

RESEARCH PAPERS

***Phytophthora cinnamomi* causing stem canker and root rot of nursery-grown *Platanus* × *acerifolia*: first report in the Northern emisphere**

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Summary. Lethal stem and root cankers were observed in nursery-grown *Platanus* × *acerifolia* trees in Rome. Externally, canker lesions appeared as bluish or blackish areas starting from the stem base and extending upward. Inner bark was necrotised. In some cases an irregularly-shaped callus reaction attempted to heal the bark lesions. Black-stained necrosis affected the primary roots and the small branch roots to different degrees. The presence of *Ceratocystis platani* was excluded in the diseased trees. *Phytophthora*-like organisms were isolated from the altered tissue. Morphological and ITS-region-based analyses identified the isolates as *Phytophthora cinnamomi*. A pathogenicity test confirmed *P. cinnamomi* as the causal agent of the disease here defined as: stem canker and root rot of plane tree. This is the first report of *P. cinnamomi* in *Platanus* spp. in the Northern emisphere.

Key words: plane trees, bark staining, healing callus.

Introduction

Platanus is a tree genus broadly used in several regions of the world for landscaping, especially in urban settings. Canker stain, caused by *Ceratocystis platani* (J.M. Walter) Engelbr. & T.C. Harr., is known to be the most destructive disease of plane tree (Pancosi, 1999; Ocasio-Morales *et al.*, 2007). A number of additional fungal diseases can cause considerable damage to plane tree, decreasing their value as a landscape tree. For instance, anthracnose, caused by *Apiognomonina veneta* (Sacc. et Speg.), Hohn [anamorph: *Gloeosporium platani* (Mont.) Oud], and powdery mildew, caused by *Microsphaera platani* (Howe) (anamorph: *Oidium* sp.), are widespread (Pilotti, 2002). Wood decay caused by Hymenomycetes is also commonly found in many mature trees, especially in those that are periodically pruned (Pilotti, 2002; Moriondo and Santini, 2002; Pilotti *et al.*, 2005;

Luchi *et al.*, 2011). The wood of plane tree can also be susceptible to perennial cankers, in which the fungus *Fusarium solani* [(Mart.) Appel and Wollenw. emend. Syd. and Hans.] plays an important role (Pilotti *et al.*, 2002).

Plane tree can also be affected by diseases caused by *Phytophthora* spp. For instance, in Australia *Phytophthora cinnamomi* Rands has been reported as the agent of severe trunk rot in *P. orientalis* L., in nurseries and street plantings (Greenhalgh and Challen, 1979). In the USA, a *Phytophthora* sp. has been reported in *P. orientalis* seedlings affected by wedge-shaped cankers, but without any description with regard to species identification (Crandall, 1936). In Argentina, *P. cinnamomi* has been detected in plane trees affected by root rot (Frezzi, 1950, 1977) or by resinous canker (Erwin and Ribeiro, 1996a). However, despite the abundance of *Phytophthora* species in Europe (Vettraino *et al.* 2002; 2005; Belisario *et al.*, 2006; Santini *et al.*, 2013), *P. cinnamomi* has yet to be isolated from the genus *Platanus*.

In this paper, we report the occurrence of *P. cinnamomi* on nursery-grown *P. × acerifolia* plants and

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describe the results of the inoculation trial aimed at determining its role as the causal agent of a disease, here defined as: stem canker and root rot of plane tree. This is the first report of *P. cinnamomi* in *Platanus* spp. in the Northern hemisphere.

Materials and Method

Symptom survey, sample collection and isolations

Investigations were carried out in a nursery in Rome (Italy) where plane trees are usually propagated and grown. Symptoms of severe stem canker and root rot were evident on 3 to 7-year-old potted trees. Isolations were performed on stems and roots of five symptomatic plants. Small portions of discoloured tissue were collected from the reaction zone of the necrotised tissues, rapidly flamed, and plated on Potato Dextrose Agar (PDA) (Oxoid) without or with antibiotics (25 mg L⁻¹ nystatin, 10 mg L⁻¹ rifampicin, 25 mg L⁻¹ ampicillin). All plates were incubated at room temperature, in the dark.

Additionally, necrotic wood samples were investigated for the presence of *C. platani* as follows: a) microscopic observation at 20 and 40 × magnification; b) incubation in moist chambers; c) isolation on PDA plates (OEPP/EPPO, 2003); or d) carrot assays as described by Moller and DeVay (1968) and by Pilotti *et al.* (2012).

Cultural and morphological characteristics of *Phytophthora*-like isolates

Five *Phytophthora*-like isolates (PC1-PC5) were obtained from single hyphal tip isolations, and their growth rates compared in 90 mm in diameter PDA plates, by incubating at 20, 25, 30, 35°C in the dark, for 8 days (five colonies *per* isolate). A one-way fully randomized analysis of variance (ANOVA) was carried out on the average of two colony diameter measurements (at right angles to each other) for each colony. The analysis included the different isolates and temperatures (software: R version 3.0.1). A post hoc multiple range test (option: Tukey HSD) was also performed considering a Bonferroni correction where new pairwise alpha values were calculated to keep the familywise alpha value at 0.05.

Isolates were also grown on a pepper seed-supplemented medium. The medium was prepared as follows: seeds were washed under running water,

autoclaved at 95°C for 15 min., and placed on actively growing PDA cultures. After 24 h the seeds were transferred onto water agar plates (agar technical 12 g L⁻¹) - 15 seeds per plates - which were then flooded with sterilized water. The mycelium-enriched surface of the seeds was scraped off after 5–10 days and observed under a microscope.

Sequencing and phylogenetic analyses

The ITS1, 5.8S and ITS2 regions of the five isolates were sequenced as described in Pilotti *et al.* (2005), and deposited in NCBI GenBank with accession numbers KC184900-4. Sequences were compared with GenBank accessions using the “nucleotide BLAST” on the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome).

Pathogenicity test

Isolate PC2 was used for the pathogenicity test. Inoculations were performed in October, on ten 6-year old plants clonally propagated from a single tree and grown in 65 L pots. PDA mycelial plugs, 9 mm in diameter derived from the edges of 5-day-old Petri dish colonies, were placed under the bark of the stem in holes made with a cork borer, at 10–15 cm above soil level. Each inoculation hole was then covered with the bark top, that had been previously excised, and a clump of wet cotton wool. The inoculation point was then wrapped in thin plastic film and aluminum foil and taped with masking tape. Negative controls were performed on five plants by placing a plug of sterile PDA at the inoculation points.

Symptoms were monitored over a period of 2 years, after which destructive analyses were carried out to assess symptom development in the internal tissues of each plant, and to detect the inoculated pathogen.

Results and discussion

Natural symptoms

The disease was recorded on 3- to 7-year-old cuttings grown in 65 L-pots. Symptoms included severe necrosis of the root system and cankering on the basal part of the stem, resulting either in plant death or in decline symptoms in the crown.

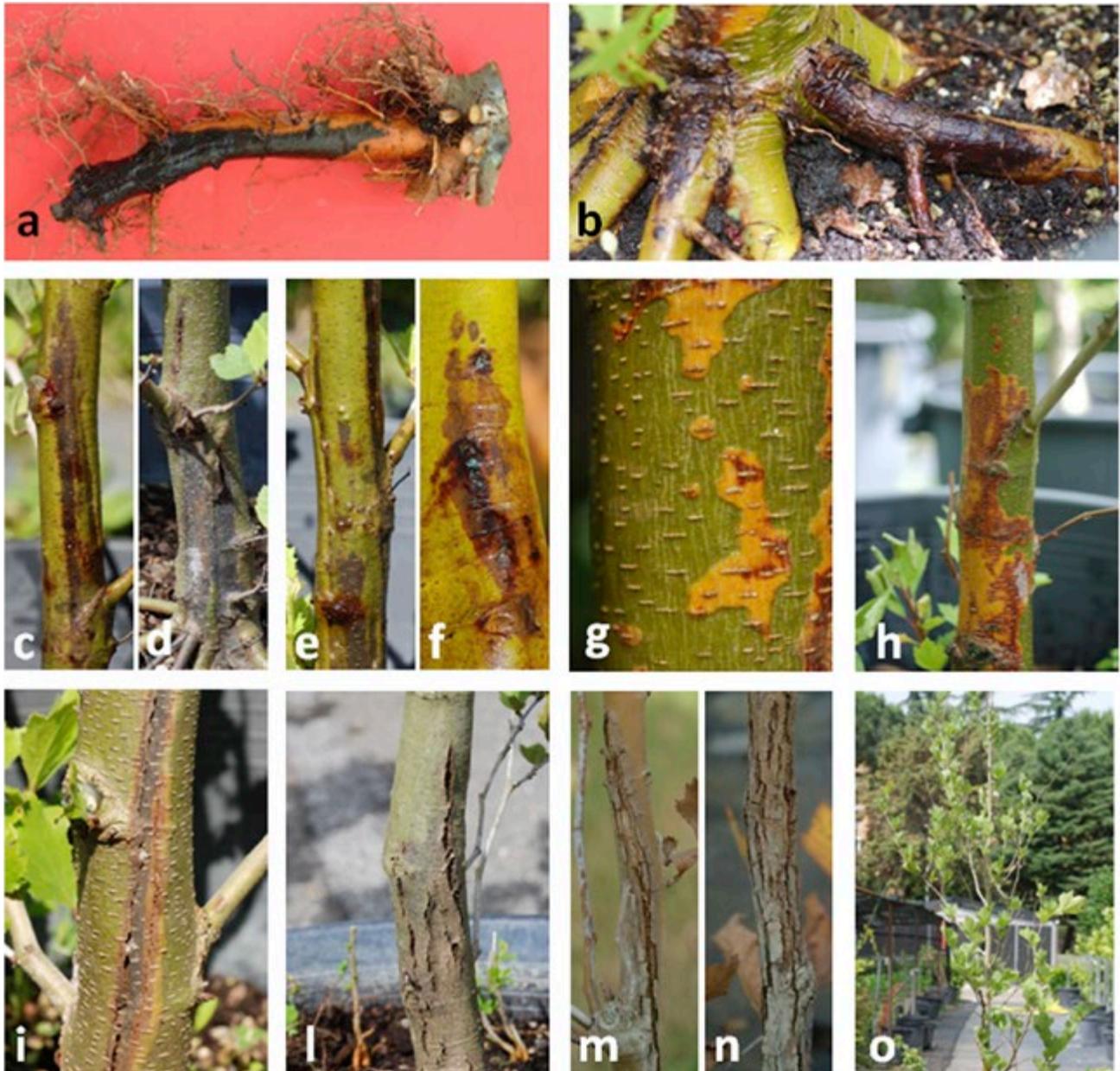


Figure 1. Symptoms of stem canker and root rot observed on nursery-grown *Platanus × acerifolia* trees, and associated with *Phytophthora cinnamomi*. Necrosis of the main roots (a, b). Bluish discolouration of the bark (c, d, e). Swollen spots turning into black and oozing blotches (e, f). Yellow /reddish/brown discolouration patterns (g, h). Healing canker with a yellow edge in the reaction zone (i). Healing/healed cankers with an irregularly-shaped callus reaction (l, m, n). Sparse foliage of a tree affected by a chronic-like course of the disease (o).

A black necrosis was evident in the roots. In the large roots connected to the collar, generally only the periderm was necrotic. In the smaller roots, the wood was also affected (Figure 1a, b). Bark cankers

occurred at the base of the main stem up to 30–50 cm. Initially, cankers appeared as discoloured areas with colours ranging from bluish, dark brown to black and spreading both longitudinally and tangentially

(Figure 1c–e). Yellowish discoloured areas frequently preceded the dark discolouration, started from lentils and progressively coalesced (Figure 1g, h). Yellow discolouration also affected the bark in the reaction zone of stem cankers (Figure 1i). Green to black oozing blotches were also observed in the cankered areas (Figure 1e, f). Bark cracks were also present. During the growing season, expanding callus reactions appeared (Figure 1i–n) but the callusing did not prevent the spread of the necrosis, which in turn triggered new callus reactions. Due to this process, the stems became deformed (Figure 1n). Cankering also spread to the lateral branches. The apparently unaffected bark surrounding the cankers and the healing callus, evidently increased rhytidome exfoliation in comparison with healthy trees.

In the worst cases, bark lesions were diffuse and no callus reaction appeared throughout the observation period (Figure 1c–f). In these cases and after 1–2 years, lesions completely girdled the stems, and death occurred as a sudden summer wilting. Trees displaying the callusing response, on the other hand, survived at least 4–5 years after the first appearance of symptoms. The crowns of these trees were thin (Figure 1o). In the plants affected by healing cankers the wood was unaffected while in the plants affected by non-healing cankers some dark discolouration slightly affected a restricted portion of the wood.

Morphological and sequence-based identification of the causal agent

Ceratocystis platani was not detected in the symptomatic trees. Instead a *Phytophthora*-like was con-

sistently isolated from these trees. Colonies showed a coralloid-type mycelium, hyphal swellings of varying sizes and shapes, ovoid non-papillate sporangia, and spherical/globose terminal chlamydospores of variable size (Figure 2, Table 1). Length and breadth of sporangia varied widely for each isolate, similarly to previously reported findings (Table 1) (Erwin and Ribeiro, 1996b). Based on these morphological characteristics, the isolates were identified as *P. cinnamomi* (PC) (Erwin and Ribeiro, 1996b).

ITS sequences matched those of *P. cinnamomi* in GenBank with 99–100% identity scores.

Isolates showed the lowest growth at 20°C and the highest at 25°C. The differences between these two temperatures were always significant with Bonferroni-corrected $P=0$. Growth rates were also significantly different between 20 and 30°C ($0.044 \geq P \geq 0$). The growth rate at 30°C was similar to that at 25°C with some significant differences ($0.003 \geq P \geq 0$, see Figure 3). Growth rate did not differ significantly among the isolates within each temperature group except for isolate PC5 at 30°C, which was different from the other isolates ($0.044 \geq P \geq 0.0000046$) (Figure 3). Incubation at 35°C caused the death of all the isolates.

Pathogenicity test

In the inoculated plants symptoms appeared at the end of the Winter following the Autumn inoculation, and included cracking of the bark, bluish depressed necroses mostly progressing in basipetal and acropetal orientation (Figure 4a–d). The inoculation points and the cracks were surrounded by yellow halos, turning brown during the growing sea-

Table 1. Sizes (μm) of sporangia and chlamydospores of *Phytophthora cinnamomi* isolates from *Platanus × acerifolia*.

Isolate	Sporangia ^a		L-B ^b	Chlamydospores ^a
	Length range (average value)	Breadth range (average value)		Diameter range (average value)
PC1	18.5–58.7 (33.4)	12.6–35.8 (23.7)	1.2–1.8	19.6–45.5 (30.1)
PC2	16.7–78.3 (36.1)	13–39 (24.3)	1.2–1.8	19.4–51.5 (28.1)
PC3	26.1–74.4 (57.5)	20–45.8 (37.9)	1.3–2.1	21.1–38.2 (30.5)
PC4	16.3–61.1 (33.4)	10.6–49.1 (23.7)	1.2–2.3	16.8–34.9 (23)
PC5	25–79.5 (43.9)	18.6–54.7 (31.4)	1.2–1.6	17.4–51.9 (29.9)

^a Twenty sporangia and twenty chlamydospores were measured for each isolate.

^b Range of Length-Breadth ratio.

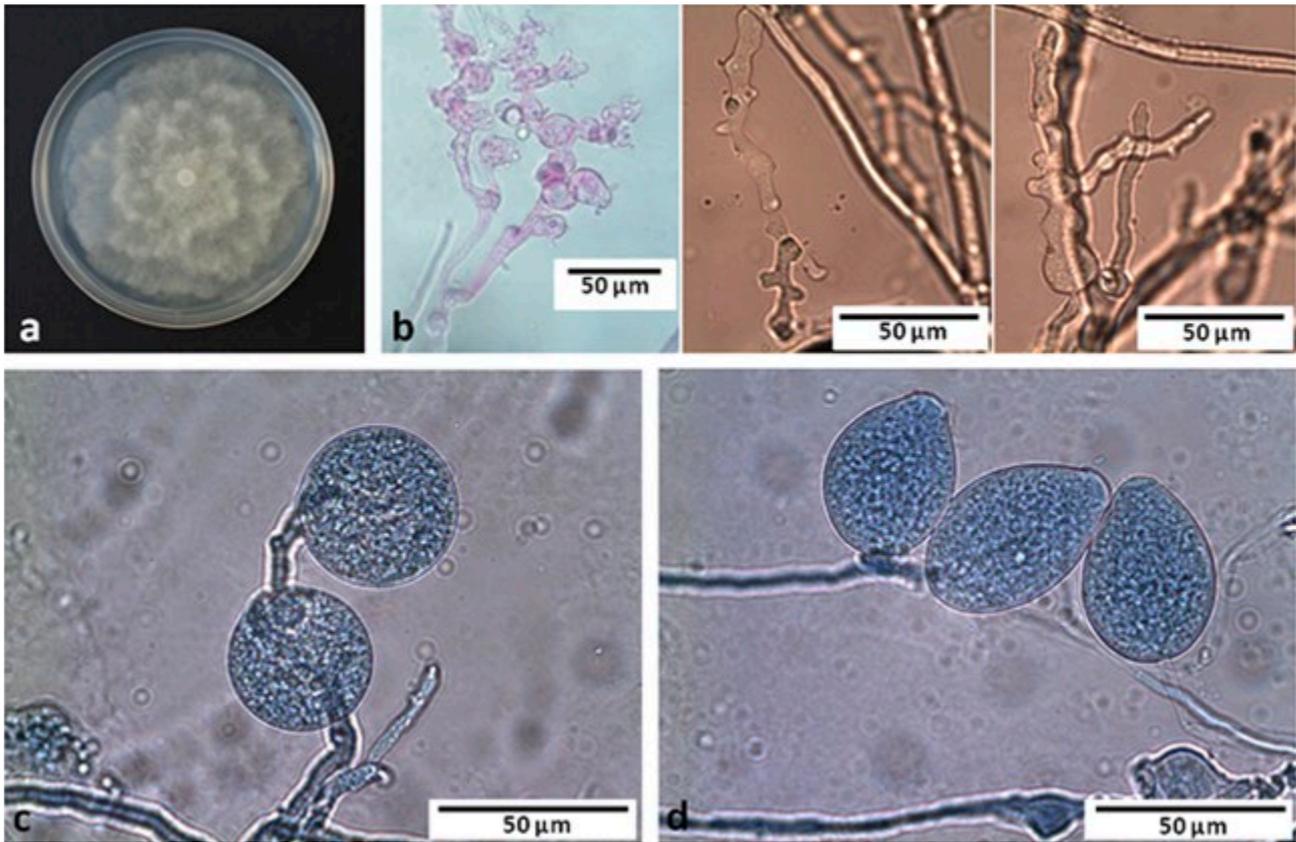


Figure 2. Morphological features of *Phytophthora cinnamomi* isolated from *Platanus × acerifolia*. Colony type (a); coralloid-type mycelium and hyphal swellings (b); chlamidospores (c); sporangia (d).

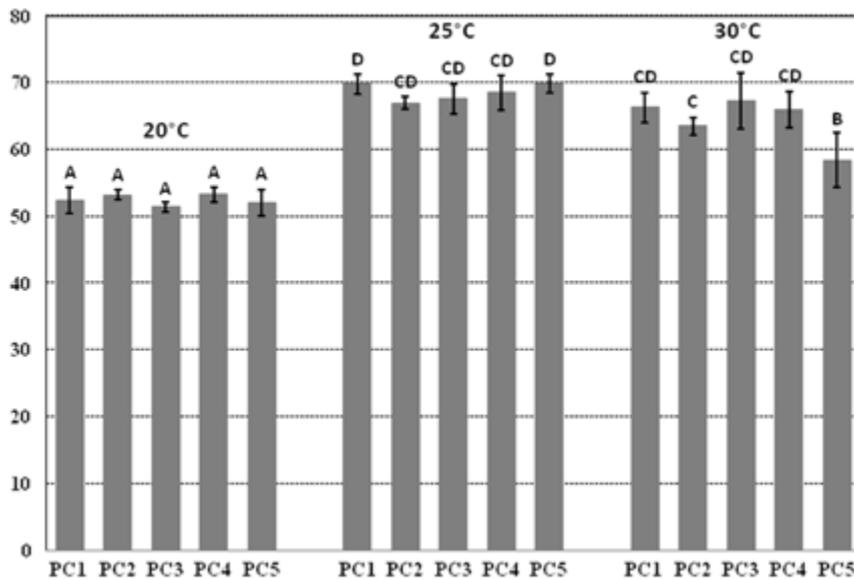


Figure 3. Average diameter growth (mm) of the *Phytophthora cinnamomi* isolates from plane tree, on PDA, after 8 days, at 20, 25 or 30°C in the dark. Growth rates of isolates marked by different letters (top of bar) are significantly different from each other (Bonferroni-corrected $P < 0.05$); vertical bars indicate standard deviation of the means.

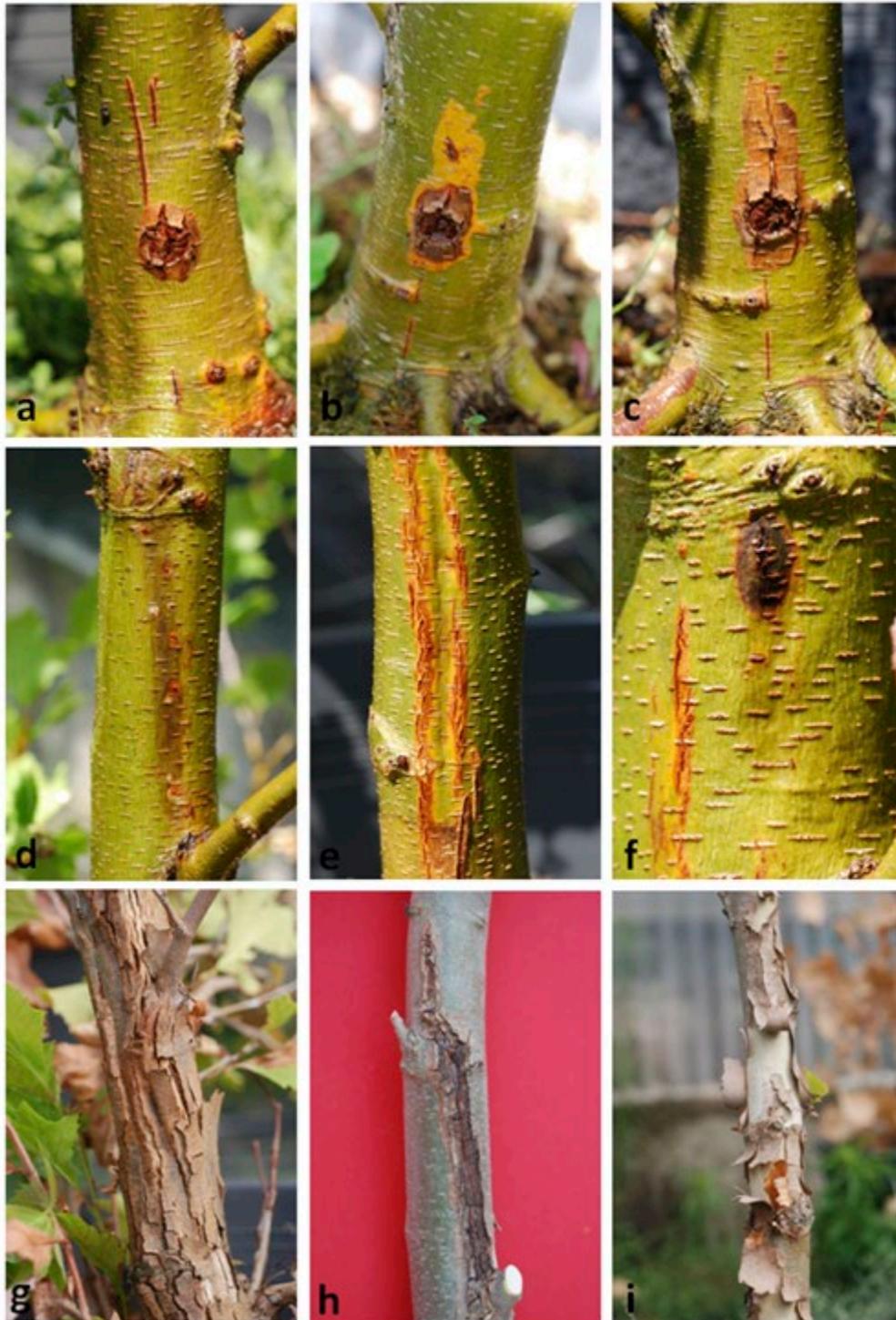


Figure 4. Stem canker on *Platanus × acerifolia* trees that have been artificially infected with *Phytophthora cinnamomi*. Bark cracks near the inoculation point (a, b, c). Yellow discolouration spreading from the inoculation point and turning into an exfoliating, brown and dried patch of tissue (b vs c). Dark staining of the bark (d). Surface, and corky cracking surrounding the edges of a longitudinal bark canker (e). A damp, black blotch (f). Callus reaction on a cankered stem, 2 years after *Phytophthora cinnamomi* inoculation (g, h). Rhytidome exfoliation in a portion of the stem far from the cankered areas (i).

son (Figure 4b, c). Over time, the bluish zones were surrounded by irregularly shaped callus reactions which nearly completely covered the lesions (Figure 4e, g, h). Damp black blotches appeared (Figure 4f). The healing callus and the stem regions far from the canker lesions were affected by an intense exfoliation of the rhytidome (Figure 4g, i). During the 2-year observation period, cankers spread on the main stems for 16–54 cm (average value: 33 cm, standard deviation: 13.5), girdled the stems and spread to the lateral branches. Bark underneath the discoloured rhytidome was always necrotic. In the regions where the callus reaction stopped disease progression, bark necrosis had been compartmentalized. In any case, the wood remained unaffected. Due to basipetal progression, a blackish necrosis affected the periderm of the roots which branched directly from the collar. At 2 years post inoculation, trees displayed thin and chlorotic crowns. Control (sterile PDA-inoculated) plants did not show any symptoms throughout the period of observation. *Phytophthora cinnamomi* was re-isolated from one tree. It was detected using molecular assay applied on tissues taken from the edges of the cankers (data not shown). Detection was positive in six trees out of ten (data not shown). Having successfully completed Koch's postulate, we establish that *P. cinnamomi* is the etiological agent of the disease under study, that we designate as stem canker and root rot of plane tree.

Symptoms of “trunk rot” in *P. orientalis*, caused by *P. cinnamomi* in Australia, have been reported as bark alterations in the form of vertical ridges, cracks, depressed areas, and necrosis of the inner bark (Greenhalgh and Challen, 1979). These symptomatic features are identical to those we report here. However, several symptomatic features of stem canker and root rot were not reported for Australasian trunk rot, including root rot, rhytidome discolouration patterns, black oozing blotches, and intense systemic rhytidome exfoliation. It is still not clear whether the symptoms that were unique to the disease on trees in Italy are due to the fact that we monitored disease development over time in a detailed manner, or were due to substantial differences in symptoms occurring in the two countries.

Acknowledgements

This research was supported by a MIPAF (the Italian Ministry of Agricultural and Forestry Policies)

project: “Armonizzazione della diagnosi e valutazione del rischio di patogeni da quarantena e nocivi ai vegetali e ai prodotti vegetali - Malattie emergenti di piante erbacee e arboree (ARON/ARNADIA)”. We thank Dr P. Menesatti (CRA-ING, Unità di Ricerca per l'Ingegneria Agraria) for his help with the statistical analysis, Dr Alessandra Belisario (CRA-PAV, Plant Pathology Research Center) for her useful comments on the manuscript, and Section Editor Matteo Garbelotto for editing the manuscript.

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Accepted for publication: July 15, 2013