Diagnosis of Ink Disease of Chestnut by Molecular Identification of Associated *Phytophthora* Species

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Abstract

For diagnostic proposes of ink disease, chestnut orchards with symptoms of decline or sudden death of trees were sampled by soil baiting techniques and selective agar media (P_{10}VPH). Thirty-six *Phytophthora* isolates were obtained. One isolate per tree and three or two isolates from the soil of the same plant were considered for molecular identification. Genomic DNA was extracted from all the isolates and from the reference strain *P. cinnamomi* CECT 2965. The ribosomal regions ITS1, 5.8S and ITS2 were amplified with the universal primer pair ITS6 (Cooke and Duncan, 1997) and ITS4 (White et al., 1990) by PCR. The amplified fragment (900 pb) was digested with restriction enzymes *Msp*I, *Alu*I and *Taq*I. Two different patterns of fingerprinting were obtained with enzymes *Taq*I and *Alu*I (type I and II) and three different patterns with *Msp*I (type I, Ia, II). The fingerprinting of each isolate was compared with database of CABI by public web access. Type I and Ia (14 isolates) were assigned to *P. cinnamomi* and type II (4 isolates) was assigned to *P. cambivora*. Molecular methods provide a rapid means of *Phytophthora* species identification associated with ink disease of chestnut and will provide a useful tool for etiological and epidemiological studies of this important disease of chestnut.

INTRODUCTION

Ink disease of chestnut, which causes decline and death of chestnut trees of all ages, is responsible for severe losses and represents a serious threat in all chestnut areas. Both, *Phytophthora cinnamomi* and *Phytophthora cambivora* are frequently associated with ink disease of chestnut (Pimentel, 1947; Crandall, 1950). More complex etiology can be envisaged as other *Phytophthora* species have been associated with ink disease of chestnut: *P. megasperma* was found on ink diseased chestnut trees by Grente (1961) and Vittraino et al. (2001) isolated *P. cactorum*, *P. citricola* and *P. cambivora* from the soil under the canopy of diseased chestnut trees in central Italy.

The objectives of this work were to obtain a great number of *Phytophthora* isolates associated with ink disease syndrome, to identify them to species level by using the ITS-RFLP approach and to elucidate the complex etiology of ink disease of chestnut.

Soil-borne *Phytophthora* are extremely difficult, if not impossible, to detect on old or decayed plant tissues, unless special techniques are used (Tsao, 1990). Unfortunately, these types of plant tissues are the most frequent in field situations when dealing with ink disease of chestnut. To overcome this constraint baiting techniques and selective agar media for detection and isolation were used.

Phytophthora identification is also a difficult subject. *Phytophthora* species identification is primarily based on morphological criteria. Plasticity of morphological characters and overlapping of metric characteristics hamper identification. It also requires

considerable expertise to produce the various stages through cultural manipulations and to recognise fine morphological differences. Molecular methods provide a new approach for *Phytophthora* species identification (Lee and Taylor, 1992; Liew et al., 1998; Cooke and Ducan, 1997; Cooke et al., 2000). In this work the method used was proposed by Cooke and Ducan (1997) and was based on the amplification PCR of the entire 5.8S rDNA gene and both ITS1 and ITS2 with specific *Oomycetes* primers, ITS6 (Cooke and Ducan, 1997) and ITS4 (White et al., 1990) followed by enzymatic restriction with *Msp*I, *Alu*I and *Taq*I and analysis of the restriction fragments.

MATERIALS AND METHODS

Detection and Purification of *Phytophthora* Isolates

Chestnut trees with ink disease symptoms were surveyed in three chestnut orchards in the south Bragança region (Rossas, Arufe and Paredes). Four old chestnut trees in adult orchards – Rossas (trees more than 30 years old), three chestnut trees in young orchards – Arufe (8-10 years old) and eight recently transplanted plants – Paredes, were sampled.

All plant tissue for isolation of *Phytophthora* species was carefully rinsed for 10-15 min under tap water (decayed tissue of roots and collar bark was rinsed overnight) blotted dry on filter paper and little segments were placed in Petri dishes on selective medium P_{10}VPH (Tsao and Guy, 1977) (PDA (Difco) 39 g L^{-1} instead of CMA, pimaricine - 10 mg, vancomycine - 200 mg, PCNB - 100 mg, hymezaxol - 50 mg). Dishes were incubated in the dark at 20-22ºC and were checked daily for characteristic coralloid and cenocytic hypha. Transfers from colonies were maid to P_{10}VPH for isolate purification. Pure isolates were kept on PDA (39 g/L, Difco) or in sterile water at 15ºC.

In recently planted orchards with a great number of dead plants (Paredes) soil near and around roots of dead plants was sampled. Soil for baiting tests was mixed and diluted with sterile distilled water (1:4) and baited with discs of chestnut leaves, floated on the surface of the water. After 48 h of incubation at laboratory conditions, the leaves were blotted on filter paper, and placed on P_{10}VPH. In adult orchards where newly infected tissue was difficult to sample the "natural bait" was applied. Young chestnut plants, germinated nuts under the canopy, with and without symptoms of the ink disease were used as natural bait for *Phytophthora* detection. The isolation and purification of the parasites on bait tissue was the same as described above.

Genomic DNA Extraction, PCR Amplification and Enzyme Digestion

Each isolate was grown on cellophane-PDA for 3-4 days. Mycelium was scraped from the surface and genomic DNA was immediately extracted as described by Cooke and Ducan (1997). PCR reactions were performed in a total volume of 25 µl with the universal primer pair ITS6 (Cooke and Ducan, 1997) and ITS4 (White et al., 1990) at 2 µM for the amplification of the entire 5.8S rDNA gene and both ITS1 and ITS2. Each reaction contained 100 µM dNTPs, 1.5 mM $MgCl_2$, 1 unit of *Taq* polymerase (Taq polymerase, Promega) 2.5 µl of buffer supplied with kit and 2.5 µl of BSA (Bovine Serum Albumin) diluted in 10 mg ml^{-1} before use and 1 µl of extracted DNA template. For the negative control, water instead of template DNA was included in each experiment.

Thermal cycler (UNOII, Biometra®) starting with a single step at 94ºC for 3 min was followed by 35 cycles of 55ºC - 30 s, 72ºC - 60 s and 94ºC - 30 s and a final single step at 72ºC for 10 min. Amplicons were analysed by electrophoreses in 1% agarose gels on TAE and visualized by ethidium bromide staining.

A 10 µl sample of the PCR product was digested with restriction enzymes – *Alu*I, *Msp*I and *Taq*I according to enzymes manufacturer. The reactions were performed at 37ºC overnight. Digested DNA was electrophoresed on 2.5% NuSieve (*Taq*I digest was electrophoresed at 3.5% NuSieve). The gels were stained with EtBr for polymorphism analyses. The size of the restriction fragments, in base pairs, was measured from the gels and compared to standard ladders. The gels were photographed under UV light and digital

images were scanned by Eagle Eye™ (Strategene). The pattern fingerprint of each enzyme digest was compared to CABI-Bioscience database (www.phytid.org) for isolate identification at species level.

RESULTS

Detection and Purification of *Phytophthora* Isolates

Thirty-six *Phytophthora* isolates were obtained from the fifteen sampled trees and soil of surveyed orchards. In all chestnut orchards and on all chestnut trees with ink disease symptoms, *Phytophthora* species were detected. Only one isolate per tree and three or two isolates obtained from the soil around the same diseased plant were included for molecular identification (Table 1).

Genomic DNA Extraction, PCR Amplification and Enzyme Digestion

The quality of the genomic DNA was assessed by gel electrophoresis. With the universal primer pair ITS6 (Cooke and Ducan, 1997) and ITS4 (White et al., 1990) all *Phytophthora* isolates yielded a single bright fragment of approximately 900 bp.

Three restriction patterns were shown after digestion with *Msp*I: I, Ia, II; two restriction patterns with *Alu*I: I, II and two restriction patterns with *Taq*I. Fragment sizes of each pattern are presented in Table 2.

Three groups of isolates are defined by restriction patterns: thirteen isolates were group I (Pr112, Pr115, Pr120, Pr123, Pr124, Pr125, Pr128, Pr129, Pr130, Ar104, R105, R106, R108), four isolates were group II (Pr135, Ar101, Ar102, R107) and one isolate, Pr122, was included in a Ia group. *P. cinnamomi* (CECT-2965) was included in group I isolates. Generated restriction patterns of each group of isolates were compared with CABI - Bioscience database of *Phytophthora* ITS fingerprint and identification at species level was obtained by percent (%) similarity with reference strain pattern in database. Isolates of Group I and II have a characteristic pattern and identification was accurately obtained. Isolate (Pr122) has a different restriction pattern with *Msp*I but this pattern did not modify the relative percentage of species similarity and so did not affect identification. Isolates of Group I and Ia (fourteen isolates) were identified as *P. cinnamomi* and the four isolates of group II have been identified as *P. cambivora*.

DISCUSSION

Ink disease of chestnut has been present in Portugal since 1838 (Fernandes, 1966). It drastically reduces chestnut area and a great number of foci are present on all chestnut regions. The disease is difficult to control and is a real constraint for establishment of new groves and conservation of old ones.

In this study, *Phytophthora* species were isolated by classical methods from all trees with ink disease symptoms and from soil around dead young plants. In old chestnut orchards with symptoms of decline or sudden death of trees, newly infected plant tissue is difficult to sample. In this situation the detection was performed on young plants which had germinated under the canopy (lost nuts of previous year) with and without symptoms of ink disease. These plants were natural bait for *Phytophthora* species in the orchard soils and were a useful technique for extensive survey in old chestnut orchards. Baiting the soil with chestnut leaves is also an efficient technique and provides many *Phytophthora* isolates from soil around decayed and dead roots.

Phytophthora identification by ITS-RFLP is an amenable method for *Phytophthora* identification at species level. *P. cambivora* and *P. cinnamomi* with fine morphological discrimination were accurately identified by this molecular approach.

In this study, *P. cinnamomi* and *P. cambivora* were identified in all sampled orchards. *P. cinnamomi* was the most frequently isolated from roots and collar stem bark of 2-3 year-old chestnuts and on recently germinated seedlings used as natural bait in old chestnut orchards. *P. cambivora* (R107) was recovered only once from these young seedlings. *P. cambivora* was predominantly obtained from chestnut trees 8-10 years old with

characteristic necrosis on collar stem, but *P. cinnamomi* (Ar104) also was isolated in this group of trees. In soil baited with chestnut leaves, the two *Phytophthora* species were also identified and once more *P. cinnamomi* was the preponderate species and only one isolate was identified as *P. cambivora*.

Molecular methods of *Phytophthora* identification which allow more rapid and accurate identification of *Phytophthora* isolates will facilitate more extensive surveys and will improve the knowledge of *Phytophthora* species distribution in soil and plants, its seasonal activity, its aggressiveness and means of survival and its ability to build up inoculum in soil.

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Tables

Table 1. *Phytophthora* isolates detected in chestnut trees with ink disease symptoms, sites, sampled tissue and orchard type.

Site	Isolate number	Sample tissue	Orchard type
Paredes	Pr112	Roots/collar bark	Recently transplanted
"	Pr115	"	(2-3 years old)
"	Pr120	"	
	Pr122	"	
	Pr123*	Bait soil technique	
	Pr124*	"	
	Pr125*	"	
	Pr128+	"	
	Pr129+	"	
	Pr130	"	
	Pr135	"	
Arufe	Ar101	Collar bark	Young
	Ar102	"	(8-10 years old)
	Ar104	"	"
Rossas	R105	"Natural bait"	Adult
	R106	(roots of young plants)	(>30 years old)
	R107		
	R108		

* + Isolates from soil around the same plant

Table 2. Restriction fragment sizes (bp) from the DNA region ITS1, 5.8S and ITS2 of *Phytophthora* isolates amplified with primer pair ITS6 (Cooke and Ducan, 1997) and ITS4 (White et al., 1990) with restriction enzymes *Msp*I, *Alu*I and *Taq*I.

Restriction patterns	Fragment sizes (bp)
Digestion with MspI	
I	147, 163, 221, 407
Ia	147, 163, 221, 394, 407
II	146, 166, 221, 294, 407
Digestion with AluI	
I	188, 207, 543
II	188, 750
Digestion with TaqI	
I	59, 90, 117, 125, 150, 180, 190
II	90, 120, 125, 150, 194