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FILAMENTOUS FUNGI ASSOCIATED WITH *MONOCHAMUS GALLOPROVINCIALIS* AND *ACANTHOCINUS AEDILIS* (COLEOPTERA: CERAMBYCIDAE) IN SCOTS PINE

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Abstract. The composition and abundance of filamentous fungi species associated with *Monochamus galloprovincialis* and *Acanthocinus aedilis* on *Pinus sylvestris* in southern Poland were studied. Fungi were isolated from *A. aedilis* adults and *M. galloprovincialis* adults and their galleries, collected from three pine stands. In total, 214 fungal isolates from *A. aedilis* insect bodies and 232 fungal isolates from *M. galloprovincialis* adults were obtained; 1569 fungal isolates representing 39 species were obtained from *M. galloprovincialis* gallery systems. The most important groups of fungi were ophiostomatoid fungi and molds, including *Mucor*, *Penicillium* and *Trichoderma* species. Among ophiostomatoid fungi, *Ophiostoma minus* and *O. piceae* were the most frequently isolated species from *A. aedilis* and *M. galloprovincialis* adults, respectively. *Ophiostoma minus* was the dominant species in *M. galloprovincialis* galleries, with a frequency of 41%. Another relatively common fungus was *Graphium* sp. 'W', whereas other ophiostomatoid species were only occasionally isolated.

Key words: Blue-stain fungi, molds, ophiostomatoid fungi, cerambycid beetles, *Pinus sylvestris*, *Monochamus galloprovincialis*, *Acanthocinus aedilis*

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INTRODUCTION

The black pine sawyer beetle *Monochamus galloprovincialis* (Oliv.) is considered a serious secondary pest of Scots pine in Poland. It attacks mainly weakened trees, but when the population level of *M. galloprovincialis* is high it may also attack healthy trees. The larvae bore galleries in the phloem, sapwood and heartwood. This species prefers the higher part of stems. Adults have their maturation feeding in the crowns of trees, where they often injure the branches and needles (Kolk & Starzyk 1996).

In contrast to the black pine sawyer beetle, the timberman beetle *Acanthocinus aedilis* (L.) infests heavily weakened trees, stumps and windthrows. It breeds in the lower part of Scots pine trunks. Larvae develop under the bark or in the wood if the bark is thin (Kolk & Starzyk 1996).

The subcorticolous insects are associated with various fungi. The entomochoric ophiostomatoid fungi are frequent associates of phloem-feeding insects infesting coniferous trees, whereas hypocrealean *Geosmithia* species are often associated with bark beetles and other subcortical insects attacking deciduous trees (Kirschner 2001; Kirisits 2004; Kolařík 2006). Very little is known about the fungal associates of cerambycid beetles in Europe. The majority of studies have focused on the fungal species carried by *Tetropium* spp. (Mathiesen 1950; Mathiesen-Käärik 1953; Kotýnková-Sychrová 1966; Jacobs & Kirisits 2003; Jacobs *et al.* 2003). The species associated with this beetle genus were *Ophiostoma tetropii* Mathiesen, *O. kryptum* Jacobs & Kirisits, *O. minus* (Hedgc.) Syd. & P. Syd., *O. penicillatum* (Grosmann) Siemaszko and *O. piceae*

(Münch) Syd. & P. Syd. However, the primary fungal symbionts of cerambycid beetles are endosymbiotic fungi, mainly true yeasts and yeast-like fungi. They play an important role as suppliers of enzymes for degradation or detoxification of organic matter, particularly wood (Schomann 1937; Kühlwein & Jurzitza 1959; Buchner 1965; Dominik & Starzyk 1989; Jones *et al.* 1999).

An association between *M. galloprovincialis* and a filamentous fungal species has not been reported yet. Pashenova *et al.* (1994, 1998) and Jacobs *et al.* (2000) reported that *Leptographium sibiricum* Jacobs & M. J. Wingf. and *Ophiostoma* species were carried by *M. urussovi* (Fischer) on Siberian fir. Wingfield (1987) and Wingfield and Blanchette (1983) found a few *Ophiostoma* species in the galleries of *Monochamus* species. Wingfield (1987) isolated *O. minus* from cerambycid beetles collected from red pine bolts in Wisconsin. Japanese studies suggested that the pinewood nematodes carried by *Monochamus* beetles could transmit blue-stain fungi to trees (Kobayashi *et al.* 1974, 1975; Maehara & Futai 2002; Maehara *et al.* 2005).

In Sweden, *O. olivaceum* Math.-Käärik and *O. floccosum* Math.-Käärik have been found in tunnels of *A. aedalis* on Scots pine (Mathiesen 1950). The aim of this study was to identify species of filamentous fungi isolated from *M. galloprovincialis* and *A. aedilis* adults and from *M. galloprovincialis* galleries. The results will contribute to our understanding of the potential of the beetles to spread phytopathogenic and blue-stain fungi, as well as the potential role of fungi in the life cycle of the beetles.

Material and methods

Sampling areas

The study was conducted in 2005–2006 in two *P. sylvestris* L. stands. The first of these was a 50–55-year-old Scots pine stand in the Mielec Forest District (50°19′25″N/21°29′39″E). The second was a mixed forest stand *ca* 90 years old, located in the Olkusz Forest District (50°14′47″N/19°31′17″E). In addition, adults were collected from a large clearcut area in a 120-year-old pine stand (Niepołomice Forest District; 50°00′09″N/20°20′40″E).

Isolation of fungi from collected insects

Monochamus galloprovincialis adults were collected in two ways. First, adults were collected during the flight period (24–31 July 2005) from surfaces of pine timber lying in a large clearcut area in the Niepołomice Forest District. In the second instance, emerged *M. galloprovincialis* adults were taken from pupal chambers in dead *Pinus sylvestris* trees. To study the fungal species from emerged adults, six dead Scots pines (50 years old) infested by *M. galloprovincialis* were selected from the Mielec Forest District. On 25 May 2005 the trees were felled, cut into 150 cm logs and laid on the forest floor. After six weeks, living *M. galloprovincialis* adults were taken from pupal chambers.

Acanthocinus aedilis adults were collected during the flight period (12–19 April 2006), from logs and stumps.

In total, 169 *M. galloprovincialis* and *A. aedilis* adults were collected and stored individually in sterile Eppendorf tubes (1.5 ml) at 5°C. Within 12 hours of their collection, each beetle, without surface sterilization, was placed on Petri dishes containing two different culture media: 2% malt extract agar (MA; Difco Laboratories, Detroit, MI, USA) amended with 200 mg tetracycline sulphate/L, and 2% malt extract agar amended with 200 mg tetracycline sulphate/L and 100 mg cycloheximide/L. The first medium was intended to isolate all culturable fungi, and the second (selective) medium to isolate *Ophiostoma* species. Each adult was crushed using sterile tweezers and then placed directly onto culture media. The Petri dishes were incubated at room temperature. Where necessary, cultures were purified by transferring small pieces of mycelium or spore masses from individual colonies to fresh 2% MA. The fungal species were identified according to morphological and physiological characteristics by classical microbial techniques.

Isolation of fungi from Scots pine infested with *M. galloprovincialis*

In order to determine the frequency and diversity of fungi associated with *M. galloprovincialis*, standing dead trees infested by these insects were examined. The trees died in the year the samples were taken. Trees were felled in September–November. In total, 31 pines were analyzed. On these trees, eleven species of insects were found: *M. galloprovincialis, Arhopalus*

rusticus (L.), *Rhagium inquisitor* (L.), *Pogonocherus fasciculatus* (Deg.), *Tomicus piniperda* (L.), *T. minor* (Hrtg.), *Trypodendron lineatum* Ol., *Hylurgops palliatus* (Gyll.), *Phaenops cyanea* (Fabr.), *Pissodes piniphilus* (Herbst.) and *Pityogenes bidentatus* (Herbst.). Among them, *T. minor*, *P. bidentatus* and *M. galloprovincialis* occurred only in the higher part of the trunk. For fungal isolation only Scots pines infested by *M. galloprovincialis* were taken; the trees most heavily attacked by bark beetles were rejected. Wood samples for fungal isolation were taken from only 12 trees.

Four sections 20 cm long with intact bark were cut from parts of the trunk infested by *M. galloprovincialis*, located 10–14 m from the base of the trunk. In the laboratory each section was cut into two discs (10 cm thick), and then all discs with symptoms of feeding by other insects were rejected. The bark was separated from the wood under sterile conditions, and gallery fragments were disinfected by covering with cotton wool saturated with 96% ethanol, for 15 sec. Isolates were made from wood adjacent to beetle galleries and from pupal chambers. After drying, small wood samples (*ca* 4 × 4 mm) were placed in Petri dishes containing MA. The methods for culturing and identifying the fungi were as mentioned above. Altogether, 1062 fragments were taken from 60 galleries examined (Table 1).

Frequency of occurrence was defined as the percentage of isolates of individual taxa versus the total number of isolates. The frequency of isolates of ophiostomatoid species was calculated from results obtained from selective media. For other fungal species the frequency was calculated from the results with non-selective media.

Results

Fungal isolates from *Acanthocinus aedilis* beetles

A total of 214 fungal isolates representing 19 taxa were obtained from the insect bodies. *Mucor* sp. and *Trichoderma* sp. were the dominant species. They were isolated from 27% and 22% of the adults, respectively. Other frequently isolated species were *Beauveria bassiana* (Bals.-Criv.) Vuill. (15%), *Penicillium* sp. 1 (9%) and *Ophiostoma minus* (8%). The frequency of occurrence of the remaining fungal species was <4% (Table 1). Apart from *O. minus*, blue-stain fungi were not commonly found on the body surface of *A. aedalis*. From *A. aedilis* adults, four species of blue-stain fungi were identified: *Hormonema dematioides* Lagerb. & Melin, *O. minus*, *O. piceae* and *Leptographium procerum* (W. B. Kendr.) M. J. Wingf. (Table 1).

Fungal isolates from *Monochamus galloprovincialis* beetles

232 fungal isolates representing 26 fungal taxa were found to be associated with the insect. *Penicillium* sp. and *Mucor* sp. were commonly isolated from the beetles. They were isolated from 29% and 21% of the adults, respectively. Other frequently isolated species were *Alternaria alternata* (Fr.) Keissl. (7%) and *Cladosporium cladosporioides* (Fresen.) G. A. de Vries (6%). Overall, five blue-stain species were isolated from the beetles. These blue-stain fungi were isolated from emerged beetles as well as from beetles collected during the flight period. The most common species among them were *O. piceae* (6%), *H. dematioides* (5%) and *O. minus* (5%) (Table 1).

Fungal isolates from *Monochamus galloprovincialis* gallery systems

A total 1569 fungal isolates representing 39 taxa were obtained from gallery systems. Fungi were isolated from 90% of 1062 plant fragments (Table 1). The most frequent group of fungi were blue-stain fungi, represented by seven species: *H. dematioides*, *O. minus*, *O. piceae*, *Graphium pseudormiticium* M. Mouton & M. J. Wingf., *G. pycnocephalum* Grosm., *Hormonema* sp. and *Graphium* sp. 'W'. Among them, three *Graphium* species and *Hormonema* sp. were isolated only from gallery systems (Table 1).

Among the blue-stain fungi, *Ophiostoma minus* occurred most frequently (41%) (Table 1). Two other ophiostomatoid fungi, *O. piceae* and *Graphium* sp. 'W', were isolated from 4–8% of the samples. Among non-ophiostomatoid fungi, *Trichoderma* sp., *Lecythophora hoffmannii* (J. F. H. Beyma) W. Gams & McGinnis and two species of Basidiomycetes were most abundant (Table 1).

Table 1. Fungi isolated from *Monochamus galloprovincialis* (Oliv.) and *Acanthocinus aedilis* (L.) adults and from *M. galloprovincionalis* gallery systems.

Taxon	Number of isolates (% frequency)		
	Acanthocinus aedilis	*Monochamus galloprovincialis*	
	beetles	beetles	galleries
Acremonium charticola (J. Lindau) W. Gams,	5(2.3)	1(0.4)	5(0.3)
Alternaria alternata (Fr.) Keissl.	8(3.7)	16(6.9)	6(0.4)
Apiospora montagnei Sacc.	2(0.9)	3(1.3)	21(1.3)
Beauveria bassiana (Bals.-Criv.) Vuill.	32(15.0)	3(1.3)	
Botrytis cinerea Pers.	1(0.5)		1(0.1)
Chaetomium sp.	1(0.5)		
Chrysosporium sp.		1(0.4)	
Cladosporium cladosporioides (Fresen.) G. A. de Vries	2(0.9)	14(6.0)	6(0.4)
Cladosporium sphaerospermum Penz.		6(2.6)	
Cytospora sp.			40(2.5)
Dipodascus aggregatus Francke-Grosm.			6(0.4)
Epicoccum nigrum Link	4(1.9)		7(0.4)
Exophiala sp.			32(2.0)
Fusarium oxysporum Schltdl.		2(0.9)	8(0.5)
Fusarium sp.	2(0.9)		
Fusicoccum sp. like			30(1.9)
Geosmithia sp.			52(3.3)
Gliocladium catenulatum J. C. Gilman & E. V. Abbott		1(0.4)	2(0.1)
Graphium pseudormiticum M. Mouton & M. J. Wingf.			1(0.1)
Graphium pycnocephalum Grosm.			31(2.0)
Graphium sp.'W'			132(8.4)
Hormonema dematioides Lagerb. & Melin	4(1.9)	12(5.2)	6(0.4)
Lecanicillium lecanii (Zimm.) Zare & W. Gams		3(1.3)	
Lecythophora hoffmannii (J. F. H. Beyma) W. Gams & McGinnis			70(4.5)
Leptodontidium beauverioides (de Hoog) de Hoog			4(0.3)
Leptographium procerum (W. B. Kendr.) M. J. Wingf.	4(1.9)	1(0.4)	
Mortierella sp.			8(0.5)
Mucor sp.	58(27.1)	48(20.7)	19(1.2)
Oidiodendron tenuissimum (Peck) S. Hughes		3(1.3)	
Ophiostoma minus (Hedgc.) Syd. & P. Syd.	16(7.5)	11(4.7)	648(41.3)
Ophiostoma piceae (Münch) Syd. & P. Syd.	4(1.9)	13(5.6)	63(4.0)
Ophiostoma piliferum (Fr.) Syd. & P. Syd.		1(0.4)	1(0.1)
Paecilomyces farinosus (Holmsk.) A. H. S. Br. & G. Sm.		4(1.7)	
Penicillium sp. 1	20(9.3)	51(22.0)	57(3.6)
Penicillium sp. 2		17(7.3)	8(0.5)
Pestalotia stevensonii Peck		1(0.4)	
Pezicula cinnamomea (DC.) Sacc.			32(2.0)
Pezicula eucrita P. Karst.			57(3.6)
Phialocephala sp.	2(0.9)	1(0.4)	14(0.9)
Phialophora sp.		3(1.3)	
Phoma sp.			4(0.3)
Rhizoctonia sp.			20(1.3)
Scopulariopsis sp.	1(0.5)		
Sepedonium chrysospermum (Bull.) Fr.			2(0.1)

Table 1. *Continued.*

Taxon	Number of isolates (% frequency)		
	Acanthocinus aedilis	*Monochamus galloprovincialis*	
	beetles	beetles	galleries
Sordaria fimicola (Roberge ex Desm.) Ces. & De Not.			1(0.1)
Sporothrix sp.			9(0.6)
Stachybotrys atra Corda			2(0.1)
Trichoderma sp.	46(21.5)	8(3.4)	90(5.7)
UNIDENTIFIED			
Basidiomycetes (2 species)			62(4.0)
Other (4 species)	2(0.9)	8(3.4)	12(0.8)
Number of isolates	214	232	1569
Number (percentage) of sterile beetles/gallery fragments	0	0	109(10.3)
Number of beetles/gallery fragments	88	81	1062

DISCUSSION

This study showed that blue-stain fungi are associated with *M. galloprovincialis* and *A. aedalis*. These fungi can be isolated from the surface of beetles as well as from gallery systems. Except in *M. galloprovincialis* gallery systems, however, blue-stain fungi were not the most common fungal associates, with molds (mainly *Penicillium*, *Trichoderma* and *Mucor* species) isolated more frequently. A similar spectrum of blue-stain fungi was found on insects and in galleries of *Tomicus piniperda* in Scots pines in Poland (Siemaszko 1939; Jankowiak 2006a; Jankowiak & Kurek 2006; Jankowiak & Bilański 2007). This was not surprising because *T. piniperda* and *M. galloprovincialis* often breed together in Scots pine trunks.

Ophiostoma minus was the most commonly isolated ophiostomatoid species from cerambycid adults. This species was also most frequently isolated from *Monochamus* galleries. A similar situation was described by Wingfield (1987) in his study of fungi associated with cerambycid beetles in Wisconsin, although he found *O. minus* as a fungal associate of other *Monochamus* species. He also found other *Ophiostoma* species not found in this study. Japanese *in vitro* studies showed that beetles carried a greater number of nematodes when *O. minus* dominated the wood around the pupal chamber of *M. alternatus* Hope (Kobayashi *et al.* 1974, 1975; Maehara *et al.* 2005).

Ophiostoma minus is known to be distributed worldwide (Upadhyay 1981) and associated mainly with different phloeophagous bark beetles (Kirisits 2004). In Poland this fungus was found associated with *T. piniperda*, *Ips sexdentatus* (Börn.), *H. palliatus* (Siemaszko 1939; Jankowiak 2006a, b; Jankowiak & Kurek 2006; Jankowiak & Bilański 2007) and *T. minor* (Jankowiak, unpublished). This species was able to kill *P. sylvestris* trees in experimental mass inoculations (Långström *et al.* 1993; Solheim *et al.* 1993).

Our isolation data showed that *O. minus* was isolated much more frequently from *M. galloprovincialis* galleries than from adult insects. This suggests that *O. minus* is introduced to pine trunks at low frequency, and then probably rapidly colonizes sapwood and adjacent uninfected gallery systems. The hyphae of *O. minus* probably are concentrated in the ray parenchyma and resin ducts of infected sapwood (Münch 1907). These results confirmed the findings of Jankowiak and Kurek (2006), who isolated *O. minus* from *T. piniperda* galleries frequently ten weeks after the beetles' main attack; after four weeks the frequency of this species was much lower.

Apart from *O. minus*, cerambycid beetles were also associated with *O. piceae* in this study. It was isolated more frequently from *M. galloprovincialis* than from *A. aedilis* adults. *Ophiostoma piceae* is widely distributed in nature and is considered a generalist, not associated exclusively with any

particular insect or tree species. Its presence on timberman beetles is not surprising because this fungus was also frequently carried by *A. aedilis* in Mathiesen's investigations (Mathiesen 1950). *Ophiostoma piceae* was commonly found as a saprophyte on conifer timber (Seifert 1993) or as a weak pathogen on *Picea* spp. and *Pinus* spp. (Nevil & Alexander 1992; Krokene & Solheim 1998; Yamaoka *et al.* 2000; Jankowiak 2006a).

Our studies showed that cerambycid adults could carry propagules of ophiostomatoid fungi on the body surface. Unlike bark beetles, however, adult cerambycid beetles have limited contact with pine trees because they do not penetrate the bark and phloem during oviposition. These insects have contact with pine trees only during maturation feeding and when the females, using their ovipositors, deposit eggs on the bark surface in cracks and underneath the scales (Kolk & Starzyk 1996; Anbutsu & Togashi 2000). Thus, phytopathogenic fungi carried by cerambycid adults may play an important role in branch dieback during maturation feeding, when they damage the bark. When female beetles lay eggs in the bark the spores are probably transmitted in a different way. It seems that nematodes and mites associated with cerambycid beetles may act as secondary vectors of these fungi to the insect gallery (Wingfield & Blanchette 1983; Moser *et al.* 1989; Ryss *et al.* 2005; Naves *et al.* 2006). Many aspects of cerambycid beetle-ophiostomatoid species associations are poorly understood, and much more research is needed.

Molds were also commonly isolated from ceramybcid adults in this study. These fungi are widely distributed in nature, and the majority of them have been isolated from *T. piniperda* and from other insects (Kirschner 2001; Jankowiak & Kurek 2006; Jankowiak & Bilański 2007). The presence of mold species may be the result of the contact of cerambycid adults with the forest floor during the flight period, especially in the case of *A. aedilis* which often colonizes pine stumps.

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