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ISOLATION, MOLECULAR IDENTIFICATION, AND PATHOGENICITY OF *BEAUVERIA HOPLOCHELI* FROM THE PINE PROCESSIONARY MOTH, *THAUMETOPOEA PITYOCAMPA* (LEPIDOPTERA: NOTODONTIDAE)

MUSTAFA YAMAN* and TUĞBA SAĞLAM GÜVENDIK

Bolu Abant İzzet Baysal University, Faculty of Arts and Science, Department of Biology, Bolu, 14030, Turkey
*E-mail: muyaman@hotmail.com (corresponding author)

Abstract

The pine processionary moth, *Thaumetopoea pityocampa* (Den. & Schiff.), is a significant defoliator in pine forests. *T. pityocampa* also causes health risks to people due to their urticating hair. Possible control options include the application of chemical pesticides, mechanical removal, and the use of predator insects. However, these control methods are insufficient to control the pest. Therefore, new environmentally friendly control methods are needed to protect pine forests. In this study, a previously unreported species of entomopathogenic fungus was isolated and identified, and its insecticidal effect against pine processionary moth larvae was determined for the first time. The fungal isolate was identified as *Beauveria hoplocheli* according to the results of fungal rDNA gene sequence analysis. The insecticidal potential of the fungal isolate was also tested against second-instar larvae of *T. pityocampa.* The fungal isolate had a high pathogenic effect, ranging from 73.4% to 100% against the *T. pityocampa* larvae.

KEY WORDS: Pine processionary moth, *Thaumetopoea pityocampa, Beauveria hoplocheli*, biological control

Introduction

The pine processionary moth (PPM), *Thaumetopoea pityocampa* (Den. & Schiff.) (Lepidoptera Thaumetopoeidae), is the most significant pest affecting pine trees in southern Europe and northern Africa, where it is found in high densities (Cebeci *et al.,* 2010; Bonsignore *et al.,* 2011). Its larvae cause defoliation by consuming leaves primarily from coniferous species such as *Pinus brutia* Ten.*, P. nigra* Arn.*, P. pinaster* Ait.*, P. pinea* L. (Atakan, 1991; Kanat, *et al.* 2005; Trematerra *et al.,* 2019). Although native to southern Europe, northern Africa, and parts of the Middle East, this pest has expanded its geographical range, now spanning

from North Africa to central Europe (de Boer & Harvey, 2020). Climate change is encouraging PPM caterpillars to intensify their attacks on pine forests (Hodar *et al.,* 2003). *Thaumetopoea pityocampa* also poses health risks to humans, pets, and livestock due to their urticating hairs (Trematerra *et al.,* 2019). Possible treatment options include the application of chemical pesticides, mechanical removal, and the use of predatory insects (Kanat & Özpolat, 2006; Goertz & Hoch, 2013).

However, these control methods are insufficient to manage the pest populations. The potential of entomopathogens in the biological control of the pine processionary moth was recently well-reviewed (Yaman, 2022). Entomopathogens are relatively specific in their targets and have a lower environmental impact (Chelkha *et al.,* 2022).

In forest ecosystems, entomopathogens such as bacteria, fungi, viruses, protozoa, and nematodes can replace non-selective chemicals that have negative effects on beneficial insects and other animals, enabling them to play a more significant role in controlling and regulating pest populations (Ahmed & Leather, 1994; Hazir *et al*., 2022). Entomopathogens offer a valuable resource for discovering novel, effective agents to control insect pests in forest trees. Among entomopathogens, fungi, in general, are less host-specific and have the advantage of infecting hosts upon contact by penetrating the host integument, eliminating the need for ingestion. Therefore, they can be utilized as contact pesticides (Ahmed & Leather, 1994).

The entomopathogenic fungi of the PPM have been studied with great interest in recent years. To date, at least ten entomopathogenic fungi have been isolated or tested for the biological control of the PPM (Yaman, 2022).

In this study, a distinct entomopathogenic fungus species, previously unassociated the pine processionary moth, was isolated, identified, and its insecticidal effect on the larvae was determined in the laboratory for the first time.

Materials and Methods

Macroscopic examinations and fungal isolation

Thaumetopoea pityocampa larvae were collected in the *Calosoma sycophanta* rearing laboratories in Selçuk (İzmir), located in the Aegean region of Türkiye, where it is commonly found as the sole species. Insect specimens suspected of having fungal disease were subjected to macroscopic examination. The most prominent symptom was larval bodies covered with fungal mycelium.

After macroscopic examination, the suspected samples were individually placed into 70% ethanol and gently shaken for 3 min. Following surface sterilization, samples were washed with sterilized water under aseptic conditions. The samples were then crushed in 5 ml of sterile phosphate buffer solution (PBS). Subsequently, a 100 µl suspension was spread on Sabouraud dextrose agar (SDA) plates. The plates were incubated at 25°C for one week. After incubation, the plates were examined, and fungal colonies with similar colony characteristics and color morphology were selected. Various colony types of fungi were chosen and purified on SDA plates through subculturing. A single colony for each isolate was selected and transferred to a fresh SDA plate, which was then incubated at 25°C for two weeks until the plates were fully overgrown and sporulated. Purification was carried out through single spore isolation. Individual colonies were subcultured twice to ensure purity. One of the subcultured colonies was utilized for the identification of species per isolate.

Morphological features of the subcultured colonies included pigmentation, shape, and form. These characteristics were revalidated microscopically. The size and shape of conidia were also determined. Mycelia were harvested for DNA isolation.

Molecular and phylogenetic analysis

Table I. Species and GenBank accession numbers for the rDNA sequences of 30 fungal species used in phylogenetic analysis.

Species	Strain code	Locality	GenBank Accession no.
Beauveria bassiana	-	Greece	JF792883.1
Cordyceps bassiana	-	Iran	AJ564886.1
Beauveria sp.	BUB 283	China	MG642828.1
Beauveria bassiana	NBAII-Bb-23	India	NBAII-Bb-23
Cordyceps locustiphila	TS881	Colombia	JF837082.1JQ958606.1
Beauveria bassiana	CILBFNKM607105B019	India	MT158869.1
Lecanicillium longisporum	-	Greece	EF512987.1
Beauveria bassiana	K8	Iran	MZ956767.1
Beauveria bassiana	I-3	Iran	OM760897.1
Beauveria bassiana	MTCC_9968	India	JQ266153.1
Beauveria pseudobassiana	SUAn12	Slovakia	MT241786.1
Beauveria pseudobassiana	SUAb28	Slovakia	MT239436.1
Beauveria pseudobassiana	RCEF6181	China	OL684599.1
Beauveria brongniartii	PAV:M ORH4A	Italy	MW447504.1
Beauveria hoplocheli	Bt99	France	KC339698.1
Beauveria hoplocheli	B507	France	KC339680.1
Beauveria hoplocheli	Bt129	France	KC339694.1
Beauveria hoplocheli	Bt123	France	KC339688.1
Uncultured *Cordyceps*	-	China	LR603988.1
Paecilomyces fumosoroseus	-	China	AF368805.1
Paecilomyces fumosoroseus	-	Japan	AB265146.1
Akanthomyces lecanii	-	Russia	MH231308.1
Lecanicillium attenuatum	NBAII-VL-22	India	JF718716.1
Paecilomyces fumosoroseus	ARSEF 4484	France	AF461748.1
Uncultured *Isaria*	-	Spain	HE605234.1
Isaria farinosa	HK7	Ukraine	KC768083.1
Cordyceps farinosa	DS24	China	KY432326.1
Purpureocillium lilacinum	A546	China	KX463002.1
Purpureocillium lilacinum	GZUIFR 22.369	China	OM423566.1
Metarhizium anisopliae	Sbz	Iran	KJ573520.1
Trypanosoma congolense	IL300	United Kingdom	FN265929.1

All isolates were further identified by amplifying the ITS1-5.8S- ITS2 rDNA sequences. For this, pure fungal suspensions were used for DNA extraction. To facilitate DNA isolation, cell walls were disrupted by agitating the fungal mycelia with glass beads. The fungal suspension was diluted with distilled water, and equal volumes of fungal suspension and glass beads were put into new Eppendorf tubes and vigorously shaken on the vortex for 1 min at maximum speed. The solutions were incubated with proteinase K at 56°C for 3 h. After that, nucleic acid extraction was performed with a DNA isolation kit (QIAGEN DNA Isolation Kit-69504, Hilden, Germany), following the manufacturer's guidelines and the modified protocol of Hyliš *et al.* (2005). The PCR fragments were amplified using the universal primers: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and

ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Siahmard *et al.* 2017). Amplification (Bio-Rad t100 thermal cycler) was performed under the following conditions: after initial denaturation of DNA at 95°C for 15 min, 45 cycles were run (94°C for 30 s, 61°C for 90 s, and 72°C for 90 s) with a 10 min extension at 72°C. The PCR-amplified products were loaded onto 0.9% agarose gel, supplemented with ethidium bromide. The PCR products and the primers used for PCR were then sent (Sentegen, Ankara, Türkiye) for determination of the base sequences. The DNA sequences (PQ573488) were analyzed using BLAST in NCBI. The GenBank accession numbers of fungal rDNA gene sequences from 30 fungi used in the phylogenetic analysis are listed in (Table I).

Multiple sequence alignment and phylogenetic analysis were performed with the maximum likelihood method using Kimura's two-parameter distance and evaluated by 1000 bootstrap replications with the MEGA X (https://www.megasoftware.net). The GC content of the rDNA sequence of the fungus was also analyzed with the % G - C Content Calculator Program with web design.

Preparation of conidial suspensions for bioassays

A single colony of the isolate was cultured on SDA and incubated at 25°C for 15 days. Fully overgrown and sporulated plates were used to obtain conidial suspensions by scraping conidia from Petri dishes into distilled water with 0.01% Tween-80. The conidial suspensions were filtered through two layers of sterile cheesecloth to remove mycelial and agar particles. The concentration of conidia in the final suspensions was determined using a hemocytometer and adjusted to five concentrations, ranging from 3.2×10^3 to 3.2×10^7 conidia/ml for the fungal isolate.

Bioassay experiments

Bioassay experiments were performed with the fungal species originally isolated from dead *T. pityocampa* larvae in this study. For the experimental infections, second-instar larvae of *T. pityocampa* were collected from pine forests. After macroscopic examination, healthy larvae were randomly selected for bioassay experiments. Twenty larvae per treatment were used in the bioassays. Insect inoculation was carried out by dipping the second-instar larvae in the conidia suspension for 10 s (Rohrlich *et al.*, 2018) at concentrations ranging from 3.2×10^3 to 3.2×10^7 conidia/ml. The control group was treated with sterile water. Three replications of each experimental and control group were used. The experimental and control groups were kept under 25 ± 1°C, 80 ± 5% relative humidity (RH), and a 16/8 h light/dark photoperiod for 21 days.

Statistical analysis

To obtain statistically significant data, groups of 20 insects per test were used for bioassays. Every test and control group were performed in 3 replicates. A chi-square test was used to compare observed results. A P value less than 0.05 was considered significant.

Results

In this study, an entomopathogenic fungus from the larvae of *T. pityocampa* was isolated and identified with phylogenetic analysis based on the internal transcribed spacer (ITS) region. To this end, fungal DNA was successfully extracted. The 503-base pair fragment of the ITS1-5.8S-ITS2 rDNA regions was successfully amplified in PCR and sequenced. BLAST analysis confirmed that the molecular identity of the fungal isolate matched the previously reported morphological identities, corresponding to the ITS sequences of various *Beauveria* strains.

The phylogenetic tree generated revealed two major clades: the *Beauveria* group and others (Fig. 1). *Beauveria* species were placed in three subgroups: *Beauveria bassiana, B. pseudobassiana* and *B. hoplocheli*. Other species settled in a scattered manner, grouping among themselves. Phylogenetic analysis revealed that our isolate clustered within a clade containing *Beauveria hoplocheli* species and was positioned as a sister to *B. hoplocheli* (KC339688.1) from Réunion Island (Fig. 1). Therefore, the fungal isolate was identified as *Beauveria hoplocheli* according to the results from the fungal rDNA gene sequence analysis. Analysis of the length and GC content of the ITS1-5.8S-ITS2 ribosomal DNA gene of the fungal isolate was also done. A 503-bp fragment was used in the analyses, and the GC content of the fungal isolate's base sequences was found to be 56.9%.

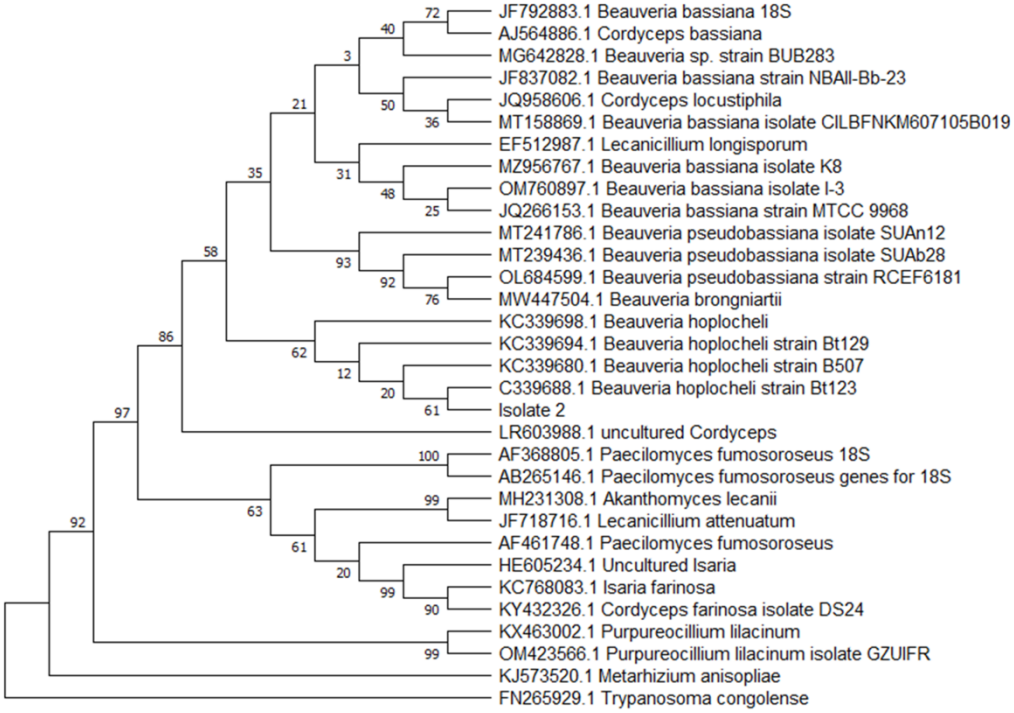

Figure 1. Phylogenetic relationships among fungi species isolated from different hosts based on ITS1-5.8S-ITS2 ribosomal DNA regions. The tree was constructed by the maximum likelihood method using Kimura's two-parameter distance and evaluated by 1000 bootstrap replications with the MEGA.X program.

We also tested the insecticidal potential of the fungal isolate against second-instar larvae of *T. pityocampa.* Bioassay tests showed that the fungal isolate had a high pathogenic effect against the larvae. The infection rate ranged from 73.4% to 100% with concentrations from 3.2×10^3 to 3.2×10^7 conidia/ml (Fig. 2). There is a statistically significant difference between the experimental and control groups (Pearson chi-square, P=0.000 <0.05).

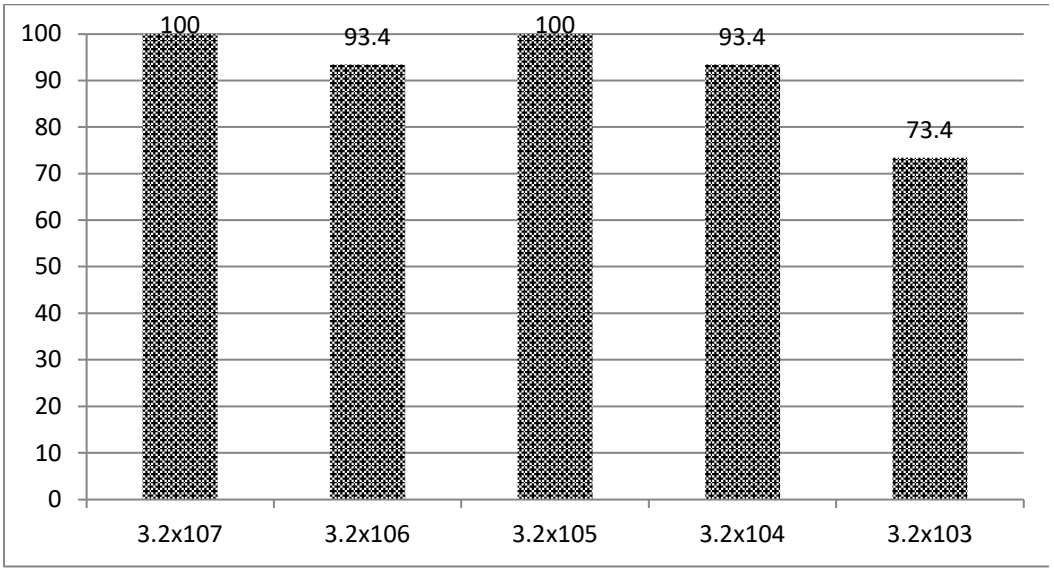

Figure 2. Insecticidal potential of *Beauveria hoplocheli* on *T. pityocampa* second-instar larvae.

Discussion

In this study, an entomopathogenic fungus from the larvae of *T. pityocampa* was isolated and identified. Phylogenetic analysis confirms that the fungal isolate is *Beauveria hoplocheli* (Fig. 2). *B. hoplocheli* was described as a new species of Réunion Island fungus, previously identified as *Beauveria brongniartii* (Robène-Soustrade *et al.,* 2015). To date, the species *B. hoplocheli* has only been isolated from the white grub *Hoplochelus marginalis* (Fairmaire) (Coleoptera: Scarabaeidae) (Robène-Soustrade *et al.,* 2015). In our study, we revealed for the first time that *B. hoplocheli* causes infection in a natural population of *T. pityocampa,* a lepidopteran pest.

To date, ten entomopathogenic fungal species have been isolated or tested against *T. pityocampa* (Table II). Sevim *et al.* (2010) identified *bassiana* from *T. pityocampa* in the Black Sea Region of Turkey. Alkan Akıncı *et al.* (2017) identified four fungi, *Isaria farinosa*, *Beauveria bassiana*, *Fusarium sambucinum*, and *Aspergillus terreus* from *T. pityocampa* larvae collected from pine forests in the same region. Barta *et al.* (2019) identified three entomopathogenic fungal species, *B. pseudobassiana*, *B. varroae* and *Purpureocillium lilacinum* from natural populations of *T. pityocampa* in Bulgaria for the first time. Georgieva *et al.* (2020) detected the hyperparasitic fungus *Syspastospora parasitica* attacking two *Beauveria* spp., which infected the larvae and pupae of the new tritrophic unit, *T. pityocampa.* So far, three *Beauveria* species, *B. bassianai B. pseudobassiana*, and *B. varroae*, have been isolated from *T. pityocampa* or tested for their insecticidal potential. As seen in Table 2, there is no record of *B. hoplocheli* from *T. pityocampa*. In the present study, we isolated and identified *B. hoplocheli* from a *T. pityocampa* population for the first time. *B. hoplocheli* was originally described from a scarab host. Our isolate is the first record of *B. hoplocheli* from a lepidopteran host.

Table II. Insecticidal effects of different fungi species on *T. pityocampa* larvae

Entomopathogen Species	Mortality (%)	References
Beauveria hoplocheli	73.4-100 (Exp.)	In this study
Beauveria bassiana	100 (Exp.)	Ozdemir *et al.,* 2019
	15-31 (Exp.)	Alkan Akinci *et al.,* 2017
	100 (Exp.)	Sönmez *et al.,* 2017
	100 (Exp.)	Sevim *et al.,* 2010
Beauveria pseudobassiana	41.8 (Field)	Georgieva *et al.,* 2020
Beauveria varroae	41.8 (Field)	Georgieva *et al.,* 2020
	100 (Exp.)	Ozdemir *et al.,* 2019
Metarhizium brunneum	100 (Exp.)	Sönmez *et al.,* 2017
	94-100 (Exp.)	Aydın *et al.,* 2018
Paecilomyces fumosoroseus	95-100 (Exp.)	Er *et al.,* 2007
Fusarium sambucinum	8-24 (Exp.)	Alkan Akinci *et al.,* 2017
Aspergillus terreus	1-5 (Exp.)	Alkan Akinci *et al.,* 2017
Isaria farinosa	51-55 (Exp.)	Alkan Akinci *et al.,* 2017
Lecanicillium lecanii	54.6 (Exp.)	Er *et al.,* 2007

On the other hand, the efficacy of *B. hoplocheli* against *T. pityocampa*, or any isolation record from a lepidopteran pest, was previously unknown. We also determined that *B. hoplocheli* has a significantly high mortality effect (73.4 to 100%) on the second-instar larvae of *T. pityocampa* (Fig 2) when compared with other entomopathogenic fungi (Table 2). In the literature, *B. hoplocheli*, formerly described as *B. brongniartii* or *B. tenella* (Rohrlich *et al.,* 2018), showed little or no virulence to *Melolontha melolontha* (Coleoptera: Scarabaeidae) (NeuveÂglise *et al.,* 1997) and *Procontarinia mangiferae* (Diptera: Cecidomyiidae). In contrast, it was pathogenic to *Galleria mellonella* (Lepidoptera: Pyralidae) (Bricca *et al*., 2014) in laboratory bioassays.

Entomopathogenic fungi used for biological control have diverse host ranges. The host range of an entomopathogenic fungus can be defined as the number and taxonomic diversity of the hosts it can infect. Host range can be discussed in terms of ecological host range and physiological host range. While the ecological host range refers to the range of species that an entomopathogenic fungus infects in field conditions, the physiological host range is the range of species that the pathogen can infect under optimized conditions, determined by laboratory tests (Hajek & Goettel, 2007; Rohrlich *et al*., 2018). Rohrlich *et al.* (2018) performed laboratory mortality bioassays to understand the physiological host range of three *Beauveria* strains belonging to two species, *B. hoplocheli* and *B. bassiana*, against nine insect pests from the orders Lepidoptera, Coleoptera, and Diptera. They found that the *B. hoplocheli* strain is less virulent on Lepidoptera and Diptera compared to the two *B. bassiana* strains. However, further studies are needed to assess the effectiveness of the isolates against *T. pityocampa* and other pests under field conditions.

Control strategies other than biological control for *T. pityocampa* are either not sufficiently effective or have harmful effects on the environment. Chemical insecticides used to control *T. pityocampa* have potentially undesirable side effects on humans, plants, and other animal species, especially predators and parasites of this pest (Kashian & Dodson, 2002). Most entomopathogenic fungi have the advantage of infecting their hosts through contact, penetrating the host integument, and do not require ingestion to establish infection. Therefore, they can be used as contact pesticides (Ahmed & Leather, 1994). As seen in (Table 2), a significant proportion of entomopathogenic fungi species causes high mortality on *T. pityocampa* larvae. The results from this study, along with the literature, reveal that the diversity of entomopathogenic fungi in *T. pityocampa* populations is significantly high. In forest ecosystems, entomopathogens fungi can replace some of the less specific and more

harmful chemical insecticides, allowing naturally occurring beneficial insects to play a greater role in controlling and regulating insect pest populations (Ahmed & Leather, 1994).

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ИЗОЛАЦИЈА, МОЛЕКУЛАРНА ИДЕНТИФИКАЦИЈА И ПАТОГЕНОСТ *BEAUVERIA HOPLOCHELI* ИЗ БОРОВОГ ЧЕТНИКА, *THAUMETOPOEA PITYOCAMPA* (LEPIDOPTERA: NOTODONTIDAE)

MUSTAFA YAMAN и TUĞBA SAĞLAM GÜVENDIK

Извод

Боров четник, *Thaumetopoea pityocampa* (Den. & Schiff.), је значајан дефолијатор у боровим шумама и представља ризик по здравље људи. Могући начини контроле укључују примену хемијских пестицида, механичко уклањање и употребу инсеката предатора. Међутим, трага се за новим еколошки прихватљивим методама. У овој студији изолована је и идентификована нова врста ентомопатогене гљиве, а по први пут је утврђено њено инсектицидно дејство на ларвама боровог четника. Гљива је идентификована као врста *Beauveria hoplocheli* на основу резултата анализе генске секвенце гљивичне рДНК. Инсектицидни потенцијал гљивичног изолата је такође тестиран на ларвама *T. pityocampa* другог ступња. Резултат је показаао висок степен патогеног дејства, у распону од 73,4% до 100%.

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