Fungal flora associated with Ips typographus: Frequency, virulence and ability to stimulate the host defence reaction in relation to insect population levels.

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Abstract: This study was aimed at determining the composition of *Ips typographus* L. (Coleoptera: Scolytidae) associated fungal flora in France, its virulence, and its ability to stimulate host defence reactions. The relationship between these parameters and the beetle population levels was also considered. The study was conducted in 2001, 2002, and 2003 in Norway spruce (*Picea abies* (L.) Karst.) stands, with different bark beetle damage levels. In each stand, the frequency of association between fungi and *I. typographus* was determined. The virulence of the most frequent species was assessed through mass inoculations on living spruce trees. The ability to stimulate the host defence reactions was estimated with low-density inoculations. The most frequent species, *Ophiostoma bicolor* Davids. & Wells, *Ophiostoma piceaperdum* Rumbold, and *Ophiostoma tetropii* Mathiesen, were all pathogenic. *Ophiostoma piceaperdum* also induced intense defence reaction zones, suggesting that it could play a role in *I. typographus* population establishment on living trees. However, significant correlations between fungal frequencies and damage of the current year were observed only with *O. tetropii* or *O. bicolor*, and no relationships between damage of the previous year and fungal frequencies were found. The effects of some fungal species on beetle population dynamics was suggested, but selection of species during epidemic condition was not confirmed.

Résumé : La composition de la flore fongique associée à *Ips typographus* L. (Coleoptera : Scolytidae), sa virulence et son aptitude à stimuler les défenses de l'hôte, ainsi que les relations entre ces paramètres et les niveaux de population de l'insecte, ont été étudiés en France. L'étude a été menée en 2001, 2002 et 2003 dans des peuplements d'épicéa (*Picea abies* (L.) Karst.) où les niveaux de dégâts dus aux insectes différaient. Les fréquences d'association entre les champignons et *I. typographus* y ont été estimées. La virulence des espèces majoritaires a été évaluée avec des inoculations massives sur des arbres vivants et leur aptitude à stimuler les réactions de défense, avec des inoculations ponctuelles. Les espèces majoritaires, *Ophiostoma piceaperdum* Rumbold, *Ophiostoma bicolor* Davids. & Wells et *Ophiostoma tetropii* Mathiesen, se sont toutes révélées pathogènes. *Ophiostoma piceaperdum* a aussi induit des réactions de défense importantes et pourrait ainsi participer à l'établissement des insectes sur les arbres vivants. Néanmoins, seules les fréquences d'*O. tetropii* et *O. bicolor* ont été corrélées avec les dommages de l'année en cours et aucune relation n'a été observée entre les fréquences d'association et les dommages antérieurs. Nos résultats suggèrent l'implication d'espèces fongiques dans la dynamique des populations d'insectes mais ne confirment pas la sélection d'espèces au cours d'une épidémie.

Introduction

Ips typographus L. (Coleoptera: Scolytidae) is the major insect pest of European spruces (*Picea* spp.). In endemic conditions, it preferably attacks stressed and wind-thrown trees (Chararas 1962; Lindelöw et al. 1991). Droughts or storms regularly trigger beetle outbreaks, during which healthy standing trees are killed and significant damage is generated. For example, in the European Union, more than 25 million m^3 of spruce was damaged by this beetle in 1992 and 1993, causing an economic loss estimated at €600 million (Lieutier 2001).

Ips typographus is associated with various ophiostomatoid fungi that are carried on the pronotum, elytra, and in the digestive tract (Furniss et al. 1990). Some fungal species help the beetles to exhaust tree defences by stimulating the host-tree

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Table 1. Locations and stand characteristics of *Ips typographus* sampling points in France.

Location	Code	Lat. (N)	Long. (E)	Altitude (m)	Stand age (years)	Stand density (trees/ha)	Year(s) of study	Date of sampling
Charleville-Mézières	CM	49°50′	4°41′	250	68	300	2001, 2002, 2003	28 May 2001, 21 May 2002
Vouziers	VZ	49°23′	4°51′	150	68	380	2001, 2002, 2003	15 May 2001, 22 May 2002
Planchez	MO	47°10′	4°01′	650	61	810	2001, 2002, 2003	21 May 2001, 6 May 2002
Servières	S	45°38′	2°50′	1200	90	350	2001, 2002, 2003	13 June 2001, 20 June 2002
Mulhbach	MU	48°02′	7°03′	600	80	550	2001, 2002, 2003	5 June 2001, 4 June 2002
Climbach	CL	49°01′	7°51′	450	40	1200	2001, 2002	29 May 2001, 5 June 2002
Labergement	L	46°46′	6°18′	1000	40	500	2001, 2002	11 June 2001, 18 May 2002
Remiremont	RE	48°05′	6°30′	450	80	400	2001	30 May 2001
Arc sous Cicon	AR	47°01′	6°14′	800	25	1600	2001	12 June 2001
Champ du Feu	F	48°24′	7°15′	1100	105	370	2001	6 June 2001
Verdun	VD	49°15′	5°23′	150	70	300	2001	15 May 2001
Ambérieu	AM	45°32′	5°22′	900	90	400	2001	22 May 2001
Remiremont	R	48°05′	6°31′	400	70	400	2002	3 June 2002
Annecy	AN	45°26′	6°16′	1100	35	1660	2002	19 June 2002
Belleherbe	B	47°14′	6°38′	800	110	280	2002	17 May 2002
Aigoual	AI	44°05′	3°18′	1200	80	400	2002	1 July 2002
Mouchet	MH	44°59′	3°21′	1400	100	310	2002	2 July 2002

defence reaction (Christiansen et al. 1987). These fungi are transferred to the tree during the beetle attack and are considered important partners in beetle population establishment (Horntvedt et al. 1983). In addition, during and after beetle establishment, fungi invade the host's phloem and sapwood, where their hyphae can cause blue staining, which has important economic consequences for wood quality (Defays 1992).

Previous studies have shown that different fungal species associated with *I. typographus* have different levels of virulence and can vary in their ability to stimulate host defence reactions (Solheim 1988, 1993*a*; Krokene and Solheim 1998). Consequently, different fungal species could have different effects during bark beetle establishment on trees; some of them, such as *Ceratocystis polonica* (Siem.) Moreau, are considered key species for insect population dynamics (Harding 1989; Krokene and Solheim 1998). However, the specific composition of the fungal flora associated with *I. typographus* varies depending on studies, even if isolation techniques are identical. It has been hypothesized that the most virulent fungal species could be selected during outbreak periods, when insects attack healthy resistant trees, but counter-selected during the latency periods, to the benefit of saprophytic species more adapted to decaying trees (Solheim 1992*b*, 1993*b*). These fungi play an important role during beetle establishment, implying that the presence of virulent fungal species at high frequency in a beetle population could contribute to its aggressiveness; that is, the amount of trees that the insect population would be able to kill (Harding 1989). Considering that different strains of the same pathogenic fungal species, with different virulence levels, can coexist in the same forest (Lieutier et al. 2003), this hypothesis may be extended to the strain level: virulent strains could play an important role or be selected during bark beetle outbreaks. As a consequence, the fungal species composition, virulence, and ability to stimulate host defence reactions in the phloem might be important parameters in interpreting *I. typographus* population dynamics.

In December 1999, two severe hurricanes generated 160 million m^3 of windfalls in France (Wencélius 2002), thus triggering bark beetle outbreaks in different areas (Nageleisen 2002). We took advantage of this situation and studied the variability of the fungal flora associated with *I. typographus* in France in terms of species composition, virulence, and ability to stimulate host defence reactions. The study was conducted in locations where insect population levels were expected to differ, in order to estimate the possible consequences of beetle outbreaks on the fungal flora, at species and strain levels, and the influence of the fungal flora on beetle population aggressiveness.

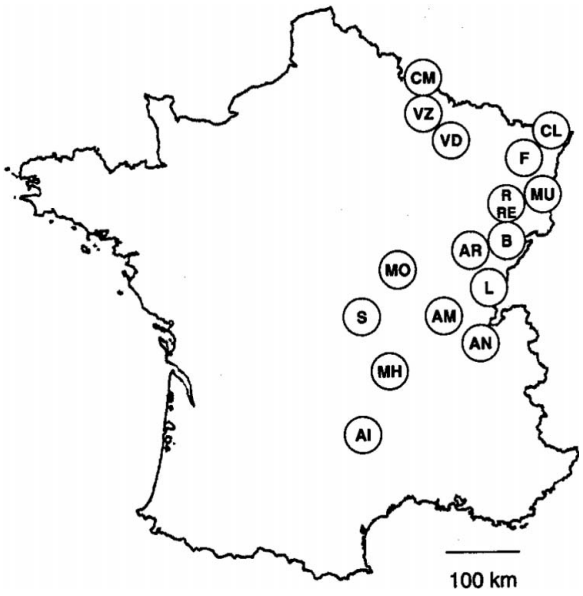
The frequency of the fungal species was studied in several spruce stands in which *I. typographus* population levels differed. The virulence and the ability to stimulate the host defence mechanisms were investigated for the most frequently isolated fungal species. The ability to stimulate the host defence mechanisms was considered both at a species and strain level.

Materials and methods

Localities and bark beetle population level estimations

Twelve Norway spruce (*Picea abies* (L.) Karst.) stands were selected in 2001 and 2002 and five in 2003 (Table 1, Fig. 1), in locations damaged or undamaged by the hurricanes. Five stands were examined for 3 years and two for 2 years. As no technique was available to estimate bark beetle population levels, we proceeded by estimating the dam-

Fig. 1. Map of France indicating study locations. See Table 1 for location codes.



age generated by the beetles. The number of standing trees freshly killed by *I. typographus* in a circular area of 500 m around the sampling points was counted in each stand at the end of autumn in 2001 and 2002. This distance is considered the maximum distance in which new damage would appear from a previous outbreak locale (Wichmann and Ravn 2001). Considering that the number of beetles colonizing a tree depends on tree size, each tree's diameter was measured at breast height (DBH), and the amount of damage in the locality was expressed as the cumulative basal area of killed trees.

Isolation, identification, and estimation of the fungal frequency association

Yearly, in every stand, 100 *I. typographus* adults were collected on trap trees 2 weeks after the beginning of spring attacks (Table 1). The associated fungi were isolated according to Furniss et al. (1990). This technique was chosen because it has been used by most authors that supported the hypothesis of selection of virulent fungal species during outbreaks (Solheim 1993*b*; Krokene and Solheim 1996; Viiri 1997; Viiri and Lieutier 2004). The day of beetle collection, three logs (1 m long, 10 cm diameter) from young spruce trees were cut in each stand. Before cutting, their foliage and bark were inspected, and the trees without any disease symptoms were selected. To prevent logs from drying, their ends were sealed with paraffin, and they were stored at 3 °C before being inoculated with beetles from the same origin. Bark plugs (5 mm diameter) were removed from the cambium level with a sterilized cork borer, and one beetle was inserted in each hole and crushed by replacing the plug in its original position. Inoculations were performed within 1 week after beetle collection. Approximately 30 inoculations per log were performed. Three weeks later, necrotic, elliptic lesions had developed around each inserted beetle. The logs were debarked and a fragment of phloem (5 mm × 5 mm) was collected at one extremity of each lesion. Fragments were placed on malt–agar medium (3% malt extract (Prolabo, Fontenay-sous-Bois, France), 1.5% agar (Merck, Whitehouse Station, New Jersey, USA)) in Petri dishes. Malt–agar medium is commonly used for ophiostomatoid isolations (Solheim 1986, 1993*b*; Viiri 1997). Emerging fungi were identified using fructifications and ascospores morphologies (Upadhyay 1981; Solheim 1986; Grylls and Seifert 1993). In case of contamination, the fungi were subcultured on malt–agar medium amended with citric acid (3% malt extract, 1.5% agar, 0.5% citric acid) or cycloheximide (3% malt extract, 1.5% agar, 0.5% cycloheximide (Sigma, St. Louis, Missouri, USA)) (Schneider 1956; Rapilly 1968). Cycloheximide was used only when there was no indication that the species isolated was *C. polonica*, as *Ceratocystis* species are generally sensitive to this compound (Samuels 1993). We did not investigate precisely whether the different isolations of the same species could be considered different strains, therefore they were qualified as isolates throughout the text.

Assessment of the virulence and ability to stimulate host-tree defences

In our study, virulence was considered to be a quantification of fungal pathogenicity (capacity to kill the host tree). Virulence was estimated through the ability of fungal species to penetrate into the sapwood and cause occlusion after mass inoculation on living trees (Horntvedt et al. 1983; Christiansen 1985*a*; Kirisits 1998; Croisé et al. 2001). The ability of fungi to cause important defence reactions in the phloem, that is, long necrotic lesions, after inoculation is sometimes considered an alternative estimator of their virulence (Raffa and Smalley 1988; Kirisits 1998). However, some species such as *Ophiostoma penicillatum* Grosm. can induce important defence reactions in the phloem, but cause almost no damage in the sapwood (Solheim 1992*a*, 1992*b*). Hence, this character is generally considered a poor estimator of virulence (Krokene and Solheim 1999). Nonetheless, it is an important factor to consider in bark beetle population dynamics, since it contributes to lowering the critical threshold of attack density. We estimated the ability to stimulate host defence reactions in the phloem after low-density inoculations for different fungal species and isolates and compared those estimates with the virulence estimates.

Experiments with mass inoculations and low-density inoculations were carried out in 2002 with fungal isolates collected in 2001, to assess virulence at species and strain levels. All isolates originated from different beetles within each locality. Experiments were conducted in two locations in France: Planchez (Nièvre, 47°17′N, 4°02′E) and La Tour de Scay (Doubs, 47°38′N, 6°23′E). The occurrence of wounds or disease symptoms on trunk and foliage of the trees was checked before experiments, and obviously unhealthy trees were discarded.

Comparison of virulence among species

Ophiostoma piceaperdum Rumbold, *Ophiostoma bicolor* Davids. & Wells, and *Ophiostoma tetropii* Mathiesen strains were collected from localities with different beetle population levels in 2001 and used for mass inoculations in 2002. Mass inoculations were performed with 2-week-old malt–agar cultures. Malt–agar plugs (5 mm diameter) containing fungal hyphae were inserted into trees (14 ± 1.4 cm

DBH) into 5 mm diameter holes previously bored into the cambium with a sterilized cork borer.

In Planchez, six isolates of *O. bicolor* (1 VD isolate, 1 RE, 2 L, 2 CL; see Table 1 for abbreviations), six isolates of *O. tetropii* (3 L, 2 S, 1 VZ), and seven isolates of *O. piceaperdum* (1 VD, 2 mol/L, 2 S, 2 CL) were each inoculated into two trees at a density of 8 inoculations/dm² on a 60 cm wide band without any branches at a minimum of 50 cm above ground. This number of inoculations is considered the critical threshold of inoculation above which trees are killed by *C. polonica*, the most pathogenic fungus associated with *I. typographus* (Christiansen 1985*a*, 1985*b*). In La Tour de Scay, five strains of each species were used (*O. bicolor*: 1 VD, 2 L, 1 RE, 1 CL; *O. tetropii*: 2 L, 2 S, 1 VZ; *O. piceaperdum*: 1 VD, 1 mol/L, 2 S, 1 CL; see Table 1 for abbreviations). Each isolate was inoculated into three trees at the same level above ground and the same density, but on a 1 m wide band, that is, above the virulence threshold of *C. polonica*, in order to distinguish the most virulent species.

All trees were harvested 3 months later. Six noninoculated control trees in Planchez and three in La Tour de Scay were also felled. For each tree, one log (2 m long) with the inoculation band was cut and stored at 3 °C. Water conductivity measurements were performed within 3 days after logging. A 20 cm long log, without any wounds or branches, was then cut from the middle of each inoculation band and used for water conductivity measurements. The loss of water conductivity was assessed by a comparison between inoculated and noninoculated trees following the technique of Guérard et al. (2000) and modified from Sperry et al. (1988). Deionized and degassed water was used at a pressure of 5 kPa. The flow through the log segment was recorded as the amount of water recovered in 1 min, at the open end of the log segment, after a 15-min circulation. Before these measurements, thin disks (2 mm wide) were removed from each end of the log; blue-stained and dried sapwood areas were recorded by transparency using translucent paper and quantified with Scion Image software® (Scion Corp.).

Ability to stimulate host defences

A total of 40 isolates of *O. piceaperdum* (9 VD isolates, 8 S, 8 mol/L, 8 L, 7 CL), 22 isolates of *O. bicolor* (5 VD, 2 S, 4 mol/L, 4 L, 3 CL, 4 RE), and 4 reference strains of *C. polonica* (E/7/9, GL/32/7, KRB/5/6/1, KRB/2/4/3) were used in this study. In May 2002 at Planchez and La Tour de Scay, low-density inoculations were performed on 15 spruce trees (25 ± 3.1 cm DBH) in each location, using 2-week-old fungal cultures on malt–agar. The inoculation technique was the same as described previously. Each isolate plus two sterile plugs of malt–agar medium were inoculated at random once into each tree. Inoculations were done between 70 cm and 2 m above the ground in six bands spaced 20 cm apart. Three weeks after inoculation the outer bark was removed around each inoculation point. Inoculations generally resulted in the formation of an elliptic, necrotic reaction in the phloem. The length of this reaction zone was recorded.

Statistical analyses

All analyses were performed with Statistica® software (StatSoft Inc. 2000). The relationships between damage caused by the beetles and the frequency of association with the main fungal species were examined by correlation. As the nature of the relationship was unknown, linear and nonlinear correlations were tested (Pearson, Spearman, Kendal's τ). Correlations among the different virulence estimators and between virulence estimators and the ability to stimulate host defence reactions were also assessed. Comparisons among fungal species, strains, location of origin, and experimental plots based on the fungal virulence and the ability of the fungi to stimulate host defence reactions were examined with univariate or multivariate analysis of variance (ANOVA) followed by mean comparisons tests of Newman–Keuls or Scheffé's when sample sizes differed. Each ANOVA was preceded by a Shapiro–Wilk's test to check for normality of data and a Levene test to check for homogeneity of variance. For multivariate analyses, the Levene test was replaced by a test of Sen and Puri. Lengths of induced reactions and values of water conductivity were log transformed to homogenize variances. Likewise, blue-stained, occluded sapwood areas and frequencies of association were arcsin square-root transformed (Zar 1996). When distributions were not normal or when variances were not homogenous, Kruskal–Wallis tests were performed. The significance level was set at $\alpha = 0.05$.

Table 2. Basal areas of tree mortality in sampling locations in France.

	No. of trees killed		Basal area (dm²)*	
Location	2001	2002	2001	2002
CM	1	0	14	0
VZ	89	14	712	141
MO	10	0	66	0
S	120	218	654	926
MU	55	16	622	253
CL	30	7	128	12
L	6	38	38	289
AR	12	—	85	—
F	119	—	1147	—
VD	146	—	1390	—
AM	7	—	79	—
RE	95	—	1037	—
R	95	3	895	29
AN	0	0	0	0
B	100	0	707	0
AI	22	0	174	0
MH	25	24	294	189

Note: For location codes see Table 1. A "—" indicates that a locality was not studied in 2002.

*Cumulative basal areas of standing trees killed by *Ips typographus* were recorded in autumn 2001 and (or) 2002 in a radius of 500 m around the sampling point.

Results

Damage estimations

In 2001, damage estimates were high (>600 dm²) in eight locations, low (<100 dm²) in six locations, and intermediate in three locations (Table 2). In 2002, damage levels were on average lower than in 2001 and were high in only one loca-

Table 3. Frequency of *Ophiostoma piceaperdum* (O.p.), *Ophiostoma bicolor* (O.b.), *Ophiostoma tetropii* (O.t.), *Ophiostoma ainoae* (O.a.), and *Ceratocystis polonica* (C.p.) isolated from 100 *Ips typographus* collected in 2001, 2002, and 2003.

	2001					2002					2003				
Site	O.p.	O.b.	O.t.	C.p.	O.a.	O.p.	O.b.	O.t.	C.p.	O.a.	O.p.	O.b.	O.t.	C.p.	O.a.
CM	26	16	0	0	4	49	3	0	0	0	55	10	0	3	1
VZ	76	28	7	0	0	17	14	0	0	0	38	8	1	4	0
MO	67	6	1	0	2	19	16	0	1	1	35	19	0	3	0
S	19	8	20	0	0	3	42	3	1	1	25	38	5	7	0
MU	26	7	1	0	0	19	16	0	1	1	31	21	1	4	0
CL	23	6	1	0	10	7	27	1	0	3	—	—	—	—	—
L	16	3	16	0	0	30	11	1	0	0	—	—	—	—	—
RE	16	37	2	0	1	—	—	—	—	—	—	—	—	—	—
AR	27	5	2	0	0	—	—	—	—	—	—	—	—	—	—
F	18	24	5	0	0	—	—	—	—	—	—	—	—	—	—
VD	37	27	15	0	2	—	—	—	—	—	—	—	—	—	—
AM	19	3	1	0	1	—	—	—	—	—	—	—	—	—	—
R	—	—	—	—	—	12	20	0	1	1	—	—	—	—	—
AN	—	—	—	—	—	18	15	0	2	0	—	—	—	—	—
B	—	—	—	—	—	27	15	3	4	0	—	—	—	—	—
AI	—	—	—	—	—	5	11	0	6	0	—	—	—	—	—
MH	—	—	—	—	—	11	18	0	0	0	—	—	—	—	—
All	30.9ab	14.1	5.8a	0*c*	1.7	20.5b	16.6	0.7b	1.4b	0.5	37.2a	19.2	1.4ab	4.2a	0.2

Note: Different letters following values indicate significant differences among years for each species observed. A "—" indicates that data were not recorded. See Table 1 for location details.

tion, intermediate in four locations, and low in the remaining six locations. Considering that no or little tree damage had been recorded by local foresters in 2000, the beetle populations that caused intermediate or high damage (>100 dm²) in 2001 and 2002 were thus considered to be at epidemic levels (Table 2).

Frequencies of association between fungi and beetles

The most common fungal species isolated in 2001, 2002, and 2003 were *O. bicolor* and *O. piceaperdum* (Table 3). *Ophiostoma ainoae* Solheim and *O. tetropii* were also frequently isolated. *Ceratocystis polonica* was found only in 2002 and 2003. *Ophiostoma piceae* Münch and *O. penicillatum* were isolated scarcely (seven and four isolates respectively). There was high variability among locations and years. In the five locations that were examined for 3 years, a significant correlation was found between years for the frequencies of *O. tetropii* (Pearson; 2001–2002: $r^2 = 0.88$, $p = 0.015$, 2002–2003: $r^2 = 0.94$, $p = 0.007$) but not for the other fungal species. Considering all locations together, the average frequency of *O. tetropii* decreased significantly between 2001 and 2002 (Kruskal–Wallis: $H_{[1,24]} = 7.14$, $p = 0.008$). The average frequency of *C. polonica* increased each year (2001–2002: $H_{[1,24]} = 9.11$, $p = 0.003$; 2002–2003: $H_{[1,17]} = 6.31$, $p = 0.012$), and *O. piceaperdum* average frequency significantly increased between 2002 and 2003 ($H_{[1,17]} = 4.44$, $p = 0.035$) (Table 3).

Each year the data from locations where beetle populations were of similar levels (either latent or epidemic) were grouped, and a Kruskal–Wallis analysis was performed between these two groups, but no significant differences were found. One positive correlation was found in 2001 between *O. tetropii* frequencies and beetle damage level (Kendal's $\tau = 0.45$, $p = 0.04$). *Ophiostoma bicolor* frequencies and beetle damage level also were positively correlated in 2002 (Pearson: $r^2 = 0.61$, $p = 0.037$). No correlation was found between damage levels in 2001 and frequencies observed in 2002. Data from 2 years showed highly significant positive correlations between beetle damage levels and frequencies of *O. bicolor* (Pearson: $r^2 = 0.55$, $p = 0.006$) or *O. tetropii* (Spearman: $r^2 = 0.31$, $p = 0.004$).

Virulence assessments

Mass inoculations and among species comparisons

At Planchez and La Tour de Scay, the three fungal species tested caused significant losses of hydraulic conductivity compared with controls, but no differences occurred among the three fungal species (Fig. 2). The fungi also induced an important sapwood occlusion, again, without differences among species. *Ophiostoma tetropii* and *O. piceaperdum* isolates induced sapwood blue staining at both field localities. *Ophiostoma bicolor* did not induce any blue staining in Planchez, whereas in La Tour de Scay it induced significantly less blue staining than *O. piceaperdum* (Fig. 2). Blue staining and sapwood occlusion induced by *O. tetropii* and *O. piceaperdum* in La Tour de Scay were significantly greater than in Planchez ($H_{[1,25]} = 4.94$, $p = 0.026$ and $F_{[1,27]} = 74.44$, $p < 0.001$ for blue staining; $F_{[1,25]} = 5.05$, $p = 0.034$ and $F_{[1,27]} = 4.19$, $p = 0.05$ for sapwood occlusion, respectively). Dead sapwood areas correlated positively with blue-stained areas (Pearson: $r^2 = 0.24$, $p < 0.001$) and negatively with hydraulic conductivity (Pearson: $r^2 = 0.35$, $p < 0.001$).

Low-density inoculations and comparisons among isolates

A tree effect on the reaction zone length was observed at both locations (La Tour de Scay: $F_{[15,968]} = 12.49$, $p < 0.001$;

Fig. 2. Means (± SE) of dead sapwood area, blue-stained sapwood area, and hydraulic conductivity (Ks) resulting from mass inoculation experiments (8 inoculations/dm²) conducted in 2002 at Planchez and La Tour de Scay, with isolates of *Ophiostoma bicolor*, *Ophiostoma tetropii*, and *Ophiostoma piceaperdum*. Values in parentheses indicate the number of isolates used for each species. Letters indicate significant differences for blue staining, and asterisks indicate significant differences for K_s.

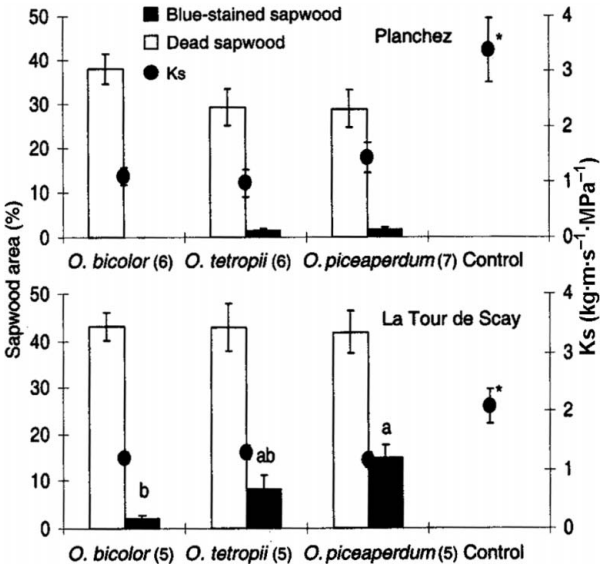
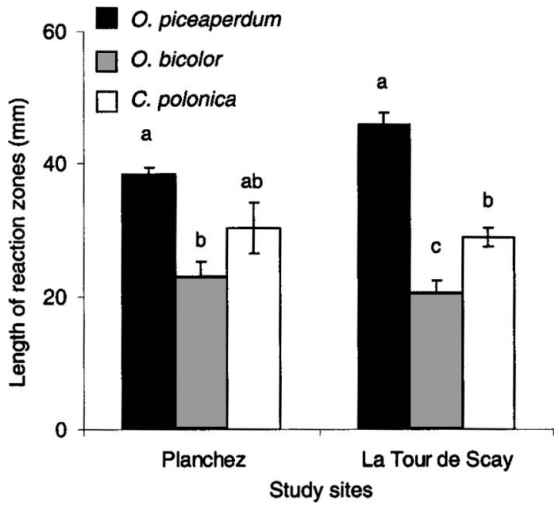
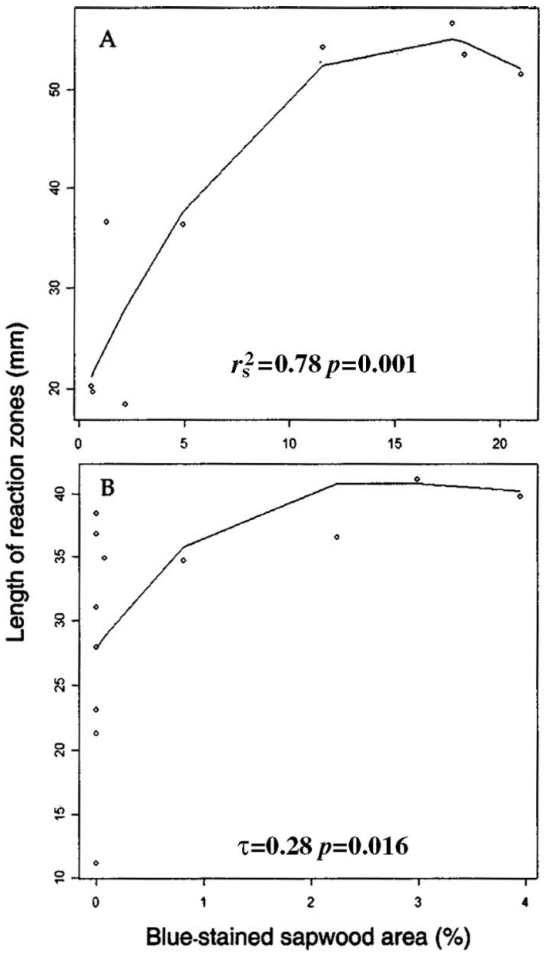


Fig. 3. Mean reaction zone length (± SE) induced 3 weeks after inoculation with isolates of *Ophiostoma piceaperdum* (n = 40), *Ophiostoma bicolor* (n = 20), and *Ceratocystis polonica* (n = 4) at Planchez and La Tour de Scay. Letters indicate significant differences among species.



Planchez: $F_{[14,973]} = 6.39$, $p < 0.001$). A weak but significant positive correlation was found only for *O. piceaperdum* isolates between the length of the reaction zones and tree diameter (Pearson: $r^2 = 0.01$, $p = 0.019$). At each location, average length of the reaction zone differed among trees, but the ranking of the isolates was the same on all trees. Hence, each tree was considered one replicate for all isolates. *Ophiostoma piceaperdum* isolates induced significantly longer reaction zones at La Tour de Scay than at Planchez. For

Fig. 4. Spearman (r_s^2) and Kendall' τ correlations between phloem reaction zone length and sapwood blue staining induced by isolates of *Ophiostoma piceaperdum* and *Ophiostoma bicolor*, respectively, after low-density inoculations and mass inoculations in La Tour de Scay (A) and Planchez (B).



this fungal species, no correlation was found between both study sites for the length of reaction zones. For *O. bicolor*, however, the lengths of reaction zones were equivalent and correlated in both locations (Pearson: $r^2 = 0.66$, $p < 0.001$).

The three species induced significantly longer reaction zones than sterile malt–agar controls. The longest reaction zones were produced by *O. piceaperdum*, the shortest by *O. bicolor*, with *C. polonica* being intermediate (Fig. 3). There was a significant among-isolate difference for each of the three fungal species, even among isolates from the same location ($p < 0.001$ for *O. piceaperdum* isolates ($F_{[38,543]} = 4.82$ in La Tour de Scay and $F_{[38,545]} = 3$ in Planchez) and *O. bicolor* isolates ($F_{[20,292]} = 7.28$ in La Tour de Scay and $F_{[20,294]} = 13.36$ in Planchez); $F_{[3, 55]} = 3.49$, $p = 0.022$ in Planchez and $p = 0.556$ in La Tour de Scay for *C. polonica* isolates). However, no significant relationships were found with beetle damage levels or isolate origin and reaction zone length. Significant nonlinear correlations were found between reaction zone lengths and sapwood blue staining (Fig. 4), when considering *O. piceaperdum* and *O. bicolor* together (the limited number of isolates for each species did not allow us to examine them separately).

Discussion

Fungal flora composition and variation with insect epidemic status

Fungal species isolated during these experiments are common associates of *I. typographus* (Kirisits 2004), considering that *O. piceaperdum* is a synonym of *O. europhioides* Wright & Cain (Jacobs et al. 2000). The relatively high frequencies of *O. bicolor* and *O. piceaperdum* are in accordance with previous observations, using the same isolation technique, in Finland and France (Viiri 1997; Viiri and Lieutier 2004), but contradicts observations in Norway (Solheim 1993*b*). In our study, *O. penicillatum* and *C. polonica* were absent in 2001 and rare in 2002 and 2003, although *C. polonica* frequency increased during the 3-year study period. This contrasts with observations by Viiri and Lieutier (2004) in three areas investigated in 1996 in France. In their study, *O. penicillatum* and *C. polonica* occurred frequently (38% and 36%, respectively). However, an important outbreak occurred in 1991–1995, during which *C. polonica*, considered as the most pathogenic associate of *I. typographus* (Horntvedt et al. 1983; Krokene and Solheim 1998; Kirisits 1998), might have been selected. Similarly in our sampling, the apparition and extension of *C. polonica* isolates was observed only since 2002 (Fig. 2), as beetle populations began to increase in 2000 on wind-thrown trees and started to massively attack standing trees in 2001. Nevertheless, this frequency was still relatively low in 2003. Moreover it did not differ between latent and epidemic populations, and no significant correlation was found between previous damage levels and the frequency of *C. polonica* or any other fungal species.

Concerning the hypothesis of virulent species selection during outbreaks, our data suggest three possible explanations. First, it is possible that a selection occurs as a consequence of tree resistance and competition among fungal species, but only during certain years and after numerous attacks on healthy spruce trees. Although the existence of different damage levels confirmed that the population levels of *I. typographus* differed among locations (Table 2), damage was lower in 2002 than in 2001, confirming the early collapse of the outbreak throughout France. Thus, the outbreak might not have been significant enough to allow selection. Second, it is possible that no selection of virulent fungal species occurs during bark beetle outbreaks. Beetle aggregation could be the reason, because it leads to the inoculation of several different fungal species all at the same time. Once dead, a tree can be invaded simultaneously by virulent and saprobic fungal species, and consequently, when the progeny beetles emerge, they can carry spores from different fungal species. Finally, the isolation technique on logs might have favoured saprobic fungi to the detriment of virulent species and thus underestimated their frequency. However, in our study, several isolations of the virulent fungus *C. polonica* were realised, and some isolates from various other fungal species showed a high virulence. Isolations from living trees or from the sapwood would have been more discriminating, but also more difficult to conduct in a sterile manner. Likewise, isolation on malt–agar media could have favoured some particular ophiostomatoid species (Upadhyay 1981). However malt–agar medium has been used in most studies of the associated fungal flora of *I. typographus* (Solheim 1993*b*; Viiri 1997; Kirisits 1998) and was thus the best suited for comparisons among studies.

An alternative hypothesis to explain the discrepancies among studies of associated fungal flora composition could be that environmental conditions can affect both host resistance and fungal development, and could then influence the fungal flora composition. The high among-location and among-year variability of fungal flora composition encountered in our study (Table 3) supports an environmental-condition effect on fungal species occurrence. Soil conditions and humidity can affect host resistance (Jakus 1995; Christiansen and Glosli 1996) and could indirectly favour virulent or saprobic fungal species. As different fungal species have different thermal growth optima (Lieutier and Yart 1989), temperature could also favour certain species. Temperature may also have indirect consequences on the fungal species, as at low temperatures beetles tend to hibernate in the litter rather than under the bark (Annila 1969); these are two environments that could have different influences on the fungal flora present on the beetle's cuticle.

Fungal flora implication in the insect population dynamics

Considering the low isolation rate of *C. polonica* in our study, one can wonder about the role of the other fungal species in the population dynamics of *I. typographus*. Would they be virulent enough or able to significantly stimulate host defence reactions to play an important role during beetle population establishment? To answer this question, it was particularly interesting to study these parameters. Different virulence estimators, sapwood blue staining, sapwood occlusion, and water conductivity were linearly correlated and were thus in agreement with each other. Using these different parameters simultaneously enabled us to separate the different biases of each estimator and thereby have a more accurate estimate of fungal virulence. The length of the phloem reaction zone also correlated positively with blue-stained sapwood area, although not linearly (Fig. 4). This finding supports the results of Lieutier et al. (2003) with *Leptographium wingfieldii* Morelet that the length of reaction zone in the phloem could be used as an estimator of virulence, but only when comparing among isolates of the same species. The reaction zone length induced by the fungal isolates correlated positively between Planchez and La Tour de Scay for *O. bicolor*, but not for *O. piceaperdum*. This suggests that *O. piceaperdum* isolates are more susceptible to environmental conditions. The reaction zone length of *O. piceaperdum* also correlated with tree diameter at one study location, confirming previous observations by Kytö et al. (1996) and Baier et al. (2002). Considering that *O. piceaperdum* isolates induced the longest lesions in the phloem (Fig. 3), it is also possible that, when extended, the length of the reaction zone induced by a fungus depends on the inoculated tree.

Mass inoculations showed that *O. piceaperdum* and *O. tetropii* were able to cause important losses of hydraulic conductivity and extended sapwood occlusion (Fig. 2). Sapwood occlusion in the present study was less important compared with what was induced by mass inoculation with *C. polonica* in previous studies (Christiansen 1985*a*; Krokene and Solheim 1998, 2001), but environmental and

technical conditions differed (climate, delays before harvesting, etc.). Nevertheless, *O. piceaperdum* and *O. tetropii* isolates were able to cause blue staining at an inoculation number equal to the critical threshold of inoculations by *C. polonica*, above which spruce trees die. Consequently, *O. piceaperdum* and *O. tetropii* can be considered pathogens of Norway spruce. This is in agreement with the fact that they caused more extended sapwood blue staining and sapwood occlusion at the La Tour de Scay study site, where the number of inoculations was higher than at Planchez, although a site effect cannot be discarded. The pathogenicity of *O. piceaperdum* is in accordance with results by Harding (1989) and Solheim (1993*a*), but contradicts those of Kirisits (1998). *Ophiostoma piceaperdum* isolates also stimulated importantly host defences as, in the low density inoculations experiments, they induced longer phloem reaction zones than *C. polonica* isolates (Fig. 3). Our results are the first report on *O. tetropii* pathogenicity in Norway spruce.

Ophiostoma bicolor isolates caused no or only little blue staining, but they were able to damage as much sapwood as the other fungal species (Fig. 2). Consequently, *O. bicolor* might also be considered a pathogen of Norway spruce, albeit a less virulent one than the other two species. The possible pathogenicity of *O. bicolor* contradicts the results of previous virulence assessment studies (Solheim 1988; Kirisits 1998), but is in agreement with Harding (1989) that *O. bicolor* is significantly more frequently isolated from living trees attacked by bark beetles than from windfalls and with the fact that *O. bicolor* can penetrate rapidly and deeply into sapwood (Solheim 1992*a*, 1992*b*). Our isolates of *O. bicolor* also induced shorter phloem reaction zones compared with those of *C. polonica* and *O. piceaperdum* (Fig. 3). Hence, this species might be less able to invade the phloem and stimulate host defences in this tissue.

The association frequency of *O. piceaperdum*, its virulence, and its ability to stimulate host defence reactions indicate that it could be an important associate for *I. typographus* during population establishment in France. However, no correlation was found between its frequency of association and the extent of beetle damage. In contrast, positive correlations were found between *O. bicolor* or *O. tetropii* frequencies and beetle damage levels, although these fungi did not exhibit a high level of virulence or a high ability to stimulate host defence reactions. Nevertheless, given the among-year and among-location variability, these correlations should be considered with caution. Further data are still needed before concluding on the degree of interference of fungal species on *I. typographus* population aggressiveness. Moreover, no difference in virulence estimations was observed among isolates from locations with different beetle population levels. Thus, the possibility that virulent fungal isolates aid in the spread and intensity of bark beetle outbreaks was not confirmed.

Fungal flora intraspecific variability

As hypothesized by Kirisits (1998), the contradictory results regarding virulence estimations among different studies might be due to differences among fungal strains. Although an effect of environmental conditions could not be discarded, the among-isolate variability observed in our mass and low-density inoculation experiments tends to confirm the hypothesis of Kirisits. The significant variation among isolates from the same origin and the absence of a significant effect of isolate origin also indicate that a certain level of among-isolate variability is present even within one locality. These observations are in agreement with results from studies with *L. wingfieldii* (Lieutier et al. 2003) and also confirm that different isolates could exhibit different levels of virulence (Kirisits 1999; Lieutier et al. 2003). It tends to put in perspective the results of previous between-species comparisons of virulence for fungal associates of *I. typographus*, in which commonly only one isolate of each species was used.

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