Eur J Plant Pathol (2009) 125:329–335
DOI 10.1007/s10658-009-9471-x

Rapid *in planta* detection of *Chalara fraxinea* by a real-time PCR assay using a dual-labelled probe

Renaud Ioos · Tadeusz Kowalski · Claude Husson · Ottmar Holdenrieder

Received: 30 October 2008 / Accepted: 6 April 2009 / Published online: 22 April 2009

Abstract *Chalara fraxinea* is a fungus currently threatening ash trees (*Fraxinus excelsior*) in several European countries. This emerging pathogen was assigned to the EPPO's alert list and therefore accurate detection and identification tools are needed. Because of its slow growth rate on agar media and the frequent presence of fast-growing saprotrophic fungi within the host tissue, classical isolation techniques are time-consuming and sometimes inefficient. In this study, we used species-specific polymorphisms observed within the internal transcribed spacer region to design a primer pair and a dual-labelled probe to be used in a real-time PCR assay for the detection of *C. fraxinea*. The test proved to be specific, based on *in silico* and *in vitro* assessments, and could detect as little as 20 fg of *C. fraxinea* DNA. A protocol was developed in order to detect the pathogen directly in plant tissue and proved to be more efficient and rapid than isolation on agar plates. This new tool should be useful both for monitoring and to conduct epidemiology research on this emerging pathogen.

Keywords Diagnosis · Fraxinus excelsior · Disease

R. Ioos (✉)
Laboratoire National de la Protection des Végétaux,
Station de Mycologie, IFR 110,
Domaine de Pixérécourt,
54220 Malzéville, France
e-mail: renaud.ioos@agriculture.gouv.fr

T. Kowalski
Department of Forest Pathology, Faculty of Forestry,
Agricultural University,
Kraków, Poland

C. Husson
INRA, Nancy Université,
UMR 1136 Interaction Arbres/Micro-organismes, IFR 110,
54280 Champenoux, France

O. Holdenrieder
Forest Pathology and Dendrology,
Institute of Integrative Biology (IBZ), ETH Zurich,
8092 Zurich, Switzerland

Chalara fraxinea (Kowalski 2006) is a hyphomycete whose teleomorphic stage was recently assigned to the ascomycete *Hymenoscyphus albidus* (Kowalski and Holdenrieder 2009a), and which causes dieback of common ash, an emerging disease of *Fraxinus excelsior* in Europe (Kowalski and Holdenrieder 2009b; EPPO, 2008; Bakys et al. 2009a). *Chalara fraxinea* sporulates infrequently on ash and its epidemiology is still unclear (Kowalski and Holdenrieder 2009b). However, given the limited prospects for eradication of established infestations (Kowalski and Holdenrieder 2009b), effective prophylactic measures to prevent spread of the pathogen are of great

importance. In particular, ash nurseries should be monitored with care, in order to avoid long-distance dissemination of infected plants (Jeger et al. 2007). Reliable enforcement of such monitoring depends on the application of rapid, specific, and sensitive detection tools. Detection by isolation of *C. fraxinea* in culture is particularly difficult and time-consuming because this fungus grows very slowly and it is easily outcompeted by faster growing saprotrophic or endophytic fungi (Bakys et al. 2009). As a consequence, 'false-negative' results are frequently obtained.

In the present study, we developed a real-time PCR detection tool that makes it possible to detect *C. fraxinea* directly *in planta* within a few hours. Publicly available internal transcribed spacer sequences (ITS) for different *Chalara* species and allied genera, including *Thielaviopsis* spp., *Xenochalara* spp. and *Ceratocystis* spp. (teleomorphic stages of several *Chalara* spp.) were aligned with additional ITS sequences obtained for several European *C. fraxinea* strains (see accessions in Table 1). A *C. fraxinea*-specific combination of dual labelled-probe and primer pair was manually designed (i) based upon species-specific polymorphisms revealed by multiple alignments, and (ii) taking into consideration the thermodynamic requirements for a probe/primer combination as described by Bustin (2000). In this research, the specificity of the probe/primer combination was firstly assessed by *in silico* PCR simulations with ITS sequences using Fast PCR software (Kalendar 2006), then was tested *in vitro* with a wide range of DNA extracts from *C. fraxinea* strains, fungal endophytes or saprophytic fungal species commonly isolated from ash tissue (Table 1). Lastly, the sensitivity of the real-time assay was assessed for its ability to detect the pathogen directly *in planta* (Table 2). In addition, the quality of the DNA extracts was assessed for each ash sample by real-time PCR using 18S uni-F/R/-P primers and probe combination that targets a highly conserved region of 18S rDNA in plant and fungi (Ioos et al. 2009). This 18S uni-F/-R/-P real-time PCR served as a DNA extraction control, and enabled us (i) to check that no DNA shearing or loss occurred during the DNA extraction process and (ii) to assess the potential presence of inhibiting compounds that would have partially (high Ct value) or completely (Ct value >40) impeded DNA amplification.

Fungal DNA was extracted from pure culture using DNeasy plant mini kit (Qiagen, Courtaboeuf, France) as previously described (Ioos et al. 2005). Plant DNA was extracted using another commercial plant DNA extraction kit (Nucleospin Plant II® miniprep, Macherey-Nagel, Hoerdt, France). Strips or pieces of symptomatic ash tissue were collected using a sterile scalpel blade and firstly roughly cut into small pieces (ca 2×2×2 mm). Then the sample (ca 200–300 µl) was transferred into a 2-ml microcentrifuge tube. Two 3-mm steel beads, 2 mg of polyvinylpolypyrrolidone (PVPP, Sigma-Aldrich, L'isle d'Abeau, France), and 400 µl of Nucleospin Plant II lysis buffer were added and the sample was subsequently ground for 2 min at 30 Hz with a beadbeater (TissueLyser, Qiagen). Genomic DNA was subsequently extracted following the manufacturer's instructions, except that the 65°C incubation was extended to 20 min.

Real-time PCR reactions were performed with a Rotor-Gene 6500 (Corbett Research, Mortlake, Australia) set with an autogain optimisation for each channel performed before the first fluorescence acquisition. Amplifications were carried out in 20-µl reaction volumes using the qPCR core kit no ROX (Eurogentec, Seraing, Belgium) and consisting of molecular-grade water, 1× reaction buffer, 5 mM $MgCl_2$, 4×0.2 mM dNTPs, 0.3 µM of the respective forward (Cfrax-F, 5′-ATTATATTGTTGCTTTAGCAGGTC-3') and reverse primer (Cfrax-R, 5′-TCCTCTAGCAGGCACAGTC-3′), 0.1 µM of the dual-labelled probe (C-frax-P, 5′-FAM- CTCTGGGCGTCGGCCTCG-BHQ1-3′), 0.5 U of Hotgoldstar, and 2 µl of template DNA (0.8–30 ng). The mixture composition was identical for 18S uni RT PCR, simply replacing the *C. fraxinea* primers and probe by 18S uni-F/-R/-P, respectively (Ioos et al. 2009), The real-time PCR cycling conditions for both assays included an initial denaturation step at 95°C for 10 min followed by 40 cycles of denaturation and annealing/elongation for, respectively, 15 s at 95°C and 55 s at 65°C. The Ct value for each reaction was determined using the Rotor-Gene software version 1.7.75. Each sample of DNA extract was tested in triplicate and a standard deviation was computed.

The specificity of the Cfrax-F/-R/-P combination was successfully checked with a collection of European fungal species, either phylogenetically close taxa or sharing the same ecological niche (Table 1). As expected, all *C. fraxinea* isolates yielded cycle thresholds (Ct), regardless of their geographical origin,

Table 1 List and characteristics of the organisms tested in this study and results following the real-time PCR assay targeting *C. fraxinea* ITS

Species	Isolate	Host plant	Origin	Provider	ITS sequence (Genbank accession)	RT-PCR[a] Cfrax-F/-R/-P
Chalara fraxinea	071026.1	*Fraxinus excelsior*	Czech Rep. (Brno)	O. Holdenrieder	FJ429386	17.5 (0.1)
C. fraxinea	080517.2	*F. excelsior*	Lithuania (Vilnus)	O. Holdenrieder	FJ429378	18.4 (0.2)
C. fraxinea	080520.2b1	*F. excelsior*	Lithuania (Palanga)	O. Holdenrieder	FJ429379	19.2 (0.1)
C. fraxinea	080518.5	*F. excelsior*	Lithuania (Klaipeda)	O. Holdenrieder	FJ429377	17.5 (0.0)
C. fraxinea	080519.3a1	*F. excelsior*	Lithuania (Palanga)	O. Holdenrieder		18.1 (0.4)
C. fraxinea	1582	*F. excelsior*	Poland (Gryfice)	T. Kowalski	FJ429380	16.5 (0.2)
C. fraxinea	1591	*F. excelsior*	Poland (Gryfice)	T. Kowalski		15.5 (0.2)
C. fraxinea	2112	*F. excelsior*	Poland (Stary Sącz)	T. Kowalski	FJ429381	16.6 (0.3)
C. fraxinea	2116	*F. excelsior*	Poland (Stary Sącz)	T. Kowalski	FJ429382	17.0 (0.2)
C. fraxinea	2245.1	*F. excelsior*	Poland (Rokita)	T. Kowalski	FJ429383	17.1 (0.1)
C. fraxinea	2249.5	*F. excelsior*	Poland (Miechow)	T. Kowalski	FJ429384	16.3 (0.2)
C. fraxinea	2252.6	*F. excelsior*	Poland (Miechow)	T. Kowalski	FJ429385	15.8 (0.2)
C. fraxinea	2269.2	*F. excelsior*	Poland (Rokita)	T. Kowalski		18.2 (0.4)
C. fraxinea	HMIPC 18364	*F. excelsior*	Poland (Limanowa)	T. Kowalski	FJ429374	17.1 (0.2)
C. fraxinea	HMIPC 18372	*F. excelsior*	Poland (Andrichów)	T. Kowalski	FJ429375	16.3 (0.0)
C. fraxinea	08.410	*F. excelsior*	France (Haute-Saône)	P. Loevenbruck	FJ429376	15.3 (0.1)
C. fraxinea	08-1128	*F. excelsior*	France (Vosges)	C. Saurat		15.3 (0.0)
C. fraxinea	26	*F. excelsior*	France (Haute-Saône)	C. Husson		16.2 (0.2)
C. fraxinea	3	*F. excelsior*	France (Haute-Saône)	C. Husson		14.2 (0.3)
C. fraxinea	34	*F. excelsior*	France (Haute-Saône)	C. Husson		14.1 (0.1)
Acremonium sp.	18372.s	*F. excelsior*	Poland (Andrichów)	T. Kowalski		–[b]
Alternaria alternata	2401	*F. excelsior*	Poland (Stary Sącz)	T. Kowalski		–
Alternaria sp.	2498	*F. excelsior*	Poland (Stary Sącz)	T. Kowalski		–
Alternaria sp.	08-554b	*F. excelsior*	France (Haute-Saône)	R. Ioos		–
Aureobasidium pullulans	08-483.9	*F. excelsior*	France (Haute-Saône)	R. Ioos		–
Chalara sp.	811	*Platanus acerifolia*	France (Gard)	J. Hubert		–
Chalara paradoxa	08-106	*Phoenix canariensis*	France (Alpes-Maritimes)	C. Saurat		–
Cladosporium sp.	08-411d	*F. excelsior*	France (Haute-Saône)	R. Ioos		–

Table 1 (continued)

Species	Isolate	Host plant	Origin	Provider	ITS sequence (Genbank accession)	RT-PCR[a] Cfrax-F/-R/-P
Cytospora sp.	2256.1	*F. excelsior*	Poland (Rokita)	T. Kowalski		–
Cytospora sp1	08-483.2	*F. excelsior*	France (Haute-Saône)	R. Ioos		–
Cytospora sp2	08-483.3	*F. excelsior*	France (Haute-Saône)	R. Ioos		–
Diplodia mutila	1880	*F. excelsior*	Poland (Miechow)	T. Kowalski		–
Epicoccum nigrum	08-483.3	*F. excelsior*	France (Haute-Saône)	R. Ioos		–
Fusarium avenaceum	2283	*F. excelsior*	Poland (Rokita)	T. Kowalski		–
Fusarium avenaceum	2629	*F. excelsior*	Poland (Miechow)	T. Kowalski		–
Fusarium equiseti	08-472	*F. excelsior*	France (Haute-Saône)	R. Ioos		–
Fusarium lateritium	08-411b	*F. excelsior*	France (Haute-Saône)	R. Ioos		–
Fusarium lateritium	2623	*F. excelsior*	Poland (Miechow)	T. Kowalski		–
Fusarium oxysporum	08-554a	*F. excelsior*	France (Haute-Saône)	R. Ioos		–
Fusarium semitectum	08-976	*F. excelsior*	France (Haute-Saône)	R. Ioos		–
Fusarium solani	08-411c	*F. excelsior*	France (Haute-Saône)	R. Ioos		–
Fusarium sp.	2386	*F. excelsior*	Poland (Stary Sącz)	T. Kowalski		–
Fusarium sp.	2626	*F. excelsior*	Poland (Miechow)	T. Kowalski		–
Fusarium sp.	081004.2	*F. excelsior*	Switzerland (Rottenschwil)	O. Holdenrieder		–
Geniculosporium serpens	2493	*F. excelsior*	Poland (Stary Sącz)	T. Kowalski		–
Mucor sp.	08-411f	*F. excelsior*	France (Haute-Saône)	R. Ioos		–
Penicillium sp.	08-411 g	*F. excelsior*	France (Haute-Saône)	R. Ioos		–
Phomopsis sp1	1942.1	*F. excelsior*	Poland (Ojcow)	T. Kowalski		–
Phomopsis sp2	1601.1	*F. excelsior*	Poland (Ojcow)	T. Kowalski		–
Phomopsis sp.	08-410	*F. excelsior*	France (Haute-Saône)	R. Ioos		–
Phomopsis sp.	080910.1	*F. excelsior*	Switzerland (Zurich)	O. Holdenrieder		–
Dematiaceous sterile hyphomycete	081004.3	*F. excelsior*	Switzerland (Rottenschwil)	O. Holderiender		–
Non-sporulating fungus	08-483.5	*F. excelsior*	France (Haute-Saône)	R. Ioos		–
Non-sporulating fungus	081004.4	*F. excelsior*	Switzerland (Rottenschwil)	O. Holdenrieder		–
Fraxinus excelsior bark	Fr brk	n.a.	France (Meurthe et Moselle)	O. Cael		–
Fraxinus excelsior leaf	Fr lf	n.a.	France (Meurthe et Moselle)	P. Loevenbruck		–

Table 1 (continued)

Species	Isolate	Host plant	Origin	Provider	ITS sequence (Genbank accession)	RT-PCR[a] Cfrax-F/-R/-P
Fraxinus excelsior petiole	Fr pt	n.a.	France (Meurthe et Moselle)	P. Loevenbruck		–
Fraxinus excelsior sapwood	Fr sw	n.a.	France (Meurthe et Moselle)	O. Cael		–
Fraxinus excelsior bud	Fr bd	n.a.	France (Meurthe et Moselle)	P. Loevenbruck		–

[a] Cycle thresholds (Ct) were computed with all the DNA extracts normalised to 1 ng μl^{-1}, each tested in triplicate. Standard deviation is indicated between brackets, [b] No Ct value yielded before completion of the 40 cycles

whereas no amplification could be observed with all the other tested taxa. The sensitivity threshold for the Cfrax-F/-R/-P combination was determined experimentally using a 10-fold dilution series of genomic *C. fraxinea* DNA (strain 08-520) (i) in deionised DNA-free water and (ii) in a DNA solution obtained from healthy *F. excelsior* bark tissue (5 ng μl^{-1}), to yield final concentrations ranging from 1 ng μl^{-1} to 0.1 fg μl^{-1}. Standard curves were constructed and identical amplification efficiencies (1.01) and R^2 (0.99) were obtained with dilutions both in water and in *F. excelsior* DNA. For both dilutions, a linear relationship between Ct values and log (initial target concentration) was observed down to a concentration of 10 fg μl^{-1} of genomic DNA, and the limit of detection of the test was estimated to be 20 fg of *C. fraxinea* DNA.

The Cfrax-F/-R/-P combination was also successfully used to detect *C. fraxinea* in naturally infected plant tissues. A series of 33 symptomatic ash samples was collected in France and Switzerland in 2008 and were analysed by both real-time PCR and isolation on malt extract agar. For each sample, a single tube was prepared from a single lesion for DNA extraction and real-time PCR analysis; while from one up to eight subsamples from several lesions were analysed separately by agar plating. The real-time PCR successfully detected the presence of *C. fraxinea* in 28 out of 33 ash samples displaying typical dieback symptoms, either in sapwood or in necrotic inner and outer bark, whereas a pure culture of *C. fraxinea* could only be isolated in 12 of the 33 samples (Table 2). Sample 081810-1.1a was the only sample from which a pure culture of *C. fraxinea* could be obtained, whereas the real-time PCR yielded a negative result. However, for this sample, *C. fraxinea* grew from only one out of eight subsamples, suggesting that more extensive sampling might have made it possible to detect the pathogen by real-time PCR. Lastly, for three samples showing symptoms, neither real-time PCR, nor isolation could detect *C. fraxinea*. However, for these samples the result of the RT-PCR DNA control test indicated that DNA was amplifiable (Ct values = 17.6, 18.1 and 17.9, respectively) thus suggesting that they displayed confusing symptoms that were not caused by a *C. fraxinea* infection.

The real-time PCR test developed in this study enables efficient detection of *C. fraxinea in planta* within a few hours and should be of great interest for phytosanitary control. Recent experiments showed that sensitivity and efficiency of the test was not affected by carrying out duplex real-time assays including simultaneously both Cfrax-F/-R/-P and 18S uni-F/-R/-P (data not shown), thus reducing the turnaround time for analysis. This test could also be used as a tool to address unanswered questions about pathogen dissemination and host infection courts. In this context, a cautious design of the primer/probe combination as well as a high annealing/extension temperature were considered in this study in order to avoid potential cross-reactions with phylogenetically close taxa that may be present outside the known ecological niche of *C. fraxinea*.

Acknowledgements The authors are very grateful to P. Loevenbruck, and C. Saurat for providing *C. fraxinea* isolates and the French forest health service (DSF) for collecting naturally infected ash samples.

Table 2 List of the symptomatic ash samples tested in this study and results following isolation and real-time PCR assays targeting *C. fraxinea* ITS and 18S rDNA region

Sample	Origin	Tissue analysed	*C. fraxinea* isolation	RT-PCR *C. fraxinea*[a]	RT-PCR DNA control[ab]
08-1128	France (Vosges)	NTE[c]	+	14.4 (0.1)	15.4 (0.0)
08-1198	France (Vosges)	NTE	–	33.4 (0.6)	16.2 (0.1)
08-1199	France (Alsace)	NTE	–	30.0 (0.2)	16.3 (0.1)
08-1200	France (Doubs)	NTE	–	34.7 (0.6)	17.0 (0.3)
08-1201	France (Vosges)	NTE	–	27.0 (0.0)	17.1 (0.1)
08-1202	France (Alsace)	NTE	–	n.a.[g]	–
08-1224	France (Vosges)	NTE	–	20.6 (0.1)	16.0 (0.2)
08-1338	France (Vosges)	NTE	+	27.4 (0.2)	19.2 (0.3)
08-1376	France (Alsace)	NTW[d]	–	21.8 (0.0)	15.7 (0.3)
08-1377	France (Doubs)	NTW	+	20.7 (0.4)	17.2 (0.1)
08-1378	France (Haute-Saône)	NTW	–	22.5 (0.1)	20.7 (0.2)
08-1379	France (Haute-Saône)	NTW	–	15.6 (0.1)	16.8 (0.0)
Mer 1.6	France (Haute-Saône)	NTE	+	21.1 (0.2)	20.3 (0.2)
Mer 1.7	France (Haute-Saône)	NTE	+	23.1 (0.0)	18.2 (0.2)
Mer 1.8	France (Haute-Saône)	NTE	–	19.6 (0.1)	15.5 (0.0)
Mer 2.7	France (Haute-Saône)	SW[e]	+	26.3 (0.2)	20.2 (0.1)
Mer 2.8	France (Haute-Saône)	NTE	–	28.2 (0.2)	18.2 (0.2)
Mer 2.9	France (Haute-Saône)	NTE	–	29.4 (1.2)	17.9 (0.1)
Mer 2.10	France (Haute-Saône)	NTE	–	26.5 (0.1)	18.2 (0.3)
Mer 2.12	France (Haute-Saône)	SW	–	26.5 (0.2)	16.8 (0.2)
Ain 1.9	France (Haute-Saône)	NTE	–	23.9 (0.1)	17.0 (0.1)
Ain 1.11	France (Haute-Saône)	NTE	+	19.9 (0.0)	16.2 (0.0)
Ain 1.12	France (Haute-Saône)	SW	–	26.4 (0.0)	14.8 (0.1)
Ain 1.13	France (Haute-Saône)	NTE	–	30.4 (0.2)	18.1 (0.1)
Ain 1.14	France (Haute-Saône)	NTE	+	24.2 (0.2)	16.5 (0.1)
Ain 2.3	France (Haute-Saône)	NTE	–	19.8 (0.1)	25.8 (0.0)
Ain 3.2	France (Haute-Saône)	NP[f]	+	22.4 (1.1)	19.4 (0.1)
081810-1.1a	Switzerland (Zurich)	NTW	+	–	18.1 (0.0)
081810-1.2	Switzerland (Zurich)	NTW	–	–	17.6 (0.1)
081810-1.3	Switzerland (Zurich)	NTE	+	15.9 (0.1)	18.2 (0.1)
081810-2	Switzerland (Zurich)	NTE	+	16.6 (0.3)	19.6 (0.2)
081810-3	Switzerland (Zurich)	NTE	–	–	18.1 (0.0)
081810-4	Switzerland (Zurich)	NTW	–	–	17.9 (0.2)

[a] Cycle thresholds (Ct) were computed with each sample tested in triplicate. Standard deviation is indicated in brackets. Dashes indicate that no Ct value was obtained after completion of the 40 cycles, [b] Quality of the DNA extracts was checked with a real-time PCR using 18S uni -F/-R/-P primers and dual-labelled probe, that target a highly conserved 18S sequence within rDNA region of eukaryotes (Ioos et al. 2009), [c] NTE: necrotic bark tissue at the edge of the lesion, [d] NTW: necrotic bark tissue within the lesion, [e] SW: coloured inner sapwood, [f] NT: necrotic petiole; [g] sample that could not be analysed due to PCR inhibition

References

Bakys, R., Barklund, P., Ihrmark, K., & Stenlid, J. (2009). Investigations concerning the role of *Chalara fraxinea* in declining *Fraxinus excelsior*. *Plant Pathology,* . doi:10.1111/j.1365-3059.2008.01977.x.

Bakys, R., Vasaitis, R., Barklund, P., Thomsen, I., & Stenlid, J. (2009). Occurrence and pathogenicity of fungi in necrotic and non-symptomatic shoots of declining common ash (*Fraxinus excelsior*) in Sweden. *European Journal of Forest Research, 128*, 51–60. doi:10.1007/s10342-008-0238-2.

Bustin, S. A. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology, 25*, 169–193. doi:10.1677/jme.0.0250169.

EPPO. (2008). http://www.eppo.org/QUARANTINE/Alert_List/fungi/Chalara_fraxinea.htm.

Ioos, R., Husson, C., Andrieux, A., & Frey, P. (2005). SCAR-based PCR primers to detect the hybrid pathogen *Phytophthora alni* and its subspecies causing alder disease in Europe. *European Journal of Plant Pathology, 112*, 323–335. doi:10.1007/s10658-005-6233-2.

Ioos, R., Fourrier, C., Iancu, G., & Gordon, T. R. (2009). Sensitive detection of *Fusarium circinatum* in pine seeds by combining an enrichment procedure with a Real-Time PCR using dual-labeled probe chemistry. *Phytopathology*, *99*, 582–590.

Jeger, M. J., Pautasso, M., Holdenrieder, O., & Shaw, M. W. (2007). Modelling disease spread and control in networks: implications for plant sciences. *New Phytologist, 174*, 279–297. doi:10.1111/j.1469-8137.2007.02028.x.

Kalendar, R. (2006). Fast PCR, PCR primer design, DNA and protein tools, repeats and own database searches program. www.biocenter.helsinki.fi/bi/programs/fastpcr.htm.

Kowalski, T. (2006). *Chalara fraxinea* sp. nov. associated with dieback of ash (*Fraxinus excelsior*) in Poland. *Forest Pathology, 36*, 1–7. doi:10.1111/j.1439-0329.2006.00453.x.

Kowalski, T., & Holdenrieder, O. (2009a). The teleomorph of *Chalara fraxinea*, the causal agent of ash dieback. *Forest Pathology.* doi: 10.1111/j.1439-0329.2008.00589.x.

Kowalski, T., & Holdenrieder, O. (2009a). Pathogenicity of *Chalara fraxinea*. *Forest Pathology, 39*, 1–7. doi:10.1111/j.1439-0329.2008.00565.x.