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Phylogenetic analysis and biocontrol potential of entomopathogenic fungi against *Tropinota* (=*Epicometis*) *hirta* (Poda) (Coleoptera: Cetoniidae) and the apple scab disease *Ventura inaequalis*

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Abstract

Background Entomopathogenic fungi have long been used as a biopeptide in the biological control of insect pests in agriculture, forestry and veterinary. At the same time, it is known that these fungi have positive effects on plant growth, health, and the antagonist effect against various plant pathogens. Entomopathogenic fungal isolation was performed from soil samples collected from apple orchards and *Tropinota* (=*Epicometis*) *hirta* (Poda) (Coleoptera: Cetoniidae) adults which is an important pest of fruit trees. The isolated fungi were identified at the species level by phylogenetic analysis based on multi-locus sequence approach using various gene sequences (*ITS*, *rpb1*, *bloc*, *EF1-a* and *β-tubulin*). The fungi obtained were tested against *T. hirta* and the apple scab disease, *Ventura inaequalis* under laboratory conditions.

Results Three (Bz isolates) and 15 (AK isolates) entomopathogenic fungi (EPF) were isolated from *T. hirta* adults and 48 soil samples, respectively. The isolated fungi were identified as *Beauveria bassiana* (Bz-1, Bz-2, AK-10, AK-14, AK-17, and AK-18), *Metarhizium robertsii* (Bz-3, AK-4, AK-5, AK-6, AK-7, AK-8, AK-9, AK-11, AK-13, AK-15, and AK-16) and *Metarhizium* sp. (AK-12). All isolated fungi were tested against *T. hirta* adults, causing the same mortality but different mycosis values. In addition, the antagonistic effects of fungal isolates against *V. inaequalis*, the important apple pathogen, were determined and the highest effect was obtained from *B. bassiana* AK-10 with 69.3%.

Conclusion This is the first study to determine the effectiveness of EPF against *T. hirta* and *V. inaequalis*, and the results obtained are thought to be useful for the biological control of both pests.

Keywords *Tropinota* (=*Epicometis*) *hirta*, *Ventura inaequalis*, *Beauveria*, *Metarhizium*, Biological control

Background

Tropinota (=*Epicometis*) *hirta* (Poda) (Coleoptera: Cetoniidae) (apple blossom beetle) is a common pest species in orchards of all European countries, including Turkey, especially in temperature parts (Toth et al. 2009). The adults of this pest cause serious damage feeding on petals, staminae and stigmae of flowers, even young leaves and flower buds at the time of flowering of fruit trees and some other plants such as strawberry, roses, wheat,

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bushes, and ornamental trees. It is a phytophagous pest and can cause damage on many economic fruit plants such as apple, pears, cherries, apricots, plums, peaches, citrus fruits, even wheatgrass, vine, ornamental plants, some vegetables, and weeds are among its hosts (Republic of Turkey, Ministry of Agriculture and Forestry 2008). Because of its high-flying capacity, it can switch to different plants during feeding and continue its damage, and as a result damaged flowers cannot form fruits (Yaşar et al. 2013). In some studies, it has been reported that this pest causes up to 70% damage to flowers of some plant species of agricultural importance (Kutinkova and Andreev 2004). Its larvae live in the soil and feed on rotting plant materials without causing any damage on plants (Toth et al. 2009).

Until now no effective control method against *T. hirta* has been developed although it is one of the most important pests of fruit trees in many parts of the world. The control of this pest is difficult as it causes damage on flowers and chemical control can only be applied when the population reaches very high levels. However, considering that honeybees and other pollinator insects also come to flowers during the flowering period, it is thought that chemical control is not suitable and other control methods have been gained importance (Vuts et al. 2009). Apart from chemical control, cultural (ensuring the reduction in egg, larva and adult population of the pest in the soil by tillage), mechanical (by laying cloth covers under the trees in the early hours of the morning when the adults are less active, shaking the trees vigorously and destroying the collected adults) and biotechnical (the use of blue colored attractant) control methods are used to control this pest (Aydın and Yaşar 2019). Among these methods, the use of color traps (especially light blue color) and various attractants such as cinnamyl alcohol, transanethol and cinnamyl acetate have been shown to be useful for the control of this pest and seasonal monitoring (Subchev et al. 2011). However, there is a very limited knowledge about the biological control agents of this pest and their use. In this sense, it is needed to develop more effective and safer control methods for *T. hirta*, which is requiring less labor and cost.

Entomopathogenic fungi (EPFs) are an important factor in the natural control of many harmful insects, and these microorganisms often cause wide-spread epizootics in many insect populations. EPFs have been used as microbial control agents over 100 years worldwide. In general, many insect orders are susceptible to fungal diseases, and EPFs have a good potential as microbial control agents against insect pests (Skalicky et al. 2014). Many EPFs directly infect their hosts through cuticle, so they do not need to be eaten by their hosts. This feature makes EPFs leading candidates in the control of insects, especially those that feed on plant sap (St Leger and Wang 2020). Today, there are many commercial preparations consisting of EPFs worldwide and they are used to control various agricultural and forest pests (Goettel et al. 2005). Among the EPFs, *Beauveria* spp. (especially *B. bassiana*) and *Metarhizium* spp. (especially *M. anisopliae*) are the most two studied species in terms of commercial production (Zimmermann 2007a, 2007b). In addition to the use of EPFs in the biological control of insect pests, they have additional roles in nature such as endophytism, plant disease antagonism, plant growth promotion, and rhizosphere colonization (Yadav et al. 2022). Therefore, it should be interesting to study the antagonistic activity of these fungi against some plant pathogens to provide their possible potential use in integrated pest management (IPM) strategies for future perspectives. Therefore, in this study, the apple scab disease (*Venturia inaequalis* (Cooke) G. Winter (1875)) was selected for the evaluation of antagonistic relationships with EPFs.

In this study, various EPFs were isolated from the field by collecting *T. hirta* adults and soil samples collected from apple orchards and characterized isolated fungal species by gene sequencing such as *ITS*, *RBP1*, *Bloc*, *EF1-α* and *β-tubulin*. Also, isolated fungi were tested against *T. hirta* adults under laboratory conditions. Moreover, the antagonistic activity of the fungi against the apple scab disease agent (*V. inaequalis*) was determined.

Methods

Collection *Tropinota hirta* adults and soil samples

Tropinota hirta adults were collected from apple orchards using the blue colored traps in Konya province, Turkey between 2020 and 2021. The collected adults were brought to the laboratory and kept in growing chamber for 5–10 days to observe whether there was a fungal infection or not. In addition, soil samples from apple orchards were collected in Kırşehir and Konya provinces, Turkey to isolate EPFs. Soil samples were collected according to the study of Ali-Shtayeh et al. (2002) and a total of 48 soil samples was collected.

Isolation of entomopathogenic fungi

Collected *T. hirta* adults were regularly checked in terms of fungal infection and fungal isolation was performed from the infected adults. EPF species isolation from soil samples was carried out according to "*Galleria* bait method" with minor modifications (Zimmermann 1986). As a trap insect, 3–4 larval instars of mealworm (*Tenebrio molitor L.* (*Coleoptera*: Tenebrionidae)) were used (Chang et al. 2021). Both dead *T. hirta* adults and *T. molitor* larvae found dead in soil samples were first subjected to surface sterilization for 3 min with 1% sodium hypochlorite and then washed with distilled sterile water

three times. After that, they were placed into moisture chamber to stimulate fungal growth and incubated at 25° for 10 days (Sevim et al. 2010a). During incubation, fungi from insect samples showing external fungal growth were purified with inoculation loop. PDAY (Potato dextrose agar +1% yeast extract) was used as the first medium during purification. Ampicillin (50 µg/ml), tetracycline (20 µg/ml) and streptomycin (200 µg/ml) were added to the medium to prevent bacterial growth (Sevim et al. 2010a). All fungal isolates were propagated from a single conidium and stocked in 15% glycerol for future studies.

Molecular identification

The isolated and purified fungal isolates were identified by phylogenetic analysis using various gene sequences. For this purpose, genomic DNA extraction was performed with the E.Z.N.A. Soil DNA kit (OMEGA-BIO-TEK, GA, USA) according to the manufacturer's recommendations. Isolated DNA was stored at −20 °C until use. After DNA isolation, *ITS*, *EF1-α*, *Bloc*, *RBP1* and *β-tubulin* gene regions were amplified by PCR, using the primers specified in (Table 1). *ITS* gene region was amplified for all isolates. *EF1-α, Bloc* and *RBP1* gene regions were amplified for *Beauveria* isolates and *RBP1* and *β-tubulin* gene regions were amplified for *Metarhizium* isolates and used in phylogenetic analysis. The PCR reaction mixture was prepared for the amplification of ITS1-5.8S-ITS2 regions to contain 200 µM from each dNTP, 50 pmol from opposing primers, 2.5 U *Taq*-DNA polymerase, 5 µl 10 × *Taq* DNA polymerase reaction buffer and 50 ng genomic DNA. The final volume was completed to 50 µl with dH_2O. PCR conditions were as followed: after initial denaturation at 95 °C for 4 min, 35 cycles of 95 °C for 1 min, 58 °C for 55 s and 72 °C for 2 min, and 72 °C for 10 min as the final extension (Sönmez et al. 2016). PCR reaction conditions for *EF1-α*, *Bloc*, *RBP* and *β-tubulin* gene regions were carried out according to the references mentioned in Table 1. After performing PCR, 5 µl from each PCR product was electrophoresed in 1% agarose gel with 0.5 µg/ml ethidium bromide for 45 min at 90 V. The remaining PCR products were sent to MACROGEN (Netherlands) for DNA sequence analysis. The resulting DNA sequences were compared to DNA sequences at NCBI GenBank using Blast search to confirm species identification and then used for phylogenetic analysis (Benson et al. 2012). GenBank accession numbers for each sequence are given in Table 2.

Insect bioassay

All isolated fungi were tested against *T. hirta* adults under laboratory conditions. To prepare conidial suspensions, 100 µl (1×10^5 spore/ml) from fungal stocks was spread on PDAY and left to incubation for 2–3 days at 28 °C. At the end of the growth period, single colonies were selected and transferred to another PDAY and incubated at 28 °C for 4 weeks. After adequate sporulation of cultures, 10 ml sterile 0.01% Tween 80 was separately added to each Petri dishes and scraped with glass stirring rod to allow spores passing into the water. Spore suspensions were then filtered into 50 ml sterile Falkon tubes through two layers of sterile muslins to remove mycelial and agar pieces. The resulting suspensions were homogenized by vortexing for 5 min and spore concentrations were adjusted to 1×10^7 spore/ml with the Neubauer hemocytometer. The viability of spores was tested by spreading of 100 µl spore suspension on PDAY and determining the germination rate after a 24 h incubation. Spores were considered as germinated if the germ tube was longer than the diameter of the spore. As a result, spores germinated 90% or more were used in virulence tests (Sevim et al. 2012).

Spore suspensions of the fungal isolates were used in virulence tests against *T. hirta* adults. In April–May of 2021,

Table 1 Primers used in this study and their sequences

Gene region	Primer name	Sequence (5′→3′)	References
ITS1-5.8S-ITS2	ITS4	5′-TCCTCCGCTTATTGATATGC-3′	White et al. (1990)
	ITS5	5′-GGAAGTAAAAGTCGTAACAAGG-3′	
Tef (*EF1-α*)	EF1T	5′-ATGGGTAAGGARGACAAGAC-3′	Rehner and Buckley (2005)
	1567R	5′-ACHGTRCCRATACCACCSATCTT-3′	
Bloc	B5.1F	5′-CGACCCGGCCAACTACTTTGA-3′	Rehner et al. (2006)
	B3.1R	5′-GTCTTCCAGTACCACTACGCC-3′	
RPB1	RPB1Af	5′-GARTGYCCDGGDCAYTTYGG-3′	Stiller and Hall (1997)
	RPB1C	5′-CCNGCDATNTCRTTRTCCATRTA-3′	
β-Tubulin	T1	5′-AACATGCGTGAGATTGTAAGT-3′	O'Donnell and Cigelnik (1997)
	T2	5′-TCTGGATGTTGTTGGGAATCC-3′	

Table 2 Entomopathogenic fungal species isolated from apple orchards in Turkey and their locality, geographic coordinates, and GenBank accession numbers for *ITS*, *Bloc*, *tef*, *rpb1* and *β-tubulin* gene regions

Isolate	Source	Species	Locality		Geographic coordinates	GenBank accession numbers				
			City	County		*ITS*	*Bloc*	*EF1-α*	*rpb1*	*β-tubulin*
Bz-1	*T. hirta* adult	*Beauveria bassiana*	Kırşehir	City center	39° 15′ 89.20″ N 34° 16′ 44.36″ E	MW689265	ON089020	ON093089	ON093083	–
Bz-2	*T. hirta* adult	*Beauveria bassiana*	Konya	Beyşehir	37° 42′ 56.5″ N 31° 43′ 16.3″ E	MW689266	ON089021	ON093090	ON093084	–
Bz-3	*T. hirta* adult	*Metarhizium* sp.	Konya	Beyşehir	37° 42′ 56.5″ N 31° 43′ 16.3″ E	MW689271	–	–	ON125474	ON125487
AK-4	Soil	*Metarhizium robertsii*	Kırşehir	City center	39° 05′ 48.7″ N 34° 09′ 22.7″ E	MW689272	–	–	ON125475	ON125488
AK-5	Soil	*Metarhizium robertsii*	Konya	Beyşehir	37° 42′ 52.5″ N 31° 43′ 21.4″ E	MW689273	–	–	ON125476	ON125489
AK-6	Soil	*Metarhizium robertsii*	Konya	Beyşehir	37° 43′ 00.3″ N 31° 43′ 18.6″ E	MW689274	–	–	ON125477	ON125490
AK-7	Soil	*Metarhizium robertsii*	Konya	Beyşehir	37° 42′ 57.6″ N 31° 43′ 14.0″ E	MW689275	–	–	ON125478	ON125491
AK-8	Soil	*Metarhizium robertsii*	Konya	Beyşehir	37° 42′ 56.5″ N 31° 43′ 16.3″ E	MW689276	–	–	ON125479	ON125492
AK-9	Soil	*Metarhizium robertsii*	Konya	Beyşehir	37° 42′ 56.4″ N 31° 43′ 12.9″ E	MW689277	–	–	ON125480	ON125493
AK-10	Soil	*Beauveria bassiana*	Konya	Beyşehir	37° 43′ 04.5″ N 31° 43′ 16.9″ E	MW689267	ON089022	ON093091	ON093085	–
AK-11	Soil	*Metarhizium robertsii*	Konya	Beyşehir	37°42′ 56.1″ N 31° 43′ 19.4″ E	MW689278	–	–	ON125481	ON125494
AK-12	Soil	*Metarhizium robertsii*	Konya	Beyşehir	37° 42′ 29.0″ N 31° 43′ 35.7″ E	MW689279	–	–	ON125482	ON125495
AK-13	Soil	*Metarhizium robertsii*	Konya	Beyşehir	37° 42′ 59.2″ N 31° 43′ 22.2″ E	MW689280	–	–	ON125483	ON125496
AK-14	Soil	*Beauveria bassiana*	Konya	Beyşehir	37° 42′ 31.0″ N 31° 43′ 38.4″ E	MW689268	ON089023	ON093092	ON093086	–
AK-15	Soil	*Metarhizium robertsii*	Konya	Beyşehir	37° 42′ 54.7″ N 31° 43′ 19.5″ E	MW689281	–	–	ON125484	ON125497
AK-16	Soil	*Metarhizium robertsii*	Konya	Beyşehir	39° 11′ 45.4″ N 34° 08′ 56.5″ E	MW689282	–	–	ON125485	ON125498
AK-17	Soil	*Beauveria bassiana*	Konya	Beyşehir	37° 42′ 28.9″ N 31° 43′ 36.9″ E	MW689269	ON089024	ON093093	ON093087	–
AK-18	Soil	*Beauveria bassiana*	Kırşehir	City center	37° 42′ 31.0″ N 31° 43′ 38.4″ E	MW689270	ON089025	ON093094	ON093088	–

T. hirta adults collected from apple orchards in Konya province were used for tests. In virulence tests, ten adults were used for each repetition and each fungal isolate. All experiments were repeated three times on different occasion. Firstly, *T. hirta* adults collected from the field were brought to the laboratory and waited for 2 days so that only healthy individuals were used in tests. Ten healthy adults for each repetition were exposed to spore concentration (1×10^7 spores/ml) with an aerosol type sprayer (airbrush). The control group were only inoculated with sterile 0.01% Tween 80. After inoculation, adults were placed into plastic boxes ($20 \times 20 \times 20$ cm) with freshly collected apple flower as food. After that, they were incubated at 28 °C under 12:12 (L: D) photoperiod for 10 days. All boxes were examined for 15 days, and adults found dead were counted and the percentage mortality values were calculated. In addition, percent mycosis values were calculated to make sure the cause of death is due to fungus. For this purpose, the dead adults were surface sterilized with 1% sodium hypochlorite solution and then washed with distilled water three times. After that, they were placed into sterile Petri dishes with damp filter paper in them and incubated at 28 °C in dark (Sevim et al. 2010b). After incubation, dead adults showing external fungal growth were counted and percent mycosis was calculated.

Antagonistic activity test

The isolated fungi were also tested in terms of antagonistic activity against *V. inaequalis*. The antagonistic effects of the fungal isolates were determined according to the "direct opposition method" described by Dennis and Webster (1971). For this, 5 mm mycelial disk was taken from the actively growing *V. inaequalis* culture and were placed 1 cm away from edge of the 120 mm PDAY medium. The same sized disks were also taken from the actively growing EPFs isolates and placed 1 cm away from the opposite side of the Petri dish. These Petri dishes were then incubated at 28 °C in dark and the tests were repeated three times. The control group only included *V. inaequalis* disk in the middle of the Petri dish. To calculate the percent inhibition, the radial growth of fungi in both the control group and inhibition tests were measured by caliper on tenth day. The percent inhibition was calculated using the following formula (Royse and Ries 1977; Landum et al. 2016).

$$I\left(\text{Inhibition percentage}\right) = \left(\frac{R1\left(\text{colony radius in control}\right) - R2\left(\text{colony radius in test}\right)}{R1}\right) \times 100$$

Data analysis

All DNA sequences were edited with BioEdit 7.09 (Hall 1999) and then were blasted at NCBI GenBank to determine their similarities with known fungal species in GenBank (Benson et al. 2012). The data obtained from this were used to confirm the morphological identification of isolates. Cluster analysis of DNA sequences were also done using the Clustal W packed in BioEdit and concatenated phylogenetic trees were then produced by the neighbor-joining (NJ) analysis with *p*-distance model using MEGA 11.0.10 (Tamura et al. 2021) phylogenetic software. Alignment gaps were considered as missing data. The substitution of nucleotide sites per site was 0.05 (scale bar underneath tree). The reliability of the generated phylograms were tested with the bootstrap analysis based on 1.000 pseudoreplicates using the MEGA 11.0.10. In phylogenetic analysis, *Beauveria* isolates were compared with the reference strains described in the study of Rehner et al. (2011) using *EF1-α, Bloc* and *RBP1* gene sequences. *Metarhizium* isolates were compared to fungal species indicated in the study of Bischoff and Rehner (2009) using *RBP1* and *β-tubulin* gene sequences. Phylogenetic analysis results were also validated using different genetic distance models such as Jukes-Cantor and Kimura 2-parameter model.

Data from the virulence tests against *T. hirta* was corrected using the Abbott formula and percent mycoses were calculated (Abbott 1925). Variance analysis (ANOVA), followed by LSD multiple comparison test, was used to compare the isolates with each other in terms of mortality and mycoses. Also, the isolates were compared to respect to percent inhibition in antagonistic activity tests using ANOVA, followed by LSD post-hoc test. All data were evaluated for variance homogeneity using Levene statistics before performing variance analyses and all percent (%) data (if some come out 0 (zero)) were subjected to arcsin transformation. All data were analyzed using SPSS 16.0 statistical software.

Results

After the isolation studies, 18 EPF species were isolated, 3 of which were from the field collected *T. hirta* adults, 15 from soil samples. A 31.25% of the collected soil samples was positive with respect to the presence of EPF species. The detailed information about the fungal isolates is given in Table 2. The fungi grown on PDAY medium were first divided into two groups based on their colony colors. White-colored and yellowish-green colonies were designated as *Beauveria* and *Metarhizium*, respectively. This discrimination was also confirmed both microscopically and by ITS gene sequencing based on Blast search in NCBI GenBank (Table 3). Finally, MLSA (multi-locus

Table 3 Percentage (%) query coverage, similarity and GenBank ID numbers of the isolated entomopathogenic fungi with their most closely related fungal species based on the Blast search in NCBI GenBank using ITS gene sequences

Isolate	The most related species	GenBank ID number	Query coverage (%)	Identity (%)
Bz-1	*Beauveria bassiana* TS35	KY515385	100	99.64
	Beauveria bassiana TS22	KY515383	100	99.64
	Beauveria bassiana KJ4	KY515369	100	99.64
	Beauveria bassiana 2548	EU821494	99	99.64
Bz-2	*Beauveria bassiana* KJ4	KY515369	100	99.63
	Beauveria bassiana KJ1	KY515355	100	99.63
	Beauveria bassiana EF_46	MT528695	100	99.63
	Beauveria bassiana EF_27	MT528676	100	99.63
Bz-3	*Metarhizium* sp. YH31	MN602855	100	98.35
	Metarhizium sp. E650	MF681582	98	98.71
	Metarhizium robertsii ARSEF 14,329	MN255810	98	98.70
	Metarhizium anisopliae CBS 127,632	MH864645	98	98.70
AK-4	*Metarhizium anisopliae* RZU-10	KM117232	100	98.71
	Metarhizium anisopliae CBS 127,632	MH864645	98	98.70
	Metarhizium sp. W11LA	MF872441	98	98.70
	Metarhizium sp. G3LB	MF872437	98	98.70
AK-5	*Metarhizium* sp. YH31	MN602855	100	98.72
	Metarhizium anisopliae SKCJ1	FJ545326	99	98.79
	Metarhizium anisopliae CBS 127,632	MH864645	99	98.79
	Metarhizium sp. W11LA	MF872441	99	98.79
AK-6	*Metarhizium robertsii* ARSEF 14,329	MN255810	100	98.89
	Metarhizium anisopliae FAFU-1	MG844433	100	98.89
	Metarhizium anisopliae KTU-49	FJ177473	98	99.25
	Metarhizium anisopliae KTU-26	FJ177485	98	99.25
AK-7	*Metarhizium* sp. YH31	MN602855	100	99.26
	Metarhizium anisopliae CBS 127,632	MH864645	99	99.44
	Metarhizium sp. W11LA	MF872441	99	99.44
	Metarhizium sp. G3LB	MF872437	99	99.44
AK-8	*Metarhizium anisopliae* RZU-10	KM117232	100	98.90
	Metarhizium robertsii ARSEF 14,329	MN255810	98	99.08
	Metarhizium anisopliae FAFU-1	MG844433	98	99.08
	Metarhizium anisopliae KTU-49	FJ177473	97	99.26
AK-9	*Metarhizium robertsii* ARSEF 14,329	MN255810	100	98.54
	Metarhizium anisopliae FAFU-1	MG844433	100	98.54
	Metarhizium anisopliae KTU-49	FJ177473	98	98.89
	Metarhizium anisopliae KTU-26	FJ177485	98	98.89
AK-10	*Beauveria bassiana* TS7	KY515364	100	98.74
	Beauveria bassiana KJ1	KY515355	100	98.74
	Beauveria bassiana TS2	KY515348	100	98.56
	Beauveria bassiana KJ4	KY515369	100	98.56
AK-11	*Metarhizium robertsii* ARSEF 14,329	MN255810	100	98.27
	Metarhizium anisopliae Eg18	MK942509	100	98.27
	Metarhizium anisopliae CBS 127,632	MH864645	100	98.27
	Metarhizium sp. W11LA	MF872441	100	98.27
AK-12	*Metarhizium robertsii* ARSEF 14,329	MN255810	99	99.45
	Metarhizium anisopliae FAFU-1	MG844433	99	99.45
	Metarhizium anisopliae CBS 127,632	MH864645	100	99.26
	Metarhizium sp. W11LA	MF872441	100	99.26

Table 3 (continued)

Isolate	The most related species	GenBank ID number	Query coverage (%)	Identity (%)
AK-13	*Metarhizium anisopliae* CBS 127,632	MH864645	100	99.63
	Metarhizium sp. W11LA	MF872441	100	99.63
	Metarhizium sp. G3LB	MF872437	100	99.63
	Metarhizium sp. G3LA	MF872433	100	99.63
AK-14	*Beauveria bassiana* KJ1	KY515355	100	99.64
	Beauveria bassiana TS3	KY515349	99	99.82
	Beauveria bassiana TS7	KY515364	99	99.64
	Beauveria bassiana TS19	KY515378	99	99.46
AK-15	*Metarhizium robertsii* ARSEF:14,329	MN255810	100	98.68
	Metarhizium anisopliae FAFU-1	MG844433	100	98.68
	Metarhizium anisopliae CBS 127,632	MH864645	100	98.50
	Metarhizium sp. W11LA	MF872441	100	98.50
AK-16	*Metarhizium robertsii* ARSEF:14,329	MN255810	100	99.26
	Metarhizium anisopliae CBS 127,632	MH864645	99	99.26
	Metarhizium sp. W11LA	MF872441	99	99.26
	Metarhizium sp. G3LB	MF872437	99	99.26
AK-17	*Beauveria bassiana* 2697	EU272501	100	99.47
	Beauveria bassiana SASRI_N41S2TNb	MF802492	99	99.64
	Beauveria bassiana IRN	MK942398	99	99.29
	Beauveria bassiana TS23	KY515387	99	99.46
AK-18	*Beauveria bassiana* KJ6	KY515373	100	97.70
	Beauveria bassiana TS12	KY515360	98	98.19
	Beauveria bassiana KJ1	KY515355	98	98.19
	Beauveria bassiana TS3	KY515349	98	98.19

sequence analysis) approach were applied to all isolates. For this purpose, the partial gene regions of *EF1-α*, *Bloc* and *RBP1* for *Beauveria* isolates and *RBP1*, *β-tubulin* for *Metarhizium* isolates were amplified from genomic DNAs, sequenced, and used in phylogenetic analysis to compare them with reference isolates. As a results, all *Beauveria* isolates were identified as *B. bassiana* (Fig. 1) and all *Metarhizium* isolates were identified as *M. robertsii*, except for AK-12 which is thought to be a new species in *Metarhizium* genus (Fig. 2).

In bioassay experiments against *T. hirta*, there was non-significant difference among isolates with respect to mortality, but all isolates were significantly different from the control group ($F=4.58$, df = 18, 38, $p<0.001$). However, the fungal isolates caused different mycoses values in comparison to each other ($F=4.89$, df = 18, 38, $p<0.001$). The highest mycoses were obtained from *B. bassiana:* Bz-1, Bz-2, AK-17 and *M. robertsii:* AK-4, AK-5, AK-6, AK-7, AK-9, AK-11, AK-13, AK-15, AK-16 and AK-18 and *Metarhizium* sp. AK-12 ($F=4.89$, df = 18, 38, $p<0.001$) (Fig. 3).

Six isolates showed an antagonistic activity against *V. inaequalis* and all caused significant inhibition rate in comparison to each other ($F=384.66$, df = 5, 12, $p<0.001$). The highest inhibition rate was obtained from *B. bassiana* AK-10 with 69.3% ($F=384.66$, df = 5, 12, $p<0.001$) (Fig. 4).

Discussion

The use of some pesticides in agriculture has been abandoned (Alewu and Nosiri 2011). In addition, insect pests in agriculture gain resistance to chemical insecticides used against them, and over time these insecticides become ineffective on target pests (Elzen and Hardee 2003). Besides, pesticide residues, which can be found in food and beverages, are a concern for human and animal health (Nicolopoulou-Stamati et al. 2016). The use of insect pathogenic fungi against *T. hirta* should be interesting, especially when it is thought that *T. hirta* damages' plants during flowering times and insecticidal application during these periods have a negative effect on the flowering of the plant.

Recent studies have shown that these EPF species can live endophytically and epiphytically with plants, thus having positive effects on plant growth and health (Mantzoukas and Eliopoulos 2020). In the present study, it was observed

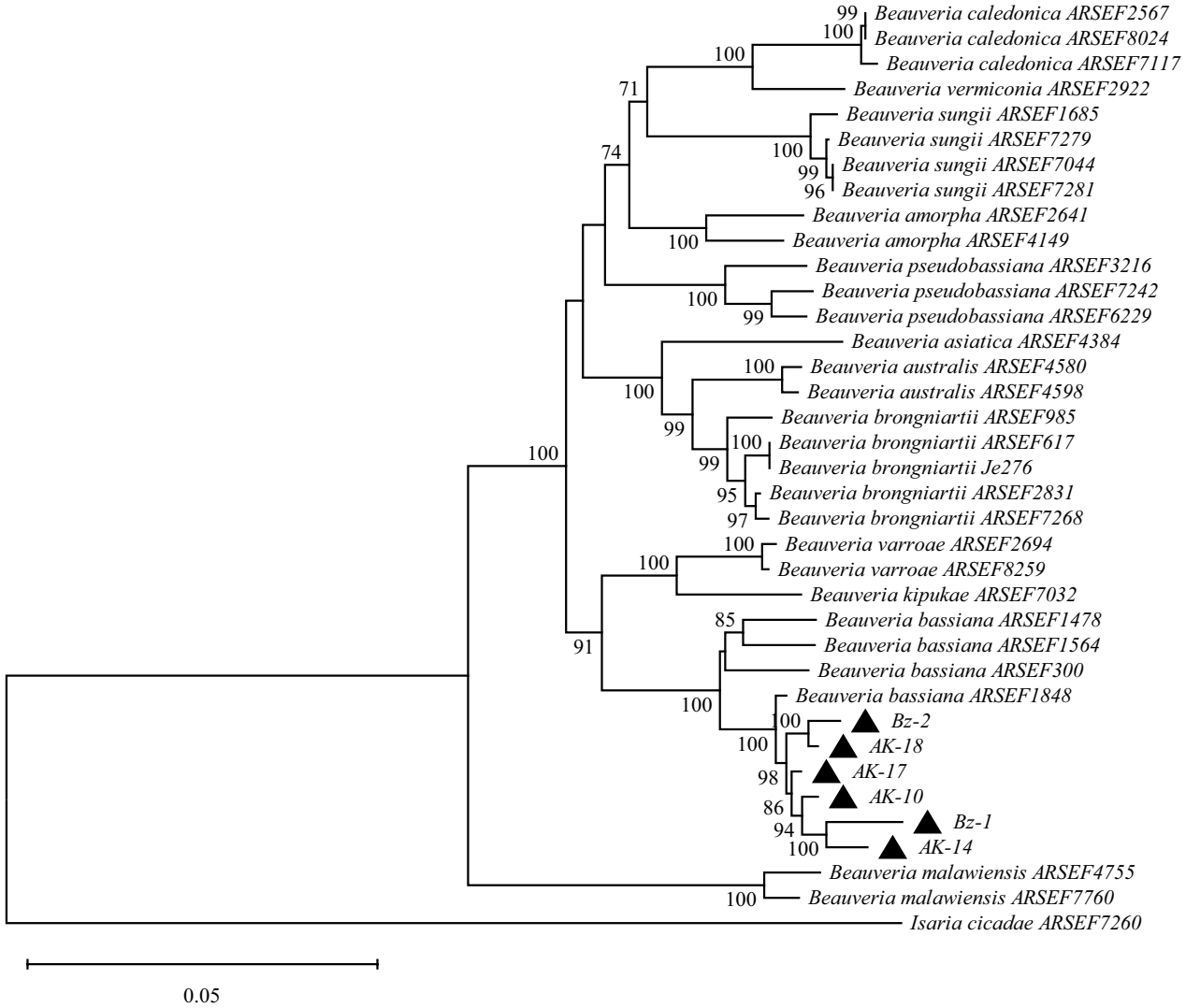

Fig. 1 Phylogenetic analysis of entomopathogenic *Beauveria* isolates obtained from *Tropinota hirta* adults (Bz-1 and Bz-2) and soil samples (AK-10, AK-14, AK-17 and AK-18) collected from apple orchards. The neighbor-joining (N-J) tree was generated using the concatenated partial sequences of *bloc*, *EF1-a* and *rpb1* gene regions. The phylogram was generated using MEGA 11.0.10 (Tamura et al. 2021) with *p*-distance model, a partial deletion of missing data and 1.000 bootstrap pseudoreplicates. The tree is rooted by *Isaria cicadae* ARSEF 7260 as outgroup. Bootstrap values of 70% and above are indicated next to the nodes. The isolates obtained from this study were indicated with black triangle. The scale at the bottom of the phylogram represents genetic distances in nucleotide substitutions per site

that various EPF species obtained from *T. hirta* adults and soil samples in apple orchards had the potential to be used against both apple blossom beetle and the apple scab disease within the scope of their biological control. During flowering period, it can be considered that the application of the fungi might also reduce the symptoms of the apple scab disease seen in leaves and fruits in future, especially in apple orchards. However, detailed field application trials are needed to support all these recommendations.

In this study, all tested fungi against *T. hirta* caused the same mortality but significantly different mycoses values. Also, the highest antagonist effect against *V. inaequalis* was obtained from *B. bassiana* AK-10. The species in the *Beauveria* and *Metarhizium* genus (especially *B. bassiana* and *M. anisopliae* or some species renamed by molecular taxonomic studies within this genus) are widely used in the control of many agricultural and forest pests worldwide due to their global distribution, easy mass production, broad host range and ease of application (Sullivan et al. 2022). In this respect, it could be considered advantageous to use local isolates obtained from this study and showing high activity within the scope of inundative,

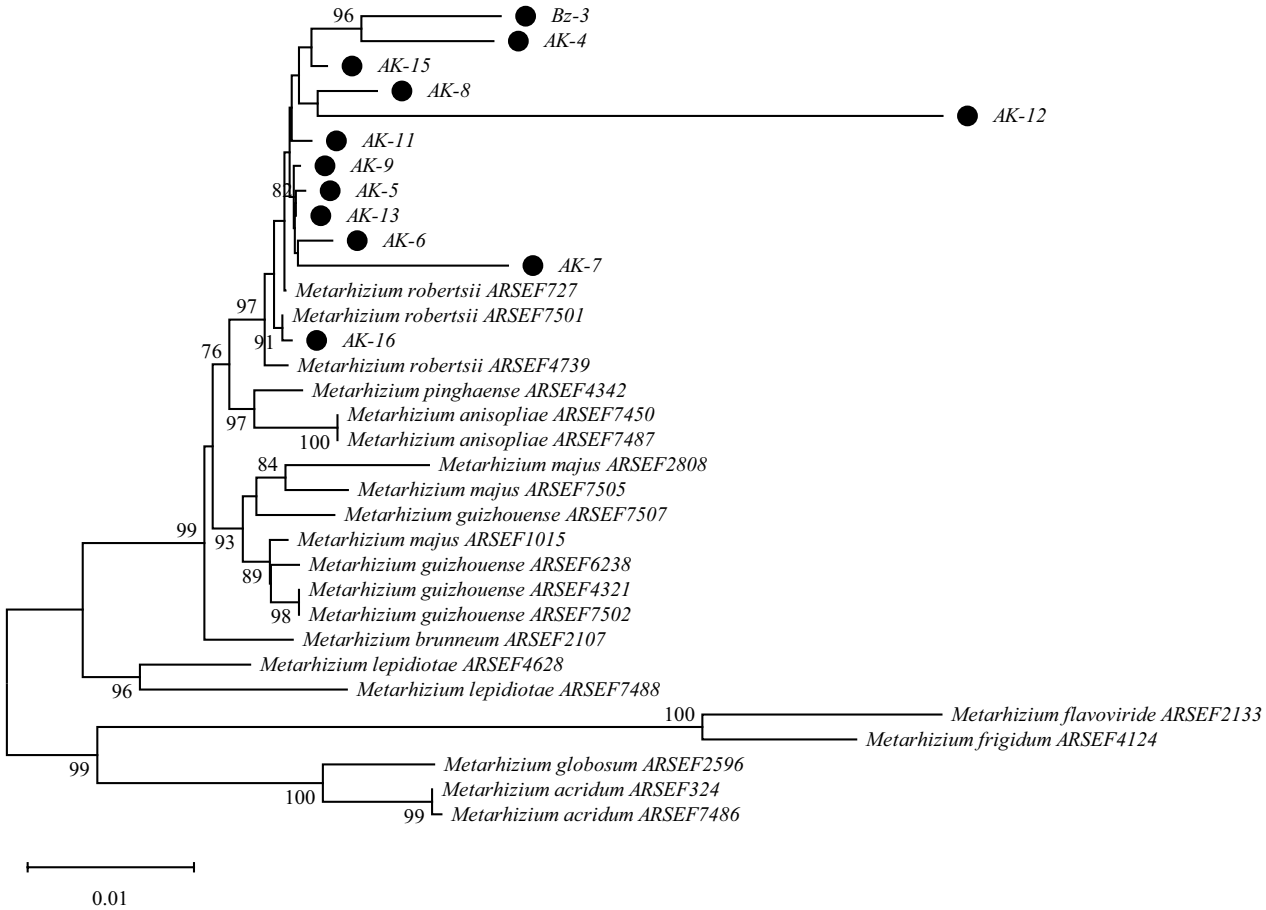

Fig. 2 Phylogenetic analysis of entomopathogenic *Metarhizium* isolates obtained from *Tropinota hirta* adults (Bz-3) and soil samples (AK-4, AK-5, AK-6, AK-7, AK-8, AK-9, AK-11, AK-12, AK-13, AK-15 and AK-16) collected from apple orchards. The neighbor-joining (N-J) tree was generated using the concatenated partial sequences of *β-tubulin* and *rpb1* gene regions. The phylogram was generated using MEGA 11.0.10 with *p*-distance model, a partial deletion of missing data and 1.000 bootstrap pseudoreplicates (Tamura et al. 2021). Bootstrap values of 70% and above are indicated next to the nodes. The isolates obtained from this study were indicated with black triangle. The scale at the bottom of the phylogram represents genetic distances in nucleotide substitutions per site

inoculative and especially conservative biological control in apple orchards.

The isolate AK-12 was only identified at genus level, and it is thought to be a new species of *Metarhizium* based on the phylogenetic analysis using *RBP1* and β-*tubulin* gene sequences. Before 2000, the species in the *Metarhizium* genus were generally identified according to their morphological characters (Mongkolsamrit et al. 2020). After that, molecular techniques were mostly used in studies related to the systematic and taxonomy of *Metarhizium* genus. Phylogenetic analyses based on multi-gene (or multi-locus approach) showed that many species of *Metarhizium* are specifically included in the *M. anisopliae* and *M. flavoviridae* species complexes, and the identification of new species has still been done from different geographical regions (Villamizar et al. 2021). It is currently known that there are 51 species identified within this genus (Villmizar et al. 2021). Within the scope of this study, it is thought that AK-12 isolate isolated from *T. hirta* is a new species within *Metarhizium* and more detailed morphological and phylogenetic studies are needed to be performed to prove this.

Conclusion

Various characterized EPF species were isolated from *T. hirta* adults and soil samples collected from apple orchards. Also, these fungi were tested against *T. hirta* adults and their antagonistic activity against *V. inaequalis* under laboratory conditions were determined. The obtained results should be beneficial for biological control of apple pests and fungal pathogens with respect to conservation biological control strategy. This is the first study of testing of EPF species against *T. hirta* and *V. inaequalis*.

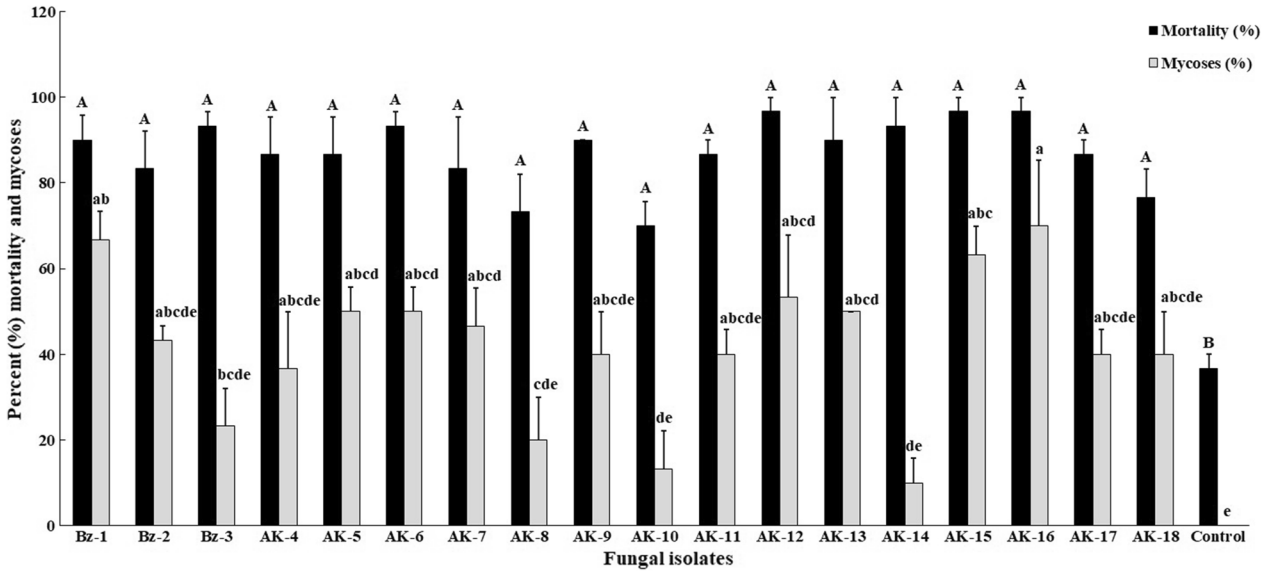

Fig. 3 Percent (%) mortality and mycoses values of *Tropinota hirta* adults after application of the fungal isolates obtained from *T. hirta* adults and soil samples in apple orchards. The conidial concentrations were applied to adults with airbrush at 1×10^7 spore/ml concentration. Mortality values were corrected using Abbott's formula (Abbott 1925). The different uppercase and lowercase letters indicated on the columns show the statistical difference in terms of mortality and mycosis among isolates, respectively. Bz isolates were isolated from *T. hirta* adults and AK isolates were obtained from soil samples. The isolates were compared using ANOVA followed by LSD post-hoc test. Bars show standard error. 0.01% Tween 80 was used as the control group

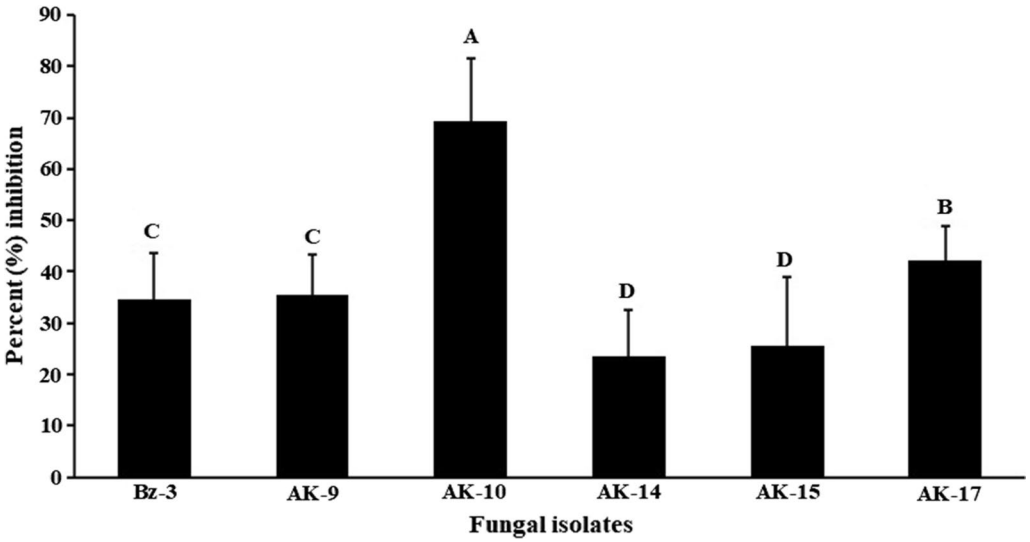

Fig. 4 Percent (%) inhibition rate of the fungal isolates against *Ventura inaequalis* according to the in vitro antagonistic activity tests. Inhibition rates were calculated based on the formula indicated in the study of Royse and Ries (1977). The different uppercase letters indicated on the column shows the statistical difference in terms of inhibition rate among isolates. The isolates were compared using ANOVA followed by LSD post-hoc test. Bars show standard error

Abbreviations

ITS	Internal transcribed spacer
rpb1	RNA polymerase II large subunit
Bloc	Bloc locus intergenic region
EF1-α	Elongation factor 1-α
β-tubulin	Beta-tubulin
EPFs	Entomopathogenic fungi
PDAY	Potato dextrose agar + 1% yeast extract

DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
dNTP	Deoxynucleotide triphosphates
NCBI	The National Center for Biotechnology Information
Blast	The basic local alignment search tool
MEGA	Molecular evolutionary genetic analysis
ANOVA	Analysis of variance
MLSA	Multi-locus sequence analysis

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Author contributions
AS participated in all parts of the study and especially, performed the study conception, design and supervised the manuscript. AK performed fungal isolation, genomic DNA isolation and virulence tests against *T. hirta*. ZT and RHŞ performed antagonistic activity tests. PCR and gene analysis were done and analyzed by all authors. AS wrote the manuscript and revised it. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed in this study are included. All strains are publicly accessible.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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