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Investigations concerning the role of *Chalara fraxinea* in declining *Fraxinus excelsior*

R. Bakys, R. Vasaitis*, P. Barklund, K. Ihrmark and J. Stenlid

Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, PO Box 7026, SE – 750 07 Uppsala, Sweden

A study was carried out to clarify the role of the fungus *Chalara fraxinea* in decline of *Fraxinus excelsior*, which is observed on a large scale in central and northern Europe with high incidence of tree mortality. The aims of this work were: (i) to check for the presence of *C. fraxinea* in various tissues of declining *F. excelsior* by agar culture isolations and by direct analysis of plant tissues using molecular techniques (DNA extraction, ITS-PCR, cloning, ITS sequencing and T-RFLP); (ii) to study fungal communities inhabiting tissues with symptoms; and (iii) to test the pathogenicity of *C. fraxinea* to *F. excelsior*. *Chalara fraxinea* was isolated from 93% of stem cankers, 91% of necrotic leaf stalks, 27–28% of bark wounds and 30% of visually healthy leaf stalks. Molecular analyses of necrotic leaves, leaf stalks and bark revealed the presence of 25 different fungal taxa, 14 of which were detected in all three types of tissue sample. *Chalara fraxinea* was the second most common species (61% of samples), and only *Cryptococcus foliicola* occurred more often (70%). All eight of the tested *C. fraxinea* isolates induced necroses in bark and cambium on each of 86 inoculated trees, and all controls remained healthy. Average length of necroses caused by different C. fraxinea strains varied from 4·2 to 8·9 cm, but the differences were statistically insignificant. Instead, differences in resistance of individual trees to *C. fraxinea* were observed.

Keywords: basidiomycetous yeasts, fungal communities, forestry, *Phytophthora*, T-RFLP, tree diseases

Introduction

Since the early 1990s, large-scale decline of *Fraxinus excelsior* has been increasingly observed in central and northern Europe, resulting in massive dieback of trees over the Baltic States, Poland, southeast Scandinavia, Germany and Austria (Juodvalkis & Vasiliauskas, 2002; Przybyl, 2002a; Barklund, 2005; Heydeck *et al*., 2005; Cech, 2006; Thomsen & Skovsgaard, 2006; Vasiliauskas *et al*., 2006). Trees are subject to dieback at various ages, in forest stands, as landscape trees and in nurseries (Kowalski & Lukomska, 2005; Schumacher *et al*., 2007; Kirisits & Halmschlager, 2008). The crowns of declining *F. excelsior* trees exhibit a wide range of symptoms: (i) wilting and premature shedding of leaves; (ii) necroses of leaves, buds, leaf stalks and bark; (iii) top and shoot dieback; (iv) cankers on shoots, branches and stems (Przybyl, 2002b,c; Barklund, 2005; Kowalski & Lukomska, 2005; Cech, 2006; Thomsen & Skovsgaard, 2006; Schumacher *et al*., 2007; Thomsen *et al*., 2007; Kirisits & Halmschlager, 2008; Skovsgaard *et al*., 2009).

*E-mail: rimvys.vasaitis@mykopat.slu.se

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A fungus from the genus *Chalara* was reported as the cause of *F. excelsior* decline (Kowalski & Lukomska, 2005; Kowalski, 2006). It was recently described as a new species *Chalara fraxinea* (Kowalski, 2006). However, the experimental evidence for pathogenicity of *C. fraxinea* to *F. excelsior* has not yet been provided, although in some cases the fungus was found to be one of the dominating species, isolated from up to 70% of examined shoots with dieback symptoms (Kowalski & Lukomska, 2005; Kowalski, 2006). In other studies only sporadic and occasional isolation of *C. fraxinea* from declining *F. excelsior* was noted [Lygis *et al*., 2005 (as *Hymenoscyphus* sp.970); Schumacher *et al*., 2007; Kirisits & Halmschlager, 2008; Skovsgaard *et al*., 2009]. Different factors could have contributed to the variable success of reported isolations, for example sampling season, phase of pathological process, symptom characteristics and isolation media. Moreover, as a slow grower in culture, the fungus may have been overgrown by other species during isolation (Kirisits & Halmschlager, 2008).

A DNA-based identification method was suggested to study fungal communities directly from tissue or environmental samples that are likely to contain some fungi that are difficult to culture (Lindahl *et al*., 2007). Here, polymerase chain reaction (PCR) amplification of

Table 1 Occurrence of *Chalara fraxinea* and other fungi in isolations from leaf and bark tissues of dieback-affected *Fraxinus excelsior*

Symptom category	Attempted isolations (no.)	Frequency of *C. fraxinea* isolation (%)	Other fungi isolated (no.)[a]
Healthy-looking leaf stalks	40	30	10
Necrotic leaf stalks	45	91	5
Small bark wounds (≤5 mm) on shoots and branches	60	27	12
Larger bark wounds with necrosis (10–15 mm) on shoots and branches	45	28	5
Necrotic bark from stem cankers	30	93	10
All categories	220	49	42

[a]Numbers in this column do not reflect fungal diversity, as they represent a total of isolated strains, including possible representatives of the same taxon.

the internal transcribed spacer (ITS) region of the ribosomal genes was combined with the community fingerprinting technique defined as terminal restriction fragment length polymorphism (T-RFLP) (Dickie *et al*., 2002). In order to identify taxa in the T-RFLP profiles, PCR products from representative samples were cloned, enabling sequencing of the ITS region of the ribosomal DNA (rDNA) genes for individual taxa within mixed samples (Landeweert *et al*., 2003; O'Brien *et al*., 2005) and subsequent identification by database comparisons (Lindahl *et al*., 2007).

The first aim of the present work was to check the presence of *C. fraxinea* in different parts of declining *F. excelsior*, with and without symptoms, by fungal isolations on different media, and by direct analysis of plant tissues using molecular techniques (DNA extraction, ITS-PCR, cloning, ITS sequencing and T-RFLP). The second aim was to study by molecular methods fungal communities inhabiting tree tissues with the advancing dieback. The third aim was to test the pathogenicity of different *C. fraxinea* strains to young *F. excelsior* trees. Moreover, as the involvement of *Phytophthora* spp. was implied as a possible cause of *F. excelsior* decline (Przybyl, 2002c), plant tissues with advancing dieback symptoms were checked for the presence of oomycetes.

Materials and methods

Study sites and sampling

Field sampling was done in September 2005 at two locations in southern-central Sweden: Örebro (59°16′N, 15°13′E) and Gnesta (59°02′N, 17°18′E), the geographic distance between which is about 200 km. Each locality comprised 20- to 30-year-old mixed natural stands dominated by *F. excelsior* showing symptoms of crown decline (Barklund, 2005; Thomsen & Skovsgaard, 2006). In both stands, branches with dieback symptoms on leaves and bark were cut and individually packed into plastic bags. In Örebro, the sampling also included portions of necrotic bark from stem cankers. In the laboratory, the collected material was sorted into the following symptom categories: (1) necrotic leaves (Fig. 1a); (2) necrotic leaf stalks (Fig. 1a); (3) necrotic shoot bark (Fig. 1b); (4) small bark wounds (≤5 mm) (Fig. 1c); (5) larger bark wounds with necroses (10–15 mm) (Fig. 1d); and (6) necrotic bark from stem cankers (Fig. 1e). Symptom categories 4, 5 and 6 included samples only from Örebro.

Isolation of fungi

Isolation of fungi to pure culture was carried out within 24 h after the collection of samples in the field, from healthy-looking leaf stalks, necrotic leaf stalks (Fig. 1a) and the bark from small wounds (Fig. 1c), larger wounds with necrosis (Fig. 1d) and stem cankers (Fig. 1e). Table 1 shows the number of isolations attempted from each symptom category. The samples for isolation from leaf stalks were prepared by cutting off 3- to 5-mm-long sections, which in the case of necrotic leaf stalks included parts with and without symptoms. Bark samples from wounds and cankers were cut off as pieces approx. 3×3 mm in size, and also included both necrotic and healthy-looking bark tissue. Surface-sterilization of all samples was done as in a previous study (Barklund & Kowalski, 1996). Pieces of stalk and bark were washed under running water, then soaked in 96% ethanol for 1 min, soaked in sodium hypochlorite (4% active chlorine) for 5 min, again soaked in 96% ethanol for 30 s, and then left to dry on sterile filter paper. For isolation, prepared samples were placed onto 9-cm Petri dishes, containing either 2% malt agar, vegetable juice agar or water agar. The Petri dishes were incubated at room temperature for 2 weeks and observed daily for the presence of fungal mycelia for subsequent subculturing into a pure culture. Mycelia were investigated using a light microscope, and the fungus *C. fraxinea* was identified according to morphological characters of its mycelium (Kowalski, 2006; Schumacher *et al*., 2007; Halmschlager & Kirisits, 2008) and by comparison of the cultures of the fungus to strains provided by Professor Tadeusz Kowalski. No attempt was made to identify other isolated fungi.

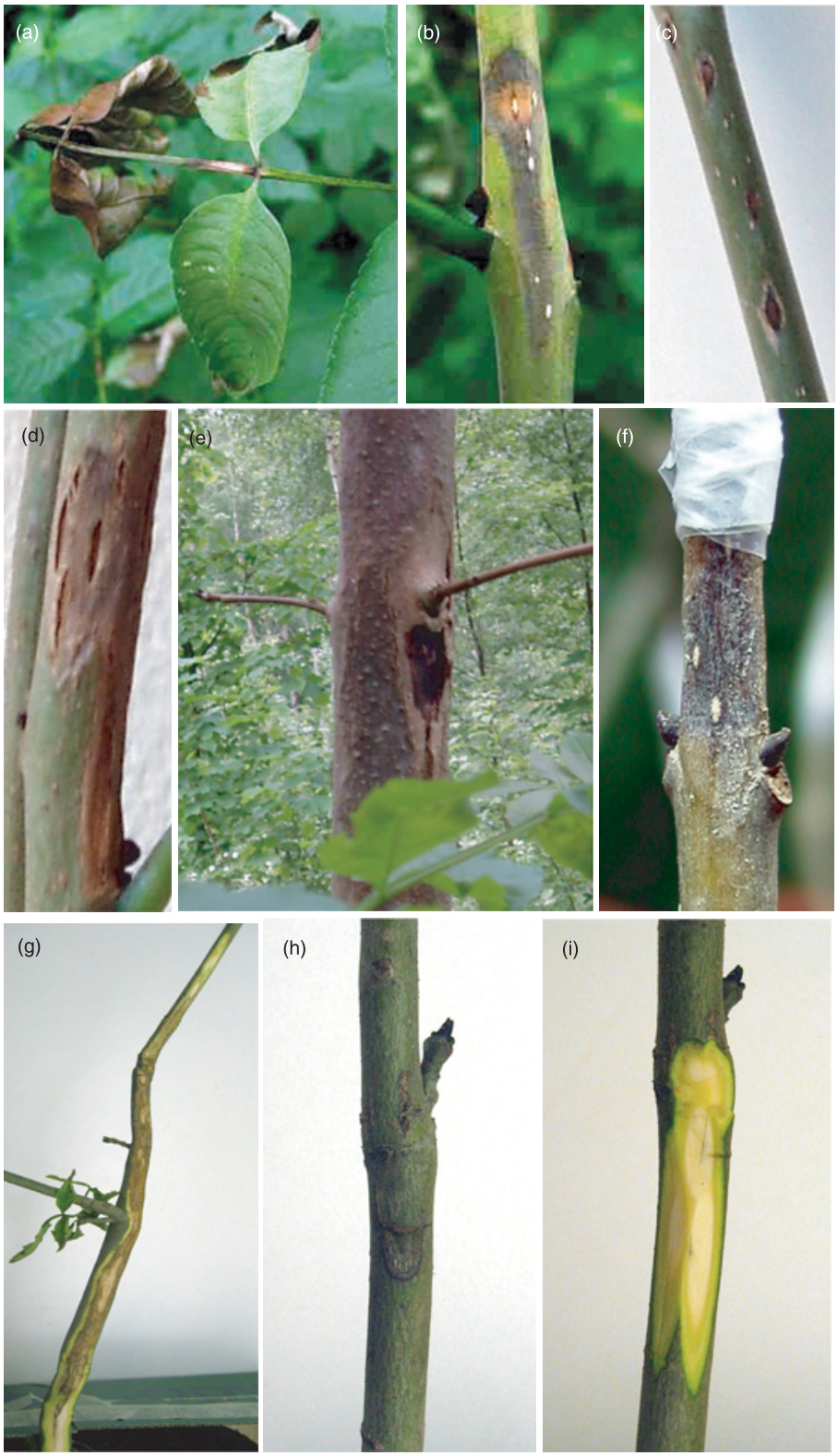

Figure 1 Dieback symptoms of *Fraxinus excelsior* and pathogenicity tests with *Chalara fraxinea*; (a) necrotic leaves and leaf stalk with necrotic and visually healthy sections; (b) necrotic shoot bark; (c) small bark wounds; (d) bark wound with necrosis; (e) stem canker; (f) artificial inoculation with *C. fraxinea* showing bark necrosis below sealed inoculum; (g) cambium necrosis following inoculation with *C. fraxinea*; (h) occluded scar following inoculation with sterile control; (i) visually healthy cambium beyond the same control inoculation.

DNA extraction from plant tissue

Analyses of fungal DNA directly from plant tissue with symptoms followed the protocol previously used in the study by Lindahl *et al.* (2007) and included 32 necrotic leaves, 32 necrotic leaf stalks and 32 discrete bark necroses on shoots (Fig. 1a,b). Each symptom category included 16 samples from Örebro and 16 from Gnesta (50:50 ratio). For extraction of fungal DNA, the tissues were prepared as follows: (i) from each leaf, a section approx. 1 cm^2 in size was cut using sterilized scissors; (ii) from each leaf stalk, a 7- to 10-mm-long portion was cut using a sterilized scalpel; and (iii) from each bark sample, a section approx. 5×5 mm in size was cut using a scalpel. All samples were taken from the zone of advancing necroses and included both necrotic and healthy-looking tissue. The samples were individually placed into Eppendorf tubes, freeze-dried and ground in a FastPrep homogenizer (MP Biomedicals) with one screw and one nut per tube without any liquid. DNA was extracted in a CTAB-buffer (3% cetyltrimetylammoniumbromide, 2 mM EDTA, 150 mM Tris-HCl, 2·6 M NaCl, pH 8) at 65°C for 1 h. After centrifugation the supernatant was extracted with chloroform and the DNA was precipitated from the upper phase with 2-propanol.

PCR, cloning, sequencing and sequence analysis

PCR was carried out using the primers ITS1-F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) with an annealing temperature of 55°C. The thermal cycling was carried out in an Applied Biosystems GeneAmp PCR System 2700 thermal cycler. An initial denaturation step at 95°C for 5 min was followed by 35 amplification cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. The thermal cycling was ended by a final extension step at 72°C for 7 min. The PCR reaction contained 200 μM dNTPs, 0·2 μM primers, 0·03 units μL^{-1} ThermoRed *Taq* polymerase with reaction buffer Y (SaveenWerner) and a final $MgCl_2$ concentration of 2·75 mM. PCR products were size-separated on 1% agarose gels (D-1, Hispanlab), stained with ethidium bromide and visualized under UV light. PCR products were cloned with the TOPO TA Cloning Kit with pCR®2·1-TOPO vector and One Shot TOP10 chemically competent *E. coli* (Invitrogen). Small amounts of bacteria were used directly for PCR, which was carried out us above, but with primers M13 Forward (GTAAAACGACGGCCAG) and M13 Reverse (CAGGAAACAGCTATGAC). The PCR products were purified with AMPure (Agencourt) and sequenced with a CEQ 8000 Genetic Analysis System with the CEQ DTCS Quick Start Kit (Beckman Coulter). Sequences from cloned fragments were compared to those of known fungi, available in the databases. Two databases – one at GenBank (Altschul *et al.*, 1997) and another at the Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences – were used to define the identity of sequences. The latter is an as yet unpublished database containing about 2000 ITS sequences of well documented fungal isolates (tree pathogenic, saprotrophic and endophytic), the majority of which (in particular microfungi) were isolated to pure cultures during previous studies here, and morphologically identified at the Central Bureau of Fungal Cultures, Utrecht, Netherlands. The ITS sequence homology for delimiting a fungal taxon (presumed species) was set at 98–100%, and for delimiting at genus level it was 94–97%.

T-RFLP

The cloning approach was combined with T-RFLP (terminal restriction fragment length polymorphism) analysis in order to enable processing of a large set of samples. All samples were used for T-RFLP analysis. In addition, all clones that were sequenced were also subjected to T-RFLP analysis, to enable matching of T-RFLP patterns with sequences. PCR was carried out as above, but the primers were labelled with WellRED fluorescent dyes, ITS1-F with D3-PA and ITS4 with D4-PA (Proligo). The PCR amplicons were digested with restriction endonucleases according to the manufacturers' instructions. The library clones were digested with *Cfo*I (Promega) and *Taq*I (Fermentas) separately. PCR products from samples were also digested with *Taq*I and *Cfo*I separately. The T-RFLP patterns were analysed with a Beckman Coulter CEQ 8000 Genetic Analysis System, using CEQ DNA Size Standard Kit-600. Sample T-RFLP patterns were compared with the reference database constituted by T-RFLP patterns from the clones using the program TRAMP (Dickie *et al.*, 2002). To account for base calling during analysis on the Beckman Coulter CEQ 8000 Genetic Analysis System and for within-taxa variation of the ITS region, a threshold level for fragment size in TRAMP was set to ±2 base pairs. Out of a total of 96 samples subjected to analysis, 89 (93%) were successfully analysed: 28 (88%) from leaves, 32 (100%) from leaf stalks and 29 (91%) from bark (Table 2).

Test for presence of oomycetes

Available DNA extractions from leaves (32), leaf stalks (32) and bark samples (30) were tested for the presence of oomycetes. PCR was carried out using the primers P1 (GAAGGATCATTACCACAC) (Geraats, 2003) and ITS4 (TCCTCCGCTTATTGATATGC) with an annealing temperature of 55°C. The thermal cycling was carried out in an Applied Biosystems GeneAmp PCR System 2700 thermal cycler. An initial denaturation step at 95°C for 5 min was followed by 35 amplification cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. The programme was completed by a final extension step at 72°C for 7 min. The PCR reaction contained 200 μM dNTPs, 0·2 μM primers, 0·03 units μL^{-1} ThermoRed *Taq* polymerase with reaction buffer Y (SaveenWerner) and a final $MgCl_2$ concentration of 2·75 mM. In all PCR runs a sample of *Phytophthora infestans* was included as a control and only if the control was positive was the run considered successful. PCR products were

Table 2 Frequencies of detection of fungal ITS clones (%) in necrotic tissues of dieback-affected *Fraxinus excelsior*

Fungal taxon	GenBank accession no.	Analysed tissues (no.) from			
		Leaves (28)	Leaf stalks (32)	Shoot bark (29)	All (89)
Cryptococcus foliicola	EU852351	100	63	48	70
Chalara fraxinea	EU852352	39	97	41	61
Phoma glomerata	EU852353	71	13	76	52
Phoma exigua	EU852354	68	13	66	47
Cladosporium cladosporioides	EU852355	79	28	38	47
Cryptococcus sp.22B	EU852356	54	28	31	37
Bullera coprosmaensis	EU852357	54	34	17	35
Unidentified sp.26A	EU852358	14	56	28	34
Cryptococcus victoriae	EU852359	50	13	24	28
Venturia fraxini	EU852360	32	6	14	17
Ramularia sp.13A	EU852361	39	–	–	12
Leptosphaeria sp.21A	EU852362	18	–	17	11
Unidentified sp.9B	EU852363	11	6	17	11
Cryptococcus dimennae	EU852364	25	–	7	10
Unidentified sp.20A	EU852365	14	–	17	10
Taphrina carpini	EU852366	21	3	3	9
Coniothyrium sp.8A	EU852367	7	9	7	8
Unidentified sp.23A	EU852368	4	4	14	7
Phaeosphaeria caricicola	EU852369	7	–	10	6
Unidentified sp.16A	EU852370	11	–	3	4
Taphrina sp.11A	EU852371	4	–	7	3
Unidentified sp.18B	EU852372	–	3	7	3
Unidentified sp.24A	EU852373	–	–	10	3
Cryptococcus hungaricus	EU852374	7	–	–	2
Leptosphaerulina sp.3	EU852375	–	–	3	1

size-separated on 1% agarose gels (D-1), stained with ethidium bromide and visualized under UV light.

Pathogenicity tests

The experiment was set up in November 2005. Pathogenicity tests included 96 1-year-old 20- to 29-cm-high *F. excelsior* trees of seed origin from a bare-root nursery. A total of eight isolates of *C. fraxinea* were used for artificial inoculations of 86 of the trees, and 10 trees were used as controls. The isolates 10B and 12A originated from necrotic leaf stalks collected in Gnesta, while the other six strains were collected in Örebro: isolate 16BO from a healthy-looking leaf stalk, isolates 22A and 23A from necrotic leaf stalks, 32A from a small bark wound, and 36A and 38C from stem cankers (Table 3).

Prior to inoculations, autoclaved pieces of *Populus tremula* wood 1·0 × 0·2 × 0·2 cm in size were kept for 2 weeks on top of pure cultures of each *C. fraxinea* isolate to be inoculated. The wood pieces were successfully pre-colonized with each respective strain, and were used as inocula. For the inoculation, wounds exposing green living cambium and wood (e.g. Fig. 1i) approx. 1·0 × 0·2 cm in size were cut using a sterile scalpel on tree stems approximately 15 cm above the soil. The pre-colonized wood pieces were applied to the wounds with forceps and sealed with Parafilm™ sealing tape. Before every inoculation, scalpel and forceps were sterilized using 70% ethanol and an open flame. For controls, sterile pieces of wood of the same size were similarly applied and sealed to similar wounds on stems of 10 *F. excelsior* trees. At the beginning of the experiment all trees were dormant. They were individually planted into pots, placed into a greenhouse, and after 2 weeks subjected to artificial inoculations. The inoculations with different isolates of the fungus and the controls were mixed in randomized blocks. The plants were kept in a greenhouse under a 12:12-h light:dark regime, at 20°C, and periodically watered.

The pathogenicity tests were scored after 6 weeks, in January 2006. At that time, leaves were emerging from about 20% of the trees. All trees were cut at the soil level, transported to the laboratory and washed with tap water. Bark at the inoculation site was removed with a sterile scalpel, and the longitudinal spread of cambium necrosis (upwards and downwards beyond the borders of the 1-cm-long inoculation site) was measured. Fungal isolations were attempted from 3- × 3-mm bark sections bordering necrotic/visually healthy tissue, as described above. The identity of isolated strains was tested against the inoculated strain by a vegetative compatibility test, thus fulfilling Koch's postulates.

Statistical analyses

Qualitative Sorensen similarity indices (Magguran, 1998) were used to analyse similarity between fungal communities

Table 3 Results of artificial inoculations of 1-year-old *Fraxinus excelsior* stems with *Chalara fraxinea*

	Inoculated trees				Length of necroses (cm)		
Isolate	No.	Without necrosis (%)	With necrosis (%)	Dead (%)	Min.	Max.	Mean ± SD[a]
32A	11	0	100	0	1·1	7·8	4·2 ± 2·5
12A	11	0	100	18	1·1	15·9	6·4 ± 6·1
16BO	11	0	100	36	1·3	14·0	7·1 ± 4·2
10B	11	0	100	18	1·2	19·3	7·2 ± 7·5
23A	10	0	100	30	1·4	15·2	7·4 ± 5·1
22A	11	0	100	36	1·1	16·8	7·7 ± 5·5
38C	10	0	100	20	1·2	22·3	8·6 ± 6·5
36A	11	0	100	27	1·5	28·7	8·9 ± 10·9
All	86	0	100	23	1·1	28·7	7·2 ± 6·5
Control	10	100	0	0	–	–	–

[a]Differences between means in this column are statistically insignificant (one-way ANOVA, $P = 0{\cdot}80$).

from different geographic locations and symptom categories. The lengths of cambium necroses caused by each inoculated *C. fraxinea* strain were compared by one-way analysis of variance (ANOVA) and the Tukey's test for multiple comparisons between all pairs of means. The analyses were performed using the Minitab™ statistical software package.

Results

Pure-culture isolations of *Chalara fraxinea*

Growth of *C. fraxinea* mycelium was recorded in each symptom category investigated, and, depending on the type of tissue, the fungus was isolated from 27–93% of samples (Table 1). Most commonly, it was found in necrotic leaf stalks (91%) and necrotic bark from stem cankers (93%), but was less often isolated from small and larger bark wounds (27–28%). The fungus was also isolated from 30% of healthy-looking (symptomless) leaf stalks. *Chalara fraxinea* was detected in 35–100% of all samples collected in Örebro, and in 25–87% of all samples collected in Gnesta. The frequencies of its isolation on malt, vegetable juice agar and water agar media were 90, 63 and 86%, respectively. Along with *C. fraxinea*, the isolations revealed the presence of 42 other fungal strains (Table 1). On average, 0·7 isolates were obtained per attempted isolation (150 strains from 220 samples): 0·6 isolates per symptomless leaf stalk, 1·0 per necrotic leaf stalk, 0·5 per small bark wound, 0·4 per larger bark wound and 1·3 per stem canker.

Molecular analyses of plant tissue

PCR and T-RFLP analyses of necrotic leaves, leaf stalks and bark revealed the presence of 25 different fungal taxa, 18 of which where identified at least to a genus level (Table 2). For the most part, the same fungi were found in samples collected in Örebro and Gnesta. There were three occasional taxa found only in Örebro and one taxon only in Gnesta, while the other 21 taxa were consistently recorded from both localities. As a result, the Sorensen qualitative index of similarity was 0·91. The amount of taxa detected per sample positively correlated with the physical size of the analysed tissue. Thus, in leaf samples (approx. 1 cm^2 in size) the method revealed the presence of 1–16 fungal taxa per sample (7·3 ± 3·5 on average), in shoot bark samples (approx. 0·25 cm^2 in size) there were 1–12 taxa per sample (5·8 ± 2·7 on average) and in leaf stalk samples (7–10 mm long) there were 1–9 taxa per sample (4·1 ± 2·1 on average). According to *t*-tests, the differences between the numbers of detected taxa in leaves and leaf stalks, and in shoot bark and in leaf stalks, were statistically significant ($P < 0{\cdot}0001$ and $P < 0{\cdot}025$, respectively). A total of 23 taxa were found in shoot bark, 22 in leaves and 15 in leaf stalks. Fourteen of those taxa were consistently detected in all three types of analysed tissue, most commonly *Cryptococcus foliicola* and *C. fraxinea*, recorded in 70 and 61% of all samples, respectively. They were followed by *Phoma glomerata*, *Phoma exigua* and *Cladosporium cladosporioides*, each of which was found in about a half of analysed samples (47–52%) (Table 2). None of the samples tested for presence of oomycetes gave any PCR product with the specific primer, while in all runs the *Phytophthora infestans* sample resulted in a PCR product.

Pathogenicity tests

All eight (100%) of the tested *C. fraxinea* isolates induced necroses in bark and cambium on each (100%) of the inoculated trees (Table 3; Fig. 1f,g). By contrast, no necroses were observed on bark or cambium on any of the control inoculations (Table 3; Fig. 1h,i). The length of the necroses on individual trees varied from 1·1 to 28·7 cm (on average, 7·2 cm), and comprised 4–99% of the total stem length. On 16 (19%) of the trees inoculated with the fungus, necrosis did not exceed 1·5 cm. Average length

of necroses caused by individual isolates of *C. fraxinea* varied from 4·2 to 8·9 cm, but the differences between means were statistically insignificant (Table 3). The pathogenicity tests resulted in mortality of 20 (23%) of *C. fraxinea*-inoculated trees, while all controls remained alive. The mortality of two to four trees (18–30%) was observed in each set of 10–11 trees, subjected to individual inoculations with seven (out of eight tested) isolates. The exception was strain 32A, where all 11 trees remained alive, and which also caused the least extensive necroses (Table 3). When the proportions of dead trees were compared between the isolate sets with the highest and the lowest mortality rates (e.g. strain 16BO or 22A, four dead out of 11 inoculated, vs. strain 32A, none dead out of 11 inoculated), the chi-squared test was statistically significant at $P = 0{\cdot}027$. After scoring the pathogenicity tests, the respective strain of *C. fraxinea* was re-isolated from 6–9 trees in each set (40–82%).

Discussion

By pure-culture isolations, mycelia of *C. fraxinea* were consistently detected in all investigated symptom categories, and on all three types of agar media used. As all samples for the isolations were taken either from healthy-looking tissue or at the zone of advancing necrosis, the results indicated that the fungus was active and perhaps even dominating other microbial species at the early stages of symptom development. The isolation data were largely consistent with the results of molecular analyses. Thus, isolation and molecular analysis of necrotic leaf stalks revealed the presence of *C. fraxinea* in 91 and 97% of samples, respectively, while in necrotic shoot bark respective detection frequencies were 27–28% and 41%. In general, molecular techniques appeared to be more powerful in exploring fungal diversity than pure-culture isolations. For example, the isolations from necrotic leaf stalks yielded an average of 1·0 strain per sample (including possible representatives of the same taxon), while the molecular analyses of the similar tissues revealed, on average, the presence of 4·1 distinct fungal taxa.

The present work consistently demonstrated frequent occurrence of *C. fraxinea* in various parts of declining *F. excelsior* trees: necrotic leaves, healthy-looking and necrotic leaf stalks, necrotic shoot bark, and wounds on shoots and stems. In previous studies this fungus was isolated at varying frequencies from leaves, buds, shoots, tree stems and roots (Kowalski & Lukomska, 2005; Lygis *et al.*, 2005; Kowalski, 2006; Schumacher *et al.*, 2007; Halmschlager & Kirisits, 2008). This demonstrates a broad capacity of the species to colonize a wide spectrum of physically and physiologically different niches, so that studies of its infection biology, enzymatic activity and metabolism might be rewarding.

In the present work, *C. fraxinea* was the second most commonly detected species. The data also showed that apart from *C. fraxinea*, the fungal community in necrotic leaves, leaf stalks and shoots of *F. excelsior* was largely composed of basidiomycetous yeasts from the genera *Cryptococcus* and *Bullera*, and that the species *C. foliicola* was the most common. However, neither of the yeasts detected in the present study are known as plant pathogens. In nature, they have a worldwide distribution reaching from the tropics to the Antarctic regions and are known as inhabitants of phylloplane and rhizosphere (Nakase, 2000; Renker *et al.*, 2004; Arenz *et al.*, 2006; Aldrich-Wolfe, 2007; Renouf *et al.*, 2007), and thus in leaves and bark of declining *F. excelsior* are likely to represent saprotrophic and/or endophytic organisms. On the other hand, some other commonly detected fungi (47–52% of samples), such as *P. glomerata*, *P. exigua* and *C. cladosporioides*, were reported as causes of leaf spots on trees and agricultural plants (Agrios, 2005; Sinclair & Lyon, 2005). Therefore, the possibility cannot be excluded that, along with *C. fraxinea*, they to some extent contribute to the observed dieback, and this needs to be evaluated in future studies. For example, experiments by Przybyl (2002b,c, 2003) demonstrated certain pathogenicity to *F. excelsior* of *Diplodia mutila* (anamorph of *Botryosphaeria stevensii*) and *Fusarium oxysporum*. However, those studies did not provide straightforward explanations of the roles of any of the fungi in tree dieback, indicating that the situation might be more complex than an attack by a single fungal species, although the present work also showed the absence of *Phytophthora* spp. from investigated samples, implying that the latter are unlikely to be involved in the decline of *F. excelsior*.

The pathogenicity tests demonstrated that each of the eight tested strains of *C. fraxinea* induced cambium necrosis on each inoculated *F. excelsior* stem. Although in one-way ANOVA the differences between average lengths of necroses caused by individual strains were not significant, the average lesion lengths of some isolates were double those of others. Moreover, the incidences of mortality following inoculation with different strains were statistically significant in certain cases. This indicates that different isolates of *C. fraxinea* might differ in aggressiveness. However, in order to elucidate this, pathogenicity tests must be carried out on clonal material of *F. excelsior*, which was not done in this work. The results of the present study explicitly demonstrated sharp differences in resistance of individual trees to inoculations of *C. fraxinea*, as differences between the lengths of necroses among trees inoculated with the same strain varied within a wide range, from 1·1 to 7·8 cm in the case of strain 32A (lowest variation), and from 1·5 to 28·7 cm in the case of strain 36A (highest variation). This is in good agreement with the results of available field inventories in clonal plantations in Sweden and Denmark, in which different severities of the disease were observed among the representatives of different *F. excelsior* clones (Stener, 2006; Olrik *et al.*, 2007).

Frequent isolations of *C. fraxinea* and its consistent detection by molecular methods in various dieback symptoms, together with the results of pathogenicity tests, clearly demonstrated the involvement of the fungus in the decline of *F. excelsior*. In addition, the results of the present study indicate some possible directions for

future research. To date, nothing is known regarding the infection biology *C. fraxinea*. Moreover, studies on population genetics could be of interest, as the fungus has been only recently described, and so far no teleomorph of this species has been observed, either in pure culture or in nature (Kowalski, 2006). Therefore, *C. fraxinea* might be a previously undetected native species in a community of microfungi on *F. excelsior*, the pathogenicity of which is triggered by changing environmental conditions. On the other hand, the fungus might be an invasive species gradually spreading over new geographic areas, its origin and mode(s) of spread remaining completely unknown. Although the pathogenicity tests in this study showed the susceptibility of *F. excelsior* to *C. fraxinea* during dormancy and early stages of seasonal growth, seasonal patterns of infection need to be clarified. The possible involvement of other fungi in *F. excelsior* decline also needs to be investigated in more detail. Of particular interest could be studies on the selection of decline-resistant *F. excelsior* clones, as this could result in recommendations to practical forestry on minimizing the damage caused by this catastrophic phenomenon.

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