

Acta entomologica serbica, 2018, 23(2): 15-25

UDC 595.782-134.2:579.8(497.2)

DOI: 10.5281/zenodo.2547665

SURVEY OF THE GUT PATHOGENIC MICROFLORA ASSOCIATED WITH CATERPILLARS OF THE BOX TREE MOTH *CYDALIMA PERSPECTALIS* WALKER, 1859 (LEPIDOPTERA: CRAMBIDAE)

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Abstract

The invasive box tree moth *Cydalima perspectalis* is an important and dangerous pest on *Buxus* spp. In this study, its destructive potential was evaluated in five areas of Bulgaria through the assessment of the defoliation levels caused by the caterpillars on the box trees. The microorganism species composition and diversity in the gut of caterpillars were investigated. In a total of 206 caterpillars, 5 bacterial (*Acinetobacter schindleri*, *Enterococcus casseliflavus*, *Klebsiella mobilis*, *Paenibacillus anaericanus*, and *Paenibacillus popilliae*) and 5 fungal species (*Metarhizium* sp., *Beauveria* sp., *Verticillium* sp., *Alternaria* sp. and *Mucor* sp.), were recovered. The most prevalent pathogen (52.4%) was the bacterium *P. popilliae*. Two entomopathogenic fungi, *Metarhizium* sp. and *Beauveria* sp., were observed in high and quite similar prevalences of 29.6% and 28.6%, respectively. The presence of these three pathogens (*P. popilliae*, *Metarhizium* sp. and *Beauveria* sp.) in relatively high levels in the gut of caterpillars indicates the possible role of gut pathogenic microorganisms as agents affecting caterpillar survival in natural populations of the pests. Further research is needed to assess the effect of the identified bacteria and fungi on *C. perspectalis* life stages.

KEY WORDS: invasive species, *Cydalima perspectalis*, defoliation levels, gut bacteria, entomophagous fungi, pathogen prevalence

Introduction

In recent years, due to intensive trade of ornamental plants and plant materials, numerous invasive pests have penetrated in the European and Mediterranean region from different parts of Asia, South and North America. One of these alien species, the box tree moth *Cydalima perspectalis* Walker, is probably the most

dangerous pest on *Buxus* spp. Since its recording for the first time in Europe (Germany) in 2007 (Billen, 2007), the box tree moth has spread across the continent and has been reported from 23 countries (Raineri *et al.*, 2017) and the Asian parts of Turkey (Hizal *et al.*, 2012) and Russia (EPPO, 2014).

In Bulgaria, *C. perspectalis* was observed for the first time in the Balchik Botanical Garden in 2014 (Beshkov *et al.*, 2015). Thereafter, the species has been recorded in: northeastern Bulgaria, regions of Ruse, Dobrich, Shumen and Targovishte; eastern Bulgaria (Black Sea coast), regions of Balchik, Varna, Burgas, Primorsko and Sozopol; central-southern Bulgaria, regions of Karlovo, Kalofer, Pazardjik and Plovdiv; and western Bulgaria, a region near Sofia (Arnaudov & Raikov, 2017; Pencheva & Yovkova, 2017). In these areas, *C. perspectalis* has shown itself to be a very destructive pest to common box, *Buxus sempervirens* L. The larvae of the moth feed on leaves of the plant causing, in some cases, complete defoliation of the trees.

Due to a lack of natural enemies of the box moth in Europe, including Bulgaria, *C. perspectalis* can reach high population density (Leudhardt, 2013). At the same time, specific control methods for this pest have not yet been established. Several chemical insecticides (deltamethrin, lambda-cyhalothrin, etc.) have been used for control of moth larvae, but the efficiency of these products has not been sufficient in any treatments (Fora *et al.*, 2016). Other approaches, such as using the bacterium *Bacillus thuringiensis* (Korycinska & Eyre, 2011), sex pheromone traps (Santi *et al.*, 2015), the egg parasitoids of the genus *Trichogramma* (Göttig & Herz, 2016) and plant oils and extracts (Göttig *et al.*, 2017), have been evaluated for regulating the box tree moth.

Apart from *B. thuringiensis*, several species of bacteria have been cited as insect pathogens, e.g. *B. megaterium*, *Proteus vulgaris*, *Serratia vulgaris* (Osborn *et al.*, 2001). There are also several genera of fungi that have been considered as important entomopathogenic agents, such as *Isaria*, *Metarhizium*, *Beauveria* (Takov *et al.*, 2012; Draganova *et al.*, 2013). A pathogenic complex consisting of bacteria, fungi and microsporidia causes a flacherie on Lepidopteran larvae and often leads to their death (Kathikairaj *et al.*, 2013; Aker & Tancer, 2016). In the context of the reduced leaf damage to *B. sempervirens* caused by *C. perspectalis* in 2017 compared with 2016 and especially 2015, the objective of this study was to obtain data about the pathogen-species composition of box tree moth caterpillars, in particular their occurrence, and relate these data to changes in defoliation levels.

Materials and Methods

In situ observations and collection of *Cydalima perspectalis*

Observations of *C. perspectalis* survival and feeding on box trees (*B. sempervirens*) were conducted within five areas near Varna, Elhovo, Svilengrad, Plovdiv (private gardens) and Burgas (Sea Garden) during 2015-2017. The levels of damage caused by caterpillars on plants were assessed on 10 naturally-infested box trees per each of the investigated areas at the end of July. Each time visual rating was done. Defoliation levels were measured using the 4-degree scale described by Hoover (2000): 0-30% – light defoliation, 31-50% – moderate defoliation, 51-70% – heavy defoliation, 71-100% – extremely heavy defoliation.

Fifth- and sixth-instar caterpillars (known to be the most destructive stages) manifesting the symptoms of loss of appetite, slow mobility, swelling of the thorax, the appearance of brown spots on the skin, were selected for isolation of microorganisms. According to Zhang *et al.* (2013) in their investigation on the larvae of *Bombyx mori* L. infected with the bacterium *Providencia rettgeri*, the mentioned symptoms are an unequivocal indication of the presence of bacterial infection and should be observed at the time of material collection. Randomly chosen live individuals were collected in labelled plastic containers and transported to

the laboratory at the Agricultural University for further study. Dead caterpillars were also collected from the gardens of Burgas, Plovdiv, Elhovo, Svilengrad and Varna.

Dissection of the caterpillars

Collected caterpillars were prepared for isolation of the gut microflora. The specimens were rinsed with tap water for 60 min, then surface-disinfected in 96% ethanol for 30 s, followed by rinsing with sterile distilled water to remove the ethanol. Dissections of the caterpillars were made under sterile conditions using a dissecting microscope. The bodies of the caterpillars were dissected as follows: the head was removed; a longitudinal lateral cut was made in the body wall; the inner content of the body was removed and placed in sterile saline (PBS); the digestive tract was separated from the internal matrix; the foregut was separated from the midgut. In order to locate the gut microorganisms, the foregut and midgut were transversally cut into several thin slices that were separately observed under a light microscope. Thereafter, the parts of the foregut and midgut were homogenized and transferred to sterile nutrient media (for bacteria – trypticase soy agar (TSA) and for fungi – rose bengal chloramphenicol agar (RBCA)) to isolate the gut community. In some cases of dead caterpillars with very soft bodies, individuals were placed in a solution of formalin 4% and ethanol 70% for 24 h before dissection.

Isolation of microorganisms

Isolation of the bacteria was made on TSA, while the fungi were isolated on RBCA, under aerobic conditions. Plates were incubated at 28°C for 5-7 days to observe the colonies of microorganisms. Single colonies were selected and re-streaked on a new plate containing the same medium to obtain pure colonies. The microorganisms were stored on the same media at 4°C. Pure cultures of bacterial and fungal isolates were obtained and used in further analyses.

Morphological characterization and microbial identification of bacteria

The morphological characteristics of bacterial isolates were limited to cellular morphology, Gram-staining and spore forming.

The MicroLog system was used for identification of bacteria (Biolog, Inc., Hayward, CA). Single colonies were obtained by streaking on media and the following steps were performed in the process of identification. Microorganisms were streaked onto Biolog universal growth (BUG) agar medium and the approximate bacterial number was quantified with a turbidimeter. The bacterial solutions were pipetted into each of the 96 wells in the Biolog microplates, incubated at 28°C for 24-48 h and then read with an automated plate reader (Biolog, Inc.), assessed visually and identified to species level.

Morphological identification of fungi

The isolates were cultured for 7 days on potato dextrose agar (PDA) at 27°C. Pigment production and the colony characteristics were noted. The identification of isolated fungi was performed by macroscopic and microscopic morphometry of conidia and conidiophores, spores, etc. using a compound microscope (LEICA DM 1000 LED) at 100X, 400X and 1000X magnifications. Observations were compared with dichotomous keys (Barnett & Hunter, 1987; Samson *et al.*, 1988; Humber, 1997) for the identification of fungi genera.

Results

The results presented in Table I describe *C. perspectalis* as the most destructive pest on *B. sempervirens* in Bulgaria. Since the box moth was recorded for the first time in 2014, the insect has rapidly invaded different parts of the country – eastern Bulgaria (Varna and Burgas) and central-southern Bulgaria (Plovdiv, Elhovo and Svilengrad). The percentage of defoliation caused by the moth on the box trees ranged from 26% to 100%. In 2015, the defoliation level in the studied gardens in Varna (100%), Burgas (95%) and Plovdiv (80%) was assessed as “extremely heavy”. In 2016, the damage level varied between 62% (Plovdiv) and 84% (Varna). In the same year, a tendency of decreasing damage was observed in Burgas, Plovdiv and Varna. In Elhovo and Svilengrad, the defoliation level by the box caterpillars on *B. sempervirens* was insignificantly higher compared with 2015. In 2017, in the regions of Burgas and Plovdiv, the damage levels of the moth were markedly lower than the previous two years and declined to “light defoliation” (26% and 29%, respectively). The levels of defoliation in Svilengrad and Elhovo did not change significantly compared with 2016 (65% and 68%, respectively).

Table I. Damage levels of *Cydalima perspectalis* on box trees in different regions in Bulgaria.

Location	Tree defoliation (%)		
	2015	2016	2017
Burgas	95	72	26
Elhovo	65	68	69
Plovdiv	80	62	29
Svilengrad	60	65	65
Varna	100	84	40

During the three-year evaluation of dead caterpillars, as well as larvae showing the symptoms of bacterial and fungal infestation, 10 microorganisms – 5 bacterial species and 5 fungal species, were identified.

The bacterial community of the gut of the caterpillars comprised *Acinetobacter schindleri*, *Enterococcus casseliflavus*, *Klebsiella mobilis*, *Paenibacillus anaericanus* and *Paenibacillus popilliae*. *A. schindleri*, *E. casseliflavus* and *K. mobiles* are natural for gut bacterial complexes in various insects. *P. anaericanus* and *P. popilliae* are known insect pathogens. *P. anaericanus* was isolated from caterpillars collected at Elhovo and Svilengrad. *P. popilliae* was observed in caterpillars collected from Burgas, Plovdiv and Varna (Table II).

Isolated fungi from the foregut and midgut of *C. perspectalis* caterpillars were identified as *Metarhizium* sp., *Beauveria* sp., *Verticillium* sp., *Alternaria* sp. and *Mucor* sp. The genera *Metarhizium* and *Beauveria* are representative of entomopathogenic species, whereas the genus *Verticillium* consists of phytophagous and entomophagous species. *Metarhizium* sp. and *Beauveria* sp. were isolated from dead caterpillars collected from Varna and Burgas. *Alternaria* sp. and *Mucor* sp. are not generally associated with insect microflora and probably came to the foregut of the caterpillars with food (Table II).

Table II. Gut species community occurred in caterpillars of *Cydalima perspectalis*.

Gut microorganisms	Location				
	Burgas	Elhovo	Plovdiv	Svilengrad	Varna
<i>Acinetobacter schindleri</i>	+	+	+	+	+
<i>Enterococcus casseliflavus</i>	+	+	+	+	+
<i>Klebsiella mobilis</i>	+	+	+	+	+
<i>Paenibacillus anaericanus</i>		+		+	
<i>Paenibacillus popilliae</i>	+		+		+
<i>Beauveria</i> sp.	+				+
<i>Metarhizium</i> sp.	+				+
<i>Verticillium</i> sp.		+	+	+	
<i>Alternaria</i> sp.	+		+	+	
<i>Mucor</i> sp.		+	+	+	

In 206 examined caterpillars of *C. perspectalis*, the predominant pathogenic species was *P. popilliae*. The bacterium was found in the gut of caterpillars during the three-year period of evaluation, with highest prevalence in 2017 (71.8%). The bacterium *P. anaericanus* was recorded in lowest prevalence (10.2%) among all other pathogenic microorganisms found in the caterpillars and the highest prevalence of the bacterium (25.9%) was observed in 2015. Most of the dead caterpillars infested with the bacteria *P. popilliae* and *P. anaericanus* collected from all investigated sites showed symptoms of swelling of the body, dark spots on the integument, deformation and in some cases, rupturing of the body wall in the area of the metathorax and anterior part of the abdomen (Fig. 1). The caterpillars infected only with *P. popilliae* showed the symptoms of “milky disease” expressed in the white color of the hemolymph (“bubbles” on integument surface) (Fig. 2).

Two entomopathogenic fungi, *Beauveria* sp. and *Metarhizium* sp., were found in similar prevalences of 28.6% and 29.6%, respectively. Whereas *Beauveria* sp. was observed in the gut of caterpillars throughout the study period, *Metarhizium* was recorded only in 2016 and 2017. The fungus *Verticillium* sp. was observed in low prevalence (14.6%) with its highest prevalence in 2017 (Table III).

Table III. Prevalence of entomopathogenic bacteria and fungi of caterpillars of *Cydalima perspectalis*. Pa-*Paenibacillus anaericanus*; Pp-*Paenibacillus popilliae*; B-*Beauveria* sp.; M-*Metarhizium* sp.; V-*Verticillium* sp.

Sampling year	Number of examined caterpillars	Pathogens found in <i>Cydalima perspectalis</i> caterpillars									
		Pa	%	Pp	%	B	%	M	%	V	%
2015	54	14	25,9	9	16,7	2	3,7	-	-	3	5,6
2016	67	5	7,5	38	56,7	18	26,7	24	35,8	11	16,4
2017	85	7	8,2	61	71,8	39	45,9	37	43,5	16	18,8
Total	206	21	10,2	108	52,4	59	28,6	61	29,6	30	14,6



Figure 1. Dead caterpillars with symptoms of bacterial infection collected from the region of Plovdiv.



Figure 2. "Milky" appearance of the hemolymph of a caterpillar collected from the region of Burgas.

Discussion

Invasive alien species are the primary threat to global biodiversity (Early *et al.*, 2016). The successful establishment of *C. perspectalis* in different parts of the world is due to changes in the composition of native communities on box trees as a result of climate change and lack of natural enemies (Sakai *et al.*, 2001; Leudhardt, 2013; Early *et al.*, 2016). Finding natural insect pathogens and studying their potential as biological control agents could lead to the development of environmentally-friendly and effective methods for the management of box tree moth (Takov *et al.*, 2012; Göttig *et al.* 2017). Over the last decade, there has been an increasing interest in finding effective pathogens and parasites to control *C. perspectalis* (Korycinska & Eyre, 2011; Belokobylskij & Gninenko, 2016; Göttig & Herz, 2016). Gut microflora is shown to play a major role in controlling most of the important processes in the host insects, such as sexual performance and oviposition preference (Sharon *et al.*, 2010). The influence of gut bacteria on insect feeding could significantly affect their development and survival (Rajendran *et al.*, 2007). The present study described for the first time the microorganisms associated with the gut of caterpillars of *C. perspectalis* and their ability to control increasing of pest populations.

There have been numerous studies related to the gut microbiota of the order Lepidoptera. Indiragandhi *et al.* (2007) reported eleven bacterial species isolated from the diamondback moth *Plutella xylostella* L. Three of the strains identified as *Pseudomonas* sp., *Brachy bacterium* sp. and *Serratia* sp. were determined to be pathogenic to the third- and fourth-instar larvae of the moth. After studying the gut community of fifth-instar larvae of the cotton bollworm *Helicoverpa armigera* Hübner, Priya *et al.* (2012) identified the bacteria *Bacillus firmus*, *Bacillus niabense*, *Paenibacillus jamilae*, *Cellulomonas variformis*, *Acinetobacter schindleri*, *Micrococcus yunnanensis*, *Enterobacter* sp. and *Enterococcus casseliflavus*. The authors noted that *Enterobacter* and *Enterococcus* as universally present in all *Helicoverpa* samples, while *B. firmus*, *B. niabense* and *P. jamilae* were only isolated from several populations of the cotton bollworm with a high percentage of natural mortality of the larvae (Priya *et al.*, 2012). The role of gut bacteria associated with tobacco cutworm *Spodoptera litura* Fabr. on the efficacy of some fungal biocontrol agents was evaluated by Subhashini (2016), who reported four bacteria, two of which were classified as "pathogenic" (*Pseudomonas* sp. and *Serratia* sp.), and two as "naturally occurring" in the midgut of *Spodoptera* larvae (*Acinetobacter* sp. and *Micrococcus* sp.). Similarly, we isolated and identified five bacterial species, three of which (*Acinetobacter schindleri*, *Enterococcus casseliflavus* and *Klebsiella mobilis*) could be considered as part of

the “nonpathogenic” gut microflora of *Cydalima* caterpillars. The other two bacteria, *P. anaericanus* and *P. popilliae*, could be classified as “pathogenic” to many coleopteran and lepidopteran species. At present, the effect of *P. anaericanus* as a pathogenic agent against the insects is not well studied (Chen *et al.*, 2016). On the other hand, *P. popilliae* is known to play a major role as the causative agent of “milky disease” in the larvae of the Japanese beetle *Popillia japonica* Newman and other scarab species (Rippere *et al.*, 1998). Natural epizootics have been observed in *P. japonica* and in some cases in various insect species (Klein, 1992), but to date *P. popilliae* has not been used as a commercial product against Lepidoptera species (Lacey *et al.*, 2015). In this study, *P. popilliae* was isolated from the midgut of caterpillars collected from Burgas, Plovdiv and Varna, three of the evaluated locations where defoliation levels on the box tree were significantly reduced in 2017 compared to 2015 and 2016. During the last two years of the survey, the prevalence of the bacterium was 56.7% (2016) and 71.8% (2017), which suggests a probably epizootic appearance of *P. popilliae*. It is interesting to note that *P. anaericanus* was isolated from the caterpillars collected from the other two investigated sites (Elhovo and Svilengrad), where the presence of *P. popilliae* was not detected. This was probably due to an antagonistic relationship between *P. anaericanus* and *P. popilliae* like the antagonism observed between *B. niabense* and *P. jamaicae* in caterpillars of *H. armigera* (Priya *et al.*, 2012). The low prevalence of *P. anaericanus* (10.4%) could also be connected to the low competitive potential of the bacterium for food source compared with the other microorganisms inhabiting the gut of *C. perspectalis* caterpillars.

The entomopathogenic fungi *Beauveria* and *Metarhizium* have been effective in controlling over 700 (Goettel *et al.*, 1990) and 200 (Puet *et al.*, 1996) arthropod species, respectively. These fungi have often been reported as causative agents of mycoses in many lepidopteran pests (Burjanadze *et al.*, 2013; Draganova *et al.*, 2013) that have appeared in natural populations of the insects, as well as being induced by the application of commercial products based on various strains of *Beauveria* and *Metarhizium* (Butt *et al.*, 2001). In Bulgaria, Panajotov *et al.* (1960) reported that the fungi *Beauveria bassiana*, *B. globulifera* and *Spicaria farinosa* (now *Isaria farinosa*) have infected the larvae of the gypsy moth *Lymantria dispar* L. Regarding the same pest, Mirchev (2004) found 24.5% mortality of pupae caused by infection with *Scopulariopsis brevicaulis*, *Penicillium frequentans*, *Mucor mucedo*, *M. globosus*, *Aspergillus* sp. and *Fusarium* sp. In addition, Draganova *et al.* (2013) isolated the fungus *B. bassiana* from natural populations of seven lepidopteran species in Bulgarian forests. Comparably, *Metarhizium anisopliae* has been isolated from the larvae of the jute hairy caterpillar *Spilosoma obliqua* Walker (Sapna Bai *et al.*, 2010) and the fall webworm *Hyphantria cunea* Drury (Burjanadze *et al.*, 2013). In our study, *Beauveria* sp. and *Metarhizium* sp. were isolated from the gut of dead caterpillars of the box tree moth collected from the regions of Varna and Burgas. Although the prevalence of the fungi was significantly lower (28.2% and 29.6%, respectively, for *Beauveria* sp. and *Metarhizium* sp.) than the prevalence of the bacterium *P. popilliae* (52.4%), their appearance certainly contributes the pathogenic process developed in the pest. Moreover, the entomopathogenic complex of the bacterium *P. popilliae* and the fungi *Beauveria* sp. and *Metarhizium* sp. developed in *C. perspectalis* caterpillars resulted in a significant reduction in the pest-damage levels on box trees in Burgas and Varna, where leaf defoliation decreased from 95% to 72% to 26%, and from 100% to 84% to 40%, respectively, over the three years.

Conclusion

After the first two years (2014 and 2015) of extremely high damages caused by the invasive species *C. perspectalis* on *B. sempervirens*, the defoliation levels on the box trees declined in the gardens with pest outbreaks and no applied chemicals in 2016 and 2017 (Burgas, Plovdiv and Varna). Subsequently, we isolated 10 microorganisms from the foregut and midgut of a total of 206 dead caterpillars showing symptoms

of bacterial or fungal infestation. The bacterium *P. popilliae* normally associated with scarab beetles was established as a dominant species affecting caterpillars of the populations of the three regions mentioned above. The occurrence of the entomopathogenic fungi *Beauveria* sp. and *Metarhizium* sp. in the caterpillars collected from two (Burgas and Varna) of the three gardens with the presence of *P. popilliae* probably led to the decrease in damages to *B. sempervirens* and resulted in significantly lower levels of defoliation of the box trees caused by the pest.

Future surveys should be directed toward continuing the isolation of microorganisms from the different life stages of *C. perspectalis*, with detailed characterization of the pathogens described in the present study, as well as laboratory and field evaluations of the virulence of some bacterial and fungal isolates against the eggs and caterpillars of the box tree moth.

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ПРЕГЛЕД ПАТОГЕНЕ МИКРОФЛОРЕ ЦРЕВА КОД ГУСЕНИЦА ШИМШИРОВОГ ПЛАМЕНЦА *CYDALIMA PERSPECTALIS* WALKER, 1859 (LEPIDOPTERA: CRAMBIDAE)

ВИЛИ ХАРИЗАНОВА, МЛАДЕН НАЈДЕНОВ, АТАНАСКА СТОЈЕВА, ИЛИЈАНА ВАЛЧЕВА,
ДОНКА ДРАГАНОВА, ЈАНКО БОРИСОВ И МЕЛИКА МОХАМЕДОВА

Извод

Инвазивна врста *Cydalima perspectalis* је важна и опасна врста која напада *Buxus* spp. У овом раду је урађена процена деструктивног потенцијала ове врсте у пет области Бугарске тако што је посматран ниво дефолијације. Истражена је заједница микроорганизама и диверзитет у цреву гусеница. У 206 јединки укупно пронађено је пет бактеријских врста (*Acinetobacter schindleri*, *Enterococcus casseliflavus*, *Klebsiella mobilis*, *Paenibacillus anaericanus*, and *Paenibacillus popilliae*) и пет врста гљива (*Metarhizium* sp., *Beauveria* sp., *Verticillium* sp., *Alternaria* sp. and *Mucor* sp.). Најчешћа врста је била бактерија *P. popilliae* (52.4%). Две врсте ентомопатогених гљива *Metarhizium* sp. и *Beauveria* sp. су забележене са сличном учесталашћу од 29.6% и 28.6%. Присуство ова три патогена (*P. popilliae*, *Metarhizium* sp. и *Beauveria* sp.) на релативно високом нивоу у цреву гусеница указује на значајну улогу ових микроорганизама на преживљавање гусеница у природним популацијама шимшировог пламенца. Даља истраживања су неопходна да се процени ефекат регистрованих бактерија и гљива на развојне стадијуме *C. perspectalis*.

Received: May 15th, 2018
Accepted: December 14th, 2018