SILVA FENNICA

Silva Fennica 44(3) research articles

www.metla.fi/silvafennica · ISSN 0037-5330
The Finnish Society of Forest Science · The Finnish Forest Research Institute

Ophiostomatoid Fungi Transported by *Ips sexdentatus* (Coleoptera; Scolytidae) in *Pinus pinaster* in NW Spain

Alberto Bueno, Julio J. Diez and Mercedes M. Fernández

Bueno, A., Diez, J.J. & Fernández, M.M. 2010. Ophiostomatoid fungi transported by *Ips sexdentatus* (Coleoptera; Scolytidae) in *Pinus pinaster* in NW Spain. Silva Fennica 44(3): 387–397.

Ips sexdentatus (Coleoptera; Scolytidae) is one of the main vectors of ophiostomatoid blue stain fungi that can cause mortality of healthy conifers. For this reason, our objective was to identify the fungal species carried by this bark beetle in Maritime pine (*Pinus pinaster*) in north-western Spain. We collected insects from naturally infected pines placed them on malt extract agar (MEA) and left to walk freely on culture plates. Plant tissues (phloem and xylem) from adult pines were cultivated in moist chambers and also on MEA. At the same time, we inoculated pine logs with living insects in the laboratory. Four ophiostomatoid fungi appeared: *Ophiostoma ips*, *Ophiostoma brunneo-ciliatum*, *Ceratocystiopsis minuta* and *Ophiostoma* sp., as well as *Graphium* and *Sporothrix* imperfect stages. Moreover there were seven saprophytic species: *Penicillium* sp., *Trichoderma* sp., *Verticillium* sp., *Mucor* sp., *Aspergillus niger*, *Gliocladium viride* and *Scopulariopsis brevicaulis*, and the pathogenic *Ophiostoma ips*. The fructification percentage of the ophiostomatoid species was low, however; its imperfect stage *Sporothrix/Hyalorhinocladiella* produced high quantity of conidiophores.

Keywords *Ips sexdentatus,* ophiostomatoid fungi, *Pinus pinaster,* mycangia, Spain
Addresses University of Valladolid, Dept of Agroforestry Sciences, Palencia, Spain
E-mail mffernan@agro.uva.es
Received 5 November 2009 **Revised** 13 January 2010 **Accepted** 9 April 2010
Available at http://www.metla.fi/silvafennica/full/sf44/sf443387.pdf

1 Introduction

Bark beetles (Coleoptera; Scolytidae) are insects causing important forest damage in Europe. Most species are secondary at endemic levels, feeding on phloem of weakened or dead trees (Balachowsky 1949, Gil and Pajares 1986, Wood and Bright 1992). However, under epidemic conditions, bark beetles populations become aggressive attacking and killing healthy trees (Levieux et al. 1989, Fernández 2006). In Vascongadas (Spain) in 1989, 92 million euros were lost to bark beetle damage (Amezaga 1993). In 2000, 25 thousand trees were killed by *Ips sexdentatus* in Castilla y León (Consejería de Medio Ambiente 2001).

Bark beetles damage trees directly but greater

problems arise from their symbiotic phytopathogenic blue stain or saprophytic fungi. Furthermore, the commensal mites can also vector blue stain fungal spores (Doberski 1978, Bridges and Moser 1983, Moser 1985).

Ophiostomatoid fungi are often associated with bark beetles and regarded responsible for many tree diseases, such as blue-stain in conifers (teleomorphs: *Ceratocystis*, *Ophiostoma* and *Ceratocystiopsis*), black-stain in roots of conifers (*Leptographium wageneri)*, or Dutch elm disease caused by *Ophiostoma novo-ulmi* (Seifert 1993, Harrington 1993). In addition, some of their associated fungi are non-pathogenic and present a specific association with bark beetles.

The association between blue-stain fungi and bark beetles was first postulated by Hartig (1878) and by Von Schrenk (1903). Many other authors have discussed this association (Francke-Grosmann 1967, Harrington 1988) and most believe that it is a symbiotic relationship. Fungi benefit from dispersal by the insects, and probably helping it later with the establishment of the population by contributing to exhausting tree resistance (Christiansen et al. 1987, Lieutier 1993, 2002, Solheim et al. 2001). However the advantages for the insect are mixed. Lombardero et al. (2003) postulated that *Ophiostoma minus* is an antagonistic fungus transported on the exoskeleton of *Dendroctonus frontalis.* Their larvae do not survive in the fungal presence, but yet otherwise they feed on *Ceratocystiopsis ranaculosus* and *Entomocorticium* sp. (Barras and Perry 1972). Recently Scott et al. (2008) found that the successful relationship between *Dendroctonus frontalis* and *Entomocorticium* sp. is likely mediated by antibiotic producing actinomycetous bacteria, which selectively inhibit *O. minus*.

Few fungus-bark beetle studies have been done in the Iberian Peninsula (Fernández et al. 2004, Romón et al. 2007). The former study describes the threshold level of pathogenecity of *Ophiostoma ips* in *Pinus sylvestris* and the latter relates fungi to bark beetles without indicating insect body parts, where spores are found.

The aim of our study was to demonstrate the effectiveness of *Ips sexdentatus* as a vector of Ophiostomatoid fungi in *Pinus pinaster* in NW Spain. We identified 1) fungal species associated with *Ips sexdentatus* colonizing trees in the forest, 2) insect body parts, in which fungi are transported, and 3) fungal species that the beetle can vector under laboratory conditions. The sex vector effect was also considered for all three objectives.

2 Materials and Methods

2.1 Insects and Pine Tissues Collection

In 2006, *Ips sexdentatus* adults and colonized plant material were collected in Quintana del Castillo forest (León province, NW Spain, UTM coordinates 29T, X-738530, Y-4729138, altitude 1100 m.a.s.l.). Three hundred bark beetles were collected from a 40-years old *P. pinaster* stand (mean DBH: 28 cm, mean height 12 m) affected by fire. Trees were dead standing but with green-yellow needles still remaining in the crown. *Ips sexdentatus* specimens were collected at the end of September, when the population was at an endemic level and when the last generation have their galleries already built. Each collected beetle was individually stored in one Ependorff tube at 6ºC and subsequently sexed.

Colonized tissues (sapwood and phloem) were obtained from four trees naturally infested by *I. sexdentatus*. 70 cm long logs with 32 cm diameter were stored at the laboratory at 6 ºC until the extraction of the tissues.

Four non-colonized healthy *P. pinaster* logs collected in the same area (12.5–18 cm in diameter and 60–73 cm in length) were inoculated in the laboratory according to the methodology described by Furniss et al. (1990). One hundred insects (50 males and 50 females) were inoculated into four logs. For each log, a 5 mm diameter cork borer was used to create twenty five holes leaving 10 cm gaps from the edges to avoid desiccation. Thereafter, one insect was placed on each wound and crushed with the removed bark disk. Inoculated logs were then stored in the lab for one month at 25ºC.

2.2 Fungal Isolation and Identification

Different methodologies were employed for fungal isolations and identification from naturally infected pine tree tissues, logs inoculated in the laboratory, and from insects. 60 samples were taken from naturally infested logs, 30 phloem samples and 30 sapwood ones, all of them from 3 different pine logs. In each log, 10 different samples of 3 cm^2 of phloem were taken and subdivided in 5 parts of 1 cm length $\times$ 0, 5 cm wide. From the xylem, we took Pressler´s samples of 5 cm length and 0.5 cm in diameter that we divided in 5 parts of 1 cm length. All the samples were kept in a dark moist chamber at 25ºC during 30 days to promote fungal fructification.

Tissue samples were cultured on MEA+ antibiotic medium (33 g of malt extracts, 16 g of agar and 250 mg of tetracycline per litre of distilled water). Petri dishes were stored for 7 days in the dark at 25ºC and then carefully examined using NIKON SMZ-2T binoculars. Fruiting bodies from the samples were identified with a microscope Nikon Eclipse E-400 model.

Insects associated fungi were cultured by two methods: 1) leaving the insect freely move on Petri dishes with MEA+antibiotic for 2 hours and, 2) placing different insect body parts (mandibles, legs, pronotum and elytra) directly onto. After two hours, the walking insects were placed in 1.5 ml Ependorff test tubes and washed in 400 µl sterile water with 1% Tween-80 and vortexed at 40 Hz for 30 seconds (Lieutier et al. 1989). The rinsing water (400 µl) was plated on MEA using a sterile pipette, incubated for 2 weeks in the dark at 25ºC, and monitored for fungal growth.

The insect body parts (mandibles, pronotum, legs and elytra) were cultured on MEA+antibiotic. Petri dishes were stored in the dark at 25ºC for one week. Fungal colonies were subcultured to sterile MEA+tetracycline, after 30 days identified based on microscopic features and classified according to biometric characteristics.

Sapwood and phloem samples from artificially infested logs were cultured in a moist chamber and on the MEA+tetracycline media at 25ºC in the dark, subcultured, and identified under microscope.

Seven different keys were used for fungal identification: Sutton 1980, Fassatiová 1986, Wolfaardt et al. 1992, Wingfield et al. 1993, Muñoz et al. 1996, Kiffer and Morelet 1997, Jacobs and Wingfield 2001. Fungal species abundance data were analyzed by a generalized lineal model using binomial distribution and the Link Logit function. Fungal diversity was analyzed by Poisson distribution with Link Log function. Data from both experiments were analyzed using STATISTICA 5.5 program ($p < 0.05$).

3 Results

3.1 Fungal Identification

Twenty-five taxa, including twelve *Sporothrix* states were identified after analysis. All identified fungal isolates belong to the Eumycotina group.

Ophiostoma ips, *Ophiostoma brunneo-ciliatum*, *Ceratocystiopsis minuta* and *Ophiostoma* sp. were isolated in their sexual state from the moist chamber experiment. Only *Ophiostoma ips* was isolated simultaneously from moist chamber and culture media. Different asexual states of *Ophiostoma* genus, such as *Graphium* (acquired from logs stored in moist chambers), or *Sporothrix* and *Hyalorhinocladiella* (obtained by both methodologies) were also isolated. The existing similarity between these genera recommends the use of *Sporothrix* "sensu lato", including the *Hyalorhinocladiella* form under that genus (Lieutier et al., 1989). Consequently, 11 conidial types were distinguished and each was categorized within *Sporothrix* 2, through *Sporothrix* 12. Isolates without conidia but with *Sporothrix* biometric mycelial characteristics were grouped to *Sporothrix* 1 (Lieutier et al. 1989). One fungal taxon was classified as Deuteromycete 1 attending to the characteristics of the asexual fruiting body (no spores were found).

Isolates belonging to *Trichoderma*, *Penicillium*, *Aspergilus niger*, *Verticillium*, *Gliocladium viride*, *Mucor* and *Scopulariopsis brevicaulis* were cultured from insect bodies and plant material on MEA medium.

3.2 Fungi Isolated from the Logs Naturally Colonized by Insects

The following six Ophiostomatoid species, including the imperfect states were identified from the sapwood and the phloem of the logs: *Ophiostoma ips*, *Ophiostoma brunneo-ciliatum*, *Ceratocystiopsis minuta*, a species identified only at the genus level (*Ophiostoma* sp.), *Sporothrix* 12 and *Graphium* sp. Also one unidentified fungus Deuteromycete 1 was detected (Table 1). Only *O. brunneo-ciliatum* and *Graphium* sp. were present on the totality of the logs, but *O. brunneo-ciliatum* appeared on both tissues (log × tissue) and occurred more frequently.

The number of isolates was higher in phloem than sapwood. O. *brunneo-ciliatum* was most abundant fungus in the phloem ($p = 0.003$), and *O. ips* was the second most frequent, seldom found in the sapwood ($p = 0.000$). The anamorphic states of *Ophiostoma sp.*, *Sporothrix* 12 and *Graphium* sp. were detected more frequently in phloem than sapwood, although the differences were significant only for *Sporothrix* 12 ($p = 0.004$). *Ceratocystiopsis minuta* and *Ophiostoma* sp. were found in phloem, and Deuteromycete 1 from sapwood was detected with low frequency.

The fungal diversity detected in the phloem (1.5 fungal species per sample) was much higher than diversity in sapwood (0.4 fungi per sample) (data not shown).

3.3 Fungi Isolated from Insects

3.3.1 Walking Insect and Dilution Method

Six fungal species were isolated using the two techniques and from both sexes of *Ips sexdentatus* (Table 2): *Trichoderma* sp., *Penicillium* sp., *Aspergillus niger*, *Verticillium* sp., *Gliocladium viride* and *Sporothrix* 1. With the exception of *Sporothrix* 1, all fungi were isolated from walking males; in addition *Trichoderma* sp., *Penicillium* sp. and *Verticillium* sp. were isolated from females. Dilution method yielded *Trichoderma* sp., *Penicillium* sp. and occasionally *Sporothrix* 1 on females.

Highest records frequency for both methodologies were found for *Trichoderma* sp. (40%

Table 1. Percentages of different fungi isolated from sapwood and phloem naturally colonized by *Ips sexdentatus* the log and the interaction tissue × log. (-)[1]: number of Petri dishes, *: significant differences ($p > 95\%$).

Fungal Taxa	Tissue		*p* value	Logs			*p* value	Presence (%)								*p* value
								Log 1		Log × Tissue Log 2		Log 3				
	Sapwood (30)[1]	Phloem (30)		Log 1 (20)	Log 2 (20)	Log 3 (20)		Sapwood (10)	Phloem (10)	Sapwood (10)	Phloem (10)	Sapwood (10)	Phloem (10)			
Ophiostoma ips	3.3	33.3	0.000*	25	–	30	0.006*	10	40	–	–	–	60			0.431
Ophiostoma brunneociliatum	23.3	60	0.003*	55	35	35	0.336	30	80	10	60	30	40			0.272
Ceratocystiopsis minuta	–	6.7	0.084	5	–	5	0.437	–	10	–	–	–	10			1.000
Ophiostoma sp.	–	6.7	0.070	–	–	10	0.104	–	–	–	–	–	20			1.000
Sporothrix 12	3.3	23.3	0.004*	25	–	15	0.021*	–	50	–	–	10	20			0.048*
Graphium sp.	6.7	16.7	0.169	10	20	5	0.322	10	10	–	40	10	–			0.018*
Deuteromycete 1	6.7	–	0.070	–	–	10	0.104	–	–	–	–	20	–			1.000

Table 2. Percentage of different fungi isolated from *Ips sexdentatus* based on different methodologies, sex, and interaction methodology × sex. (-)[1]: number of Petri dishes, *: significant differences ($p > 95\%$), ex.: exponential values.

Fungal taxa	Presence (%)										
	Methodology		*p* value	Sex		*p* value	Methodology × sex				*p* value
	Walking	Dilution					Walking		Dilution		
	(50)[1]	(50)		♂♂ (50)	♀♀ (50)		♂♂ (25)	♀♀ (25)	♂♂ (25)	♀♀ (25)	
Trichoderma sp.	40	92	0.000*	54	78	0.268	12	68	96	88	0.005*
Penicillium sp.	20	4	0.026*	12	12	1.000	20	20	4	4	1.000
Aspergillus niger	4	–	0.998	4	–	0.998	8	–	–	–	0.998
Verticillium sp.	10	–	ex.	6	4	1.000	12	8	–	–	ex.
Gliocladium viride	4	–	0.998	4	–	0.998	8	–	–	–	0.998
Sporothrix 1	–	2	0.998	–	2	0.998	–	–	–	4	0.998

Table 3. Percentages of presence and fungi diversity between sexes and different methodologies. [1]: average number of fungi isolated from each Petri dish, (-)[2]: number of Petri dishes, *: significant differences ($p > 95\%$), ex.: exponential values..

Species	Presence (%) and Fungi diversity[1]					
	Methodology					
	Walking & Dilution		*p* value	Body parts		*p* value
	♂♂ (50)[2]	♀♀ (50)		♂♂ (40)	♀♀ (40)	
Trichoderma sp.	54	78	0.268	–	–	–
Penicillium sp.	12	12	1.000	55	37.5	0.994
Aspergilus niger	4	–	0.998	–	–	–
Verticillium sp.	6	4	1.000	–	–	–
Gliocladium viride	4	–	0.998	–	–	–
Scopulariopsis brevicaulis	–	–	–	–	2.5	0.999
Mucor sp.	–	–	–	2.5	2.5	1.000
Ophiostoma ips	–	–	–	7.5	–	0.237
Sporothrix 1	–	2	0.998	35	35	0.852
Sporothrix 2	–	–	–	27.5	–	ex.
Sporothrix 3	–	–	–	5	2.5	0.553
Sporothrix 4	–	–	–	7.5	–	ex.
Sporothrix 8	–	–	–	10	–	ex.
Sporothrix 9	–	–	–	2.5	–	0.999
Sporothrix 10	–	–	–	40	40	0.858
Sporothrix 11	–	–	–	5	2.5	0.553
Fungal diversity	0.8	1.0	0.325	1.9	1.2	0.012*

walking, 92% dilution) with significant differences between methods ($p = 0.000$) and relation methodology × sex interaction ($p = 0.005$). The highest rate of recurrence was found in males isolated by dilution method with 96% success rate in contrast to walking technique, which resulted in 12% recovery. In contrast to the dilution method results, walking method generated higher incidence of *Trichoderma* sp. isolated from females than males. Percentages of abundance were much lower for the remaining fungal species; 20% of samples yielded *Penicillium* sp., 10% *Verticillium* sp. and 4% *Aspergillus niger and Gliocladium viride. Sporothrix* 1 was occasionally recovered from females by dilution method. With reference to methodology, only significant differences were noticed for *Trichoderma* sp. and *Penicillium* sp. ($p = 0.026$ and $p = 0.000$). *Trichoderma* spp. are very fast growing molds and may easily overgrow slower growing ophiostomatoid fungi.

Fungal diversity was not significantly different despite the slightly higher average number of fungi per insect in females than in males (1.0 and 0.8) (Table 3).

3.3.2 *Fungi Isolated from Ips sexdentatus Body Parts*

Twelve taxa were isolated from body parts of 10 males and 10 females: *Penicillium* sp., *Mucor* sp., *Scopulariopsis brevicaulis*, *Ophiostoma ips*, *Sporothrix* 1, *Sporothrix* 2, *Sporothrix* 3, *Sporothrix* 4, *Sporothrix* 8, *Sporothrix* 9, *Sporothrix* 10 and *Sporothrix* 11 (Table 4). *Scopulariopsis brevicaulis* was isolated only from the mandible of a female, whereas the rest of the fungi occurred in both sexes, or only on males.

Penicillium sp., *Mucor* sp., *Sporothrix* 1, *Sporothrix* 3, *Sporothrix* 10 and *Sporothrix* 11 were also isolated from females (Table 4). *O. ips* was isolated only from the male pronotum and elytra. *Sporothrix* 1 and *Sporothrix* 10 were isolated from every body part of both sexes. The recovery rates were highest for *Penicillium* sp., *Sporothrix* 10, *Sporothrix* 1 and *Sporothrix* 2 (the last, isolated only from the males), whereas the isolation frequency of the remaining fungi was low. Sex differences depend on the body part (sex × body part interaction: $p < 0.05$; Table 5). *Penicillium* sp. was recovered in highest frequency from the elytra of both sexes and *Sporothrix* 1 was found primarily on the mandibles. *Sporothrix* 10 was often isolated from male elytra and pronotum and from female pronotum. With the exception of *Sporothrix* 3 and *Sporothrix* 2, differences in abundance percentage for sex × body part interaction was not significant for any of the isolated fungi.

The fungal diversity analysis showed statistically significant differences between sexes for the body part cultures. On average, 1.9 fungi per male and 1.2 fungi per female were isolated ($p = 0.012$) (Table 3).

3.4 Fungi Isolated from Inoculated Logs

Eight fungal taxa were isolated from tissues (sapwood or/and phloem) of *I. sexdentatus* inoculated logs. *Penicillium* sp. and *Trichoderma* sp. were more commonly isolated from both tissues inoculated by males and females (Table 5).

Logs inoculated with females showed highest fungal abundance in the sapwood (*Trichoderma* sp. followed by *Penicillium* sp). The *Ophiostoma*

Table 4. Percentages of presence of the different fungi taxa isolated from *Ips sexdentatus* according to the sex, body parts, and interaction sex × body parts. (-)[1]: number of Petri dishes [2], M: mandibles, L: legs; P: pronotum; E: elytra. ex.: exponential values. n.v.: no value: the statistical program didn't find any p value), *: significant differences ($p > 95\%$).

Fungal taxa	Sex			Body parts				Presence (%)													
	♂♂	♀♀	p value	M	L	P	E	p value	M	♂♂ L	P	E	Sex × body parts M	E	♀♀ L	P	E	p value
	(40)[1]	(40)		(20)	(20)	(20)	(20)		(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	
Penicillium sp.	55	37.5	0.994	15	50	55	65	0.816	30	60	60	70	–	40	50	60		0.989
Mucor sp.	2.5	2.5	1.000	–	5	5	–	0.420	–	–	10	–	–	10	–	–		0.090
Scopulariopsis brevicaulis	–	2.5	0.999	5	–	–	–	1.000	–	–	–	–	10	–	–	–		1.000
Ophiostoma ips	7.5	–	0.237	–	–	10	5	0.091	–	–	20	10	–	–	–	–		1.000
Sporothrix 1	35	35	0.852	65	25	25	25	0.021*	50	20	30	40	80	30	20	10		0.213
Sporothrix 2	27.5	–	ex.	15	15	15	10	1.000	30	30	30	20	–	–	–	–		ex.
Sporothrix 3	5	2.5	0.553	–	5	5	5	0.622	–	10	–	10	–	–	10	–		0.047*
Sporothrix 4	7.5	–	ex.	–	–	10	5	n.v.	–	–	20	10	–	–	–	–		1.000
Sporothrix 8	10	–	ex.	5	10	5	–	n.v.	10	20	10	–	–	–	–	–		1.000
Sporothrix 9	2.5	–	0.999	5	–	–	–	1.000	10	–	–	–	–	–	–	–		1.000
Sporothrix 10	40	40	0.858	30	40	60	30	0.149	20	40	50	50	40	40	70	10		0.180
Sporothrix 11	5	2.5	0.553	–	–	15	–	0.013*	–	–	20	–	–	–	10	–		1.000

Table 5. Percentages of presence depending on the sex, the tissue and the interaction sex × tissue of the different fungi isolated from the sapwood and phloem of the inoculated logs at the laboratory with *Ips sexdentatus* insects. (-)[1]: number of Petri dishes., [*]: significant differences ($p > 95\%$).

Fungal Taxa	Presence (%)										
	Sex		*p* value	Tissue		*p* value	Sex × tissue				*p* value
	♂♂	♀♀					♂♂		♀♀		
	(20)[1]	(20)		Sapwood (20)	Phloem (20)		Sapwood (10)	Phloem (10)	Sapwood (10)	Phloem (10)	
Trichoderma sp.	40	45	0.749	45	40	0.731	40	40	50	40	0.695
Penicillium sp.	55	35	0.202	50	40	0.514	60	50	40	30	0.977
Mucor sp.	–	5	0.234	–	5	0.221	–	–	–	10	1.000
Sporothrix 1	5	25	0.066	5	25	0.053	–	10	10	40	0.570
Sporothrix 2	5	–	0.235	–	5	0.221	–	10	–	–	1.000
Sporothrix 8	5	–	0.235	–	5	0.221	–	10	–	–	1.000
Sporothrix 9	–	15	0.036[*]	5	10	0.524	–	–	10	20	1.000
*Sporothrix*10	15	20	0.679	5	30	0.018[*]	–	30	10	30	0.342

anamorphs *Sporothrix* 1, 9 and 10 were most frequently isolated from the phloem. The logs inoculated with males exhibited high recovery rate of *Penicillium* sp. followed by *Trichoderma* sp. in the sapwood, whereas the remaining taxa were only found in the phloem.

Fungal diversity recovered from phloem was greater than sapwood (1.6 and 1.1 respectively; $p = 0.172$). Furthermore, female inoculated logs had a greater fungal diversity in comparison with male inoculated logs (1.5 and 1.3; *p* value no statistically significant; data not shown).

4. Discussion

4.1 Which Ophiostomatoid Fungi are Colonizing *Pinus pinaster?*

From naturally colonized logs by *Ips sexdentatus* seven fungal species were isolated in the lab under moist chamber conditions: *Ophiostoma ips*, *Ophiostoma brunneo-cilliatum*, *Ceratocystiopsis minuta*, *Ophiostoma* sp., as well as two anamorphs (*Sporothrix* 12 and *Graphium* sp.) and one unidentified Deuteromycete (Table 1). Mathiesen-Käärik (1953) and Lieutier et al. (1989, 1991) observed a species-specific association between *O. brunneo-cilliatum* and *I. sexdentatus*. In our study, *O. brunneo-cilliatum* represented the most frequently recovered species from the phloem (60%) followed by *O. ips*, also isolated from phloem (33.3%). These results concur with Jankowiak's (2005) findings, where he states that fungal occurrence associated with *Ips typographus* was higher in the phloem galleries (99.1%) than sapwood (52.1%) of *Picea abies*.

Fernández et al. (2004) first described the presence of *O. ips* in Spain and found the highest degree of association of this fungus with *I. sexdentatus* in *Pinus sylvestris.* Romón et al. (2007) isolated *O. ips* from *I. sexdentatus* and other bark beetles species colonizing *Pinus radiata*. In other European countries, *O. ips* was more frequently isolated from *Ips* species such as *I. acuminatus* and different secondary bark beetles (Lieutier et al. 1991, Mathiesen-Käärik 1953, Kirschner 1998).

Some authors found that *Ceratocystiopsis minuta,* is an unspecific fungus commonly found in many bark beetles in Europe, such as *Ips* species (*I. acuminatus*, *I. sexdentatus*) (Kirschner 1998, Solheim 1986), or *I. typographus* found in *Picea abies* with more degree of association (Viiri and Lieutier 2004).

In our study we also found two anamorphic states: *Sporothrix* 12 and *Graphium* sp. Lieutier et al. (1989) isolated *Sporothrix* with 100% success rate from blue stain areas close to *I. sexdentatus* galleries on *Pinus sylvestris*; finding 10% sexual forms, most of them corresponding to *Ophiostoma ips.* After sampling deep-xylem and non-stained areas they recorded 90% of *Sporothrix* anamorph, of which 5% corresponded to *O. brunneo-cilliatum* and 21% to *O. ips.*

In the non-stained galleries, only 30% of our isolates were *Sporothrix* and no sexual fruiting bodies were found. In Lieutier's et al. (1989) survey of sexual fruiting state, the highest levels were detected in *O. ips* whereas in our study, *O. brunneo-cilliatum* produced the most fruiting bodies. Lieutier et al. failed to find blue stain fungi in the sapwood, or saprophytic fruiting bodies, whereas Jankowiak (2006) isolated many non-ophiostomatoid fungi, mainly *Penicillium* sp. and *Trichoderma* sp. from sapwood in quantities similar to results in our study.

4.2 Insect Body Parts Utilized for Fungal Transport

Walking and dilution methods, as well as insect body part culturing technique were used to answer the following objectives of our study: 1) detection of fungal species vectored by insects, 2) identification of sexual or asexual stages of vectored fungi, and 3) identification of fungal species successfully inoculated into logs. Seven fungi from colonized trees were identified and only *O. ips* was also detected in the lab by insect body part culturing technique.

The number of ophiostomatoid fungi isolated by the walking and dilution techniques was low (only *Sporothrix* was detected), however, many other saprophytic fungi, such as *Trichoderma* sp. and *Penicillium* sp. were present (Table 2). Disregarding the likelihood of contamination in the laboratory, we considered the possibility that insect mycangia, which are used for transport of ophiostomatoid spores (Lévieux et al. 1991), failed to produce effective inoculations because of the limited contact with the culture media, thus preventing spores to germinate. Conversely, saprophytic fungi that are commonly transported on exoskeleton grew very well (Six 2003a). Lieutier et al. (1989) found saprophytes as *Penicillium*, *Trichoderma*, *Aspergillus*, or *Cladosporium* on *Ips sexdentatus*, and, Peverieri et al. (2006) recovered these fungi from *Tomicus destruens*. Considering the low economical importance in the forest, saprophytes are not often referenced in publications; however, some of them are very important biological control agents, for example *Trichoderma* spp. Whitney and Blauel (1972) reported ascosporic masses of several species of *Ceratocystis*, *Ceratocystiopsis* and *Ophiostoma* dispersed in conifer resin but not in water. These findings substantiate the lack of ophiostomatal isolations from insect washing dilutions.

From the insect body part cultures, *O. ips* and different conidia of 8 *Sporothrix* forms were identified (Table 4). *Sporothrix* conidia were frequently isolated from pronotum and mandibles whereas *O. ips* was mainly cultured from pronotum. Moreover, 3 non-ophiostomatoid fungi were isolated from insects, most often from elytra and legs. Therefore, these findings confirm that mandibles and pronotum of *I. sexdentatus* are involved in ophiostomatoid spore dispersal, whereas elytra, pronotum and legs could be implicated in transporting non-ophiostomatoid spores. Lévieux et al. (1989) noticed spore masses of various shapes situated on pronotal sides, with limited quantities in rounded hollows of the strial punctures of elytra and its declivity, and occasionally located under the abdominal sternites, or in the punctures of the external side of mandibles.

4.3 Which Fungal Species Can Be Inoculated by the Beetle into the Trees?

Regarding the insect's ability to inoculate fungi into the pine trees, five different conidia from ophiostomatoid fungi were isolated in the sapwood and phloem (*Sporothrix* 1, 2, 8, 9 and 10) and three saprophytic fungi: *Trichoderma* sp., *Penicillium* sp., and *Mucor* sp. (Table 5). *Sporothrix* spp. was rarely occurred; *Sporothrix* 1 and 10 were more frequently found in the phloem, however, none occurred in sapwood. Out of seven identified fungi, only *O. ips* was detected also in the lab. The perithecium of this fungus was frequently recovered from cultures containing *Sporothrix* after placing wood chips in the plates.

All the ophiostomatoid fungi were the anamorphs whereas if we compared with the fungi isolated from naturally colonized tissues trees, we can see that 6 ophiostomatoid species were found, 4 of them, were teleomorphs. Several explanations could be given for these differences: the different climatic conditions for the fungi growth between the lab and the field (anaerobic

conditions could stop fungi fructification). Secondly, the technique used at the lab that consisted in crashing the beetle inside the hole made with the cork-borer. That also could be augmenting the saprophytic fungi presence due to the intestinal content of the insects. The last explanation for this difference in the frequency of the ophiostomatoid fungi could be the high presence of these two ubiquitous saprophytes (*Trichoderma* and *Penicillium* species) and their antagonistic mycoparasitic nature, that may interact in significant, but still unknown, the ways with bark beetle-fungal associates (Six 2003b). It is interesting to note that in the whole study, 0% of sexual fructification was obtained in MEA Petri dishes where these two saprophytic fungi were present; that could support the inhibition hypothesis. We have also to take account that no controls were used in this experiment and this lack didn't leave us to determine if the high presence of the saprophytic fungi could be due to contaminations in the lab inoculation procedure, but the naturally presence in logs of the mentioned fungi is well know and noticed from several authors and studies, as we mentioned before.

Using the same methodology as in our study, Viiri and Lieutier (2004) isolated 11 ophiostomatal fungi in *Picea abies* but the logs were disinfected with 70 % alcohol before inoculations with *Ips typographus*. Lieutier et al. (1989) also disinfected logs prior to inoculation with *Ips sexdentatus* and used sterilized distilled water with Tween 80, recovering S*porothrix* with 68% success rate.

5 Conclusion

We isolated twenty-five fungal taxa associated with *Ips sexdentatus*: 4 Ascomycetes, 1 Zygomycetes, and 20 Deuteromycetes, including 13 anamorphs (12 *Sporothrix* spp. and 1 *Graphium* sp). The isolates were obtained by different methodologies; 6 taxa from the walking+dilution method and 10 taxa more grew on insect body parts. Only *Penicillium* and *Sporothrix* 1 were obtained by both techniques. From the seven fungal taxa isolated from naturally infested pine logs, only *O. ips* was associated with insects using the body part culturing technique.

The highest frequency of fungal spores carried by insects corresponded to *Penicillium* sp. on the elytra. Ophiostomatoid fungi had the highest diversity on pronotum followed by mandibles, legs and elytra. However, only the values for *Sporothrix* 1 on mandibles and *Sporothrix* 11 on pronotum were significant (Table 4).

The total fungal diversity (16 fungal taxa isolated from insects) was higher in males than females. Moreover, males carried more than twice as many ophiostomatoid fungal species, (9) than females (4) but differences were not statistically significant. Only the value for *Sporothrix* 3 sex × body part interaction was statistically significant. ($p = 0.047$). In general, higher numbers of ophiostomatoid fungi appeared in phloem tissue and were associated with males.

The inoculated log results revealed 8 taxa, including 5 ophiostomatoid (Table 5), and highest abundance in females associated with *Sporothrix* 1 and 10, although the values were no significant. The value for *Sporothrix* 9, (fungus transported only by females) was statistically significant.

Further studies using molecular identification will be necessary to provide more information about the fungal diversity associated with *I. sexdentatus*, the identification of the *Sporothrix* anamorphs and their teleomorphs.

Acknowledgements

We would like to express our gratitude to Gonzalo Baz for helping in sample collection and fungal identification, as well as D. Valentín Pando for his kindly involvement with statistical analyses.

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Total of 46 references