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Biological characteristics of a new entomopathogenic fungus detected in *Cydalima perspectalis* (Walker, 1859) (Lepidoptera: Crambidae)

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Abstract. *Cydalima perspectalis* (Walker 1859), the boxwood moth, is one of the important invasive pests spreading rapidly throughout Europe within a few years. The objective of the current research was to determine an alternative biological control agent for the control of *C. perspectalis*. *Beauveria bassiana*, an entomopathogenic fungus with high insecticidal activity, was isolated from the larvae of the pest that causes substantial economic losses on boxwood (*Buxus sempervirens*) in the eastern Black Sea region of Turkey, and its virulence was tested on it. Based on the morphological data and molecular characteristics, including the partial sequence of ITS (ITS1-5.8S-ITS2 gene region) and EF1, the fungus was identified as an isolate of *B. bassiana* (CP1). In larval stage susceptibility tests, it was determined that larval mortality was higher in older stages. In concentration-response experiments using $1 \times 10^{4\text{-}8}$ conidia/ml, the LC_{50} value of the new isolate was calculated to be 1.7×10^4 conidia/ml within 10 days against *C. perspectalis* 6th instar larvae under laboratory conditions. This is a significant study to describe a novel *B. bassiana* CP1 isolate, and preliminary data suggests that the fungal isolate has considerable potential as an effective biopesticide for controlling *C. perspectalis*.

Keywords: *Buxus sempervirens, Cydalima perspectalis*, the box tree moth, biological control, *Beauveria bassiana.*

Introduction

Buxus sempervirens (Buxaceae), the boxwood, is a dense, heavily branched, evergreen, round, or broad outline and a very valuable small tree. The species is shade-tolerant, slow growing, and prefers moist and nutrient-rich soils. *B. sempervirens* is native to some areas of Europe, Africa, Asia, England, Morocco, Turkey, the Caucasus, and Iran (Decocq et al. 2004, Chadburn & Barstow 2018). Boxwood grows naturally in the Black Sea region, Thrace, the southern Marmara region, and the eastern and western Mediterranean forests in Turkey (Lehtijarvi et al. 2017). The species, which generally spreads in the forest substrate, has formed a natural population of about one thousand hectares in the forests of Turkey. Boxwood is used as an ornamental and landscape plant in parks and gardens and as a filler for bouquets and wreaths. Because its wood is very hard, it is difficult to work, but it is very durable. While the boot of the boxwood is used to make toys, kitchen utensils, household and ornamental items, and musical instruments, its leaves, which contain various chemicals, are also used in alternative medicine (Rahman et al. 1991, Neves et al. 2009).

Boxwood is under pressure from various environmental factors, pathogens, and pests. A serious pest of boxwood is *Cydalima perspectalis* (Walker 1859) (Lepidoptera: Crambidae: Spilomelinae), the boxwood moth (Kenis et al. 2013, Toker Kaygın & Tasdeler 2019). The moth was sourced in East Asia (China, Japan, Korea) and was first discovered in southwestern Germany in 2007 and the Netherlands in the same year (Billen 2007). Within three years, it spread rapidly to other European countries (England, France, Switzerland, Austria, and Italy) (Van der Straten et al. 2010). This pest was also recorded in Spain and Galicia in 2014 (Pino Pérez & Pino Pérez 2014). The pest was found to have spread between 2010 and 2014 in all Balkan countries (Romania, Hungary, Croatia, Serbia, Montenegro, Slovenia, Bulgaria, and Greece) (Iamandei 2010, Farina & Rizzo 2015, Arnaudov & Raikov 2017). Its discovery in Russia and Georgia was reported in 2010-2015 (Mally & Nuss 2010, Matsiakh et al. 2016). *C. perspectalis*, which was first discovered in gardens and parks in Sarıyer, Istanbul, in 2011 (Hizal et al. 2012), has rapidly spread to all cultural and natural boxwood fields in Turkey Turkey (Ozturk et al. 2016, Toker Kaygın & Tasdeler 2019, Alkan Akıncı & Kurdoglu 2019, Ak et al. 2021).

The effectiveness of biocontrol treatments has been tested in urban parks and gardens. Entomopathogenic microorganisms such as *B. bassiana* (SangMyeong et al. 1997), *Bacillus thuringiensis* (Berliner) (Las Heras et al. 2019, Las Heras & Arimany 2020), viruses (Oberemok et al. 2017, Rose et al. 2013), and nematodes (Las Heras et al. 2019, Göttig & Herz 2018, Miladinović et al. 2022) have been studied. In addition, some members of the family Tachinidae from the order Diptera have been reported to parasitize boxwood moths (Farahani et al. 2018, Bird et al. 2020). Salioglu & Gokturk (2021) showed that *Bacillus subtilis* from another source has a mortality effect of up to 80% on boxwood moth larvae. In another study, Usta (2020) reported that a *Bacillus thuringisensis* isolated from the pest has a mortality effect of 85% on its host. A study (Lee et al. 1997) showed that a strain of *Beauveria bassiana* did not significantly affect the larvae of the pest in Korea. Harizanova et al. (2018) demonstrated fungal contamination in the Bulgarian population of *C. perspectalis*. Zemek et al. (2020) showed that *Isaria fumosorosea* has a minor effect on boxwood moth larvae under laboratory conditions. The two studies to date that have detected an entomopathogenic fungus in the box tree borer population were the isolation of a strain of *B. bassiana* from Iran and Bulgaria, and the morphological characteristics of the strain were reported (Harizanova et al. 2018, Zamani et al. 2017). Entomopathogenic fungi, commonly used to control insect pests, play an extremely important role in controlling pest and vector populations in nature (Ansari et al. 2011).

In this study, a dead larva with natural mycosis was discovered because of observations in the areas where the boxwood moth is common in Artvin, in the eastern Black Sea region of Turkey, in 2020. A fungal isolate was isolated from

this infected larva, its biological properties were determined, and its insecticidal effect on *C. perspectalis* larvae was studied.

Materials and methods

Collection of insects

Dead *C. perspectalis* larva was collected from the boxwoods (*Buxus sempervirens*, Buxaceae) near Artvin, Turkey (latitude: 400.34N, longitude: 412.07 E) in June 2020, and transferred to the laboratory in a sterile tube, and fungi were isolated dead larva and named CP1. In addition, healthy *C. perspectalis* larvae were brought to the laboratory in aerated containers. A fungal cadaver was photographed (Canon EOS 550D) and used for fungal isolation, while healthy larvae were used for bioassays.

Isolation of fungal isolate

Fungal isolate was isolated from infected larvae by transferring spore and mycelial fragments from a cadaver and cultured on potato dextrose agar medium containing 1% yeast extract (PDAY medium) with 50 µg/ml tetracycline and 50 µg/ml ampicillin to prevent bacterial contamination at 28 °C until fungal growth. Pure cultures were maintained on PDAY media and subcultured monthly. The fungus isolate was named as CP1 and stored as glycerol stock in Karadeniz Technical University, Department of Biology, Microbiology Lab fungus culture collection at -80 °C.

Morphological identification

The cadaver's appearance, color, and condition were first evaluated in morphological identification. Then, the shape, morphology, color, and pigmentation of the colonies on the PDAY were considered. Fungal spores and micelle structures were examined under a phase contrast microscope (Nicon, Exlipse Ni). The morphology of the colonies was evaluated, and the morphological description of the isolate was done according to Humber (2012).

Molecular identification

Following the manufacturer's instructions, the DNA was isolated from mycelia of CP1 isolates grown on PDAY medium at 27 °C for 14 days using the Kit (Quick-DNA Fungal/Bacterial MiniPrep, Zymo Research, USA).

The partial sequence of the ITS1-5.8S-ITS2 gene and elongation factor 1 (EF1) of the new isolate CP1 were amplified by polymerase chain reaction (PCR) using primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990), and Fw (5'-ATGGGTAAGGARGACAAGAC-3') as the EF forward and Rv (5'-ACHGTRCCRATACCACCSATCTT-3') as the EF reverse sequence (Rehner & Buckley 2005). The PCR reaction was prepared with DNA (50 ng), reaction buffer (5X), dNTPs, primers (forward and reverse), DNA polymerase, and sterile dH_2O in a total volume of 50 µl. The reaction conditions were after a denaturation step at 98 °C for 5 min, 35 cycles of denaturation at 98 °C for 1 min, annealing at 55 °C for 55 s, extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min, using the T100 thermal cycler (Bio-Rad, UK).

PCR products were electrophoresed in a 0.7% agarose gel and examined under UV light with ethidium bromide. After the assay, the amplified fragment was purified and cleaned using a PCR clean-up kit (Marherey-Nagel, Germany) according to the manufacturer's instructions. It was quantified using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and sent out for sequence analysis (ETKA Biotechnology, Samsun, Turkey).

The isolate's ITS1-5.8S-ITS2 and EF1 gene sequences were aligned with the sequences in the NCBI GenBank database for comparison with other *Beauveria* species. Phylogeny was analyzed using the neighbor-joining method with MEGA X software (Kumar et al. 2018), and analysis for comparison with known species and bootstrap tests were performed with 1000 replicates.

Insecticidal activity tests

The new isolate's fungal conidiospores were obtained using sterile 0.1% Tween 80 to the 4 weeks old culture. The fungus isolates conidiospores were then harvested with a 0.1% Tween 80 (AppliChem), and the mixture containing conidiospores was filtered through cheesecloth to remove fungal residues and transferred to a sterile 50 ml falcon. Conidiospore suspension was counted under the microscope with a Neubauer hemocytometer to determine the concentrations and serially diluted in 0.01% Tween 80 from 1×10^4 to 1×10^8 conidia/ml. Fungal suspensions at all concentrations were applied with a sterile sprayer.

To evaluate the larval age susceptibility, a concentration of 1×10^8 conidia/ml *B. bassiana* CP1 was tested on 2nd-3rd, 4th-5th, and 6th instar larvae. Bioassays were performed on thirty healthy *C. perspectalis* larvae with three replications. The experiments were repeated three times at different times. The larvae were placed in plastic boxes with boxwood leaves, to which one ml of CP1 spores was applied with a small hand sprayer for each dilution. Tween 80 was used for the control group. The experimental group was housed in a climate chamber (ARALAB, FITOCLIMA 600&1200 BIO, Portugal) at 25 ± 1°C, 60% RH, and 16L:8D photoperiod and recorded dead larvae for 10 days (concentration–responseet al. 2020). The dried leaves were replaced daily with fresh leaves. Dead larvae were counted and removed from the boxes, and cadavers were disinfected with a 0.2% sodium hypochlorite solution for 3 min, rinsed with sterile distilled water, and placed in moistened petri dishes to encourage fungal mycosis. Mycotic cadavers were counted, and the percentage of mycosis was determined and photographed with a Canon 550 D camera.

The concentration-response test was performed on 6th instar *C. perspectalis* larvae at $1 \times 10^{4\text{-}8}$ conidia/ml concentrations. The protocol used in the concentration–response tests is the same as larval age bioassay described above.

Statistical analysis

For statistical analyses, mortality rates on the 10th day were calculated using the Schneider-Orelli formula (Püntener 1981), and LC_{50} and LT_{50} values were determined by Probit analysis using MS Excell (Finney 1971). The data from the larval age susceptibility test were subjected to ANOVA and subsequently to Duncan's test to separate means ($p < 0.001$) using SPSS 23.0 statistical software (IBM Corporation, Armonk).

Results

A fungus was isolated from the cadaver of *C. perspectalis* larva that died and mycosed naturally (Figure 1). Based on the morphological images on the agar plates, the isolate (CP1) was determined to be a new entomopathogenic fungal isolate. Colonies were round, slightly raised with a white powdery surface, and slightly downy with circular rings (Figure 1). Phase contrast micrographs of the fungal isolate are shown in Figure 2. It was morphologically identified as *Beauveria bassiana*. Genomic analysis of the ITS1-5.8S-ITS2 and EF1 partial genes also revealed that the new isolate was identical to *B. bassiana* (Figure 3 and Figure 4). The sequences of the ITS1-5.8-ITS2 and EF1 genes were deposited at the NCBI data library under accession numbers OP295701 and OP422526, respectively. CP1 was similar to the known *B. bassiana* strains registered in the genebank (ITS *B. bassiana* AF291871 and *B. bassiana* MN122431, and EF1 *B. bassiana* KM031765 and *B. bassiana* AY883710).

The new isolate was found to have a very high pathogenic effect on *C. perspectalis* 6th instar larvae. In larval stage susceptibility tests, it was determined that larval mortality

was higher in older stages. Larval mortality was significantly lower in the 2-3rd and 4-5th instar larvae compared to the 6th instar larvae. The mortalities caused by 1 × 10^8 conidia/ml concentration were 99.24, 43.25, and 23.94% against 6th, 4th-5th, and 2-3rd instar larvae, respectively (Figure 5).

The mortality rates of the isolate differed from those of the control groups within 10 days of application. The lowest concentration (1 × 10^4 conidia/ml) killed half of the insects used in this study (Figure 6). It was found that as the concentration increased, the mortality of the pest larvae increased, and the concentration of 1 × 10^8 conidia/ml resulted in 100% death of the pests. After the concentration-response test, the LC_{50} value of the new isolate was calculated to be 1.7 × 10^4 (0.1 – 23.2) (Slope ± SE= 0.343 ± 0.570, *df*= 3, χ^2=0.892) conidia/ml within 10 days against the larvae of *C. perspectalis* under laboratory conditions. Mycosis in all cadavers indicated that the deaths were due to fungal infection (Figure 7). Using the LT_{50} value at 1 × 10^8 conidia/ml concentrations, it was found that the fungal isolate CP1 killed half of the *C. perspectalis* larvae in 3.87 days (3.07 – 4.88).

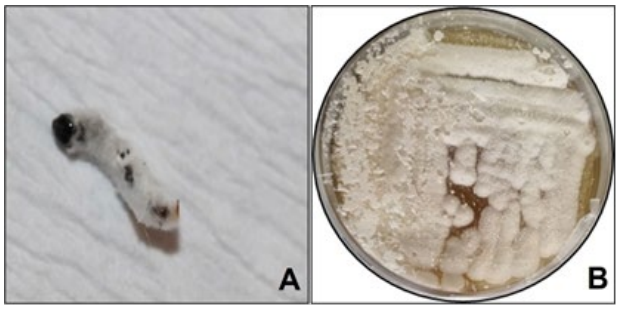

Figure 1. Macroscopy of the new *Beauveria bassiana* isolate from *Cydalima perspectalis*. A: larval cadaver showing mycosis naturally infected by the new fungal isolate, B: Macroscopic image of the new fungal isolate on PDAY.

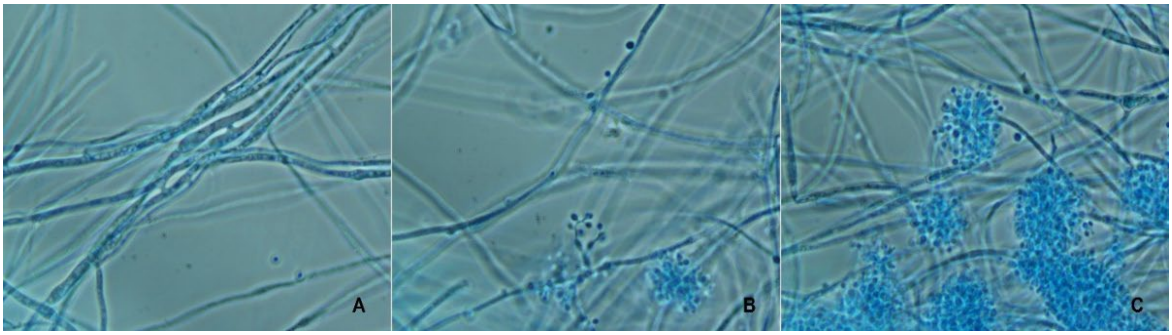

Figure 2. Phase contrast microscopy images of the new *Beauveria bassiana* CP1 isolate. A: Structure and connections of micelles, B: Conidiogenous cells with swollen bases and denticulate raches extending apically with a conidium formed successively on each denticle, C: Typical dense balls of conidia.

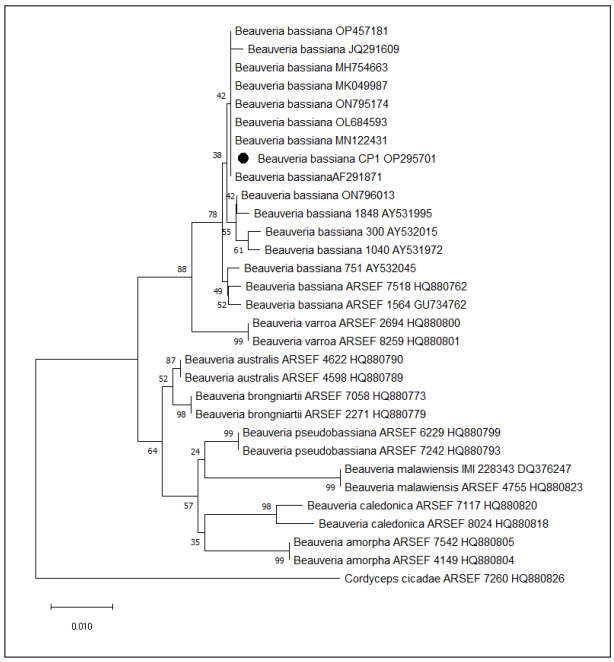

Figure 3. Neighbor-joining tree of the new *Beauveria bassiana* isolate and closely related fungal species based on the sequence of ITS1-5.8S-ITS2 gene region. A black dot indicates the isolate. The numbers at the nodes are bootstrap percentages based on 1000 replicates.

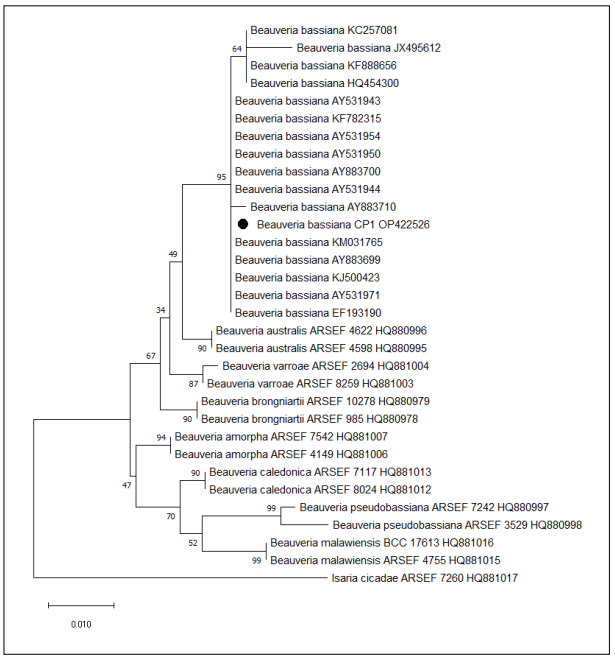

Figure 4. Neighbor-joining tree of the new *Beauveria bassiana* isolate and closely related fungal species based on the sequence of EF1 gene region. A black dot indicates the isolate. The numbers at the nodes are bootstrap percentages based on 1000 replicates.

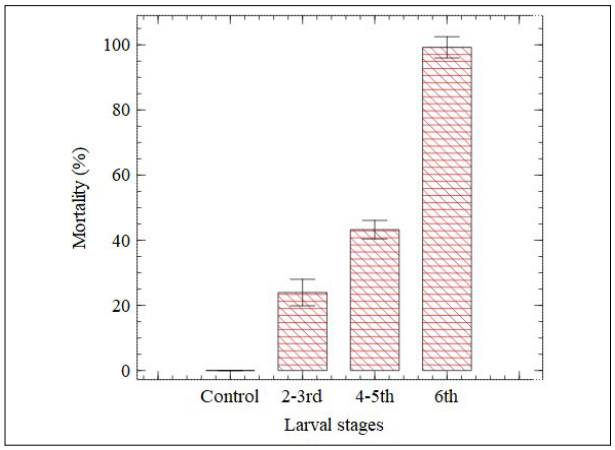

Figure 5. Insecticidal activities of *Beauveria bassiana* CP1 isolate at 1 × 10^8 conidia/ml on different larval stages of *Cydalima perspectalis* within 10 days.

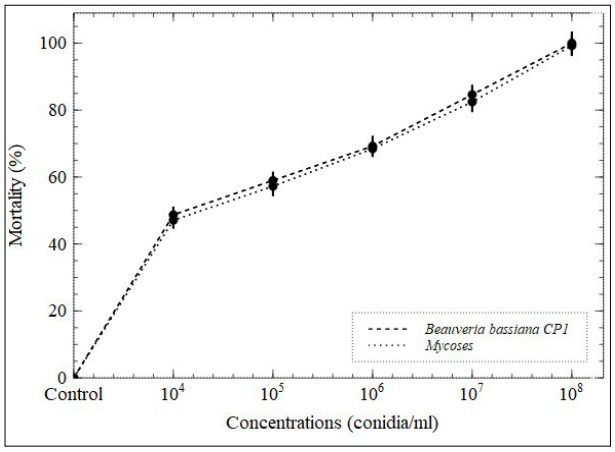

Figure 6. The insecticidal effect of the new *Beauveria bassiana* isolate on *Cydalima perspectalis* 6th larvae within 10 days. Fungal conidia concentrations tested ranged from 1 × 10^4–10^8 conidia/ml.

Figure 7. Mycosis images of *Cydalima perspectalis* larval cadavers infected with the new *Beauveria bassiana* isolate in practice.

Discussion

Although *C. perspectalis* is geographically widespread, from the Far East to the Caucasus, Central Asia, the Balkans, all of Europe, and North Africa, only two studies have been conducted to detect fungi as natural pathogens of boxwood moth until the current study (Harizanova et al. 2018, Zamani et al. 2017). In a study conducted in Iran, Zamani et al. (2017) identified a fungus they isolated from *C. perspectalis* larvae as *B. bassiana*. In another study conducted in Bulgaria (Harizanova et al. 2018), the intensity of fungal infestation in different stages of boxwood moth under natural conditions was determined, and the images of infected individuals were published. In their study, the fungal isolates were evaluated at the genus level as *Beauveria*, *Metarhizium*, and *Verticillium*, and the percentage of fungal pathogenicity detected at five different locations in the country was reported. Despite these important findings, the use of these strains in the biological control of the pest was not considered. In another study on the pest in Romania (Hulujan et al. 2021), although various natural enemies, including bacteria, parasitoids, and predators, were detected in the box tree moth, no fungal pathogenicity was found. This suggests that natural fungal infections are extremely rare worldwide in the boxwood moth population. Nevertheless, the cadaver pattern in the current study, the second report of the pest larvae in the world, is compatible with that reported from Iran. The hard-body cadaver with white color and dense mycelial structure indicates a typical *Beauveria* infection.

Insects are the most important source of entomopathogenic fungi, which exhibit varying degrees of insecticidal activity against important agricultural pests (Lacey et al. 2001). Numerous entomopathogenic fungi have been isolated from dead insects at various stages (Biryol et al. 2020, 2021), and they are highly effective in maintaining the ecological balance of agricultural and forest pests. The isolate of *B. bassiana* is obtained from cadavers that form a dense white coating on the exoskeleton, grow on agar as a white mold, and produce many dry, powdery conidia in characteristic white spore balls. The conidiogenous cells of *B. bassiana* are short and elliptical and terminate in a narrow apical process named rachis (Figure 2). The rachis lengthens after each conidia production, resulting in a long zigzag extension (Humber 2012). All symptoms appeared highly consistent with *B. bassiana* infection in *C. perspectalis* cadavers. The signs on the corpses, the growth characteristics on agar, and the microscopic images showed morphologically that the new fungus was a new isolate of *B. bassiana*.

The ITS1-5.8S- ITS and EF1 gene regions were sequenced to verify the morphological description of the new isolate. These sequences were compared with representative sequences from the study by White et al. (1990) and Rehner & Buckley (2005). While the new isolate (*B. bassiana* CP1) ITS1-5.8S- ITS (OP295701) and EF1 (OP422526) sequences were very similar to *B. bassiana* (MN122431 and AY883710) species based on the dendrogram generated using ITS and EF (Figure 3, Figure 4).

In addition to the increasing interest in the genetic diversity and molecular ecology of *Beauveria* about its pathogenic aspect, the taxonomic study is also being seriously pursued (Rehner et al. 2011).

The morphological appearance of the isolate detected in the current study and its effect on cadavers are in excellent agreement with the isolates identified in previous studies. Infections of *C. perspectalis* with *B. bassiana* have already been reported (Harizanova et al. 2018, Zemek et al. 2020, Burjanadze et al. 2019, López et al. 2022), showing this fungal genus to be an excellent candidate for the search for new mycoinsecticides to control *C. perspectalis* in parks or gardens

(López & Eizaguirre 2019).

The strain of *B. bassiana* obtained from larvae collected in the forest was identified with *B. bassiana* species and showed higher virulence than *C. perspectalis* old larvae. According to the study of López et al. (2022), they stated that *B. bassiana* has a low effect on the early larval stages of *C. perspectalis*, and this may be due to the short duration of the stage (3-5 days in the early stage 8-10 days in the late stage), and cocoon production (López & Eizaguirre 2019). In the present study, the activity of *B. bassiana* CP1 on early-stage larvae was lower than in late-stage larvae. The use of biological control agents for *C. perspectalis* is a promising new area, with several new species currently being discovered and evaluated for use as control agents (Zemek et al. 2020, Hulujan et al. 2021, Ghavamabad et al. 2021).

In summary, we have shown that *B. bassiana* isolate CP1 is the most important natural fungal pathogen of *C. perspectalis*, which was most likely introduced from Asia via the boxwood plant (*Buxus* spp.) trade and has become a serious pest on one of the most popular ornamental shrubs in Europe within a few years. *B. bassiana* CP1 is immensely important for the sustainable control of *C. perspectalis*. Ecological conditions are highly suitable for discovering a natural fungal infection in the boxwood moth populations and using entomopathogenic fungi for their control.

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