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Recovery and pathogenicity of *Phytophthora* species associated with a resurgence of ink disease in *Castanea sativa* in Italy

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Three species of *Phytophthora*, *P. cambivora*, *P. citricola* and *P. cactorum*, were found to be associated with a recent outbreak of ink disease causing high mortality of chestnut trees in central Italy. *Phytophthora cambivora* was isolated from 11·6% of the soil samples taken around symptomatic trees, and was mainly associated with heavily diseased trees. It was the most aggressive species to *Castanea sativa*, but survived poorly in the soil. *Phytophthora citricola* and *P. cactorum* showed a limited ability to induce disease on chestnut, but could be recovered from soil during most of the year. A fourth species, *P. gonapodyides*, was recovered only from mud of stream beds within the chestnut stands. Involvement of these species in the development of disease is discussed.

Keywords: *Castanea sativa*, ink disease, mating types, pathogenicity, *Phytophthora cactorum*, *P. cambivora*, *P.citricola*, *P. gonapodyides*

Introduction

Ink disease, which causes root and collar rot of seedlings and adult trees in nurseries, plantations and forests, is one of the most destructive diseases of *Castanea* spp. Since it was first recorded in Portugal in 1838, it has become widespread in Europe on sweet chestnut (*Castanea sativa*), and in the USA on American chestnut (*C. dentata*) and other chestnut species (Crandall *et al*., 1945). Two species of *Phytophthora* were shown to be responsible for the disease in Europe and the USA: *P. cambivora* and *P. cinnamomi* (Petri, 1917; Milburn & Gravatt, 1932; Day, 1938; Crandall *et al*., 1945). Other *Phytophthora* species have also been found to be associated with infected trees or to be pathogenic to *Castanea* spp. (Curzi, 1933; Smith, 1937; Cristinzio & Verneau, 1954; Wicks & Volle, 1976).

Because of the devastating epidemic of chestnut blight caused by *Cryphonectria parasitica* in the 20th century in Europe and the USA, less attention has been paid in recent decades to ink disease and to aspects of its etiology and epidemiology. Recently, high mortality of sweet chestnut caused by ink disease has been reported from different areas of Europe (Abreu, 1996; Anselmi *et al*., 1996) and the USA (Vettraino *et al*., 2000), in some cases limiting the establishment of new groves or the conservation of old ones. In Italy the disease has recently spread dramatically, apparently from infection foci, in most chestnut-growing areas (Anselmi *et al*., 1996). Large numbers of diseased trees have been found along roads and trails from which the disease has probably spread, carried by man and animals (Anselmi *et al*., 1999). Due to the high economic and environmental value of sweet chestnut in Italy, this new outbreak of ink disease has caused considerable concern.

In order to understand the causes of this new epidemic, it is important to study the epidemiology and etiology of the disease, including whether one or more *Phytophthora* species are involved in addition to *P. cambivora* and *P. cinnamomi*. The present work aimed to identify the species of *Phytophthora* present in soils of chestnut forests and orchards affected by ink disease in central Italy. Another aim was to assess the variation in pathogenicity of the *Phytophthora* species isolated.

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Materials and methods

Areas investigated

Two chestnut-growing areas were investigated in central Italy: the first in Northern Latium on the Monti Cimini; the second in Eastern Latium on the Monti del Cicolano. Both areas have extensive coppice stands and orchards devoted to fruit production, ranging in

Table 1 Isolates of *Phytophthora* spp. used as standards for identification

Species	Strain	Host	Mating type	Provided by
P. gonapodyides	AB4	*Quercus* spp.	Unknown	C. Delatour, INRA, France
P. gonapodyides	P501	*Q. ilex*	A1	C.M. Brasier, Forest Research, Forestry Commission, UK
P. erythroseptica	IMI 17028	*Solanum tuberosum*	Homothallic	G. Magnano di San Lio, University of Reggio Calabria, Italy
P. cryptogea	13·4.9	*Prunus avium*	Unknown	G.C. Adams, Michigan State University, USA
P. cryptogea	IMI 180615	Unknown	Unknown	G. Magnano di San Lio, University of Reggio Calabria, Italy
P. drechsleri	–	*P. avium*	Unknown	G.C. Adams, Michigan State University, USA
P. megasperma	27·1.5	*Juglans nigra*	Homothallic	G.C. Adams, Michigan State University, USA
P. cactorum	PH31	*Betula alba*	Homothallic	G. Magnano di San Lio, University of Reggio Calabria, Italy
P. cactorum	PH6	Unknown	Homothallic	G. Tamietti, University of Turin, Italy
P. citricola	P1013	*Quercus* spp.	Homothallic	C.M. Brasier, Forest Research, Forestry Commission, UK
P. citricola	21/25-KV	*Chamaecyparis lawsoniana*	Homothallic	S. Werres, BBA, Germany
P. cambivora	20/95–21	*Q. rubra*	A2	S. Werres, BBA, Germany
P. cambivora	21/95-KII	*C. lawsoniana*	A1	S. Werres, BBA, Germany
P. cinnamomi	69094	*C. lawsoniana*	A1	S. Werres, BBA, Germany
P. cinnamomi	8/88/92	*C. lawsoniana*	A2	S. Werres, BBA, Germany

altitude from 400 to 1000 m a.s.l. Although ink disease was not uniformly present in these areas, an increasing number of infection foci were recorded each year, sometimes with a high incidence of the disease.

Surveys were carried out between 1998 and 2000 by collecting soil samples from four different forest stands (three in the Monti Cimini and one in the Monti del Cicolano) where the disease had been recorded previously (Anselmi *et al*., 1996). Occasionally diseased trees recorded from additional stands were sampled. Samples were not taken from all stands at each sampling time. A total of 155 soil samples were collected: 119 soil samples were analysed from the chestnut area of the Monti Cimini; of these 93 were collected under symptomatic trees (both in coppice and orchards), and 26 under healthy-looking trees. Thirty-six soil samples were analysed from the chestnut-growing area of the Monti del Cicolano: of these 19 were collected under symptomatic trees, seven under healthy-looking trees, and 10 from two stream beds crossing chestnut forest sites affected by the disease.

Isolation of *Phytophthora*

Each soil sample contained fine or coarse chestnut roots, and resulted from a mix of four monoliths of soil (25 × 25 × 25 cm) collected at four compass points around a tree at a distance of about 50 cm from the collar. After collection, soil samples were moistened with sterile distilled water and incubated at 20°C for 3 days. About 200 mL soil was then flooded with 500 mL distilled water in plastic containers. Five freshly picked leaves of *Rhododendron* spp. (Themann & Werres, 1998) were placed directly on the water surface and incubated at 20°C for 1 week until spots developed on the leaves or the leaves become discoloured. The leaves were then blotted on filter paper, cut into small pieces (0·5 × 0·5 cm) and placed on PARBhy selective medium [10 mg pimaricin, 250 mg ampicillin (sodium salt), 10 mg rifampicin, 50 mg hymexazol, 15 mg benomyl, 15 g malt extract, 20 g agar in 1000 mL H_2O] (Robin, 1991). *Phytophthora* isolates were maintained on carrot agar (CA) (Brasier, 1969) at 20°C in darkness and subcultured at 4 week intervals.

Identification of isolates

Isolates were identified by comparing colony growth patterns and morphological features of sporangia, oogonia, antheridia, chlamydospores and hyphal swellings with known isolates (Table 1) and with species descriptions reported in literature (e.g. Stamps *et al*., 1990; Erwin & Ribeiro, 1996). Colony morphology was described on 10-day-old cultures grown on CA in 90 mm Petri dishes at 20°C in darkness.

Sporangia were produced by placing a disc of mycelium from a 7-day-old culture grown on CA in soil extract prepared according to Chee & Newhook (1965). Morphology was assessed by light microscopy, and the length and breadth of 100 sporangia were measured for each isolate.

Identification of the isolates was confirmed by

comparing the RFLP patterns of their ribosomal DNA (rDNA) with those of known isolates listed in Table 1. *Phytophthora* spp. DNA suitable for PCR amplification was purified according to the methodology of Cenis (1992). ITS1 and ITS4 universal primers (White *et al.*, 1990) were used to amplify *Phytophthora* rDNA. PCR amplification was performed using Ready-to-Go PCR Beads following the manufacturer's instructions (Amersham Pharmacia Biothec Inc., Uppsala, Sweden). Template DNA (10 ng), 1 μM of each primer and bi-distilled H_2O were added to each reaction tube to make a final volume of 25 μL. The mixture was subjected to thermal cycling in a Techne Progene cycler (Techne, Cambridge, UK). An initial denaturation step at 95°C for 2·5 min was followed by 35 cycles of annealing for 30 s at 55°C, extension for 30 s at 72°C, and denaturation for 30 s at 95°C. Final extension was performed for 5 min at 72°C. Amplification products were purified using a QIAquick PCR Purification kit (Qiagen Corp., Venlo, The Netherlands) following the manufacturer's directions. A 10 μL sample of the amplification product was digested with the restriction enzymes *Alu*I, *Msp*I and *Rsa*I according to the manufacturer's instructions (Takara Shuzo Co. Ltd, Shiga, Japan). Digestion products were separated on a 1·5% Separide Gel Matrix (Life Technologies, Grand Island, NY, USA), stained with ethidium bromide, and visualized under UV light.

Sexual behaviour

For heterothallic species, the mating type of each isolate was determined directly on microscope slides by placing a CA plug of the 'unknown' isolate in contact with a CA plug of an A1 or A2 tester strain. Slides were incubated at 20°C in the dark at high relative humidity and scored with the aid of a light microscope for the presence of oospores after 10–15 days. Homothallic species produced oospores in single culture on CA. Morphology was studied using light microscopy, and the length and breadth of 100 oogonia, antheridia and oospores were measured for each colony after pairing.

Pathogenicity tests

Five field isolates of *P. cambivora*, *P. citricola* and *P. cactorum* and four isolates of *P. gonapodyides* were used for pathogenicity tests. Tests were performed on excised sprouts using a method described by Browne & Mircetich (1993, 1996), with some modifications, and also by soil infestation.

One-year-old dormant sprouts (105), 2 cm in diameter and 1 m in length, were collected from a large stump, placed in test tubes with sterile H_2O, and maintained at 20°C with a 12 h photoperiod. Shoots were inoculated as the buds started to open. A cork borer was used to remove a 3 mm bark disc from the excised shoot. The bark disc was replaced by a 3 mm plug of a 10-day-old culture grown on PDA. Five replicates of each isolate were used. Controls (10) were represented by shoots inoculated with a PDA plug. After inoculation, shoots were incubated in a ventilated chamber for 1 week at 20°C and 100% relative humidity. After incubation, the length of bark necrosis was measured on each shoot.

For the soil infestation tests, *C. sativa* seedlings were obtained from surface-sterilized seeds planted in pots containing steam-pasteurized potting mix (50% peat, 25% sand, 25% ground pumice). Seedlings were grown in a greenhouse until their lower stems were well lignified and their average height was 20–30 cm. Subsequently they were transplanted into 1 L plastic containers filled with the same potting mixture that was either unamended or infested with *Phytophthora* spp. *Phytophthora* inoculum was prepared by growing isolates for 4–6 weeks at 20°C on sterilized millet seeds thoroughly moistened with V8 broth (200 mL V8 juice, 3 g $CaCO_3$, 800 mL distilled H_2O). The inoculum was repeatedly rinsed with sterile water to remove unassimilated nutrients, then added to the potting mixture at the rate of 25 mL inoculum per 1000 mL potting mixture. Mixtures of inoculum and potting mixture were then flooded for 24 h to induce sporulation of *Phytophthora* isolates There were 50 replicate pots per isolate. Plants were watered to field capacity every other day. Two months after inoculation, 50 g samples of soil from each of six randomly chosen pots for each species of *Phytophthora* were flooded with water and baited separately with 15 *Rhododendron* leaf discs 1 cm in diameter. The health status of the plants was then scored on a scale of 0–4, where 0 = healthy plant; 1 = leaves turning pale green and necrosis on up to 10% of leaf surface; 2 = yellowing of leaves on up to 50% of leaf surface and necrosis of up to 25% of leaf surface; 3 = yellowing of leaves on up to 100% of leaf surface and necrosis of up to 50% of leaf surface; 4 = dead plant. A disease index (DI) was calculated using the following formula: $DI = \Sigma(g \times n)/N$, where g = damage score, n = number of samples belonging to each class, and $N = 50$, the total number of inoculated plants.

Statistical data analysis

Statistical analysis of data was performed with the software INSTAT 3 (GraphPad, San Diego, CA, USA).

Results

Recovery of *Phytophthora* and species identification

Four *Phytophthora* species, *P. cambivora*, *P. citricola*, *P. cactorum* and *P. gonapodyides*, were identified on the basis of morphology. This was confirmed by the RFLP profiles characteristic of the rDNA of the four species (Fig. 1). Four different patterns were generated among the field isolates, corresponding to those of the standard isolates PH31 and PH6 (*P. cactorum*), P1013 and 21/25-KV (*P. citricola*), 20/95-21 and 21/95-KII (*P. cambivora*), and AB4 and P501 (*P. gonapodyides*).

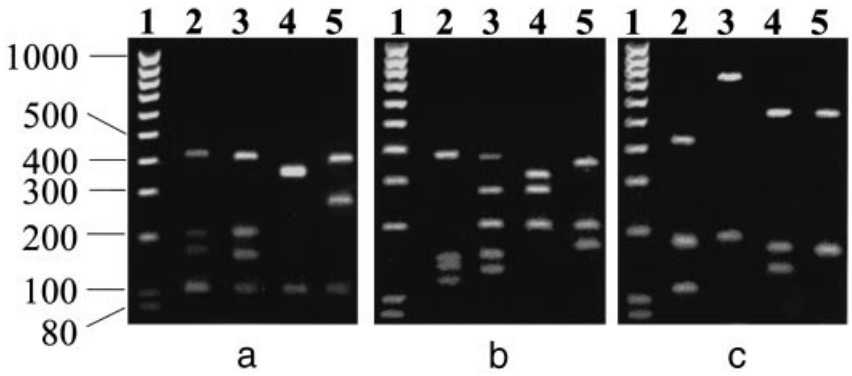

Figure 1 PCR amplification products of the ITS1/5·8S/ITS2 region of the RNA gene repeat of four species of *Phytophthora* isolated under chestnut trees (primers ITS1 and ITS4) digested with the DNA restriction enzymes *Rsa*I (a), *Msp*I (b) and *Alu*I (c). Lane 1, 100 bp ladder; lane 2, *P. gonapodyides*; lane 3, *P. cambivora*; lane 4, *P. citricola*; lane 5, *P. cactorum*. Molecular weights (bp) of the 100 bp ladder are indicated on the left.

Percentage recovery of *Phytophthora* spp. in the Monti Cimini and Monti del Cicolano chestnut forests is shown in Table 2. A total of 262 colonies of *Phytophthora* spp. were obtained by the *Rhododendron* leaf baiting technique. Up to three species were sometimes recovered from the same soil sample. The effect of sampling date on the isolation of four species of *Phytophthora* from diseased stands of *C. sativa* is shown in Table 3.

Among the heterothallic species, *P. gonapodyides* was characteristically sterile. All the colonies (98) of *P. cambivora* isolated from the 13 soil samples belonged to the A2 sexual compatibility type.

Pathogenicity tests

The mean length of necrosis produced by each species is shown in Fig. 2. All four species caused necrosis, but *P. cambivora* was by far the most aggressive species. Similarly, in the soil infestation test, by far the highest DI was obtained with *P. cambivora* (Fig. 3). *Phytophthora cambivora* also caused the highest mortality rate among inoculated seedlings: 46% died, compared with 22% for *P. citricola*, 14% for *P. cactorum*, and 14% for *P. gonapodyides*. All four species of *Phytophthora* were re-isolated from the soils in the pots 2 months after adding inoculum.

Statistical data analysis

The contingency table analysis for presence/absence of *Phytophthora* spp. and health status of trees in the sampled stands revealed a 1·292-fold ($P = 0{\cdot}015$) higher risk that a chestnut with *Phytophthora* in its rooting zone would show above-ground symptoms. This value increased to 1·333 ($P = 0{\cdot}040$) for *P. cambivora*.

Discussion

In chestnut stands in central Italy affected by ink disease, *Phytophthora* spp. were recovered from soils around both diseased and healthy-looking trees. However, the isolation frequency was much higher around symptomatic trees than around healthy-looking ones (26·8 versus 6·0%).

No ambiguous results were obtained when RFLP patterns were compared for the four species identified on the basis of morphological characters. As also demonstrated by Cooke & Duncan (1997), RFLPs of the rDNA region appear to be a relatively reliable method for species identification, especially if applied to genera such as *Phytophthora*, whose identification based on morphological characters is time-consuming and requires considerable experience.

Table 2 Frequency of isolation of four *Phytophthora* species from soil from four diseased stands of *Castanea sativa*

	Source of the sample			
	Symptomatic trees	Healthy-looking trees	Stream bed	Total
Total number of attempted isolations	112	33	10	155
Number of successful isolations (all species)	30 (26·8)[a]	2 (6)	7 (70)	39 (25·2)
P. cactorum	7 (6·3)	1 (3·0)	2 (20)	10 (6·4)
P. citricola	13 (11·6)	1 (3·0)	4 (40)	18 (11·6)
P. cambivora	13 (11·6)	0	0	13 (8·4)
P. gonapodyides	0	0	4 (40)	4 (2·6)

[a]Figures in brackets are percentage.

Table 3 Effect of sampling date on isolation of four species of *Phytophthora* from four diseased stands of *Castanea sativa*

Date of sampling		Species			
Month	Day–year	*P. citricola*	*P. cactorum*	*P. cambivora*	*P. gonapodyides*
February	4–00	–	–	–	–
	24–00	–	–	–	–
March	15–99	+	+	–	–
	19–99	+	+	–	–
April	29–99	+	+	+	–
May	03–98	+	–	+	–
	24–99	+	+	–	–
	31–99	+	+	–	–
June	12–99	+	–	–	+
	30–99	+	+	–	–
July	05–99	+	–	–	+
August	04–99	+	–	–	–
September	26–98	–	–	+	–
October	02–99	–	–	+	–
	26–99	+	+	+	+
November	12–98	+	–	–	–
	25–99	–	–	–	–
December	4–99	–	–	–	–

Three species, *P. cambivora, P. citricola* and *P. cactorum*, were present in the rooting zone of chestnut trees affected by the new outbreak of the disease. *Phytophthora cambivora* was typically associated with heavily diseased trees, and was never recovered from soils around slightly or nonsymptomatic hosts. Furthermore, it was the only species isolated from V-shaped necroses at the base of the stems (A. V., unpublished data). These necroses are typical of the latest phase of ink disease development (Petri, 1917). However, an isolation rate of 11·6% of *P. cambivora* from soil around diseased trees and the relative risk to the host of 1·333 are low. One possible explanation could be that the *Rhododendron* leaf-baiting technique is not efficient enough to detect the presence of the pathogen in the soil. However, *P. cambivora* appears to survive poorly in the soil during most of the year, and in this study was recovered only in late April–May and late September–October during periods of rain and mild temperatures. The difficulty in isolating *P. cambivora* from soil could be associated with a failure to produce resting structures. Some oospores may be formed as result of selfing stimulated by ubiquitous soil fungi (e.g. *Trichoderma*; Brasier, 1971), but in general oospore formation is likely to be poor due to the apparent presence of only a single mating type (A2) in the populations analysed. Furthermore, chlamydospores are not known to be formed by this species. The above considerations would suggest that *P. cambivora* has the ability to spread from infected roots and establish new infections for only a limited period of the year, when climatic conditions are favourable to the formation, release and survival of zoospores.

Phytophthora citricola, previously reported on chestnut by Biocca *et al*. (1993), was recovered between March and November, demonstrating its ability to survive under relatively adverse conditions, probably as oospores in the soil. This species is able to cause disease when inoculated on chestnut, although to a lesser extent than *P. cambivora*. *Phytophthora cactorum* has not

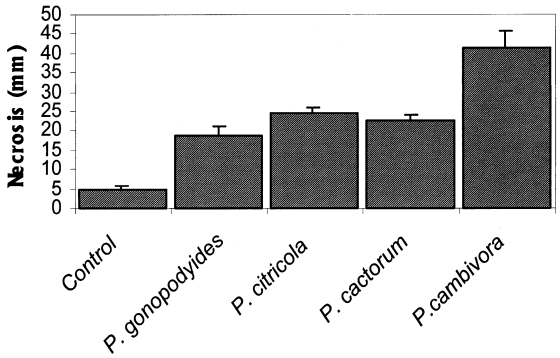

Figure 2 Mean length of necrosis (mm) produced by the four species of *Phytophthora* on excised chestnut sprouts 1 week after inoculation. Vertical bars, SD.

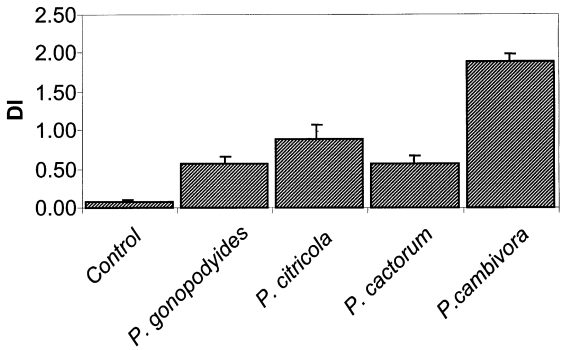

Figure 3 Index of disease (DI) (see Materials and methods for details) caused on 1-year-old chestnut seedlings growing in soil infested with four *Phytophthora* species. Vertical bars, SD.

previously been reported to occur in the rooting zone of chestnut trees in areas affected by ink disease, although it is known to be pathogenic to *Castanea* spp. following artificial inoculation (Curzi, 1933; Smith, 1937; Cristinzio & Verneau, 1954).

Whether *P. citricola* and *P. cactorum* are involved in disease development in the field remains a matter for speculation. Both species are serious pathogens of many other woody hosts (Erwin & Ribeiro, 1996), and both were able to cause necrosis in the pathogenicity tests reported here. The absence of these species in the typical V-shaped necroses (A.V., unpublished data) and the relatively low disease index (DI) suggest that *P. cactorum* and *P. citricola* are not strongly involved in ink disease of sweet chestnut. However, further isolations must be made to clarify their role in ink disease as fine root pathogens.

Phytophthora gonapodyides was isolated only from stream beds. It is typically associated with aquatic habitats in Britain and North America (Brasier *et al*., 1993) and has also been recovered, together with *P. citricola*, by Jung *et al*. (1996) and Hansen & Delatour (1999) from streams running through oak stands. An involvement of this species in ink disease seems unlikely, since it was not recovered from soil around symptomatic trees.

The present work has identified four species of *Phytophthora* present in chestnut stands affected by ink disease in central Italy. *Phytophthora cinnamomi* was not isolated in this study, but the results clearly show that, in addition to *P. cambivora*, at least two other species are associated with diseased trees and could contribute to disease development.

As in other woody hosts (e.g. walnut, apple), root rot by *Phytophthora* on chestnut could be caused by a number of different species (Cristinzio & Verneau, 1954; Browne & Mircetich, 1993). Many *Phytophthora* species from other hosts have been shown to be pathogenic on *Castanea* spp. These include *P. syringae* (Day, 1938), *P. citrophthora* (Curzi, 1933; Smith, 1937; Cristinzio & Verneau, 1954); *P. megasperma* (Waterhouse, 1963); *P. cryptogea* (Wicks & Volle, 1976); and *P. katsurae* (Uchida, 1967).

Further work is needed to identify the *Phytophthora* species present in chestnut stands in other areas of Italy and Europe, and to determine their pathogenicity and relevance in disease development.

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