Short Communication

Chalara fraxinea – Ash Dieback in the Czech Republic

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Abstract

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The causal agent of ash dieback, the hyphomycete *Chalara fraxinea*, was isolated from a *Fraxinus excelsior* cv. Pendula tree, in the Arboretum Křtiny between Křtiny and Jedovnice in Drahany Highland, and subsequently from several other locations in South Moravia in the Czech Republic. The infection was associated with severe twig dieback and symptoms of ash dieback were observed in many locations across the Czech Republic. The morphology of *C. fraxinea* is described and an ITS sequence is provided. *Hymenoscyphus albidus,* the teleomorph of *C. fraxinea,* is known as a quit common species from precedent mycofloristic studies provided in different areas in the Czech Republic.

Keywords: *Chalara fraxinea*; ash dieback; *Fraxinus excelsior*; *Fraxinus angustifolia*; ash; distribution; *Hymenoscyphus albidus*

Common ash (*Fraxinus excelsior*) is threatened in large parts of Europe by a new disease, which is associated with cankers and dieback. Many fungi occur on declining ash trees (e.g. Lygis *et al*. 2005; Cech 2006), but the hyphomycete *Chalara fraxinea* has been recently determined to be the causal agent of this disease (Kowalski 2006, 2007; Kowalski & Holdenrieder 2008, 2009a; Bakys *et al*. 2009).

Chalara fraxinea was reported in Poland (Kowalski 2006), Denmark (Thomsen *et al*. 2007), Germany (Schumacher *et al*. 2007), Austria (Halmschlager & Kirisits 2008), Hungary (Szabó 2008a, b), Finland (EPPO 2008a), Lithuania (Vasaitis – personal communication), Norway (EPPO 2008b; Solheim – personal communication), Sweden (Bakys *et al*. 2009), Switzerland (Engesser & Holdenrieder unpublished) and France (Ioos – personal communication). Symptoms of ash dieback were also reported from Slovakia in 2008 (Kunca – personal communication); the disease was noted also in Slovenia (Ogris *et al.* 2009) and Croatia (own observation) in 2008. *Chalara fraxinea* has been updated on the EPPO alert list since 2007 (EPPO 2007).

The ascomycete *Hymenoscyphus albidus* (Roberge ex Desm.) W. Phillips was identified as the teleomorph of *C. fraxinea* by Kowalski and Holdenrieder (2009b).

Ash decline has also been observed throughout several locations in the Czech Republic since the end of the 1990s, but attempts to isolate *C. fraxinea* have failed until now. Here we report the successful isolation of *C. fraxinea* from common ash in the Czech Republic.

MATERIALS AND METHODS

Ash twigs (4–8 mm diameter) with dieback (comprising a necrotic distal portion and a proximal living portion) were collected and transferred to the laboratory in plastic bags. After storage in a moist chamber for 10 days at 4°C, they were surface disinfected by spraying with concentrated ethanol and superficially dried on a clean bench. The periderm was removed locally from the necrotic lesions with a No. 11 scalpel under a dissection microscope. Tissue samples (~1 mm × 1 mm) from the inner bark and the xylem (5 samples of each tissue type per lesion) were aseptically transferred onto malt agar (MEA 2) containing 20 g/l malt extract, 15 g/l agar and 50 mg/l oxytetracycline (added after autoclaving). In total, 60 tissue samples were prepared from 6 twigs. The samples were incubated at room temperature (approx. 20°C) in diffused daylight.

Subcultures were made on MEA without oxytetracycline and on water agar on which a sterile cellophane sheet was placed. The cultures were incubated at room temperature and at approx. 10°C in darkness. The first isolate (No. 070926.1) is preserved in the collections of ETH Zurich and Mendel University of Agriculture and Forestry in Brno. The GenBank accession number is FJ429386. Herbarium specimens were deposited in the BRNL herbarium of Mendel University of Agriculture and Forestry in Brno. Dried cultures were deposited at the mycological herbarium of ETH Zurich (Z+ZT).

For light microscopy, the specimens were mounted in concentrated lactic acid, and measurements and photomicrographs were made with a Zeiss Axiophot, equipped with phase and differential interference contrast.

For internal transcribed spacer (ITS) sequencing, DNA was extracted according to Grünig *et al*. (2002). ITS regions were amplified with prITS4 and prITS5 primers (White *et al*. 1990) in a 30 µl reaction volume with ~1 ng template DNA. Single bands were gel purified with the QIAquik® gel extraction kit (QIAGEN, Basel, Switzerland), and sequences were determined with primer prITS4 (Microsynth, Balgach, Switzerland).

A modified isolation method was used at Mendel University of Agriculture and Forestry in Brno. The medium (MEA 3) was composed of malt extract (30 g/l), pepton (5 g/l), agar (15 g/l) and, according to Kowalski (2006), streptomycin (100 mg/l) added after autoclaving. Tissue samples (diameter 2–5 mm, about 2–3 mm long), were dissected from the sapwood below necrotic lesions, after bark removal. Samples were then surface sterilised by immersion in sodium hypochlorite (7–10 %) for 60–90 s, then immersed in 96% ethanol for 60–90 s, washed in sterilised water and placed on the medium.

RESULTS AND DISCUSSION

Ash dieback associated with bark necroses and withering of young shoots was recorded in several areas in the Czech Republic during 2004–2008. The locations affected include: Beskydy Mts., Jeseníky Mts., Giant Mts., Bayerischer Wald Mts., Central Bohemia, Prague, Eastern Bohemia, Czech Moravian Highland, the area at the junction of the Thaya and Morava Rivers, and along the Czech, Austrian and Slovak borders. Ash dieback has extended across the entire country since 2004. The symptoms were also noted in nurseries, especially on saplings.

The first record of *Chalara fraxinea* in the Czech Republic originated from samples collected at Drahany Highland, Arboretum Křtiny, from *Fraxinus excelsior* cv. Pendula at the entrance to the Arboretum, coordinates: 49°19'7''N, 16°44'35''E (date of collection: 26. 09. 2007). Various fungi were isolated from the necrotic lesions, but only a single lesion yielded *C. fraxinea.* The pathogen was present only in the xylem; the bark was colonised by *Phomopsis* sp. and a dark sterile mycelium (presumably *Diplodia* sp.). All other lesions yielded *Phomopsis* sp., cf. *Diplodia* sp. or *Fusarium* sp. (in 3 of 5 lesions only the bark was colonised). While multiple causes for dieback on the same tree had been observed as early as 2004, research did not demonstrate the occurrence of *C. fraxinea*.

The mycelium of *C. fraxinea* grew slowly and irregularly, filling the plate within approx. 2 months at room temperature (Figure 1). In the isolation culture, after 2–3 weeks, a few fertile phialophores

Figure 1. Ash dieback symptoms and *Chalara fraxinea.* (a) dieback of shoots; (b) leaf stalk with necroses; (c) necrotic bark in shoots; (d) necrotic areas in the bark; (e) discoloration of xylem in twigs; (f, g) cancers on the bark, cancers limited by production of remedial meristems; (h) cultures of *Chalara fraxinea* grown on MAE; (i, j) sporulating of culture at its margin – dark stromatic mycelia, following incubation at 4°C; (k, l) phialophores and conidia of *Chalara fraxinea*

were observed on the plant tissue. Spore size was 2–2.5 µm × 2.5–4.5 (–6) µm. These characteristics are in agreement with the description of Kowalski (2006); our identification was confirmed by T. Kowalski.

Subcultures remained sterile at room temperature for prolonged periods. However, on cellophane sheets, abundant sporulation and epidermoid hyphae were observed. After prolonged incubation (up to 1 year) of cultures on MEA at cool temperatures, irregular patches of dark stromatic pseudoparenchyma were formed, on which occasionally abundant sporulation occurred. Sporulation did not occur at room temperature. The same phenomenon was also observed by Halmschlager and Kirisits (2008).

The ITS sequence of the Czech isolate showed a high degree of similarity to a *C. fraxinea* isolate from Lithuania AY787704 (Lygis *et al*. 2005), displaying a difference of only two base pairs.

Chalara fraxinea was confirmed after the first record at the following locations: Ochoz u Brna, *Fraxinus angustifolia* cv. Pendula, coordinates: 49°15'24''N, 16°43'56''E; Hradčany u Brna, nursery, *Fraxinus excelsior*, coord. 49°19'36''N, 16°25'58''E; Lomnice u Tišnova, Czech Moravian Highland, *Fraxinus angustifolia*, coord. 49°24'34.51''N, 16°24'46.765''E. Necrotic lesions were also observed on *Fraxinus angustifolia* from the Rendez-Vous, Lednice-Valtice area, coord. 48°44'55.754''N, 16°47'39.651''E.

Old cankers and dead sprouts from the year 2007 were observed until mid-August 2008. Fresh necrotic lesions occurred rarely until this time. However, a great outbreak of new cankers and withering shoots was observed in many places from the end of August to October. Abnormal dropping of green leaves due to necroses on petioles was observed in many places, beginning in late August 2008.

Hymenoscyphus albidus, the teleomorph of *C. fraxinea* was commonly collected on ash petioles in precedent mycofloristic studies in the Czech Republic, including area of the first confirmation.

The pathogenicity of *C. fraxinea* has been demonstrated by inoculation experiments (Kirisits *et al*. 2008; Bakys *et al*. 2009; Kowalski & Holdenrieder 2009a). However, the ecological behavior of this pathogen and the process of canker formation are still enigmatic. Apparently the pathogen is adapted to low temperatures; canker initiations and growth occur preferentially during autumn and winter (Cech 2008). After infection, the pathogen may be replaced by opportunistic bark colonisers like *Phomopsis* sp., *Diplodia* sp. or *Fusarium* spp.

Chalara fraxinea causes a gradually emerging disease which eventually threatens its host on a large scale. However, single genotypes of ash are tolerant against the infection (Bakys *et al*. 2009). Therefore ash will probably survive in the long term, but its population may be significantly reduced over the course of several generations.

CONCLUSIONS

Ash dieback is widespread in forests, landscape and urban trees, plantations in towns and also in nurseries throughout the Czech Republic. This outbreak is particularly dangerous for alluvial stands, because ash has frequently replaced elms after Dutch elm disease.

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