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Potential of entomopathogenic fungi as biological control agents of *Yponomeuta malinellus* Zeller, 1838 (Lepidoptera: Yponomeutidae)

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ABSTRACT

The apple ermine moth, *Yponomeuta malinellus* Zeller, 1838 (Lepidoptera Yponomeutidae), is a common pest of apple trees in Asia and Europe, and it has spread to North America. In apple growing regions of Turkey, the population of this pest may increase from time to time, requiring a separate control measure. In such cases, Turkish apple growers generally rely on synthetic insecticides to control this pest. The present study aimed to evaluate indigenous isolates of some entomopathogenic fungi (EPFs) against the pest as potential biological control agents. In the pathogenicity tests, 14 EPF isolates that belong to 4 fungal species [*Beauveria bassiana* (Bals.) Vuill. – 7, *Clonostachys rosea* (Link) Schroers – 3, *Isaria farinosa* (Holmsk.) Fr. – 2 and *Purpureocillium lilacinum* (formerly known as *Paecilomyces lilacinus* (Thom) Samson) (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson – 2] were assayed against the 4th instar larvae of *Y. malinellus* under laboratory conditions. All the EPF isolates were tested against the pest with three different conidial suspensions (1×10^7, 1×10^8 and 1×10^9 conidia ml^{-1}), using the spray method. The results of pathogenicity assays demonstrated that the effectiveness of the isolates increased with increasing concentration and elapsed time up to 9 days after treatment. Of the 14 isolates tested, 3 *B. bassiana* isolates (BbDm-1, BbDs-2 and BbKm-3) were the most pathogenic, causing mortalities between 96.7% and 100% at the highest concentration 9 days post treatment. All the results suggest that the most pathogenic above-mentioned 3 isolates of *B. bassiana* have a significant biocontrol potential against *Y. malinellus*.

1. Introduction

The apple ermine moth, *Yponomeuta malinellus* Zeller, 1838 (Lepidoptera Yponomeutidae), is a member of a European group of small ermine moths (Yponomeuta), consisting of nine species (McDonough et al. 1990). Adult *Yponomeuta* species are difficult to separate from one another, even by genitalia examination. Larval foodplant and some larval and pupal features are most reliable in their separation (Kimber 2021). *Y. malinellus* occurs in the Palaearctic region (in both Asia and Europe) (Kuhlmann et al. 1988; McDonough et al. 1990). However, there is a record that this species is also found in North America (Nearctic region) (Unruh et al. 1993).

The moths of this species have pure white forewings with black dots. First instars larvae overwinter under dense thick shield. In early spring, they crawl from under the shield, penetrate into swollen buds, and then mine the top part of leaflets of apple species. After the first molt, they leave the mines and live in the open. The later larval stages (2nd - 5th instars) feed all together in a silken web from May to early June. As larvae grow, their body varies from dark grey to yellowish grey in color, with dark spots along their sides (Iren 1960; Anonymous 2012). Different larval stages can be seen in the same web. A full- grown larva may reach 18 to 25 mm in length (Kimber 2021). During outbreak years, this species can negatively impact fruit production by defoliating apple trees (Anonymous 2012).

Until the last quarter of the last century, *Y. malinellus* was the second most important pest in apple orchards in Turkey after the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) (Iren 1960; Erturk 2016). Although it is still common in apple-growing regions of Turkey, no separate control measures are required for this univoltine pest species in apple orchards in most growing seasons, due to insecticide applications against other pests, especially *C. pomonella*. However, in some years, the population of this pest may increase enough to require a separate control measure. In such cases, chemical control remains a dominant management tactic against this pest. Although the presence of some effective parasitoids of this species has been reported in Turkey, their effectiveness has been greatly diminished due to excessive use of synthetic pesticides in apple orchards in many parts of the country (Gencer 2003; Narmanlioglu and Coruh 2018). Due to the many undesirable results of chemical applications, biological control methods have become a trend in recent years (Sönmez and Mamay 2018; Mamay and Mutlu 2019). Microbial agents have an important place among biological control agents including entomopathogens such as fungi (Alramadan and

Mamay 2019a), bacteria (Alramadan and Mamay 2019b), viruses (Alramadan and Mamay 2019c) and nematodes (Alramadan and Mamay 2019d). Microbial control agents may be viable alternatives to synthetic insecticides in the control of this pest, with no hazardous effects to human health or the environment. Recently, some *Bacillus thuringiensis* (Berliner) (Bacillales: Bacillaceae) isolates were tested against *Y. malinellus* as microbial agents and some good results were obtained (Erturk 2016). Entomopathogenic fungi (EPFs) are common in terrestrial environments and play an important role in the regulation of insect populations (Alramadan and Mamay 2019a). EPFs are the most common microbial agents, and they are used in many parts of the world with great success and advantage due to the large number of virulent strains known, easy mass production and improving formulation (Butt et al. 2001; Wraight et al. 2001; Butt 2002; Goettel et al. 2010). Hence, this study had the objective of evaluating the biocontrol potential of indigenous isolates of some EPFs against this pest.

2. Materials and methods

2.1. Insect material

Insects used in the experiments were collected from the infested apple orchards in Cavdir (Burdur, Turkey) in June 2020. Silken nests containing 3rd and 4th instars larvae were carefully pruned from branchlets of trees and transported in 5-liter containers to the Entomology Laboratory in the Plant Protection Department, Akdeniz University (Antalya) for testing. The larvae were supplied with fresh apple (*Malus domestica* Borkh. cv. 'Starking') foliage under controlled conditions (25 ± 2°C, 60 ± 5 RH, and 16:8 h L: D photoperiod) in a climate room. They were used in the experiments when all of them reached the 4th instar larval stage (Baki et al. 2021).

2.2. Indigenous EPF isolates

Fourteen indigenous isolates belonging to three soil-borne EPF species, which had been previously isolated from soil samples collected from the selected agricultural habitats and their natural surroundings in Antalya province (South-western part of Turkey), and have already been maintained at the EPF Collection of Plant Protection Department of Akdeniz University, were tested in this study. The species and code names, habitats, sampling sites and geographic coordinates of the isolates tested are presented in Table 1.

2.3. Preparation of conidial suspensions

The EPF isolates were grown on PDA (Potato Dextrose Agar) in Petri dishes (9 cm diameter) and maintained in darkness at 26 ± 2°C and 65 ± 5 RH for 14 days for the completion of sporulation. Then, conidia were collected by scraping the surface of the culture of each fungal isolate gently with an inoculation needle and put into vials containing 10 ml of sterile distilled water + 0.03% Tween-80 (Sigma Chemical, St. Louis, Mo, USA). Prepared stock suspensions were filtered using a sieve (60-mesh) to remove hyphae and growing substrate and then homogenized for 3 minutes using a vortex (Yuyao Haiju Laboratory Equipment Co., Ltd., Zhejiang, China). The conidial concentration of stock suspensions was determined by direct count using a Neubauer hemocytometer (Fancelli et al. 2013). Serial dilutions (10^7–10^9 conidia ml^{-1}) were prepared in sterile distilled water containing Tween-80 and preserved at 4°C until used in the assays. Three concentrations (1×10^7, 1×10^8 and 1×10^9 conidia ml^{-1}) of each EPF isolate were tested, using the spray method. For each isolate, conidial viability was determined using the method of Goettel and Inglis (1997) before being used in the assays. The isolates with a viability of >95% were used in the assays.

2.4. Pathogenicity assays against the larvae of Y. malinellus

The assays were carried out under controlled conditions (25 ± 2°C, 60 ± 5 RH, 16:8 h L: D photoperiod) in the Entomology Laboratory of Plant Protection Department of Akdeniz University. For each treatment, randomly selected ten 4th instar larvae of *Y. malinellus* were introduced to each Petri dish (9 cm in diameter) lined with 3-layer filter paper. The insects were then sprayed through a handheld sprayer from a distance of 30 cm, using 2 ml of any conidial concentration of any EPF isolate. A control treatment (distilled water containing 0.03% Tween-80) was also included in the assays. All the treatments were replicated 3 times, and each treatment contained 10 larvae. After air-drying, treated larvae were transferred to new dishes containing clean fresh apple foliage using a fine camel-hair brush. The lids of the Petri dishes were closed and then perforated with a hot needle for ventilation (20 times per each). All the dishes were kept in the laboratory under the above-mentioned conditions of temperature, humidity and photoperiod. Surviving larvae in each of the dishes were fed on clean fresh apple foliage until the end of the experimental period.

Table 1. Indigenous entomopathogenic fungal isolates used in this study.

Isolate code	Fungal species	Sub-region	Vegetation	Latitude and longitude
BbKm-3	*Beauveria bassiana*	Kumluca	Orange	N 36°22'39.6" E 30°17'40.0"
BbKr-1	*B. bassiana*	Kemer	Forest	N 36°35'51.0" E 30°33'22.7"
BbMp-1	*B. bassiana*	Muratpaşa	Fig	N 36°53'07.2" E 30°44'30.4"
BbAl-1	*B. bassiana*	Alanya	Banana	N 36°33'40.8" E 31°56'43.7"
BbDs-2	*B. bassiana*	Döşemaltı	Pomegranate	N 37°00'02.4" E 30°38'16.1"
BbKs-1	*B. bassiana*	Kaş	Olive	N 36°12'08.8" E 29°38'46.3"
BbDm-1	*B. bassiana*	Demre	Orange	N 36°14'39.7" E 29°58'45.0"
CrFn-1	*Clonostachys rosea*	Finike	Orange	N 36°19'11.2" E 30°09'12.1"
CrFn-2	*C. rosea*	Finike	Orange	N 36°21'17.2" E 30°07'59.6"
CrKm-1	*C. rosea*	Kumluca	Orange	N 36°21'07.6" E 30°14'36.9"
IfKm-1	*Isaria farinosa*	Kumluca	Wheat	N 36°20'41.5" E 30°15'25.3"
IfDs-1	*I. farinosa*	Döşemaltı	Pomegranate	N 37°01'39.2" E 30°36'46.9"
PlKa-1	*Purpureocillium lilacinum*	Konyaaltı	Apple	N 36°53'53.5" E 30°37'51.8"
PlMp-1	*P. lilacinum*	Muratpaşa	Sassafras tree	N 36°53'42.6" E 30°39'56.7"

The dishes were observed daily under a stereomicroscope, and treatment mortalities were recorded at 3, 5, 7 and 9-days post infection. At each count, all dead larvae were removed individually from the dishes, and they were transferred to new Petri dishes containing damp filter paper individually. The dishes were incubated at 25 ± 2°C and 65 ± 5 RH in complete darkness for up to 14 days to monitor signs of mycosis in order to confirm fungal infestation as a probable cause of death. Larvae that did not display fungal outgrowths with identical characteristics to those of the applied fungus as treatment were not included in the count because their mortality was attributed to another factor or factors.

2.5. Molecular identification and phylogenetic analysis of EPF isolates

Molecular phylogenetic analyses were executed only for the three most virulent isolates of *B. bassiana* (BbKm-3, BbDs-2 and BbDm-1). For DNA isolation of the BbKm-3, BbDs-2 and BbDm-1 isolates, firstly pure cultures were developed in SDA (Sabouraud dextrose agar) medium at 25°C for 7-10 days incubation. Fungal genomic DNA was extracted from monosporic cultures of these three *B. bassiana* isolates through CTAB method described by Doyle and Doyle (1990). In this study, ITS 1/ITS 4 PCR primers of the ITS gene region, which are used in the molecular identification of many organisms White et al. (1990), and 983/2218 primers used in the amplification of the mRNA translation elongation factor 1-alpha (EF1alpha) (TEF) gene region Rehner (2001) were used in the diagnosis of high virulence entomopathogenic fungus isolates.

The classic PCR was conducted in a Peqlab Thermocycler Primus 96 device using two different primer sets, sequence and PCR conditions as shown in Table 2.

The phylogenetic analyzes were performed using the MEGA7 software (Biodesign Institute, Tempe, Arizona) using the Maximum Likelihood (ML) method based on the Tamura 3-parameter model (Tamura et al. 2011). Phylogenetic analysis was performed relative to the ITS and mRNA translation elongation factor 1-alpha (EF1 alpha) region sequence of the fungal isolates and the nucleotide sequence of other fungi from GenBank.

2.6. Data analysis

During the pathogenicity assays, no control mortality was detected; therefore, no adjustment was made for the mortality values. Prior to analysis, mortalities were arcsine-transformed and analyzed using the general linear model of the SPSS 23.0 Windows by one-way ANOVA (IBM Corp. 2015, USA). Tukey's honest significant difference test ($P<0.05$) was used to define significant differences among the treatment means. For all EPF isolates, lethal time (LT_{50} and LT_{95}) values with 95% confidence limits were also calculated using Probit analysis and the Log-probit method (SPSS® 23.0).

3. Results

3.1. Effectiveness of EPF isolates on Y. malinellus larvae

The results from the pathogenicity assays with the 4th instar larvae showed that all the EPF isolates tested had different efficacy rates against *Y. malinellus* (Table 3). Mortality was generally time- and concentration-dependent. Mortality rates caused by the isolates varied over time, and differences in mortality at each count date were generally significant among the different fungal isolates ($P<0.05$). Of all the EPF isolates tested, 4 isolates of *B. bassiana* (BbDm-1, BbDs-2, BbKm-3 and BbKs-1) and 1 isolate of *C. rosae* (CrFn-2) were most pathogenic and caused mortalities between 80% and 100% at all the concentrations tested 9 days post treatment. Even, at the shortest incubation time (3 days after application), these isolates exhibited ≥60% mortalities, except for the isolate BbKs-1 (Table 3).

For the highest concentration (1×10^9 conidia ml^{-1}) of EPF isolates tested, the time required for 50% and 95% mortality (LT_{50} and LT_{95}) of the 4th instar larvae of *Y. malinellus* varied between 0.79-4.57 days and 4.87-34.82 days, respectively (Table 4). The lowest LT_{50} and LT_{95} values were calculated for isolates BbDm-1, BbDs-2, BbKm-3, BbKs-1 and CrFn-2 (LT_{50}: 0.79, 1.83, 2.59, 2.66 and 2.36; LT_{95}: 4.87, 7.93, 9.06, 13.93 and 20.22 respectively), implying their high virulence and their biocontrol potential against *Y. malinellus*.

3.2. Phylogenetic placement of the EPF isolates tested

The accession numbers of the three most virulent EPF isolates found in this study, which belong to *B. bassiana*, and those of other isolates of the related species in GenBank are given in Table 5. After alignment analysis, the ITS and TEF region sequences of these three *B. bassiana* isolates data set consisted of 460 aligned positions. All these three *B. bassiana* isolates had 99%-100% homology with other isolates of the respective species in the GenBank (Figures 1 and 2).

Table 2. PCR primers and programs used in the identification of EPF isolates in this study.

Primers and References	Sequence	PCR conditions		
		Temperature (°C)	Time (Seconds)	Cycles
ITS1/ ITS4 White et al. (1990)	5′-TCCGTAGGTGAACCTGCGG-3' 5′-TCCTCCGCTTATTGATATGC-3'	94	300	1
		94	30	30
		56	60	
		72	90	
		72	300	1
983/ 2218 Rehner (2001)	5'-GCYCCYGGHCAYCGTGAYTTYAT-3' 5'-ATGACACCRACRGCRACRGTYTG -3'	95	300	1
		95	60	35
		58	60	
		72	60	
		72	300	1

Table 3. Mortality in the 4th instar larvae of *Yponomeuta malinellus* assayed with different conidial concentrations of EPF isolates at different time intervals after treatment

Fungal species and isolate name*	Dose (spores ml^{-1})**	Percent mortality (± SE)			
		3rd day***	5th day	7th day	9th day
Beauveria bassiana					
	1×10^7	40.0±0.0 BCDEFbIII	63.3±3.3 ABCaII	76.7±3.3 ABCaI II	90.0±5.8 AaI
BbKm-3	1×10^8	46.7±3.3 CDEabIII	73.3±3.3 ABaII	86.7±3.3 ABaI II	93.3±6.7 ABCaI
	1×10^9	60.0±5.8 BCDaII	76.7±3.3 ABCaI II	90.0±5.8 ABaI	96.7±3.3 ABaI
	1×10^7	36.7±3.3 CDEFbIII	50.0±0.0 CDEbII	63.3±3.3 ABCbI	70.0±0.0 ABbI
BbKr-1	1×10^8	50.0±5.8 BCDEabII	63.3±3.3 BCDab I II	70.0±0.0 ABCabI	76.7±3.3 CDabI
	1×10^9	63.0±6.7 BCaI	73.3±6.7 ABCDaI	76.7±3.3 ABaI	83.3±3.3 ABaI
	1×10^7	33.3±3.3 CDEFbII	40.0±5.8 EFGbII	63.3±3.3 ABCaI	76.7±6.7 ABaI
BbMp-1	1×10^8	33.3±3.3 DEFbIII	56.7±3.3 BCDabII	66.7±6.7 BCaI II	76.7±3.3 CDaI
	1×10^9	53.3±3.3 BCDaII	60.0±0.0 CDEFaII	66.7±3.3 BaI II	83.3±6.7 ABaI
	1×10^7	20.0±5.8 EFaII	33.3±3.3 FGaII	56.7±6.7 CDaI	73.3±3.3 ABaI
BbAl-1	1×10^8	23.3±3.3 FaII	36.7±6.7 EaII	60.0±5.8 CaI	80.0±0.0 BCDaI
	1×10^9	36.7±3.3 DaIII	46.7±6.7 FaII III	66.7±6.7 BaI II	80.0±5.8 BaI
	1×10^7	63.3±3.3 ABaI	70.0±0.0 ABbI	80.0±5.8 ABaI	86.7±8.8 AaI
BbDs-2	1×10^8	70.0±3.3 ABaII	73.3±3.3 ABabII	86.7±3.3 ABaI	96.7±3.3 ABaI
	1×10^9	73.3±3.3 ABaIII	83.3±3.3 ABaII III	90.0±0.0 ABaI II	100.0±0.0 AaI
	1×10^7	50.0±5.8 ABCDaIII	60.0±0.0 BCDaII III	76.7±3.3 ABCaI II	80.0±5.8 ABaI
BbKs-1	1×10^8	53.3±8.8 ABCDaII	63.3±3.3 BCDaI II	80.0±5.8 ABCaI	86.7±3.3 ABCDaI
	1×10^9	56.7±8.8 BCDaII	70.0±5.8 BCDEaI II	83.3±6.7 ABaI II	90.0±0.0 ABaI
	1×10^7	70.0±0.0 AbII	76.7±3.3 AbI II	83.3±3.3 AaI	86.7±3.3 AbI
BbDm-1	1×10^8	73.3±3.3 AbIII	86.7±3.3 AabII	93.3±3.3 AaI II	100.0±0.0 AaI
	1×10^9	90.0±0.0 AaI	93.3±3.3 AaI	96.7±3.3 AaI	100.0±0.0 AaI
Clonostachys rosea					
	1×10^7	43.3±6.7 BCDEaII	60.0±0.0 BCDaI II	63.3±3.3 ABCaI	70.0±0.0 ABbI
CrFn-1	1×10^8	50.0±0.0 BCDEaIII	63.3±3.3 BCDaII	73.3±3.3 ABCaI II	83.3±3.3 ABCDaI
	1×10^9	53.3±3.3 BCDaIII	66.7±3.3 BCDEFaII III	76.7±3.3 ABaI II	86.7±3.3 ABaI
	1×10^7	56.7±3.3 ABCaII	60.0±0.0 BCDaII	70.0±5.8 ABCaI II	80.0±0.0 ABaI
CrFn-2	1×10^8	60.0±5.8 ABCaII	66.7±3.3 BCaI II	73.3±3.3 ABCaI II	83.3±3.3 ABCDaI
	1×10^9	60.0±0.0 BCDaII	66.7±3.3 BCDEFaII	80.0±0.0 ABaI	86.7±3.3 ABaI
	1×10^7	36.7±6.7 CDEFaIII	46.7±3.3 DEFaII III	60.0±5.8 BCaI II	70.0±0.0 ABbI
CrKm-1	1×10^8	40.0±5.8 CDEFaIII	50.0±0.0 CDEaII III	63.3±6.7 BCaI II	73.3±3.3 DabI
	1×10^9	43.3±3.3 CDaIII	53.3±3.3 DEFaIII	66.7±3.3 BaII	80.0±0.0 BaI
Isaria farinosa					
	1×10^7	16.7±3.3 FcIII	30.0±3.3 GbII	36.7±3.3 DbII	56.7±3.3 BbI
IfKm-1	1×10^8	30.0±0.0 EFbIII	46.7±3.3 DEabII III	63.3±3.3 BCaI II	73.3±6.7 DabI
	1×10^9	46.7±3.3 CDaIII	53.3±6.7 DEFaII III	70.0±5.8 BaI II	80.0±0.0 BaI
	1×10^7	26.7±3.3 DEFaII	43.3±6.7 EFGaI II	56.7±3.3 CDaI	60.0±0.0 BbI
IfDs-10	1×10^8	30.0±0.0 EFaIII	46.7±3.3 DEaII III	60.0±5.8 CaI II	73.3±3.3 DabI
	1×10^9	43.3±6.7 CDaIII	50.0±0.0 EFaII III	66.7±3.3 BaI II	80.0±5.8 BaI
Purpureocillium lilacinum					
	1×10^7	43.3±3.3 BCDEaII	53.3±3.3 CDEaI II	60.0±0.0 BCaI II	70.0±5.8 ABaI
PlKa-1	1×10^8	46.7±3.3 CDEaII	60.0±0.0 BCDaI II	70.0±5.8 ABCaI	76.7±3.3 CDaI
	1×10^9	50.0±0.0 BCDaIII	60.0±5.8 CDEFaII III	73.3±6.7 ABaI II	83.3±3.3 ABaI
	1×10^7	36.7±8.8 CDEFaIII	43.3±3.3 EFGbII III	70.0±5.8 ABCaI II	73.3±6.7 ABaI
PlMp-1	1×10^8	46.7±3.3 CDEaIII	56.7±3.3 BCDaII III	70.0±5.8 ABCaI II	73.7±3.3 CDaI
	1×10^9	50.0±5.8 BCDaIII	60.0±0.0 CDEFaII III	73.3±6.7 ABaI II	83.3±3.3 ABaI
Control	dH_2O	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

*The differences between the means with different capital letters in different isolates on the same day are statistically significant (P<0.05; Tukey test).
**The differences between the means with different lowercase letters on the same day and in the same isolate are statistically significant (P<0.05; Tukey test).
***The differences between the means with different roman numerals on different days in the same isolate are statistically significant (P<0.05; Tukey test).

Table 4. LT_{50} and LT_{95} (days) values of indigenous EPF isolates tested at 1×10^9 conidia ml^{-1} to the 4th instar larvae of *Yponomeuta malinellus*.

Isolate name	Fungal species	LT_{50} (LCL-UCL)*	LT_{95} (LCL-UCL)	Chi-Square (df)	Regression equation (y=ax+b)
BbKm-3	*Beauveria bassiana*	2.59 (1.52-3.29)	9.06 (7.07-15.81)	54.942 (10)	y= -1.253+3.027x
BbKr-1	*B. bassiana*	1.59 (0.05-2.80)	34.82 (15.18-6.75)	34.330 (10)	y= -0.251+1.229x
BbMp-1	*B. bassiana*	3.00 (1.36-3.97)	32.37 (16.63-64.7)	34.569 (10)	y= -0.761+1.593x
BbAl-1	*B. bassiana*	4.57 (3.36-5.43)	21.60 (13.95-61.73)	47.433 (10)	y= -1.609+2.439x
BbDs-2	*B. bassiana*	1.83 (0.96-2.54)	7.93 (6.42-12.14)	30.332 (10)	y= 0.724+2.633x
BbKs-1	*B. bassiana*	2.66 (1.29-3.51)	13.93 (9.56-39.93)	50.951 (10)	y= -0.972+2.287x
BbDm-1	*B. bassiana*	0.79 (0.0-1.87)	4.87 (2.83-16.32)	51.006 (10)	y= 0.209+2.086x
CrFn-1	*Clonostachys rosea*	2.86 (2.07-3.45)	18.46 (13.32-34.09)	17.580 (10)	y= -0.927+2.031x
CrFn-2	*C. rosea*	2.36 (1.77-2.83)	20.22 (15.25-31.93)	14.011 (10)	y= -0.659+1.764x
CrKm-1	*C. rosea*	3.97 (3.29-4.52)	26.10 (18.01-50.73)	15.468 (10)	y= -1.205+2.011x
IfKm-1	*Isaria farinosa*	3.69 (2.53-4.50)	26.79 (16.03-96.84)	31.836 (10)	y= -1.086+1.913x
IfDs-1	*I. farinosa*	4.08 (2.96-4.92)	26.28 (15.79-95.05)	36.717 (10)	y= -1.245+2.035x
PlKa-1	*Purpureocillium lilacinum*	3.24 (2.07-4.02)	22.48 (14.14-70.46)	31.779 (10)	y= -0.999+1.956x
PlMp-1	*P. lilacinum*	3.26 (2.09-4.04)	24.88 (15.22-83.87)	29.116 (10)	y= -0.957+1.864x

*95% confidence limits (CL); LCL, lower limit; UCL, upper limit.

Table 5. GenBank accession numbers and gene regions used in phylogenetic analysis of indigenous three most virulent *Beauveria bassiana* isolates and their relatives.

Isolate name	Species	Gene	Accession no.	Isolate name	Species	Species	Accession no.
BbDm-1	***B. bassiana***	**TEF**	**OM489219**	**BbDm-1**	***B. bassiana***	**ITS**	**MT441872**
BbDs-2	***B. bassiana***	**TEF**	**OM489220**	**BbDs-2**	***B. bassiana***	**ITS**	**MT441879**
BbKm-3	***B. bassiana***	**TEF**	**OM489221**	**BbKm-3**	***B. bassiana***	**ITS**	**MT441870**
792	*B. bassiana*	TEF	AY531957	F19-N	*B. bassiana*	ITS	MG640376
3097	*B. bassiana*	TEF	AY531925	MG562497	*B. bassiana*	ITS	MG562497
ArgB33	*B. bassiana*	TEF	KT748548	SHU.M.161	*B. bassiana*	ITS	KU158472
TM1613	*B. bassiana*	TEF	LT220758	SHU.M.131	*B. bassiana*	ITS	KU158461
TMCR05	*B. bassiana*	TEF	LT220759	EABb04	*B. bassiana*	ITS	KC753382
GMCR51	*B. bassiana*	TEF	LT220745	SASRI BB444	*B. bassiana*	ITS	JX110368
CHE-CNRCB 84	*B. bassiana*	TEF	MH203473	2718	*B. bassiana*	ITS	KU364353
TMSL142	*B. bassiana*	TEF	LT220761	EABb 04/01	*B. bassiana*	ITS	DQ364698
2579	*B. bassiana*	TEF	AY531916	HHWG1	*B. brongniartii*	ITS	JX110385
GMGJ75A	*B. bassiana*	TEF	LT220749	SASRI	*B. brongniartii*	ITS	JX110388
CHE-CNRCB 414	*B. bassiana*	TEF	MH203489	FUM03	*B. varroae*	ITS	MF667767
LPSc1213	*B. bassiana*	TEF	MK047585	B5	*B. varroae*	ITS	MH374536
B47	*B. caledonica*	TEF	MK040132	ARSEF 2641	*B. amorpha*	ITS	HQ880808
BUB421	*B. caledonica*	TEF	MG642903	B518a	*B. amorpha*	ITS	HQ880806
YFCC 7025	*B. vermiconia*	TEF	MN576997	BYYC-05	*B. asiatica*	ITS	MG345071
BCC14510	*B. asiatica*	TEF	MN401502	BUB824	*B. asiatica*	ITS	MG642836
BCC12907	*B. asiatica*	TEF	MN401481	ARSEF 4622	*B. australis*	ITS	HQ880790
YFCC 5600	*B. asiatica*	TEF	MN576996	ARSEF 4598	*B. australis*	ITS	HQ880789
BCC75846	*B. asiatica*	TEF	MN401462	F585	*B. caledonica*	ITS	DQ529233
BCC2120	*B. asiatica*	TEF	MN401465	BG47	*B. caledonica*	ITS	MT180427
C18-2_b	*B. brongniartii*	TEF	KJ908277	1717	*B. vermiconia*	ITS	FJ973063
RUG50-1_b	*B. brongniartii*	TEF	KJ908276	ARSEF 7281	*B. sungii*	ITS	HQ880815
RUB11-2_b	*B. brongniartii*	TEF	KJ908275	EFCC 5657	*B. sungii*	ITS	JX463219

4. Discussion

The results of the present study showed that all indigenous EPF isolates tested had a pathogenic activity against the 4th instar larvae of *Y. malinellus* under laboratory conditions; however, three isolates of *B. bassiana* (BbDm-1, BbDs-2 and BbKm-3) were more pathogenic than others. A review of the relevant literature revealed that there is no study on the biological control of *Y. malinellus* using EPFs. So, we could not compare our results with others. In a previous study with entomopathogenic bacteria, Erturk (2016) evaluated the insecticidal effects of some *Bacillus thuringiensis* (Berliner) (Bacillales: Bacillaceae) isolates as biological control agents against *Y. malinellus*. He reported that two *B. thuringiensis* isolates (HD-1 and BTS-1) were the most pathogenic and caused 97% and 83% mortalities of 4th larvae of *Y. malinellus* at the concentration of 1.8 x 10^9 72 h post treatment under laboratory conditions. The results obtained from the Erturk's study confirmed that the biological control of the pest may be possible by using entomopathogenic agents. However, the efficiency of entomopathogenic fungi as well as bacteria is greatly influenced by many abiotic factors, such as temperature, relative humidity, solar radiation, etc. (Vidal and Fargues 2007). That is why it is necessary that EPF applications be made at a

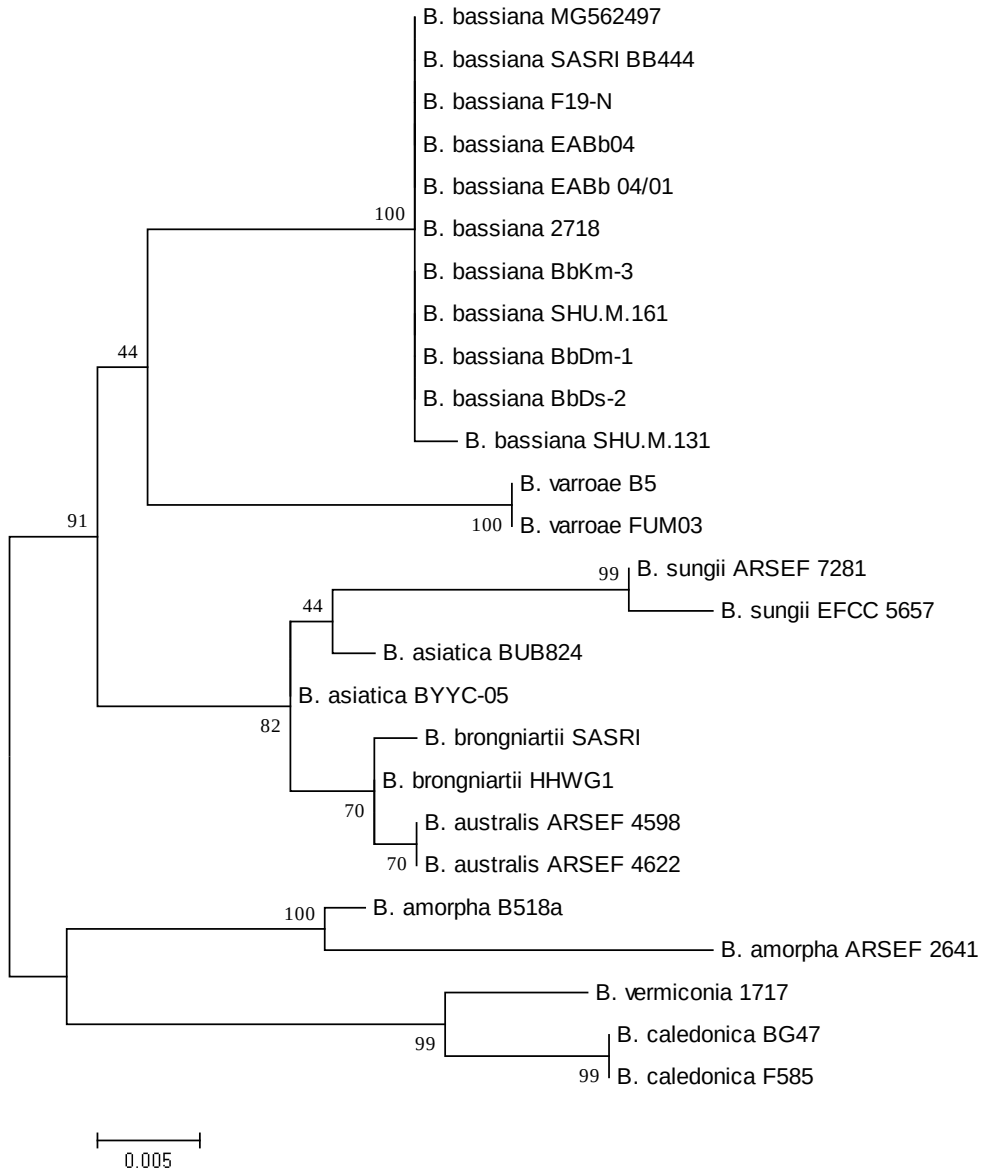

Figure 1. The Maximum Likelihood tree based on the Tamura 3-parameter model showing the phylogenetic relationship between the three *B. bassiana* isolates (BbDm-1, BbDs-2 and BbKm-3) found to have high virulence in the present study and other *B. bassiana* isolates from GenBank based on ITS region sequence.

time when ambient temperature and humidity is suitable for optimal entomopathogenic activity. Likewise, it is more appropriate to do applications in the evening or early in the morning when the solar radiation is low or nonexistent.

As for the phylogenetic placement of the three most virulent EPF isolates, which are 3 isolates of *B. bassiana* (BbKm-3, BbDs-2 and BbDm-1), the results demonstrated that these three isolates had 99% evolutionary homology with other *B. bassiana* isolates from the NCBI database. In the present study, two different gene regions (ITS and TEF regions) were used for identifying and comparing the above-mentioned EPF isolates. Rehner and Buckley (2005) reported that molecularly based sequences based on a single region can be misleading in determining *B. bassiana* isolates. Also, many researchers used the multiple gene sequencing approach for identifying and comparing *B. bassiana* isolates (Glare and Inwood 1998; Glare 2004; Rehner and Buckley 2005; Glare et al. 2008). Serna-Domínguez et al. (2019) identified 44 *B. bassiana* isolates from different pests in the west-central Mexico (The state of Colima) using a translation elongation factor 1-α (TEF) and Bayesian phylogenetic analysis of the nuclear intergenic Block region. They did not detect any significant genetic associations between any substrate, insect-host, or geographic origin combination. Their results also indicated that the TEF region was effective in identifying *B. bassiana* isolates, similar to those of the present study. Likewise, Castro-Vásquez et al. (2021) molecularly identified 32 *B. bassiana* isolates, 26 from Costa Rica, 5 from Puerto Rico and one from Honduras, using the Bloc, TEF-1α and RPB2 regions. Their results showed that the TEF region can be used effectively in the identification of *B. bassiana* isolates in molecular characterization and there is a low correlation between geographic origin and variation between isolates.

Based on the results of this study, it was concluded that three indigenous isolates (BbDm-1, BbDs-2 and BbKm-3) of

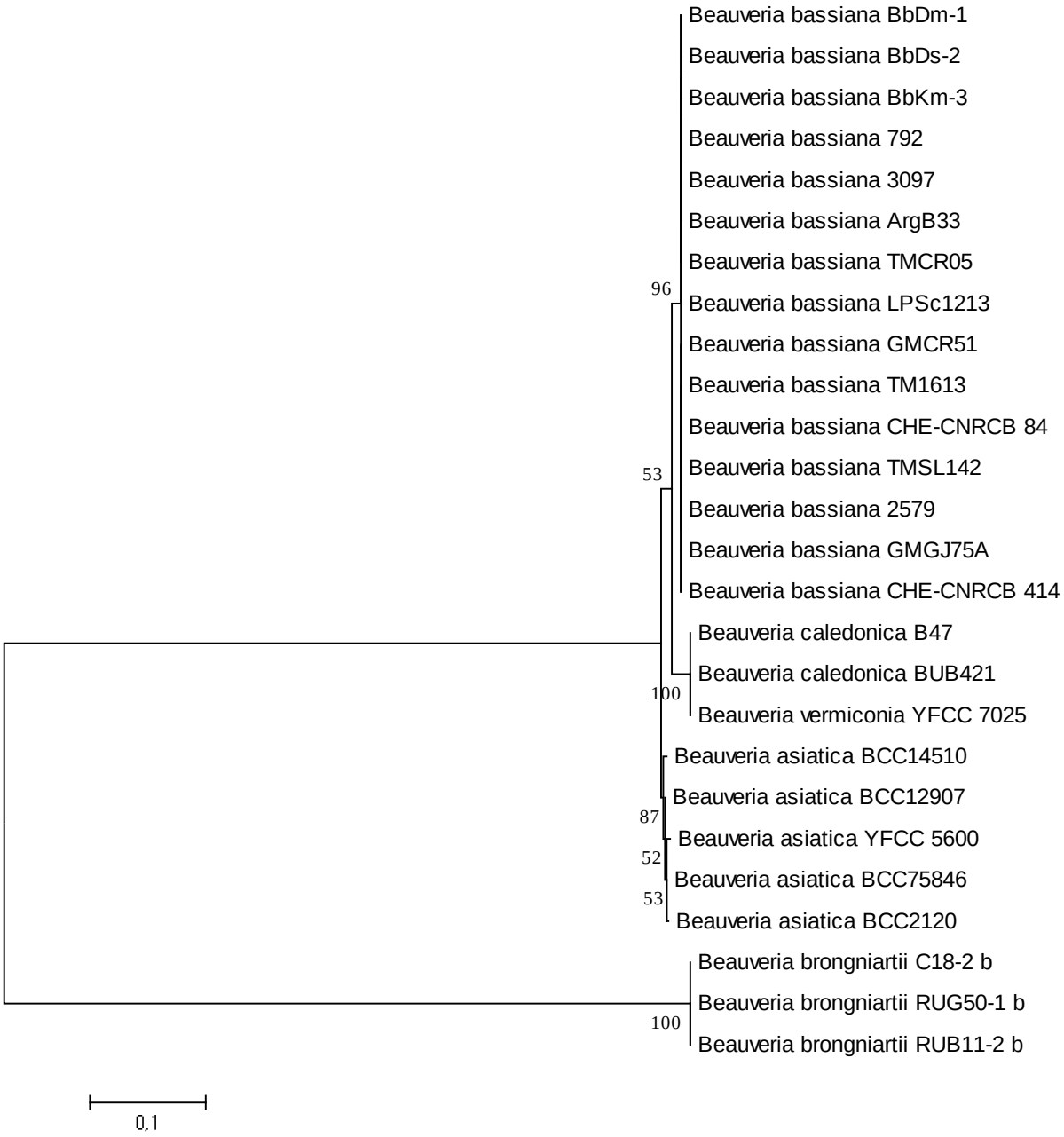

Figure 2. The Maximum Likelihood tree based on the Tamura 3-parameter model showing the phylogenetic relationship between the three *B. bassiana* isolates (BbDm-1, BbDs-2 and BbKm-3) found to have high virulence in the present study and other *B. bassiana* isolates from GenBank based on TEF region sequence.

B. bassiana can be used as potential alternatives for the management of *Y. malinellus*. However, further studies should be conducted under field conditions to better understand the efficacy of these three *B. bassiana* isolates and their potential as effective biocontrol agents within the framework of an integrated pest management (IPM) program in apple orchards.

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