



## ***Sypastospora parasitica* (Tul.) Cannon & Hawksw. (Ascomycota: Hypocreales), a Parasite of Entomopathogenic Fungi (*Beauveria* spp.) Attacking Pine Processionary Moth in Bulgaria: New Tritrophic Associations**

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**Abstract:** New tritrophic associations are reported: the hyperparasitic fungus *Sypastospora parasitica* attacking two *Beauveria* spp. that had infected larvae and pupae of the pine processionary moth *Thaumetopoea pityocampa* (Lepidoptera). Totally, 1475 samples of *T. pityocampa* were collected from three sites in the Eastern Rhodopes (Fotinovo village – 1120 larvae and pupae, Sarnak village – 117 and Kandilka village – 178) as well as 60 larvae and pupae from the town of Sandanski (SW Bulgaria). The entomopathogenic fungi *Beauveria pseudobassiana* and *B. varroae* were isolated from 15.7% of *T. pityocampa* larvae and pupae. Fruit bodies (perithecia) of *S. parasitica* appeared on 41.8% of infected by *Beauveria* spp. specimens of *T. pityocampa*. Perithecia covered completely the *Beauveria* mycelium two months after their appearing and stopped the entomopathogenic fungi development.

**Key words:** hyperparasitic fungus, *Beauveria pseudobassiana*, *Beauveria varroae*, *Thaumetopoea pityocampa*, Bulgaria

### **Introduction**

The pine processionary moth *Thaumetopoea pityocampa* (Denis & Schiffmüller, 1775) (Lepidoptera: Notodontidae) is regarded as one of the most damaging defoliator in planted and natural pine forests in the Mediterranean countries (BATTISTI et al. 2015). It is also responsible for various allergic reactions in humans and animals. In Bulgaria, the pest attacks approximately from 5000 to 27,000 ha per year (MIRCHEV et al. 2018). In recent decades, the negative economic, environmental and social impact of *T. pityocampa* has increased. The outbreaks of the pest cause severe damages to pine stands by decreasing growth of trees and making them vulnerable to other pests and disease attacks.

The fungi of the genus *Beauveria* Vuillemin are natural antagonists of insects helping to control the host population and prevention of outbreaks formation. Approximately 22 *Beauveria* spp. have been determined as pathogenic and considered as potential biological control agent attacking many insect species worldwide (SARANRAJ & JAYAPRAKASH 2017). The entomopathogenic fungus *Beauveria bassiana* (Bals.-Criv.) Vuillemin is extensively used for the control of many important pests of various crops and forests around the world and has been tested on different target insects, including *T. pityocampa* (SEVIM et al. 2010). DRAGANOVA et al. (2013) found that larvae of the pine processionary moth were affected mostly by *B. bassiana*. The reason of the observed high prevalence was that soil-dwelling

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fungus can easily come into contact with the premature stages of *T. pityocampa* during their stay in the soil. Recently, *Beauveria pseudobassiana* Rehner & Humber and *B. varroae* Rehner & Humber have been identified on *T. pityocampa* natural population in Bulgaria (BARTA et al. 2019).

*B. bassiana* is known as a host of a mycoparasitic fungus *Syspastospora parasitica* (Tul.) Cannon & Hawksw. (BEGUM & MANDAL 2015). The genus *Syspastospora* (Sordmiales: Ceratostomataceae) was erected by CANNON & HAWKSWORTH (1982) to accommodate *S. parasitica*, which was removed from *Melanospora* Corda mainly on the basis of the neck structure, parallel hyphae and distinctive ascospores (GARCÍA et al. 2002).

In Bulgaria, *S. parasitica* (syn. *Melanospora parasitica* (Tul.) was recorded as a secondary parasite of the entomopathogenic fungus *B. bassiana* on pupae of pine sawfly *Neodiprion sertifer* (Geoffroy) (Hymenoptera: Diprionidae) (ROSNEV & TSANKOV 1978). In 1988, an outbreak of the ash weevil *Styreonychus fraxini* (De Geer) (Coleoptera: Curculionidae) was registered and *B. bassiana* appeared as a biological factor reducing the beetle population (MARKOVA 1991). The hyperparasite *S. parasitica* was detected on *B. bassiana* on naturally-infected ash weevils.

The goal of the present study is to report new tritrophic associations with the hyperparasitic fungus *S. parasitica* attacking two *Beauveria* spp. that had infected pupae of *T. pityocampa* in natural populations in Bulgaria.

## Materials and Methods

A total of 1475 *T. pityocampa* larvae and pupae were collected from three sites in the Eastern Rhodopes (Fotinovo, Sarnak and Kandilka villages) and 60 pupae in town of Sandanski (SW Bulgaria) (Table 1).

The biological material (larvae and pupae of *T. pityocampa*) was collected in 30-50 year-old plantations of *Pinus nigra* Arn. between 6 February and 26 March 2019. The samples were transported to the Laboratory of Entomology at the Forest Re-

search Institute in Sofia. Collected larvae and pupae were put into sterile sand in plastic boxes in groups of 50-60 individuals per box and incubated at temperature 20-24°C. They were daily sprayed with distilled water for four months to observe pest survival and a natural prevalence of entomopathogenic fungi. All larvae and pupae with characteristic symptoms of mycosis were examined under a dissecting microscope (40×). Fungal cultures were obtained from the individuals with confirmed fungal infection. A small part of mycelia was taken from pupa using an inoculation loop and placed on malts-agar medium 2% into Petri dishes (90×15 mm), including 50 µg/ml tetracycline to prevent bacterial growth.

Twenty-seven dead larvae and pupae displaying typical macroscopic symptoms of fungal infection was placed in a 1.5 mL microtube and sent to the Molecular Laboratory of Institute of Forest Ecology in Nitra, Slovakia. The cultures were microscopically identified and subsequently analysed by a sequencing study of internal transcribed region of rDNA and a partial sequence of TEF1- $\alpha$  gene (BARTA et al. 2019).

For the identification of the hyperparasite *S. parasitica*, fruit bodies (perithecia) with emerged spores were analysed with a microscope Zeiss NU 2 at magnification 125×. Apices of perithecium necks with spores in them were cut with a sterile needle and transferred to 15-day-old colonies of *Beauveria* spp. grown on malts-agar medium at 22.4°C.

## Results

A total of 1475 larvae and pupae of *T. pityocampa* were inspected for natural infection by entomopathogenic fungi. *Beauveria* spp. was identified in 232 of them. Altogether, 27 *in vitro* isolates were obtained from the inspected *T. pityocampa* pupae and two *Beauveria* species were identified – *B. pseudobassiana* Rehner & Humber – 7 isolates and *B. varroae* Rehner & Humber – 6 isolates); *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson (14 isolates) was also found (BARTA et al. 2019).

**Table 1.** Main characteristics of studied localities.

Locality	Data of collection	Altitude, m	Latitude	Longitude	Orientation of the slope	Host tree
Fotinovo	06.02.2019 26.03.2019	459	41°37'82"	25°32'16"	S	<i>Pinus nigra</i>
Kandilka	26.03.2019	465	41°40'94"	25°58'18"	NE	<i>Pinus nigra</i>
Sarnak	26.03.2019	471	41°25'52"	25°36'29"	E	<i>Pinus nigra</i>
Sandanski	23.03.2019	127	41°31'11"	23°16'23"	S	<i>Pinus nigra</i>

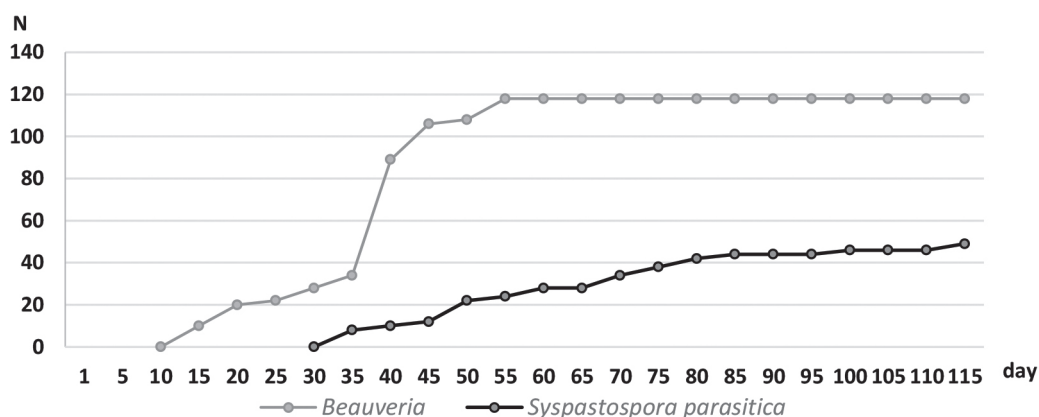


Fig. 1. *Beauveria* spp. and *Syspastospora parasitica* cumulative appearance in laboratory conditions

Table 2. *T. pityocampa* larvae and pupae parasitised by *Beauveria* spp. and *Syspastospora parasitica*

Locality	Data of collection	Number of studied larvae and pupae of <i>T. pityocampa</i>	Parasitised larvae and pupae by <i>Beauveria</i> spp., %	Hyperparasitised by <i>S. parasitica</i> , %
Fotino	06.02.2019	730	16.6	20.7
	26.03.2019	320	19.4	67.7
Kandilka	26.03.2019	178	14.6	26.9
Sarnak	26.03.2019	117	10.2	100.0
Sandanski	23.03.2019	130	8.4	100.0
Total		1475	15.7	41.8

Larvae and pupae of pine processionary moth showing characteristic symptoms of *Beauveria* mycosis were firstly observed on the 15th day after placing into the sand (Fig. 1). Incidence of infected larvae and pupae varied between 8.4 and 19.4% (Table 2), mostly found in the middle of March till the beginning of April. No new mycosis was observed after 4 April.

During the observation, a mycoparasitic fungus with dark brown ascomata emerged on the mycelium of *Beauveria* spp. infected pine processionary moth pupae 30 days after their laying into the sand (Fig. 1). The hyperparasite was identified as *Syspastospora parasitica*. In three months, perithecia of the fungus were observed on 41.8% of infected by *Beauveria* spp. pupae and stopped the entomopathogenic fungi development (Table 2, Fig. 2B).

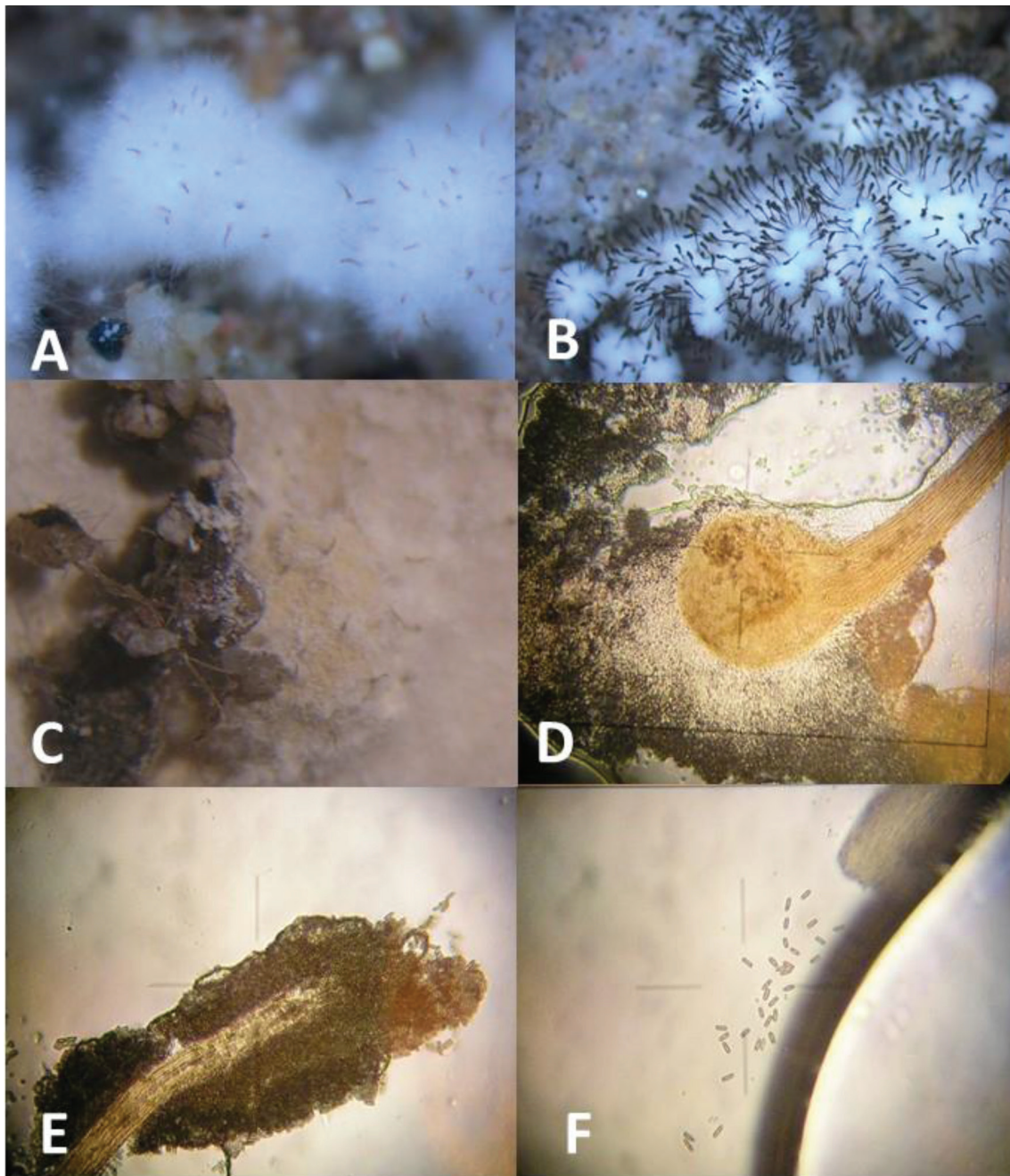
The distinguishing features of neck structure, ostiolum setae and ascospores of *S. parasitica* were noticed. The neck consists of parallel hyphae. The bases of perithecia were globose, yellow-brown in colour, and dark brown inside after the maturation of spores (Fig. 2D). Necks had the same colour as bases, 70-90 µm long, 38-44 µm wide at midpoints of neck lengths. Individual hyphae in neck were

smooth, pale brown, hyphoid, 40.2-64.5 × 2.8-4.2 µm. Asci were disintegrated with one-celled ascospores that were cylindrical, with one or two terminal fat droplets, dark brown, with an average size 8.5 × 2.8 µm (Fig. 2F). A huge number of mature ascospores were moving to the tip of the perithecium (Fig. 2E).

On malts-agar medium in Petri dishes, *Beauveria* colonies grew relatively slowly and appeared powdery, with colours ranging from white to yellow (Fig. 2C). Perithecia of the hyperparasite *S. parasitica* appeared 4 weeks after the inoculation. *S. parasitica* possessed ascomata with a long neck composed of parallel arranged hyphae and cylindrical ascospores with a large terminal slightly sunken germ pore at each end (Fig. 2F).

## Discussion

Entomopathogenic fungi are natural antagonists of arthropods helping in control of host population and prevention of outbreaks (VEGA et al. 2012). Extensive studies on the use of fungal entomopathogens as perspective biocontrol agents of insect pests in agriculture and forestry have been carried out. Fungi of the genus *Beauveria* are ubiquitous entomopatho-



**Fig. 2.** *Syspastospora parasitica*: A. An early stage of perithecia growing on *Beauveria* spp. Mycelium. B. Tritrophic association – mature perithecia growing on *Beauveria* spp. infected *T. pityocampa* pupa. C. *Beauveria* spp. culture with emergence of perithecia. D. Dissected perithecium with released ascospores. E. Ascospores moving to the tip of the perithecium. F. Ascospores.

gens, which have been found and isolated from a wide range of insects of various orders (GOETTEL et al. 1990). The fungal pathogen *B. bassiana* is a widely used for control of several insect pests, providing a biological alternative to synthetic chemical insecticides (HAJEK et al. 2001). In Bulgaria, *B. bassiana* was established as a dominating species affecting individuals of larval, pupal and imaginal stages of important forest pests (DRAGANOVA et al. 2013).

Recently, the genus *Beauveria* has been revised by molecular phylogenetic analysis (REHNER et al. 2011) and the new species *B. pseudobassiana* and *B. varroae* have been described as cryptic species, morphologically similar but phylogenetically distant from *B. bassiana*. The present study investigates the natural prevalence of *B. pseudobassiana* and *B. varroae* in pupae of natural populations of *T. pityocampa* from two regions. The results show that *Beauveria* spp. infection is consistently present.

The fungi *B. pseudobassiana* and *B. varroae* were reported from *T. pityocampa* for the first time (BARTA et al. 2019).

In this study, new tritrophic associations are reported for Bulgaria: the hyperparasitic fungus *Syspastospora parasitica* attacking two *Beauveria* spp. (*B. pseudobassiana* and *B. varroae*) that had infected larvae and pupae of *T. pityocampa*. The ascomycete *S. parasitica* is known to attack Deuteromycetes infecting insects. This hyperparasite has been detected on *B. bassiana*, *Spicaria farinosa* (Fr.) Vuill. and *Spicaria fumoso-rosea* (Wize) Vassiljevsky, developing on various hosts including sawflies, scale insects and white grubs (MÜLLER-KÖGLER 1961). It was identified on *Paecilomyces tenuipes* (Peck) Samson growing in *Bombyx mori* L. (LEE & NAM 2000) and on *B. bassiana* attacking the Colorado potato beetle *Leptinotarsa decemlineata* (Say) (POSADA et al. 2004).

## Conclusions

In recent study, the entomopathogenic fungi *Beauveria pseudobassiana* and *B. varroae* were established as dominating species affecting pine processionary moth in its larval and pupal stages. New tritrophic associations are reported for first time: the hyperparasitic fungus *Syspastospora parasitica* attacking *B. pseudobassiana* and *B. varroae* that had infected the larvae and pupae of the pine processionary moth.

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