1) was tested. Tween 80 (0.05%) containing 10^5, 10^6, 10^7 and 10^8 conidia ml^{-1} were used to inoculate each larva. The controls received distilled water with 0.05% Tween 80. Larvae were inoculated by immersing them individually in the conidia suspension for 10 seconds at the tested concentration. Individually treated larvae were transferred into Petri dishes covered with sterile filter paper, were provided with fresh *B. hyrcana* leaves and sealed with parafilm. A group of 24 larvae was used for each treatment (in a growth chamber, under the same conditions as for the insect rearing), and the experiments were conducted in 5 replicates in a completely randomized design. Mortality of the larvae due to fungal infection were recorded daily for six days. Dead individuals were moved to sterilized Petri dishes with moistened filter paper and incubated for five days in a desiccator and observed. Only larvae with white mycelia and fungal spores were considered to have died from the fungal infection.

Molecular methods and phylogenetic analyses

To confirm morphological identifications, the isolate with the highest entomopathogenic activity was molecularly identified. DNA was extracted from fungal mycelium cultivated for 96 hours at 251 °C in Sabouraud's Dextrose Broth (SDB) medium containing 2% glucose, 0.5 percent peptone, and 0.5 percent yeast. Filtration was used to extract the mycelia, which was then rinsed with distilled water, freeze-dried, and crushed in liquid nitrogen. This method was used to prepare three DNA samples. To perform the rDNA analysis, primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify the internal transcriber spacers I, II and the ribosomal 5.8S rRNA (White *et al*., 1990).

To check the successful amplification, 2 µl of the PCR product was loaded on agarose gel including DNA Green Viewer™. The PCR products were purified and sequenced in both directions with the primers ITS1 and ITS4 by the Beijing Genomics Institute (BGI), China. Nucleotide sequences were deposited in the GenBank nucleotide sequence databases (accession numbers OM413901).

Blastospore production

The experiments for blastospore production were performed according to the method described by Biryol *et al*. (2021) with some modifications. As an initial culture, the selected *B. bassiana* isolate was cultured in a small volume of the basal salt medium. Then, 10 ml sterile 0.1% Tween 80 was added to the 7-day-old plates and conidia were collected using an inoculation loop and transferred to a sterile falcon. The spore suspension was adjusted to a concentration of 10^6 conidia ml^{-1}. Then, as an initial culture in 250 ml Erlenmeyer, 15 ml of this suspension was inoculated into 150 ml basal salt medium and incubated for 3 days at 25 °C in a rotary shaker at 350 rpm. After that, each 1000 ml Erlenmeyer containing 500 ml basal salt medium, was inoculated with 5 ml initial culture containing 10^6 blastospores ml^{-1} and liquid cultures were incubated at 25 °C in a rotary shaker at 350 rpm for 3 days (Mascarin *et al*., 2015). During the spore production process, the Erlenmeyers were regularly stopped and shaken down to minimize and remove mycelial development on the walls. After the incubation, the liquid cultures were filtered through four layers of sterile cotton gauze to separate the blastospores from the mycelium, and the blastospores were concentrated using a centrifuge (10,000 g for 10 minutes). The blastospore concentration was adjusted to 10^{10} with sterile water. The blastospore suspension was mixed with skim milk powder in a ratio of 10% (wt/vol), then the mixture was dried through a two-fluid nozzle spray dryer (Biryol *et al*., 2021). The dried spores were stored at 4 °C until needed.

Development of mycoinsecticide formulations

Two types of *B. bassiana* spore powder formulations, wettable powder and oil-based formulation, have been developed to extend their shelf life before use. The wettable powder formulation was prepared by mixing naphthalene-2-sulfonic acid (3%), emulsifier OP-10 (3%), ascorbic acid (0.1%), and talc powder (83.9%) then mix with 10 g spore powder (10^9 spore g^{-1}) (Nian *et al*., 2015). Three types of vegetable oils (corn, soybean or linseed oil) were used to prepare oil formulations, which according to previous studies did not have a negative effect on the germination of *B. bassiana* spores (Luz and Batagin, 2005). Oil-based formulations were obtained from naphthalene-2-sulfonic acid (6%), ethoxylated castor oil (10%), sodium alginate (1%) and vegetable oil (73%) with 10 g spore powder (10^9 spore g^{-1}). The volume of the mixture was increased to 100 ml by adding sterile distilled water, then homogenized at 50 rpm and <10 °C for 30 minutes (Nian *et al*., 2015).

Pathogenicity of mycoinsecticide formulations of *B. bassiana* against *C. perspectalis* larvae

The wettable powder and oil-based formulations were tested against BTM third instar larvae at a concentration of 10^8 conidia ml^{-1} under laboratory conditions using the method described in the screening test. Fungus-free formulations were used as a negative control.

The field trials were conducted with *B. bassiana* B2 isolate which had the highest insecticidal effect in laboratory conditions. Field bioassays were then performed to test the formulations on larval stages of BTM in evergreen shrubs of *B. hyrcana* in two trials: trial 1 in Sisangan Forest Park, Mazandaran provinces (36°34'N 51°47'E) and trial 2 in Maskupa, Mazandaran province (36°12'N 53°19'E) in May 2021. The experiment was conducted using a randomized complete block design, five treatments included a wettable powder, three oil-based formulations (corn, linseed, soybean oil) and an untreated control five repetitions (shrubs). Fungus-free formulation and sterile distilled water were used as negative control.

Shrubs with an average height of 1.5 to 2.0 m, a life span of 5 to 10 years and a distance of about 50 m between them were selected and randomly assigned to the treatments. These trees were naturally heavily infested with a large population of different ages of BTM and had not been treated with insecticides before. In each replication, ten shoots of equal size were selected randomly from all the directions of the inspected tree for statistical counting. To determine the larval population levels, a pre-count was taken at each replication just before spraying. The treatments were sprayed using a 5-liter plastic handheld sprayer (with fully adjustable nozzle and trigger lock) at a rate of 10^8 conidia ml^{-1}. Approximately 2 to 3 litres of the solution were applied to each shrub, from the lower to the upper branches, to ensure complete coverage. After treatment, all the shoots were caged individually using a sleeve net, the open end of which was closed around the branch with a rubber band. Treated trees were sampled 14 days after treatment to check the larval mortality. The mortality rate was calculated as dead larvae per tree/total number of larvae per tree. Mortality data have been corrected to compensate for natural deaths using Abbott's formula (Abbott, 1925). No dryness, yellowish, and deformation were observed on the tested plants after spraying to consider the side effects of the tested treatments. After treatment, dead larvae were randomly collected from each treated shrub and placed in plastic containers during transport back to the laboratory. Re-isolation trials were conducted using the fungal isolation method previously described to verify larval infection with the fungus.

Data analysis

Data were analysed using analysis of variance (SAS, 2002) with insect mortality as the response variable, along with the exposure time, EPFs concentration and isolates as the main effects. Means were separated at alpha = 0.01 using Duncan's test. The LC_{50} and LC_{90} values of EPFs and the mean lethal times of LT_{50} were estimated by Probit analysis using SPSS Statistics 17.0.

Table 1. Indigenous strains of *B. bassiana* isolated in Iran.

Isolate number/ code	Locality/site	Host pest	Latitude-longitude
Isolate 1/B1	Guilan	*Cydalima perspectalis*	37°38'N-49°02'E
Isolate 2/B2	Mazandaran	*Cydalima perspectalis*	36°34'N-51°47'E
Isolate 3/F4	Mazandaran	*Cydalima perspectalis*	36°33'N-51°47'E
Isolate 4/D2	Guilan	*Cydalima perspectalis*	37°47'N-48°49'E

Results

Fungal isolates

A total of 4 pure fungal isolates from BTM larvae on *B. hyrcana* in different regions of Hyrcanian forests were morphologically identified as *B. bassiana* (table 1).

In vitro entomopathogenicity assays

The results revealed that under laboratory conditions, all four *B. bassiana* isolates (B2, F4, D2, B1) were pathogenic against third instar larvae of BTM at dosage rates of 10^6, 10^7, 10^8, and 10^9 conidia ml^{-1}, albeit mortality varied among the isolates (figure 1).

The mortality rates differed significantly depending on *B. bassiana* isolates, fungal concentrations and days post treatment ($P < 0.0001$) (tables 2 and 3). At 10^8 conidia ml^{-1} and 144 hours after inoculation, *B. bassiana* B2 had the highest mortality rate (100%), which is substantially greater than the mortalities produced by *B. bassiana* F4 (87.8%), *B. bassiana* D2 (85%), and *B. bassiana* B1 (66.4%) ($P < 0.0001$) (figure 1). At 120 hours post treatment, *B. bassiana* B2 killed 83.4% of the larvae at a concentration of 10^7 conidia ml^{-1} and 85.2% of the larvae at a concentration of 10^8 conidia ml^{-1} ($P < 0.0001$). *B. bassiana* F4 was the second most active isolate against BTM larvae, with 78.6% larval mortality at 10^7 conidia ml^{-1} and 86.8% mortality at 10^8 conidia ml^{-1} at 120 hours post treatment ($P < 0.0001$). At a concentration of 10^8 conidia ml^{-1}, *B. bassiana* D2 and *B. bassiana* B1 induced 83% and 58.8% mortality rates, respectively, at 120 hours post treatment ($P < 0.0001$) (figure 1).

At 96 hours post treatment, *B. bassiana* B2 had the lowest median lethal concentrations (LC50) of 1.2×10^7 conidia ml^{-1}, while *B. bassiana* B1 showed the highest LC_{50} of 1.0×10^8 conidia ml^{-1} on the third instar larvae of BTM (table 4). The calculated LT_{50} values for *B. bassiana* B2, *B. bassiana* F4, *B. bassiana* D2 and *B. bassiana* B1 were 73.93, 84.53, 87.84 and 130.95 hours respectively at 10^7 conidia ml^{-1} (table 5).

Pathogenicity of mycoinsecticide formulations of *B. bassiana* against *C. perspectalis* larvae

The efficacy of eight formulations of *B. bassiana* isolate B2 (linseed oil, linseed-fungi free, corn oil, corn-fungi free, soybean oil, soybean-fungi free, wettable powder and wettable-fungi free) were assessed against the larvae of BTM in Sisangan Forest Park (field trial 1) and Maskupa (field trial 2), Mazandaran province.

Factors including the trials and fungi formulations had significant effects on mortality in the BTM larvae ($P = 0.0035$ and $P < 0.0001$ respectively). The interaction of trials and fungi formulations treatment was not significant ($F = 0.62$, $df = 8$, $P = 0.755$) (tables 6 and 7).

In Sisangan Forest Park (field trial 1) corn oil formulation was considerably superior to other treatments and caused 70.4% mortality on larvae of BTM after 14 days

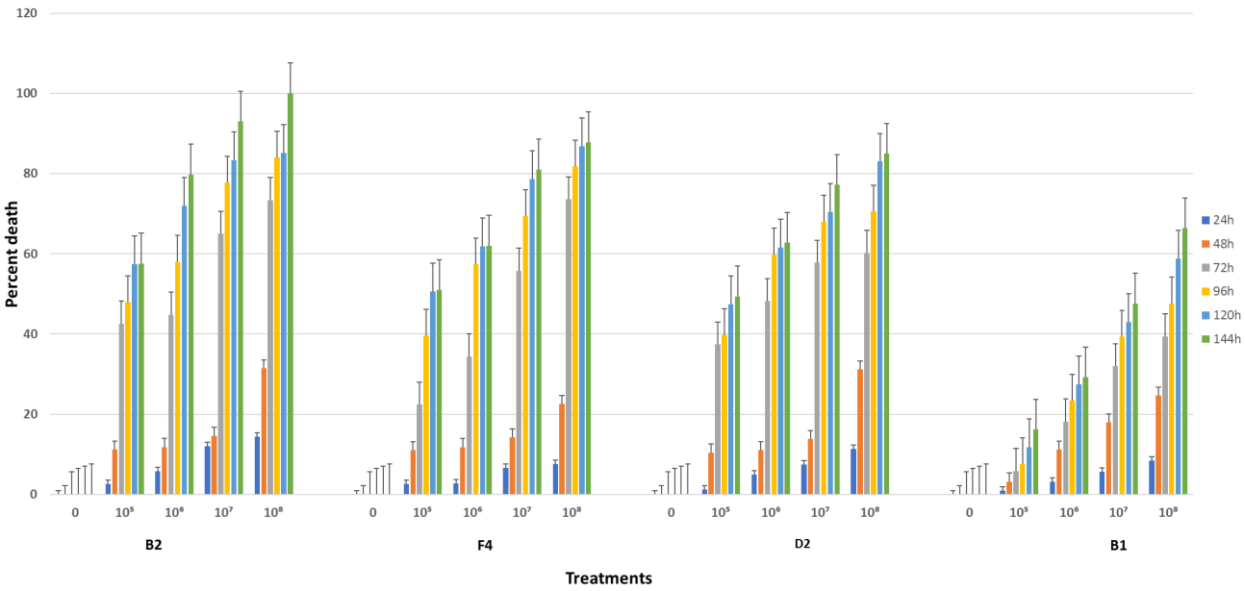

Figure 1. Efficacy of tested *B. bassiana* isolates for the third instar larvae of *C. perspectalis* at 0, 10^5, 10^6, 10^7 and 10^8 conidia ml^{-1} after 24, 48, 72, 96, 120 and 144 hours of exposure at 25 °C (mean ± SE).

Table 2. Analysis of variance of all isolates treatment effects on the third instar larvae of *C. perspectalis* after exposure to dose 10^5, 10^6, 10^7 and 10^8 conidia ml^{-1} of *B. bassiana* in 24, 48, 72, 96, 120 and 144 hours post treatment under laboratory bioassays.

Source	df	Sum of squares	Mean square	F value	Pr > F
Model	119	550206.93	4623.58	3577.24	<0.0001
Error	480	620.40	1.29		
Corrected total	599	550827.33	-	-	-
Isolates	3	38180.81	12726.93	9846.76	<0.0001
Time	5	195328.89	39065.77	30225.0	<0.0001
Concentration	4	222699.75	55674.93	43075.4	<0.0001
Isolates×Time	15	16416.82	1094.45	846.77	<0.0001
Isolates×Concentration	12	10272.97	856.08	662.34	<0.0001
Time×Concentration	20	60011.39	3000.56	2321.52	<0.0001
Isolates×Time×Concentration	60	7296.29	121.60	94.08	<0.0001

Table 3. Grouping of mean mortality for the third instar larvae of *C. perspectalis* at 0, 10^5, 10^6, 10^7 and 10^8 conidia ml^{-1} after 24, 48,72, 96, 120 and 144 hours after immersion in conidial suspensions of *B. bassiana*.

Factors	Means	N	Duncan grouping
Isolate			
B2	40.82	150	A
F4	35.74	150	B
D2	35.66	150	B
B1	19.63	150	C
Time (hours)			
144	52.25	100	A
120	48.96	100	B
96	43.61	100	C
72	35.55	100	D
48	12.55	100	E
24	4.88	100	F
Concentration			
10^8 conidia ml^{-1}	55.59	120	A
10^7 conidia ml^{-1}	47.15	120	B
10^6 conidia ml^{-1}	35.97	120	C
10^5 conidia ml^{-1}	26.11	120	D
0	0.0	120	E

N: total number of values by data set. Means with the same letter are not significantly different from each group.

post treatment, at a concentration of 10^8 conidia ml^{-1} on *Buxus* trees. Linseed oil, soybean oil and wettable powder are next with mortality rate of 51.8, 18.8 and 8.8%, respectively ($F = 44.04$, $df = 8$, $P < 0.0001$) (figure 2).

The percent death of larvae in Maskupa (field trial 2) followed a similar pattern. At a concentration of 10^8 conidia ml^{-1}, corn oil produced an 82.2% mortality after 14 days post treatment. This mortality was significantly higher than of other treatments, which included linseed oil, soybean oil, and wettable powder with 58, 24.4, and 13.2% mortality rates, respectively ($P < 0.0001$) (figure 3). For both field trials, no differences were observed between the blocks ($F = 0.64$, $df = 4$, $P = 0.636$) (table 6), so the larval mortality did not exceed 7.4% among insects treated with fungus-free formulations.

Discussion and conclusions

Our findings revealed that BTM larvae were susceptible to indigenous Hyrcanian entomopathogenic fungal isolates treated by direct immersion in laboratory conditions. Also, the effectiveness of all entomopathogenic fungus isolates was enhanced by increasing the exposure time and fungus concentration. This outcome is consistent with Burjanadze *et al*. (2019) who reported that

Table 4. LC_{50} and LC_{90} values of different *B. bassiana* isolates on the larvae of *C. perspectalis* at 96 hours post treatment.

Fungi	χ^2 (df = 6)	P-value	Intercept ± SE	Slope ± SE	LC_{50}	LC_{90}
B2	120.45	0.000	−0.15 ± 0.65	0.00 ± 0.00	1.2×10^7 (2.6×10^6-2.1×10^7)	1.1×10^8 (9.1×10^7-1.5×10^8)
F4	103.53	0.000	−0.26 ± 0.06	0.00 ± 0.00	2.0×10^7 (1.1×10^7-3.0×10^7)	1.2×10^8 (9.9×10^7-1.5×10^8)
D2	107.14	0.000	−0.24 ± 0.06	0.00 ± 0.00	2.8×10^7 (1.5×10^7-4.3×10^7)	1.7×10^8 (1.3×10^8-2.6×10^8)
B1	56.43	0.000	−0.98 ± 0.07	0.00 ± 0.00	1.0×10^8 (8.0×10^7-1.3×10^8)	2.3×10^8 (1.8×10^8-3.1×10^8)

Table 5. LT_{50} and LT_{90} values of different *B. bassiana* isolates on the larvae of *C. perspectalis* at 10^7 conidia ml^{-1}.

Fungi	χ^2 (df = 6)	P-value	Intercept ± SE	Slope ± SE	LT_{50}	LT_{90}
B2	28.46	0.000	−1.89 ± 0.13	0.02 ± 0.00	73.93 (58.33-89.19)	123.86 (105.55-158.10)
F4	33.01	0.000	−1.87 ± 0.13	0.02 ± 0.00	84.53 (66.28-103.92)	142.18 (119.01-191.90)
D2	40.23	0.000	−1.76 ± 0.12	0.02 ± 0.00	87.84 (66.02-112.93)	149.70 (121.97-218.41)
B1	17.51	0.000	−1.66 ± 0.12	0.01 ± 0.00	130.95 (108.08-179.80)	231.56 (181.82-367.86)

Table 6. Analysis of variance results for mycoinsecticide formulations effects on the larvae of *C. perspectalis* after exposure to dose 10^8 conidia ml^{-1} in 14 days post treatment in field trial 1 (Sisangan Forest Park) and field trial 2 (Mascupa).

Source	df	Sum of squares	Mean square	F value	Pr > F
Model	21	56729.933	2701.42	58.12	<0.0001
Error	68	3160.46	46.47		
Corrected total	89	59890.40	-	-	-
Trials	1	426.84	426.84	9.18	0.0035
Fungi formulations	8	55952.80	6994.10	150.48	<0.0001
Trials× Fungi formulations	8	231.55	28.94	0.62	0.755
Block	4	118.73	29.68	0.64	0.636

Table 7. Grouping of mean mortality for larvae of *C. perspectalis* at 10^8 conidia ml^{-1} after 14 days after treatment of *B. bassiana* in field trial 1 (Sisangan Forest Park) and field trial 2 (Mascupa).

Factors	Means	N	Duncan grouping
Trials			
Mascupa	23.31	45	A
Sisangan Forest Park	18.95	45	B
Fungi formulations			
Corn oil	76.30	100	A
Linseed oil	54.90	100	B
Soybean oil	21.60	100	C
Wettable powder	11.00	100	D
Corn-fungi free	8.40	100	DE
Linseed- fungi free	7.90	100	DE
Soybean- fungi free	7.00		DE
Wettable- fungi free	3.10		DE
Control	0.00		E

N: total number of values by data set. Means with the same letter are not significantly different from each group.

1×10^8 conidia ml^{-1} suspension of the local isolation *B. bassiana* -MB-103 induced 80% larval mortality against *C. perspectalis* in laboratory conditions and 60% in the field. *B. bassiana* (ET 10) was reported to be effective with 100% mortality in lab experiments using entomopathogens against the *C. perspectalis,* at the concentrations of 1×10^8 conidia ml^{-1} (Tozlu *et al*., 2022). *B. bassiana* B2 consistently outperformed all other test isolates, as indicated by the lower LC_{50} (1.2×10^7 conidia ml^{-1} at 96 hours post treatment) and LT_{50} (73.93 hours at 10^7 conidia ml^{-1}) values. Similarly, Hu *et al.* (2021) determined the LC_{50} values of *B. bassiana* strains Bb10331 and Bb7725 against *H. cunea* larvae to be 4.72×10^6 and 3.28×10^6 conidia·ml^{-1}, respectively, after 120 hours post treatment.

Our field results showed that corn oil caused the highest mortality (70.4% and 82.2% in Maskupa and Sisangan Forest Park, respectively), reinforcing the great potential of these EPFs to control pests, followed by linseed oil (larval mortality 51.8-58%). Low larval mortality was observed with soybean oil (18.8-24.4%) and wettable powder (8.8-13.2%) under spraying at a concentration of 10^8 conidia ml^{-1} on *Buxus* trees, 14 days after treatment. Oil-formulated fungi showed good results in the biological control of insect pests under field conditions (Batta, 2003). This is attributed to the oil's ability to protect fungal conidia from adverse environmental conditions, especially UV radiation, thus prolonging field persistence (Daoust *et al*., 1983; Jackson *et al*., 2009). Oil also blends better with the insect lipophilic cuticle than water does and oil spreads rapidly presumably carrying fungal spores to the insect body (Wraight *et al*., 2001). Luz and Batagin (2005) noted that *B. bassiana* conidia germinated best with corn oil (92.5%), followed by thistle (32.8%), linseed (27%) and soybean oil (19%), at 10% of the oil, as our study confirms.

C. perspectalis is one of the major destructive pests that caused severe damage to *B. hyrcana* trees in the Hyrcanian forests of northern Iran. Microbial control agents, including fungi provide a more environmentally acceptable and sustainable form of insect pest management. Environmental safety and ecosystem stability considerations lead to the conclusion that using native isolates in a microbial control program is more convenient. Fungal diversity in the moist and temperate Hyrcanian forests can vary widely due to diverse ecological conditions.

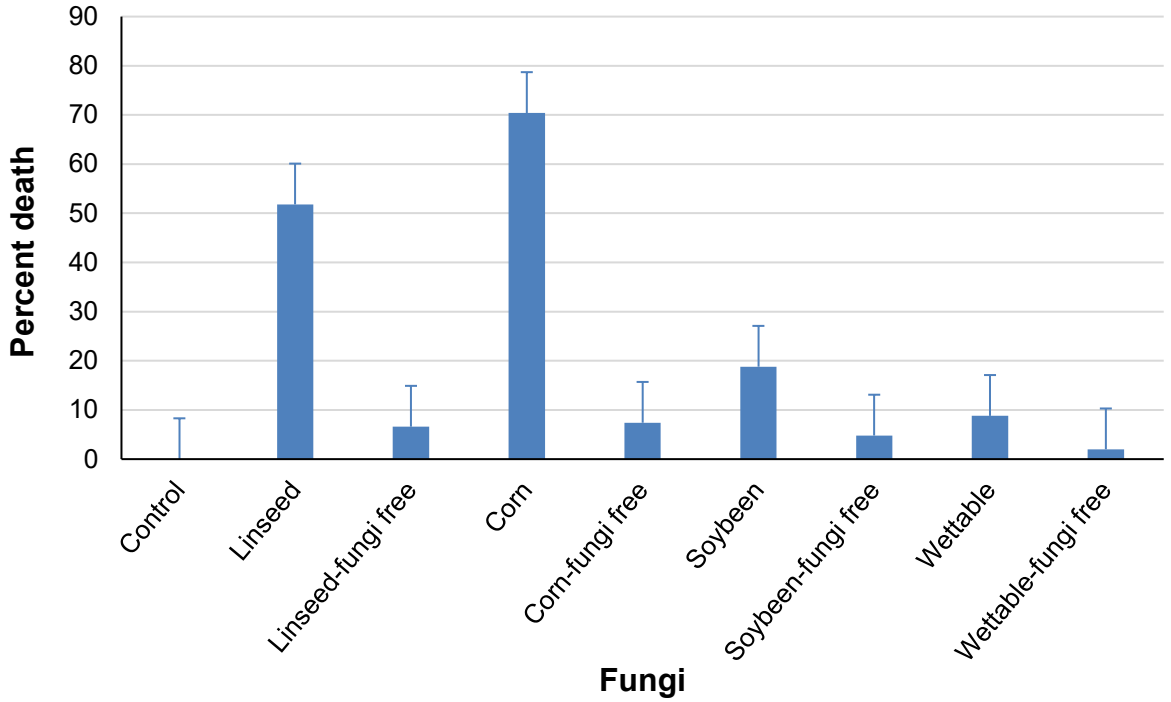

Figure 2. Corrected percent death (Abbott's formula) of larvae of *C. perspectalis* after exposure to dose 10^8 conidia ml^{-1} EPFs in 14 days post treatment in field trial 1 (Sisangan Forest Park).

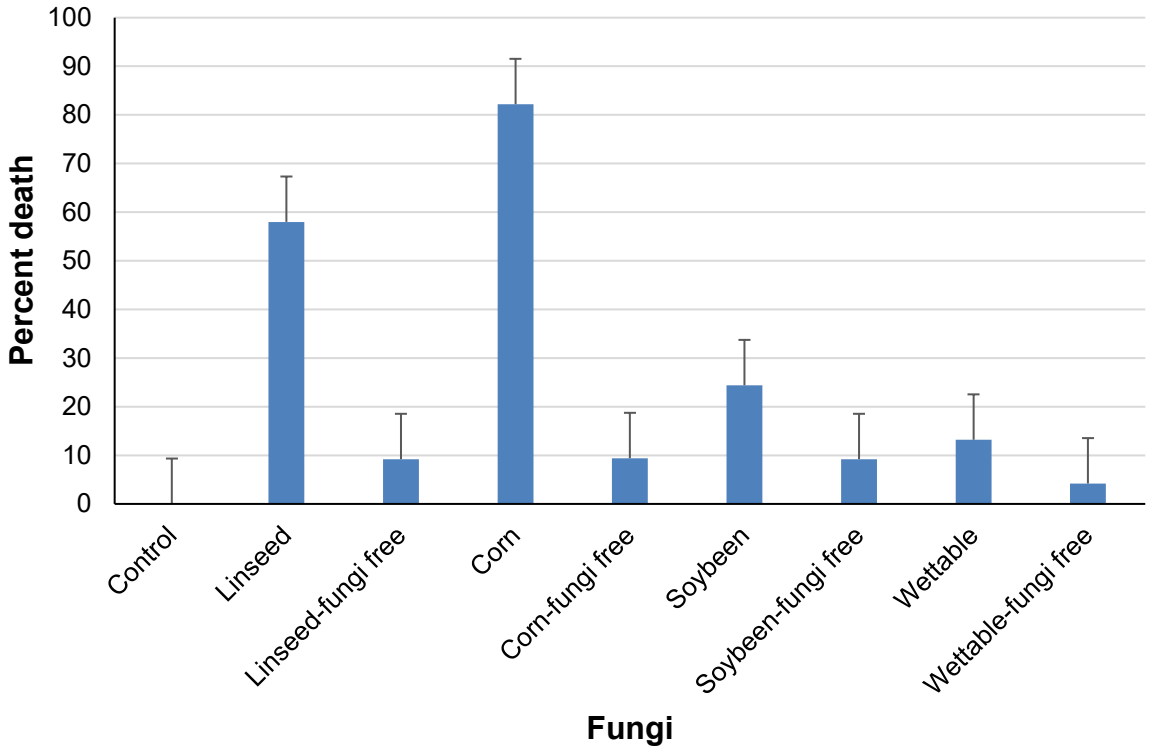

Figure 3. Corrected percent death (Abbott's formula) of larvae of *C. perspectalis* after exposure to dose 10^8 conidia ml^{-1} EPFs in 14 days post treatment in field trial 2 (Maskupa).

The results of our study demonstrate that native EPF isolate of *B. bassiana* B2 is a promising biological control agent against the larval stages of BTM. Furthermore, our results showed that two oil formulations of *B. bassiana* including corn and linseed were most effective against BTM larvae under field condition, and can be used by producers. Future research should be directed at investigating the effect of other native EPF isolate as potential biological control agents against BTM.

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