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Original article

Fungi associated with *Tomicus piniperda* in Poland and assessment of their virulence using Scots pine seedlings

Robert JANKOWIAK*

Agriculture University of Cracow, Department of Forest Pathology, Al. 29 Listopada 46, 31-425 Cracow, Poland

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Abstract – The species composition and virulence of fungi associated with *Tomicus piniperda* were studied at eight locations in Poland. The fungi were isolated from phloem and sapwood samples taken from insect galleries and then identified. The virulence of the most common ophiostomatoid species was evaluated through inoculations using two-year-old Scots pine seedlings. A great diversity of fungi were found associated with *T. piniperda*, including 4 837 cultures and 67 species. The most important groups of fungi were the ophiostomatoids and moulds, including mainly *Penicillium*, *Trichoderma* and *Mucor* species. Among ophiostomatoid fungi, *Ophiostoma minus* and *O. piceae* dominated, with a frequency of occurrence of 32.4 and 11.5% of inspected galleries, respectively. Occasionally isolated species included *Leptographium lundbergii*, *L. procerum*, *L. wingfieldii*, *Graphium pycnocephalum* and *Graphium* sp. 'W'. In general, the frequency of the ophiostomatoid species was highly variable among locations. *Leptographium wingfieldii* and *O. minus*, were the only species capable of killing whole plants and penetrated deeper into the sapwood than other species (87–100% mortality during the 11 week incubation period). Other fungi, including *O. piliferum*, *O. piceae* and *L. procerum*, were considerably less virulent.

associated fungi / *Leptographium* / *Ophiostoma* / *Pinus sylvestris* / *Tomicus piniperda* / virulence

Résumé – Champignons associés à *Tomicus piniperda* en Pologne et appréciation de leur virulence pour des plants de pin sylvestre. La composition spécifique et la virulence des champignons associés à Tomicus piniperda ont été étudiées dans huit localités polonaises. Les champignons ont été isolés d'échantillons de liber et d'aubier récoltés à partir des galeries des insectes, puis identifiés. La virulence de l'espèce d'*Ophiostoma* la plus commune a été évaluée en utilisant des plants de Pin sylvestre de deux ans. Une grande diversité de champignons associés à *T. piniperda* a été récoltée, représentant 4 837 cultures et 67 espèces. Les groupes les plus importants sont les Ophiostomatoïdes et les moisissures, dont principalement des espèces de *Penicillium*, *Trichoderma* et *Mucor*. Parmi les Ophiostomatoïdes, *Ophiostoma minus* et *O. piceae* dominent, avec une constance respective de 32.4 % et 11.5 % dans les galeries examinées. Des espèces ont été isolées occasionnellement telles que *Leptographium lundbergii*, *L. procerum*, *L. wingfieldii*, *Graphium pycnocephalum* and *Graphium* sp. 'W'. En général, la fréquence des espèces d'Ophiostomatoïdes a été très variable selon les localités. *Leptographium wingfieldii* et *O. minus* furent les seules espèces capables de tuer des plantes entières et ont pénétré plus profondément dans l'aubier que les autres espèces (mortalité de 87–100 % en 11 semaines d'incubation). Les autres champignons, dont *O. piliferum*, *O. piceae* and *L. procerum,* ont été considérablement moins virulents.

champignons associés / *Leptographium* / *Ophiostoma* / *Pinus sylvestris* / *Tomicus piniperda* / virulence

1. INTRODUCTION

Scots pine (*Pinus sylvestris* L.) is the most important forest tree species in East-Central Europe. In Poland, forest stands with Scots pine dominance occupy 68% of the total forest area [47]. The larger pine shoot beetle, (*Tomicus piniperda* (L.) Coleoptera: Scolytidae) is native to Europe, North Africa and Asia, but has also been introduced to the United States [7, 8]. *Tomicus piniperda* is one of the most destructive pests of pine forests in Europe, where it is considered to be a secondary colonizer of pine tree stems. *Tomicus piniperda*, together with its cogener *T. minor* (Hrtg.), ranks among the main insect pests of Scots pine in Poland. Other *Pinus* species also occasionally become infested. *Tomicus piniperda* completes one generation per year. The adults overwinter in the base of pine trees or into the soil and initiate flight on the first warm days of March. The adult beetles usually colonize recently fallen, weakened or dead trees but can also attack healthy trees (often in pine stands growing near sawmills and wood yards). The new adults in July emerge through the bark and attack new shoots on pine trees of all ages. The beetle attacks most of the lateral shoots near the top of pine trees, causing top damage and growth reduction [29, 40].

Phloeophagous bark beetles are associated with various fungi belonging to the yeasts, basidiomycetes, ascomycetes and anamorphic fungi without sexual states. Ophiostomatoid fungi, including the genera *Ceratocystis*, *Ophiostoma*, *Ceratocystiopsis* and their anamorphs, are the most important associates of scolytids [20, 54, 57]. These fungi include various economically important plant pathogens and agents of sapstain. Some of the ophiostomatoid fungi associated with scolytids, such as *Ceratocystis polonica* (Siem.) C. Moreau, play a role in overwhelming the resistance of vigorous trees [3, 11, 18, 26, 27].

In Europe, *T. piniperda* carries numerous species of *Ophiostoma* and their anamorphs [6, 12–14, 19, 21, 24, 32, 39,

* Corresponding author: rljankow@cyf-kr.edu.pl

Figure 1. Location of sites where *Tomicus piniperda* galleries were collected. 1: Mielec, 2: Babimost, 3: Niepołomice, 4: Krynki, 5: Opole, 6: Oleszyce, 7: Świerklaniec, 8: Kańczuga.

40, 42, 46, 48, 50, 51, 56], with *O. minus* and *Leptographium wingfieldii* being the dominant species. In Poland, there is only one report on fungi associated with *T. piniperda*. Siemaszko [50] found that *O. minus*, *O. piceae* and *O. piliferum* were associated with *T. piniperda* in pine stands near Warsaw.

The pathogenicity of blue-stain fungi has been studied by fungal inoculation of large trees (with low or high inoculation densities) and seedlings. The results of the inoculation experiments with fungal associates of *T. piniperda* indicate that *L. wingfieldii* and *O. minus* are able to overcome the defense mechanisms of Scots pine and may kill healthy trees [4, 5, 30, 31, 33, 34, 51–53].

In this study species composition and occurrence frequency of fungi associated with *T. piniperda* were investigated. In addition, the pathogenicity of several blue-stain fungi associated with *T. piniperda* was investigated by inoculating Scots pine seedlings.

2. MATERIALS AND METHODS

2.1. Study areas

All materials were collected in 2005 from eight locations: Babimost Forest District (52° 18' 01" N, 15° 46' 03" E); Niepołomice Forest District (49° 59' 53" N, 20° 19' 56" E); Krynki Forest District (53° 14' 17" N, 23° 37' 46" E); Opole Forest District (50° 40' 46" N, 18° 15' 20" E); Oleszyce Forest District (50° 07' 45" N, 22° 57' 17" E); Świerklaniec Forest District (50° 31' 50" N, 19° 00' 56" E); Mielec Forest District (50° 19' 25" N, 21° 29' 39" E); Kańczuga Forest District (50° 00' 50" N, 22° 12' 49" E) (Fig. 1). All sites were located within 40–50 years old pine stands, where *P. sylvestris* was the dominant species. The selected stands showed clear symptoms of the presence of *T. piniperda*, including dieback, yellowing, and especially dead, bored-out shoots littering the ground under infested trees. In order to determine the species richness and frequency of fungi associated with *T. piniperda* at these locations trap trees were used. In Mielec, the main study location, trap trees were placed in pine stand growing near a timber store. In this stand, the trees were heavily damaged by shot-feeding of *T. piniperda*. In contrast, the trap trees at the other locations were placed in stands where the pine trees were only slightly damaged by *T. piniperda*.

2.2. Sampling, isolation and identification of fungi

In Mielec, 24 uninfested Scots pine trees were felled in early March. In the other sampling locations only four trap trees were felled. Felled trees were laid flat on the forest floor and left for colonization by *T. piniperda*. The main attacks started the 10th of March 2005 and continued for the next three weeks. Samples from the trees were taken 6 to 8 weeks after the main attack when brood development had reached the stage where both egg and larvae were present. Four 30 cm long stem sections with intact bark were cut from infested parts of the trunk and transported to the Agriculture University of Cracow. The sections were cut from the trunk at a distance between 2 and 8 m from the base of the trees. In the laboratory the bark was separated from the wood and gallery fragments were removed and disinfected using cotton wool saturated with 96% ethyl alcohol. The disinfection lasted approximately 15 s, and then gallery fragments were dried on filter paper. Small subsamples for isolation of fungi (4 × 4 mm) were collected from the phloem around eggs and larval galleries, and from the discoloured sapwood underneath the galleries to a depth of 20 mm into the sapwood. The surface layer of phloem was removed with a sterile scalpel, and subsamples of phloem or sapwood, were cut with a sterile scalpel or a chisel and placed on culture medium in Petri dishes.

All isolations were made on 2% malt extract agar (2% MEA; 20 g malt extract, 20 g agar, 1000 mL distilled water) supplemented with the antibiotic tetracycline (200 mg per 1 L of culture medium) to inhibit bacterial growth. When necessary, cultures were purified by transferring small pieces of mycelium or spore masses from individual colonies to fresh 2% MEA. The primary isolation plates were incubated at room temperature in the dark. Emerging fungi were identified on the basis of morphological characters such as perithecia, ascospores, conidiophores, and conidia. Altogether, 4 702 subsamples of phloem and sapwood were collected in this study. Over 60% of samples were taken from Mielec.

The frequency of occurrence of each fungal species was expressed as the percentage of phloem and sapwood fragments from which the species was isolated relative to the total number of fragments from which isolations were made. Occurrence frequencies were computed together for phloem and sapwood fragments.

2.3. Pathogenicity test

In the inoculation tests the following fungi isolated from galleries of *T. piniperda* were used: *Ophiostoma minus*, *O. piliferum*, *O. piceae*, *Leptographium procerum* and *L. wingfieldii*. Two randomly selected isolates of each species were used (Tab. I).

Two-year-old seedlings of *P. sylvestris* growing in containers with a mix of peat and perlite (8.5:1.5) were used in this study. The plants were placed outside under natural lighting and temperature conditions. The seedlings were watered during the experiment.

On 5th June 2005, 15 seedlings were inoculated with each of the 10 selected isolates. In addition, 30 plants were inoculated with sterile agar as a control. Inoculations were made by cutting a bark flap (4 × 8 mm) with a sterile scalpel, placing inoculum on the exposed sapwood surface and covering it up with the bark flap and a Parafilm® M strip. Inoculum consisted of a 3 mm disc of fungus growing on 2% MEA or sterile 2% MEA. Inoculum were removed from the margin of twelve-day-old cultures. Fungal inoculums growing at 22 °C.

Observations of plant mortality were made at weekly intervals for 11 weeks. Seedlings were considered dead when all needles above

Table I. The results of the inoculation experiments with fungi associated with *Tomicus piniperda* on two-year-old plants. Depth of sapwood blue-stain and lesion length with the same letter were not significantly different according to the Fisher's test ($P = 0.05$) following ANOVA. % dead plants with the same letter were not significantly different according to the chi-square test ($P = 0.05$). ($n = 15$, except for control where $n = 30$).

Species	Isolate**	Mean depth of sapwood blue-stain (mm)	Mean lesion length (mm)	% dead plants
Ophiostoma minus	O.m 535	1.1^{F}	–*	93^{A}
	O.m 443	1.0^{EF}	–*	100^{A}
Ophiostoma piliferum	O.p 558	0.7^{BD}	14.4^{D}	33^{BC}
	O.p 559	0.8^{BE}	13.1^{CD}	0^{B}
Ophiostoma piceae	O. pic 485	0.6^{BCD}	12.0^{C}	0^{B}
	O. pic 553	0.8^{BE}	12.2^{C}	20^{B}
Leptographium procerum	L.p 122	0.5^{CD}	8.7^{B}	20^{B}
	L.p 992	0.4^{C}	9.0^{B}	7^{B}
Leptographium wingfieldii	L.w 460	1.2^{FG}	–*	87^{A}
	L.w 506	1.4^{G}	–*	100^{A}
	Control	0^{A}	6.2^{A}	0^{BC}

* Necrotic lesions length could not be measured since the plants were killed.

** All isolates were collected in Mielec, Poland, 2005, except L.p 992 which was collected in Oleszyce.

Numbers refer to the culture collection of the Laboratory of Department of Forest Pathology, Hugo Kołłątaj University of Agriculture, Cracow, Poland.

the wound were discolored. After 11 weeks all plants were harvested and the bark was removed around the inoculation site. The length of the necrotic lesion on the sapwood surface and depth of any sapwood blue-stain was measured. Necrosis lengths could not be measured on plants inoculated with *O. minus* and *L. wingfieldii*, since the whole plants were dead and necrotic below the inoculation site. The data were analysed using analysis of variance (ANOVA). Significant treatment differences were further evaluated by Fisher's (LSD) test. For fungal isolates, 2×2 tables and chi-square test were used to detect differences in plant mortality (STATISTICA® 6.0 (StatSoft, Inc., Tusla, USA).

Re-isolations of the inoculated fungi were attempted by removing a small sapwood samples near the points of inoculation and incubating them on 2% MEA at 22 °C.

3. RESULTS

3.1. Fungal isolation

In this study, 4 837 fungal isolates were isolated from gallery systems of *T. piniperda*. Overall, 55.5% of the 4 702 subsamples taken from colonized trees contained fungi. In total 67 species of fungi were isolated, including several unidentified species (Tab. II). Most isolates represented ascomycetes and anamorphic fungi, but a few zygomycetes and basidiomycetes were also isolated. The most abundant group of fungi were the blue-stain fungi and the moulds, including mainly *Penicillium*, *Trichoderma* and *Mucor* species. Twelve species of ophiostomatoid fungi were isolated, including *Ophiostoma minus*, *O. piceaperdum*, *O. piceae*, *O. piliferum*, *O. canum*, *Ceratocystiopsis minuta*, *Leptographium procerum*, *L. lundbergii*. *L. wingfieldii*, *Graphium pycnocephalum*, *G. pseudormiticum* and *Graphium* sp. 'W' (Tab. II). Among these species, *O. minus* was the most frequently isolated one, occurring in 32.4% of the subsamples. Another common fungus was *O. piceae*, whereas the other species were occasionally isolated (Tab. II).

The frequencies of occurrence of ophiostomatoid fungi varied considerably between the eight study locations (Tab. II). *Ophiostoma minus* was the most consistently occurring species, being present in all locations and dominating in Opole, Świerklaniec and Mielec. *Ophiostoma piceae* was the most common fungus at Krynki, *L. lundbergii* was dominating in Niepołomice and *L. procerum* at Oleszyce (Tab. II).

The highest number of ophiostomatoid species was found in Mielec (10 species), and *Ceratocystiopsis minuta*, *L. wingfieldii*, *O. piceaperdum*, *G. pycnocephalum* and *Graphium* sp. 'W' were detected only at this location. Only 1–5 ophiostomatoid species were found in the other locations (Tab. II).

The non-ophiostomatoid species also varied considerably between locations and they were more frequent than ophiostomatoid fungi (Table II). *Hormonema dematiodes* was the most common species at Babimost, whereas *Penicllium* spp. were most frequently isolated in Opole, and yeasts and *Peziculа eucrita* were most frequent in Kańczuga (Tab. II).

3.2. Pathogenicity test

Ophiostoma minus (isolate 535 and 443) and *L. wingfieldii* (isolate 460 and 506) killed > 87% of the two-year-old plants within 2–4 weeks after inoculation (Tab. I). Development of brownish lesions on the stems and yellowing of needles were already obvious after 2 weeks. Because the entire stem, both above and below the inoculation place, was dead, necrotic lesions could not be measured. The other inoculated fungi killed < 33% of the seedlings (Tab I), and the stem and needles below the inoculation site generally showed no external symptoms. No control plants died (Tab. I).

All the inoculated fungi caused sapwood blue-stain in the two-year-old seedlings and the most virulent species

Table II. Frequencies of occurrence (%) of fungi isolated from *Tomicus piniperda* gallery systems on Scots pine collected at eight locations in Poland.

	Locations[a]								
Fungi	1	2	3	4	5	6	7	8	Total
Ophiostomatoid fungi									
Ceratocystiopsis minuta (Siemaszko) H.P. Upadhyay & W.B. Kendr.	0.2								0.1
Ophiostoma canum (Münch) Syd. & P. Syd.						0.8			< 0, 1
Ophiostoma minus (Hedgc.) Syd. & P. Syd.	36.0	12.5	0.8	1.9	15.4	10.4	51.7	1.2	32.4
Ophiostoma piceae (Münch) Syd. & P. Syd.	13.9	7.9	8.8	32.9		1.7	1.7		11.5
Ophiostoma piceaperdum (Rumb.) von Arx				2.9					0.1
Ophiostoma piliferum (Fr.) Syd. & P. Syd.	1.2	0.4							0.8
Leptographium lundbergii Lagerb. & Melin	1.1		31,7	3.8	7.5				2.9
Leptographium procerum (W.B. Kendr.) M.J. Wingf.	1.4			2.4		42.5	2.5		3.3
Leptographium wingfieldii M. Morelet	1.6								1,0
Graphium pycnocephalum Grosm.	4.1								2.6
Graphium psedormiticum M. Mouton & M.J. Wingf.	0.4	0.4	0.4						0.3
Graphium sp. 'W'	2.2								1.4
Other species									
Acremonium sp.	0.2								0.1
Alternaria alternata (Fr.) Keissl.	0.2							0.4	0.1
Aspergillus niger Tiegh.	0.1			0.5		0.4			0.1
Chloridium viride Link	0.2								0.1
Cladosporium cladosporoides (Fresen.) G.A. de Vries	0.1			0.5			0.9	0.4	0.1
Dipodascus aggregatus Francke-Grosm.	1.2	0.8			7.1				1.2
Geotrichium sp.	0.6								0.4
Gliocladium catenulatum J.C. Gilman & E.V. Abbott	0.2								0.1
Epicoccum nigrum Link	0.2			0.5			0.4		0.2
Fusarium sp.	0.2								0.1
Hormonema dematioides Lagerb. & Melin	3.4	34.6	5.0		8.3		2.5	0.4	4.8
Lecythophora hoffmannii (van Beyma) W. Gams & McGinnis	1.8	0.4		1.4				2.4	1.6
Mortierella ramanniana var. *ramanniana* (A. Möller) Linnem.	1.1			0.5			2.1		0.9
Myrothecium cf. *indicum* P.Rama Rao	0.3			1.4					0.3
Mucor sp.	2.4	3.3		0.9		3.3	1.3		2,0
Oidiodendron tenuissimum (Peck) S. Hughes	0.6								0.4
Penicillium spp.	17.4	20.8	2.9	20.9	17.9	11.3	10.8	10.3	16.0
Pezicula eucrita P. Karst.	0.9			1.4	0.8			27.4	2.1
Phialocephala cf. *dimorphospora* Kendrick	0.2						32.9	4.4	2.0
Phialophora sp.	0.2		0.4					0.4	0.1
Phoma pinastrella Sacc.	0.1	0.8							0.1
Phomopsis occulta Trav.	1.2								0.8
Rhinocladiella atrovirens Nannf.	0.5								0.3
Sepedonium chrysospermum (Bull.) Fr.	0.2	0.4	0.8	1.9					0.3
Sporothrix sp.	0.4								0.3
Trichoderma spp.	9.5	2.9	0.8	2.4	2.1	5.0	4.2	1.2	6.9
Yeasts	5.6		6.3	15.7				38.5	6.7
Others[b]									
Unidentified									
Basidiomycota (2 species)	0.2								0.1
Other (6 species)	2.7	0.8	1.3		0.4		0.4	1.2	2
Percentage of "sterile" fragments	46.2	38.8	45.4	50.9	45.0	33.3	6.7	70.2	44.5
Number of investigated fragments	3 040	240	240	210	240	240	240	252	4 702

[a] Location number: 1-Mielec, 2-Babimost, 3-Niepołomice, 4-Krynki, 5-Opole, 6-Oleszyce, 7-Świerklaniec, 8-Kańczuga.

[b] Frequency of occurrence of species which could be isolated from 1–5 fragments galleries, include *Arthrinium* state of *Apiospora montagnei* Sacc., *Botrytis cinerea* Pers., *Ceuthospora* sp., *Cladosporium sphaerospermum* Penz., *Chaetomium globosum* Kunze, *Eladia* sp., *Geosmithia* sp., *Harposporium* sp., *Helicoma* state of *Lasiosphaeria pezicula* (Berk & Curt.) Sacc., *Leptodontidium beauverioides* de Hoog, *Mortierella isabellina* Oudem., *Nigrospora* sp., *Paecilomyces farinosus* (Holmsk.) A.H.S. Br. & G. Sm., *Phialophora bubakii* (*Laxa) Schol-Schwarz, Phialophora clavispora* W. Gams, *Rhizoctonia* sp., *Sarcinella* sp., *Trimmatostroma abietis* Butin & Pehl, *Thysanophora penicillioides* (Roum.) W. B. Kendr., *Verticillium falcatum* (Petch) W. Gams.

(*L. wingfieldii* and *O. minus*) generally caused the most severe symptoms (Tab. I).

Ophiostoma piliferum and *O. piceae* induced significantly longer necrotic lesions than *L. procerum* and all fungi induced longer lesions than sterile inoculated control plants (Tab. I).

The inoculated fungi were successfully reisolated from 20–47% of the plants, except for *O. piceae*, which was reisolated from 60–80% of the plants.

4. DISCUSSION

Isolation of > 60 species of fungi from *T. piniperda* galleries in Scots pine demonstrate that this beetle is associated with a great diversity of filamentous microfungi in Poland. Ophiostomatoid fungi were the most common fungal associates, but *Hormonema dematioides* and moulds, including mainly *Penicillium*, *Trichoderma* and *Mucor* species were also isolated frequently. In this study, 12 ophiostomatoid fungi were found in galleries of Scots pine infested by *T. piniperda*. A similar spectrum of ophiostomatoid fungi has been found associated with *T. piniperda* in other parts of its distribution range in Europe [6, 32, 39, 40, 46, 48, 50, 51]. Recently, some researchers have recorded that the mycobiota of *T. piniperda* in Asia differ from the European ones [14, 17, 25–38, 58]. The one record of blue-stain fungi associated with *T. piniperda* was made by Siemaszko in Poland, who found *O. minus, O. piceae* and *O. piliferum* [50]. All the fungi displayed by Siemaszko were also found in this study, apart from nine ophiostomatoid species.

Among ophiostomatoid fungi, *O. minus* was commonly found in galleries of *T. piniperda* on Scots pine in this study. This species occurred at all locations with frequencies ranging from 0.8 to 51.7%. The results of this study suggest that *O. minus* is the most common fungus associated with *T. piniperda* in Poland. On the other hand, this fungus had highly variable frequency of occurrence. Similar considerable variation in the occurrence frequency of *O. minus* was found by Lieutier et al. in France [32]. The association between *T. piniperda* and *O. minus* appears to be inconsistent. *O. minus* occurred at high frequencies in Sweden [51], Poland [50] and Japan [35, 36]. In contrast it was only rarely found in England [6]. In French [32] and Swedish [51] studies, *O. minus* was found to occur at highly variable and moderately high frequencies.

Ophiostoma piceae was consistently isolated from galleries of *T. piniperda*. The relatively high occurrence frequency of *O. piceae* was unexpected in comparison to the results of the earlier studies. This fungus was not recorded at all in some studies [32], but occurred occasionally in investigations conducted in Sweden [51], Poland [50], England [6] and Austria [19]. *Ophiostoma piceae* is known to be a common associate of phloeophagous bark beetles in North America and Eurasia [20, 57]. This study showed that *O. piceae* was relatively important associate of *T. piniperda* in Poland.

The pathogenic species *L. wingfieldii* was isolated at very low frequency in only one of the eight localites, and this is the first report of that fungus in Poland. Lieutier et al. [32] reported that in France *L. wingfieldii* was isolated at a low but uniform frequency. A similar situation was described by Gibbs and Inman in southern England [6]. In contrast, it was more closely associated with *T. piniperda* in Sweden [51]. Gibbs and Inman [6] reported that *L. wingfieldii* was isolated at a highly variable frequency from the various developmental stages of brood galleries. It was isolated at low frequency from young galleries, but more frequently from older galleries. This suggested that propagules of *L. wingfieldii* had been introduced by a few colonizing adults and then grew rapidly along the medullary rays and tracheids and established itself in some of the initially uninfected gallery systems. Although the results from the present study are difficult to compare with these of Gibbs and Inman, they do not seem to support the results of Gibbs and Inman [6] because the samples were taken from older galleries of *T. piniperda* at all locations, and nevertheless, *L. wingfieldii* was found only occasionally at one location. This suggests that *L. wingfieldii* is very weakly associated with *T. piniperda* in Poland.

Many authors have reported that *T. piniperda* is associated with a range of *Leptographium* species in Europe [6, 12, 13, 39, 40, 56], including *L. procerum*, *L lunbergii*, *L. huntii* (Rob.-Jeffr.) M.J. Wingf. in England [6], and *L. guttulatum* M.J. Wingf. & K. Jacobs in Austria, England and France [13]. In this study, other *Leptographium* species than *L. wingfieldii* were found frequently in galleries of *T. piniperda* at some locations, including *L. lundbergii* and *L. procerum*. Presence of *L. lundbergii* in pine tissues colonized by *T. piniperda* was not surprising because this fungus was the most frequent species associated with the bark beetle *Hylurgops palliatus* (Gyll.) on Scots pine in Poland [16]. *Hylurgops palliatus* often breeds in the neighbourhood of *T. piniperda* [41]. The data from Poland suggest that *L. lundbergii* and *L. procerum* may be a closer associates of *T. piniperda* than suggested in previous reports.

Among the ophiostomatoid fungi identified in this study, *Graphium pseudormiticum* had never been reported in association with *T. piniperda*. This species has been described in association with *T. minor* (Hrtg.), *Ips sexdentatus* (Börn.), *Orthomicus erosus* Wollaston and *O. laricis* (Fabr.) on different pine species [15, 21, 22, 43]. The unidentified species *Graphium* sp., code-named 'W' is used here in the broad sense [49]. The synnemata of *Graphium* sp. 'W' had lightly pigmented stipes with cylindrical conidia. It seems that this species is not closely associated with *T. piniperda* but with *H. palliatus* on *P. sylvestris* [16]. The taxonomic position of this species is currently under investigation and will be discussed in a later report.

This study indicates that some populations of *T. piniperda* may be more strongly connected with *Hormonema dematioides*, moulds and yeasts, than with ophiostomatoid fungi. *Hormonema dematioides* is a black yeast that causes sapstain in conifers [10]. *Hormonema dematioides* has been reported as a frequent fungal associate of *T. piniperda* in France [32], Sweden [51], Poland [50] and Japan [35]. *Penicillium* and *Trichoderma* species were also commonly isolated from galleries of *T. piniperda* in this study. *Tomicus piniperda* adults hibernate under bark at the base of the tree or in the soil. These beetles can easily introduce litter and soil fungi, such as *Trichoderma*, *Penicillium* and *Geosmithia* species, to the trunk of pine trees.

This group of fungi is not associated with any specific species of bark beetles on coniferous trees. However, species belonging to *Geosmithia* are the most important associates of some phloeophagous bark beetles (especially those attacking deciduous trees) [22, 23]. An important group of fungi isolated from gallery systems of *T. piniperda* were also endophytes, such as *Epicoccum nigrum*, *Lecythophora hoffmannii*, *Peziculа eucrita*, *Phialocephala* cf. *dimorphospora* and *Phomopsis occulta*, that are frequently isolated from symptomless and uncolonized Scots pines [25]. Other non-ophiostomatoid fungi were rarely associated with *T. piniperda* and represented various ecological groups.

Considerable differences in fungal flora between localities were detected. The highest species richness was found in subsamples taken from *T. piniperda* galleries in Mielec. This could be due to the highest number of samples taken from this location. This fact complicates comparison between localities, therefore "rarefaction" method (http://www2.biology.ualberta.ca/jbrzusto/rarefact.php) was used to correct the species list according to the locality with the fewest sample number. However, after "rarefaction" the number of species in Mielec was still considerably higher than in other localities and amounted from 24 to 30 species. Probably the variation in the spectrum of fungi between different localities depended on various factors. Among these, the high beetle population in Mielec could have a strong influence on the number and abundance of fungi associated with *T. piniperda*. This may be the reason that numerous fungi recorded at this location were not found at any other location. In other Polish studies (Jankowiak, unpublished) fungi associated with *T. piniperda* (especially ophiostomatoid species) were also more frequently recorded at localities where the trees were heavily damaged by this insect.

Ophiostoma minus and *L. wingfieldii* were much more virulent than the other fungi evaluated in this study. These fungi were capable of killing whole plant and penetrated deeper into the sapwood than other species. The results indicate that these fungi may contribute to seedling mortality in Scots pine dominated forests, where *T. piniperda* adults may feed on the stems of young Scots pine seedlings. This study confirms the results of earlier inoculation studies using larger trees [30, 31, 33, 34, 51, 52] and seedlings [44, 45], which demonstrated that *O. minus* and *L. wingfieldii* are pathogenic. In these studies *L. wingfieldii* was more virulent then *O. minus* [52, 53]. However, the results presented here do not indicate that *L. wingfieldii* is more virulent than *O. minus*. Plant morality was similar for these two species, and only one of the *L. wingfieldii* isolates caused significantly more blue-stain than the *O. minus* isolates. It seems, that various factors could be responsible for lack of differences in the virulence between *O. minus* and *L. wingfieldii*. Among these, the inoculation technique used in the present study could be very important. Other studies have showed that assessing the virulence of blue-stain fungi using seedlings may be problematic. Basham [2] suggested that fungi used in inoculation studies were more virulent to seedlings than to large trees. On the other hand, Krokene and Solheim [26], inoculating the same fungal isolates in large trees and seedlings, found that seedling inoculation could be a reliable way of determining the virulence of bark beetle-associated blue-stain fungi in Norway spruce. It seems that for obtaining similar results of fungal virulence in the case of seedlings and large trees inoculations comparable inoculation loads should be used. Studies on pathogenicity of the isolates used in this study should also be conducted on large pine trees.

Among the other inoculated fungi *O. piliferum* and *O. piceae* seemed to have some pathogenic capability. Isolates of *L. procerum* appeared to be non-pathogenic to Scots pine seedlings. The low virulence of *L. procerum* in this study was surprising, since it is responsible for white pine root decline of *Pinus strobus* L. in various parts of the United States, and has caused extensive losses in Christmas tree plantations [12]. The opinions about pathogenicity of *L. procerum* are divided. Some studies have indicated that it is non-pathogenic or weakly pathogenic [9, 55], whereas others have showed that it can kill *P. strobus* seedlings [1, 28]. This variation may be caused by differences in the virulence among isolates. Such variation has been reported for isolates of *L. wingfieldii*, which showed high individual variability in growth characteristics and virulence to Scots pine [34]. It is also possible that *L. procerum* is less virulent to Scots pine than to white pine seedlings. To fully explain these differences, further pathogenicity studies of the same fungal isolates should be conducted using large Scots pine trees and different inoculation techniques.

In conclusion, large quantitative and qualitative differences in the composition of the mycobiota of *T. piniperda* were found between localities in the present study. The association between *T. piniperda* and ophiostomatoid fungi was rather loose, but *O. minus* was the most common fungus. Among the *Leptographium*, *L. procerum* and *L. lundbergii* seem to be more closely associated with *T. piniperda* than *L. wingfieldii* in Poland. This study indicated that some populations of *T. piniperda* might be more strongly associated with moulds and yeasts than with ophiostomatoid fungi. Inoculation of Scots pine seedlings confirmed that *L. wingfieldii* and *O. minus* were more pathogenic than other fungi associated with *T. piniperda*. Of these species, *O. minus* was more consistently associated with *T. piniperda* than *L. wingfieldii* and presumably plays a more important role in overcoming the resistance of Scots pine attacked by beetles in Poland.

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