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Phytophthora cambivora causing ink disease of sweet chestnut recorded in the Czech Republic

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Sweet chestnut (*Castanea sativa*) is a non-autochthonous but commonly planted ornamental tree in the Czech Republic. However, this species is sensitive to some *Phytophthora* infections causing so-called ink disease. The disease usually occurs in warmer regions in Europe. In the Czech Republic it had not been detected until the nineties of the 20th century. In 1997 extensive decline of a sweet chestnut ornamental orchard with typical symptoms of ink disease was found at one locality in eastern Bohemia. Later the causal organism of this disease *Phytophthora cambivora* (Petri) Buisman was isolated from necrotised tissues of trunks of several declining chestnut trees. This is the first find of the pathogen causing ink disease of chestnut in the Czech Republic.

Key words: *Phytophthora cambivora*, ink disease, *Castanea sativa*, sweet chestnut, Czech Republic.

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Kaštanovník jedlý (*Castanea sativa*) je v ČR oblíbenou dřevinou, která je často pěstována v okrasných výsadbách. Kaštanovník jedlý je velmi citlivý vůči tzv. inkoustové chorobě, která se obvykle vyskytuje v teplejších oblastech Evropy a až dosud nebyla v ČR známa. V 90. letech 20. století jsme zaznamenali rozsáhlé chřadnutí okrasné výsadby kaštanovníku ve východních Čechách, které se projevovalo symptomy typickými pro inkoustovou chorobu. Později byl z nekrotizovaných pletiv kmenů chřadnoucích stromů izolován původce choroby a určen jako *Phytophthora cambivora* (Petri) Buisman. Jedná se o první potvrzený výskyt tohoto patogena způsobujícího inkoustovou chorobu kaštanovníku jedlého v ČR.

INTRODUCTION

Sweet chestnut (*Castanea sativa* Mill.) is a species originally native to south-eastern Europe and Asia Minor. The tree is widely cultivated in southern and

warmer parts of central Europe for its wood and edible nuts. Besides its rural economic importance, chestnut has an important agro-ecological role resulting in interest in chestnut cultivation and conservation of genetic resources (Vettraino et al. 2005). Sweet chestnut was introduced to the Czech Republic 300–400 years ago (Svoboda 1978). Currently the species is widely distributed in the area and regularly planted in gardens, orchards, parks and infrequently in forest stands (Haltofová et al. 2005).

Ink disease – root and collar rot of seedlings and mature trees in nurseries, plantations and forests – is one of the most destructive diseases of *Castanea* spp. It was recorded in Portugal in 1838 for the first time, after that it has become widespread in Europe. Two *Phytophthora* species are responsible for the disease: *P. cambivora* (Petri) Buisman and *P. cinnamomi* Rands (Petri 1917, Crandall et al. 1945). However, some other *Phytophthora* species, like *P. citricola* Sawada, *P. cactorum* (Lebert & Cohn) J. Schröt., *P. cryptogea* Pethybridge & Lafferty and *P. gonapodyides* (Petersen) Buisman, can also cause the disease of sweet chestnut (Erwin and Ribeiro 1996; Vannini and Vettraino 2001; Vettraino et al. 2001, 2005), but their impact is considerably lower than that of *P. cambivora* and *P. cinnamomi*. During the 20th century the attention of phytopathologists was concentrated on the devastating epidemic of *Cryphonectria parasitica* (Murrill) Barr as well (Vettraino et al. 2001), which was also found in the Czech Republic (Jankovský et al. 2004). In the nineties the unaccustomed resurgence of ink disease was observed especially in Portugal, Italy and France. This resurgence limits the establishment of new groves and the conservation of old ones (Vettraino et al. 2001). Since 2002 the sweet chestnut population in Portugal has been decreasing because new plantations have not been sufficient to exceed the number of dead chestnut trees, killed mostly by ink disease (Martins et al. 2007).

The disease develops rapidly when the soil remains excessively wet (Erwin and Ribeiro 1996). Its severity is also affected by soil compaction and organic matter level and manuring practice (Fonseca et al. 2004). The positive effect of overmanuring with nitrogen is well documented at *Rhododendron* sp. (Hoitink et al. 1986). High soil organic matter content is connected with a high content of nitrogen in plant tissues predisposing the host to the disease (Coyier and Roane 1986, Hoitink et al. 1986). On the landscape scale, disease development corresponds with higher soil tillage and higher human mobility as well (Martins et al. 2007).

P. cinnamomi and *P. cambivora* causing ink disease are distributed from Greece to Great Britain. At present the greatest impact of the disease is limited to the southwest, southern and warmer regions of central Europe (Brasier and Jung 2005, Erwin and Ribeiro 1996, Juhásová 1999, Oszako et al. 2005, Vannini and Vettraino 2001, Vettraino et al. 2005, Werres et al. 2001). Both species have a large spectrum of host species. Nevertheless, almost no attention has yet been paid to

these two pathogens in the Czech Republic. *P. cambivora* was found to cause bleeding canker of *Fagus sylvatica* here (Černý et al. 2006). Recently, both *Phytophthora* species have been found on ericaceous plants (*Rhododendron* spp., *Pieris* sp., *Vaccinium* sp.) and representative isolates are deposited in the Culture Collection of Fungi (CCF), Prague and in the culture collection of RILOG (Mrázková et al., unpubl.).

The symptoms of ink disease of sweet chestnut were first found at Nasavrky in 1997 (Gregorová 2000). The severity of the disease was assessed one year later and it was found that about one half of the 125 trees showed important damage (c. 50 % defoliation or more) and the characteristic symptoms of ink disease were found on many of them. The presence of some *Phytophthora* species in damaged stem tissues was confirmed in a laboratory, however precise species identification was not successful (Gregorová 2000). The aim of the study was to confirm the disease at the locality and to identify the causal agent of the disease.

MATERIALS AND METHODS

Study site. The damaged sweet chestnut stand is localised in Nasavrky (district Chrudim, eastern Bohemia) at 49°50'47.62" N and 15°48'9.63" E at an altitude of c. 480 m with northern exposure. The sweet chestnut park was created in 1776–1778 by conversion from an orchard where the chestnuts had grown for nuts together with other fruit trees. The stand became the basic source of cultivation material, which was at the time propagated in gardens, parks and forest stands in broad surroundings. Trees of unsatisfactory health have been regularly replaced by young ones, thus the age structure of the chestnut stand is now heterogenous. In 1992 the site „Kaštanka v Nasavrkách" was brought under legal protection and it is now called Kaštanka v Nasavrkách Nature Monument (Gregorová 2000).

Isolation, determination. Samples of damaged under-bark tissues were taken from several trees in different stages of decline in 2004–2005. They were washed under running water, cut into pieces of c. 5 × 5 × 5 mm. The surface was sterilised with 95 % ethanol, subsequently washed in deionised water, dried with pulp and plated onto a PARPNH medium (Jung et al. 1996) and cultivated at 20 °C in the dark. After several days colonies of a hyaline coenocytic mycelium around some pieces of tissues were shown. The hyphal tips from the margin of the colonies were transferred under a dissecting microscope onto a solid V8 juice medium (Erwin and Ribeiro 1996), cultivated and microscoped. All isolates belonged to a pythiaceous organism. Subsequently, the morphological and cultural characteristics of the isolates were investigated on V8 juice agar (V8A) and carrot agar (CA). Zoosporangia production was induced by cultivation of agar plate segments from colony margins in filtrated pond water (Erwin and Ribeiro 1996). All isolates belonged to a heterothallic *Phytophthora* species from the 6th morphological group according to Waterhouse (1963), thus pairing tests (Erwin and Ribeiro 1996) were carried out to acquire the sex organs.

DNA sequencing. To identify the *Phytophthora* strains, DNA was extracted and the so-called ITS region of ribosomal DNA was amplified to compare sequences with earlier published ones. For amplification of the ITS region the primer pair ITS1/ITS4 was used (White et al. 1990). The DNA was amplified with PCR, using the Mastercycler® ep thermocycler (Eppendorf, Germany). The PCR reaction was carried out in a 25 µl reaction volume; the mixture for the PCR contained 50 ng of DNA, 20 pmol of each primer, 0.2 mM dNTP's, and 1U of DynaZyme™ polymerase with the appropriate buffer (Finnzymes, Finland). PCR amplifications were performed under the following temperatures: 94 °C/3 min., 50 °C/30

s, 72 °C/1 min. (1×), 94 °C/30 s, 50 °C/30 s, 72 °C/30 s (33×) and 94 °C/30 s, 50 °C/30 s, 72 °C/5 min. (1×). The PCR products were purified with NucleoSpin Extract II (Macherey-Nagel, Germany) prior to sequencing.

The sequences were determined with an ABI PRISM 3100 Avant DNA sequencer (Applied Biosystems) at the Department of Animal Morphology, Physiology and Genetics, Faculty of Agriculture, Mendel University, Brno using the ABI PRISM BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems). All samples were sequenced with the primers used in the PCR. The sequence of the representative strain was deposited in the EMBL Nucleotide Sequence Database under accession number EF194775.

Pathogenicity test. The pathogenicity test was carried out using 1-year-old *Castanea sativa* saplings cultivated from healthy seeds collected at the study site. The infection experiment was processed in artificial conditions at 20 °C, relative air humidity 60–80 % under light simulating the intensity and wavelength of natural radiation. The stems were surface sterilised with pulp rinsed in 95 % ethanol. The surface tissues were vertically incised with a scalpel, and plugs of V8A agar from the actively growing colony margin of *P. cambivora* isolate no. CCF 3683 were inserted behind the flap. Then the wounds were sealed with Parafilm. The characteristic stem necroses developed after 4–6 weeks and the pathogen was successfully reisolated from the lesions with the method described above. The control plants were inoculated in the same way with sterile agar plugs, and remained healthy.

RESULTS AND DISCUSSION

Phytophthora cambivora was confirmed as the causal agent of ink disease of *Castanea sativa* in the Czech Republic. Koch's postulates were satisfactorily fulfilled in the infection experiment – the characteristic stem necroses developed after 4–6 weeks on all inoculated plants and the pathogen was satisfactorily reisolated from necrotised tissues. The pathogen caused blackish sunken necroses on stems of inoculated saplings, which reached usually 2–4 cm during 6 weeks. When the necroses girdled the stems of saplings, their foliage yellowed and dried. Some saplings were dying at the end of the experiment.

We have acquired none of the other species capable of parasitising on sweet chestnut, *P. cinnamomi*, *P. cryptogea*, *P. gonapodyides*, *P. citricola* and *P. cactorum* (Erwin and Ribeiro 1996, Vannini and Vettraino 2001, Vettraino et al. 2005) from stem necroses in the stand.

Phytophthora cambivora (Petri) Buisman

Description. Nearly twenty similar isolates from necrotised stem tissues of different trees were acquired during the study. The morphological and cultural characteristics of all isolates are consistent with *P. cambivora* (Erwin and Ribeiro 1996), which belongs to the 6th morphological group according to Waterhouse (1963). The colonies on V8A were homogenous, cottony, with rich aerial hyaline mycelium (Fig. 1), those on CA were rather lower with relatively sparse mycelium, but of the same general appearance (Fig. 2). Radial growth was 7.8–8.8 mm/day at 20 °C on V8A. The main temperatures were as follows: minimum

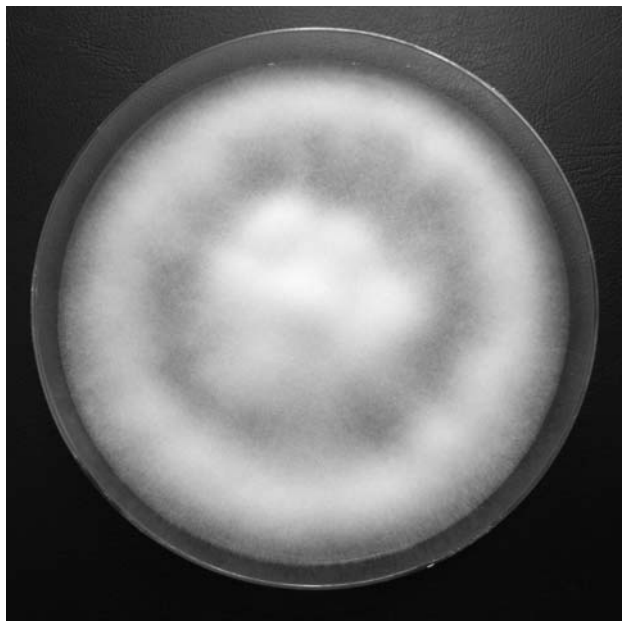

Fig. 1. *Phytophthora cambivora*, colony on V8A (10 days, 20 °C).

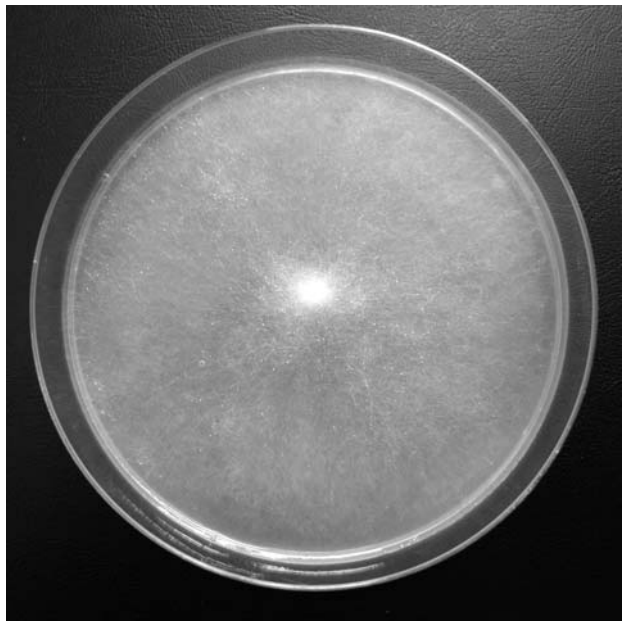

Fig. 2. *P. cambivora*, colony on CA (10 days, 20 °C).

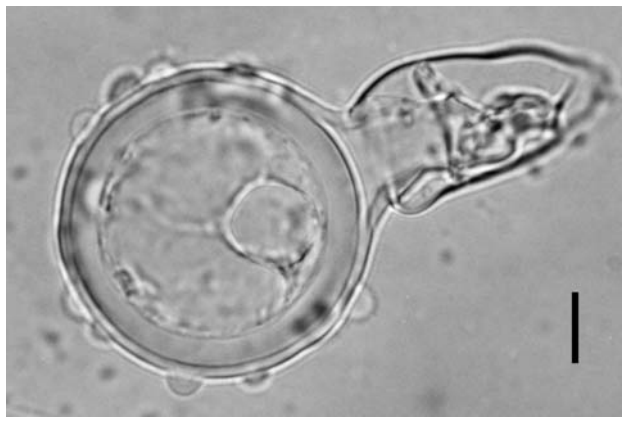

Fig. 3. *P. cambivora*, oogonium with oospore and antheridium (V8A, 15 days, 20 °C).

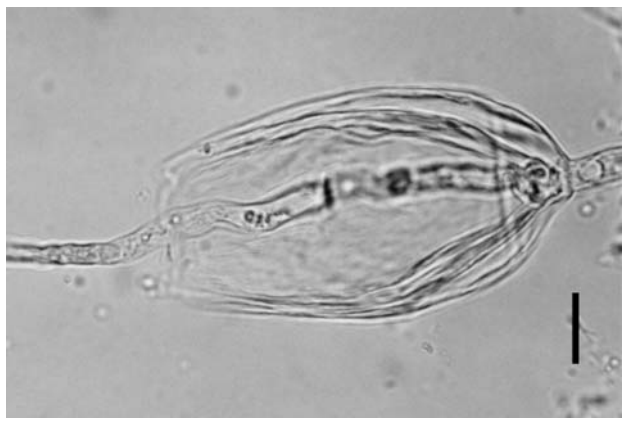

Fig. 4. *P. cambivora*, internal zoosporangium proliferation – nested and prolonged proliferation (on V8A in filtered pond water, 10 days, 20 °C).

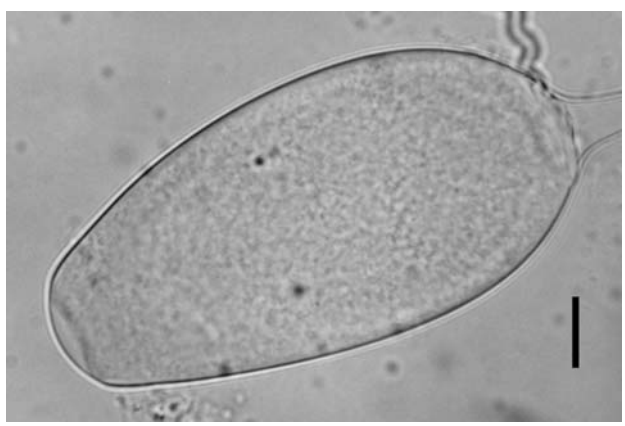

Fig. 5. *P. cambivora*, obpyriform sporangium (on V8A in filtered pond water, 10 days, 20 °C). Bars: 10 µm.

growth temperature 3 °C, optimum 23 °C, and maximum 33 °C. The mycelium was coenocytic, but older hyphae were often irregularly septate and usually measured 3–6 µm in diam. Coralloid hyphae were sometimes present, chlamydospores were not found. The isolates were heterothallic and in the paired cultures produced abundant, terminal, verrucose, spherical oogonia (Fig. 3) with a diameter of 46.9 µm on average (41.0–53.0 µm). The outer oogonial wall was distinctly verrucose or bullate (protuberances were 3–5.5 µm high), but nearly smooth oogonia were found as well. Oospores were plerotic or nearly so, with an average diameter of 42.2 µm (37–48 µm), their wall was 4 µm thick on average and ranged from 1.8 to 5.5 µm. Antheridia were bicellular, amphigynous and measured 26.8 × 19.3 µm on average (20–35 × 15–23 µm). Sporangiophores were simple, unbranched or loosely sympodially branched. Sporangia were often nested or internally proliferating (Fig. 4), usually obpyriform (Fig. 5), but sometimes oblong, limoniform or ovoid in shape, with rounded base. They were non-papillate or had a minute papilla and measured on average 80 × 42 µm (69–102 × 36–50) and the L:B ratio was 1.9 on average (1.6–2.2).

Four representative strains isolated from stem lesions of *Castanea sativa* are deposited in the culture collection at RILOG and one of them is deposited in CCF (Culture Collection of Fungi, Dept. of. Bot., Charles University, Prague) under No. CCF 3683. The ITS sequence of the strain (EF194775) is for 99 % identical with *P. cambivora* sequences deposited in GenBank.

N o t e s . *P. cambivora* is relatively well distinguishable from other *Phytophthora* species parasitising on *Castanea* and belonging to the 6th Waterhouse´s morphological group (Waterhouse 1963) – *P. cinnamomi*, *P. cryptogea*, and *P. gonapodyides*. *P. cambivora* differs from *P. cinnamomi* in its absence of characteristic swellings and chlamydospores, presence of conspicuous oogonium ornamentation, homogenous cottony character of colonies etc. (Erwin and Ribeiro 1996, Waterhouse and Waterston 1966 b, c). Furthermore, *P. cinnamomi* is more thermophilic than the other species – the species was not detected at sites characterised by a minimum temperature below 1.4 °C (Vettraino et al. 2005). The temperatures this species requires are higher than those of *P. cambivora* and other species – see Erwin and Ribeiro (1996), Zentmyer (1980), Waterhouse and Waterston (1966 c), etc. The studied area is localised in a moderately warm region (MW7 by Quitt), where the average temperature in January ranges from –3.0 to –2.0 °C (Tolasz et al. 2007). It seems that the climatic conditions in the stand are unsuitable for *P. cinnamomi*.

P. gonapodyides is distinguishable from *P. cambivora* by its rosaceous colony pattern on V8A, slower growth, smaller oospores, finely roughened and no verrucose nor bullate oogonial wall (Erwin and Ribeiro 1996). Isolates of *P. cryptogea* may be confused with those of *P. cambivora* due to their similar colony pattern, growth rate and shape and size of zoosporangia, but they differ in their smooth oogonial wall (Erwin and Ribeiro 1996, Stamps 1978, Waterhouse and Waterston 1966 b).

The isolates of *P. citricola* and *P. cactorum* are homothallic and have more appressed colonies on V8A with stellate to petallate patterns. They produce small oogonia with smooth walls and predominantly paragynous antheridia, and belong to another of Waterhouse´s taxonomic groups (Erwin and Ribeiro 1996, Waterhouse 1963). *P. cactorum* (1st taxonomic group) produces conspicuously papillate, sometimes caducous sporangia of ellipsoid, ovoid to subspherical shapes usually in close sympodia, while *P. citricola* (3rd taxonomic group) has semipapillate to papillate, sometime caducous, ovoid to obpyriform or irregularly shaped zoosporangia in loose sympodia or on simple sporangiophores (Erwin and Ribeiro 1996, Waterhouse and Waterston 1966 a, d).

All the mentioned species are well distinguishable by comparing sequences of the ITS regions of ribosomal DNA (Cooke et al. 2000), but sequences of some *P. cambivora* isolates resemble those of *P. alni* Brasier et al. (Brasier et al. 2004). Nevertheless, the host spectrum of both species is quite different – the natural occurrence of *P. alni* is restricted only to alders (Brasier et al. 2004), however a weak pathogenic potential to other tree species was found experimentally (Brasier and Kirk 2001, Santini et al. 2003). The species are distinguishable by the homothallism of *P. alni* as well (Brasier et al. 2004).

Disease development and symptoms of *Phytophthora cambivora* in the stand

The pathogen usually infects the host through the roots and collar and causes root rot and collar and stem necroses. The necroses of under-bark tissues are flame– or tongue-shaped and sometimes reach up to several metres in length. The annual growth of the stem necroses reached a size of several dozen centimetres. The necrotised host tissue produced blue-black exudates which oozed throughout the cracks in the bark. The exudates were visible as ink discolorations on the surface of stems, collars and buttresses. According to these characteristic symptoms the disease was named „ink disease“. The exudates stained the soil around the collar and damaged roots as well.

The young trees and saplings were very susceptible to this disease and often died during one season. The damage of full-growth trees developed usually several seasons. The damage of foliage and branching system corresponded to the proportion of damaged roots and/or conductive tissues in girth of collar. The foliage of diseased trees has characteristic sparse, yellowish and small leaves. Whole branches could wilt at once and then the dry, yellow, crinkled leaves remained hanging on them. The crowns of trees in advanced stage of the disease were substantially withered. They were of skeleton appearance and the remaining foliage was clumped around stems and limbs. The fruits of the diseased trees were often small and unripe when developed.

The overall distribution of sweet chestnut ink disease and its causal agent in the Czech Republic is not known. The symptoms of the disease should be observed at other *Castanea* stands.

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