European Journal of Plant Pathology (2005) 111: 169–180

Occurrence and distribution of *Phytophthora* species in European chestnut stands, and their association with Ink Disease and crown decline

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Accepted 5 August 2004

Key words: Castanea sativa, distribution map, ecology, etiology, root and collar rot

Abstract

The *Phytophthora* complex associated with *Castanea sativa* Mill. was investigated in five European countries in 35 regions and with respect to various domestication levels. Annual precipitation and length of drought season were the main parameters that regulated the presence of *Phytophthora* species in the chestnut stands. Seven species of *Phytophthora* were detected; three of these, *P. megasperma*, *P. cryptogea* and *P. syringae* had not been previously reported on sweet chestnut. *P. cinnamomi*. *P. cambivora* and *P. citricola* were most frequently isolated. *P. cinnamomi* and *P. cambivora* were the species significantly associated with declining trees with symptoms of Ink Disease. *P. cinnamomi* required distinct ecological conditions compared to the other species. *P. cinnamomi* was never detected in sites characterized by minimum temperatures below 1.4 °C, maximum temperature above 28 °C, or soil pH below 5.4. The results obtained provide useful information for modeling the probability of Ink Disease, crown decline and associated *Phytophthora* species in chestnut groves in global climatic change scenarios.

Introduction

A number of soil-borne *Phytophthora* species are potentially harmful to woody plants. These pathogens are widespread in the natural forests and hardwood plantations in Europe. Some species are host specific, such as *Phytophthora quercina* Jung (Jung et al., 2000) and the alder *Phytophthora* (Gibbs, 1995; Brasier et al., 1995, 2000), which are two serious pathogens of the genera *Quercus* and *Alnus,* respectively. Others are known as ubiquitous parasites on a broad range of hosts and are frequently associated with declining individual trees in forest stands, natural environments and plantations (Jung et al., 1996, 1999, 2000, 2002; Hansen and Delatour, 1999; Werres et al., 2001; Balci and Halmschlager, 2003a, b; Vettraino et al., 2002, 2003). In the literature two species are considered being responsible for Ink Disease of sweet chestnut (*Castanea sativa* Mill.) in Europe: *P. cambivora* (Petri) Buis and the more aggressive *P. cinnamomi* Rand (Petri, 1917; Milburn and Gravatt, 1932; Day, 1938; Crandall et al., 1945; Grente 1961). The specific symptoms of Ink Disease of chestnut are necroses of feeder and main roots, which can spread to the collar and the trunk resulting in cortical lesions with black exudates which gave the name of the disease. Root destructions lead to above ground symptoms, chlorosis, microphylly and wilting, which can be followed by a quick or a progressive death depending on the environmental conditions. The chestnut tree is a multipurpose species which is cultivated for fruit or timber production all across the Mediterranean and Atlantic Europe. Ink Disease may have dramatic economic and ecological consequences when it spreads into chestnut orchards, high forest, coppices or natural lands since

it is difficult to control; *P. cinnamomi* can threaten several plant communities. Recently, four *Phytophthora* species, *P. citricola* Sawada, *P. cactorum* (Leb. & Cohn), *P. gonapodyides* (Petersen) Buisman and *P. cambivora*, were found to be present in declining chestnut stands suffering from Ink Disease, in a restricted area in Italy (Vettraino et al., 2001). All these species proved to be pathogenic to young chestnut plants under experimental conditions, with *P. cambivora* being by far the most aggressive. However, little information is actually available on the relative impact of each of these species in chestnut stands in various European countries, and on the possible presence of additional species. The knowledge of the *Phytophthora* complex associated with chestnut sites in various geographical areas is of great relevance in order to accurately predict the impact of these species on host survival. This impact will depend not only on host susceptibility but also on the environmental conditions influencing the spread and survival of the pathogens as well as host predisposition (Davison and Tay, 1986, 1987; Shearer and Tippett, 1989; Hansen, 1996).

The aim of the present study is to assess the incidence of *Phytophthora* species in chestnut in five European countries, exploring their association with decline symptoms and domestication level, and the distribution of the various species with regard to site factors.

Materials and methods

Sites investigated

A total of 35 chestnut areas representative of the same number of geographic regions were investigated in five European countries (France, Italy, Greece, Spain, England). A description of these areas, including domestication levels present (orchard, coppice and/or naturalised stand), latitude, longitude, annual precipitation, temperatures, xerothermic index and soil pH under the litter is reported in Table 1. Annual precipitation and temperatures were obtained from local databases and referred to a time span of 10–30 years. The xerothermic index (Xi) was calculated and referred to a time span of 10 years using the following formula (Bagnouls and Gaussen, 1953); $Xi = \Sigma(TM - P)$, if $P < 2TM$; where TM is the monthly mean temperature expressed in °C, and P is the precipitation of the month expressed in mm. Each area was investigated for the occurrence of Ink Disease considering as symptoms the yellowing and lightening of crown, and/or the presence of flame shaped necroses on main roots and collar. Surveys were carried out between 1997 and 2002. A minimum of 26 trees, randomly chosen in each domestication level, were inspected for the presence of Ink Disease. In absence of symptoms, the inspection was extended to the neighbouring trees. Soil and tissue samples were collected from a minimum of 10 trees per domestication level.

Isolation of Phytophthora

Each soil sample contained fine or coarse chestnut roots, and resulted from a mix of four monoliths of soil (25 cm × 25 cm × 25 cm) collected at the compass points around a tree at a distance of about 50 cm from the collar (Jung et al., 1996). After collection, soil samples were moistened with sterile distilled water and incubated at 20 °C for 3 days. About 200 ml of soil was then flooded with 500 ml of distilled water in plastic containers (Jung et al., 1996). Five freshly picked leaves of *Rhododendron* species (Themann and Werres, 1998) or chestnut leaf disks (Robin et al., 1998) were placed directly on the water surface and incubated at 20 °C for one week until spots developed on the leaves or the leaves became discolored. The leaves were then blotted on filter paper, cut into small pieces (0.5 cm × 0.5 cm) and placed on PARBhy selective medium (per liter, pimaricin, 10 mg; ampicillin (sodium salt), 250 mg; rifampicin, 10 mg; hymexazol, 50 mg; benomyl, 15 mg; malt extract, 15 g; agar, 20 g) (Robin et al., 2001). *Phytophthora* isolates were maintained on carrot agar (CA) (Brasier, 1969) at 20 °C in darkness and sub-cultured at 4-week intervals. Tissue samples were collected from the margin of stem lesions, sterilized in ethanol and plated in PARBhy agar. Aliquots of soil from all monoliths were bulked and sub-samples were used for soil pH determination (in H_2O).

Species identification

Isolates were identified by comparing colony growth patterns and morphological features of

Table 1. Description of the chestnut areas investigated for the presence of *Phytophthora* species in Europe

Code	Region	Domestication level	Country	Long.	Lat.	P_{yr}[a] (mm)	T_{mean}[b] (°C)	T_{mmin}[c] (°C)	T_{mmax}[d] (°C)	Xi[e]	pH[f]
1	Worcestershire	C	England	2°20'W	52°18'N	652	9	0.2	20.3	0	6.5
2	Gloucestershire	C	England	2°30'W	51°46'N	795	10.3	2.2	21	0	5.6
3	Kent–East Sussex	C	England	0°44'E	51°17'N	636	10.4	1.6	22.2	0	5.7
4	Suffolk	C	England	1°03'E	51°59'N	584	9.7	1.4	20	0	5.7
5	Coruna	N,O	Spain	8°22'W	43°17'N	1148	13.2	6.2	20.8	10.6	5.4
6	Lugo	N,O	Spain	7°52'W	42°36'N	1423	11.7	3.4	21.2	30	5.5
7	Asturias	N,O	Spain	5°44'W	43°13'N	988	13.2	3.8	22	0	5.8
8	Leon	C,O	Spain	6°45'W	42°27'N	680	12.7	−6.4	36.1	38.4	5.4
9	Caceres	C,O	Spain	5°20'W	39°28'N	950	13.7	2.8	28.8	83	5.6
10	Malaga	N,O	Spain	5°18'W	36°32'N	1183	14.3	5.6	26.8	nd	6.0
11	Loire Atlantiques	C,N	France	2°05'W	47°38'N	882	12.3	2.9	25.1	0	5.4
12	Dordogne	N,C,O	France	1°02'E	44°41'N	812	13.1	1.4	28	0	7.1
13	Pyrenees Atlantiques	O,N	France	1°05'W	43°16'N	1257	13.5	2.2	26.7	0	7.1
14	Aveyron	N,C	France	2°55'E	44°04'N	745	11.1	−0.8	27.8	0	6.3
15	Gard	N,C,O	France	3°49'E	44°01'N	1479	12.2	1.4	27.4	10.3	6.1
16	Var	N,C,O	France	6°20'E	43°18'N	1231	13.4	3.2	28.3	30.9	7.0
17	Haute Corse	N,C,O	France	9°23'E	42°22'N	869	12.5	2.2	26.2	37	7.2
18	Piemonte	N,C,O	Italy	7°09'E	44°49'N	1215	10.7	−2.3	25.4	0	4.5
19	Piemonte	N,C,O	Italy	8°19'E	46°07'N	1581	9.4	−2.2	25	0	4.5
20	Friuli	N,C,O	Italy	13°29'E	46°10'N	1555	12.6	−3.2	30.5	0	4.5
21	Toscana	N,C,O	Italy	11°22'E	43°55'N	996	13.4	−0.5	30.7	10	5.1
22	Lazio	C,O	Italy	12°13'E	42°24'N	936	13.2	2.4	28.6	36	nd
23	Marche	N,C,O	Italy	13°24'E	42°44'N	881	11.9	−0.3	26.5	0	6.0
24	Basilicata	N,O	Italy	15°37'E	40°55'N	826	11.6	−0.3	27.6	11	6.2
25	Calabria	N,C,O	Italy	16°43'E	39°02'N	1489	10.1	−1	24.6	3	5.7
26	Sicilia	N,C,O	Italy	14°05'E	37°54'N	867	13.6	2.5	28.1	77	6.0
27	South–East Macedonia	N,C	Greece	23°44'E	40°32'N	649	11.2	−2.2	26.7	15	5.4
28	Eastern Macedonia	N	Greece	23°47'E	41°10'N	694	10.7	−3.7	26.4	13	5.3
29	Northern Macedonia	N,C,O	Greece	22°22'E	40°57'N	558	12	1.9	28.5	39	5.7
30	Central Macedonia	N,C	Greece	23°09'E	40°22'N	417	14.7	0.2	31.8	94	5.6
31	Southern Macedonia	N,C	Greece	22°35'E	39°58'N	697	14.5	−2.1	29.9	59	6
32	Thessalia	C,O	Greece	23°05'E	39°24'N	649	11.2	−2.2	26.7	15	5.7
33	Sterea Hellas	C,O	Greece	21°53'E	38°54'N	1031	9.4	−4.4	26.2	11	5.3
34	Lesvos Island	C,O	Greece	26°24'E	39°55'N	917	17.4	4.8	30.9	167	5.2
35	Western Macedonia	N,O	Greece	23°38'E	40°52'N	815	10.1	−4	27.4	17	5.4

[a] Annual precipitation.
[b] Mean temperature.
[c] Minimum mean temperature of the coldest month.
[d] Maximum mean temperature of the warmest month.
[e] Xerothermic index.
[f] pH in H_2O.

sporangia, oogonia, antheridia, chlamydospores and hyphal swellings with reference isolates and with species descriptions reported in literature (e.g. Stamps et al., 1990; Erwin and Ribeiro, 1996). Colony morphology was described on 10-day-old cultures grown on CA, potato dextrose agar (PDA), malt agar (MA) (Oxoid Limited, Basinstoke, England), and V8 (200 ml V8 juice; 3 g $CaCO_3$, 800 ml distilled H_2O) in 90 mm Petri dishes at 20 °C in darkness. Sporangia were produced by placing a disk of mycelium from a 7-day-old culture grown on CA into soil extract prepared according to Chee and Newhook (1965). Morphology was assessed by light microscopy and the length and breadth of 100 sporangia were measured for each isolate. For the supposed heterothallic species, the mating type of isolates was determined. Each isolate was paired, on CA Petri dishes or on microscope slides with an A1 strain and an A2 strain from the same or another heterothallic species (Vettraino et al., 2001). Presence of oogonia in one of these pairings indicated an A2 or A1 heterothallic isolate, respectively. Morphology was then studied measuring the length and breadth of 100 oogonia for each isolate using light microscopy. Identification of the isolates was confirmed by comparing the RFLP patterns of their ribosomal DNA (rDNA) with those of reference strains following the procedure described by Vettraino et al. (2001). Identification of isolates of *P. cinnamomi* and *P. cambivora* obtained from France and England was confirmed by the multiplex nested-PCR method described by Morel et al. (2003).

Statistics

Normal distribution of the data series and homoscedasticity were evaluated respectively with the Kolmogorov–Smirnov and Bartlett's tests, using the package Graphpad Instat® 3.05 (San Diego, USA). Simple correlation analysis, ANOVA and unpaired *t* test were performed with the package Systat® 7.0 (Systat Software Inc., Richmond, USA). Association between the presence of crown symptoms and the presence of *Phytophthora* species was assessed in a contingency table using the Fisher exact test (Jung et al., 2000) of the package Instat® (San Diego, USA). Diversity indexes of Shannon ($H = -\Sigma((n_i/n)\ln(n_i/n))$ where n_i is the number of individuals of taxon i, and n the total number of individuals) and Dominance ($D = \Sigma((n_i/n)^2)$ where n_i is the number of individuals of taxon i, and n the total number of individuals) were calculated with the package Past 1.10 (Hammer et al., 2001). In order to limit the number of site factors to analyze, a correlation matrix was calculated between site factors considered in the present work; probabilities were calculated with the Bonferroni method. In case of significant correlation between two factors, one was not considered for further analysis. On the basis of the results obtained, it was decided to consider the following site factors: annual precipitation (*P*), mean temperatures (T*mean*), minimum and maximum mean temperature of the coldest (T*mmin*) and warmest (T*mmax*) month, respectively, pH and Xi.

Results

Symptoms of Ink Disease were recorded from 21 areas out of 35 investigated; *Phytophthora* species were isolated from 23 areas out of 35 investigated (Table 2). Most of the areas with presence of symptoms also tested positive for *Phytophthora* (20 out of 21). Three areas, in England (#3), Spain (#10) and Greece (#35), proved positive for *Phytophthora* in the soil without any recorded symptomatic trees. No statistically significant effect of domestication levels on frequency of *Phytophthora* isolation was evidenced. *Phytophthora* species were widespread in France and Italy (respectively, 100% and 88.9% of the investigated sites); in Spain, Greece and England *Phytophthora* species were detected in 50%, 44.4% and 25%, respectively, of the investigated areas.

Up to seven species were identified from soil and tissue samples of the different chestnut areas, including *P. cambivora* (Petri) Buisman, *P. cinnamomi* Rands, *P. cactorum* (Leb. & Cohn) Schroet, *P. citricola* Sawada, *P. cryptogea* Pethyb. & Laff, *P. megasperma* (Drechsler) and *P. syringae* (Kleb.) Kleb. (Table 3). *P. cryptogea*, *P. megasperma* and *P. syringae* were limited to a single isolation from soil under non-symptomatic trees in France, Spain and Italy respectively. Three more phytophthoras could not be classified and were listed as *Phytophthora* unknown. More than one species was seldom recovered from the same soil sample. Only *P. cambivora* (27.5%) and *P. cin-*

Table 2. Presence of Ink Disease symptoms and *Phytophthora* species, sampling date, number of successful isolation from no-symptomatic (H) and symptomatic (S) trees, and origin of the sample in the investigated areas

Code	Region	Country	Symptoms[a]	*Phytophthora*	Sampling date	N° trees		N° isolations		Sample origin
						H	S	H	S	
1	Worcestershire	England	No	No	8/01	10	0	0	0	Soil
2	Gloucestershire	England	Yes	No	8/01	9	1	0	0	Soil
3	Kent-East Sussex	England	No	Yes	8/01	30	0	4	0	Soil
4	Suffolk	England	No	No	8/01	10	0	0	0	Soil
5	Coruna	Spain	Yes	Yes	9/01	7	7	0	4	Soil, tissue
6	Lugo	Spain	Yes	Yes	9/01	10	1	0	1	Soil, tissue
7	Asturias	Spain	No	No	9/01	10	0	0	0	Soil
8	Leon	Spain	No	No	9/01	10	0	0	0	Soil
9	Caceres	Spain	No	No	9/01	10	0	0	0	Soil
10	Malaga	Spain	No	Yes	9/01	11	0	4	0	Soil
11	Loire Atlantiques	France	Yes	Yes	6/01	18	2	0	1	Soil
12	Dordogne	France	Yes	Yes	9/00, 9/01	8	25	1	11	Soil, tissue
13	Pyrenees Atlantiques	France	Yes	Yes	10/98, 10/00	17	10	3	8	Soil
14	Aveyron	France	Yes	Yes	5/00, 9/00	2	27	0	7	Soil, tissue
15	Gard	France	Yes	Yes	6/97, 6/98, 10/00	27	9	0	6	Soil
16	Var	France	Yes	Yes	10/00	28	4	2	0	Soil
17	Haute Corse	France	Yes	Yes	9/00	28	3	1	1	Soil, tissue
18	Piemonte	Italy	Yes	Yes	6/00	23	7	2	0	Soil
19	Piemonte	Italy	Yes	Yes	6/00	20	12	0	8	Soil
20	Friuli	Italy	Yes	Yes	6/00	27	3	7	3	Soil
21	Toscana	Italy	Yes	Yes	9/00	27	4	1	1	Soil, tissue
22	Lazio	Italy	Yes	Yes	5-10/99, 10/00	35	124	2	34	Soil, tissue
23	Marche	Italy	Yes	Yes	10/00	26	3	3	1	Soil
24	Basilicata	Italy	Yes	Yes	5/00	19	7	2	6	Soil
25	Calabria	Italy	Yes	Yes	10/00, 4/01	27	1	0	1	Soil
26	Sicilia	Italy	No	No	3/00	28	0	0	0	Soil
27	South-East Macedonia	Greece	No	No	5/02	20	0	0	0	Soil
28	Eastern Macedonia	Greece	No	No	10/01	10	0	0	0	Soil
29	Northern Macedonia	Greece	Yes	Yes	4/01, 4/02	35	10	2	7	Soil, tissue
30	Central Macedonia	Greece	No	No	10/01, 5-11/02	20	0	0	0	Soil
31	Southern Macedonia	Greece	No	No	10/01, 5-11/02	20	0	0	0	Soil
32	Thessalia	Greece	Yes	Yes	5/01, 4-10/02	25	10	1	6	Soil, tissue
33	Sterea Hellas	Greece	Yes	Yes	9/02	20	10	2	2	Soil, tissue
34	Lesvos Island	Greece	No	No	9/02	20	0	0	0	Soil
35	Western Macedonia	Greece	No	Yes	11/02	20	0	1	0	Soil
Total						667	280	38	108	

[a] Bark lesions and/or crown decline.

Table 3. *Phytophthora* species isolated from soil (*s*) and tissue (*t*) samples in the investigated countries. H, no-symptomatic trees; S, symptomatic trees. In Italy the number of successful isolations does not correspond to number of isolates, since more than 1 species was sometimes detected from the same soil sample

	England			France			Spain			Italy			Greece		
	H	S		H	S		H	S		H	S		H	S	
		s	*t*		*s*	*t*		*s*	*t*		*s*	*t*		*s*	*t*
Totals number of attempted isolation	59	1	0	128	70	10	58	4	4	232	150	11	190	15	15
Number of successful isolation	4	0	–	7	29	5	4	2	3	17	49	5	6	10	5
P. cambivora	0	0	–	2	6	1	0	0	0	2	30	5	0	7	5
P. cinnamomi	4	0	–	2	21	4	0	2	3	0	0	0	0	0	0
P. cactorum	0	0	–	1	0	0	3	0	0	6	8	0	1	0	0
P. citricola	0	0	–	0	2	0	0	0	0	11	19	0	5	1	0
P. cryptogea	0	0	–	1	0	0	0	0	0	0	0	0	0	0	0
P. megasperma	0	0	–	0	0	0	1	0	0	0	0	0	0	0	0
P. syringae	0	0	–	0	0	0	0	0	0	1	0	0	0	0	0
Other	0	0	–	1	0	0	0	0	0	0	0	0	0	2	0

namomi (17.5%) were isolated from necrotic tissues at the collar of symptomatic trees.

Diversity indexes S and D were calculated for those chestnut areas where at least 6 positive isolations were obtained. Results are shown in Table 4. D values indicated in all the investigated areas the dominance of one or more species over the others. When present *P. cinnamomi* represented the sole or the prevalent species of the area; *P. cambivora* dominated the other species in absence of *P. cinnamomi*.

All the tested isolates (15) of *P. cinnamomi* belonged to mating type A2; 39 isolates of *P. cambivora* out of 41 tested belonged to mating type A2; 2 isolates of *P. cambivora* from site 14 (France) were A1.

A significant association was found between the presence of symptoms and the isolation of *Phytophthora* from the rhizosphere of trees, considering all the infested areas (Relative risk $= 2.8$, $P \leq 0.0001$), or limiting the analysis to the French (Relative risk $= 3.2$, $P \leq 0.0001$), the Italian (Relative risk $= 2.1$, $P \leq 0.0001$), and the Greek infested areas (Relative risk $= 12.4$, $P \leq 0.0001$) (Table 5). Analysis of contingency tables was not possible for Spain and England due to frequencies of positive isolations being too low.

With regard to the four most frequent species, crown symptoms were found to be significantly associated with *P. cambivora* and *P. cinnamomi* (Relative risk $= 2.1$, $P \leq 0.0001$ and 4.3, $P \leq 0.0001$ respectively) and not associated with

Table 4. Shannon (*S*) and Dominance (*D*) diversity indexes calculated for *Phytophthora* species in 9 chestnut areas

Region	12	15	13	14	19	22	24	29	20
Taxa	2	1	3	1	2	4	1	4	2
Individuals	12	6	11	7	11	38	8	9	12
P. cinnamomi	11	6	7	0	0	0	0	0	0
P. cambivora	0	0	2	7	8	15	8	6	0
P. citricola	0	0	2	0	3	14	0	1	10
P. cactorum	0	0	0	0	0	8	0	1	2
P. cryptogea	1	0	0	0	0	0	0	0	0
P. syringae	0	0	0	0	0	1	0	0	0
Phytophthora unknown	0	0	0	0	0	0	0	1	0
D	0.85	1	0.47	1	0.60	0.34	1	0.48	0.72
S	0.29	0	0.91	0	0.59	1.16	0	1.0	0.45

Table 5. Contingency table: occurrence of *Phytophthora* species/healthy status (crown symptoms) in the infested sites. Only soil samples were considered for this analysis

		Number of symptomatic trees	Number of non-symptomatic trees	Total
All infested sites	*Phytophthora* spp present	90	38	128
	Phytophthora spp absent	149	452	601
	Total	239	490	729
Relative risk = 2.8 95% – CI: 2.37–3.39; Fisher exact test: $P \leq 0.0001$ (extremely significant)				
French infested sites	*Phytophthora* spp present	29	7	36
	Phytophthora spp absent	41	121	162
	Total	70	128	198
Relative risk = 3.2 95% – CI: 2.34 – 4.34; Fisher exact test: $P \leq 0.0001$ (extremely significant)				
Italian infested sites	*Phytophthora* spp present	49	17	66
	Phytophthora spp absent	101	187	288
	Total	150	204	354
Relative risk = 2.1 95% – CI: 1.7 – 2.6; Fisher exact test: $P \leq 0.0001$ (extremely significant)				
Greek infested sites	*Phytophthora* spp present	10	6	16
	Phytophthora spp absent	5	94	99
	Total	15	100	115
Relative risk = 12.4 95% – CI: 4.86 – 31.5; Fisher exact test: $P \leq 0.0001$ (extremely significant)				

P. citricola and *P. cactorum* (Relative risk = 1.4, $P = 0.06$ and 0.8, $P = 0.6$, respectively) (Table 6).

The presence/absence of *Phytophthora* species in the investigated areas was partially explained by the Xi and annual precipitation values. The average Xi value of areas positive and negative for *Phytophthora* was 9.94 ± SEM 3.27 and 38.5± SEM 11.73, respectively, and was considered different at the unpaired *t* test with Welch correction ($P = 0.031$). Similarly the average annual precipitation of regions positive and negative for *Phytophthora* was 1052 mm (±SEM 48.3) and 740.8 mm (±SEM 64.7) respectively, and was considered different at the unpaired *t* test with Welch correction ($P = 0.0005$). As shown in Figure 1 *Phytophthora* species were never detected

Table 6. Contingency table: occurrence of *P. cambivora*, *P. cinnamomi*, *P. citricola* and *P. cactorum* /healthy status (crown symptoms) in *Phytophthora*-infested sites. For each *Phytophthora* species only the sites were included in the analysis where the species was present. Only soil samples were considered for this analysis

	Number of symptomatic trees	Number of non-symptomatic trees	Total
P. cambivora present	43	4	47
P. cambivora absent	162	203	365
Total	205	207	412
Relative risk = 2.1 95% – CI: 1.8–2.4; Fisher exact test: $P \leq 0.0001$ (extremely significant)			
P. cinnamomi present	23	6	29
P. cinnamomi absent	38	167	205
Total	61	173	224
Relative risk = 4.3 95% – CI: 3.0–6.0; Fisher exact test: $P \leq 0.0001$ (extremely significant)			
P. citricola present	22	16	38
P. citricola absent	168	233	401
Total	190	249	439
Relative risk = 1.4 95% – CI: 1.0 to 1.8; Fisher exact test: $P = 0.06$ (not quit significant)			
P. cactorum present	8	11	20
P. cactorum absent	140	139	828
Total	148	150	747
Relative risk = 0.8 95% – CI: 0.5–1.4; Fisher exact test: $P = 0.6$ (not significant)			

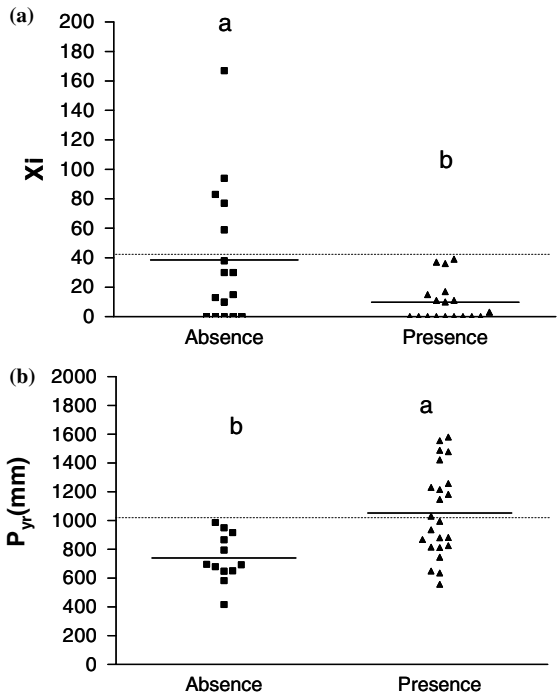

Figure 1. Xerothermic index, Xi (a) and annual precipitation, *P* (b) in chestnut areas with presence and absence of *Phytophthora* species in the soil. Mean values differed significantly at the unpaired *t* test with the Welch correction ($P < 0.05$). The dashed lines indicate the threshold over which *Phytophthora* was not detected.

at Xi values above 39; furthermore *Phytophthora* species were always detected in those regions with annual precipitation above 988 mm.

As shown in Figure 2, the geographical distribution of species was not homogeneous; *P. cinnamomi* was present in England, Spain and France but was not detected in Italy and Greece in the present work. *P. cambivora* was widespread in Italy, Greece and France but not detected in Spain or England; *P. citricola* was frequent in Italy and Greece, more rare in France but not detected in England or Spain; *P. cactorum* was not found in England.

The association with site factors was studied for the most frequent species, *P. cinnamomi*, *P. cambivora*, *P. citricola* and *P. cactorum*. The T*mmin* proved to be the only factor significantly associated with the distribution of the 4 most frequent species (ANOVA, $F = 3.695$, $P = 0.022$). *P. cinnamomi* was never recovered in areas with T*mmin* lower than 1.4 °C (Figure 3 a). The mean value of T*mmin* for the sites where *P. cinnamomi* was isolated was significantly different from those of areas where *P. cambivora* and *P. citricola* were isolated ($P = 0.035$ and 0.003 at the Fisher's LSD). Figure 3 (b and c) shows the values of T*mmax* and soil pH at which each of the 4 species was recovered in the investigated areas. No significant differences between species with regard to these site factors were found at ANOVA (T*mmax*, $F = 2.019$, $P = 0.13$; pH, $F = 1.661$, $P = 0.2$). However, *P. cinnamomi* was not present in sites characterized by T*mmax* ≥ 28 °C and soil pH ≤ 5.4.

Discussion

The present paper reports an overall picture of *Phytophthora* species associated with chestnut in 5 of the European countries most important for cultivation and completes recent reports of rich community of *Phytophthora* species in other broadleaved forest types in Europe (Brasier et al., 1993; Erwin and Ribeiro, 1996; Jung et al., 1996, 2000; Hansen and Delatour, 1999; Vettraino et al., 2001, 2002, 2003; Balci and Halmschlager, 2003a, b). Furthermore, it contributes to the knowledge about the influence of site factors on the presence of phytophthoras in chestnut stands and the geographic distribution of the various species. As shown by the results, site factors seem to affect the geographic distribution of the different *Phytophthora* species. In our survey, *Phytophthora* species were not detected in regions characterized by drought periods lasting longer than 3–4 months during the growing season (corresponding to Xi ≥ 40). It is generally accepted that failure to detect *Phytophthora* species with baiting techniques does not necessarily indicate their absence (Old, 1979; Weste, 1983; Erwin and Ribeiro, 1996; Vettraino et al., 2001). However, no symptoms of collar rot and crown decline were recorded in any of these sites, suggesting that, if present, the *Phytophthora* inoculum is unable to cause noticeable damage to trees. In fact, drought related low soil matric potentials may strongly affect pathogen activity in these sites (Pfender et al., 1977; MacDonald and Duniway, 1978; Hardy and Sivasithamparam, 1991), eventually favouring the formation of resting structures (Quitugua and Trujillo, 1998). However, several soil factors as well as the type of spores may interact with and modify the relationships between

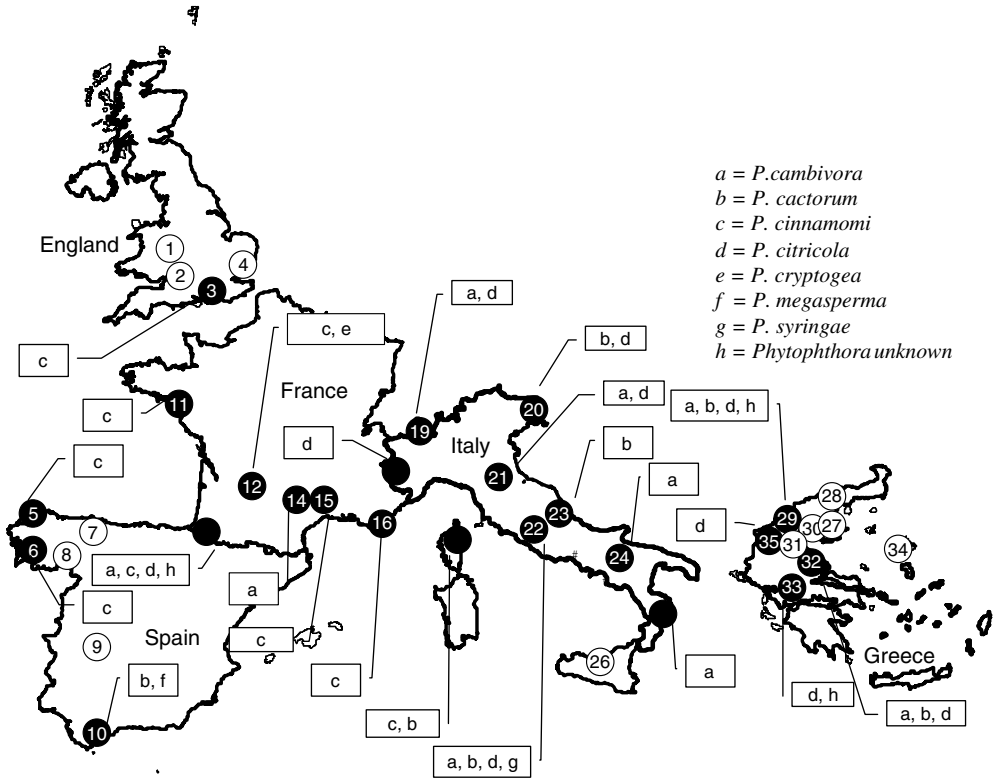

Figure 2. Distribution map of *Phytophthora* species associated with chestnut areas in Spain, England, France, Italy and Greece. The areas positive for *Phytophthora* isolation are marked in black.

soil moisture and *Phytophthora* survival, as has been demonstrated for *P. cinnamomi* (Shearer and Tippett, 1989).

More consistent is the association between *Phytophthora* detection and annual rain regimes. Phytophthoras were always detected in chestnut sites with annual rainfall above 1000 mm (about 27% of the investigated sites). These conditions increase the probability of having soil moisture levels suitable for production and spread of zoospores and infection establishment on root systems (Erwin and Ribeiro, 1996). High precipitation (above 1000 mm/year) could be a useful index in order to classify areas at risk for Ink Disease. However, seasonal or local heavy rains due to storms could result in water-logging which increases both *Phytophthora* multiplication and host susceptibility to *Phytophthora* infections (Davison and Tay, 1987; Shearer and Tippett, 1989).

Site factors also seem to affect the distribution of the different phytophthoras. In the present paper, *P. cinnamomi* is confirmed as a species confined to climates characterized by moderate temperatures and the absence of frost events (Shearer and Tippett, 1989). Most of the chestnut stands investigated in the central and eastern part of the *C. sativa* distribution area (Italy and Greece) are characterized by winter air temperatures close to or below 0 °C. The possibility for *P. cinnamomi* to colonize these areas seems to be rather limited. However, as reported by Brasier and Scott (1994), a gradual increase of temperature due to global warming could significantly increase the risk of *P. cinnamomi* in many areas previously considered not suitable for this species. In fact *P. cinnamomi* was recorded in Italy by Cristinzio (1986) in a chestnut site in the Latium region characterized by T*mmin* between 3.4–4 °C. Furthermore, unusual *P. cinnamomi* isolates were found recently in the soil of a Scots Pine forest in Northern Scotland characterized by prolonged freezing periods (below 0 °C) during the winter (Woodward, Department of Agriculture and Forestry – University of Aberdeen, UK, pers. comm.) suggesting a poten-

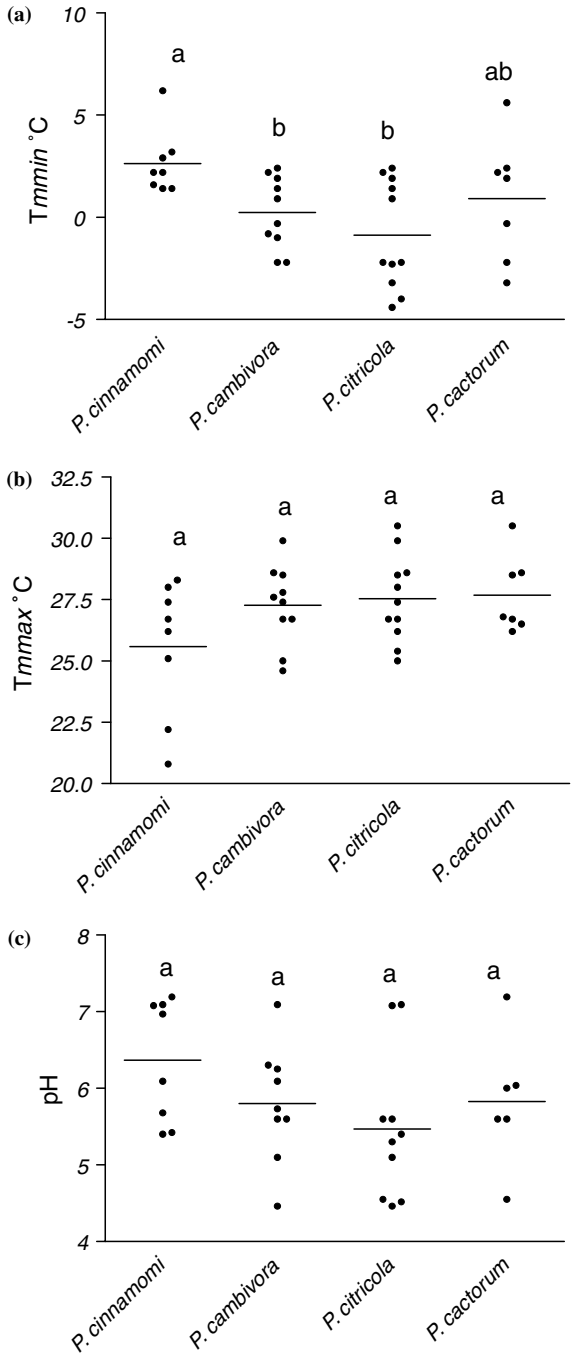

Figure 3. Climatic characteristics and soil pH of regions in which *P. cinnamomi*, *P. cambivora*, *P. citricola* and *P. cactorum* were detected. Mean minimum temperature of the coldest month, T*mmin* (a); mean maximum temperature of the warmest month, T*mmax* (b); soil pH (c). Values with the same letter are not different at the Fisher's least significant difference pair wise multiple comparison test (LSD).

tial for genetic variation of the species (Brasier, 1992). *P. cambivora*, *P. citricola* and *P. cactorum*, showed similar ecological requirements, often being found together in the same site. They were frequently detected in chestnut stands in Italy and Greece, although only *P. cambivora* proved to be significantly associated with crown symptoms and to collar and bark lesions. *P. cambivora* is by far the most aggressive species to sweet chestnut after *P. cinnamomi* (Vettraino et al., 2001). This species showed a wider adaptability to temperature extremes than *P. cinnamomi*. However, due to the inability to form resting structures in nature, the level of concentration of its soil-borne inoculum seems strictly dependent on the season (Vettraino et al., 2001). *P. megasperma*, *P. cryptogea* and *P. syringae* were never previously reported from chestnut stands. They were very rare and only isolated from the rhizosphere of apparently healthy chestnut trees. This does not exclude their ability to infect roots causing limited damage, as recently shown for walnut (Vettraino et al., 2003). Furthermore, in preliminary pathogenicity tests on 1 year old chestnut seedlings, *P. megasperma*, *P. cryptogea* and *P syringae* proved to be pathogenic to chestnut following frequent floodings. (A. Vannini, Department of Plant Protection – University of Tuscia, Italy, unpub.).

P. cinnamomi and *P. cambivora* represent the dominant species in chestnut stands in Europe. The absence or the relative low frequency of other phytophthoras in sites infested by *P. cinnamomi* and *P. cambivora* could be due to the ability of these two species to compete successfully as well as being the most aggressive to chestnut.

The results of the present work clearly confirm that *P. cinnamomi* and *P. cambivora* are responsible for Ink Disease in Europe. In fact only these 2 species were significantly associated with both crown decline and root and collar lesions of chestnut. Knowledge of the climatic and site parameters is extremely useful in predicting the risk of spread of these species, and it can be used to develop provisional models in a global climatic change scenario. Potential of genetic variation under selective pressure (Brasier, 1992) represents an additional factor for risk assessment of *Phytophthora* spread to new ecosystems. Nearly all the isolates of the aggressive heterothallic species, *P. cinnamomi* and *P. cambivora* from chestnut belong to the worldwide dominant mating type

A2; however the absence of alternate mating type (A1) does not preclude recombination events and expression of variation in *Phytophthora* populations (Brasier, 1992). Large phenotypic variation was found in asexual lineages of *P. cinnamomi* in Australia (Hüberli et al., 2001) suggesting asexual variation through mitotic recombination or other events (Brasier, 1971; Tommerup et al., 1999). Furthermore, sensible genotypic differences have been found in local A2 population of *P. cambivora* in Italy analyzed with AFLP markers (A. Vannini, Department of Plant Protection – University of Tuscia, Italy, unpub.).

A number of other *Phytophthora* species are present in the chestnut ecosystem and putatively associated with fine root necrosis as previously suggested for various shade trees in natural and semi-natural ecosystems (Brasier et al., 1993; Erwin and Ribeiro, 1996; Jung et al., 1996, 2000; Hansen and Delatour, 1999; Vettraino et al., 2001, 2002 and 2003). *P. citricola*, *P. cactorum*, *P. syringae*, *P. cryptogea* and *P. megasperma* are part of the diverse communities of soil-borne phytophthoras present in deciduous forests in temperate and Mediterranean regions of Europe (Jung et al., 2000, 2002; Balci and Halmschlager, 2003,a, b; Vettraino et al., 2001, 2002, 2003). These species are not necessarily present in the same stands, since their distribution is influenced by site factors and probably by competition, as demonstrated by Jung et al. (2000, 2002) and Vettraino et al. (2001, 2002). As suggested for diverse communities of phytophthoras affecting oak forests in Europe (Jung et al., 2000, 2002; Vettraino 2002) and walnut plantations in Italy (Vettraino et al., 2003), *P. citricola*, *P. cactorum*, *P. syringae*, *P. cryptogea* and *P. megasperma* may act as inciting or contributing factors in the progressive decline of trees subjected to other type of biotic or abiotic stresses.

Acknowledgements

This study was carried out with financial support from the project EVK2-CT1999-00006 ''Securing gene conservation, adaptive, breeding potential and utilization of a model multipurpose tree species (*Castanea sativa* Mill.) in a dynamic environment''. Special thanks go to Anne Grossmann for providing the climatic databases and to Karen Russel, Josefa Fernandez Lopez and Rafael Zas Arregui, for the help they provided in survey and sampling activities.

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