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Eco-friendly control method against invasive pest box tree moth, (*Cydalima perspectalis* (Walker) (Lepidoptera: Crambidae))

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Abstract

Background: *Buxus sempervirens* (Boxwood) is a type of plant that has economic and cultural significance, danger of extinction due to various factors. *Cydalima perspectalis* (Walker, 1859) (Lepidoptera: Crambidae), an invasive species, has an important role as a boxwood pest. The inadequacy of the pheromone trap methods recommended to control the pest or the negative effects of chemical insecticides on the environment have led to seeking alternative approaches in this regard. In this study, it was conducted to determine whether entomopathogens can be applied in pest control, the insecticidal effects of *Bacillus thuringiensis* subsp. *kenyae* (FDP-8), *Bacillus cereus* (FD-63), *Brevibacillus brevis* (FD-1) and *Vibrio hollisae* (FD-70) bacterial strains at the concentration of 1×10^8 CFU/ml and *Beauveria bassiana* (Bals.-Criv.) Vuill. (Cordycipitaceae) (ET 10) fungal isolate at the concentrations of 1×10^6 , 1×10^7 and 1×10^8 conidia/ml were tested under controlled conditions.

Results: As compared the results of the bacterial strains and fungal tested, it was determined that the best results were obtained from in the fungal isolate at the concentration of 1×10^8 conidia/ml. After 192 and 216 h. of observation, the 1×10^8 CFU/ml of bacterial strains: *B. cereus* FD-63, *B. brevis* FD-1 and *V. hollisae* FD-70; also, 1×10^8 conidia/ml concentration of ET 10 fungal isolate of *B. bassiana* control *C. perspectalis* caused mortality rate of 100% under laboratory conditions.

Conclusion: Future studies of these microorganisms against this pest in the field, may be an important alternative method to control this pest.

Keywords: Biological control, *Buxus sempervirens*, *Cydalima perspectalis*, Entomopathogens, Virulence

Background

Boxwood (*Buxus sempervirens* L. (Buxales: Buxaceae) is an evergreen plant that belongs to the box family, grows spontaneously in forest areas as natural vegetation, is generally found in the form of baskets, and rarely in the form of shrubs (Acartürk 2006). In addition to its functions as a landscape plant, it can also be used in alternative medicine, and since its wood does not rot easily and

is resistant to insects and microorganisms, it is preferred in handicrafts (ornaments such as combs, spoons, plates), increasing the economic and cultural value of the plant (Altunışık et al. 2017). The boxwood plant has been subjected to great destruction by humans over time and has faced an alarming threat of extinction as a result of the improper forest management planning processes (Toper Kaygın and Taşdeler 2019). In addition to the foregoing, disease agents such as *Cylindrocladium buxicola* Henricot, 2002 (Hypocreales: Nectriaceae) and pests such as *Monarthropalpus flavus* (Schränk, 1776) (Diptera: Cecidomyiidae), *Eurytetranychus buxi* Garman (Trombidiformes: Tetranychidae), *Psylla buxi* L. (Homoptera: Psyllidae) and *Cydalima perspectalis* (Walker, 1859)

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(Lepidoptera: Crambidae) also contributed significantly to the reduction of boxwood plants (Salioğlu 2020). Among these pests, especially invasive alien species pose as the primary threat to global biodiversity (Early et al. 2016). One of these species, *C. perspectalis* (box tree moth (box tree pyralid; BTM)) has been identified as the most important pest of boxwood as it causes a high damage to boxwood as an invasive species (Göttig et al. 2017). It has been reported that BTM (Billen 2007), the homeland of which is recorded as China, Japan, Korea and India, entered Europe for the first time from Germany and spread rapidly in Europe and Asia in the last 11 years (Suppo et al. 2020). The distribution of this harmful species in the world was reported by (EPPO 2022) and Turkey (Toper Kaygın and Taşdeler 2019).

It has been determined that BTM has become an invasive and destructive species over time (Zemek et al. 2020), and even though its host is boxwood. It has also been reported that BTM causes shedding of leaves (Zemek et al. 2020), drying of shoots and complete death of the plant in the following periods (Toper Kaygın and Taşdeler 2019).

It is essential to make a fast and effective control since the pest has the ability to multiply and spread rapidly. It is established that the suggested mechanical control to control the pest is not effective enough. In terms of chemical control, broad-spectrum insecticides are being used since there are no pesticides specific to the agent, and these insecticides cause undesirable effects on pollination and non-target organisms. In addition to this, pesticide use is prohibited in some European countries due to the importance of boxwood plants (EASAC 2015). In the use of pheromone traps, which is another recommended method for the pest control, only male individuals were attracted, resulting in an ineffective control (Plant et al. 2019). Furthermore, it has been reported by different researchers that BTM is not a good host for natural

parasitoids, so its population cannot be suppressed using natural enemies (Martini et al. 2019).

In this study, bacteria strains of *Bacillus thuringiensis* subsp. *kenyae* (Bacillales: Bacillaceae) (FDP-8), *B. cereus* Frankland and Frankland 1887 (Bacillales: Bacillaceae) (FD-63), *Brevibacillus brevis* (Migula 1900) Shida et al. 1996 (Bacillales: Paenibacillaceae) (FD-1) and *Vibrio hollisae* Hickman et al. 1982 (Vibrionales: Vibrionaceae) (FD-70) and *Beauveria bassiana* (Bals-Criv.) Vuill (Cordycipitaceae) (ET 10) fungal isolate were tested against the larvae of the pest under controlled conditions as alternatives to the recommended control of the pest.

Methods

Pest and entomopathogens

Larvae of *C. perspectalis* were collected from boxwood trees in areas where no chemical treatment was applied in Artvin province and brought to Atatürk University, Faculty of Agriculture, Pest Systematics Laboratory, Türkiye and stored in (30 × 45 × 30 cm) plastic cuvettes at 25 ± 2 °C under 65% ± 5 RH and 16-h light: 8-h dark conditions by providing daily fresh food and humidity check.

In the study, entomopathogenic bacterial strains kept in the Culture Collection of Atatürk University, Faculty of Agriculture, Department of Plant Protection, which were tested against different pests in different studies and developed in Nutrient Agar (NA; Difco) medium, and then preserved at − 80 °C in Nutrient Broth (NB; Difco) medium containing 15% glycerol, and as entomopathogenic fungus, fungus isolate kept in tubes containing Potato Dextrose Agar (PDA; Difco) in Atatürk University Faculty of Agriculture Plant Protection Department Mycology Laboratory were used (Table 1).

Preparation of entomopathogens

Conidia production was achieved by incubation of *B. bassiana* ET 10 fungal isolate in Sabourth Dextrose

Table 1 Bacterial strains and fungal isolate used in the study

Fungal isolate				
Isolate	Isolated from	ITS Identification result	ITS 1 sequences	
ET 10	<i>Sphenoptera antiqua</i>	<i>Beauveria bassiana</i>	KY806126	
Bacterial strains				
Strains	Isolated from	MIS Identification results	S*	HR
FDP-8	<i>Hypera postica</i>	<i>Bacillus thuringiensis</i> subsp. <i>kenyae</i>	0.320	–
FD-63	<i>Yponomeuta evonymella</i>	<i>Bacillus cereus</i>	0.241	–
FD-1	<i>Malacosoma neustria</i>	<i>Brevibacillus brevis</i>	0.625	–
FD-70	<i>Melolontha melolontha</i>	<i>Vibrio hollisae</i>	0.476	–

S: similarity; –: negative effect; HR: hypersensitivity

Agar (SDA; Difco) medium at 25 °C, 80% humidity for 2–3 weeks. Then, a stock suspension was prepared by washing the surface of the culture into bottles containing sterile water with 0.2 ml/l Tween-80 solution (Quesada-Moraga et al. 2006) and conidia suspension was adjusted to 3 different concentrations (1×10^6 , 1×10^7 , 1×10^8 conidia/ml) using a hemocytometer. The tested bacterial strains were cultured in 3 phases in NA medium at 30 °C for 24 h. in order to obtain fresh cultures. A single bacterial colony taken from these cultures in sterile loops was inoculated into Erlenmeyer flasks with 300 ml of NB medium and incubated for 24 h. at 250 rpm and 27 °C in a shaker with a thermostat. The bacterial density of the resulting aqueous culture was adjusted to 1×10^8 CFU/ml using sterile NB medium by spectrophotometric method and transferred to sterile spray vials.

Insecticidal activity of entomopathogenic bacterial strains and fungal isolate

Boxwood shoots and leaves and 10 larvae of *C. perspectalis* were placed in (20 × 12 × 7 cm) plastic cuvettes on which blotting paper was laid and then, the determined suspensions of the previously prepared bacterial strains and fungal isolate were applied to each tub as 5 ml sprays. In the study, Cormoran (100 g/l Novaluron and 80 g/l Acetamiprid) was used as positive control and sterile NB and sterile water (sdH₂O) was used as negative control. The treated cuvettes were then kept under controlled conditions at 25 ± 2 °C, 65–70% RH and a 16: 8 (light: dark) photoperiod. The final evaluation of the trial was carried out and the mortality rates were determined at 240 h. By isolating the larvae determined to be infected according to Koch Postulates, entomopathogenic bacterial strains and fungal isolate were obtained again. The experiment was carried out in 3 repetitions for each application on the same day. The number of dead larvae was recorded regularly every 24 h. and the death rates were determined by the formula below.

$$\text{Mortality rate (\%)} = \frac{100 \times \text{the number of dead adults in treatment}}{\text{Total adult in treatment}}$$

Data analysis

The resulting data obtained under controlled conditions were statistically analyzed using the JMP 5.0.1 software program, and the differences between the applications were determined according to the ANOVA

results and the “LSMeans Differences Students” multiple comparison test.

Results

According to the results obtained, all bacterial strains and fungal isolates were found to be effective on the larvae of the pest at varying rates. In the applications of both bacterial strains and fungus isolate, the highest mortality was obtained from the insecticide, which was the positive control, and it was observed that all larvae thereof died within the first 24 h. There was no death until the end of the experiment in the larvae that were treated with sterile water and NB which were the negative control. When each application was evaluated by hours, a statistical difference was found between the percent mortality rates obtained from the respective applications. ($F_{24 \text{ hrs}}: 216.5727$, $P < 0.01$; $F_{48 \text{ hrs}}: 168.9877$, $P < 0.01$; $F_{72 \text{ hrs}}: 42.6591$, $P < 0.01$; $F_{96 \text{ hrs}}: 53.2449$, $P < 0.01$; $F_{120 \text{ hrs}}: 78.7155$, $P < 0.01$; $F_{144 \text{ hrs}}: 56.0496$, $P < 0.01$; $F_{168 \text{ hrs}}: 41.5195$, $P < 0.01$; $F_{192 \text{ hrs}}: 40.1506$, $P < 0.01$; $F_{216 \text{ hrs}}: 39.2107$, $P < 0.01$; $F_{240 \text{ hrs}}: 30.9245$, $P < 0.01$). Dying of larvae started after 72 h in larvae treated with 1×10^7 (3.33%) and 1×10^8 conidia/ml suspensions (3.33%) of ET 10 fungal isolate, and after 96 h. in those treated with 1×10^6 conidia/ml suspension (3.7%). At 216 h., when 100% mortality was established in 1×10^8 conidia/ml suspension, this rate was 80% in 1×10^7 conidia/ml and 63% in 1×10^6 conidia/ml. At 240 h., when the last observations were taken, the mortality rate remained unchanged at 1×10^7 conidia/ml (80%), and 66.67% at 1×10^6 conidia/ml (Table 2). In larvae treated with bacterial strains, dying of larvae started in the first 24 h in all strains except FDP-8 bacterial strain. In the FDP-8 bacterial strain, death was recorded after 48 h and the mortality rate continued to increase in other strains at this observation hours. While the mortality rate at the end of 96 h. was 73.33% in larvae treated with FD-63 bacterial strain, it was recorded as 56.67% in FD-70, 53.33% in FD-1 and 30% in FDP-8. Mortality rate was 100% in larvae treated with FD-63 bacterial strain at 192 h., 100% in FD-70 at 216 h. and in FD-1 at 240 h (Table 2). When the study is evaluated in general terms; it was observed that bacterial strains FD-63 (192 h.; 100%) of *B. cereus*, FD-1 (216 h.; 100%) of *B. brevis* and FD-70 (216 h.; 100%) of *V. hollisiae* and 1×10^8 conidia/ml suspension (216 h.; 100%) of the ET 10 fungal isolate of *B. bassiana* have a high insecticidal effect against the invasive species *C. perspectalis* (Table 2).

In the study, a statistical difference was observed between 1×10^6 , 1×10^7 and 1×10^8 conidia/ml suspensions of *B. bassiana* ET 10 fungal isolates the pathogenic effects of which were tested ($F: 40.5253$; $P < 0.01$) and the

Table 2 The pathogenic effect of fungal isolate and bacterial strains on *Cydalima perspectalis*

Treatments	% Mortality at different interval periods (h)																			
	24		48		72		96		120		144		168		192		216		240	
FD-63	3.33	B	23.33	B	30.00	B	73.33	B	83.33	B	86.67	B	88.89	AB	100.00	A	100.00	A	100.00	A
FD-70	3.33	B	13.33	C	23.33	B	56.67	C	63.33	C	66.67	C	86.67	AB	90.00	AB	100.00	A	100.00	A
FD-1	3.33	B	16.67	C	26.67	B	53.33	C	70.00	C	77.78	BC	83.33	B	86.67	AB	100.00	A	100.00	A
FDP-8	0.00	B	6.67	D	20.00	B	30.00	D	30.95	D	33.33	DE	46.67	D	50.00	D	53.33	C	56.67	C
ET-10*	0.00	B	0.00	E	3.33	C	8.33	E	16.67	E	43.33	D	66.67	C	83.33	BC	100.00	A	100.00	A
ET-10**	0.00	B	0.00	E	3.33	C	3.70	E	16.67	E	23.33	EF	43.33	D	70.00	C	80.00	B	80.00	B
ET-10***	0.00	B	0.00	E	0.00	C	3.70	E	10.00	E	13.33	F	26.67	E	53.33	D	63.00	BC	66.67	BC
Cormoran	100.00	A	100.00	A	100.00	A	100.00	A	100.00	A	100.00	A	100.00	A	100.00	A	100.00	A	100.00	A
NB	0.00	B	0.00	E	0.00	C	0.00	E	0.00	F	0.00	G	0.00	F	0.00	E	0.00	D	0.00	D
sdH ₂ O	0.00	B	0.00	E	0.00	C	0.00	E	0.00	F	0.00	G	0.00	F	0.00	E	0.00	D	0.00	D
CV	30.30		26.59		35.33		23.46		16.64		17.26		16.59		14.66		14.53		16.16	
LSD	5.71		6.35		12.52		13.24		11.16		13.16		15.43		15.92		17.37		19.50	

*: 1×10^8 conidia/ml concentration, **: 1×10^7 conidia/ml, ***: 1×10^6 conidia/ml

highest mortality rate was obtained at 1×10^8 conidia/ml suspension of ET 10 fungal isolates, followed by 1×10^7 conidia/ml and 1×10^6 conidia/ml suspensions (Fig. 1). Similarly, a statistical difference was found between the percentage mortality rates obtained from the bacterial strains applied (F: 52.3907; $P < 0.01$), and the highest mortality rate was recorded in the FD-63 bacterial strain of *B. cereus*, followed by FD-1, *V. hollisae*, *B. thuringiensis* subsp. *kenyae* strains of *B. brevis*, respectively (Fig. 1). When bacterial strains and fungal isolates were compared, it was seen that there was a statistical difference between them and the pathogenic effects of bacteria were higher against the larvae of this pest (F: 52.3907; $P < 0.01$) (Fig. 1). The highest mortality percentage was obtained from FD-63, followed by FD-1, FD-70 bacterial strains. The pathogenic effect of 1×10^8 conidia/ml suspension of ET 10 fungus isolate was higher than that of FDP-8, followed by 1×10^7 and 1×10^6 conidia/ml suspensions of ET 10 (Fig. 1).

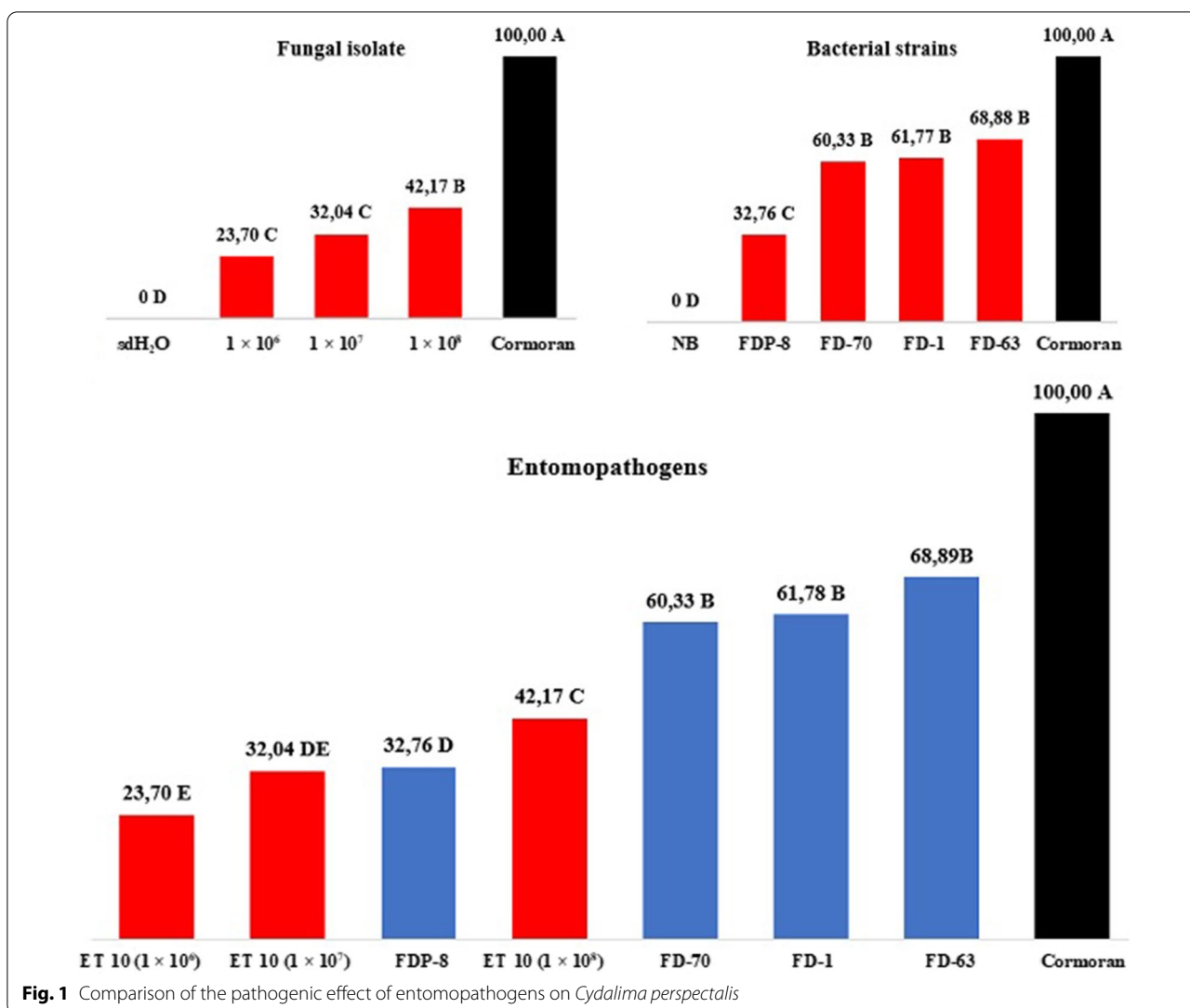
Some examples of the pathogenic effects of bacterial strains and fungal isolate used in the study on the pest are shown in Fig. 2.

Discussion

As a result of climate change and lack of natural enemies in different parts of the world, the damage of invasive species *C. perspectalis* on boxwoods has also increased (Early et al. 2016). Studies to find effective pathogens and parasitoids to control the pest have increased in recent years. Entomopathogens, which are among the most effective factors in suppressing pest populations are used in the biological control of pests. Among the entomopathogens, bacteria are the most widely used

biocontrol agent against plant pests. Species belonging to the genus *Bacillus* seem to be the most important group. *Bacillus thuringiensis* Berliner 1915 (Bacillales: Bacillaceae), *B. brevis*, *B. cereus*, *B. circulans* Jordan, 1890 (Bacillales: Bacillaceae), *B. megaterium* de Bary 1884 (Bacillales: Bacillaceae) and *B. subtilis* (Ehrenberg, 1835) Cohn 1827 (Bacillales: Bacillaceae) are used in biotechnological and industrial areas (Rooney et al. 2009). It is noted that *B. thuringiensis*, which has been studied the most, is a low-risk pesticide constituting 2% of the world insecticide market (Bravo et al. 2011). It is stated that this eco-friendly species causes especially intestinal and hemolymph poisoning and feeding cessation that leads to the death of the insect within few days after oral ingestion (Schmutterer and Huber 2005). In many studies conducted to date, *B. thuringiensis* has been found to be effective against mostly the pests belonging to the Lepidoptera order.

It is determined in some studies that *C. perspectalis* is also among the harmful species on which *B. thuringiensis* is effective (Göttig and Herz 2018). Burjanadze et al. (2019)'s study in Georgia was one of them and *B. thuringiensis* subsp. *kurstaki* Bulla et al. 1979 (Bacillales: Bacillaceae) was found to be 60.6% successful on larvae. In another study, Salioglu and Göktürk (2021) investigated the pathogenic effects of *B. thuringiensis* subsp. *kurstaki*, *B. subtilis*, *B. thuringiensis* subsp. *kenyae* and *B. brevis* bacterial species against the 2nd and 5th larval instars of *C. perspectalis* in laboratory conditions and they found mortality rates of 70, 80, 60 and 70%, respectively. In this study, it was determined that *C. perspectalis* was affected by *B. thuringiensis* subsp. *kurstaki* FDP-8 strain with a mortality rate of 56.67% during the 240 h.



Another bacterial strain, *B. cereus*, is genetically very similar to *B. thuringiensis* (Helgason et al. 2000).

B. brevis was reported to show broad-spectrum antimicrobial activity to soil borne disease-causing pathogens has been widely used in biologic control of soil borne plant diseases (Hou et al. 2015). In this study, *B. cereus*, *V. hollisiae* and *B. brevis*, were found to be effective in the control of the pest, should be approached with a distance regarding to using as biocontrol agent.

In addition to the foregoing, in a study where it was observed that the damage caused by the pest on the boxwood trees was reduced in the gardens, where chemicals were not applied, the intestinal micro-flora of *C. perspectalis* was determined, and *Acinetobacter schindler* Nemec et al. 2001 (Pseudomonadales: Morexellaceae), *Enterococcus casseliflavus* Collins et al. 1984 (Lactobacillales: Enterococcaceae), *Klebsiella mobilis* Bascomb et al. 1971 (Enterobacterales: Enterobacteriaceae), *Paenibacillus*

anaericanus Horn et al. 2005 and *P. popilliae* Dutky 1941 (Bacillales: Paenibacillaceae) bacterial species and *Metarhizium* sp., *Beauveria* sp., *Verticillium* sp., *Alternaria* sp. and *Mucor* sp. species were obtained from isolations of dead larvae and among these, *P. popilliae* was reported to suppress the pest population (Harizanova et al. 2018). The gut microbiota is associated with many essential host physiological functions (Grenie and Leulier 2020) and it has been found that bacteria in insect guts affect the life activities of insects (Ma et al. 2021).

Fungal entomopathogens are safe and can be grown on a large scale (Mantzoukas and Eliopoulos 2020), attack insects through tissue rather than orally, infect all life-cycles of insects, and remain in the soil for several seasons while continuing to attack target insects (Rajula et al. 2020) and being resistant to abiotic stresses such as salinity (Bamisile et al. 2018) increase their importance in terms of biological control. In this respect, studies

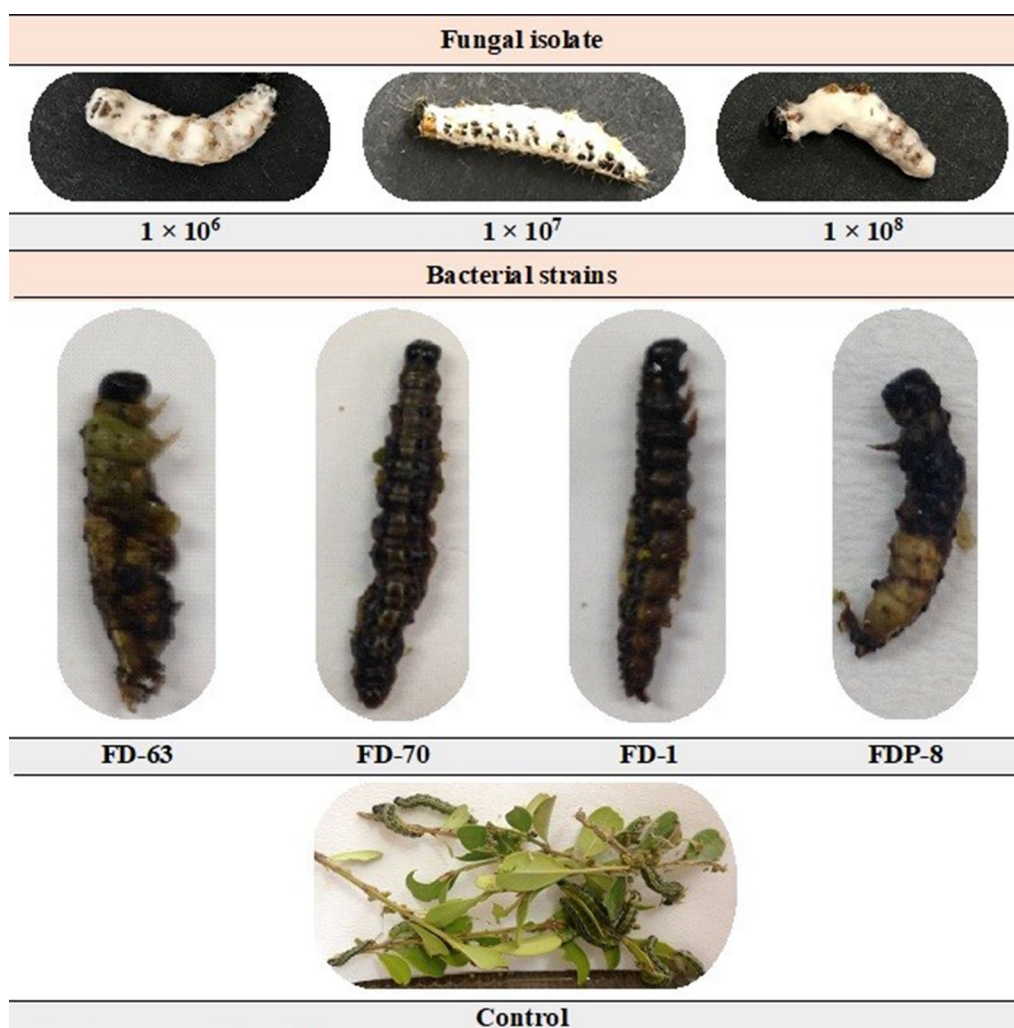


Fig. 2 Pathogenic effect of fungal isolate (three different concentrations) and bacterial strains on *Cydalima perspectalis*

have been conducted on Ascomycetous species such as *Beauveria*, *Metarhizium*, *Isaria* and *Lecanicillium* (Jan-dricic et al. 2014). In these studies, it was noted that the entomopathogenic fungus species *B. bassiana* is among the most promising species known (Akutse et al. 2013) and is the active component of many products which are under development (Tangtrakulwanich et al. 2014).

Zamani et al (2017) isolated *B. bassiana* from infected larvae in the survey study they conducted in Iran, and they also reported that this species, which has been recorded to be effective in lepidopteran larvae in many countries, was obtained for the first time through BTM in this study. Within this study, after 240 h. of observation, it was found that 1×10^6 conidia/ml suspension of *B. bassiana* was 66.67% effective, 1×10^7 conidia/ml suspension was 80% effective, and 1×10^8 conidia/ml suspension was 100% effective. In another study conducted with *B. bassiana*, it was reported that 1×10^8 conidia/

ml suspension of the fungus caused 80% larval mortality against *C. perspectalis* in laboratory conditions and 60% in the field (Burjanadze et al. 2019).

Conclusions

Cydalima perspectalis, an invasive species, has started to pose a serious problem in recent years in Turkey and across the world where boxwood grows. Different solutions have been considered to combat and control this pest. However, it has been seen nowadays that these solutions alone are not effective enough. The negative effects of the broad range of remedies used for chemical control in terms of human health, environmental pollution and sustainable agriculture have led people to seek alternative methods of pest control. Among these, entomopathogens are considered as the leading alternative. As the result of this study, it has been determined that *B. cereus*, *B. brevis*,

V. hollisiae and *B. bassiana* can be used successfully as alternatives to chemicals for the control of *C. perspectalis*. Using entomopathogens in Integrated Pest Management (IPM) programs is of great importance given their environmentally friendly nature, bio-persistence, and ability to kill pests at various developmental stages of their life cycle. It is of great importance to formulate microorganisms with proven effectiveness with suitable carriers, to determine their shelf life, and to test them in field conditions.

Abbreviations

—: Negative; °C: Degrees Celsius; *B. bassiana*: *Beauveria bassiana*; *B. brevis*: *Bacillus brevis*; *B. cereus*: *Bacillus cereus*; *B. circulans*: *Bacillus circulans*; *B. subtilis*: *Bacillus subtilis*; *B. thuringiensis* subsp. *kenyae*: *Bacillus thuringiensis* Subsp. *kenyae*; *B. thuringiensis* subsp. *kurstaki*: *Bacillus thuringiensis* Subsp. *kurstaki*; *B. thuringiensis*: *Bacillus thuringiensis*; BTM: Box Tree Pyralid; *C. perspectalis*: *Cydalima perspectalis*; CFU: Colony Forming Units; g: Gram; hrs: Hours; HR: Hypersensitivity; IPM: Integrated Pest Management; ITS: Internal Transcribed Spacer; L: Liter; ml: Milliliter; NA: Nutrient Agar; NB: Nutrient Broth; PDA: Potato Dextrose Agar; RH: Relative Humidity; rpm: Centrifugal rotation speed; *S. antiqua*: *Sphenoptera antiqua*; S: Similarity Index; SDA: Sabourth Dextrose Agar; sdH₂O: Sterile water; sp.: Species; subsp.: Subspecies; *V. hollisiae*: *Vibrio hollisiae*.

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Author contributions

This work was carried out in collaboration among all authors. GT conceived the study. TG collected pest material from Artvin. GT, ET, RK, NT and FD performed laboratory experiments. ET analyzed data. ET, GT and RK produced the manuscript. All authors corrected and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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