Türk. entomol. derg., 2023, 47 (2): 189-197
DOI: http://dx.doi.org/10.16970/entoted.1218790

ISSN 1010-6960
E-ISSN 2536-491X

Original article (Orijinal araştırma)

Isolation and identification of a fungal pathogen, *Beauveria bassiana* (Bals.-Criv.) Vuill. (Ascomycota: Hypocreales) from the Hatay yellow strain of silkworm, *Bombyx mori* L., 1758 (Lepidoptera: Bombycidae) in Türkiye[1]

Türkiye'den Hatay sarısı ipekböceği ırkı, *Bombyx mori* L., 1758 (Lepidoptera: Bombycidae)'den bir fungal patojeni olan *Beauveria bassiana* (Bals.-Criv.) Vuill. (Ascomycota: Hypocreales)'nın izolasyonu ve tanımlanması

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Abstract

The Hatay yellow strain of silkworm, *Bombyx mori* L., 1758 (Lepidoptera: Bombycidae) which produces cocoons with an extraordinary yellow color range, is one of the most important endemic and endangered cultural assets in Türkiye. In this study, intense fungal infection and many deaths were detected in the breeding trays in the Hatay yellow breed production facility. An entomopathogenic fungus was isolated from Hatay yellow strain cadavers collected in 2020. According to the morphological and molecular analysis results of the isolate, which was brought into pure culture, it was identified as *Beauveria bassiana* (Bals.-Criv.) Vuill. (Ascomycota: Hypocreales), the isolate HS1. Phylogenetic analysis results showed that the HS1 strain was very similar (99 %) to the isolates of *B. bassiana* KJ6 (Iran) and ARSEF 300 (Europe). The concentration-response test using $1 \times 10^{4-8}$ conidia/ml concentrations produced LC_{50} values of the new strain of 1.2×10^3 and 0.6×10^6 conidia/ml within 7 days against the larvae of Hatay yellow strain and hybrid strain of silkworm, respectively. The results indicated that the virulence of the *B. bassiana* HS1 strain to the Hatay yellow strain was much more severe and that the Hatay yellow strain had to fight it to survive.

Keywords: *Beauveria bassiana*, *Bombyx mori*, entomopathogenic fungi, Hatay yellow strain, silkworm

Öz

Olağanüstü sarı renk dağılımına sahip kozalar üreten Hatay sarı ipekböceği ırkı olan *Bombyx mori* L., 1758 (Lepidoptera: Bombycidae), Türkiye'nin en önemli endemik ve tehlike altındaki kültür varlıklarından biridir. Bu çalışmada, Hatay sarı ırkı üretim tesisinde, yetiştirme tepsilerinde yoğun fungal enfeksiyonu ve birçok ölüm tespit edilmiştir. Entomopatojen bir fungus 2020 yılında toplanan Hatay sarısı ırkı kadavralarından izole edilmiştir. Saf kültür haline getirilen izolat morfolojik ve moleküler analiz sonuçlarına göre *Beauveria bassiana* (Bals.-Criv.) Vuill. (Ascomycota: Hypocreales) türüne ait HS1 izolatı olarak tanımlanmıştır. Filogenetik analiz sonuçları, HS1 izolatının *B. bassiana* KJ6 (İran) ve ARSEF 300 (Avrupa) izolatlarına çok benzer (%99) olduğunu göstermiştir. Konsantrasyon-doz testi $1 \times 10^{4-8}$ konidia/ml konsantrasyonları kullanılarak yapılmış ve yeni izolatın 7 günlük LC_{50} değerleri Hatay sarısı ırkı ve hibrid ırk ipekböceği larvalarına karşı sırasıyla 1.2×10^3 ve 0.6×10^6 konidia/ml olarak belirlenmiştir. Sonuçlar, *B. bassiana* HS1 izolatının Hatay sarı ırkına verdiği hasarın çok daha şiddetli olduğunu ve Hatay sarı ırkının hayatta kalabilmek için onunla mücadele etmek zorunda kaldığını göstermiştir.

Anahtar sözcükler: *Beauveria bassiana*, *Bombyx mori*, entomopatojen fungus, Hatay sarı ırkı, ipekböceği

[1] This study was supported by Hatay Mustafa Kemal University, Scientific Research Unit, Hatay Mustafa Kemal University, Türkiye, Grant Project No: 21.GAP.001.
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Received (Alınış): 14.12.2022 Accepted (Kabul ediliş): 07.07.2023 Published Online (Çevrimiçi Yayın Tarihi): 17.07.2023

Introduction

Fungi are unicellular or multicellular eukaryotic organisms. They are found in almost all habitats, but most of them live on land, especially on soil, plant and animal material, rather than in water. A group called decomposers grow in soil or on dead biological material, where they play an important role in the cycling of carbon and other elements. Some of them are plant pathogens because they cause diseases such as mold, rust, scab, or thrush, which cause significant economic losses in agricultural production. Some of them cause significant diseases and parasitic infestations in animals, including humans. The group known as entomopathogens is one of the most important groups of arthropod infectious agents (Kocacevik et al., 2015; Sönmez et al., 2016, 2017; Biryol et al., 2020). These entomopathogenic fungi, which play a beneficial role in the control of agricultural and forestry pests (Biryol et al., 2021), are a major problem for beneficial insects. Although they have a beneficial role within populations, they cause severe epidemics leading to a sudden and rapid collapse of colonies (Wada et al., 2011; Vojvodic et al., 2012).

Bombyx mori L., 1758 (Lepidoptera: Bombycidae), the silkworm, is one of the best-known beneficial insects. It is an economically important insect as it is a major producer of silk. Silkworm breeding began in China at least 5000 years ago and spread to India, Korea, Nepal, Japan, and the West (Arunkumar et al., 2006). The domestic silk moth used in raw silk production was domesticated from the wild silk moth Bombyx mandarina, which was distributed from northern India to northern China, Korea, Japan, and even the far eastern parts of Russia (Maekawa et al., 1988; Adams & Barber, 1996). The domesticated silk moth originated in China, not Japan or Korea. As a result of thousands of years of selective breeding, it is entirely dependent on humans for its reproduction. Silk fiber is one of the indispensable textile fibers with high added value and its value and importance is increasing today as it has throughout the history. Silk fiber production is growing in parallel to meet the demand. Moreover, farmers face many problems due to the contamination of silkworms with various microbial diseases (Mishra, 2017; Sharma et al., 2020; Chopade et al., 2021). One of the most virulent microorganisms affecting *B. mori* is entomopathogenic fungi, which spread rapidly among individuals in the population and cause mass mortality (Saad et al., 2019). Various fungal diseases called muskardin, with types such as white, green and yellow, have been reported in silkworm (Mishra, 2017). Wada et al. (2010) revealed that some silkworm strains are extremely sensitive to various *Beauveria* spp. Vuill. (Ascomycota: Hypocreales) and strains. A study of commercial silkworm hybrids showed that a strain of *Metarhizium anisopliae* (Metschn.) Sorokīn (Ascomycota: Hypocreales) is an important pathogen of the silkworm (Ribeiro et al., 2017). Although governments and different organizations organize different programs and provide support to inform farmers and control these diseases, crop losses are not yet controlled as expected. The Hatay yellow strain, a privilege of Türkiye and one of the three native breeds in our country was domesticated as an insect about 5000 years ago (Ulaşlı et al., 2021). In sericulture, the only hybrid strain that weaves white cocoons has been cultivated in our country for many years. While the hybrid breed that produces only white cocoons has been widely used in our country for many years, the local breed of Hatay, the Hatay yellow strain, has not been produced for almost 50 years (İleri, 2019). Studies on the strain, which has a very high value as a biocultural heritage in our country, focus on the production of fibers naturally occurring in different shades of yellow. These fibers are in high economic demand worldwide, but research on them is limited. In a study, Ulaşlı et al. (2021) investigated some morphological and biological characteristics of the Hatay yellow strain, which is in danger of extinction. There is no study in the literature on fungal pathogens of the Hatay yellow strain, which is exceptionally susceptible compared to hybrids. The identification of fungal pathogens is important for survival of the Hatay yellow strain, which is our cultural heritage and is in danger of extinction. In this study, the entomopathogenic fungi of the Hatay yellow strain were investigated for the first time. The fungi isolated from the cadavers were identified, and their lethal effects on the Hatay yellow strain and hybrid strain were determined.

Materials and Method

Silkworm strains

Both silkworm strains were obtained from Defne Apollon İpekçilik (Harbiye Mah. Atatürk Cad. No:17 Defne, Hatay, Türkiye), a silkworm breeder operating as a family business in Hatay, Türkiye, in 2020. The Hatay yellow strain was collected as cadavers from the production tables and taken to the laboratory in sterile tubes. Cadavers showing signs of fungal infection were placed in a humid chamber. The contaminated cadavers were used for fungal isolation. Healthy silkworms of the Hatay yellow strain and hybrid strain were used for the insecticidal activity study.

Isolation of fungal strain

Fungi were isolated from the infected larvae of the Hatay yellow strain and transferred to artificial media (Sabouraud CAF agar, Liofilchem s.r.l., Italy) containing 40 µg/ml chloramphenicol to prevent bacterial contamination. Cultures were incubated at 26-28°C for 1-2 weeks to promote growth and sporulation. Pure cultures were maintained on CAF agar media and subcultured monthly. Strains were stored at -20°C in glycerol for long-term storage. Only one of the fungal strains obtained from the cadavers of mycosis larvae was cultured as an entomopathogen, named HS1, and used for bioassay studies after its identification.

Morphological and molecular identification

The appearance of fungal infection of fungal isolates on larvae and adults, colony morphology, spore size, and spore shape on CAF agar were used for initial identification. Fungal spores and mycelial structures were measured using a phase contrast microscope (Nicon, Exlipse Ni) and morphological identification was done according to Humber (2012).

Partial sequencing of the ITS1-5.8S-ITS2 gene region between the 18 S and 28 S rRNA subunits was performed to confirm the identity of the isolates. The partial sequence of the ITS1-5.8S-ITS2 gene region of the new strain was amplified by polymerase chain reaction (PCR) using primers ITS5 (5'-GGA AGTAAAAGTCGTAACAAGG-3)' and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). PCR reactions and gel imaging were performed as described in Gencer (2023). Sequencing of the amplicons was performed by MACROGEN sequencing service, Amsterdam. Sequences were compared with sequences in the NCBI (Anonymous, 2023). The sequences were assembled and edited with BioEdit version 7.0.5.3 (Hall, 1999). Multiple sequence alignment was performed, and phylogenetic trees for the ITS1-5.8S-ITS2 genes were constructed using MEGA software, version 7.0.26 (Tamura et al., 2013; Kumar et al., 2018) and phylogenetic analysis was performed to compare them to similar species (Benson et al., 2013). Finally, the sequences were compared to representative sequences described by Rehner & Buckley (2005) to determine the taxonomic position of the new strain within *Beauveria*.

Insecticidal activity tests

Preparation of conidiospore

The conidial suspension of the new strain was harvested by adding 10 ml of sterile water amended with 0.1% Tween 80 to the 4-week-old culture. The conidiospore-containing mixture was filtered through three layers of cheesecloth into sterile 15 ml Falcon tubes and vortexed, and the spores were counted using a hemocytometer. The final concentration of conidial suspensions was calculated and serially diluted in 0.01% Tween 80 from 1×10^9 to 1×10^3 conidia/ml. Fungal spores with viability greater than 95% of conidia were used for the bioassay, which was determined after 24 hours of incubation on CAF agar medium at 25°C.

Concentration-response test

Concentration-response assays were performed with the *Beauveria bassiana (*Bals.-Criv.) Vuill. (Ascomycota: Hypocreales) HS1 strain on both silkworm strains under laboratory conditions. Thirty 3rd instar larvae of both silkworm strains in each concentration and control group were used for the bioassay. The larva was placed in plastic boxes with mulberry leaves, and the conidial suspension was applied separately for each dilution using a mini hand sprayer. The control groups were sprayed water containing 0.01% Tween 80 only. Boxes were then incubated for 7 days in a climate chamber at 28°C, 60% RH, and a photoperiod of 12:12 (L:D). Boxes in the bioassay setup were checked daily and dead larvae were collected. Collected dead larvae were placed in a humid chamber to induce fungal sporulation. Mortality data were corrected for individuals with mycosis using the Schneider-Orelli formula (Püntener, 1981). The LC_{50} of the new strain was determined using probit analysis on MS Excel (Finney, 1971). The bioassay was repeated three times and the experiment was repeated three times on different days.

Results

Fungi were isolated from the larval cadavers of the Hatay yellow strain brought to the laboratory. Based on the morphological images of the isolated fungal isolates on agar plates, one of the strains (HS1) was determined to be a new entomopathogenic fungal isolate. The isolate was morphologically defined according to conidia shape, conidia size, colony morphology and color, and symptoms in cadavers (Figure 1).

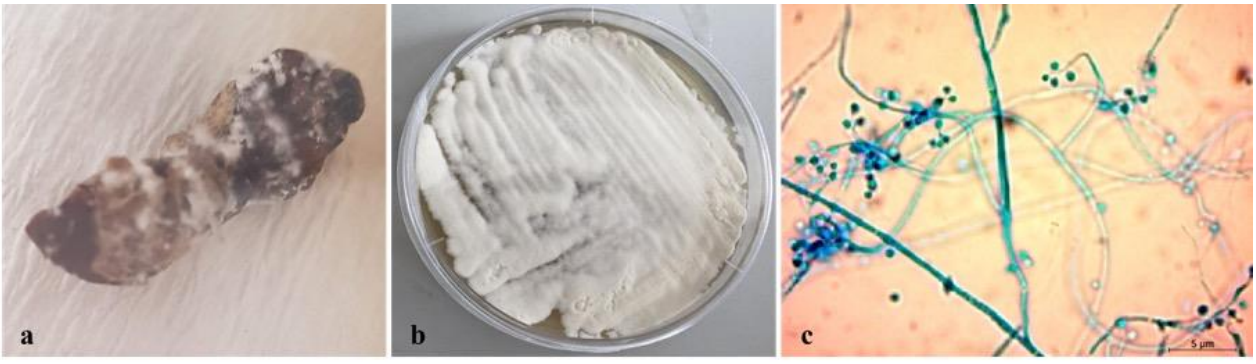

Figure 1. Macroscopy and microscopy of *Beauveria bassiana* HS1 strain. a) A larval cadaver showing mycosis naturally infected by the new strain. b) Macroscopic image of the HS1 strain on CAF. c) Mycelial and spore structures of the HS1 strain.

The new strain was morphologically identified as *B. bassiana*. Genomic analysis of the ITS1-5.8S-ITS2 gene region also revealed that the new strain was identical to *B. bassiana* (Figure 2). The HS1 strain was found to be very similar to *B. bassiana* strains KJ6 (Iran) and ARSEF 300 (Europe) in the Genbank database.

The new strain was found to be highly pathogenic on the larvae of the Hatay yellow strain (Figure 3). The mortality rates of the HS1 strain differed from those of the control groups within 7 days of application. The lowest concentration (1×10^4 conidia/ml) killed 60% of the Hatay yellow strain larvae used in the study. It was found that as the concentration increased, the mortality of the Hatay yellow strain larvae increased, and a concentration of $1 \times 10^{6-8}$ conidia/ml resulted in 100% death of the insect.

After the dose-response test, the LC_{50} value of the new fungal strain was calculated to be 1.2×10^3 conidia/ml within 7 days against the larvae of the Hatay yellow strain under laboratory conditions (Table 1). The presence of mycosis in all cadavers indicated that the deaths were due to fungal infection (Figure 4).

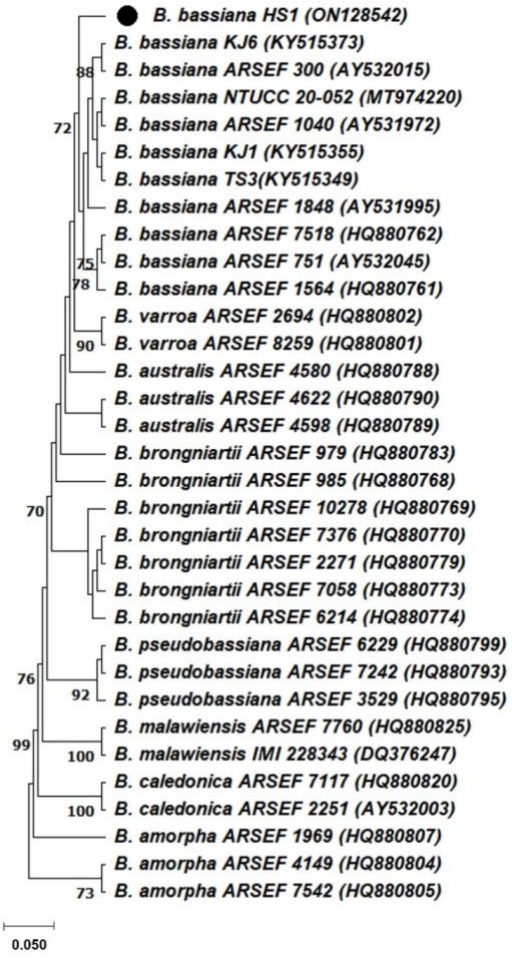

Figure 2. Neighbour-joining tree of the *Beauveria bassiana* HS1 strain and closely related fungal species based on the sequence of ITS1-5.8S-ITS2 gene region. The numbers at the nodes are bootstrap percentages based on 1000 replicates.

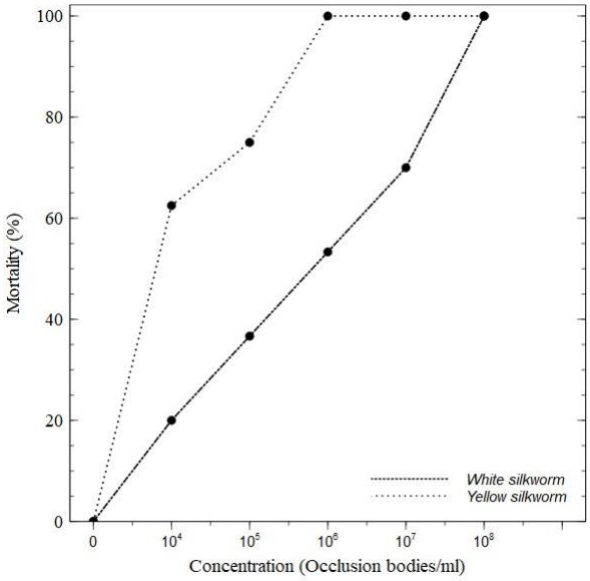

Figure 3. The insecticidal effect of the HS1 strain on *B.mori* Hatay yellow silkworm and *B.mori (*white silkworm) larvae within 7 days.

Table 1. Median lethal concentration (LC_{50}) of *B. mori* larvae exposed to five concentrations of *Beauveria bassiana* HS1 strain

Isolate HS1	N	LC_{50} (conidia/ml)	Intercept	Slope±SE	df	χ^2	95% Cl	
							Lower bound	Upper bound
B. mori (Hatay yellow silkworm)	90	1.2×10^3	3.895	0.356±0.616	3	0.548	0.07	20.4
B. mori (white silkworm)	90	0.6×10^6	2.369	0.452±0.421	3	0.764	0.09	4.3

N: number of individuals; SE: Standard Error; df: degrees of freedom; χ^2: chi-squared test; Cl: confidential limits.

Figure 4. Mycoses cadavers (Hatay yellow silkworm) at the end of the infection.

The experiments with the insecticidal activity of the white strain indicated that it was more tolerant than the Hatay yellow strain. While the lowest concentration (1×10^4 conidia/ml) caused 20% mortality, the concentrations of 1×10^5, 10^6, and 10^7 conidia/ml caused 39%, 53.33%, and 70% mortality, respectively, on the white strain. The concentration of 1×10^8 conidia/ml caused 100% mortality of the *B. bassiana* HS1 strain on the white strain of silkworm. After the concentration-response test, the LC_{50} value of the new strain with 0.6×10^6 conidia/ml was calculated within 7 days against the larvae of the white silkworm strain under laboratory conditions (Table 1).

Discussion

Fungi are one of the most important pathogens infecting insects and cause their death (Lacey et al., 2001). The strain of *B. bassiana* is obtained from cadavers that form a dense white coating on the exoskeleton, grow on agar as a white mould. The conidiogenous cells of *B. bassiana* are short and ovoid and terminate in a narrow apical process called a rachis (Figure 2). Conidiogenous cells with swollen bases and tooth-shaped, apically extending rachis with a conidium formed sequentially on each tooth are *B. bassiana*-specific morphological images under the microscope. All symptoms appeared to be highly compatible with *B. bassiana* infection in the cadavers of the Hatay yellow strain. The symptoms on cadavers, the growth characteristics on agar, and the microscopic images indicated that the pathogen was a new isolate of *B. bassiana*. This is the first detection of white muscardine in the Hatay yellow strain. White muscardine is one of the most common and best-studied fungal diseases of hybrid strain that weaves white cocoon. It causes significant problems and death of many insects during the larval and pupal periods, especially in cold and rainy seasons (Kumar et al., 1990; Lu, 1991). Symptoms such as larval inactivation, cessation of feeding, vomiting, and loss of larval elasticity were also observed in larvae of the Hatay yellow strain, which is the material of the present study. To confirm the morphological identification of the new strain, a fragment of the ITS1-5.8S-ITS gene region was sequenced. This sequence was compared with

representative sequences from the study by Rehner & Buckley (2005) and Rehner et al. (2011). Based on the dendrogram generated using ITS, the new strain was phylogenetically very close to *Beauveria* species and was included in the *Beauveria* cluster (Figure 2). In addition to recent interest in the genetic diversity and molecular ecology of *Beauveria* in relation to its role as a pathogen of insects in natural and agricultural environments, the genus has been under critical taxonomic review for several decades (Rehner et al., 2011). Specifically, *B. bassiana* includes an as yet undetermined number of cryptic lineages, many of which have an intercontinental distribution and occur as multispecies assemblages in both natural and agricultural habitats (Rehner et al., 2006; Meyling et al., 2009). In addition to the morphological data, molecular analyses and phylogenetic studies have shown, in great agreement with the literature, that the new fungus is a new strain of *B. bassiana*.

Entomopathogenic fungi are known to cause very severe infections in beneficial insects. One of the most important examples is the fungal diseases in bees caused by entomopathogenic fungi (Campano et al., 1999). One of the most striking examples of fungal infections in beneficial insects is silkworms. The first fungal infection in insects and even arthropods was white muscarine disease in silkworms caused by *B. bassiana*, described by Agostino Bassi (Bassi, 1835; Ainsworth, 1956). In a study examining the effects of *B. bassiana* on larval development and cocoon production of *Bombyx mori*, Seema et al. (2019) found significant reductions in mature larval weight, cocoon weight, shell weight, shell ratio, filament length, and unbreakable filament length as a result of fungal infection. In the current study, the new strain (HS1) isolated from the Hatay yellow strain and identified as *B. bassiana* also had a highly lethal effect on its host, which is very consistent with all the literature. All this shows that *B. bassiana* strains are a very important fungal species for many pests from different orders as well as for Lepidoptera.

The morphological appearance of the new strain of *B. bassiana* from the Hatay yellow strain that we discovered in the current study and its effect on larvae and cadavers are in excellent agreement with the isolates determined in other studies. Although there are many studies investigating the effects of *B. bassiana* strains isolated from white silkworms on their hosts and ways to control this pathogen, the fungal pathogens of the Hatay yellow strain have been neglected to date. Thus, the present study is the only one that demonstrates the virulence of a pathogenic fungus isolated from the Hatay yellow strain on its host. Virulence studies on larvae of the Hatay yellow strain showed that the fungal pathogen is highly effective on its host. Pathogenicity studies conducted with the white strain under the same conditions and concentration showed that the white strain was more resistant than the Hatay yellow strain. In one study, Lee et al. (1989) showed that the Chinese commercial silkworm strain was more resistant to *B. bassiana* than the Japanese commercial strain. A study comparing the susceptibility of three Indian commercial silkworm strains to *B. bassiana* showed significant differences in the LC_{50} value of the fungus on the insect (Lakshmi et al., 2005). In another study, Wada et al. (2011) investigated the susceptibility of twenty-two silkworm strains to *Beauveria brongniartii* (Saccc.) Petch (Ascomycota: Hypocreales), an isolate of an entomopathogenic fungus. They showed that the difference in susceptibility was in varying degrees between resistance and sensitivity. In the present study, the LC_{50} of a *B. bassiana* strain (HS1) isolated from the Hatay yellow strain was determined to be 1.2×10^3 spores/ml and 0.6×10^6 spores/ml on the third instar larvae of the Hatay yellow strain and the hybrid strain, respectively. This represents a very large difference in susceptibility of 500-fold between the two strains. Comparing this value with the susceptibility level between different silkworm breeds, it is clear that this value is extremely high. This is because in previous studies this value was found to be 20-fold (Lee et al., 1989) and 7-fold (Lakshmi et al., 2005). All these studies show that entomopathogenic fungi are important pathogens of silkworm commercial hybrids. The present study revealed that similar fungi are also very important pathogens of our cultural heritage the Hatay yellow strain. This is an extremely critical situation for the Hatay yellow strain.

In conclusion, to preserve the Hatay yellow strain, which is one of the most important cultural assets and biodiversity of Türkiye, very urgent and serious action plans must be prepared and implemented as soon as possible. Otherwise, tomorrow may be too late and the culture may be irrevocably lost. Fungal pathogens have the great advantage of spreading rapidly between individuals in populations. The climatic conditions in Hatay also favor the spread of fungal pathogens. Considering that the effects of climate change are having a negative impact on this situation, it becomes clear that the problem is much more serious.

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