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PRESENCE OF ENTOMOPATHOGENIC FUNGI AND BACTERIA IN LATVIAN POPULATION OF HORSE-CHESTNUT LEAF MINER *CAMERARIA OHRIDELLA*

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In the last 20 years horse-chestnut leaf miner has become widespread in Europe, causing defoliation of horse-chestnut trees. The first record of horse-chestnut leaf miner *Cameraria ohridella* Deschka & Dimic (Lepidoptera: Gracillariidae) in Latvia was made in summer 2002. In recent years *C. ohridella* has spread across all territory of Latvia. The aim of the study was to acquire preliminary data on mortality factors of horse-chestnut leaf miner *C. ohridella* and identify present entomopathogenic microorganisms. Causes of mortality of *C. ohridella* larvae and pupae in two sampling plots since 2010 were analysed. The developmental stage of larvae was noted and if the insect was dead, the mortality factor was referred to one of the categories: parasitism, bacteria, fungi or another cause. Specimens with symptoms of infection (reduced movement, changes of colour, cuticle covered with fungal mycelia or conidia) were used for pathogen isolation. Total mortality rate of *C. ohridella* larval stages, including spinning stages and pupae, was 16.1% - 47.1%. Eleven species of entomopathogenic fungi were isolated from collected dead specimens. Isolated and identified fungi belong to six genera: *Aspergillus; Hirsutella; Beauveria; Metarhizium; Lecanicillium* and *Isaria*. The fungi from genera *Hirsutella, Lecanicillium* and *Metarhizium* were isolated from lepidopteran pest for the first time in Latvia. We have identified bacteria, associated with field-collected larvae of *C. ohridella*. The results suggest that bacterial diversity of the surface of *C. ohridella* larvae was relatively simple - it consisted of six bacteria species and were dominated by gamma proteobacteria class bacteria.

Key words: *Cameraria ohridella,* horse-chestnut leaf miner, entomopathogenic fungi, bacteria.

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INTRODUCTION

Sustainable management of trees in urban greenery and woodlands includes effective protection against pests and diseases. Clarification whether the damaging agents are native or of exotic origin, understanding the nature of the problem, and using this knowledge for damage reduction is a core activity of tree protection. Leaf miners caused the heaviest invasion among ornamental tree pests in Europe, as an example - recent invasion of the horse-chestnut leaf miner, *Cameraria ohridella* Deschka & Dimic (Lepidoptera: Gracillariidae). In the last 20 years horse-chestnut leaf miner has become widespread in Europe, causing defoliation of horse-chestnut trees and it seems to show more climatic tolerance than it would be predicted on the basis of current knowledge. The leaf miners larvae drill into horse-chestnut leaves and feed between the two epidermis layers. The biology and distribution of this leafmining species has been described by V. Skuhravy (1998), K.Hellrigl (2001), H.Šefrova and Z.Lastuvka (2001), F.Pavan et al. (2003) and J.Freise and W.Heitland (2004). Even though natural enemies complex of *C. ohridella* includes native parasitoids (parasitic wasps), pathogens and predators (Backhaus et al. 2002), these antagonists are insufficiently adapted and not able to regulate the leafminer's population density. Nowadays, in Europe over 37 generalist parasitoid species of *C. ohridella* have been recorded (Lethmayer 2005). It is conceded that the parasitoids adaptation to horse-chestnut leaf miner life cycle may take several decades. Other leaf miner species has its own set of parasitoids that are adapted to their life cycle and the parasitism reaches more than 50% (Klug et al. 2008). However, whereas parasitoids are quite well studied, the predatory species of *C. ohridella* in Europe were never properly investigated and their impact on leaf miner populations is unknown. Highly specialized parasitoids are still to be found for biological control purposes.

A number of larval stages of *C. ohridella* natural predators have been recorded. Observations in other countries have shown that blue tits (*Parus caeruleus*), great tits (*Parus major*) and marsh tits (*Parus palustris*) feed on larvae (Grabenweger et al. 2005). Among them, three tit species are thought to predate between 2 and 4% of the leaf miner population. The southern oak bush cricket (*Meconema meridionale*) has also been proven to predate *C. ohridella*, consuming around 10 larvae per day. Overall, the predation by the southern oak bush cricket is insignificant compared to the birds. In general, ants, birds and spiders are assumed to be the most important predators of leaf miners (Skuhravy 1998, Heitland et al. 1999). Pathogens (viruses, fungi, microsporidia and bacteria) are often found to be more important in regulation of arthropod population than predators and parasitoids (Steinkrauss 2007). Like other natural enemies, insect pathogens can exert considerable control of target populations.

A.Sierpinska and K.Kubiak (2011) from Warsaw collected *C. ohridella* pupa, from which they isolated entomopathogenic fungi: *Beauveria* sp., *Isaria* sp. and *Lecanicillium* sp. It is proved in Czech Republic, that dominant entomopathogenic soil-borne fungi, collected from *C. ohridella* habitats, were *Isaria fumosorosea* and *Beauveria bassiana* (Preserve et al. 2009). Naturally occuring epizootics could be a valuable natural resource for harvesting pathogens for research or use in biological control. Many entomopathogens are specific to certain species or groups of insect pests and some have the potential to provide long-term control (Lacey et al. 2001).

Commonly used ornamental tree horse-chestnut (*Aeusculus hippocastanum* L.) has been successfully used for planting for a long time in Latvia. The first record of horse-chestnut leaf miner *C. ohridella* in Latvia was made in summer 2002, and in seven years' time *C. ohridella* has spread across all territory of Latvia (Savenkov & Šulcs 2010). Thus, the appearance of this new invasive species poses a serious threat to horse-chestnut in parks and squares of Latvian cities and this problem requires to be studied very carefully. There is a lack of information about the importance of parasites, predators and entomopathogenic organisms present in horse-chestnut leaf miner population in Latvia.

Table 1. Mortality factors of *Cameraria ohridella* collected at Salaspils and Jelgava trapping stations

Year	Trapping station	Mortality (larvae + pupae), %	Cause of death (larvae + pupae), %			
			Parasitoids	Fungi	Bacteria	Birds, predators etc.
2011	Salaspils	16.82	2.11	0.18	0.79	13.72
2012	Salaspils	16.15	1.46	0.88	0.71	13.10
2012	Jelgava	47.14	6.11	0.70	0.19	40.19

The aim of the study was to acquire preliminary data on mortality factors of horse-chestnut leaf miner *C. ohridella* and identify present pathogenic microorganisms.

MATERIAL AND METHODS

Insects collection and determination of mortality factors

Horse-chestnut leaf miner larvae and pupae were collected from natural habitats – urban greenery. Collection of insect material was started in 2010, at the trapping station located in Salaspils, but since 2012 the collection was carried out also in Jelgava.

From the beginning of June till the beginning of October, every two weeks, from five horse-chestnut (*A. hippocastanum*) trees 20 leaves per each station were taken. Leaf damage was assessed by using M. Gilbert and J. Gregoire (2002) damage scale. Every *C. ohridella* mine was dissected and examined for the presence of leaf miner individuals. The developmental stage of larvae was noted and if the insect was dead, the mortality factor was referred to one of the categories: parasitism, bacteria, fungi or another cause. Specimens with symptoms of infection (reduced movement, changes of colour, cuticle covered with fungal mycelia or conidia) were used for pathogen isolation.

Isolation and determination of entomopathogenic fungi

Collected dead larvae were surface sterilized in 1% sodium hypochlorite for 30 sec., rinsed three times in sterile distilled water and placed in humid conditions to stimulate fungal growth and sporulation. Preliminary identification of fungi was confirmed by slide preparation. For cadavers with conidial cushions preparations of conidia were obtained by film method. Shape and size of conidia were examined using a microscope fitted with a micrometer. Squash preparations of various infected insect tissues were viewed in light microscope Olympus CX41 with magnification of 400x (Lacey & Brooks 1997). Agar - coated slide technique and staining with Lactophenol Cotton Blue were used for observation of sporulating structures and spores. Fungi were isolated and maintained on Malt extract agar or Czapek media. Keys for the identification by E.Kovaly (2007) and R.A.Samson et al. (1988) were used. Method for total genomic DNA isolation from fungal spores or cultures and the *Beauveria*-specific PCR primers P1 (5'AAGCTTCGACATGGTCTG) and P3 (3'GGAGGTGGTGAGGTTCTGTT) described by T. Pfeifer and G. Khachatourian (1993) were used for distinguishing among isolates. The PCR was performed in a GeneAmp PCR System 2400 (Perkin Elmer).

Isolation and determination of bacteria

Surface microflora of 10 larvae and 10 pupae was tested. Bacterial microflora of external surfaces was estimated by putting each specimen in LB media for 24 h, and after that 0.1 ml of serial from 3-fold dilutions was spread over triplicated LB agar at neutral pH and inoculated Petri dishes were incubated at 30°C for 5 days (Frankenhuyzen et al. 2010). Isolated pure strains were classified based on morfological parametres –color, shape, margins and texture of colonies. Morphology of bacteria was examined in light microscope Olympus CX41. Gram staining, Oxidase test, Indole test and other necessary tests were performed. To determine systematic possession, BBL Crystal Identification system was applied (Anonymous 2005). Relative distribution of bacterial species was calculated.

RESULTS

The population density of *Cameraria orhidella* in traping stations was very high. In the end of September 2012 mean leaf damage area (in average) was 70.0 ± 2.3 % in Jelgava and 60.1 ± 15.2 % - in Salaspils.

Total mortality rate of *C. ohridella* larval stages, including spinning stages and pupae, was 47.14% in Jelgava and 16.15 -16.82% in Salaspils (Table 1). In Jelgava only some cases of larval death caused by fungi (0.70%) and bacteria (0.19%) were observed. Larval mortality, caused by pathogens, was higher at Salaspils trapping station than in Jelgava.

Eleven entomopathogenic fungi were isolated from collected dead specimens. Entomopathogenic fungi isolated from *C. ohridella* belong to division Ascomycota. Identified fungi belong to six genera: *Aspergillus; Hirsutella; Beauveria; Metarhizium; Lecanicillium; Isaria* (Table 2). One of the isolates is a non - sporulating mycelium.

We have identified bacteria, associated with field-collected larvae of *C. ohridella*. The results suggest that bacterial diversity from the surface of *C. ohridella* larvae was relatively simple - it consisted of six bacteria species and was dominated by gamma proteobacteria class bacteria. The most dominant bacterial species of pupae and larvae surface was *P. maltophilia* (Table 3). None of bacteria isolated from collected dead specimens in pure culture was entomopathogenic.

DISCUSSION

Obtained results showed that *C. ohridella* parasitism rate in Latvia is low: 1.6 – 6.1%, and concur with the conclusions of G.Grabenweger and C.Lethmayer (1999), that even after 20 years of moth infestation typical parasitism rates are merely 0–10%. High horse-chestnut leaf miner population density and low level of parasitism indicate inability of parasitoids to control the population of horse-chestnut leaf miner (Ferracini & Alma 2007).

M.Kalmus et al. (2008) proved in laboratory conditions that fungi *I. fumosorosea* and *L. muscarium* caused mortality of 98% and 96% on larvae, hatching from contaminated eggs. D.Richter et al. (2008) experimentaly proved that fungi *I. fumosorosea*, *L. muscarium*, *M. anisopliae* and *B. bassiana* spread on pupae and cause mortality of *C. ohridella.* He concluded, that fungus can aproximately reduce pest population to 60%. Therefore, in future the biocontrol potential of new isolates, obtained in our laboratory, will be tested.

Common insect pathogenic fungi recorded in Latvia are *B. bassiana*, *M. anisopliae* and *L. lecanii (= Verticillium lecanii*) *(*Jankevica 2004). L. Jankevica (2004) recorded associations of *M. anisopliae* and *L. lecani* only with pests from Coleoptera, Diptera, Lepidoptera, Homoptera and Thysanoptera. We recorded associations between lepidopteran pest and representatives of genus *Lecanicillium* and *Metarhizium* for the first time in Latvia. Our next step of identification will be using molecular techniques to differentiate among isolates *Isaria* sp*., Lecanicillium* sp. and *Hirsutella* sp.

Beneficial contribution of gut microbiota to host health are generally acknowleadge. Studies suggest that microorganisms provide essential nutrients or assit in important biochemical functions (Broderick et al. 2004). Insects from order Lepidoptera are herbivores and it have been proved that part of insect midgut microflora content comes from ingested plant material and diet has a significant impact on midgut microbial

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Table 2. The list of entomopathogenic fungi isolated from horse-chestnut leaf miner *Cameraria ohridella* specimens, collected at Salaspils trapping station in 2010 - 2012

#	Order: family	Genus	Species	Year of isolation
1.	Eurotiales: Trichocomaceae	*Aspergillus*	*Aspergillus flavus* Link ex Fries	2012
2.			*Aspergillus* sp. *LUBI –AS1*	2010
3.			*Aspergillus* sp. *LUBI –AS2*	2010
4.	Hypocreales: Clavicipitaceae	*Beauveria*	*Beauveria brongniartii* (Saccardo) Petch	2012
5.		*Metarhizium*	*Metarhizium anisopliae* (Metschnikov) Sorokin	2012
6.		*Lecanicillium*	*Lecanicillium sp. LUBI –VS2*	2012
7.		*Isaria*	*Isaria fomosorosea* Wize (formerly *Paecilomyces fumosoroseus*)	2010
8.			*Isaria sp. LUBI –PS2*	2012
9.	Hypocreales: Cordycipitaceae	*Hirsutella*	*Hirsutella* sp. *LUBI –HS1*	2010
10.			*Hirsutella* sp. *LUBI –HS2*	2010
11.		Non-sporulating mycelium	*Mycelia sterila LUBI –MS7*	2011

Table 3. Relative distribution of bacteria species on horse-chestnut leaf miner *Cameraria ohridella* larval and pupae surface

Identified bacteria species	Relative distribution of bacteria species, %	
	Larvae	Pupae
Leifsonia aquatica (ex Leifson 1962) Suzuki et al. 1999 = *Corynebacterium aquaticum* Leifson 1962	3.94	0.00
Enterobacter gergoviae Brenner *et al.* 1980	2.26	0.00
Enterobacter sakazakii Farmer *et al.* 1980	13.28	33.85
Pantoea agglomerans (Ewing and Fife 1972) Gavini *et al.* 1989	1.99	33.27
Pseudomonas maltophilia (*ex*Hugh and Ryschenkow 1961) Hugh 1981	78.09	32.14
Serratia plymuthica (Lehmann and Neumann 1896) Breed et al. 1948	0.43	0.74

diversity (Broderick et al. 2004, Staudacher et al. 2011) . It means that investigation of insect surface microflora can help to analyse diversity and content of midgut microlfora of insect. The present data show that all identified species from insect surface had been isolated from other Lepidoptera order insects midgut microflora and are characteristic to them. According to literature data, enteric bacteria are essential part of midgut microflora and can act as a synergist with entomopathogens (Broderick et al. 2004., Frankenhuyzen et al. 2010., Robinson et al. 2009).

Our investigation showed that there were four bacterial species, which are common for larva and pupae and in both cases dominat bacterial species were the same (Table 3). Gamma-proteobacteria group bacteria, which include *Serratia* sp., *Pseudomonas* sp. and *Enterobacter* sp. are mostly isolated from soil, plant material and animal intestines (Broderick et al. 2004, Park et al. 2007). Also, it should be noted, that this is first insight in microbial diversity of *C. ohridella* and to obtain more pricise data further studies are required.

CONCLUSIONS

Populations of horse-chestnut leaf miner *C. ohridella* in Latvia are not regulated by parasitoids (level of mortality1.6 – 6.1%. The impact of pathogens on the *C. ohridella* larvae was low – mortality rate was 0.9- 1.6 %.

Eleven species of entomopathogenic fungi, associated with *C. ohridella,* were isolated and identified. Ten of listed species belong to class Ascomycota and one isolate is a non - sporulating mycelium. Isolated entomopathogenic fungi are the representatives of genera *Aspergillus; Hirsutella; Beauveria; Metarhizium; Lecanicillium* and *Isaria.*

Further studies are necessary to analyse microflora of insects by applying more sensitive methods. All entomopathogenic fungi and opportunistic entomopathogenic bacteria isolates from current study will be further evaluated for their suitability as potential virulent entomopathogens.

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