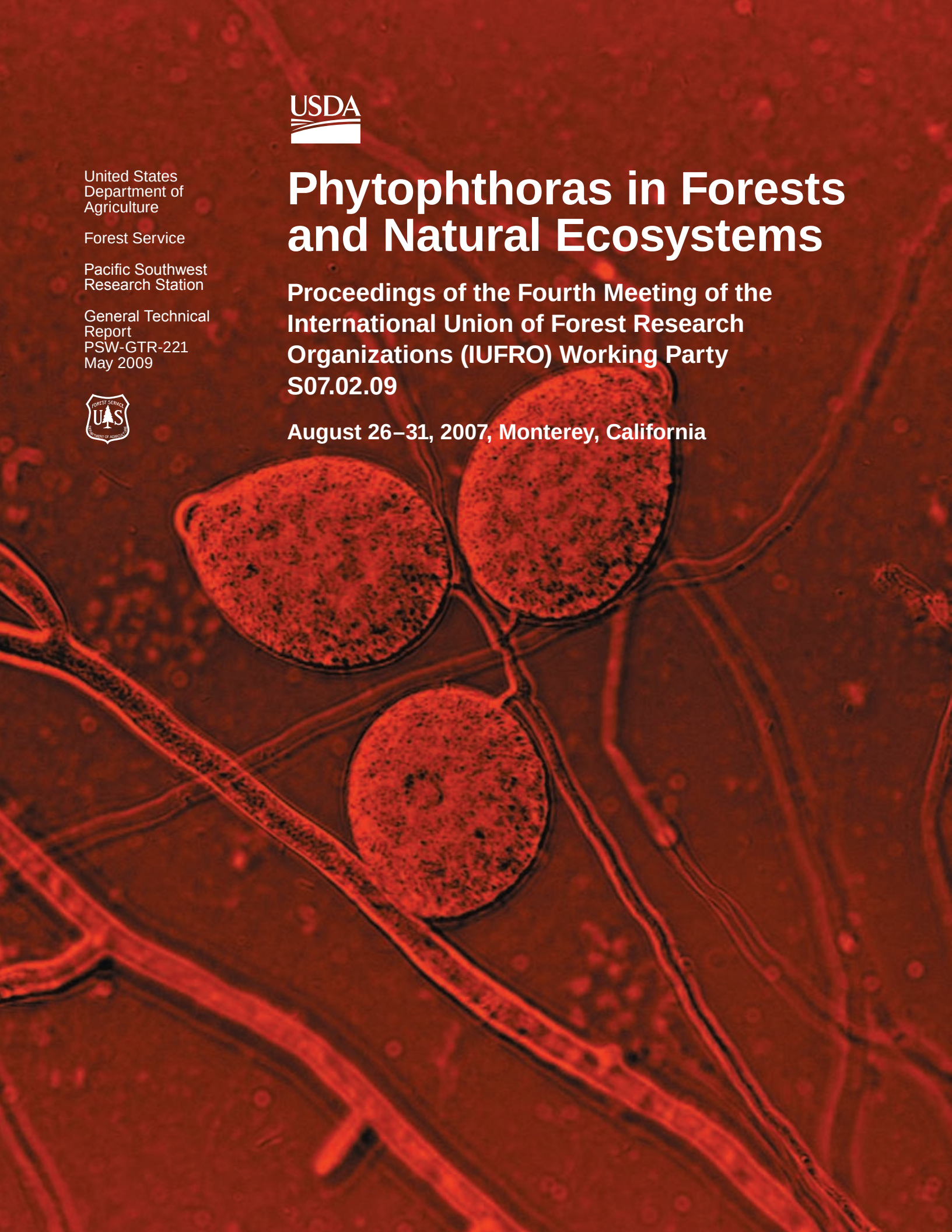

United States
Department of
Agriculture

Forest Service

Pacific Southwest
Research Station

General Technical
Report
PSW-GTR-221
May 2009

Phytophthoras in Forests and Natural Ecosystems

Proceedings of the Fourth Meeting of the International Union of Forest Research Organizations (IUFRO) Working Party S07.02.09

August 26–31, 2007, Monterey, California

Technical Coordinators

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Cover and chapter heading illustrations by Charles M. Aquilina, Vallejo, CA. Thanks also to Stephanie Tencer, Paul Reeser, Eunsung Oh, and Elizabeth Fichtner for providing photographs used on the cover and chapter pages.

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Financial and other support from:
USDA Forest Service, Pacific Southwest Research Station
USDA Forest Service, Pacific Southwest Region
Oregon State University
University of California, Davis
California Oak Mortality Task Force

U.S. Department of Agriculture, Forest Service
Pacific Southwest Research Station
Albany, CA
General Technical Report PSW-GTR-221

May 2009

Abstract

Goheen, E.M.; Frankel, S.J., tech. coords. 2009. Proceedings of the fourth meeting of the International Union of Forest Research Organizations (IUFRO) Working Party S07.02.09: Phytophthoras in forests and natural ecosystems. Gen. Tech. Rep. PSW-GTR-221. Albany, CA: U.S. Department of Agriculture, Forest Service, Pacific Southwest Research Station. 334 p.

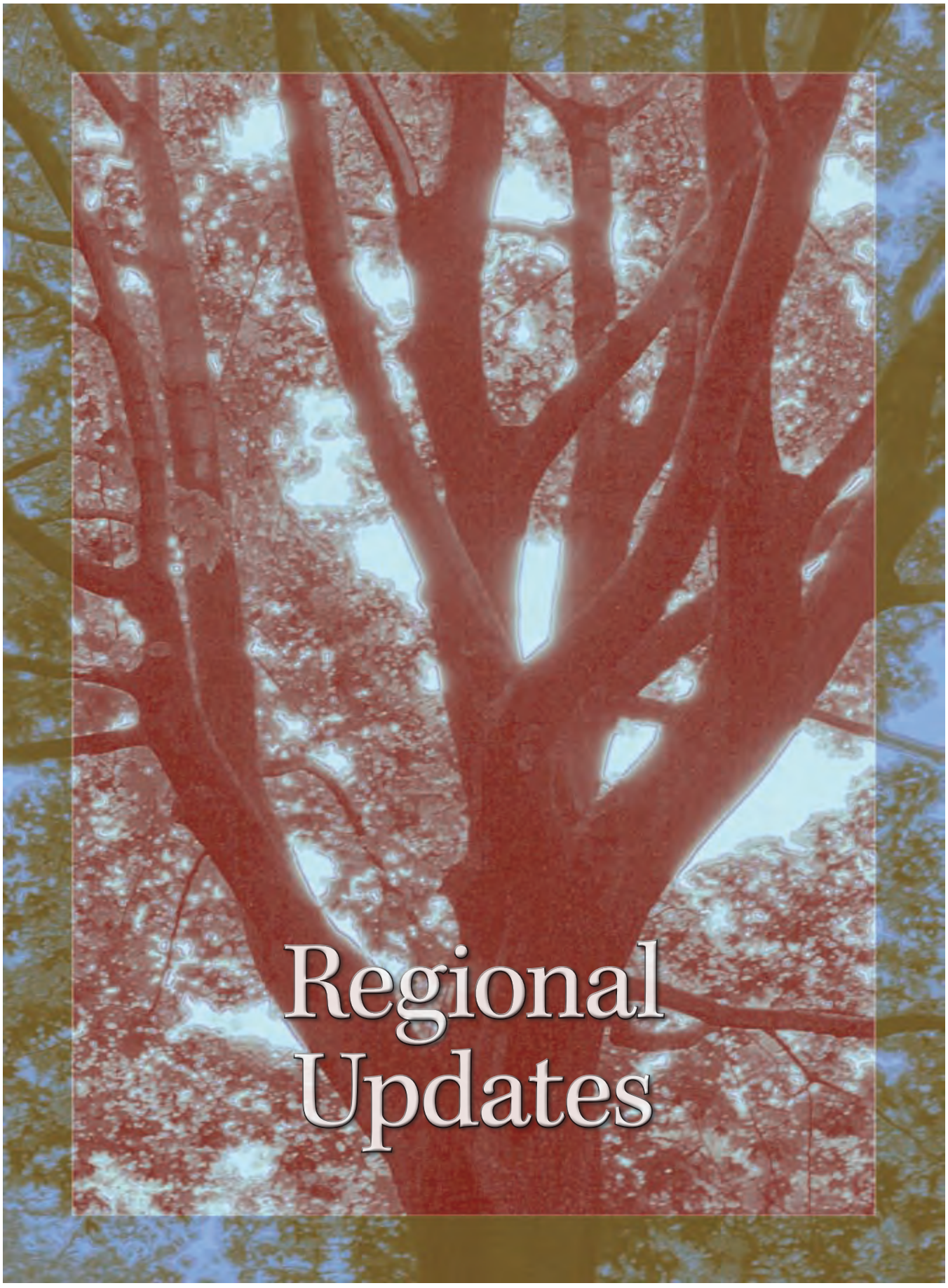
The fourth meeting of the International Union of Forest Research Organizations (IUFRO) Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems provided a forum for current research on *Phytophthora* species worldwide. Seventy-eight submissions describing papers and posters on recent developments in *Phytophthora* diseases of trees and natural ecosystems in Europe, Australasia, and the Americas are included. Research topics covered are *Phytophthora* biodiversity, ecology, epidemiology, management, and host-pathogen interactions.

Keywords: *Phytophthora* species, forest tree diseases.

Organizing Committee

Conference attendees, Asilomar Conference Center, Monterey, CA.

Contents


Regional
Updates

Progress in Understanding *Phytophthora* Diseases of Trees in Europe 2004–2007[1]

Thomas Jung,[2] Andrea Vannini,[3] and Clive M. Brasier[4]

Abstract

Much new information on *Phytophthora* diseases of trees in Europe has been produced since the 2004 meeting and is reviewed here. Topics covered include updates on *P. alni* induced dieback of alders, deciduous and mediterranean oak decline, decline and dieback of European beech and ink disease of chstnut; the nursery-plantation pathway; the status of *P. ramorum* and *P. kernoviae* in Europe; an upsurge in *Phytophthora* activity on *Tilia*, *Acer* and *Betula* species; and the current status of the remarkable array of new *Phytophthora* taxa recovered from forest and natural ecosystems in Europe over the past decade.

Introduction

Knowledge of Phytophthora diseases of trees is growing rapidly. This is timely considering the increasing threat posed by invasive Phytophthoras as a consequence of the growing international trade in living plants. Recent developments in Europe include advances in the taxonomy and phylogeny of European forest Phytophthoras; new insight into the phylogeny of the *Phytophthora alni* hybrid complex; modelling of alder dieback in France and Bavaria; surveys of alder dieback and first records of *P. alni* in Poland, the Czech Republic, Switzerland and Vienna; ecophysiological studies on the Phytophthora diseases of black alder and European beech (Koehl and others this volume); *Phytophthora* surveys in mature beech stands across Europe indicating that *Phytophthora* infection is driving European beech decline; evidence for the spread of *Phytophthora* species within tree xylem; a *Phytophthora* survey in oak stands in Sweden and the development of a conceptual model for oak decline; insights into ink disease of chestnut in Southern Europe; upsurge of Phytophthora activity in maple, linden, horse chestnut and birch stands in Germany and Switzerland; *Phytophthora* surveys in forest stands in Hungary; extensive *Phytophthora* surveys in nurseries and plantations in Germany and Austria providing further evidence for the critical role of nurseries as the pathway of Phytophthora spread; the detection of multiple *Phytophthora* species in streams and soils in Scotland; and phosphite trials in mature beech, oak, maple and linden stands in Bavaria and Austria. Developments on *P. ramorum* and *P. kernoviae* include publication of the main outcome of the "RAPRA" project (see http://rapra.csl.gov.uk); new information on the current distribution of the pathogens in Europe; increasing knowledge on environmental factors affecting spread and infection of *P. ramorum* in Europe including a comparison between Californian and European

[1] A version of this paper was prexented at the Fourth IUFRO Phytophthoras in Forests and Natural Ecosystems, August 26–31, 2007, Monterey, California.

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climates and a detailed risk analysis for Italy; new information on the susceptibility of a wide range of woody species; evidence for asymptomatic infection and sporulation of *P. ramorum* and *P. kernoviae* on leaves and fruits; evidence for infection of intact bark by *P. ramorum* and *P. kernoviae* via zoospores; and further insights into the breeding behaviour of *P. ramorum*.

Taxonomy and Phylogeny of New *Phytophthora* Taxa in European Forests

It has become customary to update the taxonomic status of the remarkable array of new *Phytophthora* taxa recovered from trees in Europe over the past decade (Brasier and Jung 2003, 2006). Since the IUFRO 2004 meeting, three known axa have been officially described as new species while five new *Phytophthora* taxa have been found in the course of different surveys of declining alder stands in Hungary and Poland (Belbahri and others and Lakatos and Szabo, this volume) enhancing the number of new *Phytophthora* taxa recovered from European forests since the mid 1990s to 23 (table 1).

Based on their ITS sequences all three newly described species fall outside the main *Phytophthora* ITS cluster described by Cooke and others (2000). *P. polonica* sp. nov. belongs to ITS clade 9 with *P. insolita* being its nearest relative; whereas *P. kernoviae* sp. nov. and *P. gallica* sp. nov. together with *P. boehmeriae* fall into ITS clade 10. Interestingly, in both clades the ancestral species are soilborne with persistent sporangia, i.e. *P. polonica* and *P. insolita* in clade 9 and *P. gallica* in clade 10, while the phylogenetically "younger" species have an airborne lifestyle and caducous sporangia, i.e. *P. captiosa* and *P. fallax* in clade 9 and *P. kernoviae* and *P. boehmeriae* in clade 10. This supports the hypothesis (Brasier 1983; Cooke and others 2000) for evolutionary development from a soilborne to an aerial lifestyle within the genus *Phytophthora*.

Some of these new species and taxa have recently been recorded from new hosts as well as from new countries or even continents (Table 1). For instance, the oak fine root pathogen *P. quercina* has recently been found widespread in US oak forests (Schwingle and others 2007a) while *P. kernoviae* and *P. europaea* were recovered from Cherimoya and Iris, respectively, in New Zealand (Beever, Jung unpublished). *P. pseudosyringae* was isolated from hornbeam (*Carpinus*) in the U.K. and Romania (Denman and others this volume; T. Jung and D. Chira unpublished). *P.* taxon riversoil has been found on fallen alder leaves in Spain (A. Moralajo, unpublished) and may shortly be described as *P. ripicola* sp. nov. New host and country records of *P. ramorum* will be reviewed later in this article.

Table 1—New *Phytophthora* taxa on trees and shrubs in Europe (new taxa and developments since 2004 are highlighted in bold)

New taxon	**Host, ecology, distribution, identified by and date**	**Taxonomic status in 2007**
P. italica sp. nov.	Myrtle, Italy. I.P.V., Palermo, 1990s	Cacciola and others 1996
P. inundata sp. nov.	Trees/shrubs (*Aesculus, Salix, Olea*), after flooding Europe, South America. FR, U.K., 1970s	Brasier and others 2003
P. quercina sp. nov.	Oak fine roots, ubiquitous across Europe and Turkey, **Eastern US**. T. Jung, 1990s	Jung and others 1999
P. ramorum sp. nov.	'sudden oak death', Rhododendron, *Viburnum*, beech, red oaks, Europe, USA. BBA, Germany, 1990s	Werres and others 2001
P. uliginosa sp. nov.	Oak forest soils, beech bark, Germany, Poland. T. Jung, 1990s	Jung and others 2002
P. psychrophila	Oak and beech soils rare, Germany, Austria, France. T. Jung, 1990s	Jung and others 2002
P. europaea sp. nov.	Oak forest soils, France, Germany, Eastern USA, **Iris New Zealand**. INRA Nancy / T. Jung, 1990s	Jung and others 2002
P. pseudosyringae sp. nov.	Roots and bark of oaks, beech, **hornbeam** and alders; Germany, France, Italy, **U.K.**, **Romania**, California, Oregon. INRA Nancy / T. Jung, 1990s	Jung and others 2003
P. alni sp. nov	Alder, swarm of species hybrids, Europe. Forest Research, U.K., 1990s	Brasier and others 2004
P. hedraiandra sp. nov.	Roots, collar and leaves of *Viburnum*. The Netherlands, Italy, Spain. **Soil of *Juglans nigra*, Hungary. Nurseries, US.** Plant Protection Service, Wageningen, NL, 2001	De Cock and Lévesque 2004
P. kernoviae sp. nov.	Bark of beech and oak; leaves and shoots of Rhododendron, Cornwall, U.K., and **Cherimoya, New Zealand**. FR/CSL, U.K., 2003	Brasier and others 2005
P. polonica sp. nov.	Soils of alder stands, Poland. University of Jussy, Switzerland, IMEDEA, Spain, Forest Research Institute, Poland, 2004	Belbahri and others 2006
P. gallica sp. nov.	Soil around oak roots and reed beds. Germany, France. T. Jung 1990s	Jung and Nechwatal 2008

Table 1 (continued). New *Phytophthora* taxa on trees and shrubs in Europe (new taxa and developments since 2004 are highlighted in bold)

New taxon	Host, ecology, distribution, identified by and date	Taxonomic status in 2007
P. taxon Pgchlamydo	Prunus, Douglas fir, beech, nursery soils, wet sites Europe, North America, Argentina. Forest Research, U.K., 1970s	Informally designated (Brasier and others 2003)
P. taxon Riversoil	Forest/riverbank soil U.K., FR, U.K., 1990s. **Fallen alder leaves, Spain. IMEDEA 2006**	
P. taxon Oaksoil	Oak soil, rare, France. INRA, Nancy, 1990s	
P. taxon Forestsoil	Forest soil (oak, Carpinus etc.) rare, France, **Hungary**. INRA, Nancy, 1990s	
P. taxon Salixsoil	Soil around roots of *Salix* and *Viburnum,* alder debris, reed beds and streams. U.K., Germany, Italy, Denmark.. Forest Research, U.K., 1970s	
Phytophthora sp. 1	Soil of *Alnus glutinosa* + *Juglans nigra* stands; Hungary, University of West Hungary	Lakatos & Szabo, this volume
Phytophthora sp. 2	Soil of *Alnus glutinosa* stands. Hungary, University of West Hungary	
Phytophthora sp. 3		
P. sp. hungarica	Soil and roots of *Alnus glutinosa* stands. Hungary, Poland. University of Jussy, CH, Hungarian Academy of Sciences and Hungarian Forest Research Institute	Informally designated at GenBank
P. sp. sylvatica		

Surveys of *Phytophthora* Populations in Natural Ecosystems in Scotland

Molecular methods are increasingly used for routine surveys of pathogens in plant tissue. They also have great potential for monitoring diversity of *Phytophthora* populations in natural habitats. In 2006, water and soil samples were taken from streams in five nature reserves in Scotland and analysed for the presence of *Phytophthora* species using a method that includes filtration, DNA extraction, nested PCR of the ITS 1, cloning and sequencing (Silvia Scibetta, David Cooke and Santina Cacciola unpublished, see Brasier this volume). Seventeen different *Phytophthora* species were detected in the water samples and only five species in adjacent soil samples. Some water samples contained up to five different *Phytophthora* species. The results of the Scottish stream survey are in good aggreement with those of stream surveys in the Eastern and Western US (Remigi and others and Hwang and others, this volume) and Australia (Smith and others, this volume) all showing rich and diverse *Phytophthora* populations in watercourses. In Europe, the use of (cheap) unfiltered surface water for irrigation of parks, gardens and nursery fields is still

widespread. Clearly this practice is likely to result in the infestation of the irrigated soils with *Phytophthora* species.

New Insights Into the Evolution of the *P. alni* Hybrid Complex

Based on morphological studies, the near tetraploid structure of *P. alni* ssp. *alni* (PAA), the intermediate chromosome numbers of the other two subspecies *P. alni* ssp. *uniformis* (PAU) and *P. alni* ssp. *multiformis* (PAM) and on AFLP and ITS DNA sequence analyses Brasier and others (1999, 2004) suggested that the progenitors of PAA were most probably *P. cambivora* and an unknown taxon close to *P. fragariae* and that PAU and PAM are most probably a result of recombination and chromosome losses in PAA. This hypothesis fits well with the observed phenotypic variation, the developmental instability, and the intermediate chromosome numbers, but fits not totally with the observed pattern of ITS polymorphisms and is not well supported by the AFLP data. Recently, Ioos and others (2006 and this volume) investigated four nuclear DNA genes (*ASF*-like, *GPA*1, *RAS-YPT*, *TRP*1), two mitochondrial DNA genes (*cox*1, *nadh*1) of a European-wide range of isolates of PAA, PAM, PAU, *P. cambivora*, *P. fragariae* and other *Phytophthora* species. Their results strongly indicate that (1) PAU and PAM or PAU- and PAM-like ancestors were most probably the progenitors of PAA, (2) PAM may also be the result of an ancient reticulation, and (3) that several hybridisation events must have occurred. It was also demonstrated that *P. fragariae* was not involved in the evolution of the hybrids and that *P. cambivora* was unlikely to be a direct progenitor of the hybrids, although it could possibly be a parental species of a PAU-like ancestor. A study of the expression of the elicitin genes of PAA, PAM, and PAU isolates and microsatellite patterns in PAA and PAM by Ioos and others (this volume) further supports these hypotheses. Due to the recent finding of 'PAU-like' isolates in streams without visible alder damages in remote areas of Alaska (Lori Trummer and Charles G. Shaw, personal communication) a further elucidation of the hybridisation events that led to the *P. alni* hybrid complex may be expected.

It was suggested by Brasier and others (1999, 2004) that the hybridisation events occurred in a nursery somewhere in Europe. Their view that nursery environments generally present suitable conditions for hybridisations are supported by the widespread finding of natural hybrids between *P. cactorum* and *P. hedraiandra* in nurseries and planted stands of Rhododendron (Man In' t Veld and others this volume). The hybrids proliferate in several European countries and seem to outcompete *P. cactorum* on Rhododendron.

Modelling Decline and Dieback of Alders Caused by *Phytophthora alni*

The serious root and collar rot of alders caused by *P. alni* continues to spread across Europe. In northeast France and Bavaria, where the disease has reached epidemic levels, two models were recently developed. The model of Thoirain and others (2006) was based on data from 78 plots located along 35 rivers in NE France. It demonstrated that the watercourse type, *i.e.* width and slope of the river and speed of water flow, mean summer temperatures of the water and the soil texture of the river

banks were the most important factors effecting disease incidence along the rivers. Infection rate and mortality of alders increased when river width, summer temperature of river water and clay content of the riverbank were higher and the slope of the river bed and water flow rate were lower. Interestingly, the total oxidised nitrogen concentration was found to be related to disease prevalence, as suggested earlier (Gibbs and others 1999). Jung and others (see abstract in this volume) tested three different models for describing the distribution of the alder disease on forest sites in Bavaria. The models were based on data on the establishment type and flooding conditions of 434 sample points of which 307 points were infested by *P. alni*; on GIS datasets on soil texture, aspect, slope and landform; and on a Vegetation Index calculated from satellite imagery. While both the spatial (only GIS datasets) and non-spatial models (only establishment type and flooding condition) were mora accurate at predicting infected sites than disease-free sites, the combined model predicted both healthy and diseased sites with high accuracy (85 percent and 86.6 percent). The French and the Bavarian models can now be used for assessing the risk of *P. alni*-induced decline and dieback of alders on riparian and forest sites, respectively, in other regions. The Bavarian variables together with additional datasets on drainage, streams, climate, distribution of alder species, distribution of wholesale and retail nurseries and urban settlements are currently being applied in a preliminary Multi-Criteria model to assess regions of the USA with alder forests potentially susceptible to to *P. alni*.

Alder Surveys and New Country Records for *Phytophthora alni*

In Poland, a statewide disease survey has been conducted and alder dieback caused by *P. alni* has been found to be widespread along more than 30 river systems (Leszek Orlikowski, personal communication). In the Czech Republic alder mortality has been widespread since severe flooding in 2002 and *P. alni* has been recovered from more than 60 alder stands in Bohemia (Cerny and others 2007). In 2007, PAU was also isolated from collar necroses of young black alders in a forest plantation in Moravia (T. Jung and L. Jankowski, unpublished results).

Recently, *P. alni* (PAA) was recovered for the first time from collar rot tissue of riparian black alders in Switzerland (Ottmar Holdenrieder, personal communication).

In 2005 a survey of Phytophthora root and collar rot was conducted along 26 rivers and streams in the wider city of Vienna, Austria. Typical *Phytophthora* symptoms were found along 16 rivers. *P.alni* (PAA and PAM) was recovered from collar rot tissues of alders growing along 4 streams with high alder mortality while in seven watercourses *P. citricola* and *P. gonapodyides* were found causing a scattered dieback of individual alder trees. Interestingly, *P. alni* was only found in river systems with infested alder plantations established in their catchments a few years ago (table 2). This confirms the results of Jung and Blaschke (2004) that the planting of infested nursery stock is the major pathway of *P. alni* spread and the primary cause of the Phytophthora root and collar rot epidemic of alders.

Table 2—Survey of root and collar rot of alders along rivers and streams in the city of Vienna (T. Jung and T. Cech, unpublished results)

Number of streams with young alder plantations in catchment	Total	Infested	*Phytophthora* species [1]				
			PAA	PAM	CIT	GON	P. sp.
Present	14	7	2	2	3	0	4
Absent	12	4	0	0	3	1	1
Total	26	16	2	2	6	1	5

[1] PAA = *P. alni* ssp. *alni*, PAM = *P. alni* ssp. *multiformis*, CIT = *P. citricola*, GON = *P. gonapodyides*, P. sp. = typical collar rot symptoms, but isolation failed because the necroses were old and dry.

Decline and Dieback of European Beech

In our last review (Brasier and Jung 2006) we presented evidence from disease surveys in Southern and Northern Germany (Hartmann and others 2006, Jung 2006, Jung and others 2005), Austria, Southern and Central Italy (Belisario and others 2005, Diana and others 2006, Motta and others 2003), Sweden and the U.K. (Brown and Brasier 2007) that an upsurge of *Phytophthora* activity on European beech has occurred. Between 2004 and 2007 most of these surveys have continued and a new survey has been conducted in Belgium (Schmitz and others this volume). New disease records have also come from Slovenia (Munda and others 2007), Switzerland (T. Jung unpublished), Romania (T. Jung and D. Chira unpublished), the Czech Republic (T. Jung and L. Jankowski unpublished) and the Netherlands (W. Man In' t Veld unpublished, http://rapra.csl.gov.uk). In total 11 *Phytophthora* species have been found to date in declining beech forests across Europe with *P. cambivora* and *P. citricola* having the widest distribution and *P. ramorum* infections of beech being restricted to southwest England and the Netherlands (Table 3).

In Bavaria, Southern Germany, where beech stands have been severely declining since the abnormally wet and and extremely dry years of 2002 and 2003 respectively, more than 300 declining beech trees in 112 stands were sampled. *Phytophthora* species were recovered from 81 percent of the trees in 93 percent of the stands (Jung, in press). In this study evidence was also presented that *Phytophthora* species, in particular *P. citricola*, may be involved in the aetiology of Beech Bark Disease in mature stands.

In Belgium a severe decline and dieback of beech stands has occurred since the late 1990s. Various hypotheses on the causes of the decline have been presented including frost events and bark beetle attacks. Recently, *P. cambivora* has been isolated from bark cankers in 19 out of 49 stands investigated (Schmitz and others this volume).

Collectively, the above studies provide accumulating evidence that interactions between introduced *Phytophthora* species, either well established or still invasive, together with climatic extremes, are a significant factor in the widespread decline and dieback of beech stands across Europe (Jung, in press).

Table 3—*Phytophthora* species associated with decline and mortality of beech in Europe

Country / region	*Phytophthora* spp.[1]										
	CAC	**CAM**	**CIT**	**GON**	**KER**	**PSE**	**RAM**	**SYR**	**CHLA**	**ULI**	**PSY**
North Germany		X				X					
South Germany	X	X	X	X		X		X	X	X	X
Austria		X	X								
Switzerland	X		X								
The Netherlands						X	X				
U.K.		X	X	X	X	X	X				
Belgium		X									
Italy	X	X				X					
Slovenia			X								
Czech Republic			X								
Romania		X									
Turkey		X									

[1] CAC = *P. cactorum*, CAM = *P. cambivora*, CIT = *P. citricola*, GON = *P. gonapodyides*, KER = *P. kernoviae*, PSE = *P. pseudosyringae*, RAM = *P. ramorum*, SYR = *P. syringae*, CHLA = *Phytophthora* taxon 'Pg chlamydo', ULI = *P. uliginosa*, PSY = *P. psychrophila*.

Colonisation of Tree Xylem by *Phytophthora* Species

The recovery of non-aerial *Phytophthora* species with persistent sporangia, *i.e. P. cambivora*, *P. citricola* and *P. gonapodyides*, from aerial stem lesions that tend to be aligned along the stem raised the question on how these pathogens are able colonise the stems, sometimes reaching up to 20 m stem height (Brown and others 2006; Brown and Brasier 2006; Jung 2006, Jung and others 2005). Between 2004 and 2006, in southwest England, Brown and Brasier (2007) investigated stems of 53 beech, oak and maple trees with aerial stem lesions in detail. They found that individual 'major' lesions and adjacent 'island' lesions in the phloem were connected by underlying strips or intermittent pits of discoloured xylem in line with the wood grain. Isolations

were made from 81 wood panels taken from below phloem lesions and 81 percent yielded *Phytophthora* spp. (*P. ramorum, P. kernoviae, P. citricola, P. cambivora* and *P. gonapodyides*). In 66 cases both a wood panel and an overlying phloem panel were sampled, and *Phytophthora* spp. were isolated from both xylem and phloem in 56 percent, from xylem only in 23 percent and from phloem only in 8 percent of cases. These results show that *Phytophthora* species are able to survive and spread within tree xylem; that it is important to attempt to isolate from discoloured xlem panels as well as from infected phloem, since the former may give a higher isolation success rate; and that debarking of infected stems, such as *P. ramorum*-infected stems, for quarantine purposes may be insufficient to prevent spread of *Phytophthora* species. The authors also suggest that apparent linear spread of a *Phytophthora* over longer distances along a stem might be due to spores being transported on embolisms within cavitated xylem vessels.

Involvement of *Phytophthora* Species in European Oak Declines

Numerous studies have been carried out on the causes of the widespread oak declines in western, central and southern Europe (Balci and Halmschlager 2003a, b; Brasier and others 1993; Gallego and others 1999; Hansen and Delatour 1999; Jung and others 1999, 2000; Vettraino and others 2002). Another study was recently carried out at the northern limit of the distribution of *Quercus robur* in Southern Sweden. Thirty two oak stands were investigated and *P. quercina* was recovered from almost 50 percent of the declining stands but only from 11 percent of the healthy stands (Jönsson and others 2005). The calculation of odds ratio showed that the probability of a stand to be in decline is more than four times higher if *P. quercina* is present. These findings are in close accordance with results from earlier studies in southern Germany and Italy (Jung and others 2000; Vettraino and others 2002) and again indicate that *Phytophthora* species are strongly involved in European oak decline. Following an analysis of many climatic and site factors within the Swedish oak stands, and accepting the experimental evidence that *P. quercina* is able to cause substantial fine root loss on oaks in a sandy acid forest soil (Jönsson 2004), a conceptual model for oak decline in Sweden has been developed (Jönsson 2006). The model addresses the overall complexity of oak decline and includes *Phytophthora* infection levels, microbial activity, secondary pathogens, site and climatic factors and ecophysiological processes.

Since the IUFRO 2004 meeting, considerable research has also been undertaken on Mediterranean oak decline associated with *P. cinnamomi*. Moreira and Martins (2005) investigated 56 cork and holm oak stands (*Q. suber* and *Q. ilex*) in Portugal and recovered *P. cinnamomi* from oaks and from 56 percent of the associated shrub flora at 27 sites demonstrating that like in the Jarrah forest in Western Australia the pathogen may represent a threat at ecosystem level in Europe, too. An analysis of various site factors indicated that *P. cinnamomi* induced oak decline was favoured by shallow soils with low fertility and low mineral nutrient levels, by a south-facing aspect, and by slopes and depressions. In another study on the survival and viability of cork oak seedlings in an oak stand naturally infested by *P. cinnamomi*, Moreira and others (this volume) found that the maternity of the progeny significantly influenced both acorn germination and seedling survival, resulting in a distinct

susceptibility to *P. cinnamomi* among half-siblings which could be the basis for a resistance screening program.

Investigations by M. Horta and colleagues in Portugal into the biochemical mechanisms of pathogenesis in *P. cinnamomi* have continued. Horta and others (this volume) demonstrated that the silencing of the beta-cinnamomin coding gene by genetic transformation of protoplasts led to a decreased virulence of the transformed *P. cinnamomi* isolate against cork oak seedlings. This result indicates that this elicitin gene might be involved in pathogenesis, a feature which could potentially have wide implications for disease control via resistance breeding or via genetic manipulation of the pathogen. Another control option for *P. cinnamomi* in mediterranean oak forests might come from the experiments of Neves and others (this volume), also in Portugal, who showed that the understory plant *Phlomis purpurea* produces substances that inhibit mycelial growth of *P. cinnamomi in vitro*. In a greenhouse trial the presence of *Phlomis purpurea* significantly protected holm oak seedlings from infections by *P. cinnamomi*. In an interesting AFLP analysis of a wide collection of *P. cinnamomi* isolates from declining holm and cork oak trees from the southwest region of the Iberian Peninsula, Caetano and others (this volume) demonstrated the presence of two clearly distinct populations, a Portuguese population which is spreading into south-western Spain and a Spanish population. Finally, *P. quercina* was recovered for the first time alongside *P. cinnamomi* from rhizosphere soil of declining cork oaks in Portugal (T. Jung unpublished).

Ink Disease of Chestnut in Southern Europe

Interesting results are also reported from several projects on ink disease of chestnut (*Castanea sativa*) caused by *P. cambivora* and *P. cinnamomi*. Fonseca and others (2004) investigated the relationship among the occurrence of ink disease, edaphic factors and management practices, and found that the probability of a stand having ink disease increases if the compaction and organic matter content of the soil are increased and manuring is the usual silvicultural practice.

Variation in susceptibility to *P. cambivora* of 23 populations of European chestnut growing at 10 locations in Italy, Greece, France, Spain and the U.K. was studied by three research groups using a root-inoculation test on seedlings of half-sib families (Robin and others 2006). The results showed significant variation in resistance to ink disease. One or more resistant trees were found in 15 populations but only three families showed resistance comparable to the resistant control clone. Variation in resistance of chestnut clones to *P. cinnamomi* was also shown by Miranda-Fontaìna and others (2007). In this study at least 2 *C. sativa* clones showed remarkable resistance to root rot and stem lesions caused by *P. cinnamomi*.

Portuguese and Italian research groups (Martins and others 2007, Vannini and others this volume) demonstrated the practical use of remote sensing and the application of geostatistical methods to data from proximal and remote sensing for monitoring and epidemiological studies on ink disease.

Upsurge of *Phytophthora* Activity on Several Broadleaved Tree Species in Central Europe

Over the past few years a severe and widespread dieback and decline of mature trees of linden (*Tilia cordata*, *T. platyphyllos*, *T. x europaea*), maple (*Acer platanoides*, *A. pseudoplatanus*, *A. campestre*), birch (*Betula pendula*, *B. pubescens*) and horse chestnut (*Aesculus hippocastanum*) has is occurred in western and southern parts of Germany and Switzerland. Symptoms include an increased crown transparency, dieback of branches, small-sized and often yellowish foliage, extensive fine root losses, dieback of tap roots and cankers on suberised roots. In some stands collar rot and aerial stem lesions were found on maples, horse chestnuts and more rarely on linden trees. Dieback and mortality rates are most pronounced in old parks and forests (>100 years). A survey of rhizosphere soil samples and, if present, of bark lesions from 125 trees in 41 stands showed almost 100 percent infestation by in total 7 *Phytophthora* species (T. Jung unpublished). *P. citricola* and *P. cactorum* were most frequently associated with the dieback (table 4).

These results extend the picture coming from research on the association of *Phytophthora* species with the current declines and diebacks of oak, beech, chestnut and alders in Europe and indicate that similar infestation levels are occurring on these other broadleaved tree species.

Table 4—Association of *Phytophthora* species with decline and dieback of linden, maple, birch and horse chestnut trees in Germany and Switzerland (T. Jung unpublished results)

Tree species	**Total no. of stands (trees)**	**No. infected stands (trees)**	**No. of stands (trees) with *Phytophthora* spp.** [1]						
			CAC	**CAM**	**CIT**	**GON**	**PSEU**	**RAS**	**SYR**
Tilia spp.	15 (61)	15 (61)	12 (49)	1 (15)	9 (31)	1 (10)	1 (1)		1 (2)
Acer spp.	11 (34)	11 (31)		1 (2)	10 (27)	3 (4)			3 (3)
Betula spp.	5 (13)	5 (13)	2 (4)		3 (9)	2 (2)		1 (2)	
Aesculus hippocast.	10 (17)	9 (15)	5 (6)		7 (13)				1 (1)
Total stands	41	40 (98%)	19 (46%)	2 (5%)	29 (71%)	6 (15%)	1 (2%)	1 (2%)	5 (12%)
trees	125	120 (96%)	59 (47%)	17 (14%)	80 (64%)	16	1 (1%)	2 (2%)	6 (5%)

[1] CAC = *P. cactorum*, CAM = *P. cambivora*, CIT = *P. citricola*, GON = *P. gonapodyides*, PSE = *P. pseudosyringae*, RAS = *P.* taxon 'raspberry', SYR = *P. syringae*.

Spread of Phytophthoras From Nurseries Into Forests and Plantations

Evidence had accumulated from meeting to meeting that Phytophthoras are spreading from infested nursery stock into forests. The first firm evidence that an epidemic in a natural ecosystem has been caused by distribution of infested nursery stock came from the investigation of riparian and forest alder ecosystems in Germany and elsewhere in Europe (Gibbs and others 1999, 2003; Jung and Blaschke 2004). At the 2004 IUFRO workshop Orlikowski and others (2006) presented results from a survey of 30 horticultural and forest nurseries in Poland showing regular infestation with a range of 9 different *Phytophthora* species. At the same meeting Hartmann and others (2006) and Jung (2006) reported widespread infestation of nursery beech fields in Germany.

Over the last few years four research groups in Germany and Austria have intensely surveyed local nursery fields and plantations erey (see abstract of Jung and others in this volume). In total 102 fields of alder (*Alnus*), beech (*Fagus*), linden (*Tilia*), maple (*Acer*), oak (*Quercus*) and horse chestnut (*Aesculus*) in 60 forest and horticultural nurseries in Germany and Austria were sampled. 64 fields (63 percent) in 48 nurseries (80 percent) were found to be infested with atotal of 13 *Phytophthora* species, among them the most aggressive ones to the respective tree species. Each nursery field yielded on average 2.1 *Phytophthora* species. In some cases up to fivedifferent *Phytophthora* species were recovered from a single field and even from the rhizosphere of an individual plant. In addition, 26 8–15 year-old plantations of oak, beech, linden and maple were screened and 24 (92.3 percent) were infested with in total 11 *Phytophthora* species, among them again the most aggressive ones to the respective tree species. Each plantation contained on average 1.7 *Phytophthora* species. Together with the finding of *P. alni* in 362 alder plantations of up to 21 years of age on non-flooded sites in Bavaria (Jung and Blaschke 2004), there is now strong evidence that the planting of infested nursery stock has a major role in the spread of Phytophthora diseases of trees into forests and natural ecosystems in central Europe. In southern Europe also, a series of *Phytophthora* problems has recently been reported in nurseries (e.g. (Alvarez and others 2007, Belisario and others 2006, Moralejo and others 2007, Munda and others 2007, Nipoti and others 2005, Pane and others 2005). Likewise, recent nursery surveys in the US have also demonstrated high infestation levels (Ferguson and Jeffers 1999, Mac Donald and others 1994, Schwingle and others 2007b).

What factors are responsible for these high infestation levels, beyond the wider problem of the international movement of plants? We suggest the following. First, over the past two decades there has been a progressive development across the European nursery sector to pyramid sales: fewer and fewer large units are producing the nursery stock which is bought in and sold by the majority of small nurseries. As a result, once a new *Phytophthora* pathogen enters the nursery production sector the intensive horizontal and vertical movement of plants between nurseries has the potential to spread it quickly and efficiently. This, together with the continuous production of nursery stock received from other nurseries, may be the prime reason for the observed accumulation of *Phytophthora* species in nursery fields. A second pathway by which *Phytophthora* species are entering the nurseries is likely to be the use of unfiltered surface water for irrigation. Thus, it was recently shown from stream surveys in Europe and in the Americas and Australia that watercourses are also

infested with an array of *Phytophthora* species (see Remigi and others, Hwang and others, Smith and others, and Brasier in this volume). Third, regular use of fungistatic chemicals, in particular phosphites, in nurseries may be resulting in infected hosts on which the expression of disease symptoms is supressed. These may then be outplanted into forests because the infestation is invisible to the buyers of the plants.

The apparent scale of the nursery problem indicates that the system of growing and distributing nursery stock in Europe needs to be overhauled. One proposal is for a significant reduction in the international movement of rooted plants to prevent the initial introduction and spread of exotic Phytophthoras (see Brasier 2008). In addition as a containment measure for existing nursery infestations, we would suggest development of a "Code of 'Good Practice for Nurseries" along the lines already proposed by the EU Concerted Action project on the alder *Phytophthora* (Gibbs and others 2003). *Viz.* the production of nursery plants in steam sterilised soil mixture in containers without soil contact; the use of tap water, filtered well water or sterilised surface water for irrigation; the avoidance of fungistatic chemicals;and the exclusive purchase by retail nurseries of nursery stock from production nurseries that produce plants according to the recommended standards.

Recent Research on *P. ramorum* and *P. kernoviae* in Europe

Results of the multitude of experiments and surveys performed within the frame of the part EU-funded 'RAPRA' project on *P. ramorum* (http://rapra.csl.gov.uk) under the guidance of Joan Webber (FR, U.K.) have substantially increased our knowledge and understanding of pathogenicity, host range, distribution, and survival of *P. ramorum* and of the biological and climatic conditions favouring disease outbreaks. Major outcomes include the following.

Distribution and Host Range of *P. ramorum* in Europe

In considering the distribution of *P. ramorum* in Europe a clear distinction must be made between the nursery environment and field situations and between foliar as opposed to stem infections of trees.

Up to IUFRO 2007 *P. ramorum* has been found in nurseries of 16 European countries: Belgium, Czech Republic, Denmark, Finland, France, Germany, Ireland, Italy, the Netherlands, Norway, Poland, Slovenia, Spain, Sweden, Switzerland and the U.K. In the U.K. alone, 580 outbreaks have been recorded from nurseries and plant retail outlets. Most records came from *Rhododendron*, *Viburnum, Camellia* and *Pieris*.

Outside nurseries *P. ramorum* has been recovered from 160 locations in the U.K. while elsewhere in Europe (the Netherlands, Germany, Ireland, Norway, Slovenia and Switzerland) the number of findings varies widely but is generally much lower than in the U.K. Most records outside nurseries are coming from Rhododendron, but leaf and shoot infections are also recorded from *Quercus ilex, Castanea sativa, Fraxinus excelsior* and *Q. cerris* as well as from ornamental species of the genera *Drimys, Magnolia, Michelia, Cinnamomum* and even *Eucalyptus*, mainly from

Cornwall, southwest England. Only in England and the Netherlands, has *P. ramorum* been found causing bleeding stem lesions on a range of tree species (table 5).

Table 5—Records of *P. ramorum* from stem lesions on trees in Europe

Country	Tree species	Family	Number of trees
England	*Aesculus hippocastanum*	Hippocastanaeae	1
England	*Acer pseudoplatanus*	Aceraceae	1
England	*Castanea sativa*	Fagaceae	1
England	*Fagus sylvatica*	Fagaceae	6
Netherlands	*F. sylvatica*	Fagaceae	6
England	*Nothofagus obliqua*	Fagaceae	2
England	*Quercus acuta*	Fagaceae	1
England	*Q. cerris*	Fagaceae	5
England	*Q. falcata*	Fagaceae	1
England	*Q. petraea*	Fagaceae	1
Netherlands	*Q. rubra*	Fagaceae	8
England	*Schima* sp.	Theaceae	2
Total			35

Data from RAPRA database on naturally infected hosts, http://rapra.csl.gov.uk. Table from Webber 2008.

The outcome of susceptibility tests with *P. ramorum* on more than 260 species and reports of 140 naturally infected host species in Europe are given in two large databases on the 'RAPRA' website.

Distribution and Host Range of *P. kernoviae* in Europe

In Europe in 2007 *P. kernoviae* was still exclusively found in the U.K. Up to July 2007, 40 outbreaks had been recorded from woodlands and gardens in Cornwall Southwest England and Wales. The pathogen was also found in two U.K. nurseries. Two outbreaks in Northern England (1 nursery, 1 single rhododendron in a managed garden) have been eradicated. Until July 2007 *P. kernoviae* has been recorded from more than 20 tree species and many ornamentals. The major hosts in the U.K. are Rhododendron and European beech. Complete host lists and additional information on *P. kernoviae* are published on the Web site http://www.defra.gov.uk/planth/pkernovii2.htm.

Factors Affecting Epidemiology of *P. ramorum* in Europe

From U.K. field and experimental data and from modelling there is now clear evidence that in Europe, as in California and Oregon, *P. ramorum* needs a susceptible foliar host for inoculum buildup and suitable climatic conditions in order to infect—and initiate lesions on—stems of trees. To date stem infections have occurred only in woodlands where *Rhododendron ponticum*, itself an invasive species in the U.K., is a significant understorey species. It was shown that due to its high susceptibility and the ability to support high levels of sporulation, *R. ponticum* plays a key role in escape of *P. ramorum* into natural and semi-natural environments and the subsequent

spread to trees (Denman and others 2006, Webber 2008). Other tree and shrub species that are abundant in the understorey of some forest types in Europe, *i.e. Vaccinium* spp., *Quercus ilex*, *Rhamnus alaternus*, *Viburnum tinus* and *Arbutus unedo*, were also shown to support abundant sporulation and might enable the spread of *P. ramorum* to trees in areas such as in Iberia.

In an extensive susceptibility test with 69 woody and herbal species belonging to 24 families and 3 vegetation zones in Italy using both a leaf dip inoculation method and a wounded detached stem test, *P. ramorum* caused leaf symptoms and stem lesion on 80 percent and 100 percent respectively, of the tested plant species (Vettraino and others this volume). Generally, species belonging to mediterranean macchia vegetation were less susceptible than those belonging to cold- and warm-temperate vegetation. However, all the plant species tested supported a high rate of pathogen sporulation, even on asymptomatic leaves. The risk of establishment and spread of *P. ramorum* in Italy, based on distribution and susceptibility of potential hosts and foliar hosts for pathogen sporulation has been predicted and mapped, and 40 percent of the Italian woodland was found being suitable. In Sardinia three areas of ca 33.000 hectares have been identified as being at high risk.

A climate matching model (CLIMEX) was used by Robert Baker (CSL) to compare southern Oregon with Europe, and the comparison then revised according to Meentemeyer and others (2004). The model identified the west of the U.K., Ireland and north-west parts of France, Spain and Portugal as regions with the closest eco-climate matching in relation to *P. ramorum*. This is a similar result to CLIMEX modelling of the potential activity of *P. cinnamomi* on trees in Europe (Brasier and Scott 1994).

Potential for *P. ramorum* and *P. kernoviae* to Enter Unwounded Bark and Cause Lesions

Generally, there is good agreement between wound inoculation tests with *P. ramorum* and *P. kernoviae* on potential host species and data on host susceptibility in the field. However, there are some discrepancies in particular with *Quercus robur* and *Acer pseudoplatanus* indicating that resistance might operate at least at two levels: resistance to initial zoospore penetration on the bark surface and resistance to phloem invasion after initial infection. This conclusion was supported by several log inoculation experiments conducted by Brasier and Brown (2007).

In two *in vitro* experiments with freshly cut logs of five host species *P. ramorum* zoospores could penetrate unwounded bark—and cause lesions—on *Fagus sylvatica, Castanea sativa, Quercus rubra* and *Picea sitchensis*. However, bark of *Q. robur* was penetrated but very few lesions developed. In another experiment freshly cut logs of *A. pseudoplatanus, F. sylvatica* and *Q. robur* were placed below Rhododendron trees with multiple natural foliage infections by *P. ramorum* or *P. kernoviae*. Again, *P. ramorum* could infect bark of *F. sylvatica* and *Q. robur* but not of *A. pseudoplatanus,* and caused substantial lesions on *F. sylvatica* but not on *Q. robur*. *P. kernoviae* could penetrate unwounded bark and cause lesions on *Fagus* but not on *Acer* and *Quercus*. These results are consistent with lesion occurrence on standing trees in the field.

New Insights Into the Breeding System of *P. ramorum*

In mating studies between A1 and A2 isolates of *P. ramorum* a high frequency (*ca* 57 percent) of gametangia were abnormally developed or contained visibly aborted oospores, and an even higher proportion were classified as non-viable following vital staining (Brasier and others 2007). Nevertheless, there is clearly a potential for genetic recombination of *P. ramorum* via the sexual stage, and the recent discovery of at least three isolates in Belgium which appear to be of European lineage but of A2 mating type is alarming (Webber 2008).

Dissemination of *Phytophthora* spp. via People

From 2004–2007 *ca* 400 samples were collected from walkers' boots in the *P. kernoviae* Management Zone in Cornwall, southwest England and baited using apple traps. *Phytophthora* spp. were recovered from more than 30 percent of the samples. The most frequently isolated species was *P. citricola*, but 10–15 percent of the samples contained either *P. ramorum* or *P. kernoviae* (Webber and Rose 2008). *Phytophthora* recovery followed a seasonal pattern with spikes in June–July and October–November**.** These results indicate a high potential for spread of the species in infested soil via movement of humans, animals and machinery.

Asymptomatic Infection and Sporulation of *P. ramorum* and *P. kernoviae*

In infection trials asymptomatic infection and sporulation of *P. ramorum* and *P. kernoviae* on leaves and fruits of *Crataegus monogyna, Laurus nobilis, Q. ilex, Rosa sempervivens* and *Smilax aspera* was demonstrated and endured at least 8 and 10 days, respectively (Denman and others 2008). Also Vettraino and others (this volume) found that *P. ramorum* was able to sporulate on asymptomatic leaves of plants from Mediterranean macchia. This asymptomatic sporulation of *P. ramorum* and *P. kernoviae* raises serious questions about international plant quarantine protocols which typically rely on visual inspection of plants.

Management of *Phytophthora* Diseases of Trees in Europe

There is much valuable long term expertise on the management of *Phytophthora* diseases of trees in Australia (*P. cinnamomi* / Jarrah dieback) and the US (*P. lateralis*/Port-Orford cedar dieback). At previous meetings of this IUFRO group this has been represented by many talks, posters and exciting excursions. Steadily increasing knowledge of the ecology and epidemiology of different *Phytophthora* species, pathways, dissemination mechanisms, aetiology, host ranges and efficiency of direct control measures is leading to the first integrated management plans for *Phytophthora* diseases of trees in Europe. In the U.K. Denman and others (this volume) investigated the potential of disease control practices for the integrated management of *P. kernoviae* on mature magnolias and developed simple and easily comprehensible decision systems for landowners. Also in the U.K., an area of about 5.5 square miles in southern Cornwall with an extensive outbreak has been defined as

the *Phytophthora kernoviae* Management Zone (PkMZ). Here an intensive integrated management process is being applied in an attempt to contain this invasive pathogen.

In Germany, Austria and Italy extensive field trials were started in 2006 to test whether or not phosphite application is a useful tool for controlling *Phytophthora* diseases of mature trees of beech, linden, oak and chestnut. In southern Germany 130 mature trees of *F. sylvatica*, *Q. robur*, and *T. cordata* in five stands and in Austria 20 mature trees of *F. sylvatica* in three stands are being sprayed annually at the stem base with 50 percent potassium phosphite amended with 2.5 percent of an organosilicatious surfactant in an attempt to prevent infection. In order to discover which application method has the highest efficacy, in southern Germany a 14 ha forest with mature (80–400 years old) trees of *F. sylvatica*, *Q. robur* and *Q. petraea* is being treated from helicopters with 50 percent potassium phosphite + 0.1 percent organosilicatious surfactant. While in central Italy chestnut stands in six areas with a total of 30–35 ha are being treated with stem injections of potassium phosphite. Preliminary results from all trials show considerable improvements of the crown conditions of treated trees as compared to untreated control trees (T. Jung and M. Blaschke unpublished results, T. Cech personal communication, A. Vannini unpublished results).

Resistance screening can also be an option for the management of *Phytophthora* diseases, as has been shown *P. lateralis* onPort-Orford cedar (Hansen and others 2000, Sniezko this volume; Sniezko and Hansen 2003). As discussed earlier in this review, Robin and others (2006) found *P. cambivora* resistant trees in 15 chestnut populations from different European countries while Moreira and others (this volume) found that maternal progeny significantly influenced seedling survival in *P. cinnamomi* infested cork oak stands. Jung and Blaschke (2006) demonstrated that surviving alder trees in riparian ecosystems affected by the aggressive *P. alni* ssp. *alni* for more than 10 years often have a significantly lower susceptibility than declining alder trees. Such results are very promising and could yet be the basis of resistance screening programmes for chestnut and black alder in the future.

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Phytophthora Research and Management in Australasia[1]

Giles E.St.J. Hardy [2]

Abstract

This article provides a brief overview of the status of *Phytophthora* diseases in Australasia. *Phytophthora cinnamomi* remains the key species contributing to the decline of many wildland ecosystems throughout Australia. Recent molecular studies indicate that many new species exist in culture collections that need describing and their role as pathogens fully ascertained. An overview of the science, management and communication processes being undertaken to reduce the impact of *P. cinnamomi* is also covered for each Australian State and for New Zealand. Activities across the region vary considerably and reflect the amount of resources that are being provided, which are often minimal.

Introduction

In Australia, *Phytophthora cinnamomi* remains the major *Phytophthora* pathogen and continues to impact on the function and health of many wildland ecosystems. For example, in the South-West Botanical Province of Western Australia it impacts on over 3000 of 5710 described plant species (Shearer and others 2004). It is widespread throughout Australia and is located mainly in regions with higher than 600 mm rainfall (fig. 1). Unless significant advances are made in control, *P. cinnamomi* will continue to move autonomously and by inadvertent anthropogenic spread through many susceptible wildland plant communities for many decades to come.

Phytopthora ramorum is listed as a Category 1 quarantine pest in Australia. The CRC National Plant Biosecurity and the Department of Environment, Water, Heritage and Arts are currently funding a Ph.D. research project (see Hüberli and others this proceedings) to determine the possible host range of Australian flora and their ability to support sporulation by *P. ramorum.* This work is being conducted in David Rizzo's laboratory at the University of California, Davis.

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26–31, 2007, Monterey, California.

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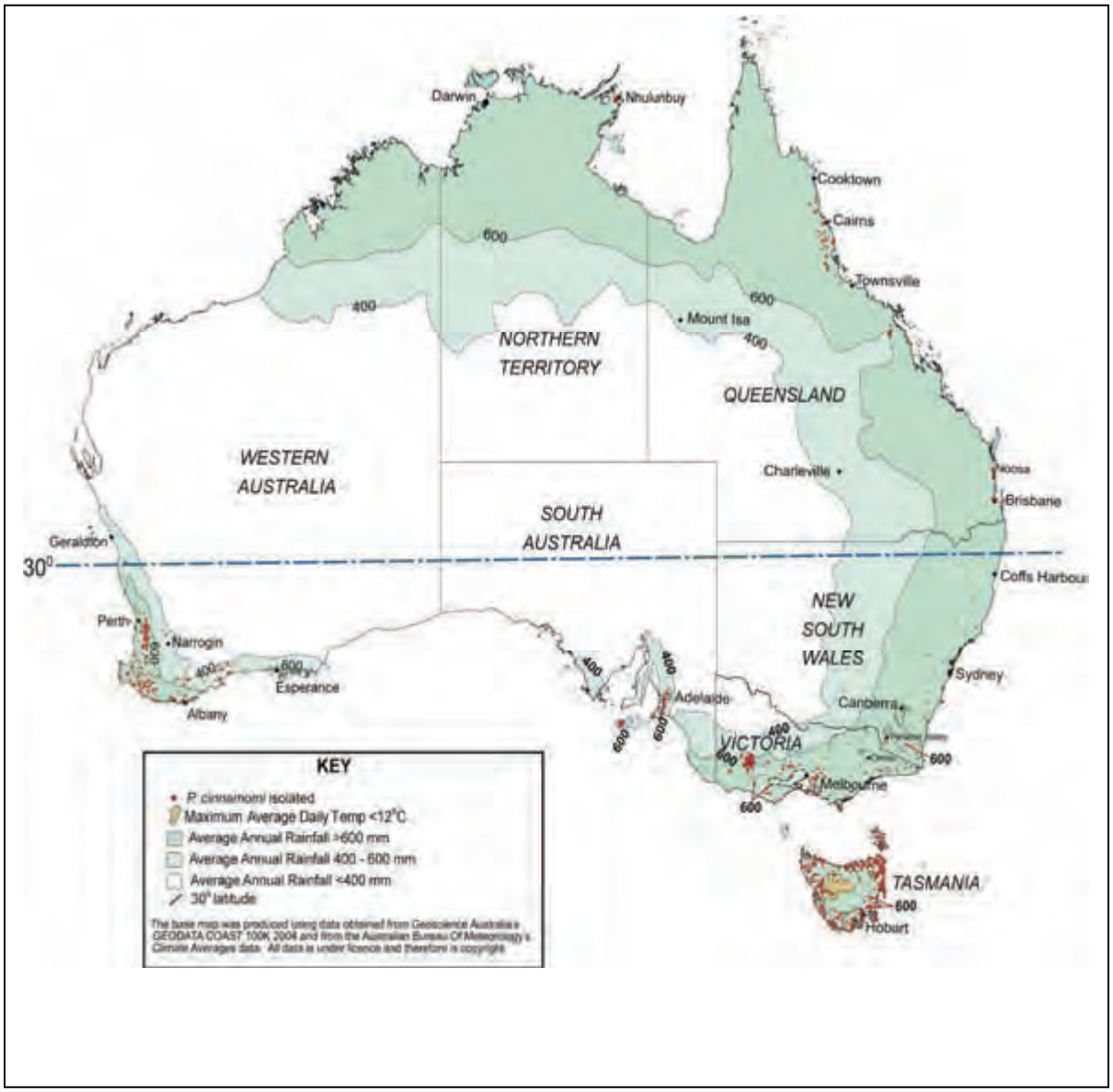

Figure 1ð *Phytophthora cinnamomi* isolations and broad climatic envelope of *P. cinnamomi* susceptibility in Australia (O'Gara and others 2005).

Western Australia

New Phytophthora *species*

Recently many isolates in the culture collections of the Centre for Phytophthora Science and Management and the Department of Environment and Conservation (DEC) have been sequenced using the ITS region of genomic rDNA (Cooke and others 2000) to confirm previous morphological identification and to determine what *Phytophthora* species are present in the State. This work indicates that there are at least 10 new undescribed species (fig. 2).

Prior to the availability of molecular tools for use in diagnostics, a number of these isolates had been submitted to world culture collections (IMI and CBS) and allocated to already designated species based on morphological features. For example, many P. sp. 4 isolates had previously been designated as *P. citricola,* P. sp. 3 as *P. drechsleri*, P. sp. 1 as *P. boehmeriae* and P. sp. 10 as *P. megasperma* var. *sojae* (table 1). All new species were isolated from plants with disease symptoms or from the soil rhizosphere of plants expressing disease symptoms. Pathogenicity tests to confirm Koch's Postulates are currently being undertaken. It is likely that all of these putative new species are pathogens; however, this needs to be confirmed. It will be interesting in the future to determine whether these *Phytophthora* species are endemic or introduced to Australia. This knowledge will help provide clear management and quarantine goals. However, irrespective of whether they are endemic or not, they should be managed to reduce their spread around the country. This will help reduce the chance of them being introduced to areas free of *Phytophthora* species and the risks of hybridization occurring. More importantly it is critical to keep in mind that global climate change may create more conducive conditions for many of these *Phytophthora* species to impact on wildlands in a similar fashion to *P. cinnamomi.* For example, P. sp. 4 has a wider distribution than *P. cinnamomi* and has been associated with plant deaths on calcareous soils where *P. cinnamomi* does not cause disease. It is also associated with deaths of *Eucalyptus gomphocephela* (see Scott and others, this proceedings).

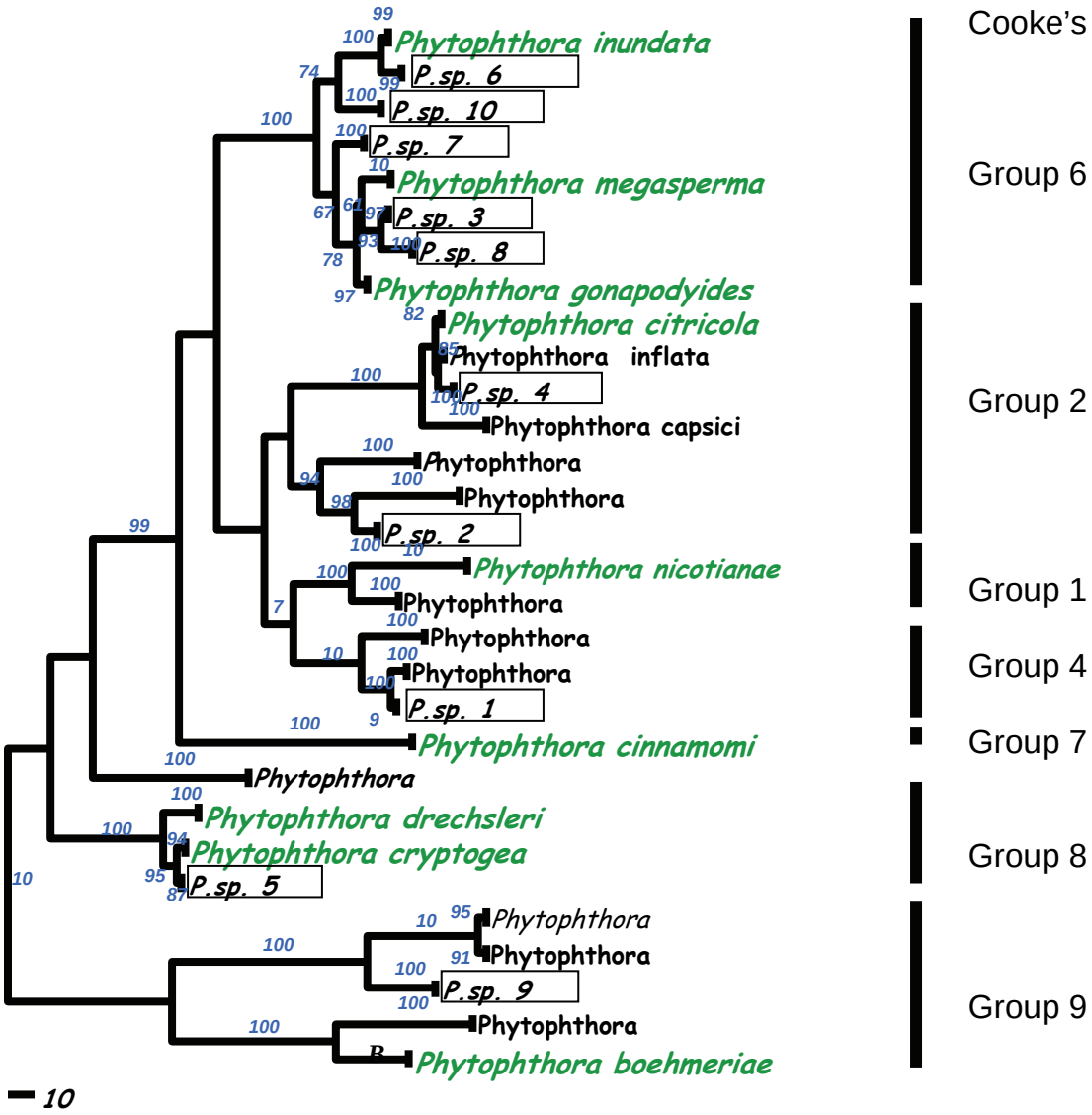

Figure 2ð *Phytophthora* species (in italics) that are present in Western Australia and where they sit with regards to Cooke's ITS Clades (Cooke and others, 2000). P. sp. isolates (Psp1 – Psp10) remain undescribed (Tree Courtesy Treena Burgess).

Table 1ð Examples of new Western Australian *Phytophthora* species based on molecular traits previously described as other *Phytophthora* species based on morphological traits (IMI = International Mycological Institute, U.K.; CBS = Centraalbureau voor Schimmelcultures, Netherlands).

Isolate	Morphological	DNA
DDS 1450	*P. citricola* (IMI 329674)	P. sp. 4
DCE 444	*P. drechsleri* (A1) (IMI 329666)	P. sp. 3
TCH 009	*P. megasperma* var. *sojae* (IMI 329669)	P. sp. 10
DDS 3884	*P. boehmeriae* (CBS 100410)	P. sp. 1

P. sp. 10 previously described as *P. megasperma* var. *sojae* has been observed to have a high impact on many Proteaceous species in the Fitzgerald River National Park in the south-west of Western Australia, particularly after very wet rainfall events. Whilst, P. sp. 4 appears to have a wider distribution than *P. cinnamomi* as it is also associated with plant deaths on calcareous soils. Therefore, with the availability of molecular diagnostic tools there is no doubt more species will be found, and whilst *P. cinnamomi* remains the major *Phytophthora* pathogen in wildlands, it is critical that attention is also paid to new species as they are identified.

Bell Track Infestation

The Fitzgerald River National Park (FRNP) in the south-west of Western Australia is one of the World's 25 Biodiversity 'hotspots' with large numbers of endemic flora and fauna. There is a 195 ha *P. cinnamomi* infestation in the park known as the 'Bell Track Infestation' that threatens in the immediate future some 6,000 ha of wildlands with many additional thousands of ha in the long-term if it breaks out of its existing catchment. The Department of Environment and Conservation have established two key goals to manage this infestation: first, to contain the further spread of *P. cinnamomi,* and second, to protect the unique biodiversity values of the FRNP from *P. cinnamomi*. In order to achieve this, extensive aerial mapping, digital elevation models and hydrological investigations have been conducted of the infestation together with extensive and intensive mapping of the pathogen through soil baiting and molecular diagnostics (see Anderson and others, this proceedings). Many thousands of soil samples have been baited to ensure accurate mapping of the pathogen. These activities have allowed detailed information to be gathered of stream paths, and where water will accumulate and be shed from given 50 and 100 year extreme rainfall events. Natural sumps were also identified. This information has in turn allowed for the planning of storage gully dams and flood diversion drains across catchments to be installed. These are intended to be able to manage extreme rainfall events and prevent the movement of *P. cinnamomi* infested water into adjacent disease-free catchments. The information has also facilitated the installation of a 13 km fence around the infestation. This has been installed to prevent movement of the pathogen by native fauna such as kangaroos and to prevent any unintentional anthropogenic movement. In addition, approximately 2 km of high density polyethylene sheeting has been buried at points adjacent to uninfested catchments at

approximately 80 cm depth to act as a barrier to prevent root to root movement of the pathogen. Currently, there are discussions on whether to incorporate the eradication and containment methods developed by Dunstan and others (see this Proceedings) to reduce the impact of *P. cinnamomi* at Bell Track.

The infestation at Bell Track has been treated regularly over the past decade with aerial applications of phosphite (phosphonate) (24 kg/ha a.i.) at low volume. These applications have been highly efficacious in reducing the impact and spread of *P. cinnamomi* at Bell Track. In addition, at Bell Track along approximately 30 ha of the immediate disease front and at two other infested sites the trunks of woody Proteaceous species have been sprayed to run-off with 30 percent a.i. phosphite together with Pulse. This has been done to address four questions; 1) does high intensity phosphite application prevent or reduce the rate of spread of the pathogen? 2) does high intensity phosphite application reduce inoculum levels ahead of a disease front? 3) does high intensity phosphite application reduce mortality of susceptible plant species? and, 4) does high intensity phosphite application effect the epidemiology of the pathogen?

Finally, due to the existing high impact of *P. cinnamomi* in the Bell Track and the loss of many of the deep rooted mid and upper story canopy species, many areas of the infestation have a changed hydrological balance and will be subject to massive surface water flow in a high rainfall event. Therefore, to re-establish natural hydrological balance across the infestation, DEC have put in place a revegetation program with an emphasis on deep rooted, long lived, greater than 0.5 m in height, rapidly growing, easily cultivated and *P. cinnamomi* resistant species that are local to the region. After extensive screening, *Acacia saligna*, *Calothamnus quadrifidus* and *Eucalyptus pleurocarpa* have been selected for establishment on the site.

Mapping and Awareness

In Western Australia, ‘Project Dieback’ funded by the National Heritage Trust through the Department of Environment, Water, Heritage and the Arts have made significant contributions to *Phytophthora* dieback issues in the State. In particular, they have put substantial effort into raising and obtaining community awareness and engagement, and towards mapping accurately the distribution of *Phytophthora* throughout the south-west of Western Australia. The mapping will provide managers and community groups with an accurate ‘Dieback’ occurrence map which will allow for assessment methodology for strategic regional planning over the next 5-10 years. Based on the mapping and knowledge of susceptible and threatened species and communities, key uninfested areas of wildlands that are considered protectable can be allocated resources to ensure that they remain protected. In addition, the mapping can be used to predict autonomous spread and to help determine where controls such as phosphite applications can be applied. ‘Project Dieback’ has also developed signage (fig. 3) that is being utilised throughout the region by government and local government agencies. Mining companies and other industries that manage activities in wildlands are also utilising the signage. This will ensure the community in general is not confused by different dieback signs on different land tenures.

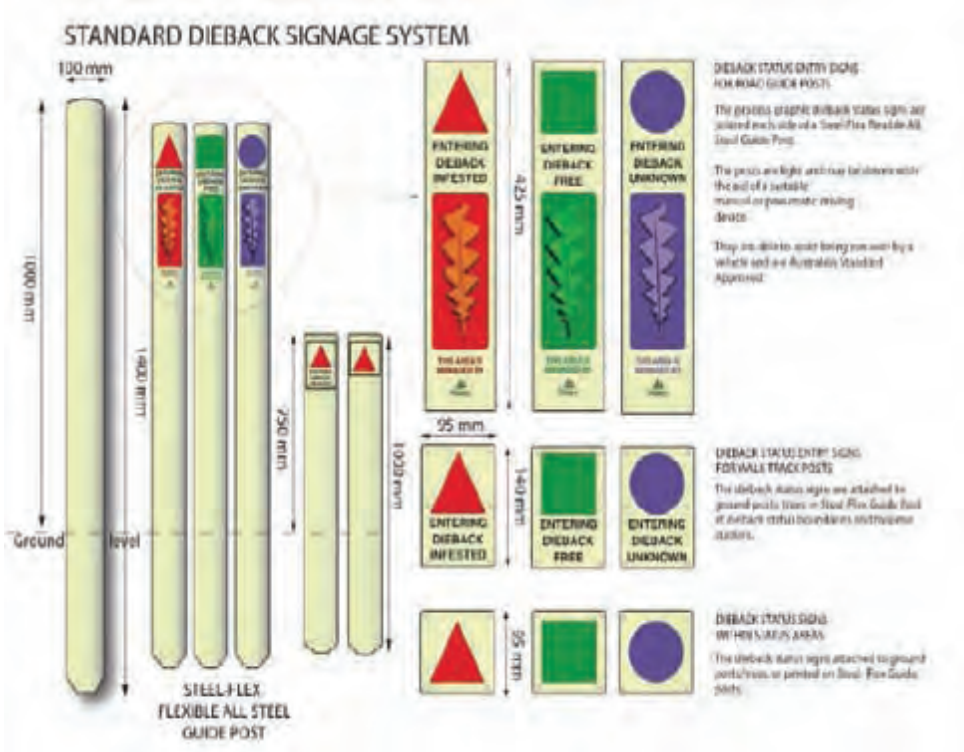

Figure 3ð Signage now in place to clearly articulate to land users the 'Phytophthora dieback' status of an area (Courtesy Project Dieback and the Department of Environment and Conservation).

Other Research in Western Australia

A number of other research activities are in progress and these include: 1) Influence of physiological stresses (fire/waterlogging/drought) on phosphite applications and pathogen control (Hüberli and others), 2) Eradication and containment from infested sites in wildland ecosystems (Dunstan and others (see this proceedings), 3) Screening Australian native plants for susceptibility to *P. ramorum* (Ireland and others (see this proceedings), 4) Taxonomy of new *Phytophthora* species in Australia, 5) Role of fire and fire frequency and its impact on *P. cinnamomi* in wildland ecosystems, 6) To investigate the effects of phosphate and phosphite on *P. cinnamomi* control using *Arabidiopsis thaliana*, 7) Understanding the mechanisms by which phosphite affects the pathogenicity of *P. cinnamomi*, 8) New methods for measuring phosphite *in planta*, 9) Impacts of *P. cinnamomi* on biodiversity values in wildlands, 10) Microarray analysis of the effect of phosphite on *Phytophthora cinnamomi.*

Victoria

Research

Current research activities include examining the durability of resistance of susceptible eucalypts regenerating on ex-dieback sites. These if shown to be tolerant or resistant to *P. cinnamomi* have the potential to be utilised as potential seed sources for further restoration work on dieback sites. Surveys are being conducted in streams, rivers and other water bodies in natural ecosystems to determine what *Phytophthora* species are present in Victoria. Finally, an assessment of the risk posed by *Phytophthora* species to natural ecosystems is on-going.

A large research project conducted out of D. Cahill's laboratory is aimed at defining plant resistance against *P. cinnamomi* and the application of resistance to revegetation. This work aims to: 1) determine at the cellular, biochemical and molecular levels what constitutes resistance of native plants to *P. cinnamomi*, 2) identify the pathways within plants that regulate resistance and then explore the potential to manipulate these, 3) examine resistance in native stands affected by the pathogen, 4) use knowledge of resistance to implement field trials in sites that encompass a range of environments, and 5) utilise model systems such as *Arabidiposis*, *Populus deltoids* and *P. tremula.*

Management

In Victoria, *P. cinnamomi* is listed as a 'potentially threatening process' under the Flora and Fauna Guarantee Act 1988. A Strategic Management Plan to address the threat of *P. cinnamomi* on public land is in place. The key elements of the plan are (1) the protection of the most important assets at highest risk, (2) development of Regional Management Plans to zone areas by level of risk, (3) clear cross-agency and cross-tenure governance arrangements are being proposed, and (4) the development of 'Best Practice Guidelines'. As part of these actions and to reduce the spread of the pathogen portable hygiene (soil wash-down) modules for operations such as fire or forestry operations are being developed. Footwear washing units have also been built and placed on walking tracks to reduce the spread of the pathogen.

New South Wales

Research

Susceptibility trials have commenced on *Eucalyptus imlayensis* an endangered mallee of south-eastern New South Wales. Eradication of a *P. cinnamomi* front moving towards a Wollemi Pine (*Wollemi nobilis*) stand is being attempted. Wollemi Pine was discovered in 1994 and is a member of the Araucariaceae and less than 100 mature trees are known to exist. It is susceptible to *P. cinnamomi.* Eradication of the pathogen is being attempted ahead of the front, a site of approximately 50 m, using soil drenches of fungicides and trunk injections of phosphite (phosphonate).

Management

A Statement of Intent is in preparation for *P. cinnamomi*, this is the first to be done in New South Wales under new legislation. A phosphite spray program has been instigated in an infested area of the South East Forests National Park to protect the habitat of the smoky mouse (*Pseudomys fumeus*), an endangered species. Many of the plants in its preferred habitat are susceptible to *P. cinnamomi*. Hygiene protocols are also in place to reduce infested soil movement between diseased and disease-free areas of its habitat.

A survey and management plan have being conducted and developed, respectively, for the Sydney and Metropolitan and Hawkesbury Nepean Catchments.

South Australia

Management of *Phytophthora* in infested areas together with preventing disease-free areas becoming infested remain priorities. Research at the University of Adelaide is being conducted on understanding the impact of *P. cinnamomi* on native vegetation in South Australia with an aim to gain knowledge on the susceptibility of threatened plant species, patterns of *Phytophthora* spread and the role of soil microbiota in disease suppression.

Tasmania

In addition to the eradication trials of Dunstan and others (this Proceedings), eradication trials of infested sites are being proposed on a tin mine. Due to the changing of plant species names a reassessment of species susceptibility is in progress with the aim to upgrade the species susceptibility list.

New Zealand

A major collaborative project between Landcare Research, Ensis and HortResearch has been using ITS sequences to verify the *Phytophthora* species present in New Zealand and to develop a rapid DNA based identification scheme. This program will continue with on-going surveillance as part of routine diagnostic work. The presence of 21 species in the country has been confirmed with two newly described species, *P. europaea* and *P. kernoviae* being confirmed, with an additional two putatively new species to be described. *P. inundata* was also recorded as a new-to-New Zealand record in 2007.

A number of other research projects are in progress. These include research on: the epidemiology and population dynamics of *P. kernoviae* (Ramsfield and others, see this Proceedings); the identity and epidemiology of *Phytophthora* taxon Agathis (Beever and others, see this Proceedings); the treatment of nursery-grown *Nothofagus* seedlings for planting into *P. cinnamomi* infested soils (Johnston and others see this Proceedings); the control of *Phytophthora* in forest nurseries through induced resistance (Dick and others, see this Proceedings).

Conclusion

Many new species are being recorded in Australasia which is not surprising given the availability of molecular diagnostic tools. With increased activity in baiting streams and water sources (Ian Smith and Daniel Hüberli, *pers com.*) it is likely many more will be found. There have been examples of successful containment and eradication of *P. cinnamomi* from sites in Tasmania and Western Australia. There has been a very active increase in the Strategic Management of *Phytophthora* across Australia, together with a massive increase in community and agency involvement in the process. The National Resource Management regions across Australia have been incorporating *Phytophthora* into their management plans and developing 'cross regional' coordination. All of which are major advances in the last three years. Despite these activities there is an urgent need for adequate funding for management, communication and research to adequately manage *P. cinnamomi* in wildlands. A recent cost-benefit analysis study estimates that the Net Present Value of biodiversity assets at risk from *Phytophthora* dieback is approximately Aus $6.4 billion. Loss of carbon sequestration due to the death of woody plants is not included in this calculation. It is also estimated that an investment of Aus $58 million over 7 years will reduce losses by Aus $500 million. Therefore, reasonably modest investments into science, communication and management of *Phytophthora* diseases are required to derive considerable outcomes in managing biodiversity assets from *Phytophthora* dieback.

Acknowledgments

Many thanks to Keith McDougall (NSW), Rosalie Daniel (NSW), David Guest (NSW), Ian Smith (Victoria), David Cahill (Victoria), Renata Velzeboer (South Australia), Annabelle Bushell (WA), Joanna Young (WA), Greg Strelein (WA), Tim Rudman (Tasmania), Margaret Dick, Tod Ramsfield, Ross Beaver and Ian Horner (New Zealand) who kindly contributed information about *Phytophthora* from their respective States or New Zealand. Daniel Hüberli kindly reviewed this paper.

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Phytophthora in Forests and Natural Ecosystems of the Americas[1]

David Rizzo[2] and Elizabeth Fichtner[2]

Abstract

This article provides a brief overview of the status of *Phytophthora* diseases in the Americas. Although *Phytophthora ramorum* has stolen many of the recent headlines, much work has advanced on many other Phytophthoras ranging from old (e.g., *P. cinnamomi*, *P. lateralis*) to many recently described species such as *P. siskiyouensis* and *P. austrocedrae*. *Phytophthora cinnamomi has* been shown to threaten native vegetation in the rare Ione chaparral ecosystem in the western Sierra Nevada foothills of California. Resistance programs developed in Oregon have made significant advances in identifying resistant lines of Port-Orford cedar (*Chamaecyparis lawsoniana*). *Phytophthora siskiyouensis*, described from coastal Oregon and California streams has been found associated with urban tree decline in central California and in Australia. In Argentina, mortality of *Austrocedrus chilensis* in Patagonia "Mal del Cipre´s" has been studied for many years and while several *Phytophthora* species were initially cultured from soils associated with declining *A. chilensis* forests, *P. austrocedrae* a new species, was isolated from necrotic lesions of stem and roots of *A. chilensis*. Research on all *Phytophthora* species has been conducted at multiple spatial and temporal scales and encompasses all parts of the host-pathogen interactions.

Introduction

Since the first meeting of the IUFRO Phytophthora group in 1999, our knowledge of the biology, diversity, ecology and epidemiology of Phytophthoras in forests of North America has greatly expanded. Although, *Phytophthora ramorum* has stolen many of the recent headlines, much work has advanced on many other Phytophthoras ranging from old (e.g., *P. cinnamomi*, *P. lateralis*) to many recently described species. Research has been conducted at multiple spatial and temporal scales and encompasses all parts of the host-pathogen interactions. The genome of *P. ramorum* has been sequenced leading to a number of papers on comparative genomics with *P. sojae* and *P. infestans*. Field studies have begun to elucidate the epidemiology and management of *Phytophthora* from the plot to the landscape level. One of the most important developments is the increased emphasis on the connections between the ornamental horticultural industry and native ecosystems. At each meeting of the IUFRO working group, increasingly more presentations have discussed the role of nursery plants in the spread and establishment of *Phytophthora* in forests.

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Monitoring for Phytophthoras in North America

Following the finding of *P. ramorum* in California, there have been increased levels of monitoring of both nurseries and natural ecosystems throughout the United States, Canada and Mexico. Such monitoring efforts have been designed to support regulations and to enhance the application and efficacy of mitigation actions (e.g., eradication efforts) by enabling early pathogen detection. Monitoring has led to the discovery of a number of new species as well as increasing the geographic and host ranges of a number of previously described *Phytophthora* species. The diversity of *Phytophthora* in natural ecosystems is quite astounding and new species have often been found in forests and nurseries that have already been extensively examined. Table 1 illustrates examples of new species of *Phytophthora* associated with trees or shrubs that have been described since the first IUFRO *Phytophthora* meeting in 1999. One fascinating, and somewhat disconcerting, finding has been that several of these species were already known from other continents (*P. pseudosyringae, P. europaea, P. hedraiandra, P. ramorum*) at the time they were first observed in North America or were noted on another continent soon after they were first described in the Americas (*P. siskiyouensis*).

Table 1. Examples of *Phytophthora* species that have been described in the Americas since the first IUFRO Phytophthora conference in 1999. Those marked with an asterisk (*) represent species that had previously been described from another continent or were noted on another continent soon after they were described in the Americas.

Phytophthora species	Habitat	Location	Reference
*P. ramorum**	forests, nurseries	CA, OR	Rizzo and others 2005
*P. europaea**	forests	eastern US, CA	Balci and others 2007; Rizzo, unpublished
*P. pseudosyringae**	forests, nurseries	CA, OR, NC	Hwang and others 2007; Wickland and others 2008
P. nemorosa	forests, nurseries	CA, OR	Hansen and others 2003, Wickland and others 2008
*P. hedraiandra**	nurseries	MN, NC	Schwingle and others 2007
P. foliorum	nurseries	CA, TN	Donahoo and others 2006
P. austrocedrae	forests	Argentina	Greslebin and others 2007
*P. siskiyouensis**	forests, urban	CA, OR	Reeser and others 2007, Rooney-Latham and others 2007, Smith and others 2006
P. quercina-like	forests	eastern US	Balci and others 2007, Schwingle and others 2007b

In forests, broad scale sampling has been accomplished through soil (Balci and others 2007, Schwingle and others 2007a) and stream monitoring (Hwang and others 2007, Wamishe and others 2007). Much of this has been accomplished via a national survey utilizing stream monitoring (Oak and others 2006). In the eastern United States, a number of Phytophthoras common to temperate forest regions of Europe have been found in soils and streams including *P. cinnamomi* and *P. cambivora*. *P. pseudosyringae* and *P. europaea* have only recently been described in Europe, but have turned out to be fairly common in eastern US forests. To date, *P. pseudosyringae* and *P. europaea* have only been observed in water courses or soils in the eastern US, but not from infected plant material. In contrast, both of these species have also been described from infected plant material in California and Oregon forests; *P. pseudosyringae* from cankers and foliar infections (see below) and *P. europaea* from infected foliage of bay laurel (*Umbellularia californica*). *Phytophthora quercina* was originally described in Europe in the mid-1990s in areas associated with oak decline (Jung and others 1999). Balci and others (2007) recently described a *P. quercina*-like taxon from soils in the central US; Schwingle and others (2007a) described a similar isolate from Missouri soils as *P. quercina.* At this time it is not clear of the final identity of these taxa (Balci, personal communication). New species descriptions of this and other taxa will most likely result from this work (Balci, personal communication).

Because of quarantine regulations, a much greater effort has been made to identify Phytophthoras recovered in ornamental nurseries to the species level. Recent nursery surveys have turned up new species (e.g., *P. foliorum*) as well as a number of surprising species formerly unknown in the nursery industry. Yakabe (2007) conducted a survey of aerial Phytophthoras in California nurseries, finding many of the usual suspects such as *P. cactorum, P. syringae,* and *P. citricola* that have long been associated with ornamental plants. However, she also identified a number of species that have typically been associated only with forest plants and streams, including *P. nemorosa, P. pseudosyringae,* and *Pg* "chlamydo".

New Phytophthoras

Three recently described species illustrate how much more we need to learn about *Phytophthora* biodiversity and biogeography. Reeser and others (2007) have described *P. siskiyouensis* from southwestern Oregon. This species was recovered from leaf baits while monitoring streams and soils for *P. ramorum*. It was also isolated from a blighted bay laurel shoot and tanoak cankers. Based on their research, Reeser and others considered *P. siskiyouensis* be native to coastal Oregon and possibly the Pacific Northwest. However, around the time it was described, *P. siskiyouensis* was isolated from Italian alder (*Alnus cordata*) in an urbanized area in Foster City, California (Rooney-Latham and others 2007). It was also isolated from *Alnus glutinosa* in a garden in Melbourne, Australia and reported as *Phytophthora* sp. at the 3rd IUFRO meeting (Smith and others 2006). Interestingly, *P. siskiyouensis* has not been isolated from alder in the area from which it was first described.

Another previously unknown species, *P. foliorum*, was discovered during nursery surveys because its genomic DNA cross-reacted with the ITS-based diagnostic PCR primers used to screen plants for the presence of *P. ramorum* (Donahoo and others 2006). *Phytophthora foliorum* was isolated from evergreen hybrid azalea leaves

collected in nurseries in both California and Tennessee. Sequence data indicate that *P. foliorum* fits into the same clade as *P. ramorum, P. lateralis* and *P. hibernalis* (Donahoo and others 2006, Blair and others 2008).

In Argentina, mortality of *Austrocedrus chilensis* in Patagonia 'Mal del Cipre´s'has been studied for many years and while several *Phytophthora* species were initially cultured from soils associated with declining *A. chilensis* forests, none were shown to have a clear relationship with tree death (Greslebin and others 2007). Eventually, a new species, *P. austrocedrae,* was isolated from necrotic lesions of stem and roots of *A. chilensis*. Once again, as with many of the other Phytophthoras discussed here, there appears to be strong linkages to nursery crops leading to the establishment of *P. austrocedrae* in the forest (Greslebin and Hansen, this volume).

Phytophthora nemorosa and *P. pseudosyringae*

While *P. ramorum* gets most of the attention, two of the most common Phytophthoras isolated from forest trees in coastal areas of California and Oregon are actually *P. nemorosa* and *P. pseudosyringae*. As with many of the species described above, they were initially found during surveys for *P. ramorum*. In California both species are primarily isolated from foliage of bay laurel and symptoms of *P. nemorosa* and *P. pseudosyringae* on all hosts are indistinguishable from the symptoms caused by *P. ramorum* (Wickland and others 2008).

Phytophthora nemorosa is found from Monterey Co. in central California to Coos Co. in central Oregon. These sites are primarily distributed within 50 km of the Pacific Ocean. However, *P. nemorosa* has also been isolated from bay laurel foliage in Yosemite Valley in the central Sierra Nevada Mountains. *Phytophthora nemorosa* is primarily found infecting trees in cooler, wetter coast redwood forests and less often in drier, warmer mixed-evergreen forest dominated by coast live oak. In California, the pathogen is most often isolated from bay laurel leaves, stems and twigs of tanoak, and leaf and small stem tissue of redwood. *P. nemorosa* has occasionally been isolated from bleeding cankers on coast live oak stems and one time each from necrotic branch tissue on a Douglas-fir (*Pseudotsuga menzesii*) sapling and from leaf tissue of hazelnut (*Corylus cornuta*), evergreen huckleberry (*Vaccinium ovatum*) and honeysuckle (*Lonicera hispidula*).

Phytophthora pseudosyringae is found a bit further south than *P. nemorosa* and ranges from San Luis Obispo Co. to Curry Co. in southern Oregon. It also was isolated from bay laurel leaves in Yosemite Valley and in the northern Sierra Nevada Mountains (Butte County). In contrast to *P. nemorosa*, *P. pseudosyringae* is primarily isolated from hosts found in coast live oak woodlands in California and only occasionally found in more mesic coast redwood forests. Bay laurel is the most common host while infection of coast live oak stems is less frequent. *Phytophthora pseudosyringae* has been isolated a single time each from foliage of big leaf maple (*Acer macrophyllum*) and manzanita (*Arctostaphylos* sp.)

Much more needs to be determined regarding the life histories of *P. nemorosa* and *P. pseudosyringae* including their complete host range, methods of dispersal, and summer survival in California's Mediterranean climate. For example, in European oak and beech forests *P. pseudosyringae* is described as a soil inhabitant causing root

and crown rot. Although this species was occasionally isolated from crown rot on coast live oak in California, it was primarily isolated from foliage on bay laurel. The soil phase of *P. pseudosyringae* in California forests needs additional investigation as do the aerial aspects of its biology in European forests.

Phytophthora cinnamomi Update

Phytophthora cinnamomi has long been important in forests, orchards and ornamental settings in the Americas and much of the history of this species has been reviewed in previous IUFRO proceedings (Hansen 1999, Goheen and Hansen 2006). *Phytophthora cinnamomi* continues to be important on oaks and chestnut in the southeastern USA, in southern California on coast live oak, and in oaks in forests of central and southern Mexico. For example, more recent studies on oak decline in Mexico have been carried out in five states of Mexico: Aguascalientes, Colima, Guanajuato, Jalisco and Nayarit. Based on their incidence, *Phytophthora cinnamomi*, *Hypoxylon antropunctatum*, and drought were considered the most important contributors to the decline and death of the oaks (Alvarado-Rosales and others 2007). Furthermore, *P. cinnamomi* was the most frequently recovered *Phytophthora* from soils surveyed in association with oak species in the northeast and mid-western United States (Balci and others 2007).

Given California's Mediterranean climate, it has been somewhat surprising that *P. cinnamomi* has not been reported more often from native ecosystems. *Phytophthora cinnamomi* has long been known in California from fruit tree orchards, vineyards, Christmas tree plantings, and in ornamental settings. It was first reported on ornamental oaks (*Quercus agrifolia, Q. suber*) in the state in 1975 (Mircetich and others 1977). In 2001, *P. cinnamomi* was first shown to cause disease of oaks in natural oak woodlands of California (Garbelotto and others 2006), and recent studies have further elucidated the importance of *P. cinnamomi* in other California natural systems.

Phytophthora cinnamomi has been shown to threaten native vegetation in the rare Ione chaparral ecosystem in the western Sierra Nevada foothills of CA (Swiecki and others 2003 2005). *Phytophthora cinnamomi* causes root and crown rot of Ione manzanita (*Arctostaphylos myrtifolia*), a rare plant limited to the unusually acidic "Ione" soils containing cement-like crusts of yellow iron-oxide, a characteristic typical of tropical soils. *Phytophthora cinnamomi* has caused large patches of ione manzanita mortality, and genetic evidence suggests that the pathogen has been introduced on multiple occasions, with subsequent spread emanating from the initial points of introduction. The long-term implications of these infestations on the ione manzanita are unknown, but the plant is currently considered threatened.

In the San Francisco Bay area, *P. cinnamomi* has been found in coast live oak forests in areas impacted by *P. ramorum* (Swiecki, unpublished). The interesting aspect of this finding is that the main hosts appear to be madrone (*Arbutus menziesii*), manzanita (*Arctostaphylos sp.)* and bay laurel rather than the native oaks. *P. cinnamomi* has been isolated from the roots of declining bay laurel and madrone on the edge of the dieback zone (Swiecki and Fichtner, unpublished), but also from soils in areas outside the dieback zone. It is not yet known whether *P. cinnamomi* is widely distributed in these native forests, but only causing mortality after *P. ramorum*-

related disturbance, or whether it was recently introduced. The linkage of *P. ramorum* and *P. cinnamomi* on individual sites has very important implications for forest dynamics and ecosystem restoration. Furthermore, the purchase of nursery-reared plants for laboratory inoculations has uncovered other, common *Phytophthoras* on madrone; highlighting the potential for introduction of other forest pathogens through revegetation programs (Fichtner, unpublished).

Phytophthora ramorum Update

So much has happened in *P. ramorum* research, management, and regulation over the past three years that it would take a review article to discuss all of the new findings and research directions. Many papers have been presented at this conference on *P. ramorum* and these proceedings include topics spanning pathogen biology and genetics to epidemiology and management. We will highlight several recent lines of research.

Recent evidence of xylem infection of *P. ramorum* in naturally-infected trees suggests a reconsideration of the distinction between canker diseases and vascular wilts (Parke and others 2007; Brown and Brasier 2007). Historically, canker-causing *Phytophthora* species were generally believed to incite disease by killing the phloem; however, Parke and others (2007) have demonstrated that colonization of tanoak xylem by *P. ramorum* results in reduced water transport and may be a physiological explanation for the "sudden" death of tanoak under conditions of water stress. The group proposes that the colonization of non-living vessels offers a conduit for lateral movement of the pathogen within the host with protection from host defenses.

The survival potential of *P. ramorum* in soils and soilless media and the potential for the pathogen to initiate root infections have been investigated both to enhance understanding of pathogen transmission pathways and assess the risk of establishment in new ecosystems. The pathogen can oversummer in forest soils in California, but survival is limited by soil-drying conditions (Fichtner and others 2007). Chlamydospores have been shown to survive adverse temperature conditions for a few days, suggesting the potential for pathogen survival in many eastern United States ecosystems and highlighting the risk associated with potential introduction of the pathogen to these systems (Tooley and others 2008). Natural infection of tanoak roots by *P. ramorum* has been reported in infested California forests (Parke and others 2005), but the range of forest plant species supporting root infections and the prevalence of root infections is unknown. Shishkoff (2007) has demonstrated that *P. ramorum* can infect roots of multiple ornamental perennial trees and shrubs and also some species native to invaded California forests, including bay laurel. *Phytophthora ramorum* may persist for months in soilless potting media (Shishkoff 2007, Linderman and Davis, 2006), and infested potting media may serve as an inoculum source for root infections in container–grown plants (Parke and Lewis 2007). Chlamydospores have been shown to germinate to form sporangia in the vicinity of roots and serve as primary inoculum for root infections (Shishkoff 2007).

The sequence of the *P. ramorum* genome was published in 2006 (Tyler and others 2006). This data has lead to a number of comparative genomics papers looking at *P. ramorum* and *P. sojae*. More importantly from a sudden oak death perspective, the genome sequence has allowed for the development of microsatellite markers for use in *P. ramorum* population genetic studies (Ivors and others 2006, Prospero and others

2007). Linkage of spatially-defined, population genetic analyses has allowed for indirect assessment of pathogen dispersal distances and the reconstruction of the spread of *P. ramorum* across landscapes (Mascheretti and others, in press; Hansen and others 2008).

Extensive ground-based monitoring, aerial images, GIS technology, and modeling have also been put to use in epidemiological research. Through a comparison of recent and historical aerial images, Meentemeyer and others (2008) examined how changes in land-use (mostly fire suppresion) over the past 60 years have increased susceptible forest area at the expense of grassland and chaparral in Sonoma County, California. This increase in forest area has primarily been associated with increases in bay laurel, the main driver of *P. ramorum* epidemics in California mixed-evergreen forests. Other modeling papers have utilized biological and climate data from California and Oregon to model potential locations for invasion by *P. ramorum* in other locations in North America (e.g., Venette and Cohen 2006; Kelly and others 2007). The combination of population genetic studies and landscape modeling will become increasingly important in designing management actions at spatial scales ranging from individual forest stands to regulatory actions at continental scales.

Phytophthora lateralis Update

Phytophthora lateralis has long been the primary *Phytophthora* species associated with extensive tree mortality forests in the Americas; it is still an ecologically and economically important disease. But we would like to end this *Phytophthora* update with some positive news. Resistance programs developed in Oregon have made significant advances in identifying resistant lines of Port-Orford cedar (*Chamaecyparis lawsoniana*) (Sniezko and colleagues, this volume). As a result of this research, Monrovia has recently released two *P. lateralis*- resistant varieties ('Silberstar' and 'Golden King') for ornamental plantings (see http://monrovia.com/). This is not the same as outplanting in native environments as part of forest restoration efforts. But it is still an excellent result and justifies continuing research efforts.

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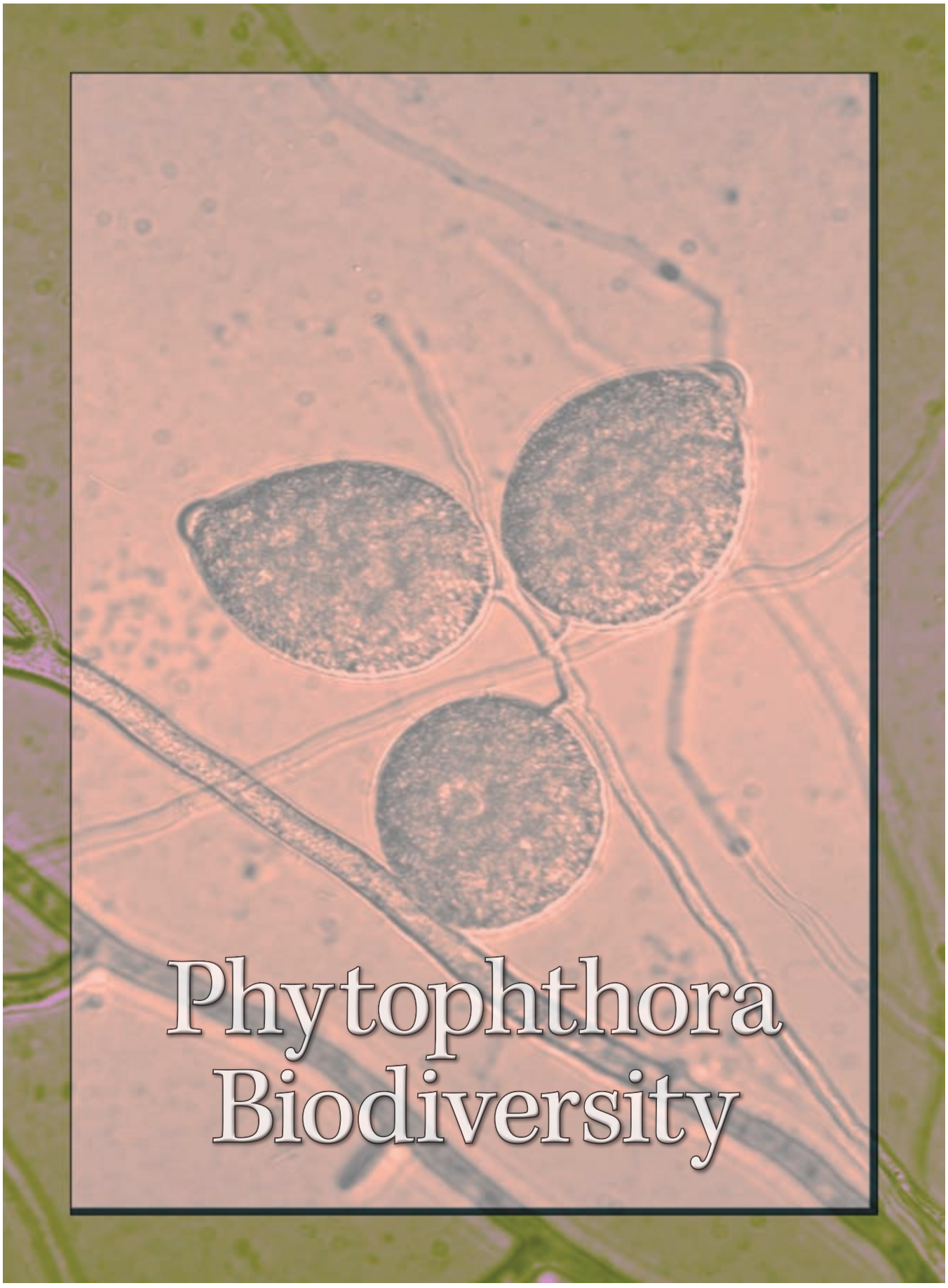
Phytophthora
Biodiversity

Phytophthora kernoviae in New Zealand[1]

T.D. Ramsfield,[2] M.A. Dick,[2] R.E. Beever,[3] I.J. Horner,[4] M.J. McAlonan,[5] and C.F. Hill[6]

Abstract

Phytophthora kernoviae was first recognised in New Zealand in 2005 by DNA sequencing of an isolate that had been recovered from diseased *Annona cherimola* (cherimoya or custard apple) in an abandoned orchard in Northland in 2002. Subsequent investigation has recovered *P. kernoviae* from the soil in Northland, Auckland, Bay of Plenty and Taupo regions. Similarity between *P. kernoviae* and descriptions of an undescribed *Phytophthora* sp. found previously in New Zealand indicates that the organism has been present here since at least 1953. This, along with the geographic range of *P. kernoviae*, and a polymorphism in the ITS sequence, suggest that the pathogen has been present in New Zealand for an even longer time. Little is known of the ecological behaviour of the pathogen in New Zealand; disease has only been recorded on *A. cherimola*.

Introduction

Phytophthora spp. are some of the most invasive and serious plant pathogens and are of concern to regulatory agencies in many countries, including the New Zealand Ministry of Agriculture and Forestry (Biosecurity NZ). In a study in 2005-6, to clarify which species of *Phytophthora* are present in New Zealand, ITS DNA sequence data obtained from cultures that had been stored in culture collections maintained by Landcare Research (ICMP), NZ Forest Research Institute (NZFS) and HortResearch were compared with authentic sequence data from GenBank (Beever and others 2006). One of the findings from this study was the presence of an isolate in New Zealand that was similar to *P. kernoviae*. *Phytophthora kernoviae* has been recently described from Cornwall in the U.K. (Brasier and others 2005), and is a serious pathogen that has been causing significant disease of *Fagus sylvatica* (European beech) and the exotic *Rhododendron ponticum* in woodlands since 2003. The pathogen is also capable of causing lesions on trunks of *Quercus robur* and *Liriodendron tulipifera* as well as the foliage of *Magnolia* spp. and *Pieris* spp. In Cornwall, the host range and pathogenesis process of *P. kernoviae* are similar to the invasive *Phytophthora ramorum*, but these two species are phylogenetically distant.

In view of the disease caused by *P. kernoviae* in Cornwall on *F. sylvatica*, it was considered desirable to further investigate the presence of this pathogen in New Zealand, especially because of the importance of the indigenous *Nothofagus* spp.,

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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which are closely related to *Fagus* spp., in New Zealand forests. Herein we describe the results of follow-up investigations into the presence of *P. kernoviae* in New Zealand and provide evidence that suggests *P. kernoviae* has been in New Zealand for over 50 years.

Initial Discovery

The first isolate to be identified as *P. kernoviae* in New Zealand (ICMP 14761) was isolated in September 2002 from rotting fruit of *Annona cherimola*, growing in an abandoned orchard close to native shrublands and forest in Northland (Braithwaite and others 2007). At the time this isolate was obtained, *P. kernoviae* had yet to be described, and the isolate was identified as *P. hibernalis* based on morphological characteristics. The isolate was revived during the study by Beever and others (2006), in which the sequence data from the internal transcribed spacer (ITS) region of the ribosomal DNA repeat suggested the isolate was the newly described species *P. kernoviae*. Biosecurity New Zealand was notified and the affected cherimoya orchard was visited in December 2005 to investigate. It was found that trees in the orchard had shoot dieback and leaf lesions and mummified fruit were present. One isolate (ICMP 16306) collected in December 2005 from shoots with dieback had an identical ITS sequence to the isolate collected in 2002 and matched it morphologically. It was thus postulated that *P. kernoviae* was causing leaf, shoot and fruit disease of cherimoya in Northland. No other plant species at the site showed obvious symptoms of infection.

Historical Presence of P. kernoviae in New Zealand

Following the recognition of *P. kernoviae* on cherimoya, a further isolate of this species was identified in the study by Beever and others (2006) using ITS sequence data. Isolate ICMP 15082 had been baited from soil that was collected in kauri forest at Trounson Kauri Park (Northland), a native forest remnant, in 2003. This collection site is approximately 50 km from the affected cherimoya orchard.

Perusal of early work in New Zealand led to the proposal that an undescribed *Phytophthora* species, that was the focus of a M. Sc. thesis by one of us (McAlonan), might also be *P. kernoviae*. This thesis (McAlonan 1970) describes a *Phytophthora* sp. (referred to as the "Tokoroa *Phytophthora*"), that was morphologically similar to *P. kernoviae*. It was recovered from under plantation *Pinus radiata* stands on pumice soils in the vicinity of Tokoroa, c.400 km south of the Northland sites. Although isolates from the thesis study were not available, the collection sites were listed in the thesis and these were revisited in 2006. Isolates matching the description of the "Tokoroa *Phytophthora*" were recovered by baiting soil that was collected from these sites. Surveys of the understorey vegetation showing possible disease symptoms of unknown cause at these collection sites have, to date, not recovered *P. kernoviae*. The "Tokoroa *Phytophthora*" was first recognized as distinct by FJ Newhook in 1953 (McAlonan, 1970) and was likely the focus of a study of non-lethal root infection by an unnamed species of *Phytophthora* described as 'widespread in pumice soil' by Newhook (1961).

Assessment of P. kernoviae in New Zealand

The morphology of the recently collected isolates, ICMP 14761 from Northland and ICMP 16723 from Tokoroa was studied in detail and compared with the original description of *P. kernoviae* recorded in Brasier and others (2005) and the description of the "Tokoroa *Phytophthora*" by McAlonan (1970). The isolates were grown on carrot agar (CA) and pea extract agar (PEA) and compared with the descriptions recorded by Braiser and others (2005) and McAlonan (1970).

In addition to cultural characteristics and morphology, DNA sequence data was generated for all isolates recovered from the Northland and Tokoroa sites. The ITS was PCR amplified and sequenced following the methods of Cooke and others (2000). Representatives of *Phytophthora* clades 1 – 8, as recognized by Cooke and others (2000), were selected and their ITS sequences were obtained from GenBank. ITS sequence data from other species of *Phytophthora* that are present within clades 9 and 10 (Dick and others 2006), and also *P. quininea*, because of its relationship with *P. richardiae* as determined by Kroon and others (2004), were also included. The phylogenetic relationship of *P. kernoviae* to the other members of clades 9 and 10 was assessed using a neighbour-joining analysis with the maximum composite likelihood model and 5000 bootstrap replications using *MEGA* version 4 (Tamura and others 2007), based on ITS sequence data that was aligned using Clustal W within *MEGA* 4.

Results

As indicated in Table 1, despite subtle differences in oogonia size, sporangial dimensions and pedicel length, other characteristics such as the low optimum temperature for growth and the lack of chlamydospores are uniform. All isolates were homothallic. Although there are some small differences in the descriptions of "Tokoroa *Phytophthora*" and *P. kernoviae*, some of these differences may have arisen through differences in culture technique. McAlonan (1970) grew his isolates on pea extract agar and soil extract agar, media that are less commonly used now. The New Zealand isolates were therefore grown on media used by McAlonan (1970) for comparison. The characteristic 'mouse' shaped sporangia of *P. kernoviae*, illustrated by Brasier and others (2005), did not form on the media used by McAlonan (1970), who described the sporangia as pyriforme, but they comprised a considerable proportion of sporangia formed on carrot agar, the medium used by Brasier and others (2005).

Table 1. Morphological comparison of *P. kernoviae* as in Brasier and others (2005), "Tokoroa *Phytophthora*" as in McAlonan (1970), ICMP14761 and ICMP16723.

	P. kernoviae (Brasier and others, 2005)	*P. kernoviae* NZ isolate ICMP 14761	McAlonan (1970)	*Phytophthora* Thesis site 2006 ICMP 16723
Colonies				
In dark on carrot agar (CA)	mycelium largely submerged with central aerial area	mycelium largely submerged with central aerial area	little or no aerial hyphae on 3 media tested	little aerial hyphae on 3 media
Diurnal	alternating rings of mycelium	alternating rings of mycelium	Not mentioned	alternating rings of mycelium
Growth rate in dark	3.8-4.6 mm/day @ 20°C on CA	2.7-4 mm/day @ 20°C on CA, 3.0 mm/day @ 20°C on PEA	3.1 mm/day @ 18°C on PEA	3.0 mm/day @ 20°C on CA 2.9 mm/day @ 20°C on PEA 2.7 mm/day @ 18°C on PEA
Optimum temperature	18°C	18°C	21°C	20°C
Upper temperature limit	*ca* 26°C	*ca* 26°C	*ca* 24°C	*ca* 25°C
Gametangia				
Diameter oogonia (μ)	21 – 28	20 – 26.5	24.5 – 28	23 – 26
Diameter oospores (μ)	19 – 25	16.5 – 20	22 – 26	21 – 22
Position oospore	plerotic	plerotic	plerotic	plerotic
Antheridia	amphigynous	amphigynous	amphigynous, occ. paragynous	amphigynous, occ. paragynous
Antheridial dimensions	10 – 14 x 9 – 12	8 – 14 x 8 – 12.5	10 – 11 x 10 – 12	9 – 10 x 10 – 11
Sporangia				
	papillate and caducous	papillate and caducous	papillate and caducous	papillate and caducous
shape	ovoid, limoniform to asymmetric	ovoid, limoniform to asymmetric	pyriforme	ovoid, limoniform, pyriforme to asymmetric
vacuole	common	common	not mentioned	common
dimensions (μ)	34 – 52 x 19 – 31	32 – 48 x 16 – 25	25 – 37 x 17 – 23	28 – 34 x 22 – 24
pedicel (μ)	5 – 19	10 – 22	4 – 7	7 – 10
Chlamydospores	None	none	none	none

When the ITS sequences were BLAST searched in GenBank, it was found that the isolates from New Zealand matched 812/813 base pairs with AY940661, the representative U.K. isolate. The difference was a one base substitution at position 679. *Phytophthora kernoviae* isolate AY940661 (Brasier and others 2005) has adenine (A) at position 679, while all isolates from New Zealand have guanine (G) at that position except for two isolates from the Tokoroa sites which were polymorphic for adenine and guanine (A/G) at that position. We have not recovered an isolate of *P. kernoviae* from New Zealand that is monomorphic for adenine (A) at position 679. Our phylogenetic analysis confirmed the results of Brasier and others (2005) and Blair and others (2007) that *P. boehmeriae* is the closest relative of *P. kernoviae* (fig 1).

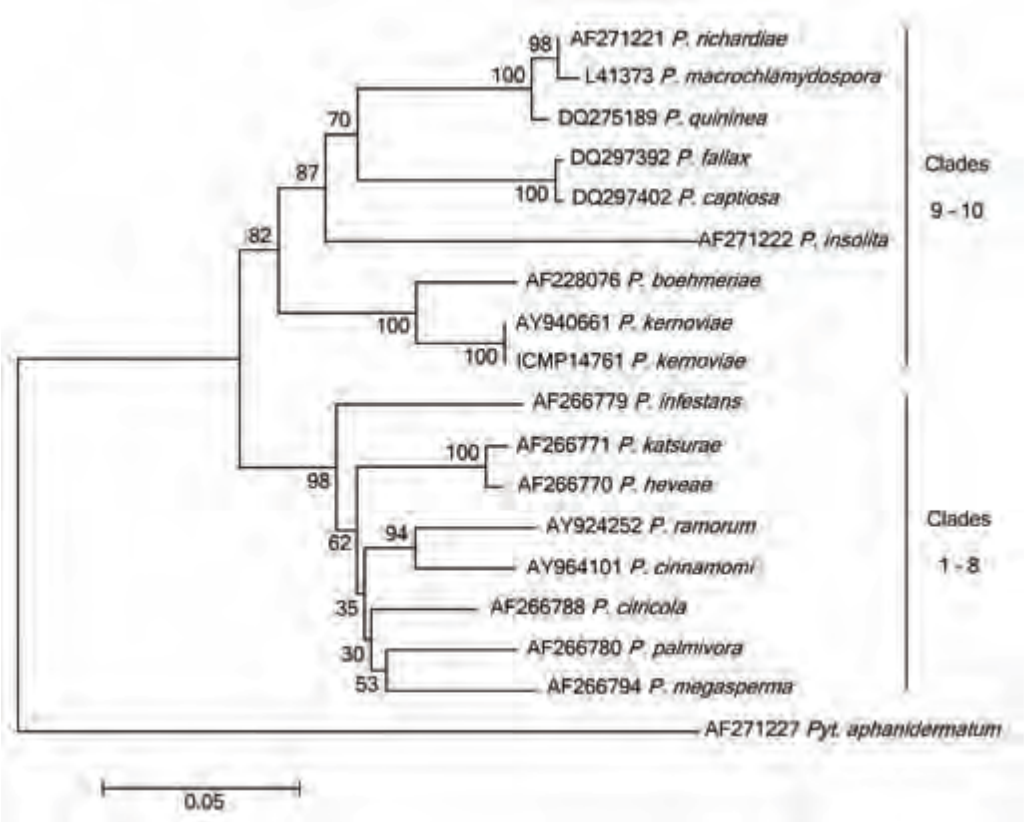

Figure 1-Neighbour joining analysis of ITS sequences from authentic *Phytophthora* spp. that represent clades 1-8 (*sensu* Cooke and others, 2000) and selected species that have been recorded in clades 9 and 10 to show the relationship of *P. kernoviae* to other *Phytophthora* spp. Bootstrap support values following 5000 replications are indicated at the nodes.

Discussion

Phytophthora kernoviae has resulted in extensive damage to woodlands of south-west England (Brasier and others 2005) and is a phytosanitary concern for many countries. Regulatory authorities in the U.K. have responded to *P. kernoviae* as an invasive organism and are attempting to eradicate it (Brasier and others 2005). The geographic origin of *P. kernoviae* is unknown; however, because of its low optimum

temperature and host range Brasier and others (2005) speculated that the organism may be native to the south-west China, the Himalayas or perhaps even Patagonia. The phylogenetic position of *P. kernoviae* in clade 9 / 10 with species known predominantly from the southern hemisphere may be further evidence for a southern hemisphere origin for this species.

McAlonan (1970) studied the pathogenicity of the "Tokoroa *Phytophthora*" on *Pinus radiata* and found that the organism was "mildly pathogenic" to the roots of this conifer, causing less damage than *P. cinnamomi* or *P. citricola*. When he assessed the pathogenicity of the "Tokoroa *Phytophthora*" on *Pseudotsuga menzesii*, the roots remained healthy (McAlonan, 1970). The species of *Phytophthora* described as 'widespread in pumice soils', and likely to have been *P. kernoviae*, was observed by Newhook (1961) to infect roots of *P. radiata* causing some root mortality and reduced growth, but infections were non-lethal and the root systems would have passed as healthy if there were not controls for comparison. He noted that *Phytophthora* spp. are an "important, but not easily recognized biotic factor of the environment" in New Zealand.

We conclude that *P. kernoviae* is present in New Zealand, and that it has been here for more than 50 years because: its presence was first recorded here over 50 years ago, it is relatively widespread in the North Island, it is present in soil in native forests or recently converted native forests, and the polymorphism in the ITS sequence at position 679 indicates some genetic diversity in the New Zealand population. To date, we have not recovered an isolate that is a 100 percent match with the U.K. isolate and the difference in disease expression between the New Zealand and U.K. populations of this organism is striking. The phylogenetic position in clade 9, with other species that are present in the Southern Hemisphere, suggests that the species may have an origin in this region.

Our study of *P. kernoviae* in New Zealand is ongoing and we are planning a survey coupled with a population genetic study that will assess the hypothesis that *P. kernoviae* is native to New Zealand. At the present time, we consider it premature to suggest that the pathogen is native to New Zealand.

Acknowledgements

The authors acknowledge funding from MAF Biosecurity New Zealand and the contributions of MAF BNZ staff Mark Braithwaite, Mark Bullians, George Gill and Heather Pearson.

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The Search for *Phytophthora* Centres of Origin: *Phytophthora* Species in Mountain Ecosystems in Nepal.[1]

Andrea Vannini,[2] Anna Brown,[3] Clive Brasier,[3] and Anna Maria Vettraino[2]

Abstract

Asia is a centre of diversity for many plant genera that are hosts of invasive *Phytophthora* species in other parts of the world. Examples of prominent invasives include *P. ramorum* and *P. kernoviae*, recently spreading in Europe and North America (Brasier and others, 2005; www.rapra.csl.gov.uk). One hypothesis is that these pathogens originated in a specific geographic area of in Asia, such as Yunnan or the Himalayas, and were then transferred via commercial movement of plants to other parts of the world. To investigate the presence of Phytophthoras in Himalayan forest ecosystems a preliminary expedition was undertaken in October 2005 to the forests of Western Nepal. During this expedition 47 soil samples were collected between Kolti (1396m) and Rara Lake (3500m) in the Bajura district. The samples came from 2 distinct ecological zones: the temperate forest, sub-tropical forest. Sixteen target broadleaved and coniferous trees and shrubs were sampled. The geographic co-ordinates of the samples were recorded by GPS and used to elaborate a survey map (fig.1)

Figure 1- purple spots correspond to the geographic position of the samples collected between Kolti (a) and Rara Lake (b)

A total of 39 *Pythium* and 89 *Phytophthora* isolates were obtained through soil baiting. A *Phytophthora*, with some morphological similarities to *P. meadii*, but also many differences, was associated with sub-tropical forest vegetation including Lithocarpus, *Cupressus*, *Cornus*, *Carpinus* and *Castanopsis* species (fig.2a). This taxon has been informally labelled '*P. meadii*-like' while its properties are being investigated and may be a previously undescribed species. A second species, *P. nicotianae*, was found only in the sub-tropical agricultural zone around *Ficus*, *Persea* and *Olea* species (fig.2b). P. citricola was the most frequently isolated species (fig.2c), and was confined to the soil around temperate forest trees including *Acer*, *Aesculus*, *Juglans*, *Ulmus* and *Viburnum* species.

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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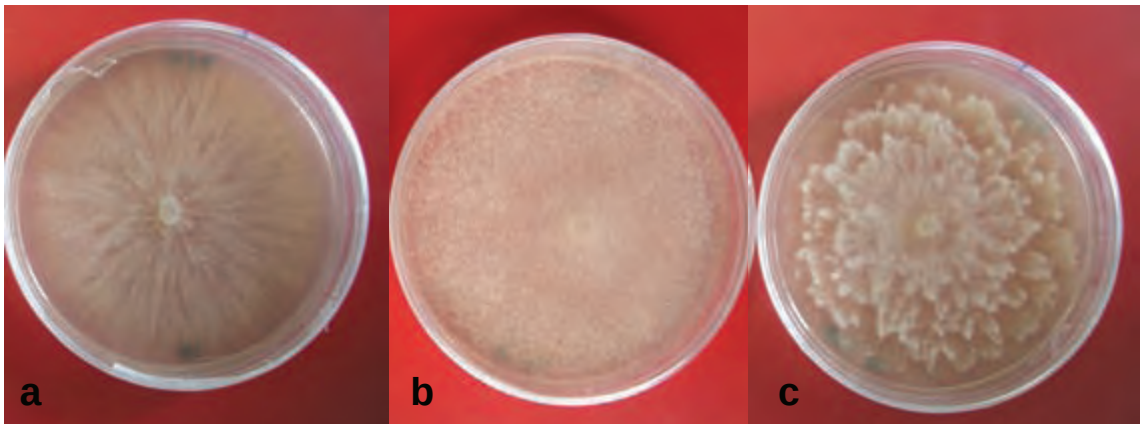

Figure 2-Isolates of *P. meadii-like* (a), *P. nicotianae* (b) and *P. citricola* (c) grew at 20°C on PDA for 1 week

The distinct zoning of the two possibly endemic Himalayan forest Phytopthoras, *P. citricola* and *P. meadii*-like, may lead to further understanding of the role of the genus in forest ecosystems. Whether *P. meadii*-like presents a threat to forest ecosystems in other parts of the world has yet to be determined. The discovery of *P. nicotianae* in association with native *Olea cuspidata* trees in Nepal may have significance for proposed commercial cultivation of European olive, *O. europaea*, in this region.

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Brasier, C.M.; Beales, P.A.; Kirk, S.A.; Denman, S.; Rose, J. 2005. *Phytophthora kernoviae* sp. nov., and invasisve pathogen causing bleeding stem lesions on forest trees and foliar necrosis of ornamentals in the U.K. Mycological Research 109, 853-859.

Natural Hybrids of Resident and Introduced *Phytophthora* Species Proliferating on Multiple New Hosts in Europe[1]

Willem Man in 't Veld,[2] Arthur de Cock and Richard Summerbell

Abstract

In 1996 we encountered some atypical *Phytophthora cactorum* strains. They all possessed numerous abortive oospores, amphigynous and paragynous antheridia and had a maximum growing temperature of 35^0 C, whereas *P. cactorum* itself generally has healthy oospores, paragynous antheridia only and a maximum growing temperature of 32^0 C. At first we tried to identify these strains with isozyme analysis, since the isozyme alleles of *Phytophthora cactorum* are known (Oudemans & Coffey, 1991). Using the dimeric enzymes malate dehydrogenase (MDH) and malic enzyme (MDHP) the population was divided into two groups: the first group (I) had with both enzymes three-banded patterns, one band having the same mobility as the typical *P. cactorum* band. The second group (II) had the same three-banded patterns with MDHP as group I, but with MDH it generated only the typical *P. cactorum* band. Three-banded patterns with dimeric enzymes are the result of two different gene products, wich can combine in three different ways creating two different homo-dimeric bands and one hetero-dimeric band in between. This characteristic pattern usually indicates that outcrossing has occurred. Restriction enzyme analysis of the PCR amplified ITS region using *Alu I* also divided the atypical *P. cactorum* population into the same two groups (fig 1). Isolates of group I contained fragments of both *P. nicotianae* and *P. cactorum* whereas isolates of group II only generated the typical *P. cactorum* band. Isolates of group I were identified as hybrids of *P. nicotianae* and *P. cactorum*, also based on RAPD evidence (Man in 't Veld and others 1998). Isolates of group II were hypothesized to be the result of intercrossing of *P. nicotianae* x *cactorum* hybrids or backcrossings to *P. cactorum*. In order to collect more evidence for either hypothesis, mitochondrial DNA was digested with restriction enzymes (fig 2). Hybrids of *P. nicotianae* x *cactorum* contained mtDNA of *P.nicotianae*, confirming the parenthood of *P.nicotianae*. Isolates of group II, however, possessed neither mtDNA of *P.nicotianae* nor that of *P.cactorum*. Instead, an unknown mtDNA fragment profile was revealed. It was concluded that there was no connection of isolates of group II with *P.nicotianae* x *cactorum* hybrids.

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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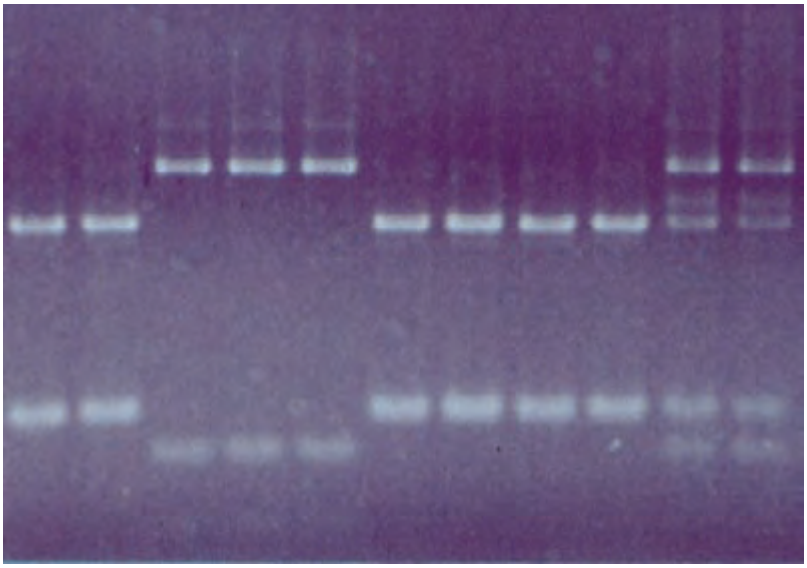
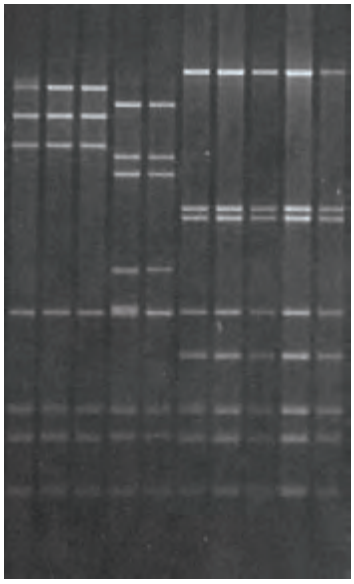


Fig.1. Restriction enzyme analysis (Alu 1) of the internal transcribed spacer sequence of the ribosomal DNA gene repeat (ITS).
1, 2: *Phytophthora cactorum*
3, 4, 5: *Phytophthora nicotianae*
6, 7, 8, 9: group II (=*Phytophthora hedraiandra* x *cactorum* hybrids)
10, 11: group I (=*Phytophthora nicotianae* x *cactorum* hybrids)

1 2 3 4 5 6 7 8 9 10 11



1 2 3 4 5 6 7 8 9 10

Fig. 2. Restriction enzyme patterns of mitochondrial DNA of *Phytophthora* spp. digested with Hind II
1, 2, 3: *P. cactorum*
4,5: group II (= *P. hedraiandra* x *cactorum* hybrids)
6, 7, 8: group I (= *P. nicotianae* x *cactorum* hybrids)
9, 10: *P. nicotianae*

After some years of no activity concerning the isolates of group II, we decided in 2004 to start up again the investigations. In a final attempt to elucidate the true identity of isolates of group II we sequenced the ITS region of these isolates. This was the key experiment since, in contrast to *Phytophthora* species, wich have generally homogeneous ITS sequences through concerted evolution, we discovered double bases at four distinct positions in the sequences of all isolates of group II. Furthermore, the double bases were exactly at those four positions were *P. cactorum* and the newly discovered *P. hedraiandra* contained different bases and they were even composed of those present in *P. cactorum* and *P. hedraiandra* (table 1).

Table 1ð Features of *Phytophthora* species, variable positions in ITS sequences, Cytochrome oxidase I (*Cox I*) type and malic enzyme (*Mdhp*) alleles.

Phytophthora species	ITS variable positions				*Cox I* type	Isozyme alleles *Mdhp*
	74	100	101	686		
cactorum	G	G	T	T	*cactorum*	AA
hybrid (=group II)	R	R	Y	K	*cactorum*	AAB
hybrid (=group II)	R	R	Y	K	*hedraiandra*	AAB
hedraiandra	A	A	C	G	*hedraiandra*	BB

It was hypothesized that isolates of group II possibly represented hybrids of *P. cactorum* and *P. hedraiandra*. Isozyme analysis was also in line with this hypothesis since the three banded MDHP pattern of isolates of group II was the result of the *P. hedraiandra* and the *P. cactorum* MDHP alleles and *P. hedraiandra* and *P. cactorum* possessed the same MDH band, which explained the single band of group II isolates. In order to confirm the parenthood of the present hybrids we sequenced the maternally, inherited mitochondrial encoded cytochrome oxidase I (*Cox I*). Most hybrid strains appeared to have the *Cox I* gene of *P. hedraiandra* and in one strain *Cox I* of *P. cactorum* was found, confirming the parenthood of both presumed parents (Man in 't Veld and others, 2007). Most hybrid strains were trisomic for the chromosome carrying the *Mdhp* locus. Two strains, having homozygous ITS sequences but containing isozyme alleles of both parents, were hypothesized to originate from intercrossings of the hybrids or from backcrossing or mitotic genome rearrangements.

The present hybrids were discovered in the Netherlands, Belgium, Germany, Switserland and Slovenia altogether from six hosts (*Allium cepa*, *Allium porrum*, *Idesia*, *Kalmia latifolia*, *Penstemon* and *Rhododendron*). All hosts, except *Rhododendron* have not been reported as host of either parent. Since 1992 only the present hybrids were isolated from *Rhododendron* indicating that the present hybrids are proliferating more successfully than *P. cactorum* on this host.

Rhododendron is a common host for *P. cactorum* and *P.hedraiandra*. Two *Phytophthora* species can not diverge in the same geographical area when they have a common host, which is in this case *Rhododendron*. Hence, since *P. cactorum* is well known in Europe for a long time, *P. hedraiandra* must have been introduced recently. In fact in the Netherlands the first *P. hedraiandra* strain was isolated in 2001.

P. hedraiandra and *P. ramorum* are both considered to be recently introduced species from hitherto unknown origin, but they followed different strategics to survive. *P. ramorum* proliferates predominantly clonally both in America and Europe presumably by means of its wind dispersed sporangia. In contrast, *P. hedraiandra* is hardly spreading; instead this species hybridized with the resident *P. cactorum* and the hybrids proliferated successfully, especially in the Netherlands (30 strains) and Belgium (40 strains).

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New Hypotheses on the Origin of the Interspecific Hybrid Oomycete *Phytophthora alni*[1]

Renaud Ioos,[2,3] Axelle Andrieux,[3] Benoît Marçais,[3] and Pascal Frey[3]

Abstract

An emergent disease of alder is caused by a complex of three taxa belonging to the genus *Phytophthora* (Oomycetes): *P. alni* subsp. *alni* (*Paa*), *P. alni* subsp. *multiformis* (*Pam*) and *P. alni* subsp. *uniformis* (*Pau*). *Paa* was previously hypothesized to be an allopolyploid hybrid between two genetically close species, *P. cambivora* and a taxon close to *P. fragariae*, whereas *Pau* and *Pam* were believed to be genetic breakdowns of *Paa* (Brasier and others 1999).

In order to examine these hypotheses, we studied the occurrence and the allelic distribution of four nuclear and two mitochondrial genes on a wide collection of *P. alni* and closely related species. *Paa* possesses three different alleles for each of the nuclear genes we studied, two of which are present in *Pam* as well, whereas the third matches the single allele present in *Pau*. Moreover, *Paa* displays common mtDNA patterns with both *Pam* and *Pau*. A combination of the data suggests that *Paa* may have been generated on several occasions by sexual hybridization between *Pam* and *Pau*, and that *Pam* may result itself of an ancient reticulation (Ioos and others 2006).

In addition, we studied the expression of elicitin genes, a multigenic family specific to the genus *Phytophthora*. The cumulative patterns of *Pau* and *Pam* in regard with *Paa* confirmed our first results and demonstrated that for this allopolyploid taxa, distinct genomes may be co-expressed (Ioos and others 2007a).

Last, in order to study the genetic variability of the different taxa, microsatellite markers were isolated in *Paa* and characterized (Ioos and others 2007b). These markers revealed a low genetic diversity in all three taxa. The cumulative microsatellite patterns observed in *Paa* confirmed the hybridization scheme and confirmed that *Paa* was generated by several hybridization events. Genotypes resolved for *Pam* suggest that this taxon may also be allopolyploid.

Altogether, our results clearly demonstrate that (i) *P. cambivora* and *P. fragariae* are not the progenitors of *P. alni*, and (ii) *Paa* was generated at several occasions by hybridization between *Pam* and *Pau*. This study raises new issues about the geographic origin, host range, ecology and aetiology of *Pam* and *Pau*.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Population Structure of the Emerging Plant Pathogen *Phytophthora ramorum* on the West Coast of the United States[1]

S. Prospero,[2] E.M. Hansen,[3] N.J. Grünwald,[3,4] J. Britt[3], and L.M. Winton[5]

Abstract

Phytophthora ramorum is a devastating pathogen in native forests in California and southwestern Oregon and in nursery crops in California, Oregon and Washington. In this study we analyzed the population structure of *P. ramorum* in the west coast (CA, OR, and WA) of the United States by screening 579 isolates recovered from 2001 to 2005 at 10 microsatellite loci.

The overall *P. ramorum* population was composed of 73 multilocus genotypes. Based on allele patterns, 72 genotypes belonged to the North American clonal lineages NA1 or NA2 (both A2 mating type) and one nursery genotype (OR and WA) belonged to the European clonal lineage (EU1, A1 mating type). Within the North American types, 71 genotypes clustered into the same clonal lineage (NA1), while one nursery genotype (CA and WA) belonged to NA2. None of the genotypes detected shared alleles of NA1 or NA2 and EU1, indicating that sexual reproduction is currently not occurring in the west coast population. Twenty out of 73 genotypes were present in more than one population. The lowest incidence of genotypes shared among populations was observed in the OR-forest population (7 out of 30), which was dominated by a genotype not found elsewhere. Using assignment tests, a high percentage of the genotypes could not be assigned to the population from where they originated. The number of genotypes shared between two populations was not correlated with the minimal geographic distance between the two populations. The overall estimated F_{ST}-value among the four populations was 0.183 (95 percent confidence interval: 0-0.377). When using clone-corrected data sets, pairwise differentiation was significant only between the OR-forest and the three other populations. Accordingly, genetic distances among OR-nursery, CA, and WA populations were very small.

Our study demonstrates how the spread and the population structure of an invasive plant pathogen can be strongly affected by human activity. Accepting the hypothesis of a recent introduction of *P. ramorum* into western United States, the movement of infected nursery stock has probably played a major role in the rapid expansion of the geographical range of the pathogen over long distances. Thus, the current *P. ramorum* NA1 population is genetically similar from California to Washington. The dominance in southwestern Oregon of a genotype not found elsewhere may account for the significant differentiation between the OR-forest and the other three populations.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26 - 31, 2007, Monterey, California .

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The Decline of *Austrocedrus* Forests in Patagonia (Mal del Ciprés): Another *Phytophthora*-caused Forest Disease[1]

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Abstract

Austrocedrus chilensis, an indigenous Cupressaceae of the Patagonian Andes forests, is suffering a disease that has been called "Mal del Ciprés" (MDC). This disease was first reported more than 50 years ago but, in spite of many studies, its causes remained unclear until recently. The disease begins in the root system, the distribution and pattern of spread of mortality in a stand is consistent with a soil-borne pathogen, and it is associated with seasonally poorly drained soils. Symptoms include defoliation, basal resinous exudates and red-brown necrotic lesions in the inner bark extending up the bole from killed roots. Brown cubic rots in roots and sapwood caused by wood-decomposer fungi are frequently- but not always- associated with dead or dying trees. These characteristics have led several workers to suggest that a *Phytophthora* species might be the causal agent of the disease. Several attempts to find a *Phytophthora* species responsible for the disease have been made. Five species were isolated from soil and/or associated streams: *P. syringae, P. cambivora, P. gonapodyides* and the undescribed taxa "Pgchlamydo" and "P. taxon raspberry" and another two species -*P. pseudotsugae* and *P. cactorum*- were reported from soil and/or fine roots in a previous study. None of them showed a clear relationship with the disease. Isolations from the margins of the necrotic lesions in the inner bark using *Phytophthora*-selective media initially failed, but an ELISA test on necrotic phloem tissues was positive for *Phytophthora*, and subsequent DNA extraction from necrotic bark and amplification of ITS DNA using *Phytophthora*-specific primers was successful. Thus encouraged, isolation attempts were renewed and were finally successful. The isolated species was an undescribed taxon of *Phytophthora* that was formally named *Phytophthora austrocedrae*. It is homothallic with amphigynous antheridia and semi-papillate sporangia, very slow growing with a maximum radial growth rate ranging from 1.0–1.8 mm/day in V8A at optimal temperature (17.5C). ITS rDNA sequence places it near *P. syringae* in phylogenetic clade 8 of the genus. It was isolated from symptomatic trees in all localities affected by MDC throughout the range of the disease, showing that the pathogen is widely distributed. Pathogenicity tests fulfilled Koch's postulates demonstrating it is the primary cause of the disease. This work presents the results of pathogenicity tests and their implications on the aetiology of the disease. A discussion of subjects that should be addressed in future work is also presented.

Introduction

Austrocedrus chilensis (D. Don.) Pic. Serm. & Bizarri (Ciprés de la cordillera) is an endemic tree of the Cupressaceae of southern Argentina and Chile. Among the few

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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conifers inhabiting southern Argentina it has the largest distribution, covering ca. 140,000 ha. (Bran and others 2002). It is a very valuable species because of it's ecological role, the high quality of it's wood and it's scenic appeal. Throughout its range, *A. chilensis* suffers a lethal disease known as "Mal del Ciprés" (MDC). This disease was first detected 60 years ago and, since then, the affected area has been constantly increasing. Several studies on biotic and abiotic factors related to the disease have been done but the origin and causes of MDC remained unclear until recently. For a detailed description of the disease and background see Greslebin and others (2005).

The symptomatology of the disease was traditionally described as a chlorosis, withering and subsequent progressive defoliation of the crown (Varsavsky and others 1975, Havrylenko and others 1989, Rajchenberg and Cwielong 1993), and a decline of radial growth of the tree (Filip & Rosso 1999). The disease originates in the root system, where the death of the tissue precedes defoliation of the crown (Havrylenko and others 1989). Death of the roots is followed, many times, by the development of brown rots in the sapwood (Varsavsky and others 1975, Havrylenko and others 1989, Rajchenberg and Cwielong 1993, Barroetaveña & Rajchenberg 1996). Resin exudation has been pointed out either as related (Varsavsky and others 1975) or as unrelated (Havrylenko 1989) to the disease. The death of the tree may occur slowly, as a progressive defoliation, or rapidly, in which case foliage changes from chlorotic to red with little or no defoliation (Filip & Rosso 1999, Greslebin & Hansen 2006). Affected stands are usually associated with poorly drained sites (La Manna & Rajchenberg 2004a, b). The origin of the disease in the roots and its association with poorly drained sites suggested the action of a Pythiaceous organism (Pythiaceae, Oomycota). Several species of *Phytophthora* were isolated from streams and soils in affected stands (Greslebin and others 2005), but attempts to isolate from necrotic tissues were negative up to recently when a new *Phytophthora* species was detected on them (Greslebin and others 2007).

The new species, *Phytophthora austrocedrae*, was the first organism consistently associated with cypress mortality suggesting it might be the primary cause of MDC. To confirm this hypothesis and fulfill Koch's postulates, pathogenicity of *P. austrocedrae* on *Austrocedrus chilensis* was evaluated. *P. syringae*, the principal species recovered from soil near symptomatic trees, was also tested. This work presents the results of pathogenicity tests and their implications on the aetiology of the disease.

Materials and Methods

Isolation

Isolates of *P. austrocedrae* (Table 1) used for pathogenicity tests were obtained from the advancing necrotic zone of phloem lesions of symptomatic trees studied in previous work (Greslebin and others 2007). The isolate of *P. syringae* (Table 1) was recovered from soil in an *Austrocedrus* stand exhibiting symptoms of MDC. Isolates were identified by their morphological features and ITS sequences (Greslebin and others 2007).

Koch's Postulates

Pathogenicity tests were performed:
a) In the field through stem and root inoculation of adult trees; and
b) In the laboratory through stem inoculation of five-year-old seedlings; and
c) Through soil infestation.

Field root and stem inoculation

Field inoculations were performed in an *A. chilensis* stand located at INTA (National Institute of Farming Research) Trevelin, where *P. austrocedrae* had been previously recorded. Ten trees showing no symptoms were selected for stem inoculation. Inoculations were made in 2 different seasons: summer (January 2005) and fall (May 2005). Five trees were inoculated (stem and roots) in each season. Results were combined for analysis.

Root inoculation

Up to four superficial main roots of each tree were excavated and four treatments [T0: control; T1: *P austrocedrae* (strain CIEFAP Py 190); T2: *P. austrocedrae* (strain ATCC MYA-4074); T3: *P. syringae* (strain CIEFAP Py 5)] (Table 1) were randomly assigned. Two inoculations per root were made in those trees with less than 4 main roots. Cores (7 mm diam) of bark were aseptically removed using a borer. V8-agar discs from the edges of 15 day old cultures of *P. austrocedrae* and 7 day old cultures of *P. syringae* were placed in the holes and covered with the removed bark. A piece of sterilized, moist cheese-cloth was placed over each inoculation point, covered with aluminum foil, and sealed with adhesive tape. Controls received uninfested V8-agar discs. After inoculation roots were covered again with the removed soil.

Stem inoculation

Each tree received 4 inoculations corresponding to the following treatments: T0: control; T1: *P austrocedrae* (strain CIEFAP Py 190); T2: *P. austrocedrae* (strain ATCC MYA-4074); T3: *P. syringae* (strain CIEFAP Py 5) (Table 1). Inoculations were made on the sides of the tree facing each cardinal direction following the same procedure as in roots. Treatments were randomly assigned to each side of the tree. After 4 months bark was removed to expose the phloem, and the length and width of the lesion (necrotic phloem) was recorded. Re-isolation was attempted from the top and the bottom edges of the lesions and ELISA immunoassays to detect *Phytophthora* were performed on necrotic tissues associated with each treatment including controls. A Kruskal-Wallis Nonparametric Analysis of Variance was applied in order to detect significant differences between mean lesion size of treatments. Significant differences between mean lesion size of each treatment and control were tested through a Mann-Whitney test. Homogeneity of variances was tested using the Levene test.

Stem inoculation of seedlings

Thirty 5-year-old seedlings were selected for stem inoculation. Two strains of *P. austrocedrae* were tested (Table 1): CIEFAP Py 190 (T1) and CIEFAP Py 232 (T2). Treatments were randomly assigned to each seedling (ten seedlings for each treatment and ten controls). Cores (5 mm diam) of bark were aseptically removed using a borer. T-agar discs from the edges of 15-day-old cultures of *P. austrocedrae* were placed in the holes and covered with the removed bark. A piece of sterilized, moist cheese-cloth was place over each inoculation, covered with aluminum foil and

sealed with adhesive tape. Controls received uninfested T-agar discs. Lesion length and width in phloem were recorded after 2 months. Re-isolation was attempted from the edges of the lesions, and ELISA immunoassays to detect *Phytophthora* were performed on necrotic tissues associated with each treatment and controls. A Kruskal-Wallis Nonparametric Analysis of Variance was applied in order to detect significant differences between mean lesion sizes of treatments. Significant differences between mean lesion size of each treatment and control were tested through a Mann-Whitney test. Homogeneity of variances was tested using the Levene test.

Soil infestation

Inocula consisted of agar discs with sporangia. Agar discs were cut from the edges of 15-day- old *P. austrocedrae* cultures (T1: strain CIEFAP Py 190, T2: strain CIEFAP Py 232) (Table 1) and placed in T-broth for 4 days. Then, agar blocks were rinsed 10 times in distilled water to remove nutrients and placed in soil extract for 4 days. Pots with a 1:1 mix of tindalized soil and sterilized volcanic sand were used for planting the seedlings. Twenty pots were assigned to each treatment and the control. Two seedlings were planted in each pot and one agar block with sporangia was placed in the same hole where each seedling was planted. Pots were flooded for 48 hours every 14 days. Controls received uninfested T-agar discs in the same hole where each seedling was planted. Mortality of seedlings was recorded weekly.

Table 1ð Isolates used for pathogenicity tests

Strain number	Species	Origin
ATCC MYA-4074 CIEFAP 203	*P. austrocedrae*	Argentina, Chubut, Los Alerces National Park, Braese stream, isolated from necrotic inner bark of *A. chilensis*, Oct. 2005
CIEFAP 190	*P. austrocedrae*	Argentina, Chubut, Futaleufú, Río Grande Valley, "La 106" Ranch, isolated from necrotic inner bark of *A. chilensis*, Set. 2005
CIEFAP 232	*P. austrocedrae*	Argentina, Chubut, Los Alerces National Park, near to Los Pumas stream, isolated from necrotic inner bark of *A. chilensis*, Oct. 2005
CIEFAP 5	*P. syringae*	Argentina, Chubut, Futaleufú, Río Grande Valley, "Los cerezos" Ranch, isolated from soil of *A. chilensis* stand. June 2001

Results

Pathogenicity of Phytophthora austrocedrae *on* Austrocedrus chilensis

Root and stem inoculations of adult trees

Since Levene test showed unequal variances (P-value < 0.001) for lesion size in both roots and stems, non parametric analyses were applied. All roots inoculated with *Phytophthora austrocedrae* developed brown, necrotic lesions that affected the phloem reaching and staining the sapwood (fig 1). Lesions developed after *P. austrocedrae* inoculations (both T1 and T2) were significantly longer and wider (one-

sided P-value < 0.0001) than those in the Controls (fig. 2). Mean lesion length and width of *P. syringae* lesions did not differ from those of the controls (fig. 1). Stem inoculations with *P. austrocedrae* developed lesions similar to those observed in root inoculations. Two exceptions were found: one tree developed small and superficial lesions and another did not develop lesions in the stem but it did in the roots. Lesions developed after *P. austrocedrae* inoculations (both T1 and T2) were significantly longer (one-sided P-value < 0.0001) and wider (T1: one-sided P-value < 0.0001, T2: one-sided P-value = 0.003) than those in Control inoculations (fig. 2). Mean lesion length and width of *P. syringae* inoculations did not differ from Control lesions (fig. 2).

Mean lesion length and width of T1 treatments were greater than mean lesion length and width of T2 treatments in both root and stem inoculation but these differences were not significant except for mean lesion width of stem inoculations (one-sided P-value = 0.02).

Figure 1ð Lesion caused by *Phytophthora austocedrae* on a root on an adult tree of *Austrocedrus chilensis*. Left: bark removed showing necrotic phloem. Right: phloem removed showing sapwood superficially affected.

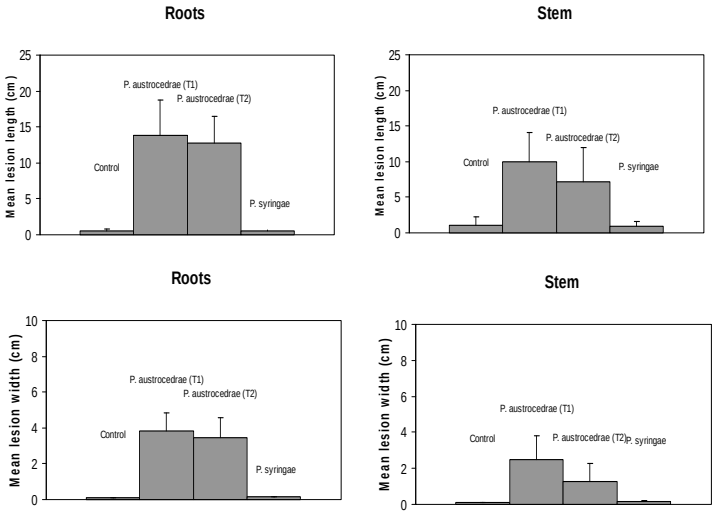

Figure 2—Mean length and width of necrosis (cm) produced by *P. austrocedrae* and *P. syringae* on roots and stems of *A. chilensis* adult trees.

Trees showed no external foliar symptoms at the end of the study. A few trees did exhibitresin exudation from the inoculation point.

Phytophthora austrocedrae was re-isolated from 78 percent of the lesions and ELISA tests were positive from 100 percent of them. *Phytophthora syringae* was not reisolated from inoculation points, and ELISA tests were negative for both *P syringae* and control treatments.

Stem inoculation on five year old seedlings

After two months all seedlings inoculated with *Phytophthora austrocedrae* developed brown, necrotic lesions that affected the phloem, reaching and staining the sapwood. A few seedlings showed external symptoms (i.e. dark red foliage) (fig. 3) but most showed no symptoms even though they developed lesions similar to those of symptomatic seedlings. Since Levene test showed unequal variances (P-value < 0.002) non parametric analyses were applied. Lesions of both treatments were significantly longer (one-sided P-value < 0.0001) than those in Controls (Fig. 4). *Phytophthora austrocedrae* was successfully re-isolated twice (from one plant of each treatment) the other re-isolation attempts failed, being over-grown by Deuteromycetes. ELISA tests were positive for 100 percent of the lesions and negative for the Controls. Mean lesion length of T2 was greater than T1 one (fig. 4) but the difference was not significant (one-side P-value = 0.11)

Figure 3ð Five year old seedlings inoculated with *Phytophthora austrocedrae* after two month of inoculation. A) Symptomatic seedling and it respective lesion. B) Asymptomatic seedling and it respective lesion.

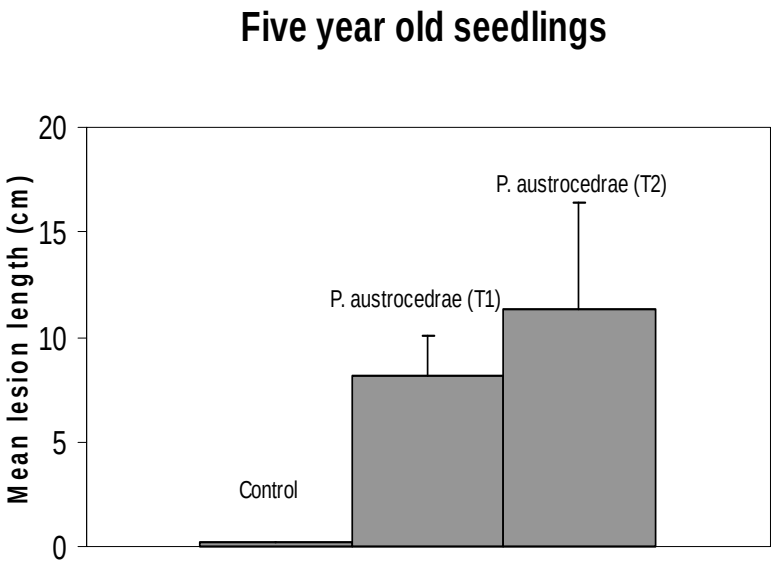

Figure 4ð Mean length of necrosis (cm) produced by *P. austrocedrae* on stems of *A. chilensis* seedlings.

Soil Infestation

In *P. austrocedrae* treatments (T1 and T2) 95 percent of the seedlings were dead after the second flooding (20 days of treatment). Mortality was mostly observed after each flooding (about 40 percent of the seedlings after the first flooding and about 55 percent after the second one). Roots of dead seedlings were brown or were severely rotted. Only two control seedlings were dead at the end of the study (one month) and they died the first week of treatment.

Isolations from rotted roots of dead seedlings were overgrown by *Mortierella* spp. and *P. austrocedrae* could not be recovered, but ELISA tests were positive. ELISA tests from the two dead seedlings found in the Controls were negative.

Discussion

The experimental evidence confirms that *Phytophthora austrocedrae* is the causal agent of MDC. The pathogenicity tests indicate that *P. austrocedrae* is an aggressive pathogen of *Austrocedrus chilensis*. Adult trees and seedlings were susceptible to infection and lesions developed quickly (up to 4.7 cm $month^{-1}$ in adult trees and up to 11.5 cm $month^{-1}$ in seedlings). A root pathogen has been frequently suggested as cause of MDC (Havrylenko and others 1989, Rosso and others 1994, Rajchenberg and others 1998, Filip & Rosso 1999), but a specific organism had not been implicated. *P. syringae* has been isolated from soil and streams in diseased stands, but never from trees (Greslebin and others 2005), and was not pathogenic in stem or root inoculations in these tests.

The apparent aggressiveness of *P. austrocedrae* was unexpected. MDC has traditionally been described as a slow decline, with progressive defoliation (Rajchenberg and others 1998), where death of individual trees may take several

decades (Calí 1996, Filip & Rosso 1999). It is not unusual to find apparently healthy trees very close to dead or dying ones. This also disagrees with an aggressive pathogen. The questions are: why do some trees die quickly while others die decades after they begin to decline, and why do some trees remain unaffected even when they are next to dying trees? We have observed that some lesions in the stem become inactive and trees begin to wall off old lesions with callus tissues (fig. 5). Whether the lesions become inactive because of tree defenses or because of ambient conditions unfavorable for the pathogen is an issue that remains unclear and should be considered in future research. *P. austrocedrae* growth in vitro is affected by high temperatures (Greslebin and others 2007), showing no growth at 25 C. Maximum air temperature in summer in areas where *Austrocedrus* grows can reach 35 C (exceptionally more). High summer temperatures could be responsible for the inactivation of the lesions on some trees. The effect of ambient temperatures on lesion development as well as the variation of *A. chilensis*' resistance/susceptibility to *P. austrocedrae* should be evaluated in future work.

Figure 5—Callus tissues walling off an old, inactive lesion.

MCD symptomatology is consistent with a *Phytophthora* disease but similar crown symptoms may result from other causes. We propose that the disease name Mal del Ciprés be reserved for the disease of *Austrocedrus* caused by *Phytophthora austrocedrae* characterized by root infection and necrotic lesions extending up the bole. This pathogen has now been isolated from dying *A. chilensis* trees in Isla Victoria (Nahuel Huapi National Park) the place where MDC was first reported, and in most of the other areas throughout the tree's range that have been affected by MDC (Greslebin and others 2007).

Another very important issue that should be resolved is the geographic origin of *P. austrocedrae*. Is it a native species or an introduced pathogen? The area where the disease was first observed (Isla Victoria) was planted early in the 20th century with exotic tree species from many places in the world and a nursery was built to provide seedlings to many other places in Patagonia. *P. austrocedrae* could be an exotic pathogen that was introduced in Isla Victoria and spread from this point. On the other hand, today it is a widely distributed species present throughout the cypress

distributional range, and its closest genetic relative is *Phytophthora syringae*, another species that is also present in *A. chilensis* forests. Perhaps it is a native species that acts as a pathogen under favorable site conditions including poor soil drainage (La Manna & Rajchenberg 2004a,b) or has increased it activity because of the climate change. To elucidate the origin of *P. austrocedrae* as well as *P. syringae* will be the objective of our future work.

Acknowledgments

We want to thank Dr. Charles G. Shaw III and Dr. Paul Hennon, USDA Forest Service, Alaska Region, Forest Health Protection and ICSU-TWAS-UNESCO-UNU/IAS Visiting Scientist Programme for making possible this collaborative work by supporting AGG and EMH visits to OSU and to CIEFAP respectively. Dr. Carlos Buduba, Instituto Nacional de Tecnología Agropecuaria (National Institute of Farming Technology) INTA of Argentina, is warmly acknowledged for kindly permits the realization of pathogenicity tests in Cypress stands of INTA Trevelin. This work was supported by Agencia Nacional de Promoción Científica y Técnica (ANPCyT, FONCyT) of Argentina (PICT 1-13305) and National Research Council (CONICET) of Argentina (PIP 5660). AGG is researcher for CONICET.

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Kauri (*Agathis australis*) Under Threat From *Phytophthora*?[1]

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Abstract

Five species of *Phytophthora* have been recorded from *Agathis australis* (kauri) or soil in kauri forests: *P. cinnamomi*, *P. cryptogea*, *P. kernoviae*, *P. nicotianae* and *Phytophthora* taxon Agathis (PTA) initially recorded as *P. heveae*. *P. cinnamomi* has been found widely in natural stands and has been linked with ill-thrift and occasional tree death, especially in regenerating stands on poorly drained sites. PTA, which is known from fewer natural stands, is associated with a collar rot, causing large bleeding lesions near the ground, yellowing foliage, and tree death. The other three species have only been reported once. ITS sequence studies of PTA show it belongs with, but is distinct from, *P. heveae* in ITS clade 5. Cultural and molecular studies indicate a close relationship with *P. katsurae*. It is proposed that PTA may be introduced to New Zealand, but too few isolates are available to determine whether genetic variability of this species provides support for this hypothesis. Recent surveys have found collar rot is widely distributed across the natural range of kauri. Typically, affected stands are relatively small in size. Size class distributions indicate trees of many ages are affected and a disease front can sometimes be detected. Pathogenicity tests show PTA is highly pathogenic to kauri. We propose that collar rot caused by PTA is an emerging disease caused by an introduced pathogen that is spreading slowly from widespread disease foci. It poses a threat to kauri, both at the individual icon level and at the population level, with flow-on effects to kauri ecosystems.

Introduction

Kauri (*Agathis australis*), a conifer in the Araucariaceae, is a dominant tree of lowland stands in northern New Zealand, frequently occurring in a mosaic of mixed forests of diverse composition (Ecroyd 1982, Ogden and Stewart 1995, Wardle 1991). Following excessive exploitation for timber during the 19th and early 20th centuries, it is today virtually restricted to relatively small reserves. Giant individual trees, which can reach over 4.5m in trunk diameter and exceed 1000 years age, are accorded special status by Maori, Pakeha and tourists. Tane Mahuta ('God of the Forest') and Te Matua Ngahere ('Father of the Forest') are major tourist attractions in Waipoua Forest, the largest remaining kauri stand in the country. Kauri seeds are winged allowing wind dispersal (Ecroyd 1982). Vegetation patterns indicate kauri regeneration occurs following disturbance, which can range from large-scale wind and fire events to small-scale canopy gaps resulting from fall of giant trees (Ogden and Stewart 1995). Many present day stands result from human-induced fires

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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associated with European settlement from the mid-1800s to the mid-1900s. These stands are often seral in character, with kauri establishing in a shrubland 'nursery' dominated by the myrtacean tea trees, *Leptospermum scoparium* and *Kunzea ericoides* (Ogden and others 1987).

Five species of *Phytophthora* have been recorded from kauri and/ or kauri ecosystems (Gadgil 2005, McKenzie and others 2002, Ramsfield and others 2008): *P. cinnamomi* (var. *cinnamomi*), *P. cryptogea*, *P. kernoviae*, *P. nicotiana*e, and a species we refer to here by the tag name *Phytophthora* taxon Agathis (PTA). Erwin and Ribeiro (1996) in error cite Gadgil (1974) as reporting *P. hibernalis* from *A. australis*. He does not, nor does Gadgil (2005) list *P. hibernalis* from this host.

P. cinnamomi is found widely in natural stands and has been linked with ill-thrift and occasional tree death, especially in regenerating stands on poorly drained sites (Podger and Newhook 1971). Symptoms included severe chlorosis, canopy thinning and dieback, and sometimes tree death. *Phytophthora* taxon Agathis (PTA) was first reported from a natural stand of unhealthy kauri on Great Barrier Island (fig. 1), a small island off the northern New Zealand coast (Gadgil 1974). Symptoms included yellowing of foliage, canopy thinning and occasional tree death. Additonally, affected trees frequently had lesions on the lower trunk and main roots. These lesions sometimes encircled the stem and were bleeding copious amounts of resin (kauri gum). The affected trees occupied c. 1.5 ha in a block of forest regenerating after disturbance associated with forestry operations in 1931–32 and trees up to 30 cm diameter at breast height (DBH) were affected. A homothallic *Phytophthora* was isolated from the lesions and from soil samples collected in the vicinity of affected trees and determined, albeit with some reluctance, as *P. heveae* on the advice of IMI staff. Pathogenicity tests on kauri seedlings using both wound and soil inoculation methods showed it was highly pathogenic.

Of the species associated with kauri, *P. cinnamomi*, *P. cryptogea,* and *P. nicotianae* were likely introduced to New Zealand following human contact about 600 years ago. Whether or not PTA, and *P. kernoviae* (Ramsfield and others, these proceedings), are indigenous or have been introduced is problematic. Recent observations indicate PTA is more widespread than previously realised and may pose a significant risk both to kauri ecosystems and to individual iconic trees. This paper summarises these studies and reviews them in relation to existing knowledge of the associations of *Phytophthora* species with kauri.

Methods

Culturing and Morphological Studies

Isolations from lesion margins were made to common lab media including P_5ARP and from soil using lupin baits and apple baiting (Erwin and Ribeiro 1994). The strains used are listed in Table 1. ICMP isolates are maintained long term in liquid nitrogen storage. Cultures were grown (18–20^{0}C in the dark) on V-8 juice agar for morphological studies, and PDA for DNA sequencing studies. Gametangial images were taken by interference light microscopy using a 40x objective.

Tree Health Studies

Health status in relation to tree diameter was examined in a highly affected stand of about 1.4 ha at Huia, Waitakere Ranges. All trees were examined in a 20m x 20m plot in the middle of the stand, as well as along two transects extending at right angles from the plot to the disease margin of the affected stand. DBH was measured with a diameter tape and the health status assessed as: healthy, living but with bleeding lesions, or dead (often also with evidence of old oozing gum indicating past lesion presence).

Phylogenetic Analysis

DNA was extracted from mycelium using a QIAGEN DNeasy Plant Mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The internal transcribed spacer (ITS) region of the ribosomal DNA repeat was PCR amplified using the primers ITS-4 and ITS-6 following the PCR protocol of Cooke and others (2000).

Table 1—List of clade 5 isolates used in this study, including those for which GenBank sequences were downloaded

Species*	ICMP**	GenBank	Other collection numbers***	Country of isolation	Host	Substrate
heveae (isotype strain)	-	AF266770	IMI180616 = CBS296.29	Malaysia	*Hevea brasiliensis*	Fruit
heveae	16914	-	SCRP361 = IMI131093	Malaysia	*Theobroma cacao*	-
heveae	16691	-	DAR27023	Australia	-	soil under *Eucalyptus*
katsurae	-	AF266771	IMI360596	Ivory Coast	*Cocos nucifera*	-
katsurae	16948	-	Uchida H1024	Hawaii	*Cocos nucifera*	Fruit
katsurae	16950	-	Uchida H1027	Hawaii	*Cocos nucifera*	Fruit
katsurae	16951	-	Uchida H1031-1	Hawaii	*Cocos nucifera*	Fruit
katsurae	16915	-	SCRP388 = Panabieres F441 = CBS587.85 = ATCC36818 = IMI325914	Taiwan	-	soil
taxon Agathis (PTA)	16471	EF067922	ATCC32256 = FRI135	New Zealand	*Agathis australis*	
taxon Agathis (PTA)	17021	-	Beever REB316-1	New Zealand	*Agathis australis*	Bleeding lesion

* names as used in this study, but see text for further discussion.
** International Collection of Microorganisms from Plants, maintained by Landcare Research.
*** the first number quoted is the immediate source of the ICMP isolate.

Following PCR, the PCR products were column purified and sequenced in both directions with ITS-4 and ITS-6. *Phytophthora katsurae, P. heveae* and *P. citricola* ITS sequences were retrieved from GenBank to represent authentic isolates of the species that were being analysed. The DNA sequence data were trimmed to (5'-CCACACC….TCTCAA-3') to anchor the alignment and the sequences were aligned with Clustal X (Thompson and others 1997). Gap opening and gap extension parameters were adjusted to achieve the best visual alignment. The aligned sequences were then imported into Mega 4 (Tamura and others 2007) and a Neighbour-Joining analysis, using the Kimura 2-parameter model was used to construct a phylogeny, which was rooted with *P. citricola*.

Results

Field Observations

PTA was found in 2006 in a stand of regenerating kauri on the Maungaroa Ridge in the Waitakere Range near Auckland city, following an enquiry from the public about unusual kauri deaths. As at the Great Barrier site, affected trees showed foliage yellowing, canopy thinning and bleeding lower trunk lesions sometimes girdling the trunk and extending to the major roots. PTA was isolated from the margin of bleeding lesions. A distinct disease front could be identified along some parts of the stand. These observations are very similar to those of Gadgil (1974), who in addition demonstrated rapid death of saplings following wound inoculation on the lower stem. We conclude that PTA causes the trunk and root lesions and we designate the disease caused by PTA as kauri collar rot. As such, this designation does not exclude the possibility that more field work may link kauri collar rot with other species of *Phytophthora* in the future. Kauri collar rot has been observed in two other stands of regenerating trees in the Waitakere Range and in addition, large relatively isolated trees in mature forest have been observed with collar rot symptoms. For example, in the Cascades region of the Waitakere Range, which has not been modified by past logging, large mature trees with partly dead stag-like canopies and a sector of their trunks dead occur sporadically. Examination of some such trees shows extensive gummosis and lesion development at the margins of the dead tissue at base of the trunk, which we interpret as collar rot infection established many years earlier and gradually ring-barking the tree. On the other hand, other trees showing putative collar rot symptoms show no obvious leaf or canopy symptoms, and in some instances, it appears as if the putative lesion has been walled off as only old gummosis is apparent. No attempts have yet been made to isolate *Phytophthora* from such mature trees.

Detailed examination of trees at a regenerating site near Huia in the Waitakere Range where symptoms are abundant indicates that trees of all size classes are affected (fig 1). PTA was obtained from both trunk lesions and soil apple baiting at this site. The vegetation at this site includes senescent trees of *Kunzea ericoides* and is interpreted as a seral successional site following timber extraction and fire perhaps >100 years ago (Ogden 1983). The death of kauri at this site is likely to lead to a shift towards dominance by podocarp tree species such as *Dacrydium cupressinum* (rimu), which is present at the site but appears unaffected.

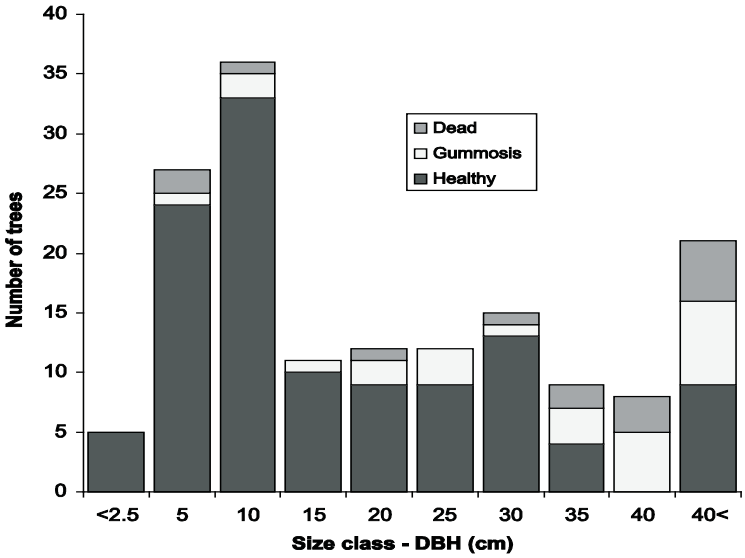

Figure 1—Size class distribution of *Agathis australis* (kauri) in a collar rot-affected forest stand at Huia, Waitakere Range. In total, 156 trees were scored. Health status was assessed as: healthy, showing gummosis (lesions oozing gum) or dead.

Collar rot symptoms have also been observed in Waipoua Forest and nearby Trounson Kauri Park, and PTA was recovered by soil baiting at Trounson (M. Braithwaite pers. comm. 2007). However, no symptoms were observed in two brief surveys in forests in the Coromandel Peninsula (fig 2).

The Great Barrier site described by Gadgil (1974) was revisited in 2006 by one of us (REB). Affected trees covered an area of c.10ha, representing a 5~10-fold increase since 1972. This corresponds to a rate of spread of c.3m per year on the assumption of circular areas of infection, a rate comparable to that of *P. cinnamomi* spread in southwestern Australia (Strelein and others 2006). An ill-defined disease front could be identified from a distance. PTA was recovered from a bleeding lesion (ICMP 17027).

Based on symptomatology, the occurrence of PTA in soil samples, the estimated rate of spread at the Great Barrier site, and the presence of distinct disease fronts at some localities, we propose that PTA is a soil-borne species that spreads predominantly through soil and soil water movement.

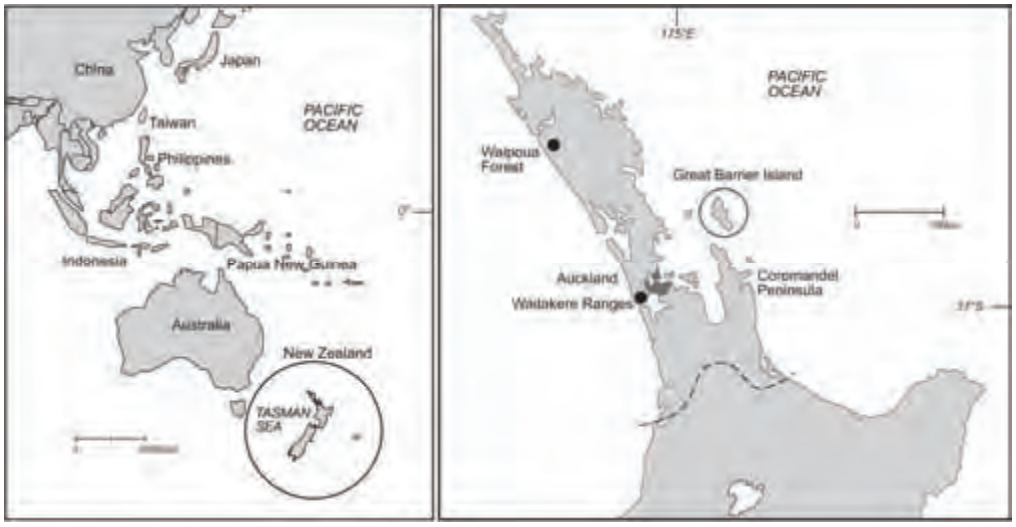

Figure 2—Map showing location of New Zealand in the Pacific and northern New Zealand showing main locations mentioned in text. Trounson Park is close to Waipoua Forest. The approximate southern limit of kauri is shown by the dotted line.

Taxonomic Studies

The recent revolution in *Phytopthora* taxonomy warranted a re-examination of the nature of PTA. Recent isolates from the Waitakere Ranges, from Gt Barrier Island and from Trounson were compared with an isolate from the 1970s study (Gadgil 1975) that had been stored by ATCC (Table 1). All isolates were culturally and morphologically similar, being homothallic with large relatively smooth to slightly rugose oogonia resembling those of *P. heveae* (fig. 3). All isolates had identical ITS sequences and phylogenetic analysis places them close to, but distinct from, *P. heveae* in the ITS clade 5 of Cooke and others (2000) (fig 4). PTA is closer in ITS sequence to isolates identified as *P. katsurae*, being identical to one isolated from soil in Taiwan (ICMP16915) and differing by 3 base pairs (substitutions) from isolates from *Cocos* (AF266771, ICMP 16948, ICMP 16950, ICMP 16951).

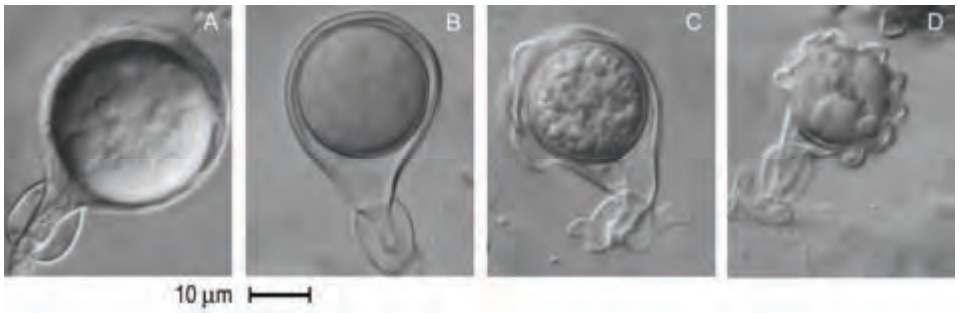

Figure3—Gametangia of selected *Phytophthora* isolates from clade 5 of Cooke and others (2000). Cultures were grown for 19 d in the dark on V8 agar. (A) PTA (ICMP 17021), (B) *P. heveae* (ICMP 16914), (C) *P. katsurae* (ICMP 16950), (D) *P. katsurae* (ICMP 16915).

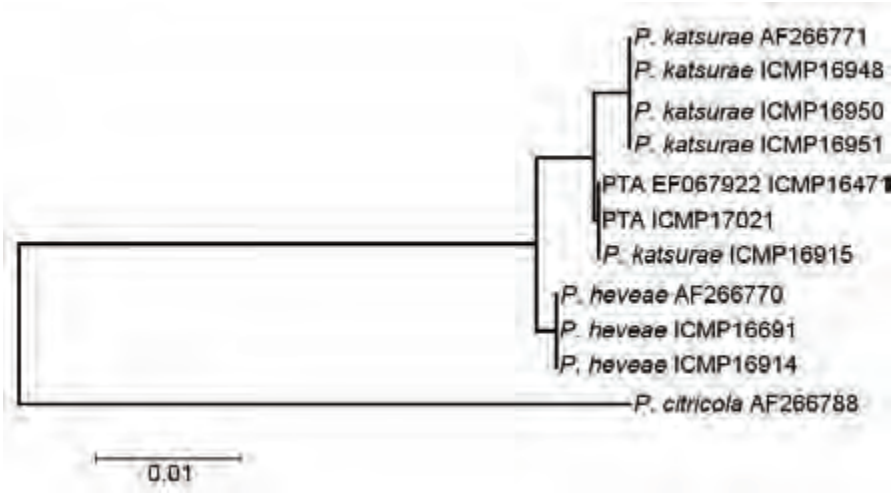

Figure 4—Neighbour joining phylogenetic tree based on ITS sequences of selected *Phytophthora* strains obtained in this study. Representative GenBank sequences used by Cooke and others (2000) for clade 5 (AF26671 for *P. katsurae*, AF26670 for *P. heveae*) are included along with AF266788 (*P. citricola*) as an outgroup.

Discussion

Despite PTA's high pathogenicity relative to *P. cinnamomi*, Gadgil (1974) did not conclude it posed a particular threat to kauri, at least in part because it was known only from one relatively isolated island site. This assessment must be reviewed in the light of our findings of its more widespread occurrence on the mainland including the Waipoua region. We consider it unlikely that these new occurrences represent recent translocations from Gt Barrier Island. Rather we suspect it has been present, but undetected, at these sites for many years. Our rationale is that while the bleeding lesion symptoms are relatively obvious, kauri responds to any injury by producing resin and indeed this feature has been used in the past to promote commercial gum production by purposely injuring the trees. Thus examples of collar rot-affected trees are likely to be overlooked by the casual observer. More work is needed to better clarify the relationships between PTA presence, lesion production and rates of spread. In this regard, we note that Gadgil (1974) reported PTA from soil under apparently healthy trees on Gt Barrier some distance from the collar rot-affected site, and concluded that the fungus was widespread and that some environmental factor was involved in triggering disease expression. This site has not been revisited to observe whether or not disease was expressed subsequently. Nevertheless, we conclude there is sufficient *a priori* evidence to propose that PTA poses a threat to stands of regenerating kauri, where its effect may be to change the composition of the forest to one dominated by podocarps. Additionally, we propose that PTA poses a threat to large iconic kauri, where infection may lead to premature death, albeit over a period of many years.

Three of the species associated with kauri have only been recorded once. *Phytophthora cryptogea* was reported from dying kauri seedlings in a nursery in the 1950s (Newhook 1959) and there is no reason to suspect this was more than an opportunistic infection associated with poor nursery hygiene. Similarly, *P. kernoviae*

was recovered only once, from soil beneath a large recently dead kauri at Trounson Kauri Park during a survey of northern kauri forests (see below).

However, little significance can be attached to this finding in relation to kauri health, especially as *P. kernoviae* has now been found, also in soil, at a number of other locations encompassing the natural range of kauri (Ramsfield and others 2008). *Phytophthora nicotianae* has been reported once from a bleeding lesion on a young tree (Brien and Dingley 1959). No collections or isolates are available to confirm this identification, and we conclude this species plays no more than a minor role in kauri health.

In contrast, *P. cinnamomi*, is widespread both in mature and regenerating kauri forests (Newhook and Podger 1972, Podger and Newhook 1971). Furthermore, *P. cinnamomi* has also been associated with seedling mortality in nursery beds (Newhook 1959), and pathogenicity tests indicate kauri seedlings and sapling are indeed susceptible to *P. cinnamomi* (Horner 1984, Johnston and others 2003, Podger and Newhook 1971). Death of trees associated with unnatural disturbance such as track and road building has sometimes been attributed to *P. cinnamomi* infection. Nevertheless, in natural communities, *P. cinnamomi* has seldom been associated with tree ill-health. For example, in May 2003 Beever and Horner (unpublished) surveyed *Phytophthora* associated with adult kauri in Waipoua Forest and the nearby Trounson Park using lupin-baited soil sampling. *P. cinnamomi* was recovered frequently at both Waipoua (47 percent of 36 samples) and Trounson (25 percent of 25 samples). However, no association was detected between recovery of *P. cinnamomi* and tree health, with *P. cinnamomi* recovered in similar numbers under both healthy and non-thrifty trees. PTA was not recovered during this survey.

However, Podger and Newhook (1971) do implicate *P. cinnamomi* in a disease outbreak in natural stands in the Waitakere Range where kauri was showing symptoms similar to those of littleleaf disease of *Pinus echinata* in the southern US. These symptoms included severe chlorosis, canopy thinning and dieback, and sometimes death. Affected trees were observed in two small areas of dense stands of regenerating forest. *P. cinnamomi* was isolated from kauri roots as well as the roots of many associates in the stands. The authors attributed the disease to severe fine root damage caused by *P. cinnamomi* associated with abnormally heavy summer rainfall and unusual waterlogging of the soil at the affected sites. While Podger and Newhook (1971) point out that stands of naturally regenerating kauri are common and raise the possibility that *P. cinnamomi*-associated mortality might increase in such stands in the future, casual observation suggests this has not occurred to date. We (Horner and Beever) revisited the primary site studied by Podger and Newhook (1971) in August 2006 and, while rotting fallen kauri trunks were observed, the remaining trees look in good health. We conclude that *P. cinnamomi* probably plays a minor role in health of adult kauri, perhaps as part of a root disease complex that under abnormal conditions at some sites leads to disease. Nevertheless, *P. cinnamomi* may be playing a more subtle role in forest dynamics through pre- and post-emergence damping off of kauri seedlings (Johnston and others 2003). Experimental treatment with metalaxyl in regenerating forest plots suppressed *P. cinnamomi* root rot in kauri seedlings, and resulted in a substantial increase in seedling growth (Horner 1984).

Phytophthora katsurae was described from Japan (as *P. castanea*), where it was associated with lower trunk lesions of chestnut (Katsura 1976). Subsequently it was recorded from natural forest soil in Taiwan and renamed (Ko and Chang 1979). Based on cultural and morphological characters, including strongly bullate oogonia, Ko and Chang (1979) concluded the Taiwan soil fungus (ATCC 36818 = ICMP16915) was identical with a 'type' culture of *P. katsurae* obtained from K. Katsura, which they also examined. ICMP16915 shows these strongly bullate oogonia (fig. 3). *P. katsurae* coconut isolates have moderately bullate oospores (fig. 3, Ko and others 2006). Recently, Oh and others (2007) report a chestnut trunk lesion pathogen from Korea resembling the type of *P. katsurae* in its strongly bullate spores and pathogenicity to chestnut and with ITS sequence identical to both PTA as well a chestnut isolate (C. Hong 22H6) from Japan (E. Oh pers. comm. 2008, these proceedings). Isolate C. Hong 22H6 is a derivative of P. Tsao P990, which was received from Japan in 1970 as #031 (M. Gallegly, C Hong pers. comm. 2008) and is linked by the latter number to the holotype (Kyoto Herbarium no. 1971-031), and is thus very likely an ex type culture. The Korean chestnut pathogen is thus reasonably determined as *P. katsurae*. However, despite PTA having an identical ITS sequence, we suspect PTA is not this species based on its smooth to slightly rugose oogonial ornamentation (fig. 3) and different cardinal temperatures (Horner unpublished). We propose that PTA may be introduced to New Zealand, in view of its relatively recent first report (Gadgil 1974) coupled with its distinctive symptoms on kauri, and the cultural similarity of isolates recovered to date along with their identical ITS sequences. However, we have not located reports of isolates matching PTA in both spore ornamentation and ITS sequence from other parts of the world. Whether or not it is introduced, we suggest it should be treated as introduced for management purposes until the situation is clarified. Such management could include restricting access to affected sites and ensuring high health plants are used in restoration projects.

In considering the origin of PTA, it is useful to consider the records of *P. heveae* and *P. katsurae*, especially those from apparently natural plant communities. Arentz (1986) reports both species are present in soils in Papua New Guinea, including rainforests containing *Agathis robusta* and *Araucaria cunninghamii* and *Araucaria hunsteinii*, although without obvious linkage to disease. Brown (1999), in a study focused on the association between *P. cinnamomi* and patch death in tropical rainforests of northern Queensland (containing both *Agathis* and *Araucaria*), also reports both *P. heveae* and *P. katsurae*, with *P. heveae* more frequent at higher elevations under dead patches of rainforest. Ko and others (2006) report both species from natural forests in Taiwan, neither in obvious association with disease. However, none of these authors provide DNA sequence data for their isolates and so their precise relationships are not clear, although the oogonial ornamentation of the Taiwan *P. katsurae* isolates matches ICMP16915, also from Taiwan. Ko and others (2006) point out that their Hawaiian *Cocos* isolates of *P. katsurae*, which include isolates H1024 and H1027 for which we obtained sequence data, are much less ornamented than the Taiwan isolates and indeed some approach *P. heveae* and PTA in their lack of ornamentation, although differing from both in ITS sequence. While it is reasonable to conclude both *P. heveae* and *P. katsurae* are indigenous to eastern Asia and Australasia, whether records from further afield (Erwin and Ribeiro 1996) represent indigenous populations or recent introductions must await further study. A recent multi-locus study (Blair and others 2007) confirms that *P. heveae* and *P. katsurae*, the only named members of clade 5 of Cooke and others (2000), are closely

related but phylogenetically distinct species. Morphologically, they are distinguished largely by the ornamentation on the oogonium (Erwin and Ribeiro 1996, Ko and others 2006, Oudemanns and Coffey 1991). It is apparent that a more comprehensive study of cultural, morphological and molecular variation of isolates from around the world, including in particular areas of possible natural occurrence, is warranted to better clarify the number and relationships of the clade 5 species, including PTA.

Acknowledgments

Maureen Fletcher, Duckchul Park, Sarah Tsai, Nic Faville (Landcare Research), Mark Braithwaite, Mark Bullians and Heather Pearson (MAF Biosecurity), Jenni Stanley and Asher Jones (Auckland University) assisted with laboratory and field work and figure preparation. Alex Nathan (Te Iwi O Te Roroa), Stephen King (Waipoua Forest Trust), and Dept of Conservation (Northland) facilitated our sampling at Waipoua and Trounson. David Cooke (Scottish Crop Research Institute, Scotland), Janice Uchida (University of Hawaii, US) and Michael Priest (Orange Agricultural Institute, Australia) supplied cultures. Auckland Regional Council (Jack Craw) supported our work in the Waitakere Range. ATCC cared for the PTA isolate deposited in 1970s, and facilitating our obtaining it for the present study. Don Woodcocks (Dept of Conservation, Gt Barrier), who assisted in the 1972 field work on Gt Barrier, guided us to the original site in 2006. Peter Gadgil encouraged the continuation of his pioneering study on PTA. This work was funded by New Zealand's Foundation for Research, Science and Technology and Ministry of Research, Science and Technology, and MAF Biosecurity New Zealand.

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Pythiaceous Fine Feeder Root Pathogens Associated With *Eucalyptus gomphocephala* (Tuart) Decline in Southwest Western Australia[1]

Peter M. Scott,[2,3] Bryan L. Shearer,[4] Paul A. Barber,[2,3] and Giles E.St.J. Hardy[2,3]

Abstract

Eucalyptus gomphocephala is a keystone canopy species endemic to a narrow (5-10 km wide) coastal strip approximately 300 km in length in south-west Western Australia. *Eucalyptus gomphocephala* is undergoing a significant decline that was first identified as a spot decline in 1994 and now occurs throughout large sections of its remnant distribution within Yalgorup National Park, in some areas resulting in 100 percent mortality. Multiple factors, including soil-borne pathogens, have been identified as possibly contributing to the decline. Fewer fine roots are associated with trees on declining sites compared to those on healthy sites. Foliar analysis indicates that declining trees have lower concentrations of some micronutrients, including zinc, which uptake is typically impaired by fine feeder root loss. A range of Pythiaceous microorganisms have been isolated from declining roots and these may be contributing to the loss of fine roots. Glasshouse trials are currently underway to determine whether these isolates are indeed pathogenic.

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Phytophthora Species Associated With Tanoak Stem Cankers in Southwestern Oregon[1]

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Abstract

From 2001 through 2006 stem cankers on tanoak (*Lithocarpus densiflorus*) were sampled during surveys to detect and eradicate *Phytophthora ramorum* from forests in southwestern Oregon. Pieces of bark from stem canker margins were plated on cornmeal agar amended with 10 ppm natamycin, 200 ppm Na-ampicillin, and 10 ppm rifampicin. *P. ramorum* was usually identified on the isolation plates by the presence of characteristic hyphae, chlamydospores, and sporangia. Other colonies resembling *Phytophthora* were isolated into pure culture and identified by examination of morphological features and by DNA sequence analysis. *Phytophthora* species other than *P. ramorum* isolated from tanoak stem cankers include *P. cambivora*, *P. cinnamomi*, *P.* species "Pgchlamydo," *P. gonapodyides*, *P. nemorosa*, *P. pseudosyringae*, and *P. siskiyouensis*. Additional isolates may represent new species of *Phytophthora* and have not yet been identified. Selected isolates were tested for pathogenicity by inoculating healthy tanoak stems. Lesion margins were plated for re-isolation of the pathogen.

Introduction

Since 2001 the Oregon Department of Forestry (ODF) has conducted an intensive quarantine and eradication effort in southwestern Oregon to limit the spread of *Phytophthora ramorum*, cause of sudden oak death (SOD). Diseased tanoak (*Lithocarpus densiflorus*) trees were sampled to verify infection by *P. ramorum*. In some cases sampled infections yielded a species of *Phytophthor*a other than *P. ramorum*. We identified these species and tested some of them for their pathogenicity on tanoak.

Materials and Methods

Aerial surveys were conducted by the Oregon Department of Forestry twice yearly (2001 through 2006) to locate dead or dying tanoak trees. Cause of death or disease was determined by follow-up ground surveys. Cankers or lesions in tanoak stems were sampled for the presence of *P. ramorum* by plating bark from the leading edge in corn meal agar amended with 10 ppm Na-natamycin, 200 ppm Na-ampicillin, and 10 ppm rifampicin (CARP). *Phytophthora* colonies were purified by re-plating on fresh CARP and then sub-cultured on corn meal agar amended with 20 ppm β-

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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sitosterol (CMAβ) for DNA extraction and long-term storage. Isolates were stored at room temperature as agar plugs in sterile de-ionized water.

For morphological identification isolates from 2006 were grown on 15 percent clarified V8 agar amended with 20 ppm β-sitosterol (V8S). Plugs from colony margins were transferred into stream water to generate sporangia. Isolates which did not form oogonia in single culture on V8S were paired with standard A1 and A2 tester strains on V8S to generate oospores and determine mating type. Structures for microscopic observation were fixed and preserved in 3.7 percent formaldehyde. Isolates were identified by comparison of features with published descriptions (Brasier and others 2003, Hansen and others 2003, Jung and others 2003, Reeser and others 2006, Waterhouse 1963). Representative isolates from each species identified were selected for DNA sequencing. DNA extracted from mycelium grown on CMAβ was used as template to amplify nuclear rDNA internal transcribed spacer (ITS) for sequencing. ITS sequences were compared with Genbank database using BLAST Search to aid identification.

Isolates from 2001 through 2005 were identified by sequencing the mDNA COX spacer and comparing with sequences of known reference isolates (Frank Martin, personal communication). Representatives from each group identified were confirmed by morphological examination as described above.

Five isolates of *P. cambivora,* four isolates of *P. gonapodyides,* one isolate of *P. nemorosa,* and one isolate of *P. siskiyouensis* were inoculated into green twigs of tanoak seedlings by placing a CMAβ culture plug over a pinprick wound and sealing with parafilm. Each inoculated twig also received an agar control. Lesion size was measured after 10 to 15 days, and lesion margins were plated on CARP for re-isolation of the pathogen.

One isolate each from *P. cambivora, P. cinnamomi, P. gonapodyides, P. nemorosa, P. siskiyouensis,* and *P.* species 'Pgchlamydo' was tested for pathogenicity on tanoak stems in the field. A 5 mm diameter plug from the margin of a V8S culture was placed in a hole in the bark, covered with wet cheesecloth and sealed with aluminum foil and duct tape. Each isolate was inoculated into five different stems. Each stem received three different isolates and an agar control. After four weeks bark was removed to reveal lesion development. Lesions were measured (length by width), and pieces from four points on the lesion margin were plated in CARP to re-isolate.

Results

Results of tanoak stem canker isolations are listed in table 1. More than one half of the tanoak cankers sampled were culture negative for *Phytophthora*. *P. ramorum* and *P. nemorosa* were the species most frequently isolated. Eight additional species were identified, three of them being un-named ('Pgchlamydo') or undefined ('ilicis clade' sp.1 and sp.2). Each of these eight species occurred only rarely.

Infection in green twigs of tanoak seedlings was variable. All four isolates of *P. gonapodyides* tested produced relatively large lesions in green tanoak twigs. Only one of the five isolates of *P. cambivora* tested produced lesions in green tanoak twigs. The one isolate of *P. nemorosa* tested produced moderately sized lesions in

green tanoak twigs, and the one isolate of *P. siskiyouensis* tested did not form any lesion.

Table 1—*Phytophthora* species isolated from tanoak stem cankers sampled during sudden oak death surveys in southwest Oregon from 2001 through 2006.

Species isolated	**Number of stem cankers**
Negative culture for *Phytophthora*	575
P. cambivora A1	4
P. cinnamomi A2	1
P. species 'Pgchlamydo'	1
P. gonapodyides	6
P. nemorosa	102
P. pseudosyringae	3
P. 'ilicis clade' sp.1	1
P. 'ilicis clade' sp.2	1
P. ramorum	359
P. siskiyouensis	4
Total stem cankers sampled	1057

In contrast, when inoculated in stems of field-grown tanoak, *P. cambivora, P. cinnamomi, P. gonapodyides, P. nemorosa, P. siskiyouensis,* and *P*. species 'Pgchlamydo' were all pathogenic after 4 weeks, causing substantial bark lesions relative to agar control (table 2). In all cases, the species used for inoculation was recovered on re-isolation from lesion margins.

Table 2—Pathogenicity of selected isolates of *Phytophthora* from tanoak stems. Isolates were inoculated into tanoak stems in the field.

Isolate	**Species**	**n**	**mean lesion area (cm^2) [a]**	**s.d.**
control		12	0.2	0.10
9641.1	*P. cinnamomi*	5	11.5	10.48
9569	*P. siskiyouensis*	5	13.4	84.63
9675-BK	*P. cambivora*	5	16.5	5.55
9364	*P*. sp. 'Pgchlamydo'	5	18.1	6.13
9684.2	*P. nemorosa*	5	18.4	14.75
9616	*P. gonapodyides*	5	18.6	7.59

[a] lesion area computed as approximate rhomboid (l x w)/2 - inoculum area

Discussion

Prior to the recent SOD epidemic, no species of *Phytophthora* were known to cause cankers in forest grown tanoak. The discovery of *P. ramorum* as a pathogen of tanoak in California was quickly followed by the discovery of *P. nemorosa* and *P.*

pseudosyringae associated with tanoak cankers. Six years of diagnostic support of survey and detection for *P. ramorum* in tanoak forests of southwest Oregon has revealed the occurrence, at very low frequency, of at least five additional species of *Phytophthora* causing stem cankers in tanoak.

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Monitoring Occurrence and Distribution of *Phytophthora* Species in Forest Streams in North Carolina Using Baiting and Filtration Methods[1]

Jaesoon Hwang,[2] Steven W. Oak,[3] and Steven N. Jeffers[2]

Abstract

The recent epidemics of sudden oak death, caused by *Phytophthora ramorum*, in the coastal forests of California and southwest Oregon have drawn attention to other species of *Phytophthora* present in natural ecosystems that may threaten forest tree health. Since *Phytophthora* species are well adapted to aquatic environments, the species present in a strategically-selected stream may represent the occurrence and distribution of *Phytophthora* species over the relatively large land area drained by this stream. Based on this assumption, five streams in three watersheds in the Pisgah National Forest in western North Carolina were monitored monthly for *Phytophthora* species. from April 2005 to March 2006. Filtration and two baiting methods were used to detect propagules of *Phytophthora* species in stream water. A 1-liter water sample was collected from each stream and vacuum-filtered with Nuclepore and Durapore membrane filters. In addition, wounded or non-wounded leaves of *Rhododendron maximum* were used as baits and were exposed in a stream for 3 days or 2 to 3 weeks, respectively.

Phytophthora species were detected from all five streams throughout the 12-month monitoring period. *P. cambivora, P. cinnamomi, P. citricola, P. citrophthora, P. gonapodyides, P. heveae, P. pseudosyringae*, and seven morphologically and genetically distinct groups of isolates were identified from 1560 isolates collected. *P. gonapodyides* was the most prevalent (1353 isolates) and was detected consistently throughout the monitoring period. *P. citricola, P. gonapodyides*, and *P. pseudosyringae* were widely distributed and occurred in all five streams. Diversity of *Phytophthora* species was greatest in July, when 11 species/groups were detected and least in February when only one species was detected. *P. gonapodyides* and *P. pseudosyringae* were the only two species recovered over winter months (from November to February). Thirteen of the 14 species/groups were detected by filtration while only eight species/groups were isolated with each baiting method. Overall, filtration was superior to baiting for detecting *Phytophthora* species in forest streams: it provided quantitative data on propagule density; it was more efficient than either baiting method; and it was more effective at detecting greater species diversity.

Introduction

The recent epidemics of sudden oak death, caused by *Phytophthora ramorum*, in the coastal forests of California and southwest Oregon have drawn attention to other species of *Phytophthora* present in natural ecosystems that may threaten forest tree

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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health. Since species of *Phytophthora* are well adapted to aquatic environments, the species present in streams may represent the occurrence of *Phytophthora* species over most or all of the relatively large land area drained by them. Consequently, five streams were sampled monthly for 12 months to determine if streams could be used to monitor occurrence and diversity of *Phytophthora* spp. in a forest watershed.

Materials and Methods

Five streams in four distinct watersheds in the Pisgah National Forest in western North Carolina were sampled monthly from April 2005 to March 2006: Bent Creek, Big Creek, Fletcher Creek, Davidson River, and South Mills River. Filtration and two baiting methods were used to detect propagules of *Phytophthora* spp. in stream water. For filtering, a 1-liter sample of water was collected from each stream, and the samples were processed within 10 h of collection. Nine 100-ml sub-samples of water were vacuum-filtered through two types of membrane filters (47 mm in diameter) with three pore sizes: Nuclepore filters with 1- and 3-μm pores and Durapore filters with 5-μm pores. The filters were inverted onto PARPH-V8 selective medium in 100-mm-diameter disposable petri plates, and the plates were placed in the dark at 20°C for two to three days. Filters then were removed and colonies of *Phytophthora* spp. were counted. Representative colonies were subcultured and stored for later identification.

For baiting, either four wounded leaves or four non-wounded leaves of *Rhododendron maximum* were placed in a mesh bag rigged to float just below the water surface. Leaves were wounded by making cuts (5 to 10 mm in length) perpendicular to the leaf edge at approximately 5- to 7-mm intervals along the leaf edge (fig 1). Wounded leaves were exposed in a stream for three days while non-wounded leaves were exposed in the same stream for two to three weeks. To isolate *Phytophthora* spp. from wounded leaves, five rectangular pieces (approximately 5 to 7 mm on a side) with water-soaked lesions (fig 1) were cut from the edge of each leaf and embedded in PARPH-V8 medium. To isolate from non-wounded leaves, five pieces (approximately 5 mm × 5 mm) were removed from the margin area of water-soaked or necrotic lesions on each leaf and transferred to PARPH-V8 medium. Isolation plates were held at 20°C in the dark for 14 days and examined regularly for mycelia characteristic of *Phytophthora* spp. Representative colonies were subcultured and stored for later identification.

Figure 1—Water-soaked lesions on a wounded rhododendron leaf after 3 days of exposure in a forest stream.

Phytophthora species were identified based on morphological and molecular characters. Isolates grown on PARPH-V8 medium were examined for the presence of sporangia, chlamydospores, and oospores. In addition, agar plugs with actively growing hyphae were removed, flooded with 1.5 percent non-sterile soil extract solution, and placed under fluorescent lights at room temperature (22 to 24°C) for 24 h to induce sporangium production. For molecular characterization, each isolate was placed in pea broth medium at room temperature for five to seven days to produce a mycelium mat for DNA extraction. The ITS region of ribosome DNA and *cox* region of mitochondrion DNA were amplified using ITS4 and ITS6 or FM75 and FM77/83 primers sets. RFLP analysis was done with *Alu*I and *Msp*I restriction enzymes for ITS region DNA and with *Alu*I, *Rsa*I, and *Taq*I restriction enzymes for DNA from the *cox* region (fig 2).

Over the course of monitoring these streams, native plants of *Rhododendron maximum* and *Kalmia latifolia* with symptoms of tip dieback and leaf blight were observed in the riparian zone along all five streams. Shoots on some of these plants appeared to have been submerged in the streams during periods of high water flow. Foliage samples from symptomatic plants were collected, and tissue pieces from lesions were embedded in PARPH-V8 medium to isolate *Phytophthora* spp. as described above. In addition, composite soil samples were collected from the riparian zone of all five streams and assayed for *Phytophthora* spp. using a baiting bioassay. Approximately 1-liter of soil was collected along each stream. Samples were mixed thoroughly, and three replicate 100-ml aliquots of each soil sample were flooded with distilled water (200 ml of water per 100 ml of soil) and baited with leaf pieces of camellia and rhododendron. After three days at room temperature, leaf-piece baits were transferred to PARPH-V8 medium and plates were placed in the dark at 20°C for seven to ten days. Colonies of *Phytophthora* spp. were isolated and identified as described above.

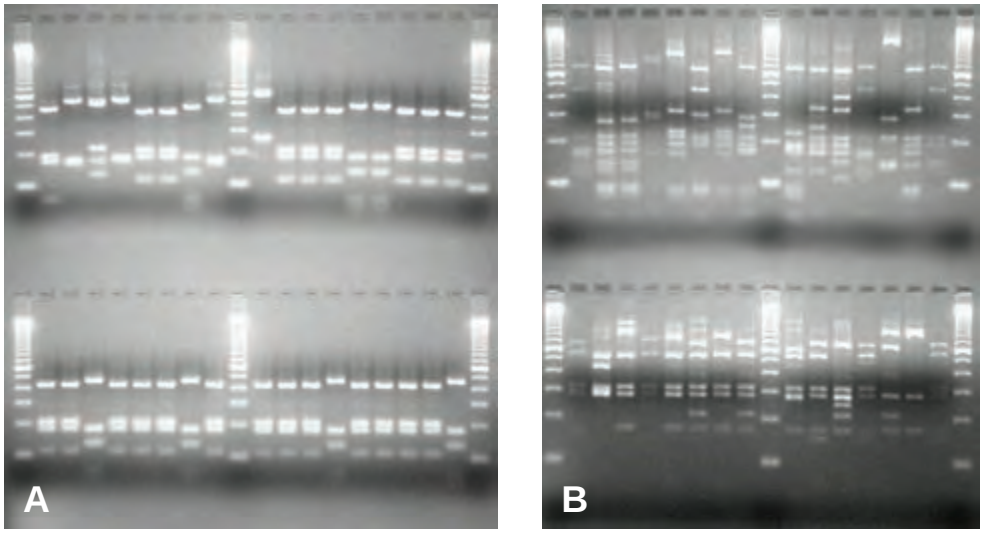

Figure 2—Identification of isolates of *Phytophthora* spp. by RFLP analyses: **A**, DNA from the ITS region of 34 isolates was amplified and digested with *Alu*I; **B**, DNA from the c*ox* region of 15 isolates was amplified and digested with *Alu*I (top) and *Rsa*I (bottom). Lanes (both top and bottom) 1, 10, and 20 in **A** and lanes 1, 10, and 18 in **B** contain a molecular marker.

Results and Discussion

Species of *Phytophthora* were detected in all five streams throughout the 12-month monitoring period. From 1,560 isolates collected, seven recognized species—*P. cambivora, P. cinnamomi, P. citricola, P. citrophthora, P. gonapodyides, P. heveae,* and *P. pseudosyringae*—and seven other morphologically and genetically distinct groups were identified. *P. gonapodyides* was most prevalent (1,353 isolates) and was detected consistently throughout the monitoring period. *P. citricola, P. gonapodyides,* and *P. pseudosyringae* were widely distributed and occurred in all five streams. The number and diversity of species of *Phytophthora* recovered varied depending on the month, location, and detection method. Diversity of *Phytophthora* spp. was greatest in July, when 11 species and groups were detected, and least in February—when only one species was detected. *P. gonapodyides* and *P. pseudosyringae* were the only two species recovered in winter months (November to February). The greatest diversity in a single stream occurred in the South Mills River where ten species and groups were detected over the 12-month sample period. Diversity was least in Big Creek where only four species and groups were found. Thirteen of the 14 species and groups were recovered by filtration while only eight species and groups were recovered with each baiting method. Overall, filtration was superior to either baiting method for detecting *Phytophthora* spp. in forest streams; it provided quantitative data on propagule density, recovered a greater diversity of species, and was more efficient because only one trip was needed to sample each stream. *P. citricola* and *P. heveae* were the only two species isolated from the samples of symptomatic plants collected along the five streams, and only *P. cinnamomi* and *P. heveae* were recovered from the riparian soil samples. In conclusion, *Phytophthora* spp. were abundant throughout the year in the five North Carolina forest streams we monitored, and there appeared to be a greater diversity of

species of *Phytophthora* in the streams than in the soils and plants along these streams—based on the methods employed in this study.

Acknowledgments

We thank Lynn Luszcz, Frank Martin, Patricia de Sa, Pedro Uribe, and Drew Zwart for technical assistance in isolation and species identification; Jill Baker for assistance with stream sampling; North Carolina Arboretum and Pisgah Ranger District in Pisgah National Forest for providing access to study locations. This project was funded by USDA ARS Foreign Disease-Weed Science Research Unit, USDA Forest Service Pacific Southwest Research Station, and Armstrong Wood Products Inc.

Phytophthora Species in Forest Trees in Hungary: A Genetic Approach[1]

Ferenc Lakatos[2] and Ilona Szabó[1]

Abstract

Disease symptoms caused by different *Phytophthora* species were studied in Hungary since 1999. A collection of more than 300 cultures from 6 main localities and from four different hosts (*Alnus glutinosa*, *Juglans nigra*, *Quercus petraea* and *Q. cerris*) was established. The identification of the isolates presented sometimes difficulties because of the absence of adequate morphological characters. In 2006 we have started to use molecular markers for species identification based on the methods and results published on the identification of different *Phytophthora* species and the advances of the available molecular methods at our institute.

Altogether 80 isolates were analyzed. DNA extraction and PCR assay were performed using the REDExtract-N-Amp kit from Sigma. The amplification of the DNA was carried out followed the manufacturer's instruction with the primers "ITS4" and „ITS6". Initial denaturation step of 3 min at 94° C, followed by 35 cycles of 94° C (30 sec), 55° C (30 sec) and 72° C (90 sec) and a final extension step at 72° C (10 min). PCR was performed in an Eppendorf Mastercycler and the sequence reactions on an AB 3730XL automatic sequencer respectively.

The DNA sequence was alignment by ClustalX using default settings. Distance analysis was performed by the Neighbor Joining NJ algorithm. The distance matrix was calculated based on the Tamura-Nei substitution model, while the robustness of the topology was tested by bootstrapping with 1000 repetitions. For comparison also several GenBank (http://www.ncbi.nlm.nih.gov/) entries of different *Phytophthora* taxa were taken.

74 percent of the amplified DNA resulted in sequences (59 isolates). Altogether seven already described and at least three undescribed (or at least in the GenBank as *Phytophthora* sp. documented) *Phytophthora* taxa were found. Eight of them were found in *Alnus* (*P. alni*, *P. citricola*, *P. gonapodyides*, *P. inundata*, *P. megasperma* and *P.* sp1, *P.* sp2, *P.* sp3), four in *Juglans* (*P. cactorum*, *P. citricola*, *P. hedraiandra*, *P.* sp1) and two in *Quercus* (*P. citricola*, *P. gonapodyides*). The sequences of the Hungarian isolates represents 4 major clades: 1: *P. inflata/citricola*, 2: *P. cactorum/ideae/hedraiandra*, 3: *P. inundata/megasperma/gonapodyides* and 4: *P. alni*. The mostly widespread is the *P. citricola/inflata* group, having four haplotypes (17 isolates) from all four investigated hosts. *P. gonapodyides* showed the highest polymorphism, having 7 haplotypes (11 isolates) on two hosts (*Alnus glutinosa* and *Quercus petraea*).

Three undefined taxa (*P.* sp1, sp2, sp3) didn't result in an exact species determination even after an intensive BLAST search in the GenBank. All haplotypes found were submitted to the GenBank and are under revision at the time of abstract submission.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Introduction

Disease symptoms caused by different *Phytophthora* species were studied intensively in Hungary since 1999 (Szabó and others, 2000). In the recent years a collection of more than 300 cultures from 8 localities (Barcs, Csorna, Hévíz, Jánossomorja, Homorúd, Kaszó, Sopron and Tornyosnémeti) and from four different hosts (*Alnus glutinosa*, *Juglans nigra*, *Quercus petraea* and *Q. cerris*) was established. The identification of the isolates presented sometimes difficulties because of the absence of adequate morphological characters. In 2006 we have started to use molecular markers for species identification based on the methods and results published on the identification of different *Phytophthora* species (Cooke and Duncan, 1997; Schena and Cooke, 2006; Garnica and others, 2006) and the advances of the available molecular methods at our institute.

Materials and Methods

The molecular identification was performed by sequencing the ITS1 and ITS2 regions of rDNA and comparing with the known *Phytophthora* sequences accessible in GenBank. Altogether 80 isolates were analyzed so far. The DNA was extracted from pure culture using REDExtract-N-Amp kit from Sigma-Aldrich. PCR was performed using specific primers ITS6 and ITS4 (Cooke and Duncan 1997) in an Eppendorf Mastercycler and the sequence reactions on an AB 3730XL automatic sequencer respectively.

The DNA sequence was aligned by ClustalX using default settings. Distance analysis was performed by the Neighbor Joining NJ algorithm with Kimura 2 parameter. The distance matrix was calculated based on the Tamura-Nei substitution model, while the robustness of the topology was tested by bootstrapping with 1000 repetitions. For comparison also several GenBank entries of different *Phytophthora* taxa were taken.

Results

Seventy-four percent of the amplified DNA resulted in sequences (59 isolates). Altogether seven already described and further 4 undescribed (in the GenBank as *Phytophthora* sp. documented) *Phytophthora* taxa were found. Ten of them were found in *Alnus* (*P. alni*, *P. citricola*, *P. gonapodyides*, *P. hungarica*, *P. inundata*, *P. megasperma* and *P.* sp1, *P.* sp2, *P.* sp3, *P.* sp4), three in *Juglans* (*P. cactorum*, *P. citricola*, *P.* sp1) and two in *Quercus* (*P. citricola*, *P. gonapodyides*) (Table 1.).

Table 1—*Phytophthora* species found in different forest stands

***Phytophthora* species**	***Alnus glutinosa***	***Juglans nigra***	***Quercus petraea***	***Quercus cerris***
Phytophthora alni	X			
Phytophthora cactorum		X		
Phytophthora citricola	X	X	X	X

Phytophthora gonapodyides	X		X	
Phytophthora hungarica	X			
Phytophthora inundata	X			
Phytophthora megasperma	X			
Phytophthora sp1	X	X		
Phytophthora sp2	X			
Phytophthora sp3	X			
Phytophthora sp4	X			
Total	10	3	2	1

P. alni was found at two locations in *Alnus* stands solely. The three sequenced isolates from one location were genetically identical. *P. cactorum* was found on one location in a *Juglans* stand. The four DNA fragments showed minimal genetic variation (1bp, 0.16 percent). *P. citricola* was the most widely distributed species found in all four forest types. Also the investigated 17 isolates showed the highest genetic diversity (11bp, 1.44 percent). *P. gonapodyides* was found on two hosts on *Alnus glutinosa* and *Quercus petraea*. The investigated 11 isolates showed moderate genetic diversity (5 bp, 0.61 percent). *P. hungarica*, *P. inundata* and *P. megasperma* was found only in *Alnus glutinosa* stands with no (*P. hungarica* and *P. megasperma*; two isolates each), or limited (*P. inundata*; 8 isolates) genetic variation.

Four additional *Phytophthora* taxa were found in *Alnus glutinosa* stands. None of them (*P.* sp1, sp2, sp3 and sp4) turned out as a known species even after an intensive BLAST search in the GenBank. These 15 investigated isolates have a fragment length between 817-822bp. Some of them showed some similarities to closely related species in the clade 6 (regarding Cooke and others, 2000), which is one of the most complicated clade within the genus (Brasier and others 2003).

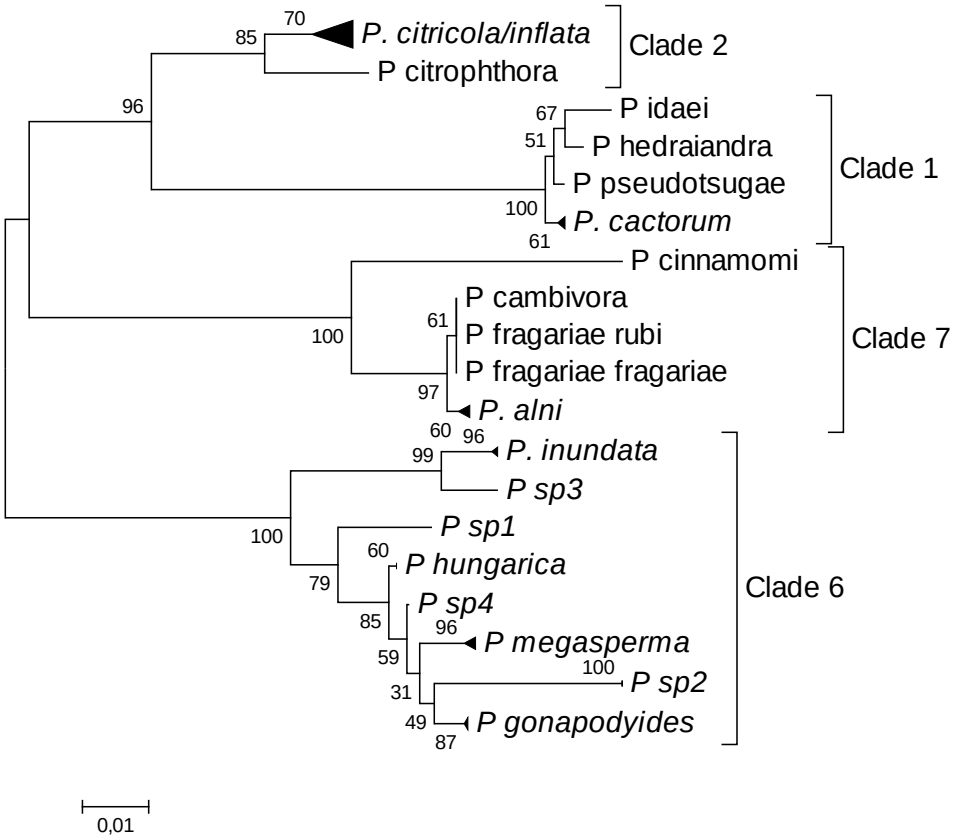

Figure 1—The Neighbour-Joining tree of the investigated *Phytophthora* isolates (Kimura-2 parameter). In *Italics*: species found in Hungary, others were taken from GenBank for comparison.

The sequences of the Hungarian isolates represent 4 caldes regarding the classification of Cooke and others (2000) (fig 1.): Clade 1: *P. cactorum* ; Clade 2: *P. citricola/inflata*, Clade 7: *P. alni* and Clade 6: *P. inundata/hungarica/megasperma/gonapodyides* including all undescribed species (P. sp1, sp2, sp3 and sp4). The mostly widespread is the *P. citricola/inflata* group, having four haplotypes (17 isolates) from all four investigated hosts. *P. gonapodyides* showed the highest polymorphism, having 7 haplotypes (11 isolates) on two hosts (*Alnus glutinosa* and *Quercus petraea*).

All haplotypes found were submitted to the GenBank and are under revision at the time of submission.

Conclusions

Seven described and further four until now undescribed species of *Phytophthora* were found in Hungary: *P. alni, cactorum, citricola, gonapodyides, hungarica, inundata* and *megasperma*. Large genetic diversity was found in the investigated *Phytophthora* isolates regarding the host tree species and location. Large intraspecific variabiliy was found in *P. citricola* and *P. gonapodyides*. The analysis of the ITS

fragment doesn't allow the species determination in some cases. Here the analysis of further DNA parts is desired.

Acknowledgment

Our research was supported by the Hungarian Research Fund (OTKA T049077).

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Phytophthora Biodiversity: How Many *Phytophthora* Species Are There?[1]

Clive Brasier[2]

Introduction

Many new *Phytophthoras* species are currently being identified associated with the nursery trade or natural ecosystems. Many of these may be recent invasives: a result of the increasing international movement of plants leading to export and spread of exotic *Phytophthora* species. Examples include *P. ramorum* and *P. kernoviae*. The frequency of the new discoveries, the obvious taxonomic and phylogenetic interest involved, and the possible plant health risk to forests prompted me to take a look at the theoretical question: how many *Phytophthoras* species are there? To explore this question, it seems best to begin with the history of *Phytophthora* species over time.

Phytophthora species 1876–1999

The numbers of known *Phytophthora* species represented in various taxonomic treatise's between de Bary's (1876) description of *P. infestans* and Erwin and Ribeiro's (1995) encyclopaedic compilation of *Phytophthora* knowledge '*Phytophthora* Diseases Worldwide' is shown in table 1.

Table 1—*Phytophthora* species over time 1876–1996

Date	Author(s)	Number of known or described species
1876	Anton de Bary	*P. infestans*
(1876 ~1920s)		(uncertain ~ some generic confusion)
1917	J. Rosenbaum	ca 11 species
1931	C.M. Tucker	ca 21 species
1963	G.M. Waterhouse	ca 41 species
1976	F.J. Newhook, G.M. Waterhouse and D.J. Stamps	ca 50 species
1996	D.C. Erwin and O.K. Ribeiro	ca 54 species

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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The "early period" *ca* 1880–1920 involved a considerable degree of taxonomic uncertainty as criteria to separate Oomycete genera were consolidated. Among the historic land marks, Rosenbaum (1917) made one of the first scholarly compilations of *Phytophthora* species using morphological criteria such as antheridial type, sporangial papillum type, chlamydospore presence and biometric measurements such as sporangial length/breadth ratio. Tucker (1930), in a sizeable monograph and key, investigated both the morphological and physiological aspects of described *Phytophthoras* species including host specificity and temperature–growth relationships. Waterhouse (1963) organised the described species into her six well known morphological groups, based mainly on the antheridial and sporangial criteria used by Rosenbaum and Tucker. She also produced a workable key based on the same criteria. Newhook, Waterhouse and Stamps (1976) updated the Waterhouse approach with a tabular key. Erwin and Ribeiro's (1995) volume bought all the available information on *Phytophthora* species together.

Erwin and Ribeiro (1995) was the ultimate word on biology and pathology of *Phytophthora* species prior to the era of *Phytophthora* population biology and molecular genetics. It marked the end of the period in which Phytophthoras were treated primarily as morphological entities for taxonomic purposes. A curve fitted to the number of species known and described over the period 1876–1995 is shown in figure 1. It shows a steady but unremarkable rate of increase, rising to about 54 described species at the time Erwin and Ribeiro's volume went to press. This figure had increased only to 55 species by 1999 (table 2), with the description of *P. quercina* (Jung and others 1999).

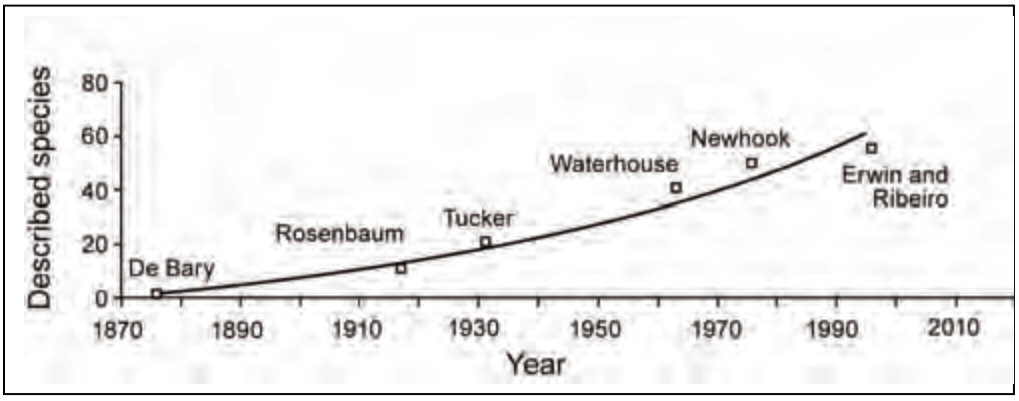

Figure 1—Number of described *Phytophthora* species over time, 1876–1995.

How Many Phytophthoras?

Hawksworth (2001) estimated that only 10 percent of fungi were known to science. Crous and Groenwald (2005) proposed that only 7 percent of fungi were known. One could use these authors' estimates as a simple, though crude, rule of thumb from which to estimate the possible total number of extant *Phytophthora* species, albeit that they should be treated with caution, since they are hedged around with various assumptions.

A necessary assumption for the present purpose is that species diversity in Oomycete fungi follows similar principles to that in "true fungi." The estimation process also involves some rather fanciful play with dates and numbers. In the world of estimates it depends which figures you use and how you use the figures. As a starting point in the process, I am also going to assume that there were ~60 *Phytophthora* species known at the end of 1999. This is slightly higher than the figure of 55 species deriving from the 54 taxa listed by Erwin and Ribeiro (1995) plus *P. quercina* (table 2). Even the figure of 54 species for Erwin and Ribeiro is not an absolute, since various equivocal taxa are listed in their treatise, such as possibly synonymous species (e.g. *P. arecae* and *P. palmivora*), varieties (e.g. *P. cinnamomi* var *parvispora*), and unconfirmed or improperly described spcies such as *P. eriugena*.

Table 2—*Phytophthora* species described Pre 2000

P. arecae	*P. erythroseptica*	*P. japonica*	*P. phaseoli*
P. boehmeriae	*P. fragariae frag.*	*P. katsurae*	*P. porri*
P. botryosa	*P. fragariae rubi*	*P. lateralis*★	*P. primulae*
P. cactorum★	*P. gonapodyides*	*P. macrochlamy.*	*P. pseudotsugae*★
P. cajani	*P. heveae*	*P. meadii*	*P. quercina*★
P. cambivora ★	*P. hibernalis*	*P. medicaginis*	*P. quininea*
P. capsici	*P. humicola*	*P. megakarya*	*P. richardiae*
P. cinnamom★	*P. idaei*	*P. megasperm*★	*P. sinensis*
P. citricola★	*P. ilicis*★	*P. melonis*	*P. sojae*
P. citrophthora	*P. infestans*	*P. mexicana*	*P. syringae*
P. clandestina	*P. inflata*★	*P. mirabilis*	*P. tentaculata*
P. colocasiae	*P. insolita*	*P. multivesiculata*	*P. trifolii*
P. cryptogea★	*P. iranica*	*P. nicotianae*	*P. vignae*
P. drechsleri	*P. italica*	*P. palmivora*	~11/55 = 20 percent ★

★ Damaging to nursery trees and/or forests and natural ecosystems.

Taking Hawksworth's 10 percent as the proportion of known fungal species, then, using the figure of ~60 described species at the end of 1999, one arrives at a possible total of 600 extant *Phytophthora* species i.e. currently living species including both the knowns and unknowns. That implies a further, rather daunting possibility that there may have been as many as ~540 *Phytophthora* species still unknown (meaning not yet discovered and not yet formerly described) at the beginning of the current millennium!

However, instead of applying the raw figure of about 600 extant species, I propose using a more conservative, bracketed figure of between 200–600 extant species. On this basis, there may have been anything between 140 and 540 unknowns at the end of the last millennium. Another way to view this 140–540 range is to consider it as 340 ± 200 unknowns. This figure seems to me more comfortable, although not necessarily more comforting. If it is a reasonable approximation to the truth, then whatever the precise figure is, the large numbers of still undiscovered *Phytophthora* species are going to be a significant challenge to our diagnostic and taxonomic expertise, our phylogenetic expertise, and perhaps even to the patience of those who will have to write their descriptions. Such a large number of unknowns may also represent a threat to our plant health regimes, as I shall mention later.

Developments Since 2000

While the number of described *Phytophthora* at the end of 1999 was about 55 (table 2), already, since 2000, a remarkable 50+ new species (species that have been morphologically and molecularly identified as unique though not yet necessarily formally described) have been discovered. These are listed in table 3. Again, the figure of 50+ species is an approximation. The precise number is unknown, and I have heard of more potential additions since this paper was originally prepared in August 2007. Also, some taxa, such as *P. alni* and its subspecies, could either be counted as one species or as multiple taxa. Nonetheless, the fact that the number of species described or under description in the seven years since the year 2000 is about the same as the number described in the previous 120 years is, surely, extraordinary.

In figure 2, a new data point has been added to the species versus time diagram of figure 1. I have used the IUFRO *Phytophthora* meeting of 2007 as the cut-off point. The amended diagram clearly shows the dramatic upswing since 1995. What is the explanation for this upswing? I suggest that four main factors are responsible:

- The emergence of more holistic, population-based species concepts since the 1980s, leading to greater awareness of the subtlety and complexity of *Phytophthora* species, including the existence of uniquely evolutionarily adapted species within the old taxonomic morphospecies, and so to a greater willingness to identify and describe novel taxa.
- The rapid application of molecular tools for defining evolutionary units in fungi.
- Increasing international trade in rooted plants, which is having the effect of distributing more pathogens beyond their natural endemic ranges. Consequently, some of them become noticed as a problem within the nursery trade, or as threats to forests and natural ecosystems.
- Increased environmental screening for *Phytophthora* species based on PCR-based detection of molecular signals.

Table 3—*Phytophthora* species / taxa identified Post 2000 (morphologically and molecularly unique species, some not yet formally described)

P. alni (x 3)★	*P. kernoviae*★	*P. frigida*★	*P.* taxon *salixsoil*★
P. andina	*P. nemorosa*★	*P. austrocedrae*★	*P.* taxon *pgchlamydo*★
P. asparagi	*P. niederhauserii*	*P. lagoariana*	*P.* taxon *riversoil*★
P. bisheria	*P. pistaciae*	*P. cuyabensis*	*P.* taxon *oaksoil*★
P. brassicae	*P. polonica*★	*P. cact* x *hedr*	*P. parvasperma*★
P. captios ★	*P. pseudosyringae*★	*P. foliorum*★	*P. hungarica*★
P. europae ★	*P. psychrophila*★	*P. sulawesiensis*	*P. sylvatica*★
P. fallax★	*P. ramorum*★	*P. siskiyouensis*★	*P. parsiana*
P. gallica★	*P. sansomea*	*P. uliginosa*★	*P. quercetorum* ★
P. glovera	*P.* taxon Banksia★	*P. rosacearum*	*P.* taxon meadii-like★
P. hedraiandra	*P.* taxon. chicory	*P. sansomeana*	*P.* taxon Acer★
P. inundat ★	*P. cactorum* x *nicotianae*		*P.* taxon Agathis★
P. ipomoeae	*P. tropicalis*		*P.* taxon orphan
P. kelmania	*P. alticola*★		~ 30 / 52 = 58 percent ★

★ Associated with, or with research on, nursery trees, forests and natural ecosystems.

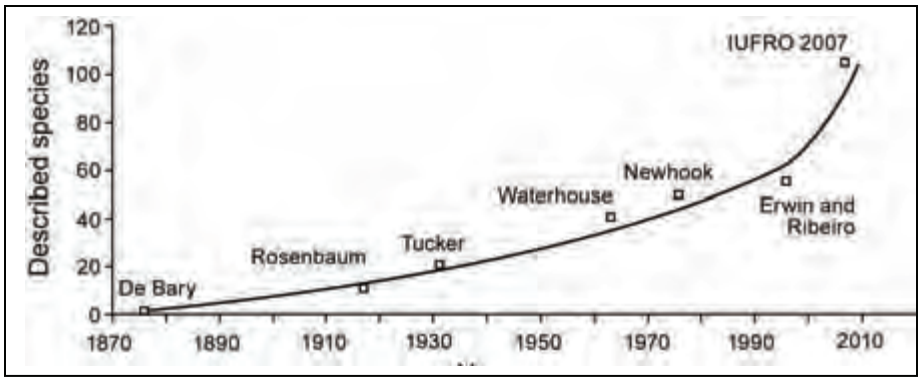

Figure 2—Number of described *Phytophthora* species over time, extended beyond 1995 (Erwin and Ribeiro) to IUFRO 2007, showing the remarkable increase over this period.

If the surge in identification of new *Phytophthora* species continues for another decade or so, we will soon be on the way towards the minimum of 200 extant *Phytophthora* species estimated above.

Demonstration of Phylotaxa

A valuable practical application of modern PCR technology is the ability to sample environmental substrates such as soil, water and plant material for microbial diversity. Such studies are beginning to reveal unknown phylotaxa: taxa which are apparently unique on the basis of their molecular profile, but for which no living culture exists. During the 1990s, unique bacterial phylotaxa were shown to be abundant during investigations of the ecology of bacterial populations in soil.

Phylotaxa are now beginning to be identified in comparable sampling for *Phytophthora*. Arcate, Kemp and Nelson (2006) recently applied PCR-based methods to search for Oomycetes in the rhizosphere of tomatoes and other crops in New York State. They found many Pythiums, a couple of Phytophthoras, and greater biodiversity than they obtained by direct plating methods. They were also able to sample the dormant oospore flora as well as active mycelium and propagules.

Two research groups associated with IUFRO have recently began to apply these methods to screen for Phytophthoras in natural ecosystems, so far mostly in stream water. Hansen, Reeser and Sutton (these proceedings) have examined streams in south west Oregon using combined SSCP data of ITS and *Cox* loci. They have identified 30 phylospecies, of which ten are believed to be new or unknown taxa based on BLAST searches. In a similar study of a central Oregon stream near Corvallis, Remigi, Sutton, Reeser and Hansen (these proceedings) identified eleven phylospecies, of which five are probably unknown taxa.

Scibetta, Cooke and Cacciola (also unpublished) have used nested PCR of ITS1, followed by cloning and sequencing, to examine water samples from streams in

western Scotland. They found 17 phylospecies in the streams, of which three are believed to be new or unknown taxa. Scibetta and Cooke (unpublished) used the same methods to survey streams in Ecuador at 2–3000 m above sea level. They found ten phylospecies, including *P. infestans*, three *Peronospora* 'species', and three new or unknown taxa.

These pioneering studies show that significant numbers of currently unknown *Phytophthora* species may be present in streams, even in relatively well documented locations such as western Oregon. Since, as yet, we have no cultures of these unknowns, these results are tantalising, to say the least! Particularly intriguing is the fact that DNA of typically aerially dispersed taxa with caducous sporangia, such as *P. infestans* and *Peronospora* species—not expected to be stream inhabitants—were detected in the streams in Ecuador. This raises interesting questions about how the DNA of these taxa came to be in the water. Does it perhaps reflect the 'raining' of aerially dispersed sporangia from the atmosphere into streams; or the percolation of plant debris containing inoculum into the stream from the surrounding environment? Or does it reflect a genuine inhabitation of the stream by the *Phytophthora* (or *Peronospora*) species concerned?

New Species Revealed by Re-examination of Culture Collections

Additional information is coming from the application of molecular diagnostics to *Phytophthora* culture collections. This is especially true where a collection has not previously been subjected to a rigorous taxonomic screening. A good example is the collection of the Vegetation Health Service (VHS) at CALM, western Australian. This has recently been subject to ITS sequencing by Stukely, Burgess and Hardy (unpublished). In this still ongoing analysis, 30 taxa have been identified of which seven (23 percent) are considered new or unknown taxa. Since (in contrast to phylotaxa) cultures are available, the unknowns can now be examined for their phenotypic and other molecular characteristics. In a similar study of the isolates in the ICMP *Phytophthora* culture collection of Landcare Research in New Zealand, Beever and others (2000) identified around 30 species on the basis of ITS sequencing. Among these were two new or unknown taxa. These have now been partly characterised and informally named *P*. taxon Agathis and *P*. taxon orphan (see also Beever, this volume).

What Proportion of New *Phytophthora* Species are Associated With Forests and Natural Ecosystems?

Of the ~55 *Phytophthora* species known in 1999, eleven could be considered as damaging to nursery trees, forests and natural ecosystems (my assessment). These are shown in bold in table 2. This represents about 20 percent of the total. About the same proportion might therefore have been expected among the 50 or so species characterised since 2000. But this is not the case. As can be seen in table 3, the proportion for these post-2000 species is ~30/50, or 60 percent.

This difference between the pre- and post-2000 proportions (20 percent versus 60 percent) might be explained by three factors: (1) before 2000, more species of

significance to agriculture may have been identified because of a research emphasis on agricultural problems, (2) since 1990, there has increasing interest and concern about the impact of *Phytophthoras* in forests and natural ecosystems, and (3) since the 1990s, there may have been an increased spread of new species from exotic habitats into nurseries and into natural environments as a result of growing international trade in plants.

Whatever the precise factors involved, the fact that a greater proportion of the 'new' species appear to be associated with nurseries, forests and other natural environments clearly has implications for international plant biosecurity and plant health practice. That current international biosecurity protocols are inadequate has been discussed elsewhere (Brasier 2005, 2008).

Phylogenetic Implications of the Increasing Number of New *Phytophthora* Species

Numbers again

The first comprehensive molecular phylogeny of *Phytophthora* and other Oomycetes (Cooke and others 2000) was, based on ITS sequences. It covered some 47 of the *ca* 55 *Phytophthora* morphospecies known at the time (table 2). The taxa were assigned to ten major Clades numbered 1–10 (fig 3). This overall structure is still retained in recent multi-gene trees, such as four locus tree of Kroon and others (2004) and the seven locus tree of Blair and others (2008; fig 4). It is interesting to consider how the many new species described since Cooke and others (2000) (table 3), or indeed the possible 200–600 species that may eventually be found, will affect the structure and interpretation of the trees.

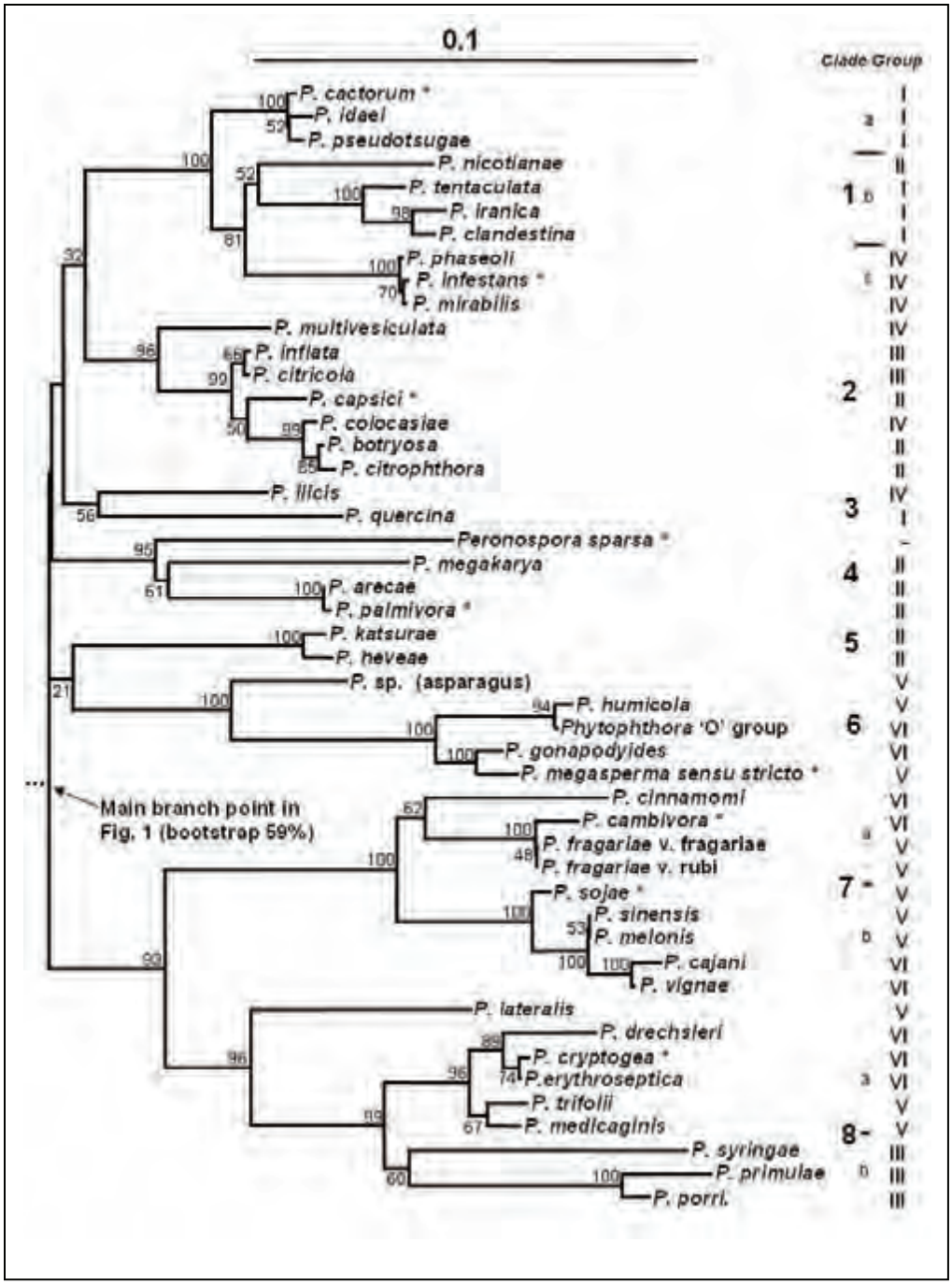

Figure 3—*Phytophthora* Clades 1–8 as originally identified by Cooke and others (2000) from ITS sequences. Their Clades 9 and 10 are not shown.

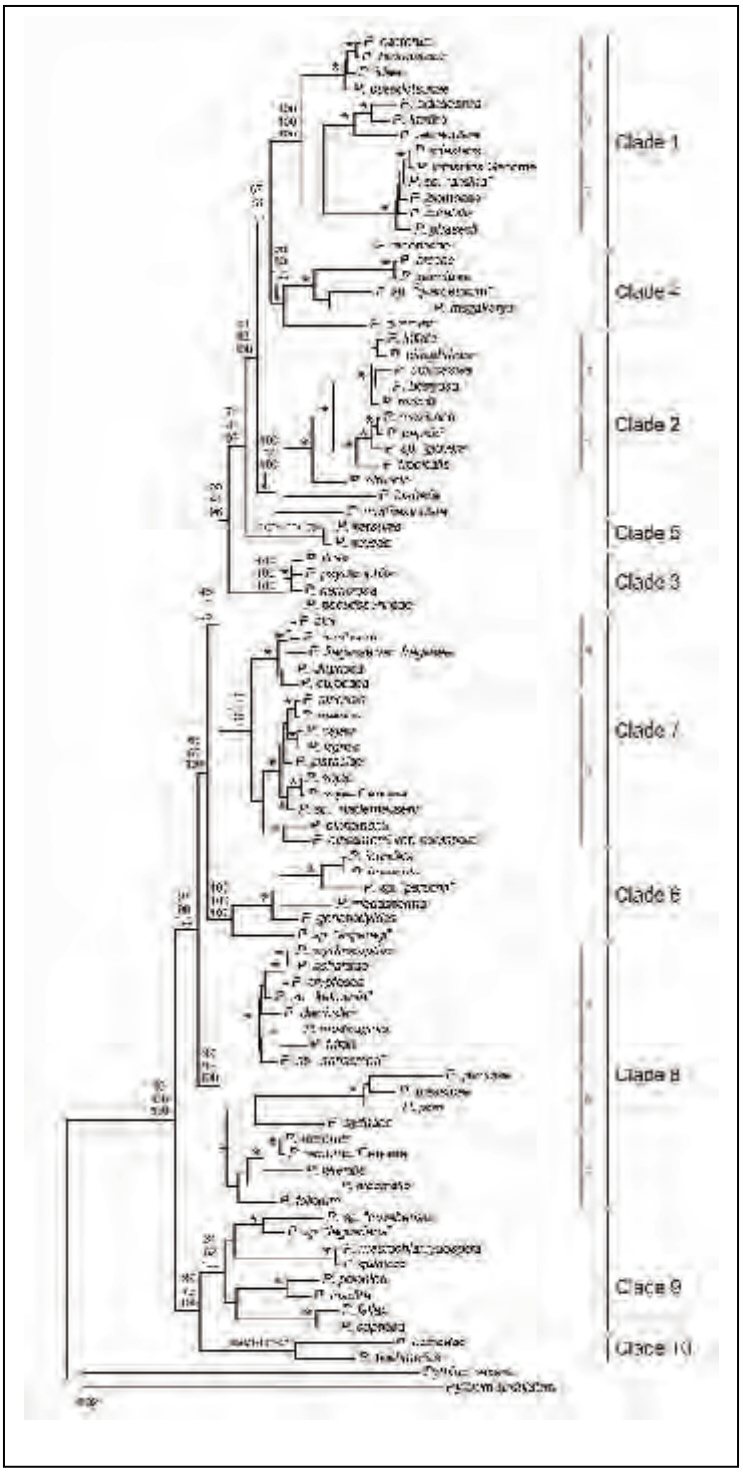

Figure 4—*Phytophthora* Clades 1–10. A recent seven locus concensus tree constructed by Blaire and others (2008).

A typical example of the continuing numerical increase in species is represented by the expansion of Clade 8. This has already gone from nine species in Cooke and others (2000) to about 16 species today (fig. 4). In the Cooke and others tree, *P. lateralis* was something of a lone outrider. Now this species is part of a significant subcluster (subclade 8c, fig. 4), having been joined by *P. hibernalis* (cultures of which were not available to Cooke and others) and two new species *P. ramorum* and *P. foliorum*. Presumably, more new species will follow.

The Ever Growing Clade 6

The considerable increase in the number of Clade 6 species is one of the most interesting developments. When first constructed by Cooke and others (2000), Clade 6 contained only *P. megasperma*, *P. humicola*, *P. inundata* (as P. sp. 'O-group') and *P. gonapodyides*. Subsequently, a combined phenotypic and molecular study was carried out on putative Clade 6 isolates of uncertain status from culture collections in Europe and North America (Brasier and others 2004). This study identified a number of unique taxa, many of them high temperature and/or sexually sterile species. It also extended the number of known Clade 6 species to 13. Their phylogenetic relationships are shown in figure 5. Some of these new taxa have yet to be formally described e.g. *P.* taxon salixsoil, *P.* taxon rasberry and *P.* taxon walnut (table 2). Another, *P.* taxon forestsoil, has recently been described as *P. sylvatica* sp. nov (table 2 and J. Bakonyi, personal communication).

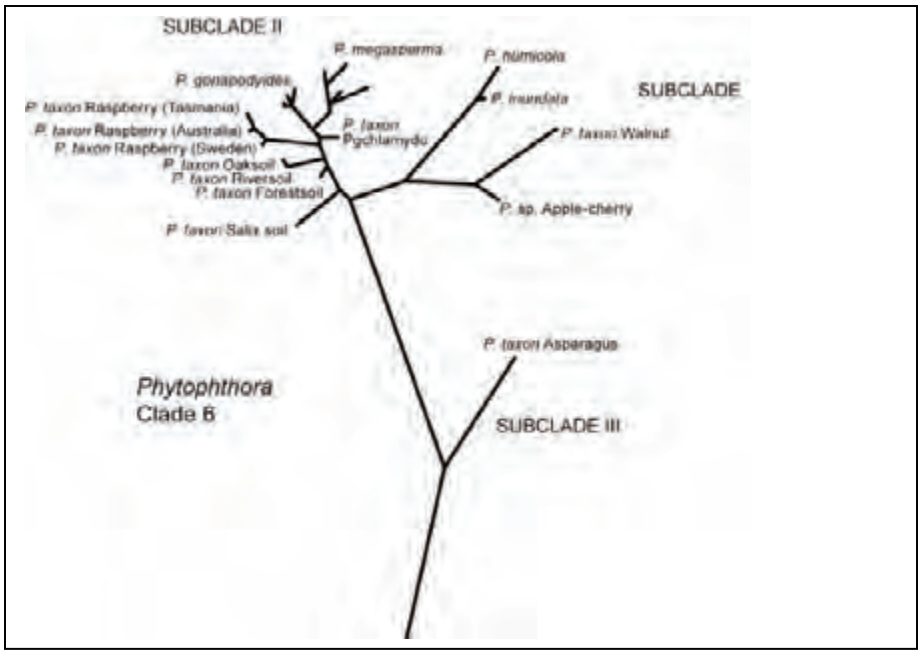

Figure 5—Phylogenetic relationships of the thirteen taxa of Clade 6 recognised by Brasier and others (2004). Based on ITS sequences.

To these 13 species can now also be added *P. hungarica* sp. nov. (table 3 and J. Bakonyi, personal communication) and *P.* taxon orphan identified in the ICMP culture collection in New Zealand (table 3 and Beever and others 2006). And that is not all. Five of the seven 'new' taxa recently identified by Stukely, Burgess and

Hardy in the VHS culture collection also fall within Clade 6, bringing the current number of species in Clade 6 to about 20. Overall, these additions represent an increase of 16 taxa since Cooke and others (2000).

Of course, not all the additions are new discoveries. Some have languished in the culture collections owing to the considerable difficulty in identifying species units within this group before population-based species concepts and molecular phylogenetic tools were applied see Brasier and others 2004. And the Clade 6 saga does not end there. Of the ten unknown phylotaxa recently identified by Hansen and colleagues in south west Oregon streams, the majority probably also belong in Clade 6.

So, how many extant species are there in Clade 6, including the current unknowns? Let's play again! If we start with a figure of ~15 known or described *Phytophthora* taxa in Clade 6 at the time of this meeting (based on data up to and including Cooke and others 2000, Brasier and others 2004, Beever and others 2006 plus *P. sylv*atica and *P. hungarica*) and use the figure of approximately 105 currently known or described *Phytophthora* species overall (Tables 2 and 3 combined), then the number of *Phytophthora* species presently assigned to Clade 6 is *ca* 14 percent of the total. If we allow my previous estimate of 200–600 extant species in the genus, then 14 percent would indicate there may be between 28 and 84 extant species in Clade 6. This means that the number of species in Clade 6 might even be greater than the number of *Phytophthora* species known to science in 1999 … surely a figure to conjure with!

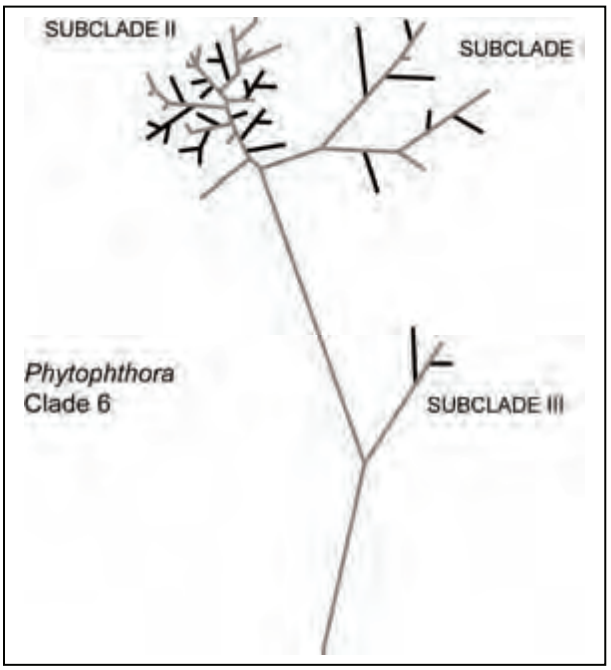

Figure 6—How Clade 6 might look, theoretically, if the minimal estimate of 28 extant species were represented (compare with figure 5). Note the now very crowded taxa of Subclade II. Consider what the tree might look like with the maximum estimate of 84 extant species.

Many of the Clade 6 taxa in the ITS tree shown in Brasier and others (2004) are very closely related i.e. separated by only a few base substitutions, indicating recent divergence, especially those in Subclade II (fig. 5). It is therefore likely that many of the so far unidentified species in Clade 6 will be closely related to existing species and to each other. In which case Clade 6 could become very crowded indeed, as indicated in figure 6! Subclade 6 is already becoming too crowded to show all the taxa in a rooted tree without double or triple parking the labels. Furthermore, if the 'missing' taxa also prove to be phenotypically somewhat similar (aquatic, sterile or inbreeding, high temperature) species, such as *P. gonapodyides*, *P*. taxon

Pgchlamydo, *P*. taxon salixsoil etc (Brasier and others 2004), then we may have to rethink our *Phytophthora* species concept, at least for Subclade II. We will also face the challenge of identifying the evolutionary processes which have bought about such a high level of phylogenetic diversity.

Phylogenetic Pitfalls?

Since the ITS tree of Cooke and others (2000), we have been faced somewhat unexpectedly with a rapidly rather than a gradually expanding *Phytophthora* phylogenetic tree. For the foreseeable future, trees produced every few years are likely to acquire more 'twigs and branchlets'. They will be more sophisticated in terms of genetic analysis and will look increasingly impressive in size. But what do these trees provide in terms of our fundamental understanding of *Phytophthora*?

Obviously, trees constructed from DNA should be an order of magnitude better than the morphological groups of Waterhouse (1963), or an earlier morphology-based phylogeny of my own (Brasier 1982). While Waterhouse's groups and key provided a valuable taxonomic framework for forty years, and were presented by her as necessarily artificial constructs, we can be reasonably confident that our molecular phylogenetic trees are much more representative of 'natural relationships'. However, can we also be confident about what they tell us of the evolutionary history of the known taxa?

I suggest we should treat our fast growing phylogenetic trees with a degree of caution. First, many taxa, and therefore much data, are missing because of past extinctions. Some of these missing taxa could be key ancestral taxa that would have lain at or closer to the nodes and branches of the main clades, but disappeared during major evolutionary jumps and radiations. Second, much evidence of past reticulation (inter-specific hybridisation) events may be missing. Where new taxa have arisen via interspecific hybridisation or introgression in the ancient past, the genetic footprints of the process will often have been lost over time through genetic rearrangement and concerted evolution. Third, much information is missing because we may still have to add to the trees the estimated 100–500 unknown *Phytophthora* species.

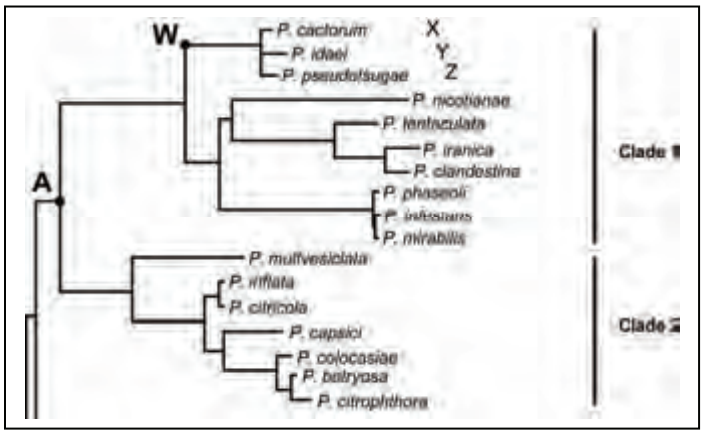

Figure 7—The real issues? Why did ancestral species 'A' give rise to Clades 1 and 2? Why did ancestral species 'W' give rise to species X, Y and Z (*P. cactorum*, *P. idae*i, *P. pseudotsugae*) ? Diagram adapted from Cooke and others 2000.

The addition of some of these new taxa may well alter the substructure within some of the Clades, as already seen for Clades 6 and 8. There might even be missing Clades.

What is surely important in terms of interpreting the trees is not so much that species X and Y are closely related e.g. that *P. ramorum* and *P. lateralis* are related within Clade 8. So what? What does that really tell us? In Darwinian terms, what is more important is to understand are the processes and events that gave rise to their divergence: the processes that were responsible for the nodes or branches. *Why* did ancestral species A give rise to Clades 1 and 2? (fig. 7). Or why did ancestral species W give rise to three W species X, Y and Z? (fig. 7). Traditional fungal taxonomy lost its way in the middle of the last century by concentrating too much on the what (morphological and descriptive) and not enough on the how and why issues (the parameters which defined species boundaries in evolutionary terms; cf. Brasier 1997). Maybe *Phytophthora* molecular phylogenetics is in danger of doing the same?

Concluding Comments

To me, this dip into *Phytophthora* numerical biodiversity and phylogenetic uncertainties has been another reminder of how little we know about the origins of the genus. Indeed, it seems reasonable to suggest that the construction of a phylogenetic tree is simply the point at which the real evolutionary challenges begin. Given that there are likely to be between 200 and 600 *Phytophthora* species out there, I see two key challenges for the future.

One is to understand *why* there are so many *Phytophthora*s. What environmental processes and events (geographic, climatic, biotic) drove their evolution? Why did e.g. *P. ramorum* and *P. lateralis* diverge from a common ancestor, or from each other? Addressing this set of questions is the job of the evolutionary biologist.

Another is to understand, at a basic genetic level, *how* the species are different e.g. how *P. ramorum* differs from *P. lateralis*. Addressing this set of questions is the job of the classical geneticist and the genomicist. In the context of the present paper, it was also the job of the next speaker (Howard Judelson, Chapter X).

Fortunately, these two challenges are, of course, not mutually exclusive, and answers obtained for one could enormously benefit understanding in the other.

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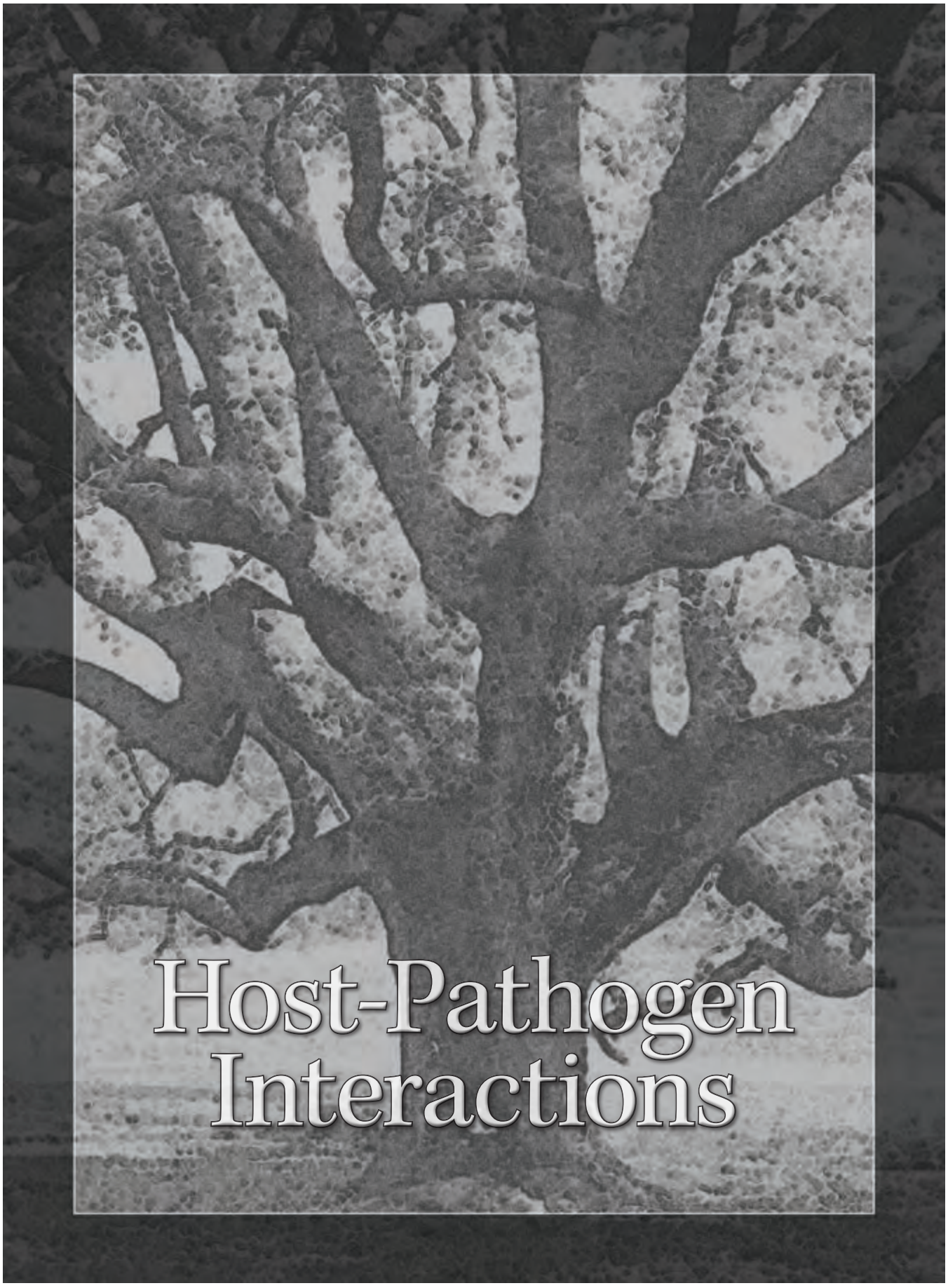

Host-Pathogen Interactions

First Report of Ink Disease on Chestnut Caused by *Phytophthora katsurae* in Korea[1]

Eunsung Oh,[2] Keum Chul Shin,[3] Sang Hyun Lee,[2] Kyung Hee Kim,[2] and Jong Kyu Lee[3]

Abstract

Phytophthora is well known as a destructive pathogen on crops and nursery stocks. In the late 1990s *Phytophthora* species, newly described or suddenly lethal to mature forest trees, have appeared in the United States and Europe. Some cultivars of chestnut (*Castanea crenata* X *C. mollissima*) in Korea have also died without knowing the causal agent in last two years. The typical symptom was black-oozed, reddish, sunken tissue on trunk. When bark was peeled off, a distinct necrotic region on basal trunk and the vertical spread of the discoloration were observed. In November 2006, we were able to isolate the agent from the necrotic areas, and thirteen isolates were obtained from three different areas. The isolates produced numerous homothallic oogonia (34.0~46.2 x 21.9~26.7 um) with warty protuberance on the surface and a long, funnel-shaped stalk enclosed by antheridium (amphigynous) at the base. No chlamydospores were observed, but the formation of papillate, ovoid to obpyriform sporangia (17.0~38.9 x 14.6~29.2 um) were induced by cold treatment in filtered creek water or de-ionized water. Based on morphological characteristics and rDNA ITS sequencing, the isolate showed 99.6 percent similarity with *P. katsurae* (AF266771), indicating three base pair differences. All isolates from 3 different areas were completely identical in the comparison of rDNA ITS sequence. Resistance or susceptibility of different cultivars of chestnut against the isolate showed variations among cultivars, resulting in potential impact on chestnut plantations with many cultivars in Korea.

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Development of Molecular Diagnostic Tools for the Detection of *Phytophthora cinnamomi* From Cryptic Soil Samples in Southern Australia[1]

Nari M. Williams,[2] Emer O'Gara,[2] Giles E.St.J. Hardy,[2] and Phillip A. O'Brien[2]

Abstract

Phytophthora cinnamomi is responsible for the widespread destruction of native forest and heathlands across southern Australia. In these ecosystems, *P. cinnamomi* is responsible for the death of a broad range of susceptible species including members of the Proteaceae, Epacridaceae, Papilionaceae and Myrtaceae, and results in significant changes in species composition, community structure and ecosystem function. Management of the pathogen in native ecosystems is centred on pathogen containment and relies on the identification of dieback boundaries and detection of the pathogen directly from soil. Once an infestation is defined, preventative measures may be taken to ensure infested soil and plant material are not spread during land management activities. Ongoing monitoring of the pathogen then forms an integral component of sustainable ecosystem management.

Efficient management of *P. cinnamomi* is impeded by the inability to consistently detect the pathogen from infested soil samples. This is especially the case for cryptic sites in which there is no apparent expression of plant symptoms. Such situations commonly occur where sites are excavated during mining activities or are disturbed by fire. In the absence of a better alternative, land managers still use baiting analysis of soil samples in formulating management plans for *P. cinnamomi* containment. This is despite the fact that recoveries are often low and there is a high risk of false negatives. This in turn limits the confidence placed on the results of baiting analysis. DNA based detection offers improved sensitivity and higher sample throughput for the detection of *P. cinnamomi* than baiting assays. Through our research, comparative analysis using PCR based methods in parallel to baiting assays has shown a significant increase in the detection of *P. cinnamomi* by nested PCR. However, although the benefits of DNA based diagnostic tools have good promise for future disease management, low and variable target populations mean that sampling strategy and confidence levels remain the key issues in delivering a reliable and consistent diagnostic service.

This presentation will examine the challenges encountered during the development and validation of nested and real time PCR protocols for the detection of *P. cinnamomi* from soil collected from native ecosystems throughout southern Australia. The implications for using molecular diagnostic assays as management and research tools for *P. cinnamomi* will be discussed.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26ï 31, 2007, Monterey, California.

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Host-Pathogen Interaction of *Phytophthora citricola* and *P. pseudosyringae* With European Beech (*Fagus sylvatica*)[1]

Julia Koehl,[2] Roberto Portz,[2] Vera Martin,[2] Angela Simonsmeier,[2] and Wolfgang Oßwald[2]

Abstract

Fagus sylvatica is an important tree species and due to its immense tolerance towards different climate conditions and habitats it is one of the major tree species in European forests. Over the past years the number of trees infected with *Phytophthora* increased dramatically. Particularly *P. citricola* was described as an important and an aggressive species in this context, that besides *P. cactorum* and *P. cambivora* lead to destruction of trees. Noticeable differences could be observed regarding virulence and symptom development: Infection with *P. citricola* that was identified as most aggressive species resulted in massive reduction of fine root mass. Before trees showed any wilting symptoms, rates of photosynthesis and transpiration decreased. Intriguingly also the nonaggressive *Phytophthora* species *P. pseudosyringae* reduced fine root system likewise, but neither wilting nor die back of plants occurred (Jung and others 2003; Fleischmann and others 2004). The root system of beech seedlings infected with *P. pseudosyringae* was reduced in length but far less destructed than *P. citricola* infected roots which points out some kind of development of resistance in beech seedlings after infection with *P. pseudosyringae.* This project aims to investigate these mechanisms by employing an aggressive and a non-aggressive *Phytophthora* species on beech, since until now any resistance mechanisms involved in *Fagus sylvatica -Phytophthora* interaction are completely unknown. Besides the above ground symptoms we investigated the extent and mode of action of the infection process of both *Phytophthora* species in beech roots. We applied histological, physiological (photosynthesis, water potential, transpiration) and molecular (qPCR and gene expression) methods to determine nature and progression of the infection in beech seedlings with *P. citricola* and *P. pseudosyringae* as well as a temporal dependence of symptom development on quantity of infestation. A biphasic course of infection of the root tissue could be observed after infection with *Phytophthora*, at which the first increase was due to zoospore encystment within the first 36 hours. The second peak is correlated with penetration and growth in beech root tissue. Most remarkably was the fast and immense colonization of xylem vessels by hyphae of *P. citricola*, from where rapid propagation of this aggressive *Phytophthora* species into other parts of the plant evolves. The activity of hydrolytic enzymes (as glucanases and invertases) in both *Phytophthora* species was investigated and content of soluble sugars, as well as starch were determined to elucidate their role for colonization by *Phytophthora* species with different pathogenic impact. At the present time our group is about to transform *P. citricola* and *P. pseudosyringae* with GFP (green fluorescent protein) to acquire three-dimensional data sets from intact, individual, pathogen encounter sites *in planta* and to open possibilities for analysis of functional genes involved in infection processes.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26ï 31, 2007, Monterey, California.

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Investigation of the Effects of *Phytophthora alni* subsp. *alni* Collar Inoculation on CO_2 Uptake, Transpiration, and Carbohydrate Content of *Alnus glutinosa* Saplings[1]

Julia Koehl,[2] Frank Fleischmann[2], Rainer Matyssek[3] and Wolfgang Oßwald[2]

Abstract

Three-year-old *Alnus glutinosa* trees were single or double-inoculated at the stem basis with *Phytophthora alni* subsp. *alni* under natural climate conditions to clarify local and systemic pathogenic effects on young alder saplings. Lesion formation on the bark showed a biphasic pattern with moderate extension rate in spring and strong development during late-summer. However, large variability was encountered in pathogen development within the collective of the infected plants, ranging between high susceptibility and almost resistant. Infection resulted in strong growth retardation, and finally 75 percent of all inoculated trees had died after two years of infection. During disease development, rates of CO2 uptake and transpiration were significantly reduced. Consequently, minimum levels of leaf water potential were less negative in infected than control trees, reflecting stomatal narrowing under infection. A linear regression was found, when all girdling values of the single and double inoculated saplings were plotted versus the corresponding mean values of photosynthesis measured in 2004, proving the inhibitory systemic effect of stem girdling on leaf photosynthesis. In the second year of infection the amount of girdling of all surviving saplings decreased to values below 50 percent. All these saplings did no longer differ from controls in terms of photosynthesis, transpiration and water use efficiency (WUE). Starch levels of leaves of infected trees were significantly increased relative to control trees, possibly indicating destruction of the bark tissue by the pathogen to cause blockage of phloem transport from leaves to roots. Given such a syndrome scenario, stomatal closure appears to result from product-inhibited photosynthesis upon phloem disruption, preventing WUE to decline. Hence, the found gas exchange responses are systemic responses of the local pathogen infection at the stem basis.

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26ï 31, 2007, Monterey, California.

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Phytophthora ramorum Tissue Colonization Studied With Fluorescence Microscopy[1]

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Abstract

The continual worldwide spread and the expanding host spectrum of *P. ramorum* has become a serious threat to natural plant communities. To counter this threat, detailed knowledge about infection pathways and tissue colonization is essential. To analyze these issues, histological studies of infected tissue with epifluorescence microscopy have been started. For first infection tests the *Rhododendron* has been taken as a model host. Roots of potted *Rhododendron* cuttings were infected with the *P. ramorum* isolate BBA 9/95 (ex-type strain). Inoculation of the non-injured plants was done by application of a zoospore suspension onto the surface of the pot soil. The plants were incubated with 16 hours of light at 20°C in a quarantine chamber. Samples of healthy looking plants and plants with typical symptoms were taken and fixed. Unstained hand-cuttings were analyzed with fluorescence microscopy. The development of zoosporangia on leaf surfaces was analyzed using the vital stain FUN®1 (Molecular Probes). Natural autofluorescence of *P. ramorum* and plant tissue is enhanced using the fixing fluid FAA (formaline-aceto-alcohol).

Epifluorescence images showing *P. ramorum* structures in different tissues and in different stages of disease development are presented. An overview of the development of *P. ramorum* in *Rhododendron* root, twig and leaf tissue is presented for discussion.

Introduction

In Europe *Rhododendron* plants present the main risk for the spread of *P. ramorum* in natural ecosystems. Using *Rhododendron* as a model system for the study of woody plants was a logic consequence. In preceding infection trials with irrigation water in containered *Rhododendron* stands (Werres and others 2007) *P. ramorum* was detectable in healthy looking root balls of asymptomatic plants. At this time only limited knowledge of root infection in rhododendron plants was available. Furthermore little is known about the whole issue of *P. ramorum* latency. To approach these problems infection trials to investigate *P. ramorum* colonization in root-infected *Rhododendron* plants were started.

Traditionally in fungal phytopathology transmission light microscopy is used to analyze tissue colonization and host-pathogen interaction. This technique usually

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26ï 31, 2007, Monterey, California.

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requires laborious pre-treatments of the samples like clearing, staining, embedding and thin sectioning (Pogoda and Werres 2004). To highlight fungal or oomycetic structures a wide range of stains including fluorescence dyes have been used (Zhang and Dickinson, 2001). Most of these dyes fail to specifically stain only the pathogen structures. Most often host tissue is stained too and not all of the pathogen structures showed the same staining intensities.

In this study we found that *P. ramorum* tissue expresses a very strong and specific natural autofluorescence which is distinguishable from surrounding host tissue. Here we report a simple method for analyzing host-pathogen interaction at a histological level using the natural autofluorescence of *Rhododendron* tissue and *P. ramorum* structures.

Material and Methods

Plant material and inoculations

As host plants, rooted *Rhododendron* cuttings of the cultivar `Cunninghams White´ were maintained in a quarantined chamber at 20 °C with 16 hours of light. Inoculations were done using a 5ml aqueous suspension containing 30.000 zoospores of the European *P. ramorum* isolate BBA 9/95. The inoculum was applied directly onto the freshly irrigated potting medium.

Sample preparation and microscopic analysis

Samples (segments of root stem and foliar tissue) from asymptomatic and symptomatic plants were taken at regular intervals starting seven days after inoculation. The samples were fixed using the fixing fluid formaline-aceto-alcohol (FAA). Prior to hand sectioning, the samples were washed in de-ionized water for 15 minutes. Whole fine roots or hand sections were mounted in water on microscopic slides and then used for microscopic observation.

For microscopic analysis a Zeiss Photomicroscope II with epifluorescence equipment was used. Observations were made using the × 10, × 16 or × 40 lenses and the Zeiss fluorescence filter set No. 05 (excitation filter: 395–440 nm, 460 nm beam splitter and 470 nm long pass filter). Emission spectra profiles (lambda scan) were generated using the Leica TCS SP2 confocal laser scanning microscope (CLSM) and the Leica LCS software. Excitation was done with a 488 nm Argon Krypton laser at power levels of 10 percent to 50 percent. Settings for the gain and offset were 700 to 750 and 0 to -20 respectively.

Results and Discussion

P. ramorum emits a strong natural autofluorescence when excited with blue light (430 to 500 nm). Using confocal laser scanning microscopy (CLSM) the emission maximum could be located in the green-yellow area at approx. 550 nm (fig. 1). Excited with the same light the different types of host tissue show a specific emission

spectra too. Using this emission, pathogenic structures were clearly distinguishable from host tissue.

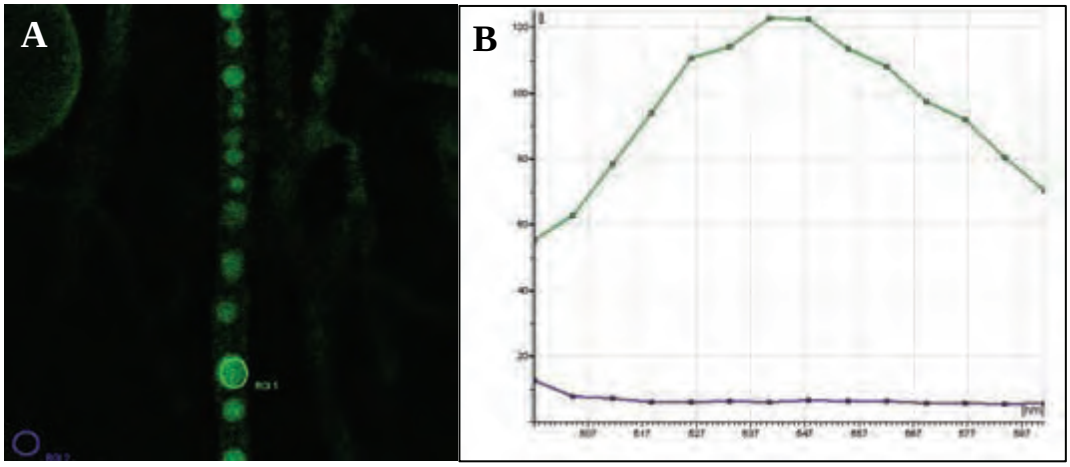

Figure 1—Natural *P. ramorum* autofluorescence, emission spectra profile (CLSM lambda scan), **A**: CLSM image of a hypha, **B**: typical emission curves with maximum at approx. 550 nm

Furthermore, dependent on the state of infection (asymptomatic, discolouration, necrosis, etc.) the spectra of host tissue exhibit specific variations. The red autofluorescence of chlorophyll indicates the asymptomatic state of green tissue in leaf palisade mesophyll or the cortex of stems. In pith and cortex, asymptomatic stages are marked by light green and light yellow autofluorescence respectively. With the beginning of discolouration the fluorescence shifts to yellow and brownish colours. Necrotic and collapsed tissue exhibits a typical brown colouration. Thus the determination of tissue type and state can be evaluated by shape and colour.

In asymptomatic plants the presence of chlamydospores in roots was observable 7 to approx. 60 days after inoculation. The spores were predominantly located in the root exodermis on lateral sites of fine roots (fig. 2). The majority of chlamydospores exhibited a bright yellowish autofluorescence which enabled very easy detection in the root tissue. Occasionally chlamydospores with an unusual red autofluorescence were found. The germination of chlamydospores could be observed in some cases. First phaenotypic visible disease symptoms appeared 15 days after inoculation. In early stages of infection hyphae are the predominant *P. ramorum* propagules found in pith, xylem, phloem and the cortex of roots and stems (fig. 2). Especially the xylem is most extensively colonized by hyphae. In vessels, hyphae grow mainly intracellularly but penetration of cell walls (intercellular growth) occurs regularly. In later stages of infection massive colonization by hyphae was found in nearly all types of host tissue. Furthermore in xylem of infected plants an increased production of tyloses can be observed. The production of tyloses is often referred to as a possible reaction of plants to progressing infections.

In progressed stages of infection the production of chlamydospores can be observed in the tissue of pith and cortex. While in pith tissue chlamydospores can be observed only occasionally, in cortex chlamydospores they can often accumulate in high numbers. The majority of these chlamydospores show, similarly to the spores found

in roots, a typical bright yellow-greenish colouration. But also chlamydospores exhibiting red autofluorescence were found. Most abundantly these red spores were found in discoloured or necrotic tissue.

Colonization of foliar tissue follows pathways similar to the situation observed in stems. Early stages of infection are characterized by the spread of hyphae via veins. Later a massive colonization of mesophyll tissue can be seen and hyphae start to grow out of stomata (fig. 2). At this stage foliar tissue still appears to be intact which is indicated by the red autofluorescence of chlorophyll. Under optimal conditions (humid and cool) zoosporangia start to develop on hyphae branching out of stomata (fig. 2). When leaves start to show signs of discolouration, chlamydospores can be found inside foliar tissue. In necrotic tissue chlamydospores can be found accumulated in huge numbers (fig. 2).

The occurrence of chlamydospores with unusual red autofluorescence is, except for the roots, linked to the presence of discoloured or necrotic tissue. In vitro, cultured chlamydospores never exhibited such red autofluorescence. In progressed states of infection when host tissue starts to collapse most of the chlamydospores show red fluorescence (fig. 2I). Additionally at this stage of infection in the tissue of roots, stems and leaves, areas were found where a similar red autofluorescence occurs (figs. 2C and 3B). With CLSM lambda scans it could be shown that both cases of red autofluorescence have similar emission spectra profiles (fig. 3). It can be assumed that substances like phenolics which are emitted by the plant during the process of plant defence or in the progress of tissue-collapse, represent the origin of the red autofluorescence.

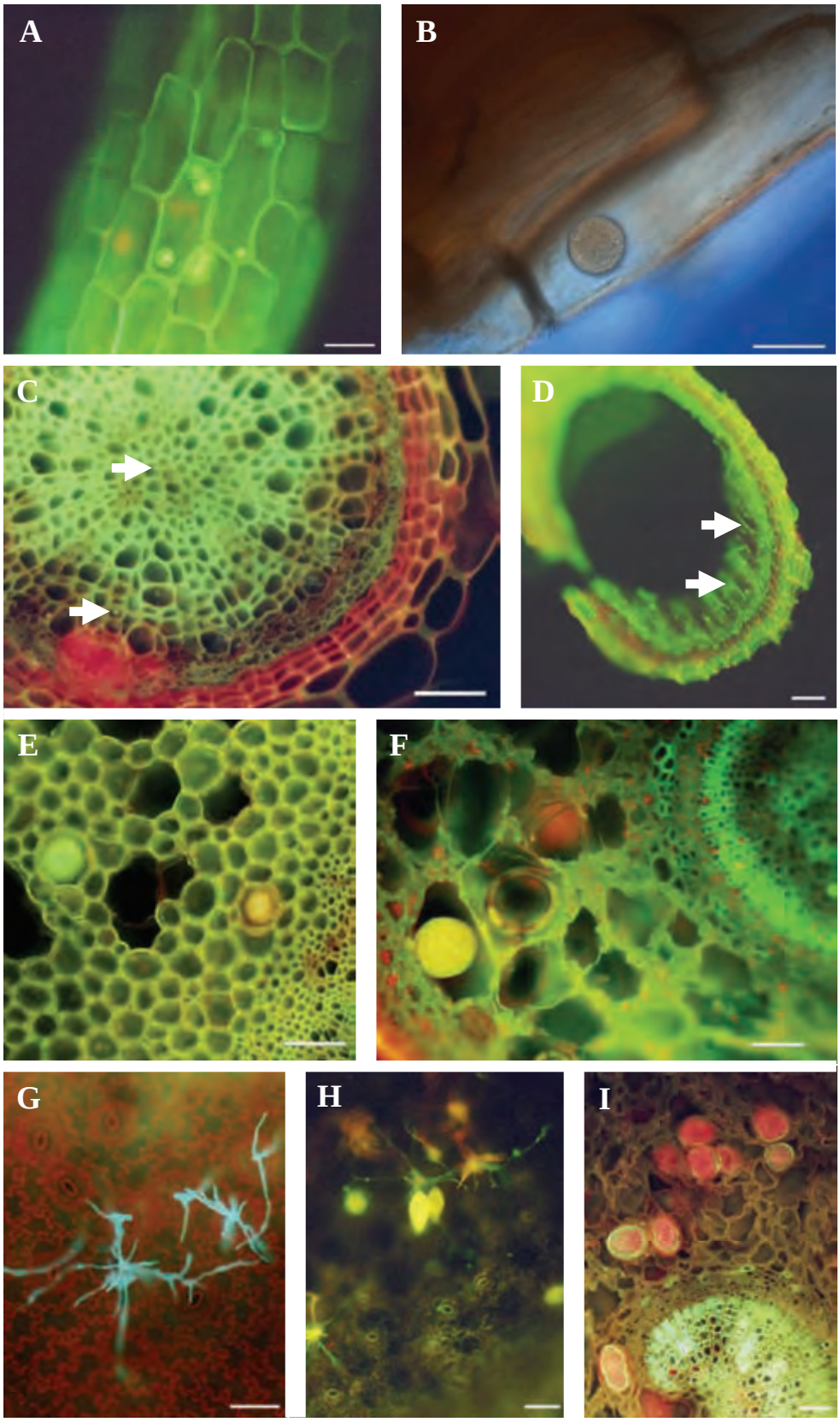
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Figure 2 (preceeding page)ð Microscopic images of *P. ramorum* propagules in rhododendron tissue. **A, C–I**: epifluorescence images, **B**: transmission light image
A, B: Chlamydospores in exodermis of healthy looking roots, **C**: Growth of hyphae (arrows) inside xylem of discoloured roots and accumulation of substances with red autofluorescence. **D**: hyphae (arrows) growing alongside of vessels, **E**: production of chlamydospores in pith tissue of discoloured stems, **F**: chlamydospores with yellow and red autofluorescence in collapsing cortex of discoloured stems, **G**: hyphae growing out of stomata on asymptomatic leaves, **H**: zoosporangia produced on hyphae growing out of stomata of discoloured leaves, **I**: accumulation of chlamydospores in necrotic mesophyll cells of foliar tissue, white bars indicate 50 µm

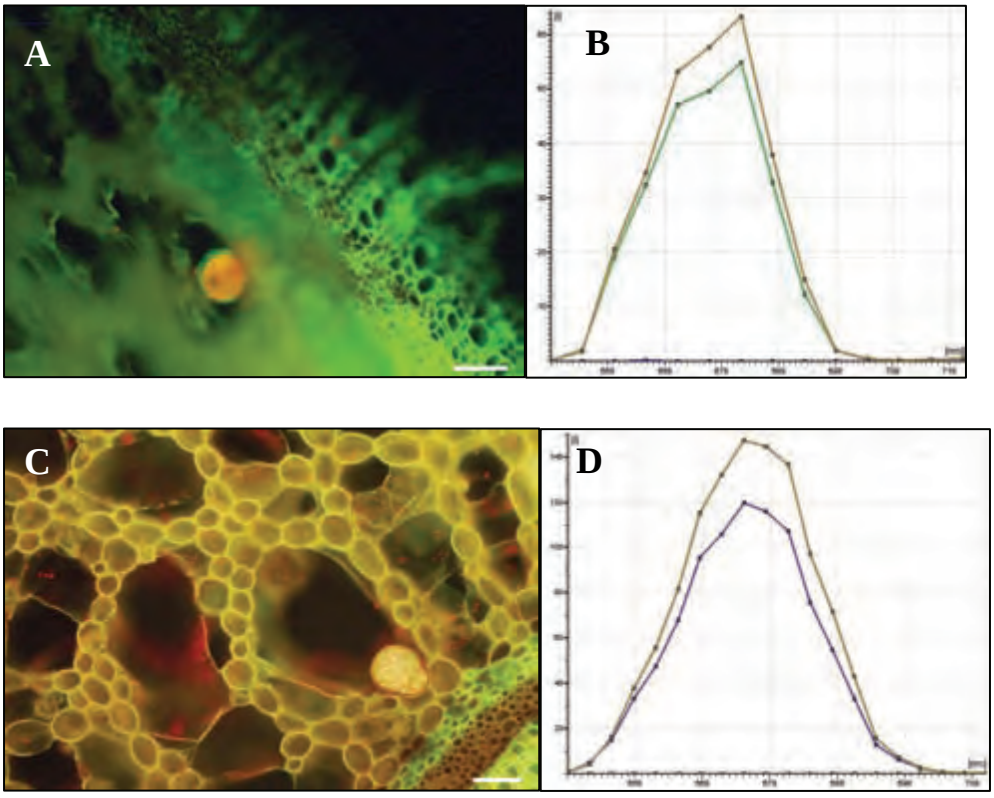

Figure 3ð Red autofluorescence in *P. ramorum* chlamydospores and infected rhododendron tissue. **A**: chlamydospore in cortex of discoloured stem showing unusual red autofluorescence, **B**: CLSM emission spectra profile of red chlamydospore, **C**: red autofluorescence in cortex of discoloured rhododendron stem, **D**: CLSM emission spectra profile of stem tissue exhibiting red autofluorescence, white bars indicate 50 µm

Acknowledgments

We would like to thank USDA-Forest Service, Pacific Southwest Research Station for funding the project.

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Spread of Infection Within Tanoak *(Lithocarpus densiflorus)* Trees Inoculated With *Phytophthora ramorum*[1]

Jennifer Parke,[2,3] Bradley Collins,[3] Gerri Buckles,[2] Everett Hansen,[3] and Barb Lachenbruch[4]

Abstract

In an experiment with mature naturally infected tanoak trees, *Phytophthora ramorum* was observed in sapwood and appeared to interfere with stem water transport (Parke and others 2007). We postulated that colonization of the xylem could contribute to rapid spread within the tree and lead to development of crown mortality symptomatic of sudden oak death. In this field experiment, we compared the initial spread of *P. ramorum* within xylem and phloem of tanoak trees inoculated at specific locations in the bole, basal sprouts, and belowground tissues. We determined hydraulic conductivity of sapwood from inoculated vs. wounded boles and investigated the spatial and temporal development of tyloses in response to infection.

The experiment was conducted in a redwood-tanoak stand at El Corte de Madera Open Space Preserve in the Midpeninsula Regional Open Space District in San Mateo Co., California. Boles of understory tanoak trees (10-15 cm DBH) were inoculated in May 2006 with mycelial plugs of *P. ramorum* placed at the cambium. Bole controls received agar plugs without the pathogen. Twigs of basal sprouts and belowground tissue were inoculated similarly. There were twelve replicate trees for each of the four treatments (bole wounded control, bole-inoculated, sprout-inoculated and root-inoculated). Half of the trees were harvested in September 2006 and the remaining trees were harvested in 2007. Sapwood samples were excised for hydraulic conductivity assays, and adjacent sapwood and phloem tissues were used for pathogen isolation, diagnostic PCR, and microscopy to determine the extent and location of *P. ramorum* infection.

For both sampling times, inoculated boles with xylem infections had significantly more tyloses compared to wounded, non-inoculated boles (Collins, 2008). In addition, the increase in tyloses was associated with a decrease in specific hydraulic conductivity, suggesting that tyloses induced by infection with *P. ramorum* could interfere with stem sap flow. Over time, tylosis development increased in tissues further away from the inoculation site, preceding the vertical spread of infection. Results suggest that infected sapwood contains numerous tyloses which could significantly impede stem water transport. This is consistent with the hypothesis that tanoak mortality associated with *P. ramorum* infection results, at least in part, from impaired hydraulic conductivity (Collins and others, *submitted*). In this study, infections did not spread from inoculated basal sprouts to the boles, nor did they spread from inoculated belowground tissues to aboveground tissues.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26ï 31, 2007, Monterey, California.

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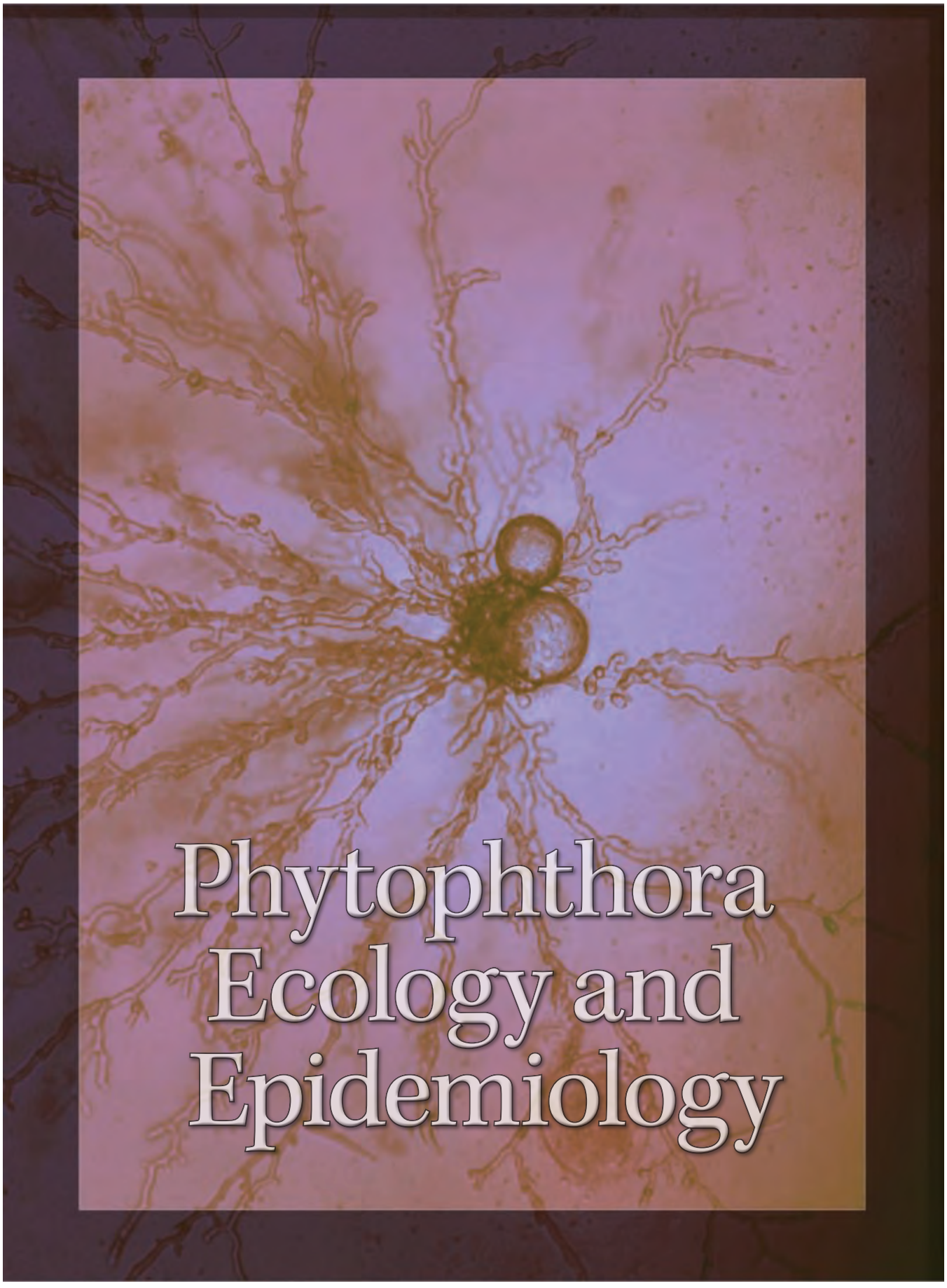
Phytophthora
Ecology and
Epidemiology

Studying Ink Disease Distribution Patterns on Heterogeneous Landscape by Means of Remote Sensing Activities and GIS Technology[1]

Andrea Vannini,[2] Giuseppina Natili,[2] and Anna Maria Vettraino[2]

Abstract

Forest disease monitoring by means of remote sensing, using satellite or digital cameras carried by aircrafts, is getting more and more popular. The main advantages of this technique are the high image resolution (less than one meter), the possibility to acquire information on large surfaces in short time, the utilization of pre-defined spectral bands chosen to best discriminate the reflectance signature of healthy and diseased individual trees. Combination of remote sensing with GIS technology and geostatistic modules represents a powerful tool in order to develop risk maps based on disease variables, landscape features and climate, and to obtain important epidemiological information.

Monitoring of Ink disease in Italy was carried by mean of spectral images acquisition with ASPIS technology. Following image interpretation and definition of foci, a disease map was developed and resolved in a GIS. The association of disease presence with landscape features including slope, presence of roads and drainages was investigated by analysing the different informative layers. A significant effect of road density (expressed as meters/hectare) and slope on disease presence was evidenced. Furthermore disease gradient from roads and drainages followed the Kiyosawa and Shiyomi model. Patterns of disease spread was studied at small scale (two hectares) suggesting that more factors, in addition to drainage and roads architecture, are associated to the foci expansion.

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26ï 31, 2007, Monterey, California.

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Modelling the Potential Distribution of *Phytophthora alni* Root and Collar Rot of Alders in Bavaria and Preliminary Application of the Model to Estimate the U.S. Susceptibility to *P. alni*[1]

Thomas Jung,[2] Marla Downing,[3] Vernon Thomas,[4] Markus Blaschke,[5] Michael F. Tuffly,[6] and Robin Reich[7]

Abstract

Phytophthora alni formerly known as the "alder *Phytophthora*" is a recently evolved soil- and waterborne hybrid pathogen causing root and collar rot of *Alnus* species. *P. alni* has quickly spread across Europe via planting of infested nursery stock and has yet not been recorded from other continents. A model was developed to predict the potential distribution of *P. alni* in Bavaria, Southern Germany in order to have a tool for assessing the potential hazard posed by *P. alni* to forests in other continents. Bavaria was chosen because of (i) being a hot spot of the disease, and (ii) the existence of both a large disease data base and expert knowledge. Logistic regression was used to identify environmental and site conditions important in describing alder infestations. Three models were evaluated. The models were based on categorical variables describing the establishment (planted/natural) and the flooding conditions (flooded/non-flooded) of 434 sample points of which 307 points were infested by *P. alni*; on GIS datasets on soil texture, aspect, slope and landform; and a Normalized Difference Vegetation Index (NDVI) calculated from satellite imagery. The non-spatial model included only the flooding condition of the sites and the establishment type while the spatial model included only GIS datasets and the vegetation index. The third model was a combination of the spatial and non-spatial model and included the flooding condition and establishment type of the stands and selected GIS datasets. Receiver Operating Curves (ROC) were used to assess the predictive accuracy of models. Both the spatial and the non-spatial model were highly accurate at predicting infected sites (95.8 and 82.1 percent accuracy) but their accuracy in predicting healthy sites was insufficient (66.1 and 61.1 percent). In contrast, the combined model predicted both healthy and diseased sites with high accuracy (85 percent and 86.6 percent, respectively). Preliminary results of an application of the Bavarian rules along with additional datasets on drainage, streams, climate, distribution of alder species, distribution of wholesale and retail nurseries, and urban settlements in a Multi-Criteria model to the United States indicate that there are regions with a considerable susceptibilty of alder forests to *P. alni*.

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Susceptibility of Some Italian Ornamental and Forestry Species to *Phytophthora ramorum*[1]

Anna Maria Vettraino,[2] Barbara Ceccarelli[2] and Andrea Vannini[2]

Abstract

Phytophthora ramorum is the fungal-like pathogen which is the causal agent of a condition known in the USA as sudden oak death. Initially observed in a container-grown *Rhododendron* and *Viburnum* plants in nurseries, *P. ramorum* is now known to infect a wide range of hosts both in nursery and forest in Europe (Werres and others 2001; www.rapra.csl.gov.uk). Symptoms varied on different hosts and they are often restricted to leaf spots, stem and twig blight or occasionally seedling blight or tip dieback and bleeding cankers (Davidson and others 2003). The aim of this work was to assess the susceptibility of Mediterranean potential hosts. We assessed the reaction of 69 coniferous and broad-leaved tree species to stem and leaf inoculation with this quarantine pathogen. Three different *P. ramorum* isolates have been used: isolates P1376 (from *Viburnum tinus*) and P1577 (from *Rhododendron catawbiensis*), both A1 mating type, European isolates and isolate P1403 (from *Vaccinium ovatum*), A2 mating type, American isolate. Leaves were inoculated with $4x10^5$ zoospores mL^{-1}. Stems were inoculated by placing a plug of mycelium in a wound cut, diameter 7mm. Foliar and stems lesions were examined after 10 days. Moreover the asexual sporulation 'in planta' was examined on each host.

Leaf lesions caused by *P. ramorum* appeared variable in colour and size depending on the host. Symptoms ranged from scattered spots to large necrotic areas; sometimes lesions appeared surrounded by diffuse margins and a halo of yellow (fig. 1).

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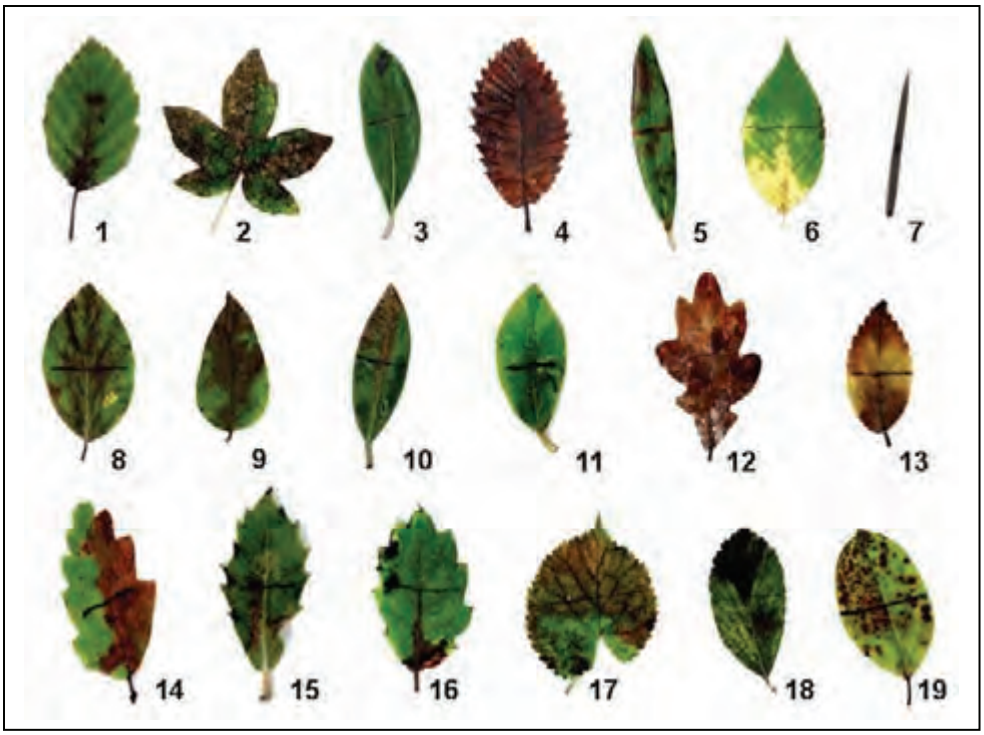

Figure 1ð Symptoms of *P. ramorum* infection on inoculated hosts:1) *Alnus incana*; 2) *Acer campestre*; 3) *Arbutus unedo*; 4) *Castanea sativa*; 5) *Olea europea*; 6) *Celtis australis*; 7) *Juniperus communis* L.; 8*) Fagus sylvatica*; 9) *Fraxinus ornus*; 10) *Rhododendron* sp.; 11) *Arbutus unedo*; 12) *Quercus robur*; 13) *Ulmus minor;* 14) *Quercus cerris*; 15) *Quercus ilex*; 16) *Quercus macrolepis*; 17) *Tilia cordata*; 18) *Sorbus aria*; 19) *Cornus sanguinea*.

The 65 hosts varied widely in susceptibility, ranging from 0 percent to 100 percent leaf area infected. No symptoms appeared on *Phyllirea angustifolia, Phyllirea latifoglia, Photinia fraseri, Pistacia lentiscus, Rhamnus alaternus* and *Myrtus communis*. Hosts varied widely in their reaction to inoculation regarding both disease incidence ($\chi 2=109$; $P<0.0001$) and lesions size (ANOVA; F= 15,978; P<0,0001). Stem inoculations resulted on all host species in necrotic lesions extending beyond the margin of the inoculation wound. Disease incidence was 100 percent for all the 65 hosts species analysed. The lesion length was highly variable among individual plants (ANOVA; F=16.2; P<0.001). *Phytophthora ramorum* was always re-isolated form symptomatic tissues and never from controls. Sporangia were observed on all the 51 species tested including the 6 symptomless. Significant variability in sporangia production was observed among hosts (ANOVA; F=37.9; P<0.0001).

This study provides the first record of the reactions to *P. ramorum* of a broad range of host species growing from sea level to 1200 a.s.l. Moreover it contributes to update the list of the potential hosts of *P. ramorum*, with 35 species not hitherto tested and defines a list of SOD risk Mediterranean hosts. Our results indicate that most of the species tested can support both infection and sporulation following artificial inoculations with *P. ramorum* (Hansen and others 2005). Not always host species with high leaves susceptibility showed to be greatly sensible to stem inoculation, confirming results from other authors.

The presence of wide range of symptoms, even similar to those caused by other *Phytophthora* spp., the broad host range associated with *P. ramorum* together with the presence of sporangia on symptomless hosts species could make difficult to detect the pathogen. For this reason potentially there is a very high risk of inadvertent introduction of *P. ramorum* plant through trade due to a hidden infection.

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Widespread *Phytophthora* Infestations of Nurseries in Germany and Austria and Their Role as Primary Pathway of *Phytophthora* Diseases of Trees[1]

Thomas Jung,[2] Jörg Schumacher,[3] Sindy Leonhard,[3] Günter Hartmann,[4] and Thomas Cech[5]

Abstract

Between 2001 and 2007 extensive nursery surveys were carried out across Germany and Austria. In Northern Germany beech, oak and maple fields of 14 nurseries were investigated and 54 percentwere found infested by *P. cactorum* (23 percent), *P. syringae* (23 percent) and *P. cambivora* (15 percent). Other *Phytophthora* spp. were isolated infrequently. In Lower Saxony, Northwestern Germany beech fields were surveyed in six nurseries and five were found infested by *P. cactorum*, *P. cambivora*, *P. citricola*, *P. gonapodyides* (each two nurseries) and *P. pseudosyringae* (one nursery). In Bavaria, Southern Germany the beech fields of all nine nurseries tested were infested by a range of eight *Phytophthora* species with *P. citricola*, *P. cactorum* (each seven nurseries) and *P. cambivora* (five nurseries) being most widespread. In Southern and Western Germany all oak fields (*Q. robur, Q. rubra, Q. petraea*) of the eight tested nurseries were infested by *Phytophthora* spp. *P. quercina, P. citricola* (each five nurseries) and *P. cactorum* (four nurseries) were most common.

Alder fields were investigated in Bavaria, Southern Germany, in Brandenburg, Eastern Germany and in Austria with the specific purpose of detecting *P. alni* which is responsible for the epidemic alder mortality across Europe. In Bavaria *P. alni* was recovered from rootstocks of alders from three out of four nurseries which regularly bought in alder plants for resale, but not in rootstocks from four nurseries that grew their own alders from seed. In addition, *P. cambivora*, *P. cactorum*, *P. gonapodyides* and *P.* taxon 'Pgchlamydo'(each 37.5 percent), *P. megasperma* (50 percent) and *P. citricola* (62.5 percent) were isolated. In Brandenburg *P. alni* was found in five out of ten nurseries. In addition, *P. cambivora, P. cactorum* and *P. syringae* were recovered with annually changing isolation frequencies. In both countries the infested nurseries used water from infested water courses for irrigation. In Austria *P. alni* was found in one of the four nurseries studied. As a result alders in Bavaria and Brandenburg were produced according to a code of good practice. Control isolations showed that *P. alni* but not the other *Phytophthora* spp. could be eliminated.

Extensive field studies in young forest and amenity plantations in Southern and Northwestern Germany, and in more than 3000 alder and more than 200 mature beech and oak stands across Germany demonstrate the ubiquitous involvement of *Phytophthora* species in the devastating

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broadleaf tree declines, and the role of infested nursery stock as a primary pathway of Phytophthora diseases of trees. The implications of our results for the nursery and the forest industries are discussed.

Survival and Chlamydospore Production of *Phytophthora ramorum* in California Bay Laurel Leaves[1]

Elizabeth J. Fichtner[2], David M. Rizzo,[2] Shannon C. Lynch,[2] Jennifer Davidson[3], Gerri Buckles,[4] and Jennifer Parke[4]

Abstract

Sudden oak death manifests as non-lethal foliar lesions on bay laurel (*Umbellularia californica*), which support sporulation and survival of *Phytophthora ramorum* in forest ecosystems. The pathogen survives the dry summers in a proportion of attached bay leaves, but the propagules responsible for survival are unknown. This study focuses on summer pathogen survival associated with bay laurel in redwood-tanoak and mixed-evergreen forests with specific objectives including:i) detection of *P. ramorum* in leaf litter and soils throu ghout the annual disease cycle, ii) quantification of chlamydospores on and within attached symptomatic leaves, and in fresh and aged litter, iii) determination of chlamydospore germination, and, iv) assessment of pathogen survival within litter and canopy leaves, addressing the location of viable inoculum within foliar tissues. Ten trees were tagged for repetitive sampling in four redwood-tanoak and four mixed-evergreen forests. Sampling was conducted at four times between May 2006 and September 2007 to target different points during the disease cycle. To determine pathogen presence in leaf litter and soil, three soil samples and 20 symptomatic litter leaves were collected and independently bulked from each tree. Samples were then baited for *P. ramorum* with rhododendron leaves. Chlamydospore populations on surfaces of attached leaves, and fresh and aged litter were determined by scrubbing individual leaves with a moistened toothbrush and filtering the resulting suspension through 35µM nylon mesh. Chlamydospores were then counted under a dissecting microscope and a subsample of chlamydospores was placed on selective medium to observe germination potential. To evaluate chlamydospore production within tissue, leaves were cleared with KOH and then observed with light microscopy. Pathogen survival and colonization was determined by subdividing symptomatic tissue from each leaf for detection by PCR, culture, and microscopy. Chlamydospore populations on attached leaf surfaces were higher in redwood-tanoak than in mixed-evergreen forests, but chlamydospore germination was never observed. Pathogen recovery was highest in the late spring, but declined by the end of the summer survival period. Isolation recovery was higher in attached leaves than in freshly cast leaves, but was rare from aged leaf litter tissues. High pathogen detection frequency was achieved by PCR, even when recovery in culture was not possible. Pathogen detection by PCR and isolation did not correspond with the heightened chlamydospore production observed at some sites. Though bay laurel supports chlamydospore production, the quantity of chlamydospores produced per leaf varies between sites and the lack of germination of these survival propagules contributes to the mystery of their potential role in the epidemiology of sudden oak death.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26ï 31, 2007, Monterey, California.

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Introduction

Phytophthora ramorum, the cause of sudden oak death (SOD) in California (CA), is an exotic plant pathogen that has caused extensive mortality of susceptible oak (*Quercus* spp.) and tanoak (*Lithocarpus densiflorus*) in coastal California forests since the mid-1990s (Rizzo and others 2002, Rizzo and others 2005). The pathogen causes non-lethal infections on numerous hardwood and coniferous forest tree species, understory shrubs, and herbaceous plants (Davidson and others 2003, Garbelotto and others 2003). The non-lethal foliar lesions formed on California bay laurel (*Umbellularia californica*) by *P. ramorum* support prolific sporulation during rain events and summer survival of the pathogen in forest ecosystems (Davidson and others 2005b).

Though symptoms of *P. ramorum* on bay laurel are limited to foliar lesions, the sporulation and survival potential of the pathogen on this host elevate its epidemiological significance in the forest ecosystem. At an ecological level, presence of bay laurel correlates with occurrence of *P. ramorum* (Rizzo and others 2005), and on individual leaves the pathogen may produce as many as $5x10^3$ zoospores during a single rain event (Davidson and others 2005a). Foliar infections of bay laurel generally precede the infection of adjacent oaks (Rizzo and Garbelotto, 2003) and isolate aggressiveness on bay laurel correlates directly with aggressiveness on oak (Hüberli and others 2005). The pathogen survives the hot, dry summer months in a proportion of symptomatic bay leaves (Davidson and others 2005a); but summer survival potential in bay leaves varies between sites. The phenomenon of site-influenced survival of *P. ramorum* within bay laurel has been documented in both a redwood-tanoak forest and a mixed-evergreen forest (dominated by coast live oak) at Jack London State Park (Glen Ellen, CA) and Fairfield Osborne Preserve (Pengrove, CA), respectively. Summer foliar survival in attached symptomatic leaves is higher in the redwood-tanoak forest (70 percent recovery) than in the mixed-evergreen forest (20 percent recovery), yet these two sites are located only 5km apart on opposite sides of Sonoma Mountain (J. Davidson, unpublished data). Furthermore, sporangia production is detected earlier and both disease spread and severity are higher in the redwood-tanoak forest than in the mixed-evergreen forest. The difference in the disease progress curve between the two sites suggests a disparity in amount of primary inoculum at the onset of the rainy season; however, the propagules responsible for pathogen survival are yet unknown.

Based on the hypothesis that primary inoculum is more abundant in redwood-tanoak forests than in mixed-evergreen forests at the onset of the fall rainy season, the overall focus of this work is to assess summer pathogen survival associated with bay laurel in the two forest types. Because infected bay laurel leaves are more likely to abscise than uninfected leaves, our investigations target both symptomatic attached leaves, and symptomatic leaves in the litter layer. Specific objectives include: i) detection of *P. ramorum* in leaf litter and soils throughout the annual disease cycle, ii) quantification of chlamydospores on attached symptomatic leaves, and in fresh and aged litter, iii) determination of chlamydospore germination, and, iv) assessment of pathogen survival within litter and canopy leaves, addressing the location of viable inoculum within foliar tissues.

Materials and Methods

Ten trees were tagged for repetitive sampling in four redwood-tanoak (Jack London State Park, Pfeiffer Big Sur State Park, Henry Cowell Redwoods State Park, and Samuel P. Taylor State Park) and four mixed-evergreen forests (Fairfield Osborne Preserve, Pacheco, Skyline Regional Park, and China Camp State Park). The redwood-tanoak and mixed-evergreen forest sites spanned latitudinal gradients of over 225 km, and 125 km, respectively. Sampling was conducted in May and August 2006 and in March and September 2007. At each sampling event, all samples were collected from the eight sights within two days. Three types of bay foliage were collected: symptomatic attached leaves, freshly cast litter leaves, and aged litter leaves. The September 2007 collection diverged from the normal sampling strategy because freshly cast leaf specimens were not available at all sites.

Baiting of Litter and Soil

To determine pathogen presence in leaf litter and soil, 20 leaves were collected and bulked from the forest floor and three soil samples were collected and bulked from under the canopy of each tree. Litter and soil samples were then baited with rhododendron cv. Colonel Coen leaves for *P. ramorum*. Lesion margins from symptomatic baits were placed on PARPH selective medium containing 0.05 g hymexazol/L for determination of presence of *P. ramorum*. A subsample of soil from each tree was passed through a 2 mm sieve and a 30-40 g sample was weighed and oven-dried for determination of soil moisture content.

Determination of Chlamydospore Populations

At each sample time, one symptomatic attached leaf, one symptomatic freshly cast leaf and one symptomatic aged litter leaf were collected for determination of chlamydospore presence on leaf surfaces of each of the 80 tagged trees. Additionally, one asymptomatic leaf was sampled from each of two trees at each site to assess for asymptomatic sporulation. Individual leaves were scrubbed on both sides with a moistened toothbrush and rinsed with deionized water before filtering the resulting suspensionthrough a 35 μM nylon mesh. Chlamydospores were then counted under the dissecting microscope and the total number of chlamydospores per leaf was recorded.

To look for chlamydospores within leaf tissue, another set of symptomatic attached-, freshly cast-, and aged litter leaves was gathered from each tagged tree and cleared in 1N KOH to render tissue translucent. After approximately three weeks in the KOH solution, leaves were clear enough to observe under the compound microscope.

Determination of Chlamydospore Germination Potential

At each sample time chlamydospore germination potential was determined by individually selecting 10 mature chlamydospores from the symptomatic attached-, freshly cast-, and aged litter leaves at each of the eight sites. For some sets of leaves, particularly those collected from mixed-evergreen forests, fewer than ten chlamydospores were available for germination studies. Individual chlamydospores were placed on PARPH selective medium, incubated in the dark at room temperature, and observed periodically over two weeks for chlamydospore germination.

Additionally, chlamydospores dislodged from symptomatic attached leaves in September 2007 were transferred to uninfected bay laurel and rhododendron leaves to

determine infection potential. Immediately after scrubbing leaves from Samuel P. Taylor State Park and Henry Cowell Redwoods State Park, chlamydospores were counted and 10 chlamydospores were placed on three detached, moistened bay laurel and rhododendron leaves. Leaves were then stored in moist chambers for approximately 2 weeks for observation of symptom development and subsequent isolation of symptomatic and asymptomatic tissue onto PARP.

Pathogen Survival and Colonization

To assess pathogen survival and colonization in leaf tissue, one symptomatic attached-, one freshly-cast-, and one aged litter- leaf was collected from each tagged tree. For each leaf, the area along the lesion margin was subdivided for detection by PCR, culture, and scanning electron microscopy (SEM). For detection in culture, tissue from attached symptomatic leaves was placed in PARP, whereas tissue sampled from the forest floor was placed in PARPH. Isolation plates were then stored in the dark for up to three weeks at room temperature for assessing recovery of *P. ramorum* and other *Phytophthora* spp. present. Only samples lacking recovery of *P. ramorum* in culture were assessed for pathogen presence using PCR. For visual observation of colonization, samples were fixed and preserved in formalin acetic acid (FAA) for future observation using scanning electron microscopy.

Results

Baiting of Litter and Soil

P. ramorum was baited from 60–90 percent of soil samples at all sites in May 2006 (fig. 1A), but was undetectable by August 2006. In May 2007, *P. ramorum* was baited from 0-70 percent of the soil samples (fig. 1B), with samples from redwood-tanoak forests exhibiting generally higher recovery than those from mixed-evergreen forests. In September 2007, the pathogen was only baited from one soil sample at Henry Cowell Redwoods State Park, a redwood-tanoak site. *Phytophthora ramorum* was only baited from the bulk leaf litter in March 2007, and only at sites experiencing a rain event concurrent with collection.

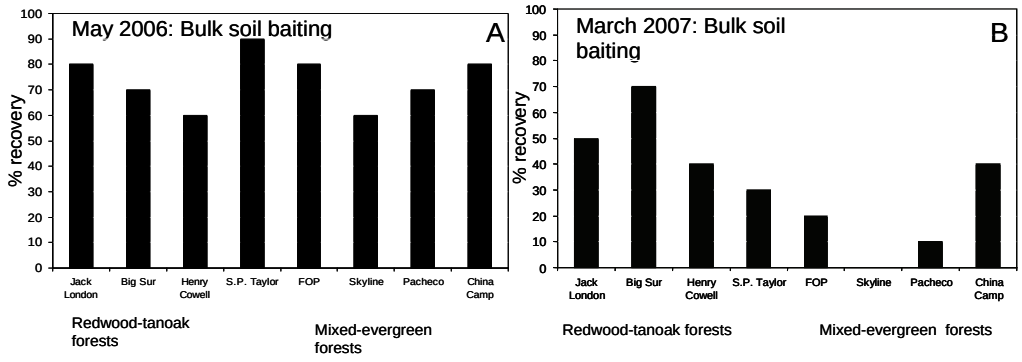

Figure 1ð Percent recovery from soil baits sampled at the end of the winter/spring rainy seasons. Graph A represents recovery in May 2006 and B from March 2007.

Determination of Chlamydospore Populations

Chlamydospore populations on attached leaf surfaces were higher in redwood-tanoak than in mixed-evergreen forests at all sample dates (fig 2). Chlamydospore populations were consistently higher at Henry Cowell Redwoods and Samuel P. Taylor State Park over all sample times. Chlamydospore levels were variable in fresh and aged litter.

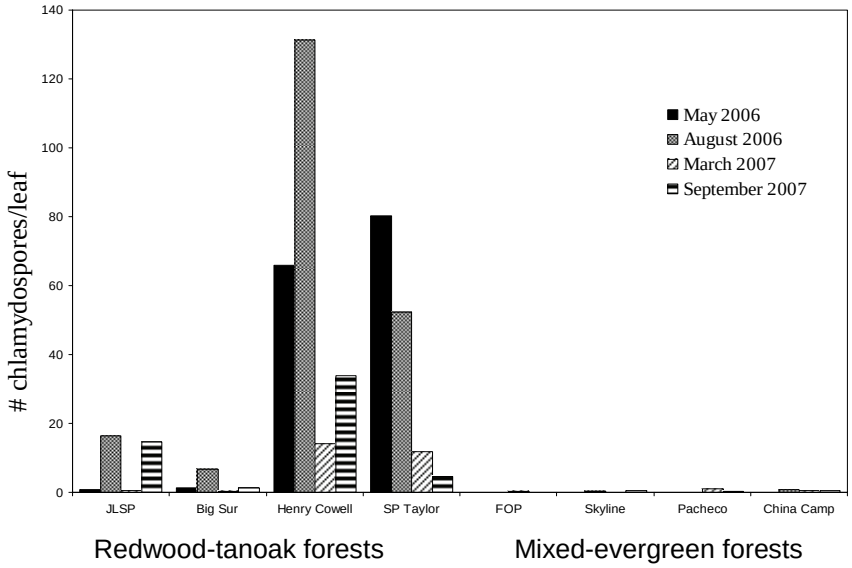

Figure 2ð Chlamydospore populations on attached symptomatic bay laurel leaves. Values represent average number of chlamydospores per leaf at each of 8 sites and over four sample dates.

Chlamydospores were not observed in leaf tissues cleared in KOH, even in leaves from sites supporting high chlamydospore loads. To determine whether chlamydospores were sloughed off leaf surfaces while incubating in KOH, a drop of KOH was pipetted from the bottom of multiple test tubes and observed under the compound microscope. Chlamydospores were observed to accumulate at the bottom of test tubes containing leaves incubating in KOH.

Chlamydospore Germination

Chlamydospore germination was never observed on selective medium at any collection times. Similarly, chlamydospores placed on bay laurel and rhododendron leaves in September 2007 did not initiate infections of either leaf tissue.

Pathogen Survival and Colonization

Pathogen isolation from attached leaves ranged from 40-100 percent at each site in May 2006 and declined to a range of 0–40 percent in August 2006 (fig. 3). In August 2006 (fig 3B) and September 2007 (fig 3D), *P. ramorum* was not recovered from

attached leaves at four sites. The summer of 2006 was characterized by excessive heat, with numerous days having high temperatures over 38º C at most sites. Over the rainy season between August 2006 (fig 3B) and March 2007 (fig. 3D), recovery from attached leaves increased at five sites and remained static at three sites.

PCR resulted in higher detection of *P. ramorum* than isolation in culture (figs. 3, A and B).

Banksia attenuata

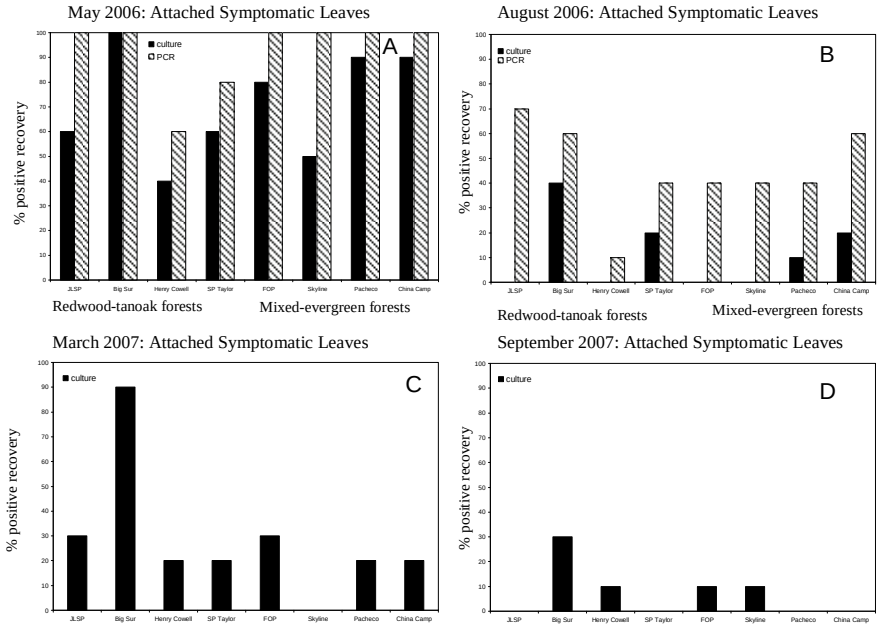

Figure 3ð Recovery of *Phytophthora ramorum* from attached symptomatic bay laurel leaves. Graph A and B represent recovery by PCR and isolation in culture for samples collected in May and August 2006, respectively. C and D represent recovery in culture for samples collected in March and September 2007, respectively.

Fresh leaf litter was only collected at all eight sites in August 2006 (fig. 4A) and March 2007 (fig 4B), whereas aged litter was collected from all eight sites in August 2006 (fig. 5A), March 2007 (fig. 5B), and September 2007. *Phytophthora ramorum* was recovered from freshly cast leaves with higher frequency in March 2007 than in August 2006 (fig 4). Difference in isolation recovery of *P. ramorum* between attached leaves and freshly cast leaves was not more than 30 percent, with the exception of samples collected from Henry Cowell Redwoods in March of 2007. At this time, isolation recovery from fresh litter was 50 percent higher than recovery from attached leaves.

P. ramorum was isolated in culture from one aged leaf at each of two sites in March of 2007 (fig. 5B); however, the collection at these two sites was concurrent with a

heavy rain event. PCR detection of *P. ramorum* was possible in both fresh litter (fig. 4A) and aged litter (fig. 5A), even when the pathogen was not recovered in culture.

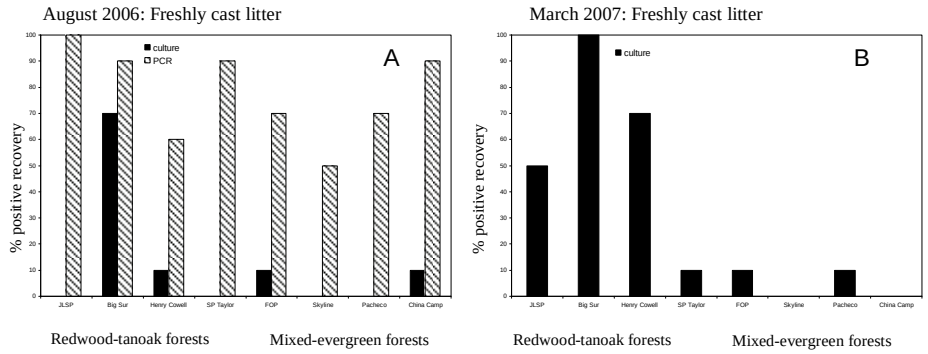

Figure 4ð Recovery of *Phytophthora ramorum* in freshly cast leaf litter. A represents recovery by PCR and isolation in August 2006 and B represents isolation recovery in March 2007.

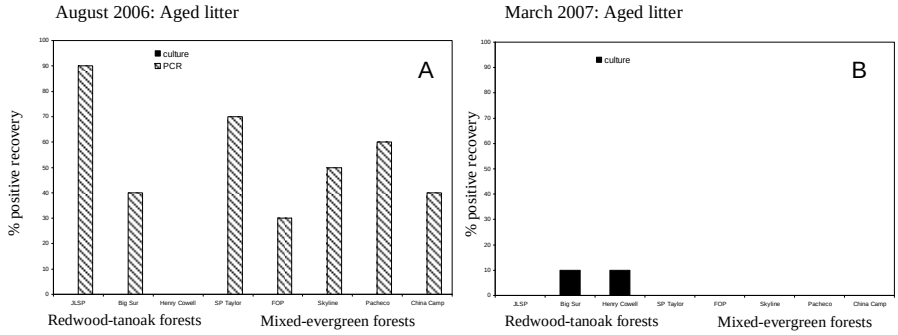

Figure 5ð Recovery of *Phytophthora ramorum* in symptomatic, aged leaf litter. Pathogen detection by isolation in culture and PCR was completed for the sampling in August 2006 (A), and recovery by isolation was completed for samples collected in March 2007 (B).

Discussion

After four collection events spanning different times within the annual disease cycle of *P. ramorum*, our data suggest that bay laurel in redwood-tanoak forests support higher levels of foliar chlamydospore production than bay laurel in mixed-evergreen forests. Two sites in particular, Henry Cowell Redwoods and Samuel P. Taylor State Parks, exhibit higher chlamydospore loads on bay laurel than the other sites examined in this study. Presumably the heightened chlamydospore loads at these sites result in more primary inoculum at the onset of the disease cycle; however,

pathogen recovery at the end of the summer survival periods was not higher in sites exhibiting high chlamydospore loads. Furthermore, because chlamydospores produced on naturally-infected bay leaves did not germinate in vitro, one can only speculate on their viability, survival potential, and infectivity.

Infected bay laurel leaves are more likely to abscise than uninfected leaves, resulting in deposition of inoculum at the forest floor (Davidson and others 2005a). Pathogen recovery by isolation was higher in freshly cast litter than in aged litter, suggesting that survival declines rapidly after abscission. Detection of *P. ramorum* by PCR in fresh and aged litter remained high, even when the pathogen could not be recovered by isolation. PCR positives, however, may result from detection of non-viable pathogen tissue. Though PCR positives may result from presence of chlamydospores, the two sites exhibiting high chlamydospore loads did not have a higher frequency of PCR positives in aged or fresh litter than sites with low chlamydospore loads.

P. ramorum survives the hot, dry summers in a portion of infected bay laurel leaves, providing a source of primary inoculum at the onset of the fall rainy season. Chlamydospores are produced on infected leaves, however, their potential role in survival and infectivity is yet unknown. Chlamydospore production on bay laurel foliage varies between sites, but their potential role in shaping disease progress in epidemics in different forest types remains open for conjecture.

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Survival of *Phytophthora cinnamomi* in Soil After Prescribed Fire in a Southern Appalachian Mountain Forest[1]

Inga M. McLaughlin,[2] Steven N. Jeffers,[2] and Thomas A. Waldrop[3]

Abstract

The use of prescribed fire has become a common management strategy in fire-adapted forests of the southern Appalachian Mountains. Little is known about the direct effects of fire on survival of species of *Phytophthora* naturally present in forest soils. Therefore, the effects of fire on survival of *P. cinnamomi* was assessed during two low-intensity prescribed fires that were set in a mixed oak-pine forest within the Jocassee Gorges natural area in northwestern South Carolina during February 2007. *P. cinnamomi* is known to occur throughout this forest site. Before the first fire, six plots were established in the area to be burned and two plots were established in adjacent areas that would not be burned. Before the second fire, four plots were established in the area to be burned and one plot was established in an area that would not be burned. In each plot, seven 100-ml aliquots of forest soil naturally infested with *P. cinnamomi* in aluminum-mesh packets were placed at 2 cm and 10 cm below the soil surface. Temperature sensors attached to dataloggers were placed with each packet and recorded temperature in the soil at 1.5-second intervals. Sensors also were used to measure above-ground temperature during the second fire. After the fires, the soil in each packet was assayed for *P. cinnamomi* using a baiting bioassay procedure.

During the second fire, maximum temperatures 30 cm above the soil surface in each plot ranged from 47 to 111°C. Soil temperatures in the non-burned plots during both fires ranged from 7 to 13°C at both 2 cm and 10 cm below the soil surface. However, in the burned plots, soil temperatures ranged from 7 to 14°C at 10-cm depths and from 11 to 42°C at 2-cm depths. After the two fires, *P. cinnamomi* was recovered from all 42 soil aliquots from both depths in the three plots in non-burned areas. *P. cinnamomi* also was recovered from all 70 soil aliquots buried at 10 cm and from 69 of 70 aliquots buried at 2 cm in the burned areas; it was not recovered from one soil aliquot placed 2 cm beneath the soil surface during the first fire—where the soil temperature reached 42°C for 40 min. In this study, the direct effect of low-intensity, prescribed fire had minimal impact on the survival of *P. cinnamomi* in soil.

Introduction

The use of prescribed fire has become a common management strategy in fire-adapted forests of the southern Appalachian Mountains to reduce fuel accumulation and encourage regeneration of declining forest ecosystems (Barden and Woods 1976; Brose and Waldrop 2000; Waldrop and others 1987). Little is known about the effects of fire on survival of species of *Phytophthora* naturally present in forest soils. Therefore, two prescribed fires were used to assess the direct effect of fire on the

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26–31, 2007, Monterey, California.

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survival of *P. cinnamomi* in soil in a mixed oak-pine forest within the Jocassee Gorges Natural Area of northwestern South Carolina in February 2007. *P. cinnamomi* is known to occur throughout this forest site (Wood and others 2001).

Materials and Methods

Before the first fire, six plots were established in the area to be burned and two plots were established in adjacent areas that would not be burned. Before the second fire, four plots were established in the area to be burned and one plot was established in an area that would not be burned. In each plot, seven 100-ml aliquots of forest soil naturally infested with *P. cinnamomi* in aluminum-mesh packets were placed at 2 cm and 10 cm below the soil surface (fig. 1) depths at which *P. cinnamomi* has been recovered consistently (McLaughlin and Jeffers, *unpublished*; Wood and others 2001). Temperature sensors attached to dataloggers were placed within each packet and recorded temperature in the soil at 1.5-second intervals (fig. 1). Sensors also were used to measure temperature approximately 30 cm above the soil surface during the second fire (fig. 1). After the fires, the soil in each packet was assayed for *P. cinnamomi* using a baiting bioassay. Soil from each packet was placed into a plastic freezer box (450 ml) and flooded with approximately 200 ml of distilled water. Six camellia leaf disks (5 mm in diameter) were floated on the surface of the water in each box as baits for *P. cinnamomi*, and boxes were kept at room temperature (22 to 25 °C) for 3 days. Baits then were removed, blotted dry, and embedded in PARPH-V8, a medium selective for *Phytophthora* spp. (Ferguson and Jeffers 1999), to recover *P. cinnamomi*. Numbers of baits and soil packets that were positive for *P. cinnamomi* were counted and percentages were calculated.

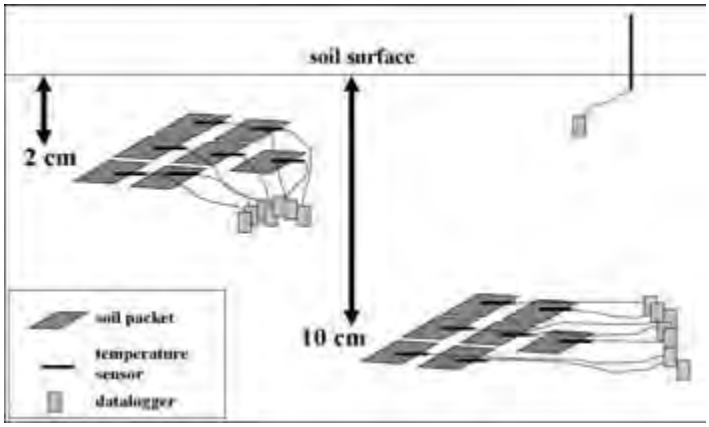

Figure 1—Diagram of the layout of each plot in the study site at the Jocassee Gorges Natural Area in northwestern South Carolina.

Results and Discussion

During the second fire, maximum temperatures 30 cm above the soil surface in the four burned plots were 47, 62, 91, and 111 °C. Maximum soil temperatures were slightly elevated at 2 cm beneath the soil surface in the burned plots (table 1) and ranged from 13 to 42 °C during the first fire and 9 to 16 °C during the second fire. Maximum soil temperature at 10 cm beneath the soil surface in the burned plots ranged from 9 to 18 °C during the first fire and from 11 to 13 °C during the second fire. Maximum soil temperatures in the non-burned plots during both fires were similar and ranged from 7 to 15 °C at 2 cm and 10 cm below the soil surface.

After the two fires, *P. cinnamomi* was recovered from 99 to 100 percent of leaf baits and soil packets at 2 and 10 cm beneath the soil surface in the non-burned, control plots (table 1). *P. cinnamomi* also was detected from 100 percent of leaf baits and soil packets at 10 cm beneath the soil surface in the burned plots. However, presence of *P. cinnamomi* was slightly less in soil packets (99 percent) and leaf baits (97 percent) at the 2-cm depth in the burned plots (table 1) where soil temperatures in some plots reached 42 °C for 40 min, temperatures adequate to inactivate propagules of *P. cinnamomi* (Benson 1978; Jaurez-Palacios and others 1991). In this study, soil temperatures were not elevated consistently for a long enough period of time to inactivate propagules of *P. cinnamomi*. Therefore, typical prescribed fires may not be adequate to eliminate or significantly reduce propagules of *Phytophthora* spp. from forest soils in the southern Appalachian Mountains.

Table 1—Summary of survival of *P. cinnamomi* in soil after and soil temperatures during two prescribed burns in the Jocassee Gorges Natural Area in northwestern South Carolina in 2007

Treatment	Depth (cm)	Plots (no.)	Packets[y]			Baits[z]			Max. soil temp. (°C)	
			Total	No.	Percent	Total	No.	Percent	Mean	Range
Burn	2	10	70	69	99	420	408	97	17	9-42
	10	10	70	70	100	420	418	100	10	9-18
Control	2	3	21	21	100	126	126	100	11	9-13
	10	3	21	21	100	126	125	99	10	7-15

[y] Seven packets were used in each plot, and each packet contained 100 ml of naturally-infested soil. Values are the numbers (No.) and percentages of packets in which *P. cinnamomi* was detected after the fires.

[z] The soil in each packet was baited with six camellia leaf disks; values are the numbers (No.) and percentages of baits from which *P. cinnamomi* grew.

Acknowledgments

This research was funded by the U.S. Department of Agriculture, Forest Service. Special thanks to Mark Hall and Richard Baker for technical assistance and to William Bridges, Jr., for statistical advice.

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Persistence of *Phytophthora lateralis* After Wildfire: Preliminary Monitoring Results From the 2002 Biscuit Fire[1]

Frank Betlejewski[2]

Abstract

Port-Orford-cedar (*Chamaecyparis lawsoniana*) is an economically and ecologically valuable tree species that occurs on approximately 107,044 ha in southwest Oregon and northwest California. Roughly 35,400 ha are infested with *Phytophthora lateralis*, the pathogen that causes Port-Orford-cedar root disease. The pathogen has been shown to kill more than ninety percent of the Port-Orford-cedar stocking on an infested site. Biologically valuable overstory Port-Orford-cedar are especially prone to infection and mortality. Loss of large Port-Orford-cedar can reduce the ecological function of the species and limit economic availability.

Phytophthora lateralis is sensitive to heat. Hansen and Hamm (1996) showed that survival of the pathogen is minimal in soil exposed to temperature of 40 °C (104 °F) or greater, especially if conditions are dry. The Biscuit fire burned approximately 196,800 acres in southwest Oregon and northwest California in 2002 including over 1,180 ha infested with *P. lateralis*.

In the spring of 2004, twenty-five monitoring plots were established inside the Biscuit fire perimeter on sites known to be infested with *P. lateralis*. The nineteen Silver Creek plots were baited at 0.61 m (two foot) spacing with forty-eight susceptible seedlings. The six Game Lake plots were baited at the same spacing with forty-four susceptible seedlings.

Baiting is a bio-assay that uses Port-Orford-cedar seedlings to determine the presence of *P. lateralis*. Non-resistant Port-Orford-cedar seedlings are planted in soil where *P. lateralis* is suspected to occur. After an exposure period of six to eight weeks, the seedlings are recollected and analyzed for the presence of *P. lateralis.* The Biscuit fire seedlings were removed from the bait plots after eight weeks and analyzed to determine the number of infected seedlings and establish a baseline for *P. lateralis* presence.

The plots were baited again in the fall of 2004. Fifty percent of the bait seedlings were the same susceptible stock used spring, 2004 and fifty percent of the bait seedlings were resistant to *P. lateralis*. The same contractor was used and seedlings were planted in the same holes as the spring, 2004 baiting. Dead seedlings were collected during the summer in 2005, 2006, and 2007 and analyzed to determine the number infected with *P. lateralis*.

Twenty-two of the original twenty-five plots remain in 2007. Twenty-one of twenty-two plots planted in spring 2004 had mortality caused by*P. lateralis* (table 1). *Phytophthora lateralis* mortality in the fall 2004 planting has declined from that seen in spring, 2004. Fewer plots showed *P. lateralis*-caused seedling mortality and fewer seedlings overall were infected. *Phytophthora lateralis* mortality declined to thirteen, nine, and six plots respectively in 2005, 2006, and 2007.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26–31, 2007, Monterey, California.

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Table 1ð Decline in number of plots showing *P. lateralis* mortality

	Plots with Infected Port-Orford-cedar
2004	21
2005	13
2006	9
2007	6

Table 2ð Decline in number of seedlings showing *P. lateralis* mortality:

	2004	2005ï 2007
Game Lake	63	21
Silver Creek	123	71

Phytophthora lateralis requires a live host to reproduce. Port-Orford-cedar mortality due to the Biscuit fire has diminished the live host pool. Other factors potentially affecting *Phytophthora lateralis* survival include the heat pulse of the Biscuit fire itself and subsequent warming and drying of infested sites due to loss of canopy and darkening of soil post fire.

In laboratory studies, Ostrofsky and others (1977) were unable to recover *P. lateralis* from infested organic matter after sixteen weeks at 25 °C (77 F). Hansen and Hamm (1996) showed *P. lateralis* survival is minimal in dry soil at temperatures greater than 40 °C (104 F). And DeBano, Neary, and Folliott (1998) estimated a heat pulse between 200 and 300 °C (392 and 572 °F) in high severity fire at one centimeter (0.4 inch) soil depth.

Preliminary indications are that the soil heat pulse and soil surface darkening from the Biscuit fire, Port-Orford-cedar fire mortality, and overall loss of canopy resulting in higher temperatures and lower relative humidities may be combining to create an unfavorable environment for persistence of *P. lateralis.*

There are additional factors that require further analysis:

1) The resistance effect: How does survival vary when comparing fall 2004 susceptible seedling survival versus fall 2004 resistant seedling survival?

2)The fire/time effect: How does survival vary when comparing spring 2004 susceptible seedling survival to fall 2004 susceptible seedling survival?

Management Implications: Results from the Biscuit fire monitoring may lead to the design and implementation of silvicultural and prescribed fire treatments that potentially could reduce or eliminate *P. lateralis* from currently infested sites. USDA Forest Service Port-Orford-cedar management has focused on reducing *P. lateralis* spread and resistance breeding. Little work has been done to remove the pathogen from sites where it currently exists. If *P. lateralis* eradication treatments can be developed and utilized routinely, the need for other management practices could be reduced in the long term.

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Operational Program to Develop Resistance to *Phytophthora lateralis* in Port-Orford-cedar (*Chamaecyparis lawsoniana*): the First Ten Years[1]

Richard A. Sniezko,[2] Leslie J. Elliott,[3] Everett M. Hansen,[4] Frank Betlejewski,[5] Donald J. Goheen,[5] Kirk Casavan,[6] Scott E. Kolpak,[2] Charles L. Frank,[7] Peter A. Angwin,[8] and Jim Hamlin[9]

Abstract

The USDA Forest Service and USDI Bureau of Land Management (BLM) operational program to develop populations of Port-Orford-cedar (POC) with genetic resistance to the non-native root pathogen *Phytophthora lateralis* has been underway since 1996. Earlier, smaller scale efforts focused on making a few hundred field selections from a portion of the native range and confirming the presence of genetic resistance. The Forest Service, BLM, Oregon State University and others have all played an important role in the rapid development of this resistance program. The Forest Service provides genetic and pathology expertise, coordinates and designs the overall development of the program and manages the containerized seed orchards. The Forest Service and BLM have supplied program funding and have made many of the field selections, and established field trials to test resistant families and delineate and validate breeding zones. Oregon State University provides research pathology expertise and oversees the large annual artificial inoculation and assessment of seedlings and rooted cuttings. Many other landowners have permitted tree selections from their lands or have provided sites for field resistance trials. Program goals include developing populations of resistant trees, maintaining genetic variation and adaptability, and producing resistant seed for reforestation and restoration.

Through 2006, over 12,500 field selections have been made in Oregon and California (fig 1). The first cycle of selection and testing (fig 2) is well underway. The field selections have been evaluated in the first phase of testing, using a stem dip inoculation test. Approximately ten percent of the parent trees in each stem dip trial are selected for a second phase of testing using a root dip inoculation test. Nearly a third of the stem dip selections have been root dip tested. In this second test phase, rooted cuttings or seedling progeny of a parent are inoculated and assessed for mortality to rate the resistance of the parent. In addition, root dip testing of controlled crosses (including selfs) is underway as part of the second cycle of selection and to examine the inheritance of resistance and to increase resistance.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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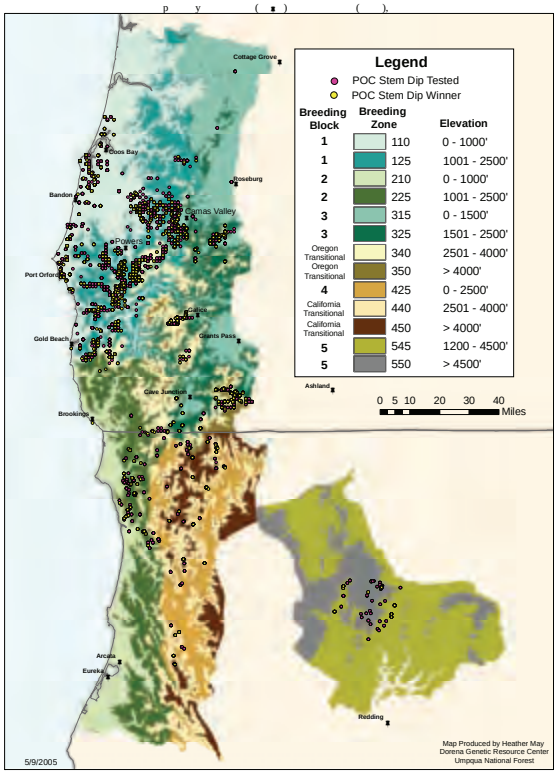

Figure 1—Geographic range of Port-Orford-cedar and location of field selections for testing for *Phytophthora lateralis* resistance.

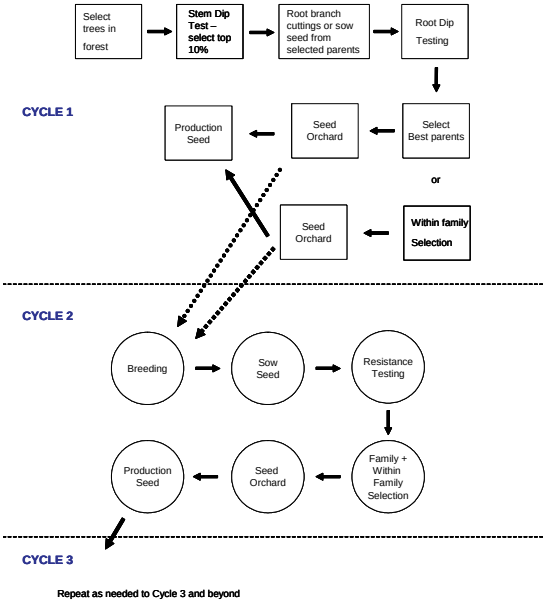

Figure 2—Overview of the steps in breeding cycles to develop *Phytophthora lateralis* resistance for Port-orford-cedar.

The native range of Port-Orford-cedar has been divided into 13 breeding zones (USDA Forest Service 2006), and screening for resistance is underway in all zones. For eight breeding zones, first-generation containerized seed orchards have been established using rooted cuttings from selected parents. Additional parent trees are needed to increase genetic diversity in most zones. Parent trees will be added or removed from orchards as additional test data become available. Since 2003, seed from several of these orchards has been utilized by federal and private landowners for restoration and reforestation. The top families show 40+ percent higher survival in short-term greenhouse testing than the most susceptible families (Sniezko 2004, USDA Forest Service 2006). Field tests have been established on over 30 sites, to validate the greenhouse and raised bed results, examine durability of resistance, and test the first orchard seed. Early results from these tests are encouraging (Sniezko and others 2006).

The POC program is one of the fastest moving resistance programs in forest trees (Sniezko 2006). In many areas, genetic resistance offers the main avenue for restoring POC in ecosystems with high mortality as well as keeping it as a component of reforestation for public and private landowners. An examination of some of the underlying mechanisms of resistance has been completed (Oh and Hansen 2007). At least two types of resistance seem to be present, and additional research is needed on the types and number of mechanisms of resistance and their inheritance (Sniezko 2004, 2006). Work in the next few years will be focused on completing the backlog of parent trees for root dip testing and adding trees to the containerized orchards. Increasing the level of resistance from the orchards and further study of resistance mechanisms and their inheritance will be undertaken as funding permits. Updates of the program are available at http://www.fs.fed.us/r6/dorena/publications/poc.

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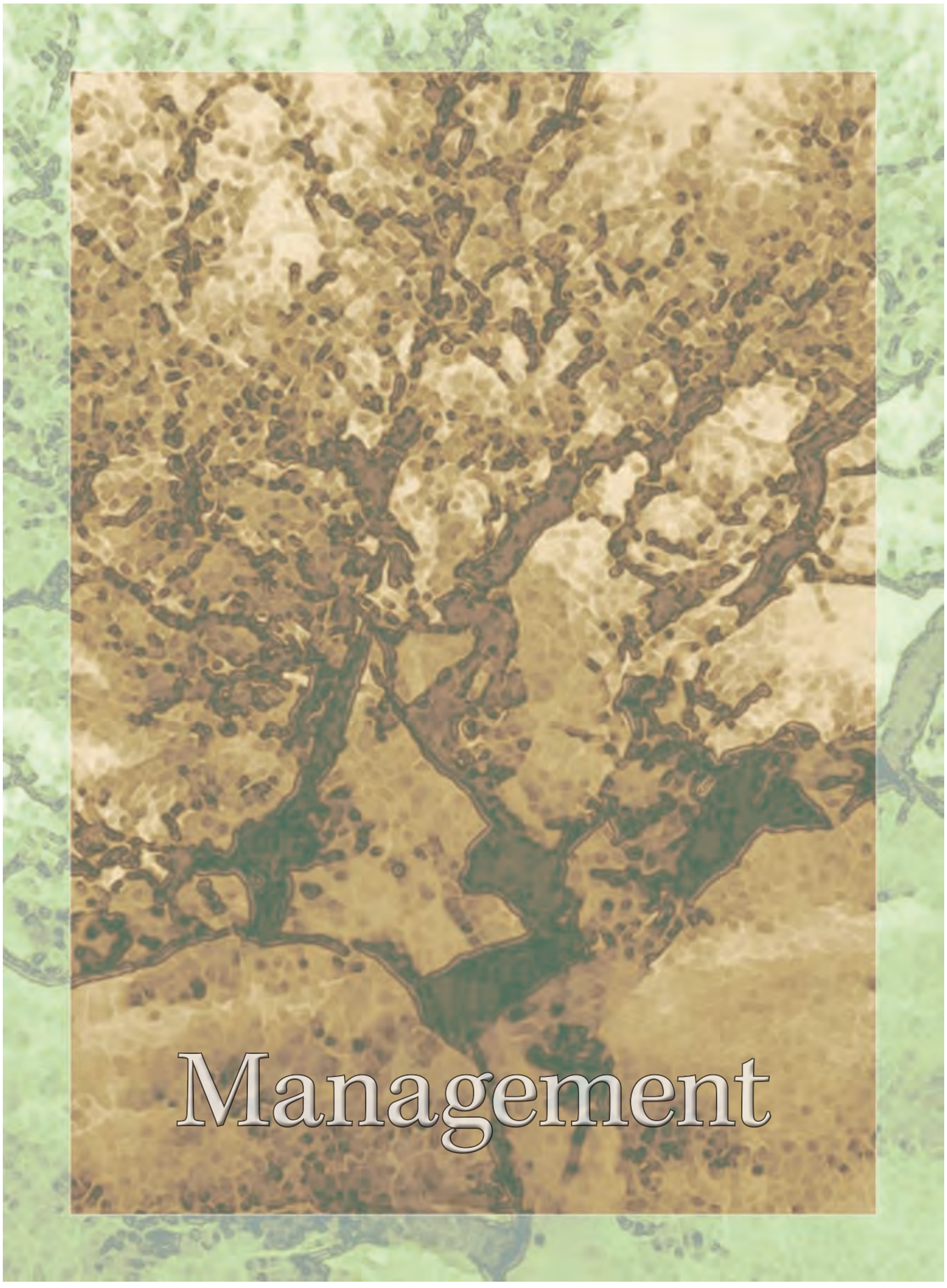

Management

Managing *Phytophthora* Species in the Forest: A Perspective[1]

Donald J. Goheen[2]

Introduction

The main responsibility of the Forest Health Protection organization in the United States Department of Agriculture, Forest Service, is to provide assistance on insect and disease concerns and issues to federal forest managers and cooperators. The Southwest Oregon Forest Insect and Disease Service Center covers an area particularly noted for having a large number of management problems associated with forest *Phytophthora* spp., and in fact, we probably have more of these to deal with than any similar-sized area in the United States. The six person staff of the Southwest Oregon Forest Insect and Disease Service Center spends roughly 60 percent of its time working on *Phythophthora* related activities. Major *Phytophthora* issues in Southwest Oregon are Port-Orford-cedar root disease, sudden oak death, golden chinquapin decline, *Phytophthora*–caused sugar pine mortality in the Sprague Seed Orchard, *Phytophthora* root diseases at the J. Herbert Stone Nursery, and *Phytophthora* diseases at the Dorena Genetics Resource Center.

Port-Orford-cedar Root Disease

Port-Orford-cedar root disease affects Port-Orford-cedar (*Chamaecyparus lawsoniana*), a unique and valuable tree species with a very limited natural range in southwestern Oregon and northwestern California. The disease is caused by *P. lateralis*, a non-native pathogen first reported in the Seattle, Washington area on nursery stock in 1923. It was introduced into the native range of the host in 1952. Long distance spread of *P. lateralis* involves chlamydospores that are carried in mud and soil adhering to machinery, vehicles, animals, and humans. Once the pathogen has been introduced into a new area, spread via motile zoospores in water becomes important. Port-Orford-cedar root disease is very damaging and causes substantial levels of host mortality on certain kinds of sites. Cool wet conditions greatly favor infection. High risk sites are the vicinities of streams and ditches, low-lying places, and poorly drained areas that are down slope from existing infested areas or below roads where new introductions can occur. In attempting to manage Port-Orford-cedar root disease, the objective of federal foresters is to maintain Port-Orford-cedar as an ecologically and economically significant species on Forest Service and Bureau of Land Management lands. Techniques used in management include: excluding vehicle entry into yet uninfested areas, road closures (either permanent or temporary), roadside sanitation treatments, vehicle washing (especially before traveling from infested to uninfested areas), operations planning and scheduling to minimize movement of vehicles and equipment from or through infested areas to uninfested

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areas during all kinds of projects, timing access into stands with Port-Orford-cedar during dry warm weather, selection of healthy or treated water sources for use in fire suppression and dust abatement, featuring Port-Orford-cedar on sites with characteristics unfavorable for establishment and spread of the pathogen, regulating harvest of non-timber forest products, public education, and development and use of resistant Port-Orford-cedar planting stock (host resistance enhancement appears to be particularly promising with Port-Orford-cedar). Management prescriptions involve combinations of the above mentioned approaches in integrated strategies.

Sudden Oak Death

In Southwest Oregon, *P. ramorum* kills tanoak (*Lithocarpus densiflorus*) and, occasionally, California black oak (*Quercus kelloggi*). The pathogen also causes leaf and tip damage on a variety of other species, most commonly Pacific Rhododendron (*Rhododendron macrophylum*) and evergreen huckleberry (*Vaccinium ovatum*). *P. ramorum* was first detected in Oregon in 2001 and now occurs at scattered locations within an approximately 6000 hectare area near the coastal town of Brookings. How the pathogen was introduced into Oregon is unknown. Since its discovery, the objective of forest managers has been to eradicate *P. ramorum* from Oregon forests. This has involved a major cooperative effort by state of Oregon and federal agencies. There is an intensive on-going monitoring and survey program that includes aerial and ground surveys and water baiting in streams to detect the pathogen. As soon as a new infection center is confirmed, it is treated by eliminating all hosts within the center and a buffer area. This usually involves cutting, herbicide treatments, and burning. Federal and state quarantines are in place to prevent movement of infected plant material and soil from the entire area where infections occur. There is also a substantial public education program. To date, management treatments have not succeeded in eradicating *P. ramorum*, but they do appear to have been successful at keeping the pathogen in a fairly small area and greatly limiting the amount of damage it causes relative to that in areas in other regions where no treatments have been done.

Golden Chinquapin Decline

A very noticeable decline of golden chinquapin (*Chrysolepis chrysophylla*) has been observed in a number of locations in Southwest Oregon mostly since 2000. Affected host trees develop root necrosis, substantial cankers on their lower boles, and chlorotic crowns. They soon die. Most impacts are noted on hosts growing close to roads and in poorly drained locations. Recent work at Oregon State University (Saavedra et al. 2007) demonstrated that golden chinquapin decline is caused by *P. cambivora*. The origin of the pathogen on this host in Southwest Oregon is not known, but it probably represents another *Phytophthora* introduction, at least at a local level. Though it is a common and widely distributed tree in Southwest Oregon and one that may be of substantial importance in providing food for a number of wildlife species, golden chinquapin is not a significant timber tree. Concern about golden chinquapin decline among forest managers is just beginning to develop. Current objectives are to determine the distribution and potential severity of the disease, develop an understanding of the ecology of the causal pathogen, and identify possible techniques that could be used if management is deemed appropriate.

Phytophthora-caused Sugar Pine Mortality in the Sprague Seed Orchard

The Sprague seed orchard located near Merlin, Oregon was intended to provide seed for regeneration of sugar pine (*Pinus lambertiana*) on Bureau of Land Management and Forest Service lands throughout the northern portion of the species range. A fast-growing and very valuable tree, sugar pine is the largest of all pines and has a wide ecological amplitude. It is greatly desired for management. Trees at the Sprague Orchard represent tested families that are resistant to white pine blister rust, caused by the fungus *Cronartium ribicola* an extremely virulent exotic pathogen of five-needle pines including sugar pine. Unfortunately sugar pine orchard trees at Sprague have experienced substantial mortality due to infection by *P. cryptogea* which was apparently introduced on the site in water used for summer irrigation shortly after the orchard was established. The pathogen subsequently was spread widely across the orchard in water and via equipment. Approximately 4,000 of the high value orchard trees have been killed since 1992. Clearly, the objective of orchard managers is to ensure survival of the valuable *C. ribicola*-resistant sugar pine seed trees, but suggested management recommendations have not been rigorously followed. Recommendations included water treatment, limiting or suspending summer irrigation, avoiding planting of seed trees in low-lying portions of the orchard grounds, roguing infected trees, and employing chemical treatments where appropriate. These should have been incorporated into management from the time that the *Phytophthora* situation was recognized. The current plan is to phase out the Sprague Seed Orchard, transfer the *C. ribicola*-resistant families to distant uninfested sites using seed and cuttings from the Sprague Orchard and exercising great caution not to introduce *P. cryptogea* with them, and accept the disruption that this will cause in the timeline of the white pine blister rust resistance program.

Phytophthora Root Diseases in the J. Herbert Stone Nursery

The J. Herbert Stone Nursery is the main federal bare-root nursery facility for the northwestern United States. It supplies seedlings for use in tree planting throughout Oregon and Washington. The nursery is located in Southwest Oregon in the town of Central Point. A number of *Phytophthora* spp. have caused significant mortality of conifer seedlings, especially Douglas-fir (*Pseudotsuga menziesii*) and five-needle pines, in the nursery in the past. *Phytophthora* spp. identified at the J. Herbert Stone Nursery included *P. cryptogea*, *P. drechsleri*, *P. megasperma*, *P. pseudotsugae*, *P. gonapodyites*, and *P. citricola*. In attempting to deal with *Phytophthora* diseases at the nursery, the management objectives are to minimize losses of seedling crops and prevent spread of disease to field planting sites on infected seedlings. In the past, treatments involved irrigation water chlorination, fumigation of nursery beds, avoidance of planting in poorly-drained beds, and monitoring and culling of infected seedlings. This combination was quite successful. Unfortunately, the water chlorination system has been shut down for safety reasons and fumigation has been largely discontinued. Now most treatment involves concentrating planting in well-drained beds, spot-treatments with fungicides, and monitoring. There has not yet been a resurgence of *Phytophthora* spp. at the nursery, but the possibility is a

significant concern. Because the nursery is large (about 120 hectares) and the demand for conifer seedlings currently is quite low, nursery managers have leeway to plant seedlings on only the least hazardous, best drained portions of the nursery and also do not have to plant the same beds repeatedly without crop rotation or fallowing. Constant vigilance is practiced in monitoring seedling crops for evidence of disease.

Phytophthora Diseases at the Dorena Genetic Resource Center

The Dorena Genetic Resource Center is responsible for developing disease resistant conifers for federal land management agencies and cooperators throughout Oregon and Washington. It has extensive, very important screening and breeding programs aimed at developing stock resistant to such diseases as white pine blister rust and Port-Orford-cedar root disease. Unfortunately, scattered unexpected occurrences of *Phytophthora* diseases have been detected at the Dorena Center. Several species including *P. citricola*, *P. gonapodyidaes*, *P. pseudotsugae*, *P. nicotianae*, *P. lateralis*, and *P. cinnamomi* have been identified. The last two are especially worrisome. The objectives of the managers of the Genetic Resource Center are to completely eliminate impacts of *Phytophthora* diseases on their resistance testing and breeding programs, and prevent loss of test trees, breeding stock, and containerized seed orchard trees. So far, *Phytophthora* diseases have been detected on few trees in very limited areas and have been immediately treated when found, but managers recognize that with a program like theirs, it is critical to have zero tolerance for any additional introductions of pathogenic *Phytophthora* spp. Current management strategies include development of a water treatment plant, stringent sanitation efforts (including improvement of growing and storage facilities), strict measures to prevent additional introductions on equipment, vehicles, and personnel, and constant vigilance by Center employees and Forest Service and Oregon State University pathologists.

Concluding Comments

Based on our experience with the above mentioned forest *Phytophthora* issues, the staff of the Southwest Oregon Forest Insect and Disease Service Center has developed a list of what we consider to be some of the significant challenges associated with management of forest *Phytophthora* spp. In no particular order they include:

- Many of the *Phytophthora* diseases that affect forest trees involve non-native, invasive *Phytophthora* spp. This has substantial implications regarding unexpected, rapidly developing impacts, difficulties in detecting and identifying the pathogens when they are first introduced, lack of prior knowledge of pathogen biology and ecology, initial lack of knowledge of potential management approaches and techniques, and hosts that have not evolved with the pathogens and thus have very little genetic resistance. Management campaigns against non-native pathogens are most successful when detection and response are extremely rapid but this is often more easily said than done. Once a new *Phytophthora* is established, eradication is very difficult and expensive. It would be so much preferable to prevent non-native species from being introduced into our forests in the first place.

- Many of our *Phytophthora* issues have involved various kinds of facilities such as nurseries, seed orchards, and breeding centers. Given the large concentrations of hosts in such places, *Phytophthora* diseases have great potential to intensify, cause substantial damage, and possibly be spread from such facilities to other areas, including the forest if they are not managed correctly. Suites of several *Phytophthora* species have often been found in facilities. Facilities can potentially be places where hybridization occurs. Facility managers need constant education on the dangers of *Phytophthora* spp. (as well as other pathogens). There is room for significant improvement in considering *Phytophthora* risks in the planning of facility location, layout, and management regimes.

- Spread of forest *Phytophthora* species involves water, roads, and the air, not easy avenues to manage.

- The commitment of administrators and higher level resource managers to addressing *Phytophthora* diseases is frequently less than we might like and response is all too often not as rapid as would be desirable. Our managers are probably typical of forest managers everywhere. They want to do the right thing but they are generally quite conservative and risk averse. Many also don't understand *Phytophthora* spp. They need to learn more but often feel that they don't have the time. We've found that managers can be very impressed by well-done risk assessments and impact models. Close cooperation among a number of managers or even agencies is important for some treatments. The good cooperation that we've enjoyed so far with the sudden oak death eradication program in Oregon is a model for such projects.

- The American public owns the public lands managed by the federal government in the United States and makes use of them for many, often highly diverse activities. Public users can have a significant role in spread of some forest *Phytophthora* spp. (such as *P. lateralis*). They also have major (and sometimes counterproductive) opinions on forest management policies. Many in the public have little or no knowledge of *Phytopthora* spp. Public education and information transfer about these pathogens is very important.

- Costs of *Phytophthora* spp. management activities are often high (and are almost always perceived as very high by managers).

- Management activities for *Phytophthora* diseases may appear to conflict with other forest objectives. For example, clearing to attempt to eradicate *P. ramorum* may be viewed as negatively impacting some kinds of important wildlife habitat. It is essential to communicate well with other resource specialists in all *Phytophthora* management strategies.

- Use of various chemicals (fungicides, resistance enhancers, herbicides) can be very desirable in some kinds of *Phytophthora* management strategies. Unfortunately in the United States, use of chemicals on federal forest lands is viewed with suspicion and fear and is actively opposed by sectors of the public. What can be done to improve this situation?

- The importance of accurate monitoring and good quality, relatively frequent surveys for keeping track of forest *Phytophthora*-caused diseases cannot be overstated. Developing good designs, having well-trained surveyors available, and paying for surveys and monitoring efforts can be challenging.

- *Phytophthora* spp. can be complex and difficult to identify and work with even for trained plant pathologists. Pathologists working with these pathogens need to have laboratory facilities. Diagnostics in some cases require rather sophisticated laboratory equipment. Extension pathologists working with forest *Phytophthora* spp. are well advised to develop relationships with Universities, other research organizations, and *Phytophthora* specialists. We have benefited greatly from our association with Oregon State University.

- There is a great need to support quality research on forest *Phytophthora* spp. There also must be flexibility so that research on newly emerging forest *Phytophthora* issues and newly introduced forest *Phytophthora* spp. can be undertaken when needed. There aren't enough research facilities that can do work on forest *Phytophthora* spp.

- Global climate change has the potential to greatly impact the ecology of pathogenic forest *Phytophthora* spp. and affect possible management strategies for them. What will be the ramifications of predicted changes? How can we prepare ourselves to deal with them?

Port-Orford-cedar Family Variation in Resistance to *Phytopthora lateralis*: Greenhouse Test of 125 Families[1]

Richard A. Sniezko,[2] Everett M. Hansen,[3] Angelia Kegley,[2] Scott E. Kolpak,[2] and Paul Reeser[3]

Abstract

Artificial inoculation of young Port-Orford-cedar (*Chamaecyparis lawsoniana*) seedlings in containers provides a method of potentially evaluating hundreds of seedling families or rooted cuttings over a relatively short time period for resistance to the root pathogen *Phytophthora lateralis*. This method has now been used as a tool for more than ten years in an operational screening program to select resistant trees to establish seed orchards.

The trial reported here evaluates 125 seedling families of Port-Orford-cedar (POC) inoculated with *P. lateralis* in February 2005. The seedlots were a mixture of control crosses (full-sib and selfs) and open-pollinated half-sib families from 88 parents (87 unrelated). The seedlots represent a mixture of untested parents and previously tested parents. Seedlings were approximately 11 months old at time of inoculation, and were grown in 164 cm^3 tubes in soil-less medium. Families were arranged in seven blocks in a randomized complete block design, generally with seven trees per family in each block. A mixture of two isolates of *P. lateralis* was used to inoculate the roots. Seedlings were kept in the greenhouse after inoculation.

Mortality of seedlings has been assessed thirteen times through January 30, 2007 (approximately 24 months after inoculation). Dead trees were recorded by date of assessment. A seedling was considered 'dead' when a stem lesion was evident around the stem above ground level following scraping of the bark. The first symptom was often wilted or slightly off-color foliage. Mean trial mortality was 54.1 percent at 12 months and 60.3 percent at 24 months after inoculation. Families ranged in mortality from 0 to 100 percent. Several previously well documented resistant and susceptible parents performed as expected, showing low, moderate, and high levels of mortality.

Rates of mortality varied widely for families with high levels of mortality. Of the three families with 100 percent mortality, two reached 100 percent mortality within 130 days from inoculation, while the third family took 217 days. Traditionally, mortality was assessed for only up to 12 months in our trials. However, the longer trial period here appears to separate families with moderate levels of mortality and putative major gene resistance from those that are 'slow dying'. There may be at least two types of resistance, and this separation will allow us to classify families for selection and breeding. Field trials are underway to ascertain the utility of both types of resistance. In addition, future greenhouse trials will examine the repeatability of the 'slow dying' type of resistance.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Phytophthora ramorum in Oregon Forests: Six Years of Detection, Eradication, and Disease Spread[1]

Alan Kanaskie,[2] Everett M. Hansen,[3] Ellen Michaels Goheen,[4] Michael McWilliams,[2] Paul Reeser,[3] and Wendy Sutton[3]

Abstract

Phytophthora ramorum was first discovered in Southwest Oregon forests in 2001, where it was killing tanoak (*Lithocarpus densiflorus*) and infecting Pacific rhododendron (*Rhododendron macrophyllum*) and evergreen huckleberry (*Vaccinium ovatum*). At that time there were nine infested forest sites totaling 16 ha. Aerial photographs suggest that *P. ramorum* probably was killing tanoak trees at one of these sites as early as 1998. In fall of 2001 eradication of the pathogen from infested sites began by cutting, piling and burning infected plants and all host vegetation within 30 to 100 m. of infected or symptomatic plants. Upon completion of burning most sites are planted with non-host or conifer seedlings. After one year it was clear that tanoak stumps sprouted prolifically following treatment and that *P. ramorum* occasionally was recovered from these sprouts. Follow-up treatments were necessary to destroy residual host material and stump sprouts that potentially harbored the pathogen. In 2003 and subsequent years all tanoaks in treatment areas were injected with herbicide prior to cutting in order to prevent sprouting (except on USDI-BLM lands where herbicide use was banned). Eradication treatments have been completed or are underway on approximately 560 ha of forest land, at a cost of $2.6 million dollars. Nearly all eradication costs have been paid by federal and state agencies, with much cooperation by landowners. There is no compensation to landowners for loss of timber or other values as a result of the eradication treatments.

Early detection surveys are conducted year-round using a combination of aerial surveys, ground surveys, and stream water baiting with rhododendron and tanoak leaves. During the first 4 years of the eradication effort (2001-2004), the number of new infested sites and infected trees decreased each year, suggesting modest success at containment and eradication. That trend ended in 2005. In 2006 and 2007 the extent of the disease increased considerably. In 2007, six new sites were found outside of the quarantine area, and several of these sites were 3 to 4 km from the nearest other infested site (the quarantine area will be enlarged in 2008). Most of the other new sites were small (less than 0.2 ha) and scattered near the center of the quarantine zone. We attribute this disease expansion to consecutive years of unusually wet spring and early summer weather which appears to favor long distance spread of *P. ramorum*. All surveys to date have failed to detect the pathogen anywhere in Oregon forests except in or very near the 65 km^2 Curry County quarantine area (figs. 1 and 2). The nearest known SOD infestation outside of Oregon is 200 km to the south in southern Humboldt County, CA.

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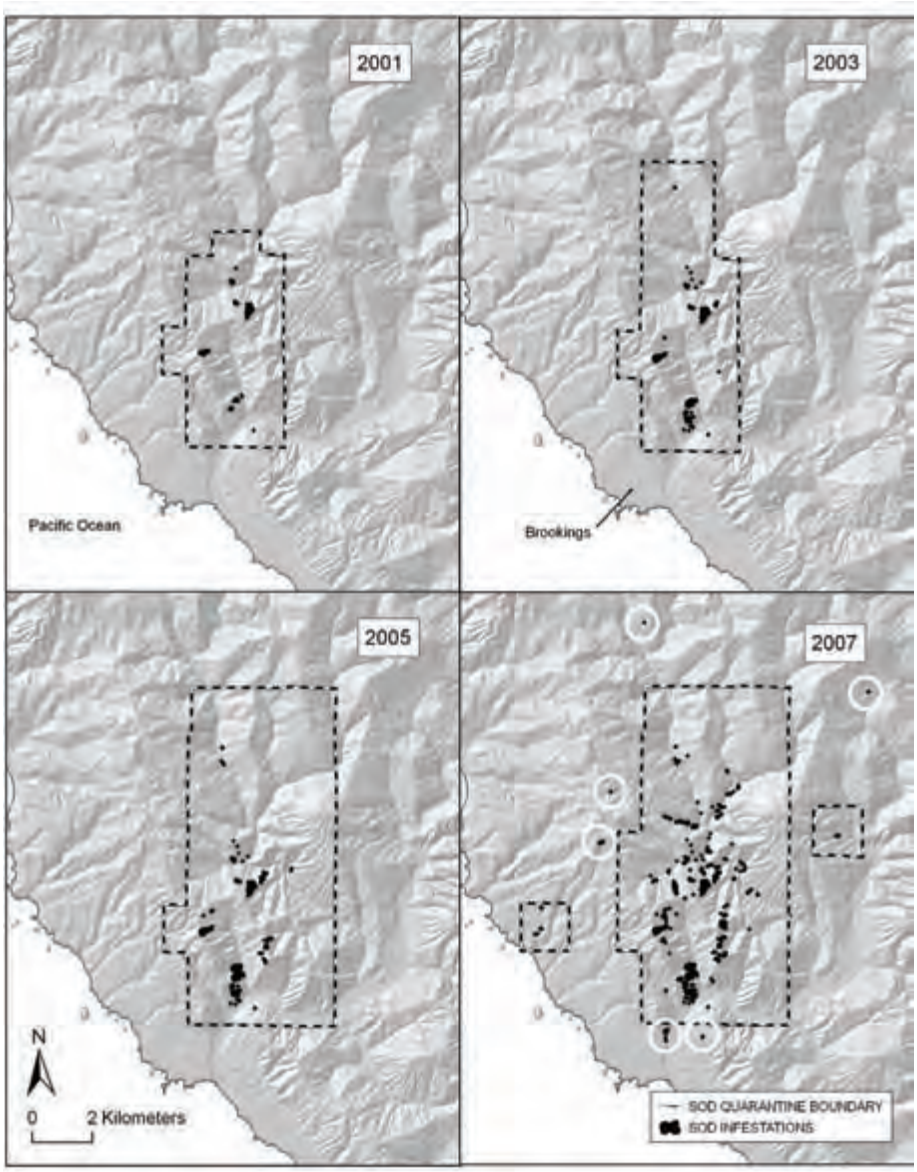

Figure 1—Expansion of sudden oak death in Oregon forests: 2001 2003, 2005, and 2007. Black blotches indicate infested areas, all of which have been treated in an effort to eradicate the pathogen. Dashed line is the *P. ramorum* quarantine boundary. White circles highlight 2007 infestations outside of quarantine boundary. Curry County, Oregon.

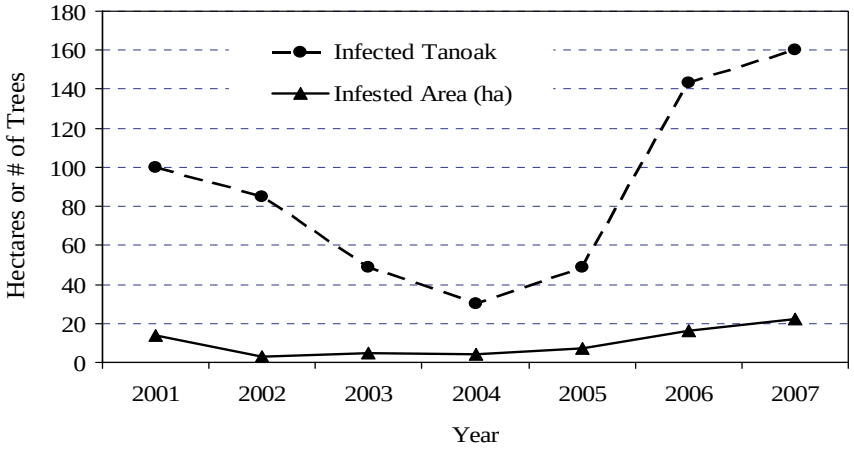

Figure 2—Number of new infected tanoaks and the area of new infestations each year in southern Curry County, Oregon.

Each year most new infested sites occurred very close to previously discovered infested sites. Post-treatment surveys indicate that disease is absent on most sites and that the pathogen, when it is present, can be recovered only at extremely low frequencies from soil and host plants. It is unlikely that new infestations in the adjacent forest occur because the pathogen survived the eradication treatments. It is more likely that the new infestations occur because the pathogen has already spread to these areas but was not detectable in our surveys. Future success of the program will require earlier detection of infected plants, reduction in the time between detection and treatment, and larger treatment areas.

The net effect of the eradication program thus far has been to eliminate the pathogen from some sites and greatly slow spread of *P. ramorum* in Oregon forests. When comparing expansion of the Oregon infestation (aggressive eradication program) to a similar area in Humboldt county California (no comprehensive control program), it is clear that the disease can expand rapidly in the absence of control measures (fig. 3). It is equally clear that containment and eradication of *P. ramorum* from Oregon forests will require a much more substantial and aggressive program of early detection and treatment.

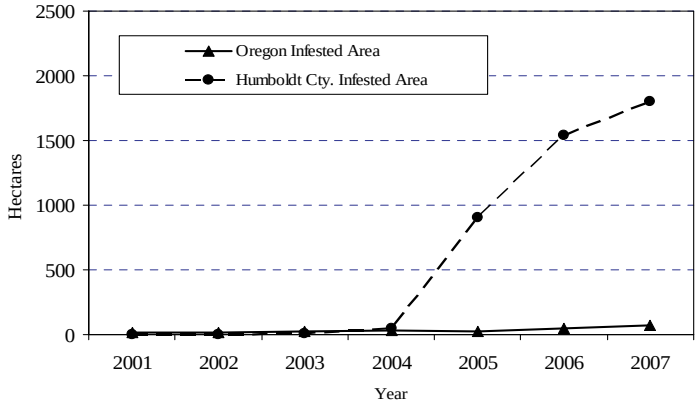

Figure 3—Comparison of the expansion of sudden oak death infestations in Curry County OR and Humboldt County, CA.

Persistence of *Phytopthtora ramorum* After Eradication Treatments in Oregon Tanoak Forests [1]

Ellen Michaels Goheen[2], Everett Hansen[3], Alan Kanaskie[4], Wendy Sutton[3], and Paul Reeser[3]

Abstract

Sudden oak death, caused by *Phytophthora ramorum*, was identified in late July 2001 in forest stands in Curry County on the Southwest Oregon coast where it was killing tanoak (*Lithocarpus densiflorus*) and infecting Pacific rhododendron (*Rhododendron macrophyllum*) and evergreen huckleberry (*Vaccinium ovatum*). Affected stands occurred on industrial forest land, non-industrial forest land, and federal forest land. Treatments to eradicate the pathogen from affected sites were started in the fall of 2001 and consisted at that time of cutting, piling, and burning all infected host vegetation and any known Oregon host species within a 15 to 30 meter buffer around all infected plants. While a number of plant species on the official host or associated host lists occured in Oregon forests, only those plant species that had a history of being infected in Oregon were treated. Patch size of the initial treatment areas ranged from 0.2 to 4.5 hectares. Since that time, additional disease centers have been identified and eradication treatments have been completed at every site. Some treatments were adjacent to sites treated previously while others involved distinct new centers. Size of treated sites has varied widely. Over the last five years, treatment methods have been altered to reflect increased understanding of host susceptibility and pathogen survival and spread. Additional treatment components have included various combinations, where possible, of backpack herbicide spraying to kill stump sprouts, stump-top treatments with herbicides to prevent tanoak sprouting, injecting all tanoaks greater than 2.5 cm diameter with herbicides to prevent sprouting, raking, piling, and burning all Oregon host material, and increasing buffer width to 100 meters. Some sites have been planted with conifer seedlings while others have not.

The end result is a mosaic of treatment patch sizes, shapes, and structures including sites where 1) Douglas-fir *(Pseudotsuga menziesii*) logs were removed from the sites and the tanoaks and understory species were cut and destroyed in broadcast burns, 2) sites where 10 to 20 percent of the conifer overstory remains and the treatment area has been replanted with conifer seedlings, 3) a site where little change has occurred in an old-growth coast redwood (*Sequoia sempervirens*)/Douglas-fir overstory but more than 90 percent of the midstory and understory trees and shrubs were removed, and 4) small (less than 0.2 ha) openings with non-host mature alder trees left on site.

Most affected stands in Southwest Oregon occur in the Douglas-fir/Tanoak forest type and have an original overstory component comprised predominantly of tanoak and Douglas-fir, with lesser amounts of red alder (*Alnus rubra*), Oregon myrtle (*Umbellularia californica*), cascara (*Frangula purshiana*), Pacific madrone (*Arbutus menziesii*), and bigleaf maple (*Acer macrophyllum*). In 2006, an infested site in a Coast Redwood/Tanoak forest type was

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confirmed and subsequently treated. On that site, Douglas-fir and coast redwood make up the overstory and tanoak was the predominant component of the midstory prior to treatment. The understory species mix in both forest types treated includes substantial amounts of tanoak, Pacific rhododendron, Oregon myrtle, and evergreen huckleberry, as well as a number of other shrub and herb species. Many of the species in both the overstory and understory produce prolific stump sprouts following cutting or burning.

Treated sites are being monitored for the presence of the pathogen using a variety of techniques including stream-water baiting with tanoak and rhododendron leaves, soil sampling and baiting using pear and leaf baits, and collecting vegetation samples on site and subjecting them to isolation and PCR-based diagnostic tests. While a few of the sites treated initially are now evidently free of the pathogen, on many sites *P. ramorum*, is still present in the soil or can be baited from streams flowing through treatment areas.

There is also a strong interest in monitoring both planted and natural vegetation on these sites. This interest stems from the desire to quantify and qualify how vegetation responds to treatment protocols, how vegetation responds in the presence of the pathogen, and whether the resulting vegetation structure and composition on the sites following treatments is consistent with overall landowner objectives.

A series of variable-radius and fixed area plots designed to sample between 5.0 to 7.5 percent of the treated areas were established to describe vegetation condition before and after eradication on a selection of sites. Trees greater than 13 cm dbh were accounted for in variable-radius plots. Trees less than 13 cm dbh were tallied and percent cover of shrub species was estimated on 0.02 ha circular plots. In some cases, pre-eradication conditions were determined from plots just outside treated areas but in similar forest types. Plants on plots and on transects between plots were examined for symptoms of *P. ramorum* infection. ELISA tests using field diagnostic kits were used to prescreen some symptomatic tissues for *Phytophthora* species infection. Most suspicious samples were sent to the laboratory at Oregon State University for identification.

Vegetation data five years after treatment show little change in overall species composition on treated sites, with the obvious exceptions that the overstory tanoak component on treated sites was eliminated in all cases, and on two sites, conifers not present before treatment were planted after treatment. In situations other than industrial forest lands where merchantable Douglas-firs within treated sites were cut and logs were removed to a nearby mill, conifers such as Douglas-fir or coast redwood, or non-host hardwoods such as red alder were left standing on sites. There were substantial changes in the percent cover occupied by understory tanoak, evergreen huckleberry, and Pacific rhododendron after treatment; however, these species were not entirely eliminated on any treated site. Tanoak sprouts were sometimes missed in post-treatment herbicide sprays. Evergreen huckleberry and Pacific rhododendron were not routinely subjected to herbicides and many sprouted back after cutting. Percent cover of sword fern (*Polystichum munitum*) generally increased after treatments. No samples collected on plots or along transects between plots were found to be infected by *P. ramorum*.

Early eradication prescriptions involved only the cutting and burning of infected and buffer host plants. When resprouting tanoaks and evergreen huckleberry plants on several sites were confirmed to be infected, herbicide treatment to kill stump sprouts was immediately done. In 2003, three-meter-diameter plots on five of the treatment areas were located around 43 stumps resulting from the cutting of known infected trees. Douglas-fir and redwood seedlings were deliberately planted as *P. ramorum* susceptible baits around each of the stumps on some sites while on others, seedlings planted as a part of post-eradication reforestation were used as bait. Sprouting tanoak, Pacific rhododendron, and evergreen huckleberry were also monitored for infection. These stump-based plots have been revisited and vegetation condition of all host plants on each plot has been reassessed several times since the initial plot installation, with the most recent evaluation done in January 2007. Since the initial identification of infected tanoak

and evergreen huckleberry sprouts, no vegetation on these plots, planted or natural, has been found infected.

In May 2003 soil samples were collected at the base of the selected stumps and baited for the presence of *P. ramorum*. Soil sampling at these stumps has continued through the present on most sites. *Phytophthora ramorum* has been recovered from soil samples periodically since 2003 at the base of selected stumps on three of the sites, was recovered only in the initial sampling on one site, and was never recovered from soil samples on one site (Table 1)

Table 1ð Number of samples where *Phytophthora ramorum* was baited from soil collected adjacent to stumps of previously infected trees at five eradication treatment sites in Curry County, Oregon

Site	Sample date				
	5/2003	7/2004	12/2004	6/2005	1/2007
10	2/8*	0/8	0/7	0/8	0/8
11	0/5	0/5	0/1	1/4	0/3
18	1/8	0/8	1/8	3/8	2/8
33	3/17	0/5	7/16	4/17	8/13
36	0/5	0/5	Not sampled	0/5	Not sampled

* Number of stumps where *P. ramorum* was recovered from adjacent soil/Total number of stumps sampled at the site

Baits consisting of Pacific rhododendron and tanoak leaves placed in mesh bags and floated in streams running through treated sites are used to monitor the presence of *P. ramorum.* Recovery of *P. ramorum* from leaf baits in these streams varies from site to site and by sampling period. On some sites, *P. ramorum* can still be recovered from stream water several years after initial treatment. However, recovery success is greatest on those sites where the pathogen appears to still be active (where new infections adjacent to treated areas have been confirmed). No new infections have been observed that would appear to be associated with downstream, waterborne movement of the pathogen or other stream related factors.

After herbicide injection to prevent resprouting of tanoak was incorporated into eradication treatment prescriptions on selected sites, monitoring to determine if the pathogen was present and sporulting in tanoak canopies and slash pies was instituted. Tanoak and Pacific rhododendron leaf baits are floated on distilled water in open plastic bags suspended in buckets. Buckets have mesh lids to prevent through-flow of leaves and debris and rainwater is captured in the buckets after it flows through the canopy. Over the course of monitoring several sites in several years these rainwater capture buckets have been placed under known infected green tanoaks, known infected herbicide-treated tanoaks, healthy herbicide-treated tanoaks, slash piles with infected leaf material incorporated, under trees on the perimeter of treated areas, under healthy tanoaks, and out in the open (no canopy). While inconsistent from sampling period to sampling period and site to site, *P. ramorum* has been recovered in an bait buckets in all situations except undwhen buckets are placed under healthy “control” tanoaks or out in the open.

Conclusions from monitoring soil, water, and vegetation response to *P. ramorum* eradication treatments in Southwest Oregon five years after treatment include the following:

- *Phytophthora ramorum* can still be recovered from treated sites approximately five to six years after treatment.
- New infections resulting from stream water are not apparent.
- Most components of the native flora found in Douglas-fir/Tanoak and Coast Redwood/Tanoak ecosystems are essentially intact on eradication sites after

treatment, excepting tanoak which has been removed from the overstory and midstory of all treated sites.

- Openings create conditions apparently unfavorable for *P. ramorum*. No new infections have been found on host species regenerating or surviving within treated areas. This includes host species in close proximity to known infested stumps or where soil remains infested.
- Landowner objectives for growing conifer species are not compromised. Planted Douglas-fir and coast redwood seedlings/saplings associated with known infested stumps and in areas where soil remains infested are healthy.

Vegetation monitoring on treated sites will continue.

Management of *Phytophthora kernoviae* and *P. ramorum* in Southwest England[1]

Joan F. Webber[2]

Abstract

Phytophthora ramorum and *P. kernoviae* are considered to be two of several invasive tree Phytophthoras recently arrived in Britain. Both have established to some extent and are found in planted ornamental woodland-gardens and woodlands where rhododendrons (mainly *Rhododendron ponticum*) dominate. The infected foliage of rhododendron supports abundant sporulation by both these aerial Phytophthoras and there is little doubt that this host has played a key part in the spread of these pathogens in the natural environment and subsequent infection of trees. Whilst *P. ramorum* has been found primarily in woodland-gardens, *P. kernoviae* has been found more often in mixed species woodlands with rhododendron as an understorey component. A key part of efforts to contain and eradicate both pathogens therefore centers on the removal of infected rhododendron because it acts as the primary inoculum source on most outbreak sites.

Much eradication and containment effort has focused on *P. kernoviae* because it has a very confined distribution (almost exclusively in south west England) and has also been found in four nurseries. However, the process of rhododendron removal on infested woodland sites can be very costly. It has been estimated that full-scale removal from infested woodland sites, just in Cornwall alone, could cost £millions. Therefore high priority sites for clearance are selected on the basis of several criteria including public access, plant movement (some woodland gardens have associated plant retail areas), numbers of infected rhododendron and the number of infected trees. Studies at both *P. ramorum* and *P. kernoviae* infested sites show that both pathogens may persist for many months in naturally infected foliage in the litter layer in woodlands. These results suggest that removal of infected rhododendron is just the first stage in any attempt to eradicate these pathogens, as they have the potential to persist for years and infect any re-growth of rhododendron that may occur.

Introduction

Phytophthora ramorum and *P. kernoviae* are considered to be two of several invasive Phytophthoras that have recently arrived in Britain (Brasier and others 2004) and pose a threat to trees and woodland ecosystems. Their origins are uncertain although *P. ramorum* is now well known as the cause of widespread mortality oak mortality in California (Rizzo and others 2005; Frankel, 2008). Since 2002, *P. ramorum* has also been reported from twenty European countries, primarily in nurseries, where it is usually found infecting the foliage of ornamental plants of the genera *Rhododendron*, *Viburnum* and *Camellia* (see European RAPRA project on *P. ramorum* at http://rapra.csl.gov.uk). This is in marked contrast to *P. kernoviae*

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which has only been reported from two countries: Britain (with records in England, Scotland and Wales) and New Zealand (Ramsfield and others 2008).

The first reports of *P. ramorum* in Britain came in 2002; initially the only plants affected were ornamental species and findings were limited to nurseries. Since then however, the pathogen has been found at more than 200 garden/woodland sites, mainly infecting *Rhododendron ponticum* or other rhododendron species and cultivars (Defra 2008). If the outbreak is widespread and severe on rhododendron, then many spores are produced and nearby trees can become infected and develop symptoms of stem bleeding, also known as bleeding canker (Brown and others 2006). In these circumstances, the tree species most likely to succumb to *P. ramorum* incited bleeding canker is *Fagus sylvatica* (European beech), although other species such as *Quercus cerris* (Turkey oak), *Q. rubra* (red oak) and *Nothofagus obliqua* (southern beech) have all proved to be susceptible and become naturally infected in the field (Webber 2008). Whilst surveying for *P. ramorum* in south west England in 2003, another new and unrelated species of *Phytophthora* was discovered, later named *Phytophthora kernoviae* (Brasier and others 2005). It soon became apparent that *P. kernoviae* was an aggressive aerial pathogen which also attacked the leaves and shoots of *R. ponticum* and then sporulated on the leaves. Furthermore, just as with *P. ramorum*, it could spread from rhododendron onto mature beech trees causing bleeding stem lesions. However, unlike *P. ramorum*, the majority of *P. kernoviae* findings have been in the natural or semi-natural environment with around 50-60 outbreaks sites identified (Defra 2008) and only a handful of findings in nurseries. Thus for both pathogens, the occurrence of rhododendron in gardens and woodlands has played an essential part in their spread into the natural environment and the subsequent infection of trees.

Outbreaks of *P. ramorum* and *P. kernoviae*

Climatic analysis, initially using the model CLIMEX (R. Baker, CSL; U.K.: RAPRA project http://rapra.csl.gov.uk) and then later revised based on Meentemeyer and others (2004), identified the western British Isles as most suitable for the establishment of *P. ramorum.* Essentially the climate of this region which is mild and tends to have high levels of precipitation appears to be very conducive for infection of aerial plant parts by this pathogen. The same environment also seems to be ideal for *P. kernoviae* with episodes of mild wet weather promoting infection and sporulation on susceptible foliar hosts.

The south west of England, particularly the county of Cornwall, has several features which appear ideal for the two species of *Phytophthora*: this includes high levels of precipitation, infrequent frosts and acidic soils which favour the growth of rhododendrons. Circumstantial evidence suggests that plant trade and the transportation of plants have accidentally introduced both pathogens to this area, and the combination of suitable climate and availability of epidemiologically important hosts has allowed them both to establish to a limited extent. Since the discovery of *P. kernoviae* in November 2003, it has been found extensively in an area of about 5.5 square miles in southern Cornwall between the towns of Redruth and Falmouth which is now defined as the *Phytophthora kernoviae* Management Zone or PkMZ (Anon. 2004). In addition, a small cluster of outbreaks in southern Wales and also in

Argyll, Scotland have been identified, and these areas have similar climatic, soil and vegetation characteristics to Cornwall.

Both *Phytophthora* pathogens are treated as quarantine organisms although their status differs. The risk that *P. ramorum* poses to the nursery trade and potentially to woodlands and other ecosystems within Europe resulted in the European Commission decision of 19 September 2002. This put emergency measures in place to counter further introductions of *P. ramorum* and prevent its spread. It requires eradication action whenever the pathogen is detected in nurseries, and either eradication or containment if it is found in natural or semi-natural environments. In Britain, similar measures are applied to *P. kernoviae*, but as most of the findings have been in Cornwall, the efforts at eradication and containment have centred on infested woodlands and woodland-gardens in this region. For both pathogens this process involves removal and destruction of rhododendron but this does pose a number of problems. In many of the woodland gardens where infestations occur and trees are infected by *P. ramorum* or *P. kernoviae*, the infected rhododendrons are often specimen ornamental plants and a valued feature of the gardens. In Cornwall alone there are 30-40 of these historic parks and gardens and collectively they cover more than 3,500 ha. In contrast, the woodlands which contain infected trees and understorey *R. ponticum* are frequently neglected and unmanaged, and therefore of low commercial value, but often have difficult terrain for rhododendron clearance activity as well as public access. There are also environmental constraints (no burning on site) which adds to the difficulty and expense of disposing of the infected material safely. Calculating the cost and impact of eradication action is difficult but it has been estimated it could be as much £8-10 million over a five year period to eradicate *Phytophthora* from within the PkMZ alone (Snowden and Thompson 2005).

Clearance of Infected Rhododendrons

The limited distribution of *P. kernoviae* in particular provides the opportunity to contain and possibly even eradiate this pathogen and prevent its spread throughout Britain as well as into plant trade pathways. However, in the absence of sufficient funding to undertake the complete eradication of all infected rhododendron, clearance is currently underway on affected sites that pose the greatest risk of pathogen spread. Thus each site is given a priority ranking for clearance based on specific available information. Key details which inform the selection of sites include:

- The amount of rhododendron cover in a woodland or wood-land garden and the proportion that is infected. Sites with many rhododendron bushes coupled with heavy infestations are considered most likely to act as infection reservoirs that allow escape of these invasive pathogens to new locations. The occurrence of infected trees on the site is a further indicator that the infestation is intense and of longer standing.
- The value of the site, particularly its status as a heritage garden or ancient semi-natural woodland.
- The potential for both pathogens to transported from the site, possibly through plant sales of susceptible species from an on-site retail outlet or by plant exchanges.

- Amount of public access to the outbreak area, either as a tourist attraction in the case of the heritage woodland gardens or via public footpaths and use for dog walking and recreation. Evidence has shown that human activity can spread *P. ramorum* and *P. kernoviae* (Cushman and others 2008; Webber and Rose 2008).
- The presence of water courses on the site, as water is known to facilitate the movement of Phytophthoras. Many streams, water courses or ponds have tested positive for these pathogens on infested sites in Britain.

Sites which meet most or all of these criteria and thereby pose the greatest risk (for example, those with heavy infestations combined with high levels of public access and plant sales) are scheduled for rhododendron removal as quickly as possible. If the whole site cannot be cleared immediately, then areas around footpaths and public access are cleared as part of disease management. In infested woodland the process not only includes the removal of all infected and potentially infected rhododendron bushes, but clearance of all the fallen litter which often contains infected rhododendron leaves and could be a source of pathogen inoculum in future. In addition, re-growth of *R. ponticum* often occurs from the cut stumps even after herbicide treatment, so repeat herbicide treatments are usually needed (Edwards 2006).

Impact of Rhododendron Removal on Disease Development

In the case of *P. kernoviae*, extensive monitoring of some infested woodlands within the PkMZ has been undertaken before and after clearance of the infected rhododendrons. It has concentrated on two small woodland sites, in which 25 percent and 17 percent of the total beech tree population has become infected with *P. kernoviae.* The presence of *P. kernoviae* in soil and litter layers was assessed just prior to clearance around the infected trees, as these were the areas with the most heavily infected rhododendron. Testing methods involved baiting the samples of litter and soil and then isolation on Phytophthora selective medium. One and three years after the clearance of the rhododendron understorey, the same locations in the woodland were again tested for the presence of *P. kernoviae* (fig. 1).

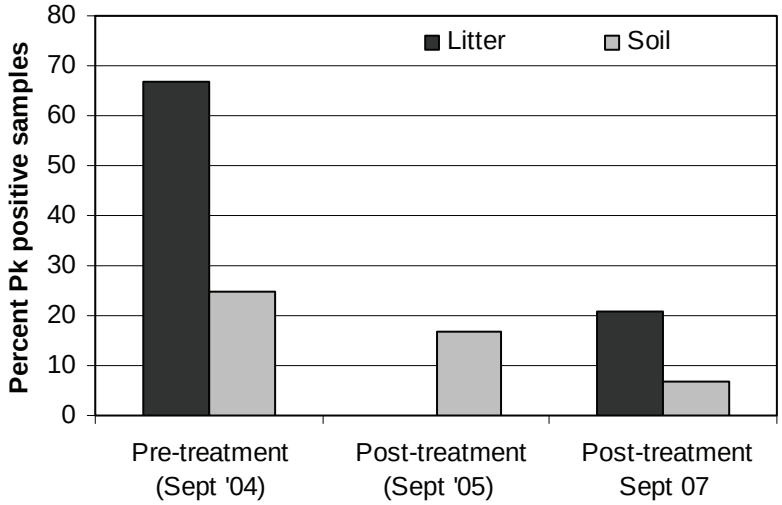

Figure 1—Outcome of soil and litter sampling pre- and post- *Rhododendron ponticum* removal in a *Phytophthora kernoviae* infested woodland (September 2004 and 2005 unpublished data from A. Brown and C. Brasier).

Removal of infected *R. ponticum* caused a marked decline in inoculum levels in the woodlands (fig. 1) and no further trees became infected although the disease progressed on many of the already affected trees. However, even following the removal of all infected rhododendrons, *P. kernoviae* apparently persisted in soil and litter, albeit at reduced levels, for at least three years and possibly longer. In addition, some of the cut rhododendron stumps continued to sprout with some of the shoots becoming infected by *P. kernoviae* years after the original clearance process. Therefore, regular herbicide treatment to kill the stumps and prevent re-sprouting is an essential part of any eradication process.

Persistence of *P. kernoviae* in soil and leaf litter presumably follows when oospores have been formed in infected foliage. Experiments looking at the persistence of both *P. kernoviae* and *P. ramorum* in naturally infected rhododendron leaves have shown that both pathogens are long-lived even when the leaves have been shed. After more than year, viable *Phytophthora* could be detected in 5-10 percent of air suspended leaves, and in a much higher proportion of leaves that formed part of the litter layer. Monitoring beyond a year was impossible as the leaves break down and become comminuted, and are subsumed into the soil layer, but the likelihood is that these leaf fragments may still contain long-lived spores; chlamydospores in the case of *P. ramorum* and oospores with *P. kernoviae*.

Conclusions

Successful eradication of both *P. kernoviae* and *P. ramorum* from infested gardens and woodlands will inevitably be a long-term process, because of the ability of both pathogens to persist in the environment several years after the main foliar host, rhododendron, has been removed. In the addition, the ability of rhododendron to regenerate from the remaining stumps following removal (even after herbicide

regenerate from the remaining stumps following removal (even after herbicide treatment) coupled with the emergence of new seedlings, emphasises that the process of eradication will not be achieved in a single action. However, removal of infected rhododendrons markedly reduces the risk of trees succumbing to infection by *P. kernoviae* even when the pathogen continues to persist in the environment. Moreover, removal of *R. ponticum* from woodlands has added benefits apart from combating *P. kernoviae* and *P. ramorum*. As an introduced plant species it has proved to be highly invasive so removal provides biodiversity benefits as well as returning woodlands to improved status.

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Australasia is at High Risk of a *Phytophthora ramorum* Epidemic[1]

Daniel Hüberli,[2,3] Kylie Ireland,[4] Ian Smith,[5] Bernard Dell,[2] Michael Ormsby,[6] David Rizzo,[7] Matteo Garbelotto,[3] and Giles E.St.J. Hardy[2]

Introduction

With an increase in global trade of plants and plant products it is paramount for Australasia to identify emerging plant diseases, carriers of these diseases, and the role of hosts in the transmission of disease. In Australia, a cause for concern and currently listed as a category 1 emergency pest pathogen is *Phytophthora ramorum*. It has a wide host range and causes widespread damage in nurseries and private estates across Europe and is devastating coastal forest ecosystems of western USA, mainly in California (Rizzo and others 2002, Werres and others 2001).

Several Australasian plant species, including *Griselinia littoralis* (New Zealand broadleaf), *Eucalyptus haemastoma* (Australian scribbly gum), and *Pittosporum undulatum* (Victorian box), have already been listed as natural hosts of *P. ramorum* based on field observations and pathogenicity tests in the USA and Europe (Hüberli and others 2006, RAPRA 2007). While *P. ramorum* has not been detected in New Zealand or Australia, a preliminary study has identified ecosystems that could be conducive to disease development in Australia (W. Smith unpublished data). It is a pathogen that the region cannot afford as the threat and management implications of this pathogen on natural ecosystems, agriculture and horticulture may potentially be far worse than that currently posed by *P. cinnamomi* (O'Gara and others 2005).

The study aims to provide knowledge of potential hosts and therefore carriers of the pathogen, provide data for the establishment of robust quarantine practices and reduce the risk of an introduction of *P. ramorum* into Australasia.

Materials and Methods

For the New Zealand study, excised branches and leaves of 17 endemic plants and three commercial species (*Eucalyptus globulus*, *Pinus radiata* and *Acacia*

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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melanoxylon) as well as two known *Rhododendron* host species were inoculated with *P. ramorum* during 2005–2006 at the University of California, Berkeley. Branches and leaves were either underbark wound or zoospore dip inoculated. A second foliar inoculation experiment of seven species, including two *Rhododendron* spp., was conducted using three different zoospore concentrations. Lesions and recovery were recorded after six days incubation in humid chambers at 19°C for all experiments. The number of sporangia produced on foliage was counted for the first inoculation.

For the Australian study, an intensive screening of indigenous plants located in regions identified as highly conducive to disease using a CLIMAX model will be conducted in California. Initial screens will assess susceptibility to the pathogen using detached leaves, followed by further work that focuses on whole plant tissue to give a more accurate assessment of natural infection potential. Studies of the sporulation potential and asymptomatic transmission of these species will also be investigated.

Results and Discussion

In branch inoculations, *Nothofagus fusca* and *Pinus radiata* were identified as susceptible (fig. 1), while *Fuchsia excorticata* was extremely susceptible in foliage inoculations (fig. 2). Additionally, F. excorticata was the only species that supported high foliar sporulation. *Pinus radiata*, *N. fusca*, and *F. excorticata*, should be added to the potential host list for *P. ramorum*. They should be monitored carefully in New Zealand for symptoms in high risk incursion areas as well as within gardens and nurseries in the United States and Europe for symptoms and/or sporulation. The results from the New Zealand study and those forthcoming from the Australian study will be highly beneficial in creating risk maps of any incursion of *P. ramorum* into Australasia.

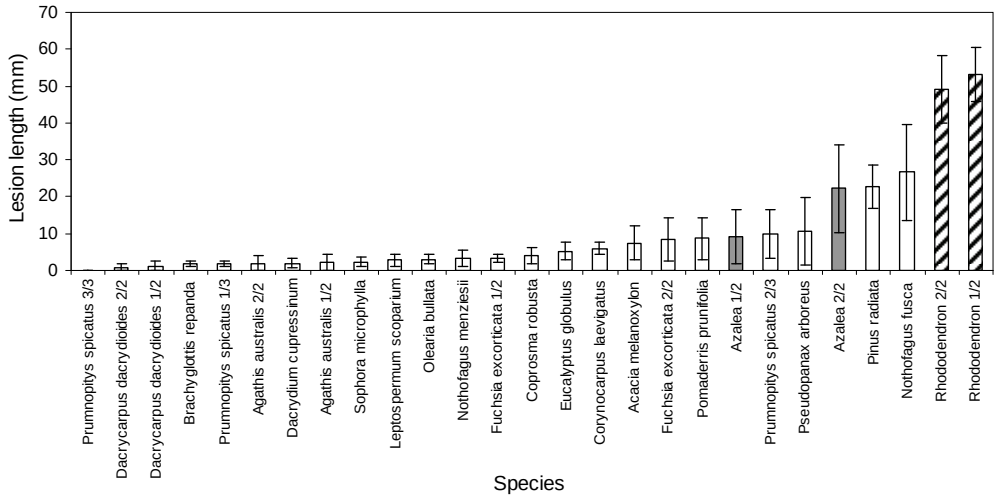

Figure 1—Mean lesion length (± standard error) of excised branch inoculations of endemic and commercial plant species to New Zealand after underbark wound inoculation with Phytophthora ramorum mycelium agar plugs. Branches were incubated in a misting chamber in the greenhouse for 10 days.

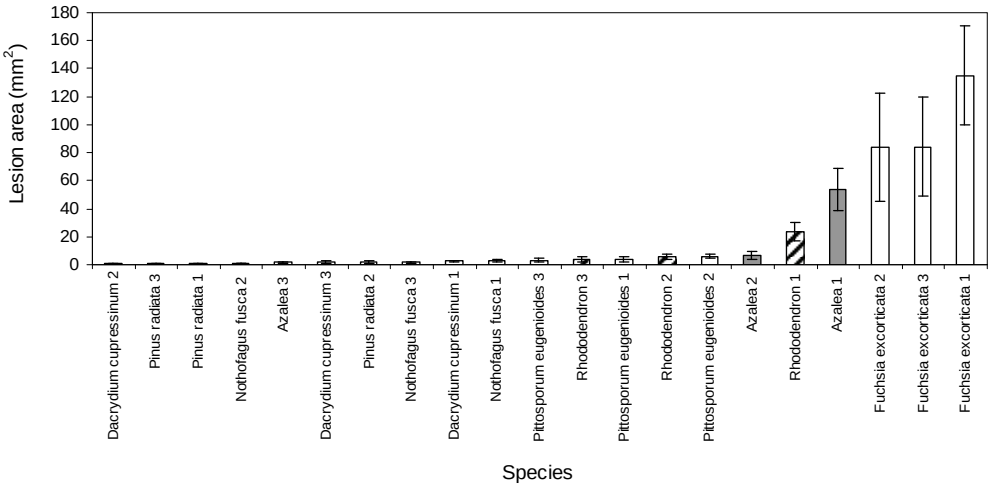

Figure 2—Mean lesion area of excised leaves of endemic and commercial plant species to New Zealand after dip nonwounded inoculation with Phytophthora ramorum zoospores. Leaves were incubated for 6 days in a humid chamber.

A more detailed account of the New Zealand project can be found in the subsequent journal publication: **D. Hüberli, B. Lutzy, B. Voss, M. Calver, M. Ormsby and M. Garbelotto (2008)** Susceptibility of New Zealand flora to *Phytophthora ramorum* and pathogen sporulation potential: an approach based on the precautionary principle. Australasian Plant Pathology, in press.

Acknowledgements

For the New Zealand project, we thank B. Lutzy, T. Harnik, K. Maeda, A. Smith and B. Voss for assistance with inoculations, University of California Botanical Garden at Berkeley for the plant material, and for funding support from Biosecurity New Zealand, the Betty and Gordon Moore Foundation and the Pacific Southwest Research Station USDA Forest Service. For the Australian study, the Department of Environment and Water and CRC Plant Biosecurity funded K. Ireland.

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Screening for Resistance to *Phytophthora cinnamomi* in Hybrid Seedlings of American Chestnut[1]

Steven N. Jeffers,[2] Joseph B. James,[3] and Paul H. Sisco[4]

Abstract

American chestnut (*Castanea dentata*) once was one of the primary hardwood tree species in forest ecosystems in the eastern USA. However, in the 1800s, Phytophthora root rot (PRR; also known as ink disease), caused by *Phytophthora cinnamomi*, resulted in widespread death of chestnut in the Piedmont region of southeastern states where clay soils are dominant. This was followed in the early 1900s by chestnut blight, caused by *Cryphonectria parasitica*, which almost eliminated chestnut from its primary mountain habitat. Since 1989, the American Chestnut Foundation (TACF) has been producing hybrid chestnut seedlings by crossing Chinese chestnut (*C. mollissima*) with American chestnut and then backcrossing progeny to *C. dentata* in an attempt to produce American-type chestnut trees resistant to *C. parasitica*. In recent years, hybrid seedlings planted in the field in southeastern states have died from PRR before they could be challenged by *C. parasitica*. Therefore, in 2004, we began screening hybrid seedlings for resistance to *P. cinnamomi*. In 2004 to 2006, hybrid seeds from known crosses were obtained from TACF cooperators, and seeds from *C. dentata* and *C. mollissima* were collected in the field. Seeds were stratified and then planted outside in April in replicate 568-liter plastic tubs filled with soilless container mix at a field site in Oconee Co., SC. Inoculum was produced by growing two isolates of *P. cinnamomi*, originally recovered from chestnut seedlings, on autoclaved rice grains. Seedlings were inoculated 12 to 14 weeks after planting. Inocula were combined, mixed thoroughly, and then evenly distributed in 1- to 3-cm-deep furrows between rows of seedlings. Seedlings were watered as needed throughout the study period, and the container mix in each tub was brought to saturation at least once while plants were actively growing. Plants were evaluated for PRR symptoms in December when fully dormant.

Each year, seedlings started dying approximately 3 weeks after inoculation and continued to die throughout the summer months; symptoms were typical of PRR. *C. dentata* seedlings consistently were susceptible, *C. mollissima* seedlings consistently were resistant, and hybrid seedlings varied from susceptible to resistant. Resistant seedlings were planted in the field for further evaluation. Preliminary results suggest that resistance is incompletely dominant and regulated by one gene. Moreover, the genes for resistance to *P. cinnamomi* and *C. parasitica* do not appear to be linked. Screening efforts have been expanded in 2007 and will continue in coming years.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Introduction

American chestnut (*Castanea dentata*) once was one of the primary hardwood tree species in forest ecosystems in the eastern United States (Freinkel 2007). However, in the 1800s, Phytophthora root rot—also known as ink disease and caused by *Phytophthora cinnamomi*—resulted in widespread death of chestnut trees in the Piedmont region of the southeastern states where clay soils are dominant (Crandall and others 1945; Zentmyer 1980). This was followed in the early 1900s by chestnut blight, caused by *Cryphonectria parasitica*, which almost eliminated chestnut from its primary mountain habitat (Anagnostakis 1987; Freinkel 2007). Since 1989, The American Chestnut Foundation (TACF) has been producing hybrid chestnut seedlings by crossing Chinese chestnut (*C. mollissima*) with American chestnut and then backcrossing progeny to *C. dentata* in an attempt to produce American-type chestnut trees that are resistant to *C. parasitica* (The American Chestnut Foundation 2007). In recent years, hybrid seedlings planted in the field in several southeastern states have died before they could be challenged by *C. parasitica*. In 2003, we diagnosed Phytophthora root rot as the cause of this seedling mortality (fig 1). Therefore, we initiated a project in 2004 to screen hybrid chestnut seedlings for resistance to *P. cinnamomi*—with the objectives of identifying families with high levels of resistance and establishing a population of resistant trees for future breeding efforts.

Figure 1—Phytophthora root rot symptoms on hybrid American chestnut seedlings. **Left**, above ground: chlorosis and wilting of foliage; **right**, below ground: dead and decayed roots and a necrotic lesion advancing up from the root crown area (arrow) on the lower stem (bark removed).

Materials and Methods

Trials began in 2004 and have been conducted each year thereafter; results from trials conducted in 2004, 2005, and 2006 are summarized here. For each trial, hybrid chestnut seeds from known crosses were obtained from TACF cooperators, and open-pollinated seeds from *C. dentata*, *C. mollissima*, and *C. pumila* (chinkapin) were collected from trees in the field. Seeds usually were received during the fall and early winter prior to planting in the spring. Seeds were mixed with moist peat and placed in a perforated plastic bag, and bags were stored in a refrigerator to stratify. In early April, when the risk of frost was minimal, germinated seeds were washed free of peat (fig. 2A) and planted outside in 568-liter plastic tubs filled with soilless container mix (Fafard 3B; Conrad Fafard, Inc., Agawam, MA) at Chestnut Return farm—a field site in Oconee Co., SC. Seeds from the same family were planted together in a row and families were replicated in multiple tubs (fig. 2B); seeds were planted in two tubs in 2004 and in six tubs in both 2005 and 2006. Plants were watered regularly throughout the growing season so that the container mix stayed moist and did not dry out. Each year, tubs were top-dressed once early in the growing season with a complete time-release fertilizer.

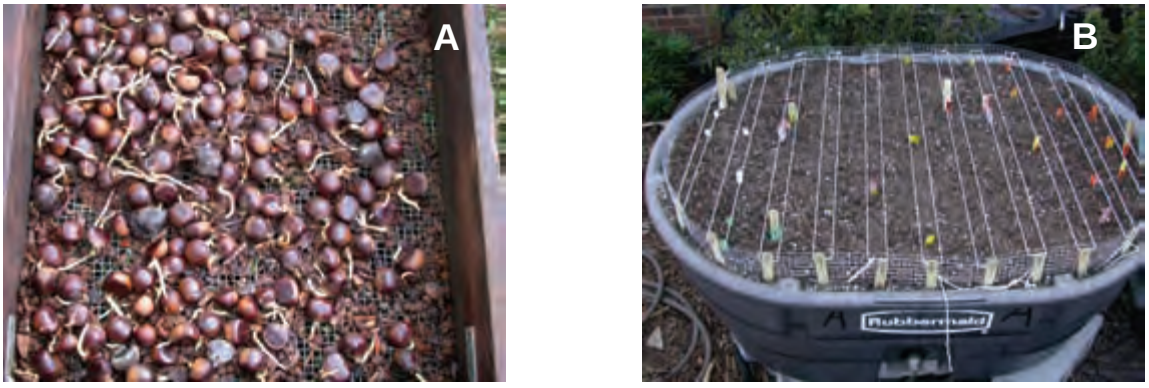

Figure 2—**A**, Germinated chestnut seeds ready for planting; **B**, plastic tub filled with container mix after planting chestnut seeds—strings identify rows and stakes delimit families.

Hybrid chestnut seedlings were inoculated 12 to 14 weeks after planting with two isolates of *P. cinnamomi* that originally had been recovered from chestnut seedlings growing at the field site. Inoculum was produced by growing each isolate axenically on autoclaved rice grains (Burns and Benson 2000) at 25°C in the dark for 10 to 14 days. Equal volumes of rice grains colonized by each isolate were combined and mixed thoroughly to produce a composite inoculum. To inoculate plants, a thin layer of rice inoculum was evenly distributed in 1- to 3-cm-deep furrows between rows of seedlings. Furrows were covered and the tubs were watered thoroughly to prevent the inoculum from desiccating. Several weeks after inoculation, the container mix in each tub was flooded once for 4 to 6 hours to promote disease development. Plants were evaluated for Phytophthora root rot symptoms in late December or early January when seedlings were fully dormant. Each plant was removed carefully from a tub—taking care to recover as much of the root system as possible. Individual plants were rated for symptom severity using a 0 to 3 scale:

0 = healthy, no visible lesions on roots
1 = lesions on at least one lateral root
2 = lesions on the tap root
3 = severe root rot, plant dead

Surviving seedlings, primarily those rated 0 or 1, were planted in an orchard at Chestnut Return farm. These plants were monitored annually for performance in the field.

Results and Discussion

Each year, seedlings started dying approximately 3 weeks after inoculation and continued to die throughout the summer months; symptoms were typical of Phytophthora root rot (fig. 1). *C. dentata* and *C. pumila* seedlings consistently were susceptible to *P. cinnamomi* and died; *C. mollissima* seedlings consistently were resistant and grew vigorously in the infested soil. Although hybrid seedlings varied from susceptible to resistant, most were susceptible and died (1262/1693 = 75 percent; table 1). The numbers of families and hybrid seedlings evaluated increased each year, but the proportion of seedlings surviving with symptom severity scores of 0 or 1 varied depending on the genetics of the families evaluated (table 1). Over the three-year period, 43 families and almost 1700 seedlings were evaluated (table 1).

Table 1—Numbers of hybrid chestnut families and seedlings evaluated in 2004, 2005, and 2006 and the frequency of seedlings in four symptom severity (SS) classes[z]

Year	Families (no.)	No. seedlings				
		Evaluated	SS = 0	SS = 1	SS = 2	SS = 3
2004	5	360	21	31	18	290
2005	15	596	60	34	111	391
2006	23	737	8	24	123	581
Total	43	1693	89	89	252	1262

[z] 0 = healthy; 1 = lesions on lateral roots; 2 = lesions on tap root; 3 = plant dead

The strength of resistance in these 43 families was determined by calculating the Survival Quotient (SQ), which is expressed as percentage:

$$SQ = ([(1 \times n0) + (0.5 \times n1) + (0.25 \times n2)] / \text{total number of seedlings}) \times 100$$

where n0, n1, and n2 = no. seedlings rated 0, 1, and 2, respectively.

Of the 43 families evaluated (Table 2), 11 families (26 percent) had no survivors and a SQ of 0 percent; 18 families (42 percent) had a SQ between 0.1 and 15.0 percent; 10 families (23 percent) had a SQ between 15.1 and 30.0 percent; three families (7 percent) had a SQ between 30.1 and 40.0 percent; and one family had a SQ over 50 percent (Hyko x JB575, SQ = 56.3 percent).

Over the three-year period, 189 resistant seedlings were planted in the field for further evaluation. To date, 77 of these have survived; these 77 survivors represent

40.7 percent (77/189) of those planted in the field and 4.5 percent (77/1693) of all the hybrid seedlings evaluated. Seedlings with a symptom severity rating of 0 or 1 have survived better than those with a rating of 2. Consequently, resistance to *P. cinnamomi* was present in some of the hybrid chestnut families that had been selected for resistance to *C. parasitica*. Preliminary results suggest that resistance to *P. cinnamomi* is incompletely dominant and may be regulated by more than one gene. Moreover, the genes for resistance to *P. cinnamomi* and *C. parasitica* do not appear to be linked. Screening efforts have been expanded in 2007 and will continue in coming years.

Table 2—Strength of resistance in 43 hybrid chestnut families screened over three years, 2004 to 2006: a survival quotient was computed for each family based on the number of seedlings in each of three symptom severity classes[z]

Family	Source of Resistance	Generation	Survival Quotient
CL-50	Clapper	B2F2	0.0
Andover x BX39	Mahogany	BC3	0.0
HP - A4 (CL248)	Clapper	B2F3	0.0
HP - C7 (CL248)	Clapper	B2F3	0.0
HP - L (CL248)	Clapper	B2F3	0.0
Milliken D5 op	Clapper	B2F3	0.0
Milliken D6 op	Clapper	B2F3	0.0
VKN x VA307	Clapper	B3F1	0.0
Uxbridge KD-1 x GL356	Graves	B3F1	0.0
Spanish Oak Rd x IL201	Clapper	B4F1	0.0
VKS x CB582	Clapper	B4F1	0.0
Fitchburg KJ19 x BG318	Graves	B4F1	0.5
Swallows x TM616	Graves	B3F1	0.5
Pike Co Marinero x GL367	Clapper	B3F1	0.6
Lincoln, RI-1x QG85	Graves	B4F1	1.1
Fitchberg x GL96	Clapper	BC3	1.2
Sudbury x BE138	Graves	BC3	1.4
Newton CS19 x HE416	Clapper	B4F1	2.4
HP - B4 (CL248)	Clapper	B2F3	3.1
rc97-107 (JoScxGR210) x opBC3	Clapper	B3F2	3.2
Uxbridge x GL356	Graves	BC3	4.5
James D-20 x Maddox MS8-12 and 9-12	Clapper	B2F3	6.3
rc97-m (FhSo x opAM) x opBC3 (Gr210)	Clapper	B4F1	6.5
ob00-m (JLCe x VA307) x opBC3	Clapper	B3F2	8.0
ob00-025 (TRTCm x AB427) x opBC3	Clapper	B3F2	9.1
hu97-m (Ort x GR137) x opBC3	Graves	B3F2	9.3
CL-149	Clapper	B2F2	10.6
HP - F1 (CL198)	Clapper	B2F3	12.2
CL-112	Clapper	B2F2	12.9
Milliken E7 op	Clapper	B2F3	17.6
Frye Mtn. x B3_176	Graves	B4F1	19.2
ob99-199 (StT1 x GR210) x opBC3	Clapper	B3F2	20.0
HP - F2 (CL198)	Clapper	B2F3	20.6
Sudbury x AB247	Graves	BC3	20.7
Milliken D1 op	Clapper	B2F3	23.3
HP - F12 (CL198)	Clapper	B2F3	24.8
CL-326	Clapper	B2F2	25.0
Rn88-38 x Hind	Douglas Hybrid	B2F1	25.0
Franklin EMC x B2_214	Mahogany	B3F1	29.2
CL-248	Clapper	B2F2	32.5
Boston SB1 x JB575	Mahogany	BC3	37.2
Milliken Tree D1 op	Clapper	B2F3	37.5
Hyko x JB575	Mahogany	BC3	56.3

[z] Survival Quotient (in percent) = ([(1 × n0) + (0.5 × n1) +(0.25 × n2)] / total number of seedlings) × 100 where n0, n1, and n2 = no. seedlings rated 0, 1, and 2, respectively

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Field Evaluation of Cork Oak Seedling Viability in a Soil Naturally Infested With *Phytophthora cinnamomi*[1]

A.C. Moreira,[2] J. Albuquerque,[3] E. Melo,[2] J.M.S. Martins,[2] R. Tapias,[4] and A. Cravador[5,6]

Abstract

The present work describes the evaluation of the tolerance or susceptibility of cork oak seedlings to *Phytophthora cinnamomi* infection in the field, and discusses the possibility of using their genetic diversity to control the cork oak (*Quercus suber*) decline in areas highly infested with this pathogen in Portugal and Spain. Acorns were colleted from trees located in six geographically distinct regions of Portugal (Trás-os-Montes, Estremadura, Ribatejo, Alto Alentejo, Baixo Alentejo and Algarve) and in two of Andalucía, Spain (Huelva and Málaga), and sown in 2004 in a soil naturally infested with *P. cinnamomi* situated in Algarve, the southern region of Portugal. The percentage of acorn germination was evaluated, the mortality was assessed and the height of the seedlings was measured twice a year (June/July and December) during three consecutive years. The highest percentage of germination was observed within families of the Portuguese regions of Alto-Alentejo and Estremadura. Mortality in the first year after sowing (2004) reached 12.3 percent and doubled during the following two years. Moreover, results show a high variability in the percentage of survival after germination between half-sib seedlings, which was not found to be correlated with geographical nor maternal origin. In 2006, seedlings with disease symptoms or already dead were harvested and *P. cinnamomi* was detected in 85 percent of them. Seedling height in December 2006 was significantly different between families of same maternal origin and between regions. Those from Alto-Alentejo, Baixo-Alentejo and Algarve produced the highest seedlings at the end of the third year. In summary, both maternal and regional origin significantly influences both acorn germination and seedling growth, indicating a distinct susceptibility to *P. cinnamomi* among maternal genotypes.

Introduction

Cork (*Quercus suber* L.) and holm (*Q. ilex* subsp. *rotundifolia* Lam.) oak trees are two evergreen species with high economic importance for the Iberian Peninsula. "Montado" is an agro-silvo-pastoral system created by man, populated mainly by cork and holm oak trees and peculiar to southern Iberian Peninsula. It faces a serious ecological, socio-economic and landscaping problem due to the oak decline that is

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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threatening the stability of the entire ecosystem. *Phytophthora cinnamomi* Rands has been associated with extensive decline and death of these species (Brasier and others 1993 a, b, Moreira and Martins 2005, Sánchez and others 2002, Sánchez and others 2003, Tuset and others 1996). In Portugal, the isolation frequency of *P. cinnamomi* from cork and holm oak roots and associated soils has increased in the last decade, namely in Alentejo region (from 35percent to 90 percent, non published results). This soil-borne plant pathogen has been introduced in Europe, where it is known since the early 19th century (Brasier 1993, Zentmeyer 1980); it causes the death of the fine roots, reducing the capacity of absorption of water and nutrients, leading to plant decay and death (Moreira and Martins 2005, Robin and others 1998).

The control of this pathogen is very difficult, as it presents a large host range and may persist in soil for long periods as resistant structures (Moreira and Martins 2005, Zentmeyer 1980). Several methods have been proposed to control *P. cinnamomi*. Chemical treatments were tested in Spain (Fernández-Escobar and others 1999, Navarro and others 2004) and are being used in some areas of Portugal to treat adult trees, but their long-term efficiency has not yet been clearly established.

Resistance to *P. cinnamomi* has been reported in some tree species (Butcher and others 1984; Stukely and Crane 1994). The possibility of using cork oak plants more tolerant or resistant to *P. cinnamomi* infection can be an important tool to the reforestation of highly infested areas. Tree survival in several dieback affected sites suggests the existence of genetic variability in this species that may be used to select more tolerant individuals.

The present work reports the results of a preliminary experiment designed to assess the diversity of response of cork oak plants to *P. cinnamomi* infection in field conditions and to evaluate their resistance or tolerance potential.

Material and Methods

Acorns from 20 individual trees were collected at several places in each of six geographically distinct regions of Portugal (Trás-os-Montes, Estremadura, Ribatejo, Alto Alentejo, Baixo Alentejo and Algarve) and in two of Andalucía, Spain (Huelva and Málaga) (fig. 1). Trees were selected imposing at least 100 m distance from one another. Acorns were collected during November and December 2003. Seeds were preserved at low temperature (4 ºC) until they were sown. Sowing was carried out in January 2004 in Barranco do Velho, Algarve (south of Portugal), in a loamy soil naturally infested with *P. cinnamomi* where all the maquis vegetation was cut. None of the extant adult cork oak trees, healthy, diseased or already dead, was uprooted during the period of observation. The field was divided in ten 160 m^2 plots. In each plot sowing was carried out in such a way that acorns collected from each tree were represented in each plot and randomly distributed. Acorns were sown in rows spaced 1 m from each other, and at 1 m intervals along the rows. Soil of six plots (1 through 6; see fig. 2) was mechanically ripped to improve root system development and water-flow; the other four plots, located in an area with a high slope, were left untreated to prevent soil erosion. The percentage of germinated acorns was assessed six months after sowing. Seedling mortality and their stem height were recorded twice a year (June or July and December) during three consecutive years. During 2006 and 2007, eighty seedlings having contrasting dieback symptoms and development were

removed to observe the root system and to detect the presence of the pathogen. The roots were cut into 1 cm sections and plated onto selective medium (PARBHy; see Robin 1991). Recovery of the pathogen was assessed after three and five day incubation at 24 ºC.

Figure 1—Geographical origin of the tested acorns.

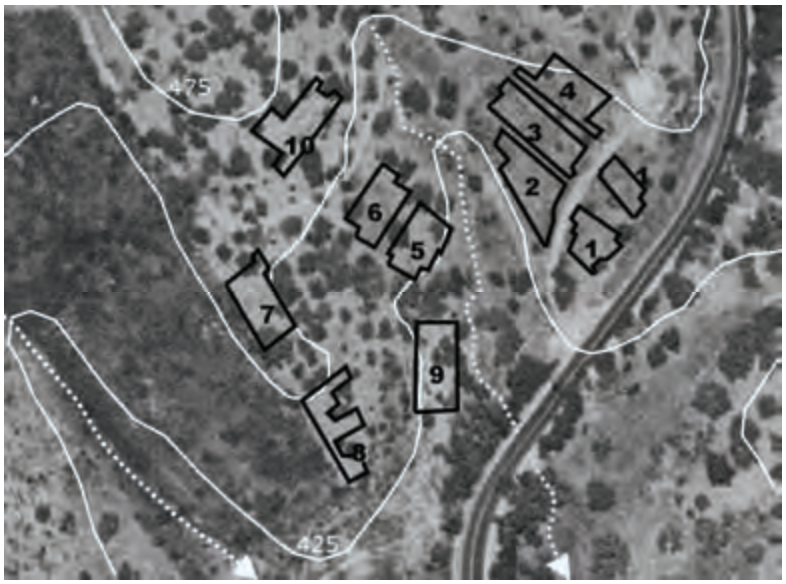

Figure 2—Distribution of the experimental plots. Solid lines: counter curves (equidistance = 25 m); dotted lines: seasonal brooks.

Results and Discussion

Results showed that germination was highly dependent on the family and geographic origin (contingency analysis, P≈0 in both cases). Acorns from populations of Alto Alentejo and Estremadura showed the highest percentage of emergence, those from Trás-os-Montes, Ribatejo and Huelva the lowest (fig 3A, Table 1).

Table 1—Differences of percentage germination of acorns and cumulated mortality (three years) of seedlings in half-sibling populations according to geographical origin

Origin	Germination	Mortality	Origin	Germination	Mortality
Alto Alentejo	0.76	0.19	Baixo Alentejo	0.61	0.23
Estremadura	0.73	0.29	Ribatejo	0.60	0.27
Algarve	0.62	0.28	Trás-os-Montes	0.53	0.27
Málaga	0.62	0.22	Huelva	0.47	0.28

Location and soil treatment of the experimental plots seemed to influence the germination and the growth – plots 1 through 4 allowed a higher percentage of seed germination and a better development of the seedlings than plots 7, 8 and 9 (extreme differences ranging from 33 percent to 81 percent; Table 2). Eventually, mechanical soil preparation and other local factors (such as slope, exposition, distance to the seasonal brook, uneven distribution of the inoculum in the soil) may have contributed to the lack of homogeneity of the ten plots. The shallow and rocky soil nature of these plots certainly contributes to a lower germination rate and may be a contributing factor to *P. cinnamomi* infection (Brasier 1993; Moreira and Martins 2005). To test the significance of this constraint, the plots were ordered by the overall percentage of germination, and separated in two groups as in Table 2. A Spearman's correlation was calculated between the ranks of each half-sibling in both groups of plots. The correlation coefficient was found to be highly significant ($P(r_s)<0.01$). Thus, it can be concluded that, despite the very high differences in percentage germination among plots, the relative position of each half-sibling respecting to the others was maintained.

Table 2—Percentage of acorn germination in ten experimental plots

Plot	Germination	Plot	Germination
1	0.81	5	0.58
3	0.79	10	0.56
2	0.78	8	0.51
4	0.69	7	0.47
6	0.61	9	0.33

By the end of autumn 2004 (first year) global mortality was 12.3 percent and decreased during the two subsequent years (6.3 percent in 2005 and 6.0 percent in 2006; see details in fig 3B). The observed mortality followed the same trend in all three years of observation, its incidence seeming to be randomly distributed by the half-siblings, and also by their geographic origin (contingency analysis, not

significant). As observed for germination, the heterogeneity of the plots seemed not to interfere with the comparative mortality: Spearman's correlation was also highly significant when applied to the comparison of the ranks of the percentage of mortality in half-siblings at the end of the three years of observations. The evolution of mortality can be understood having in mind the lower than normal precipitation that occurred in the years 2003-2004 and 2004-2005 (570 and 370 $L \cdot m^{-2}$, respectively; normal: 800-1000 $L \cdot m^{-2}$ per year). In 2005 the precipitation was lower than in 2004, and this may have influenced survival and development of the seedlings, in spite of their having been irrigated twice each summer. During 2006 precipitation values were normal, mortality decreased to 6 percent in that year.

Seedling height in December 2006 was significantly different among half-sibling populations and among regions ($P<0.001$; fig 3C). Acorns from Alto Alentejo, Baixo Alentejo and Algarve gave origin to the tallest seedlings, which were significantly different in height from those from Trás-os-Montes and Ribatejo (Table 3). This suggests that, as for germination, seedling development is connected with geographical origin.

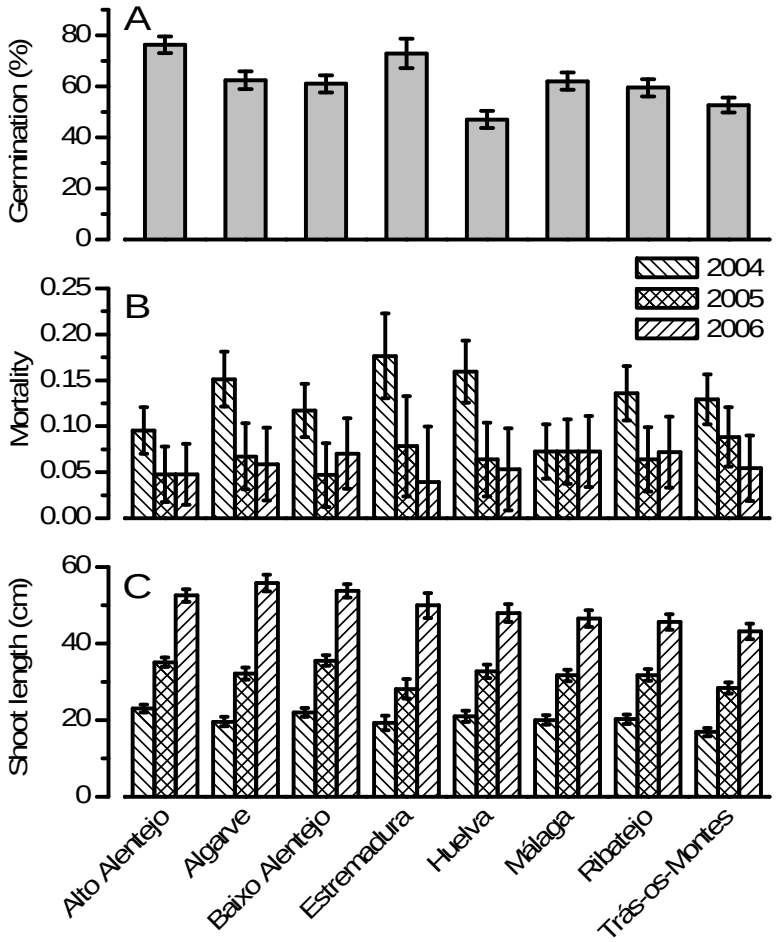

Figure 3–Germination (A), mortality (B) and growth (C) of cork oak acorns according to their provenance and sown in a *P. cinnamomi* naturally infested soil.

Field observations from 2004 to 2006 showed a high variability on germination and on growth within families. Ten seedlings represented each family, which is a very small sample to be representative. The families listed in Table 4 were those with bigger shoots (>44 cm average) and higher survival rates (> 8 out of 10 plants). However, there was a continuous variation of those two measures within each half-sib family, as would be expected in segregating descents.

Table 4—List of families that include the best-developed individuals (2003-2006)

Family	Origin	Survival in 10 plants	Average growth (cm)		
			Min	Max	Mean
015	AL	9	41	104	60
045	AA	9	12	62	44
011	AL	8	28	70	46
041	AA	8	34	68	55
044	AA	8	22	74	50
054	AA	8	23	60	44
059	AA	8	17	63	50
047	AA	8	16	80	50
030	BA	8	30	76	53

In 2006 and 2007 eighty seedlings representing 57 half-sib families and all levels of development were harvested to assess infection. *Phytophthora cinnamomi* was recovered from the roots of 85 percent of cork oak seedlings, irrespective of their development; 25 percent showed non aerial symptoms and a good development, and 50 percent showed dieback and poor development; all recently dead seedlings (10 percent) were infected. In 15 percent of seedlings *P. cinnamomi* was not detected.

The soil selected for this experiment was highly infested with *P. cinnamomi* and its inoculum was largely distributed, even in places where almost all the adult cork oak trees disappeared (e.g., plots 2, 3 and 4). It was previously shown that many shrub species growing in cork oak stands are infected, contributing to the inoculum build up. *Cistus populifolius,* for instance, which is very abundant in the area, is a species very susceptible to the infection (Moreira and Martins 2005). This suggests that maquis species and cork oak adult trees may provide the initial *P. cinnamomi* inoculum to the seedling infection affecting oak reforestation.

Plots 7, 8 and 9 showed the worst results on germination and development of the seedlings. They were located on a hilltop with a very shallow and rocky soil that was not mechanically prepared. This is in agreement with previous observations identifying hilltops as places favoring infection by *P. cinnamomi* (Brasier 1993, Moreira and Martins 2005). This result suggests that mechanical soil preparation, soil variability, and the plot location may have had an influence on the emergence success.

Results here reported illustrate the response diversity to the infection, which can be interpreted as resulting from the genetic diversity of cork oak species (Coelho and others 2006). A similar conclusion was drawn from a study conducted under controlled conditions by Tapias and others (2007).

Maternal and geographical origin significantly influences both acorn germination and seedling development, indicating a potential to exploit the differential susceptibility to *P. cinnamomi* in future breeding programs.

Acknowledgments

This study was supported by the PO-AGRO 8-Programme for Experimental Development and Demonstration from Ministério da Agricultura, Pescas e Desenvolvimento Rural (Project 416).

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National Best Practice in the Management of *Phytophthora cinnamomi* for Biodiversity Conservation in Australia[1]

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Abstract

Disease in natural ecosystems of Australia, caused by the introduced plant pathogen *Phytophthora cinnamomi*, is listed as a key threatening process under the Australian Government *Environment Protection and Biodiversity Conservation Act 1999* (EPBC Act). The Act requires the Australian Government to prepare and implement a threat abatement plan for nationally coordinated action to mitigate the harm caused by *P. cinnamomi* to the Australian environment particularly threatened flora, fauna species and ecological communities.

The 'National Threat Abatement Plan for Dieback Caused by the Root-Rot Fungus *Phytophthora cinnamomi*' (NTAP) was released in 2001, and was reviewed and revised in 2006. The NTAP is designed to promote a common understanding of the national threat *P. cinnamomi* poses to biodiversity in Australia.

The Centre for Phytophthora Science and Management is a key stakeholder in the management of *Phytophthora* in the State of Western Australia. The Centre has also played a key role in undertaking some of the most significant projects of the National Threat Abatement Plan, and undertook the review and revision of the Plan in 2006. This has placed the Centre in a unique position to critique the mechanisms for management of *P. cinnmomi* in Australia, and the impact that the three-tiered (Commonwealth or Federal, State and Local) system of government has on developing, attaining and maintaining 'best practice' management. This paper examines national and state management frameworks and discusses the outcomes for biodiversity conservation in Australia.

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Development of U.K./EU/EPPO Pest Risk Analyses for *Phytophthora kernoviae, P. ramorum and P. lateralis*[1]

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Abstract

Pest Risk Analysis (PRA) is an internationally recognised, structured process of determining whether plant pests and pathogens (known generically as 'pests') that are normally absent from a country or area could enter, establish and cause an economic or environmental risk that is deemed unacceptable. If required, PRA is also used to help identify potential phytosanitary measures to reduce the identified risk to an acceptable level. The WTO Sanitary and Phytosanitary Agreement requires that a scientific assessment of the risk posed by a plant pest is undertaken before any phytosanitary measures are set. Such measures must be the minimum needed to reduce the risk without unnecessarily impacting on trade. Before this process can commence an organism must be identified as being a unique taxonomic entity. In recent years, new plant diseases have emerged in the natural environment that have not been immediately connected with a single identifiable cause. Subsequently these diseases were found to have been caused by new species of *Phytophthora.* PRAs have been produced and developed over several years for two of these: *Phytophthora kernoviae* and *Phytophthora ramorum*. Both pathogens have been identified as posing a risk to the environment, private and managed gardens and woodlands as well as to the ornamental plant trade in the U.K./EU/EPPO region. As a result of the PRAs, emergency legislation has been implemented in the U.K. and EU respectively for these pathogens to allow action to be taken against them wherever they are found. This is due for review in 2008. Following a trend of increasing environmental risks posed by new *Phytophthora* species that can be moved in the ornamental plant trade, an EPPO Panel produced a PRA for an '*old*' *Phytophthora*, *P. lateralis*. This PRA resulted in a recommendation for regulation in the EPPO region to protect the ornamental plant trade alone, as no special environmental areas were identified as being at risk in the region. There are more '*old*' *Phytophthora* species that are known to pose environmental/economic risks and there are likely to be more '*new*' species emerging. With the globalisation of the plant trade these are increasingly likely to be moved into new areas. Identifying these threats before they become a reality is a challenge but there is now a heightened awareness of the likely increase in circulation of *Phytophthora* species in trade. The PRA framework can be used to determine the risk potential and to communicate the risk and the risk management options to policy makers, who have the option to consider implementing appropriate preventative measures in a timely way.

Pest Risk Analysis—the FAO and the WTO

Pest Risk Analysis (PRA) is a structured process by which the identification and assessment of the risk of entry, establishment and spread of exotic plant pests (including pathogens) is determined and the potential economic and environmental

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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damage arising from their introduction in a given country or area is assessed. PRA became formally recognised by the Food and Agriculture Organisation (FAO) Secretariat to the International Plant Protection Convention (IPPC) in 1996, by the publication of guidelines (FAO, 1996) for the PRA process. PRA is defined as '*the process of evaluating biological or other scientific and economic evidence to determine whether a pest should be regulated and the strength of any phytosanitary measures to be taken against it'* (FAO, 2007). At the outset of the development of standards for PRA, consideration of the risks posed to the environment by pest risk analysts was limited, with most PRAs produced concentrating on the risks to crop plant species.

Subsequent to the publication of PRA guidelines (FAO, 1996) more detail on what elements need to be considered when preparing a PRA were agreed by the Interim Commission on Phytosanitary Measures (ICPM) in April 2001, with a supplement to include an analysis of environmental risks being approved in April 2003 (and one to account for risks from Living Modified Organisms in April 2004), leading to the publication of the International Standard for PRA for Quarantine Pests (FAO, 2004). The analysis of risks posed by plant pests to the environment and biological diversity within this standard includes full consideration of those that might threaten uncultivated/unmanaged plants, wild flora, habitats and ecosystems contained in the PRA area.

PRA is the fundamental process which underpins the setting of phytosanitary regulations and is prescribed by the World Trade Organisation Sanitary and Phytosanitary Agreement (WTO, 1995) for this purpose, as well as the IPPC (IPPC, 1997). A fundamental principal when phytosanitary regulations are set, is that the measures that are imposed should be the minimum necessary to protect plant health whilst continuing to facilitate, or at least ensure, minimal disruption to trade.

Pest Risk Analysis Schemes and Processes

Different national, regional and international schemes have evolved since the publication of the first international standard for PRA (FAO, 1996) with the most recent being based upon the framework presented in FAO (2004). At the regional level, the European and Mediterranean Plant Protection Organisation (EPPO) have published a detailed decision support scheme (EPPO, 2007), with shorter schemes being developed by some of the 49 EPPO member countries.

FAO, 2007a (a revision of FAO, 1996) lists the main reasons (initiation points) for preparing a PRA as:

1. A request is made to consider a pathway that may require phytosanitary measures
2. A pest is identified that may justify phytosanitary measures
3. A decision is made to review or revise phytosanitary measures or policies
4. A request is made to determine whether an organism is a pest

Initiation point 2 is triggered when a pest is detected that poses an immediate or potential danger, which under the IPPC (IPPC, 1997) has to be reported, usually to the National Plant Protection Organisation (NPPO). This is the main initiation point for producing PRAs for new and emerging plant pathogens.

The PRA process commences with gathering as much information as possible to enable the pest risk analyst to answer a series of questions which will determine whether the pest fulfils the definition of a quarantine pest, defined as: '*A pest of potential economic importance to the area endangered thereby and not yet present there, or present but not widely distributed and being officially controlled*' (FAO, 2007). This is followed by a determination of the risk of entry, establishment and economic/environmental impact culminating in an assessment of the overall risk, and a decision as to whether and which phytosanitary measures to apply (pest risk management). At each stage of the PRA the analyst is required to identify areas of uncertainty in their responses. For pathogens new to science, the level of uncertainty is likely to be great, especially in the determination of the risk of establishment, the likely impact, as well as the efficacy of measures that could be taken to contain or eradicate the pathogen. Where the magnitude of the threat and the level of uncertainty are both great, it may be necessary to commission research, the results of which can be used to develop the PRA, thus reducing the uncertainty. The revision of a PRA in this way may result in a change to the initial phytosanitary policy. Meanwhile, in the short-term, once an organism has been identified as a potential risk, there is a requirement for good sampling, detection and diagnostic techniques to be developed. These can be used by the NPPO as a first barrier to entry. This joined-up process is effective in developing our understanding of the risk but also in developing risk management strategies to deal with interceptions and outbreaks of plant pests.

Emerging Pathogens and the Plant Trade—a Challenge for the Pest Risk Analyst

Recently, pest risk analysts have had to consider the risks posed by previously unidentified pathogens new to science. This is a challenge, as without full taxonomic identity it is difficult to proceed with a PRA. FAO (2004) requires that '*the identity of the pest should be clearly defined to ensure that the assessment is being performed on a distinct organism, and that biological and other information used in the assessment is relevant to the organism in question. If this is not possible because the causal agent of particular symptoms has not yet been fully identified, then it should have been shown to produce consistent symptoms and to be transmissible. The taxonomic unit for the pest is generally species…'*.etc.

Defra Statistics

(http://statistics.defra.gov.uk/esg/publications/bhs/2006/default.asp) show a significant increase in imports of various ornamental plants into the U.K. between 1995 and 2005. For example *ca*. £6.4 million worth of trees in 1995 increased to *ca*. £64 million in 2005; *ca*. £5 million '*cuttings, slips and other young plants*' increasing to *ca*. £16 million. This increase in trade (which has occurred around the world) has led to new opportunities for plant pathogens to be moved to new areas or countries. For those previously undescribed pathogens, without prior knowledge of their existence and if symptom development in plant material is discrete, there is a risk that they can enter new areas or countries undetected and establish away from their centres of origin. The flora present in the new area may differ from that in the area of origin of the pathogen. It is feasible therefore that these '*new*' pathogens could infect and cause disease in a wider range of plant species than was previously available to

them, since there will be no inherent resistance in many of the potential host plants. This is especially significant for *Phytophthora* species which can have a very wide host range. On entering a new area, there are many opportunities presented to them to increase their host range and to perpetuate themselves there for the foreseeable future.

The emergence of previously unknown *Phytophthora* species in the ornamental trade, e.g. *Phytophthora ramorum*, or, with the potential to be moved in such a trade e.g. *Phytophthora kernoviae*, both of which pose a threat to the environment, has proved challenging for pest risk analysts and their colleagues. In the U.K. there has been strong scientific co-operation between Government Agencies including the U.K. Central Science Laboratory (CSL) and Forest Research (FR); funding bodies (Defra, Forestry Commission (FC), U.K. levy bodies, the European Commission (EC)), policy makers, and inspectors in Defra and the FC, as well as the equivalent European and North American organisations, to develop a programme of research that could be used to enhance our understanding of these new species in order to progress the PRAs, particularly for *P. ramorum*. This has been essential to inform policy makers on the risks and strategies that could be deployed against these new species.

Phytophthora ramorum

Phytophthora ramorum was first described as a new species of *Phytophthora* in 2001 (Werres and others 2001) and is now known to occur in Europe, the USA and Canada where it is subject to official control. *Phytophthora ramorum* is considered to be an exotic pathogen introduced separately to both the USA and Europe from an unknown area, or areas, of origin, speculated but not proven to be Asia. The history of its identification and the risk it poses to the environments of Europe and North America ties-in with an increasing awareness of the risks posed by plant pathogens that can be moved in the plant trade. Prior to being formally named and described, the pathogen was first observed infecting rhododendron and viburnum in Germany and rhododendron in the Netherlands in 1993; however, these observations were not formally reported at the time (Werres and others 2001). Separately, in 1995, tree death was first observed on tanoak (*Lithocarpus densiflorus*) in California, USA. The causal agent was unknown, but since then, substantial mortality of tanoak as well as several *Quercus* (oak) species became apparent in California and the phenomenon became known as '*sudden oak death*'. Investigations into the cause finally led to the isolation of a new *Phytophthora* species in California in July 2000. Although no connection had been made between the European and North America scenario, because of the massive scale of tree mortality in the USA and the fact that a unique causal agent had finally been identified, an assessment of the risk that this new *Phytophthora* posed to the U.K. was produced in September 2000 (Brasier 2000). This assessment did not propose specific phytosanitary measures as it was undertaken using the EPPO risk assessment scheme (pre-dating the EPPO PRA scheme, thus not including the third stage of PRA, i.e. risk management). This risk assessment concluded that the pathogen had potential to establish in the U.K., possibly entering on nursery stock, and that there was a significant risk posed to (at least) U.K. native and exotic oaks. Although there was much uncertainty identified, the author felt that urgent research was required to address this; meanwhile suggesting that a decision be taken on the status of the pathogen before more information became available.

In January 2001, Brasier determined that the new *Phytophthora* species causing sudden oak death in California and that which had been isolated from rhododendron in the Netherlands and Germany (and viburnum) was the same species. EPPO added the pathogen to their Alert List (an early warning, without a full PRA) in January 2001. A second PRA was produced by the U.K. in April 2001 (published June 2001; Jones and Sansford 2001) with an accompanying Datasheet (unpublished). The PRA highlighted the risks to the U.K., EU and EPPO region, again identifying uncertainties and research needs, but also recommending surveys in the EU/EPPO region to determine distribution as well as consideration of phytosanitary measures including controls on imports of known susceptible hosts and their products into and within the EU/EPPO region from areas/countries where the pathogen has been found, to try to prevent entry. It was recommended that consideration should be given to continuation of EPPO Alert listing and to making the pathogen an EU/EPPO quarantine pest. As a result of the PRA, in the summer of 2001, Defra's Plant Health and Seeds Inspectors (PHSI) for England and Wales commenced limited surveys for the as yet unnamed *Phytophthora*.

After the pathogen was formally named as *P. ramorum* in October 2001 (Werres and others 2001) a third formal U.K. PRA was published in January 2002 (Jones 2002) with an accompanying Datasheet (unpublished). In February 2002, as a result of the ongoing survey work, Defra's Plant Health and Seeds Inspectors (PHSI) submitted one symptomatic plant of *Viburnum tinus* to CSL from a garden centre in southern England, and it was confirmed to be infected with *P. ramorum* (Lane and others 2003). This was the first U.K. record of *P. ramorum.*

Following on from the PRA work, U.K. (England) legislation aimed at *P. ramorum* was enacted in May 2002 (Anon. 2002a). This was somewhat earlier than EC legislation which came into force in September 2002 (Anon. 2002) based largely on U.K. actions. The U.K. (England) legislation required that susceptible plant material (listed as 11 genera at this stage) entering England from the USA must be accompanied by a Phytosanitary Certificate (PC) with an Additional Declaration (AD) that it originated in an area free from *P. ramorum.* Post-entry controls were also required. The U.K. (England) legislation was revoked and replaced in November 2002 (Anon. 2002b) reflecting the first EC requirements (Anon., 2002). (NB: Generally speaking, once enacted, EC legislation is usually adopted by individual EU Member States in domestic legislation; thus the Devolved Administrations of the U.K. (England, Wales, Scotland and Northern Ireland) each have their own legislation). The EC legislation broadened controls on imports of susceptible material not just from the USA, but also from other non-EU countries and had requirements for controls on movement of susceptible plants within the EU, as well as controls on outbreaks, and a requirement for EU Member States to conduct surveys to be reported back to the EC by December 2003. One other European PRA was prepared by the Netherlands in October 2002 (de Hoop and others 2002) to ensure that phytosanitary measures arising from the new EC legislation to be taken in that country were technically justified.

Meanwhile, to address the uncertainties in the U.K. PRA, the U.K. research programme for *P. ramorum* commenced in the spring of 2002. The programme dealt initially with the immediate needs, which were to develop an effective diagnostics capability and to undertake host range testing, both of which were needed to support the inspection and surveillance efforts for interceptions and outbreaks. The long-term

research effort was aimed at developing our understanding of the risk of establishment and spread. This was done by laboratory and field studies of the biotic and abiotic factors affecting sporulation, dispersal, germination, infection processes, survival and population behaviour. Some work on climatic matching was undertaken to develop the prediction of the risk of establishment. One area that was not fully developed was an examination of the economic implications in terms of the direct effects that the pathogen would have in the long-term. An evaluation of risk management strategies was also done by laboratory and field experiments. All of the information generated was intended to be used to develop the PRA and to inform the policy makers of the risk and the management options. Details of Defra-funded research can be found at http://www.defra.gov.uk/planth/pramorum6.htm.

Accounting for ongoing research results, literature, and findings of *P. ramorum* in the EU and North America, the PRA (and Datasheet) were updated again and the revised PRA was published in March 2003 (Jones and others 2003) and revised further and published again in October 2003 (Sansford and others 2003). This last revision pre-dated the first tree findings in the U.K. and the Netherlands in late October 2003. A full update of the Datasheet was prepared during the following year (Sansford and Brasier 2004) and a draft PRA, but prior to completion, the first U.K. findings of the new pathogen *Phytophthora kernoviae* (also in October 2003, see below) and the expansion in its host range over the following year led to changing priorities and the PRA work for *P. ramorum* was put on hold.

EC legislation for *P. ramorum* was amended in April 2004 (Anon. 2004) accounting for changes in host range and assessed risk and again in March 2007 (Anon. 2007). Current measures still require official surveys to be reported back to the EC at the end of the year, and broadly-speaking, import and internal movement controls of rhododendron, viburnum and camellia (the three most commonly affected traded genera in the EU) with statutory action to be taken on findings.

In 2007 a full update of the U.K. Datasheet accounting for the results of the full U.K. research programme, key aspects of the EU and USA research programme, as well as EU and North American survey findings was prepared (Woodhall and Sansford 2007) to re-examine the risks to the U.K. and to suggest risk management options in preparation for a U.K. policy review for *P. ramorum* in 2008. The pathogen is now known to affect more than 70 plant genera in 33 different families. Its origin is still unknown and it is still only recorded in North America and the EU where it is found both outdoors and in the nursery trade which is where the majority of U.K. findings have been made. CSL/FR studies in the U.K. undertaken to date support the view that rhododendron foliage is acting as the primary inoculum source for infection of tree stems in the U.K. leading to bleeding cankers (summarised in Woodhall and Sansford 2007). Clearance of invasive *Rhododendron ponticum* in affected woodlands has reduced inoculum loads and reduced the risk to trees; this is one of a number of risk management options for current and future consideration in the U.K. In the EU, only the U.K. and the Netherlands have reported trees with bleeding cankers caused by *P. ramorum* and these are all associated with infected rhododendron. In contrast to the USA, tree mortality is minimal. *Phytophthora ramorum* is likely to continue to be locally damaging in the U.K., especially in managed ornamental gardens with established susceptible plants, or woodlands infested with rhododendron, especially *R. ponticum*, and coastal valleys in the south and west of the U.K. The current U.K. PRA for *P. ramorum* is at http://www.defra.gov.uk/planth/pra/sudd.pdf

A PRA to examine the risks from *P. ramorum* to trees, woodland ecosystems and other environmentally important habitats (e.g. heathlands) in the EU and the hardy ornamental nursery industry as well as to public gardens will be finalised in 2008. This PRA will be the culmination of a three year EU-funded research project 'RAPRA' (http://rapra.csl.gov.uk/) which aimed to provide scientific information to produce a comprehensive risk assessment for Europe as well as risk management options. The consortium that has undertaken the work to complete the PRA comprises 8 organisations in 5 European countries and one in the USA. The RAPRA Project commenced in January 2004. Work has been done on pathogen distribution and spread, host range, epidemiology, and mating types as well as economic impact and risk management. The RAPRA PRA will be used to inform the review of EC legislation in 2008.

North American risk assessments/analyses undertaken by various Government Agencies can be found at http://www.cnr.berkeley.edu/comtf/html/pest_risk_assessments.html#EU

Phytophthora ramorum continues to pose a threat to the managed and unmanaged environment (woodlands, gardens, possibly heathland), the timber and ornamental plant trade and the tourism industry (i.e. associated with historic gardens) in the U.K., EU, North America and to other countries where it is yet to be reported or introduced.

Phytophthora kernoviae

A climate-matching model using the CLIMEX programme (Sutherst and Maywald 1985) was run by CSL in March 2003 (R. Baker, CSL, U.K., *personal communication*, 2003) to identify which areas of the U.K. were most at risk from *P. ramorum*. This work was based upon identifying climatic similarities between the U.K. and Oregon/California where *P. ramorum* occurs. The outcome, was that based upon a more similar match with Oregon alone (compared to California), the south and west of the U.K. were identified as potentially at high risk of *P. ramorum* establishing there. This information was used to inform the Defra PHSI surveys for *P. ramorum* required under emergency EC (and U.K.) legislation since 2002 (Anon. 2002, 2004, 2007). Surveillance by Defra PHSI was intensified in Cornwall and as a result of this a new and unknown *Phytophthora* species was isolated by CSL from symptomatic rhododendron (*R. ponticum* and *Rhododendron* spp.) from an established woodland area adjoining a commercial nursery in Cornwall in late October 2003. Concurrently, FR isolated a similar *Phytophthora* from a large bleeding canker on a mature beech tree (*Fagus sylvatica*) and from an adjacent rhododendron with foliar symptoms adjacent to the tree at a second woodland site located 23km from the first site. The pathogen was causing widespread foliar necrosis and shoot dieback of the rhododendrons. By December 2003 the isolates were considered to be the same organism and all samples submitted to CSL by the PHSI since that date for testing for *P. ramorum* were also tested for it.

Having identified a new problem, a joint CSL/FR PRA for *Phytophthora* taxon C sp. nov. (the first informal name for the pathogen) was completed (February 2004) (Sansford and others 2004) examining the risks that the pathogen posed to the U.K. As with the first PRA for *P. ramorum*, there was much uncertainty because this was a

new species of *Phytophthora*, but clearly the pathogen posed a risk to at least beech and rhododendron and had potential to spread geographically as well as to other as yet unidentified hosts. It was also considered to be an exotic introduction but its origin was unknown; consequently management of entry pathways was difficult. The PRA recommended destruction of infected plants along with further surveys to determine the true distribution of the pathogen both in the U.K. and the EU. Host-range testing was suggested to determine which other species were at risk and to inform future inspection and surveillance work. Accurate diagnostic methods were developed by CSL to differentiate the pathogen from *P. ramorum*. Long-term research akin to that being undertaken for *P. ramorum* was also recommended to understand the epidemiology of the pathogen and to develop control strategies. Up to September 2004 approximately 5000 samples submitted by the PHSI to CSL for testing for *P. ramorum* were also tested for *P. taxon* C sp. nov. The pathogen was found on a wider range of hosts and sites; new findings emerged in south Wales and the first nursery finding occurred in Cheshire. The PRA was updated several times to account for these findings culminating in September 2004 (Sansford and others 2004a). Emergency legislation aimed at containing the pathogen within a specified area of the south-west of England with a view to eradication was implemented in 2004 (Anon., 2004a). Named in 2005 as *P. kernoviae* (Brasier and others 2005) CSL and FR have mainly concentrated on undertaking host-range testing and studying the efficacy of rhododendron clearance (the main source of inoculum) as a way of reducing the risk the trees, with limited epidemiological studies. Of particular significance was the 2006 report of its presence in New Zealand on cherimoya (*Annona cherimola*) since at least 2002 (Anon., 2006; NAPPO, 2006; EPPO, 2006) which now dates back to at least the 1950s. Ramsfield and others (2007) reported[1] that an unknown *Phytophthora* known as the Tokoroa *Phytophthora* is in fact *P. kernoviae* and was first found in the 1950s, under stands of symptomless *Pinus radiata* (radiata pine). This has identified a potential past/future pathway for entry to the U.K. and other countries. A new PRA was completed in November 2007 (Sansford 2007) accounting for all new information since September 2004 to support a U.K. policy review in 2008. n examining the risk of entry, detailed CSL records of imports of ornamentals into the U.K. from New Zealand permitted under a derogation from a prohibition up until 1987 shows large quantities of known susceptible hosts (magnolia, rhododendron, *Pieris*) entering the U.K. between 1977 and 1985. Imports continued after 1985 but no details were documented as they were no longer banned, provided they were accompanied by a PC. Whilst none of these ornamentals have been recorded as hosts in New Zealand no surveillance of nurseries has been reported there. *P. kernoviae* has a more limited host-range than *P. ramorum*; by January 2008 it was known to affect 16 genera in 10 families. Unlike *P. ramorum* it has been found on very few (3) U.K. nurseries with most findings being outdoors in the south-west of England, south Wales and one (eradicated) location in the north-west of England. As with *P. ramorum*, CSL/FR studies in the U.K. support the view that rhododendron foliage is acting as the primary inoculum source for infection of tree stems in the U.K. leading to bleeding cankers (summarised in Sansford, 2007). Clearance of invasive *R. ponticum* will reduce the inoculum load in woodlands and gardens for *P. kernoviae* (as well as *P. ramorum*). The 2007 PRA is now being updated to account for the first finding in Scotland and the first finding of the pathogen on an environmentally important species *Vaccinium myrtillus* (bilberry) both of which occurred in December 2007. *Phytophthora kernoviae* continues to pose

[1] Not reported in the abstract; reported in the platform presentation.

a threat to the managed and unmanaged environment (woodlands, gardens, possibly heathland), the timber and ornamental plant trade and tourism (i.e. associated with historic gardens) in the U.K., as well as New Zealand. There is a risk to other countries where it is yet to be reported or introduced.

Phytophthora lateralis

Understanding that existing ('*old*') *Phytophthora* species can also move in trade and have potential to pose new risks, co-operation at the regional level under the auspices of EPPO has facilitated the development of the first ever EPPO PRA for *Phytophthora lateralis* and recommendations for appropriate regulation, this time to protect the ornamental trade.

EPPO added *P. lateralis* to their Alert List in January 2001 as a result of a literature search for '*sudden oak death*' which elicited various papers reporting significant tree mortality on *Chamaecyparis lawsoniana* (Port-Orford-cedar) in the U.S. in forests as well as in nurseries and gardens. The pathogen is assumed to have been introduced to North America in 1923 from an unknown origin (Roth and others 1972, Kliejunas and Adams 1981, Erwin and Ribeiro 1996). *Phytophthora lateralis* is considered to be an exotic introduction to North America from an unknown source (Hansen and others 2000). France and the Netherlands produced draft (unpublished) PRAs in 1999 and 2001 respectively in response to findings in France, and the U.K. prepared a draft Datasheet in early 2006 which was provided to EPPO to support their first ever EPPO '*Performing PRA*' panel who also met in early 2006. *Phytophthora lateralis* was isolated and identified from *C. lawsoniana* in 1996 and 1998 in different parts of France (Hansen and others 1999); its introduction to France is suspected to have been from North America but this could never be confirmed. It was suggested that this probably stemmed from a single infestation of young, potted, greenhouse-propagated cedars in a commercial nursery. Introduction to the Netherlands was first noticed in 2004 when a survey was conducted of 350 nurseries with *C. lawsoniana*. *Phytophthora lateralis* was isolated from the stem bases of *C. lawsoniana* plants from one isolated nursery but the origin of the pathogen was unknown, especially as there were no associated imports of the affected plant material and propagation material originated from the affected nursery (Meffert 2005). Both the French and Dutch outbreaks are considered to be eradicated. The EPPO Panel considered that *P. lateralis* has been introduced occasionally to new areas from unknown origins but that it was not established in the EPPO region. The Panel agreed that there was a risk of entry (based upon the French and Dutch outbreaks) although imports of the main host, *C. lawsoniana* into EU countries (within the EPPO region) is banned, as with the exception of specific derogations for bonsai, the importation of plants of *Chamaecyparis* from countries outside of Europe is prohibited under EU legislation (Anon. 2000). Soil from North America is also prohibited. Entry is most likely on roots of *Taxus brevifolia* (Pacific yew, the other main host) and possibly other ornamental plants from affected areas of North America (or from the unknown origin of the pathogen). Entry may also be possible as a contaminant of growing media or soil associated with non-host ornamental species.

The probability of establishment in the EPPO region was evaluated as high (host plants are cultivated in the PRA area, some parts of the PRA area have very favourable climatic conditions, nursery production practices are favourable to the pathogen) and economic impacts would mainly arise from losses of host plants on

specialist nurseries. Environmental impacts were thought likely to be low because the main hosts (*C. lawsoniana*, *Chamacyparis* spp. and *T. brevifolia*) are not key components of natural ecosystems in the PRA area. We agreed that P. lateralis fulfilled the criteria of a quarantine pest. Having identified the main pathways of entry, the Panel proposed risk management options. *Phytophthora lateralis* was moved from the EPPO Alert List to the EPPO A1 list of pathogens as a result of the EPPO PRA.

The U.K. PRA (Woodhall and Sansford 2006) which followed the draft Datasheet recommended that consideration be given to EU surveys of susceptible hosts to ensure country freedom and, if deemed appropriate, listing *P. lateralis* as a II/AI quarantine pest for the U.K./EU with specific requirements for the relevant pathways. This PRA and the proposed measures are subject to consultation though the Defra website.

Discussion and Conclusions

PRA is a structured process that allows us to evaluate risks from exotic pests, to identify uncertainties, to recommend suitable phytosanitary measures and to ensure that phytosanitary legislation enacted to manage the risk is re-evaluated in the light of new knowledge resulting from research targeted at addressing the uncertainties and other sources of information. In the U.K., the link between pest risk analysts, researchers, funders, inspectors and policy makers is a strong one. Under the current international phytosanitary framework, led by the Secretariat of the IPPC and required by the WTO SPS Agreement (WTO 1995) there is no process by which phytosanitary measures can be enacted against an unidentifiable cause. Brasier (2005) is critical of this system and states (Brasier 2007) that '*international plant health protocols are list-based, allowing regulation only of named organisms*'. However, it is possible to regulate at the genus level provided the organism is identified as being unique (FAO 2004). Thus the first PRAs for *P. ramorum* and *P. kernoviae* were for '*an unknown Phytophthora*' and emergency legislation was enacted in the U.K. for both organisms as quickly as possible following PRA production. Our experience with these pathogens has led to a joined-up and proactive approach to tackling new phytosanitary problems as far as the legislative process allows.

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Containment and Eradication of *Phytophthora cinnamomi* in Native Vegetation in South-Western Australia and Tasmania[1]

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Abstract

The aim of our experiments was to develop protocols that can be used to contain and eradicate spot infestations of *P. cinnamomi* that, if untreated, are likely to threaten extensive areas of native vegetation or areas of high conservation value. Treatment regimes were guided by two assumptions: 1) within the selected sites, transmission of the pathogen is by root-to-root contact, and 2) the pathogen is a weakly competitive saprotroph. In Western Australia (WA), treatment and control plots were set-up along an active disease front within scrub-heath vegetation dominated by *Banksia* spp. Treatments, applied sequentially and in combination, included: 1) destruction of the largest plants within disease free vegetation forward of the disease front; 2) destruction of all plants to create a 'dead zone'; 3) installation of physical root barriers and subsurface irrigation for the application of fungicide/s; 4) surface applications of fungicides selective against oomycetes (triadiazole and metalaxyl-M), and 5) surface injection and deep (± 1 m) treatments with Metham-sodium. In a separate experiment in Tasmania (TAS), combined treatments including vegetation removal, Ridomil and Metham-sodium and root barriers, or Ridomil and root barriers alone, were applied to experimental plots within active disease centres in *Eucalyptus-Banksia* woodland.

In the WA experiment, *P. cinnamomi* was not recovered (by soil baiting) from plots after treatment with Ridomil and metham-sodium. In the TAS experiment, similar results were achieved with combined treatments (vegetation removal + Ridomil + metham sodium) but in plots treated with Ridomil alone, recoveries of *P. cinnamomi* increased after initially showing a significant reduction in recoveries.

Introduction

Phytophthora cinnamomi, and disease caused by it, is listed as one of five key threatening processes affecting biodiversity in Australia. The area of native vegetation affected by *P. cinnamomi* exceeds many hundreds of thousands of hectares in Western Australia (> 635 000

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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ha; Department of Environment & Conservation 2006), Victoria and Tasmania, and tens of thousands of hectares in South Australia (Environment Australia 2001). Although infestations within the conservation estate are widespread and frequently extensive, large areas remain free of the pathogen and may be protectable. The control and management of *P. cinnamomi* in natural ecosystems raises considerable challenges in terms of managing the impact of the pathogen in diverse plant communities (Hardy and others 2001). Apart from the application of control measures designed to prevent human vectoring of the pathogen, there has been little testing and operational use of control measures in native vegetation apart from the use of phosphite (Hardy and others 2001). Phosphite has been shown to slow, but not stop, the autonomous spread of P. cinnamomi in native vegetation (Shearer and others 2004).

The aims of the experiments were to contain and eradicate *P. cinnamomi* within natural infestations in native vegetation. To achieve the aims a combination of methods were applied sequentially to experimental plots within sites naturally infested with *P. cinnamomi*. Methods and the rationale for their employment included: 1) localised destruction of vegetation to deny food resources to the pathogen, 2) physical root barriers, to prevent root intrusion into pathogen infested soil, particularly by plants with extensive lateral root systems, and 3) direct attack on the pathogen with fungicides and fumigant/s, starting with oomycete specific compounds and progressing to more robust treatments. The vulnerable or endangered grass-tree *Xanthorrhoea braceata* occurs within the site experimental site in Tasmania. A fungicide + root barriers only treatment was included in the experiment with the aim of assessing its potential as a non-destructive method of protecting individual plants at risk of destruction by *P. cinnamomi.*

Methods and Material

Experimental Sites

Tasmania (TAS)

The experimental site (146.62ºE, 41.16ºS) is located within Narawntapu National Park on the north central coast of Tasmania. The soil is an Aeric (or Semiaquic) Podosol (after the Australian soil classification of Isbell 2002), derived from Quaternary sands that are greater than 3 m deep. The vegetation is classified as *Eucalyptus amygdalina* coastal forest and woodland (Harris and Kitchener 2005), with scattered *Eucalyptus viminalis* and an understorey of *Banksia marginata* and scattered to dense *Xanthorrhoea australis* in heath comprised principally of species within the families epacridaceae and papilionaceae. The climate is temperate. Estimates for the mean maximum in the hottest month is ca. 21ºC, mean minimum in the coldest month ca. 5ºC, and mean annual rainfall is ca. 800 mm.

Western Australia (WA)

The experimental site (118.72ºE, 34.57ºS) is located near Cape Riche. The soil is a Rudosol, derived from fine (< 0.8 mm) aeolian Quaternary sands that are greater than 2.5 m deep. The vegetation is a scrub-heath dominated by *Banksia* spp., principally *B. attenuata* and *B. baxteri*, with an understorey that includes a diverse range of other woody shrubs from species within the families myrtaceae, proteaceae, epacridaceae and papilionaceae. Mean annual rainfall is ca. 490 mm.

Isolates of *P. cinnamomi* from both sites were all of the A_2 mating type.

Site treatments

Western Australia

Treatment and control plots (*n* = 7 each) were set-up along an active disease front, with each plot covering 10 m of disease front, and separated from adjacent plots by a buffer of not less than 5 m. Treatments, applied sequentially and in combination, included: 1) Destruction of the largest plants (principally *Banksia* spp.) to a distance of 10 m on the disease free side of the disease front, by felling and application of glyphosate to stumps (May 2006). 2) Removal of all plants to a distance of 4 m from the disease front, by slashing and applications of herbicides (triclopyr and glyphosate (Aug-Sep 2006). 3) Installation of HDPE root barriers (90 cm deep x 1 mm thick) to ca. 80 cm depth and, subsurface irrigation for the application of fungicide/s (Mar 2007). 4) Surface applications of Terrazole (triadiazole, 10 g/m^2 a.i.; Jun and Aug 2006). Applications of Ridomil 25G (metalaxyl-M, 2.5 g/m^2 a.i.; Jun and Aug 2007). 5) Surface injection, at 15 x 25 cm injection point spacings and to ca. 20 cm depth with Metham-sodium (90 g/m^2; Jun 2007). 6) Deep (± 1 m) treatments with Metham-sodium, 0.5l/m^2 applied via 40 mm PVC vertical tubes installed at 1 m centres, between the disease front and root barrier (Sep 2007).

Tasmania

Experimental plots (5 m x 5 m) were selected from areas showing signs of recent pathogen activity within an extensive area of infestations. The experiment was an unbalanced design, with four plots receiving all treatments (vegetation removal + fungicide + fumigation), four plots were treated with fungicide only (after minor clearance of organic litter and woody debris), and seven plots were untreated. Root barriers (120 cm deep x 1 mm thick) were installed at 80-90 cm deep around all treated plots (complete treatments and fungicide only) with the aim of preventing reinfestation of plots by root borne *P. cinnamomi.* Six of the 11 larger species of terrestrial mammals known to occur within the park could be considered as potential vectors of *P. cinnamomi* because of their digging habits. Given the large number of animals present, and the significant amount of soil disturbance caused by them, exclusion fencing was installed around each plot. Vegetation removal, barrier and fence installation, fungicide application, and injection with metham-sodium, were completed in Apr 2007. Initial treatments were followed by a further fungicide treatment (Aug 2007) and deep application of metham-sodium (Sep 2007). Ridomil 25G (2.5 g/kg Metalaxyl-M) was applied at 100 g/ m^2. Surface injection with Metham-sodium (90 g/m^2) to 15- 20 cm deep was at 150 cm x 250 cm spacings. Deep treatment to ± 1 m with Metham-sodium, (0.75 l/m^2) was applied via 40 mm PVC vertical tubes installed at 1 m spacings. The calculated potential mass of methylisothiocyanate (MITC; the active product from the hydrolysis of metham-sodium) applied to the plots was 180 g/m^2.

Assessment

In the WA experiment, soil sampling was systematic and stratified in two ways: (1) soil depth; sampled at 0-25 cm, 25-50 cm, 80-100 cm and 1.5 m, and (2) distance from disease front (as surveyed in Feb 2006); sampled at 0.5 m and 2.5 m from the disease front, with three sample points at each distance. In the TAS experiment, plots

were sampled systematically at five points, and at three depths per sampling point (0-25 cm, 25-50 cm, and 80-100 cm). Soil samples with roots (120-150 g wet wt.) were flooded and baited with *Lupinus angustifolius* seedlings. Seedling radicles were plated onto NARPH medium (Hüberli 2000), and *P. cinnamomi* was identified by colony form and micro morphology.

Results

Recoveries of *P. cinnamomi* from the WA experiment are shown in Figure 1. A trend in increasing recoveries of *P. cinnamomi* in untreated plots, at 2.5 m from the original disease front (figs 1A-D), showed that the pathogen was active and the infestation was expanding. Surface treatments with terrazole had no significant effect on recoveries of *P. cinnamomi* (recoveries at 0.5 m in treated plots, figs 1A and B). There were no recoveries of *P. cinnamomi* from any of the treated plots after ridomil treatments and surface injection with metham-sodium (fig 1C), or after the additional deep treatment with metham-sodium (fig 1D). There were no recoveries from the 0.5 m mark in treated plots in the last two assessments (figs 1C and 1D), in contrast to the previous harvest where recoveries of *P. cinnamomi* at the same distance in treated plots were similar to untreated controls (fig 1B).

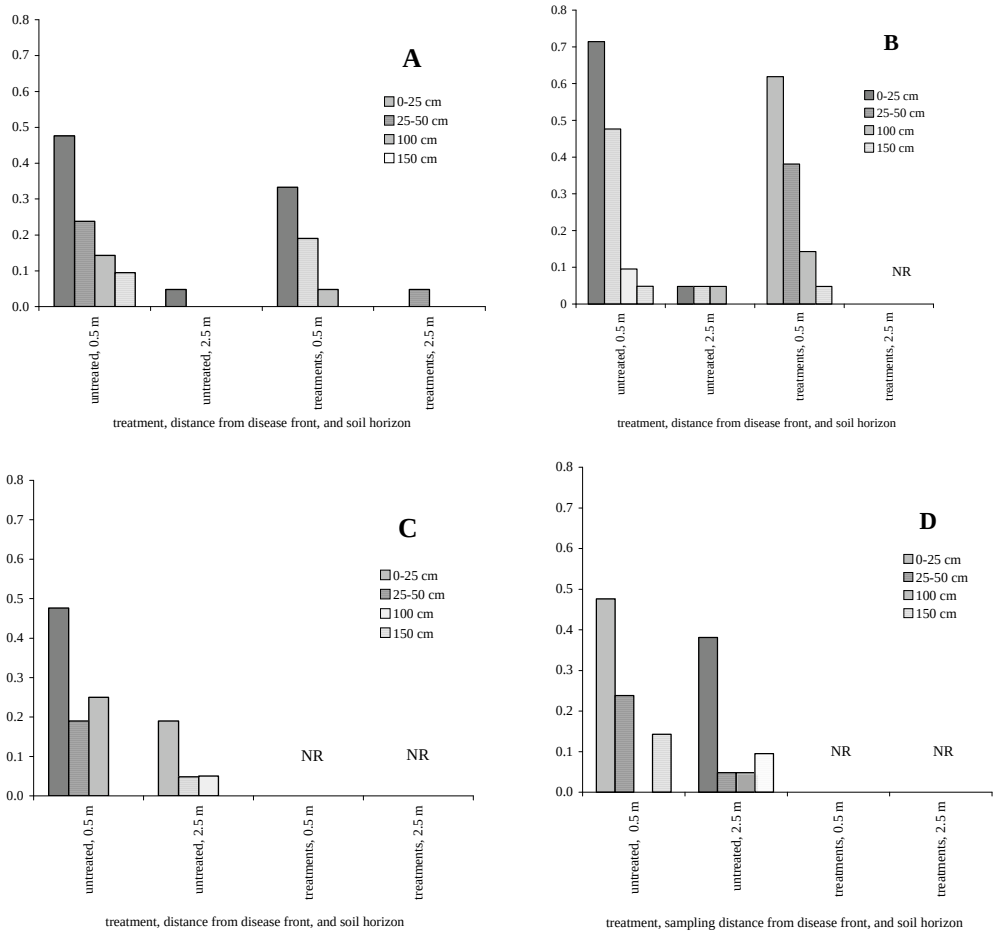

Figure 1—Effect of treatment on recovery of *Phytophthora cinnamomi*, Cape Riche, Western Australia. Untreated plots (controls, + *P. cinnamomi*) *n* = 7, treatments (+ vegetation clearance + fungicide + fumigation) *n* = 7. *n* = 21 for each histogram bar. A. After first Terrazole application and partial vegetation clearance (Jul 2007). B. After second Terrazole application and completion of vegetation clearance (Dec 2006). C. After ridomil treatments and surface injection with metham-sodium. (July 2007). D. After deep treatment with metham-sodium (Nov 2007). Distances (m) are from the active disease front (surveyed Feb 2006). NR = no recovery of *P. cinnamomi.*

Results from four assessments from the TAS experiment are shown in Figure 2. Recoveries of *P. cinnamomi* in the pre-treatment assessment showed a similar distribution of the pathogen between plots and within the soil profile to 1 m (fig 2A). In plots treated with Ridomil alone, recoveries of *P. cinnamomi* were reduced significantly ($p < 0.001$), with a trend for increased recoveries with soil depth (fig 2B). In fungicide only treated plots, *P. cinnamomi* was recovered at all assessments and post-treatment deaths of *X. australis* had also occurred. *Phytophthora cinnamomi* was recovered at low frequency at 1 m in treated plots after the first treatment (ridomil + metham-sodium surface injection; fig 2B) but no further recoveries were made from any of the complete treatment plots after further application of ridomil and deep treatment with metham sodium (figs 2C-D).

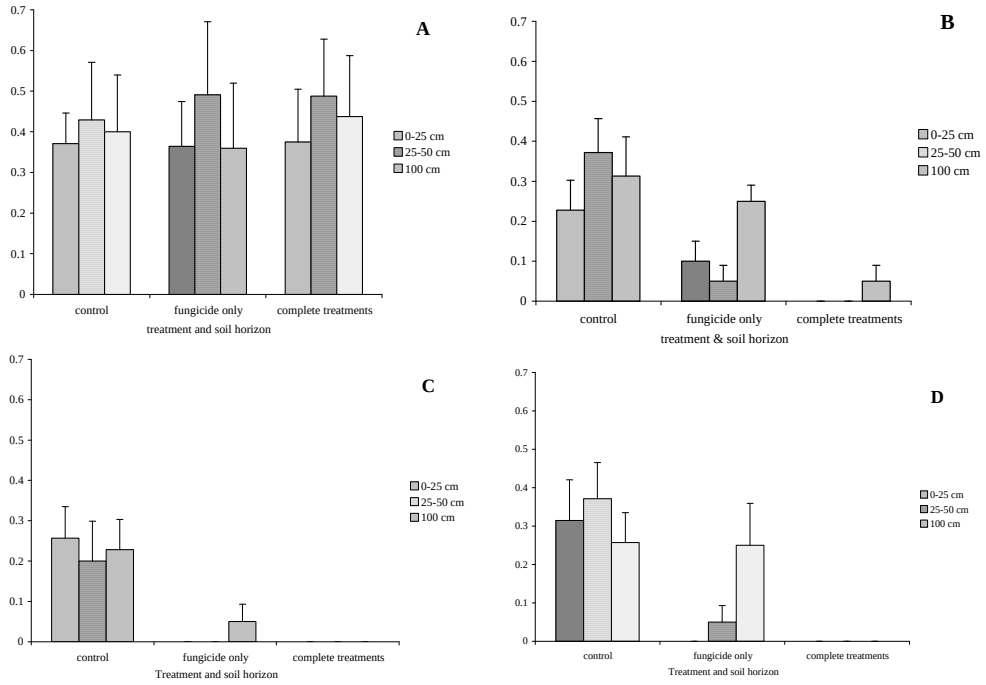

Figure 2—Effect of treatment on recovery of *Phytophthora cinnamomi*, Narawntapu National Park, Tasmania. Control (untreated) plots *n* = 7, fungicide only *n* = 4, complete treatments *n* = *4.* *n* = 20 for each histogram bar in fungicide only and complete treatments, and *n* = 35 in each histogram bar in controls, Error bars are one standard error from the mean. A. Assessment before treatment, April 2007. B. After initial treatment, June 2007. C. October 2007, after second fungicide application, and deep treatment with metham-sodium. D. December 2007.

Discussion

Physical root barriers at both sites remain untested. From the results in the WA experiment so far, the pathogen has been stopped short of the barriers by the combination of other treatments. The rate of progress of disease over the last 4-5 years is estimated to be 1-2 m/yr, too slow to expect deaths of susceptible plants beyond root barriers, if the system has failed. A separate experiment is in progress, designed to test the efficacy of root barriers alone. The WA experiment was not a fair test of the efficacy of terrazole because there was almost certainly inadequate rainfall after both applications of the fungicide to enable its infiltration into the soil profile. Rainfall at the site over any 24 h period within the critical period after either application of Terrazole was not more than 6 mm. In the WA experiment, incidental observations on unintended treatment effects included: 1) A decline in plant health, including deaths, of individual plants in some understorey species where large plants had been removed, that was probably caused by increased exposure. 2) In treated plots, an increase in soil moisture within the zone where vegetation was completely removed. Increased soil moisture is likely to be more favourable to the survival of chlamydospores (Weste and Vithanage 1979) but, given the soil type, would be unlikely to enhance lateral movement of the pathogen within the soil profile. 3) Soil erosion by wind and water within the 'dead zone' of treated plots has been minimal (after > 1.5 yr), but could be a problem in the longer term.

Based on recoveries of the pathogen in both experiments, a combination of vegetation destruction, ridomil and metham-sodium applications shows promise as a method to at least contain, and possibly eliminate, the pathogen within small infestations. In neither experiment was fumigation with metham-sodium alone used as a treatment. Therefore, we cannot make any conclusions about the efficacy of metham alone, or metham x metalaxyl interactions. Metham-sodium was used alone in an attempt to eradicate *P. cinnamomi* from plots within infested eucalypt forest by Weste and others (1973), with some success, where *Phytophthora cinnamomi* was not recovered from two of three sites for at least 18 months after treatment, but reinfestation from adjacent infested forest occurred at one site. Vegetation removal and metalaxyl was used, among other treatments, in an eradication experiment in *Banksia* woodland by Hill and others (1995). Very large reductions in recoveries of *P. cinnamomi* were achieved at 0.1-0.4 m soil depth for up to at least 20 months after treatment, however the pathogen was always recovered at later assessments (up to 20 months). Persistence of metalaxyl (half-life 10-82 d; Davison and McKay 1999) and MITC (2 percent of applied amount after 24 d; Zhang and Wang (2002)in agricultural soils is low, but further assessments will be required to determine whether the pathogen has been eliminated or has only been suppressed by residual fungicide/s. From long-term monitoring of *P. cinnamomi* in native vegetation (Victoria, Australia), Weste (2003) did not recover the pathogen in four of six sites after 30 years, and observed regeneration of highly susceptible species. If the autonomous spread of the pathogen can be arrested, and adequate measures to prevent vectoring of the pathogen off-site by animals and people can be implemented, then there is some prospect for limiting damage and recovery of vegetation to its original state. In both experiments we chose sites where a combination of characteristics that included topography, soil type, vegetation (species composition and structure), and climate, were favourable to achieving positive results. However, extensive areas within south-western Australia, south-eastern Australia and Tasmania have similar site

characteristics to those used in both experiments, therefore the methods described could be applied on a broader scale, with modification to suite individual sites.

Acknowledgements

This project was supported by the Department of Environment and Water Resources (Commonwealth of Australia). We wish to acknowledge the following for assistance in the field and laboratory: Tasmania. Brigid Morrison, Mark Dobrowolski, Tim Wardlaw, Mike Ikolowski, Anthony Timmerman, Gary Sutton, Judy Westbrook, Alena Hrasky, Andrew Ford, Mark Hedditch, Dave Widger. Western Australia. John Watson, Sarah Barrett, Greg Freebury, Deon Utber, Joanna Young, Rodney Armistead, Chen Yinglong, Daniel Huberli, Nari Williams, Dianne White, Joshua Gilovitz, Trudy Paap, Peter Scott, Georgia Scott, Christine Davis, Jessica Davis, Bryoni Palmer, Sarah Jackson, Nathalie Long.

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Does Abiotic Stress on a Plant Influence Phosphite Protection to *Phytophthora cinnamomi*?[1]

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Introduction

Large areas of indigenous forests, *Banksia* woodlands and heathlands in Australia are devastated by Phytophthora dieback disease caused by *Phytophthora cinnamomi* (Weste 1994). In southwestern Australia, some 50 percent of the 5710 plants endemic to the region are susceptible (Shearer and others 2004a). Phosphite has been shown to be effective in controlling this pathogen's impact on a wide range of plant species across different families (Hardy and others 2001).

Recently, disease extension was reduced after phosphite treatment even after fire (Shearer and others 2004b). However, very little is known about the influence of a plant's physiological status at the time of phosphite application on the subsequent efficacy of phosphite treatment to control Phytophthora dieback disease. The key seasonal stresses in an Australian ecosystem of fire and flooding are explored.

Materials and Methods

Adenanthos cuneatus (resprouter), *Banksia attenuata* (resprouter) and *B. baueri* (reseeder) are all susceptible to *P. cinnamomi* and are responsive to phosphite treatment. These species were selected within four plots in an area of the Stirling Range National Park (Western Australia) that was scheduled for a fuel-reduction burn. Treatments of the plots were: 1) phosphite spray without fire, 2) phosphite spay with fire, 3) no phosphite spray without fire, and 4) no phosphite spray with fire. Phosphite treatment was applied six weeks before the fire or nine months post-fire when all resprouter species had sufficient foliage. Periodic measurements during the experiment include leaf water potential, leaf gas exchange, lesion lengths on inoculated stems, and phosphite concentration in leaves, stems, lignotubers and roots.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Waterlogging trials were conducted in the greenhouse using *B. attenuata* and *B. baxteri* (reseeder). The response of these plants and subsequent recovery from waterlogging was examined. In the main trial, a phosphite spray treatment was applied before (Experiment 1) and after (Experiment 2) one waterlogging event of either 3 or 10 days (8 days for Experiment 2) duration. Plant physiology traits, lesion development and phosphite concentrations in leaf, stem and root were taken 1 week, 1 month and 4 months after the phosphite treatment.

Results and Discussion

Preliminary data for the fire experiment show that phosphite was present in the roots in the pre- and post-fire spray treatments. These data also suggest that lesions were reduced in all three species treated with phosphite. Further work is ongoing in this experiment.

For the waterlogging experiments, phosphite in plant tissue was at similar levels for each species and was not affected by waterlogging in Experiment 1, Week 1 harvest (fig 1), but lesions on *B. baxteri* stems were not reduced in treated plants as they were for *B. attenuata*. Photosynthesis and water potentials were reduced for waterlogged *B. attenuata* especially at the ten day waterlogging treatment, but were not impacted in waterlogged *B. baxteri*. Leaf water potentials, leaf gas exchange, lesion lengths on inoculated stems, and phosphite concentration in leaves, stems, and roots measured at one and four months for Experiment 1 and all measurement periods for Experiment 2 will be presented in a future publication.

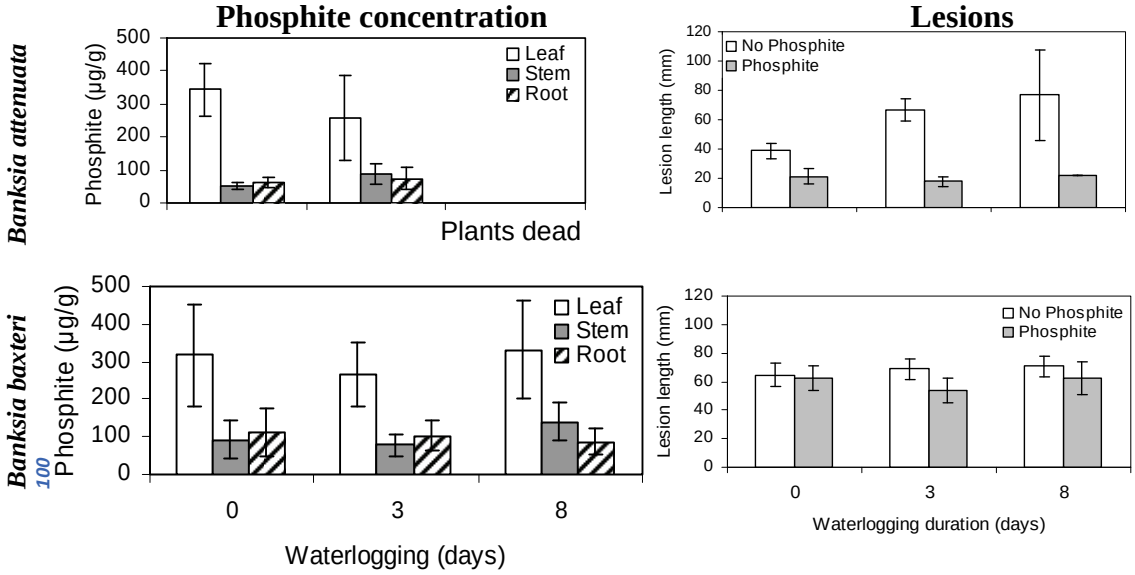

Figure 1—Mean (± standard error) phosphite concentrations in plant tissue and lesion lengths in stems 1 week after inoculation with *Phytophthora cinnamomi* of *Banksia attenuata* and *B. baxteri* seedlings that had been sprayed or not (controls) 1 week prior to the inoculation. Plants were treated with phosphite 1 week after waterlogging for 0, 3 and 10 days.

Acknowledgements

We thank O. Fry, S. Jackson, T. Johnson, J. O'Brien, B. Palmer, G. Scott, and P. Scott for assistance in the field and laboratory. The project 19.2.3 was funded by the Department of Environment and Water.

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In Vitro Activity of Plant Crude Extracts and In Situ Protective Effect of *Phlomis purpurea* Against *Phytophthora cinnamomi*[1]

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Abstract

The effect of ethanol extracts of *Arbutus unedo* (Ericaceae), *Helichrysum stoechas* (Compositae) and *Phlomis purpurea* (Lamiaceae) was evaluated against mycelial growth of *Phytophthora cinnamomi*. All plant extracts exhibited anti-*P. cinnamomi* activity. The most effective extract was from roots of *P. purpurea*, with a maximum percentage of inhibition of 78.3 percent at 1 mg/ml. *P. purpurea* was shown to significantly protect the susceptible *Quercus ilex* L. (holm oak) from *P. cinnamomi* infection, in a glasshouse trial. This spontaneous plant reduces the soil *inoculum* potential suggesting a possible supressive ability to reduce the ocurrence of root diseases caused by *P. cinnamomi*.

P. purpurea, is endemic to southern Iberian Peninsula and Morocco and is present in the understorey of *Q. suber* and *Q. ilex* habitat. The present results suggest that it could be a potential candidate to control *P. cinnamomi*, but further research is required, including field trials.

Introduction

Cork oak and holm oak trees are essential components in the agro-ecosystems of the Iberian Peninsula (*montado* in Portugal, *dehesa* in Spain) supporting rich wildlife populations and simultaneously used by farmers to grow cereals, graze animals and, as far as cork is concerned, harvest cork. Cork production in the Iberian Peninsula accounts for 80 percent of mean annual world production (DGRF 2006, APCOR 2006).

Today, these ecosystems are threatened by a decline disease whose main agent has been identified as the soil born phytopathogen *Phytophthora cinnamomi* (Brasier and others 1993, Moreira 2001, Sánchez and others 2002, 2005, Tuset and others 1996). Despite the ecological and socio-economic importance of these systems, efficient control measures are still missing. To control this disease, the use of synthetic fungicides has had limited success and the use of genetically modified organisms is still controversial (Kremer and Motavilli 2004, Motavalli and others 2004, Priest

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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2000). Moreover, in the last few years it has become evident, as a result of public awareness and environmental laws, that new and safer alternatives to synthetic pesticides are needed. The plant world can provide alternatives to existing chemicals as a source of biological natural chemicals extracted or delivered from them (Bais and others 2002, Molina-Torres and others 2004). In particular, the use of resistant native flora to control *P. cinnamomi* could be an environmentally friendly approach.

Several plants from the *Q. suber* and *Q. ilex* habitat were surveyed and *Phytophthora cinnamomi* was not isolated from *Helichrysum stoechas* and *Phlomis purpurea* (Moreira-Marcelino 2001, Moreira and Martins 2005). These plant species could be a potential control of *P. cinnamomi* reducing the amount of *P. cinnamomi* inoculum by producing anti-*P. cinnamomi* compounds.

In the present study, we examined the activity of 3 plant extracts on *P. cinnamomi* mycelial growth and determined whether those plants were or not *P. cinnamomi* hosts. The protective effect of *Phlomis purpurea* towards *Q. ilex* was also determined.

Methods and Materials

Plants and Phytophthora *Isolates*

The plants used to prepare the extracts were collected in *Q. suber* stands in Algarve, south of Portugal (Table 1).

Table 1—Species, collection site, date of collection and plant material used to prepare the extracts

Plant	Collection site	Date	Plant material
Arbutus unedo	Barranco-do-Velho	November, 2005	Leaves
Helichrysum stoechas	Barranco-do-Velho	November, 2005	Whole plant (with dried flowers)
		January, 2006	Whole plant
		June. 2006	Whole plant (with flowers)
Phlomis purpurea	Moncarapacho	January, 2006	Whole plant

Plant material for pathogenicity tests and *in situ* protective effect study of *Phlomis purpurea* against *Phytophthora cinnamomi* was obtained from nurseries (Table 2).

Table 2—Provenence and age of plants used for pathogenicity tests and *in situ* protective effect study of *Phlomis purpurea* against *Phytophthora cinnamomi*

Species	Nursery	Age	Experiment
Arbutus unedo	"Viveiros Florestais Monte Gordo (VFMG)" - Portugal	10 months old	2.1, 2.2
	"Viveiros Florestais de Huelva (VFH)" - Spain	11 months old	2.3
Helichrysum stoechas	"Viveiros Monte-Rosa (VM-R)"- Portugal	6 months old	2.1, 2.2
		6 weeks old	2.3
Phlomis purpurea	"Dir. Reg. Agric. do Algarve (DRAAG)" - Portugal	4 and 2 months old	2.1, 2.2
	"Viveiros florestais de Huelva" - Spain	7 months old	2.3
Quercus ilex	"VFMG"- Portugal	10 months old	2.2
	"Viveiros florestais de Huelva" – Spain	11 months old	2.3
Quercus suber	Aliança Florestal - Portugal	7 months old	2.2

The two *P. cinnamomi* isolates used in the present work, PA37 and PA45, belong to the mycological library of the Laboratory of Molecular Biotechnology and Phytopathology of the "Universidade do Algarve" (UAlg). Their origin, site and isolation date are described in Table 3. Both are A2 mating type.

Table 3—Identification of *Phytophthora cinnamomi* isolates used in the assay.

Isolate	Site	Date of isolation	Origin
PA37	Lagos	May 1995	Soil from *Quercus suber*
PA45	S. Brás de Alportel	May 2000	Soil from *Q. suber*

Plant extracts

The plant extraction protocol was adapted from Carini and others (2001). The dry mass of the plant part used (roots, leaves, flowering tops) were chopped with a grinding-master (Moulinex® 1,2,3). Fifty grams of the chopped leaves or flowering tops were suspended in 350 ml of 70 percent aqueous ethanol or methanol (containing 0.1 percent ascorbic acid to prevent oxidation) and shaken for 72 hours (Güllüce and others 2004). Roots were shaken for seven days (Molina-Torres and others 2004). Ethanol was eliminated by evaporation (Heidolph 94200, Bioblock Scientific I) under reduced pressure at 35°C and, in the case of *H. stoechas,* the aqueous residue washed with methylene chloride (1:1, v:v), to eliminate free aglycons and other non phenolic substances. The extract was concentrated under vacuum (Savant, SC 110A) to almost dryness to obtain a brownish sticky suspension and weighted to constant weight. The crude extract was dissolved in water or 35

percent aqueous ethanol and filtered through a 0.45 μm nitrocellulose filter (Millipore®). The extraction yield for *H. stoechas* was 16 percent whereas for *P. purpurea* was 15 percent.

Assessment of foliar and root symptoms

At the end of each infestation experiment, plants were removed from pots, soil was removed from roots and the latter washed with tap water and kept in water until they were evaluated (in the same day).The severity of aerial and root symptoms was assessed for each plant on a 0-4 scale basis (0= without symptoms, 4= dead plant). The aerial part of the plant was evaluated according to the percentage of leaves with yellowing, necrosis or defoliation and the root part by the amount of roots and percentage of necrotic roots. Plants were kept with roots wrapped in a wet absorbent paper in the dark at 4ºC, until needed, but no longer than 12 days (Streito and others 2002).

Phytophthora cinnamomi *Re-isolation*

Segments of roots from infested and control plants were plated on NARPH [Corn meal agar – CMA (17 gL^{-1}) amended, after autoclaving (at 0.7 Kp/cm^2 for 15 min.) and cooled down to 45 ºC, with nystatin (0.03 gL^{-1}), ampicillin (0.25 gL^{-1}), rifampicin (0.10 gL^{-1}), pentachloronitrobenzene - PCNB (0.10 g/l) and Hymexazol (197.2 μlL^{-1})] medium to re-isolate *P. cinnamomi*. Segments of roots were washed into running tap water for 1-2 hours, aseptically and transversally cut into small segments (5-8 mm long) on a sterile filter paper. The exposed cortex was directly plated (six to eight root groups), using sterile forceps, on NARPH medium. For each plant and isolate 5 Petri dishes were used and sealed with parafilm®. The plates were incubated at 24ºC in the dark for 2-3 days. Colonies isolated from the tissues were classified on the basis of cultural characteristics and identified by the morphology of vegetative and reproductive structures under a light microscope (Sánchez and others 1998). Percentage of *P. cinnamomi* recovering was calculated. Samples from which the oomycete was not recovered were processed until being recovered or up to three times.

In vitro *Activity of Plant Crude Extracts*

To evaluate the activity of various plant extracts on *P. cinnamomi* growth, preliminary assays were performed to optimize the technique. These included the comparison of *P. cinnamomi* growth using two different isolates, with different shaking times, two different solvents and root *versus* aerial part extract (leaves or leaves and flowers). All the experiments were done using the following procedure: a mycelial agar plug (5 mm in diameter) of each isolate was cut from an actively growing colony and placed on Potato Dextrose Agar (PDA) culture media (Park 2003). PDA culture media (Difco®) was prepared according to the manufacturer's instructions. The plant extracts were incorporated into the PDA culture media, prior to inoculation: after autoclaving, PDA was placed in a water bath at 45ºC before addition of plant extracts to avoid thermal degradation. The mixture was homogenised in a magnetic stirrer. Twelve millilitres of culture media, was poured into 90 mm Petri dishes and allowed to solidify. Control Petri dishes received the same amount of culture media and the corresponding amount of solvent. Petri dishes were sealed with parafilm®, put into plastic bags, grouped according to the plant extract and incubated, in the dark, at 24 ºC. Length and width of colonies were measured every day until colonies reached the edge of the plate (about 10 days).

These assays were performed in triplicate per isolate and product (plant extract) and were conducted three times. At the end of the incubation period, the 50 percent effective concentration (EC50) that caused an inhibition of 50 percent of the mycelial growth, when compared with the control, was determined. The EC90 was also calculated.

The percentage of inhibition of each tested substance was calculated after subtracting the diameter of each agar plug (5mm) from the diameter of each colony, as follows: inhibition (percent) = 100 [(Ut-Tr)/Ut], whereby, Ut = mean value for untreated control and Tr = mean value for media supplemented with plant extracts (Mekuria 2005).

Effect of Plants Extracts from Helichrysum stoechas and Phlomis purpurea on Phytophthora cinnamomi Mycelial Growth: Experiment 1.1.

Plant extracts used were from *Helichrysum stoechas* (root and aerial part) and *Phlomis purpurea (*root and aerial part) in ethanol. Aerial part (leaves and flowers for *H. stoechas* and just leaves for *P. purpurea*) were shaken for 72 hours and roots for 7 days. The effect of those extracts, at 1.0, 5.0 and 10.0 $mgml^{-1}$, on *P. cinnamomi* (isolates PA37 and PA45) mycelial growth was assessed.

Efficiency of Plant extracts from Helichrysum stoechas, Phlomis purpurea and Arbutus unedo on Phytophthora cinnamomi Growth: Experiment 1.2.

Phytophthora cinnamomi, isolates PA37 and PA45 were inoculated in PDA media supplemented with *A. unedo* (leaves), *H. stoechas* (leaves and flowers) and *P. purpurea* (roots) ethanol extracts. The concentrations used were 1 and 5 $mgml^{-1}$. Mycelial growth was measured.

Efficiency of Three Concentrations of Helichrysum stoechas Plant Extracts on Phytophthora cinnamomi Growth: Experiment 1.3.

Helichrysum stoechas (leaves and/or flowers) ethanol extract at 1.0, 5.0 and 10.0 $mgml^{-1}$ was tested on growth of isolates PA37 and PA45.

Efficiency of Two Concentrations of Phlomis purpurea Plant Extracts on Phytophthora cinnamomi Growth: Experiment 1.4.

Phytophthora cinnamomi, isolates PA37 and PA45 were inoculated in PDA media supplemented with *P. purpurea* (roots) ethanol extract at 1 and 5 $mgml^{-1}$.

Pathogenicity Tests

Inoculum preparation

Axenic cultures of PA37 and PA45 were grown in the dark at 24ºC on V8A. After 4-5 days, 5 mm agar plugs were taken from the edge of individual colonies and transferred into a 9 mm Petri dish containing V8® broth (V8b). Isolates were grown for 4 weeks at 24ºC, to allow the production of abundant infective structures. Inoculum suspensions were prepared by mixing the content of six agar plates, previously washed and agar free, with 600 ml of sterile deionised water in a blender (Philips twister) for 3 min at high speed. *Phytophthora cinnamomi* suspensions for

plant infestations were prepared in water because they are easier to prepare, and are as effective as culture media inoculated with the isolates (Sánchez and others 1998, 2005).

Inoculation—Experiment 2.1.

Inoculation of *Arbutus unedo*, *Helichrysum stoechas* and *Phlomis purpurea* using *P. cinnamomi* isolates PA37 and PA45 was performed by placing 5 mm agar plugs from the edge of individual colonies on the top of the elongation zone (around 5 mm above the root tip) and wrapped by foil. Control plants received a V8A plug. Plants were incubated at 24.5ºC. Segments of roots were cut 1 cm above the infested point and inoculated every 24 h for 96 hours. After 96 h all agar plugs were removed to NARPH to check viability.

Infestation—Experiments 2.2. and 2.3.

For infestation, plants were taken out from their containers and the root ball was homogeneously covered with the inoculum of PA37 and PA45 isolates before transferring them into a four litres plastic pot (13 x 13 x 30 cm) containing potting soil (PlantaFlor, Humus verkausfs, GmbH, D-49377 Vechta, Germany). One hundred millilitres of inoculum suspension in water was added per plant. All controls were treated in the same way, except for the addition of inoculum which was substituted by water. To avoid contamination controls were treated first. Replicate pots per species, per isolate and controls were gathered in plastic trays (40 x 30 x 24 cm) and placed in a glasshouse. To avoid cross contamination, infested and control pots were placed in different trays. The plants were sprayed and watered every day for one week, to avoid stress. After one week, every tray, including control ones, were filled with tap water and plants submitted to soil waterlogging two days per week. The water level in the trays was maintained at 5 cm below the soil surface, by adding water each time that plants were submitted to waterlogging, as described (Sánchez and others 1998). Maximum and minimum temperatures were recorded every day. Two experiments (2.2. and 2.3.) were carried out:

Experiment 2.2

Six replicates of each plant species (except for *P. purpurea* of which only three plants per isolate were available due to rupture of stock at the nursery) were inoculated with a 100 ml suspension of PA37 isolate and another six with the PA45 one. This experiment was kept in the glasshouse during spring till mid summer for 11 weeks with an average temperature of 28.1ºC (average of maximum and minimum temperature was 36.1ºC and 20.1ºC respectively). The last two weeks were very hot, with maximum temperatures around 40ºC. The experiment was, then ended.

Experiment 2.3

A second pathogenicity test was carried out differing from the precedent one for the number of replicates (10 plants per species, including *Phlomis purpurea*), the exclusion of cork oak plants and the inoculum (used as a mixture of PA37 and PA45 isolates). This experiment was kept in the glasshouse during autumn till the end of winter, for 16 weeks with an average temperature of 20.3ºC (average of maximum and minimum temperature was 28.0ºC and 12.5ºC respectively).

In situ *Protective Effect of* Phlomis purpurea *Against* Phytophthora cinnamomi

A mixture of stock pure cultures, at identical quantities, of PA37 and PA45 isolates was used.

Experiment 2.4.: Influence of Phlomis purpurea on the Infection of Quercus ilex Plants by P. cinnamomi

Inoculum preparation and infestation were done by the same procedures as described above. Three agar plates of each isolate (total six) were used. Groups of ten replicate pots were infested with the mixture of *P. cinnamomi* isolates (positive control), *P. purpurea* alone, *Q. ilex* alone and one *Q. ilex* seedling planted between two *P. purpurea* plants. Negative controls consisted of identical replicate groups to which sterile distilled water was added. All pots were gathered in plastic trays and placed in a glasshouse. The average temperature was 20.3ºC (average of maximum temperature was 28.0ºC and average of minimum temperature 12.5ºC). The experiment lasted for 16 weeks.

Soil Inoculum Potential

After plant removal from pots, soil from i) *P. purpurea* alone, ii) *Q. ilex* grown alone, and iii) *Q. ilex* seedling grown between two *P. purpurea* was analysed for *P. cinnamomi* inoculum population. Two and half grams of soil, taken out from several places of a total mass of soil of *ca* 650 g, were placed in an Erlenmeyer containing 50 ml of 0.2 percent sterile agar solution and homogenised with a magnetic stirrer. Once the mixture was homogeneous, 1 ml was plated in 20 Petri dishes containing NARPH culture media (2.2.1). Plates were incubated in the dark at 24.5ºC. After 24 hours, plates were washed with running tap water to eliminate the soil. Plates were incubated in the dark at 24.5ºC for another 2 to 3 days (D'Souza and others 2004, Rodríguez and others 2004). *P. cinnamomi* population was expressed as colony forming units per gram of soil (c.f.u./g soil).

Statistical Analysis

Results were analysed using "Statistix for windows version 7". The mean values were compared with those from control plates by Fisher's protected LSD (Least Significant Difference) test (Steel and Torrie 1985).

Results and Discussion

In vitro *Activity of Plant Crude Extracts*

Ethanol extract from leaves and flowers of *H. stoechas* significantly inhibited ($p<0.05$) mycelial growth when compared to the root extract (Plate 1, fig 1). *H. stoechas* root extract slightly stimulated growth at 1 and 5 $mgml^{-1}$. For this reason, *H. stoechas* aerial parts were selected for further tests. On the contrary *P. purpurea* root extract inhibited significantly ($p<0.05$) mycelial growth when compared to the leaf extract (Plate 2, fig 2). For this reason, *P. purpurea* root extract was chosen for further tests.

Phlomis purpurea root extract caused a maximum percentage of *P. cinnamomi* inhibition of 93.9 percent, while *H. stoechas* leaf and flower caused 69.1 percent inhibition and *A. unedo* leaves 61.7 percent, all at 5 $mgml^{-1}$. The three means were

also significantly different from each other. There was no significant difference between replicates. As ethanol extract from *A. unedo* leaves caused the least inhibitory effect on *P. cinnamomi* growth and because it is a host for this oomycete, it was decided to exclude *A. unedo* and focus on the other two plant extracts.

Helichrysum stoechas extracts at concentrations of 1.0, 5.0 and 10.0 $mgml^{-1}$ showed maximum inhibitory effects of 38.8, 76.6 and 86.7 percent respectively, against both isolates. The effective concentration of *H. stoechas* extract that inhibited mycelium growth by 50 percent (EC_{50}) at day 9 was 7.4 $mgml^{-1}$ and EC_{90} was 13.4 $mgml^{-1}$.

Phlomis purpurea root extracts at concentrations of 1 and 5 $mgml^{-1}$ caused significantly different mean maximum percentage of inhibition─ 78.3 and 93.1 percents respectively. The EC_{50} and EC_{90}, at day 9, were 4.1 $mgml^{-1}$ and 8.0 $mgml^{-1}$, respectively.

In culture media supplemented with plant extracts, it was often found a reduction in colony density, which was probably due to the toxic effect of the plant extracts. Although being the commonest method used, the agar supplemented with the compound or extract to be tested may not be the most suitable, because it does not take into account the colony density. An alternative method could be to grow the pathogen in a liquid medium followed by determination of dry mass.

Phlomis purpurea is very rich in saponins (both leaf and root produces froth) which are glycosidic compounds characterized by their detergent-like properties. Many saponins are recognized by their ability to haemolyse erythrocytes (Hostettmann and others 1991). Saponins in plants are considered as having the function of phytoantecipins, a group of compounds with constitutive or preinfectional defence properties, or of phytoalexins, assuring postinfectional defences against pathogens (Hammerschmidt 1999). Saponins are responsible for the resistance of *Avena sativa* (oat) roots to the fungus *Gaeumannomyces graminis* (Hostettmann and others 1991). However, in the present study, the leaves did not have anti- *P. cinnamomi* activity, but the roots did. Probably, other compounds are involved in the inhibition of mycelial growth, since *P. cinnamomi* contains few membrane sterols, the target of saponins (Kamoun and others 1999). These authors observed that oomycetes are unaffected by saponins. To understand what compound caused such a strong inhibition it is important to know the extract composition.

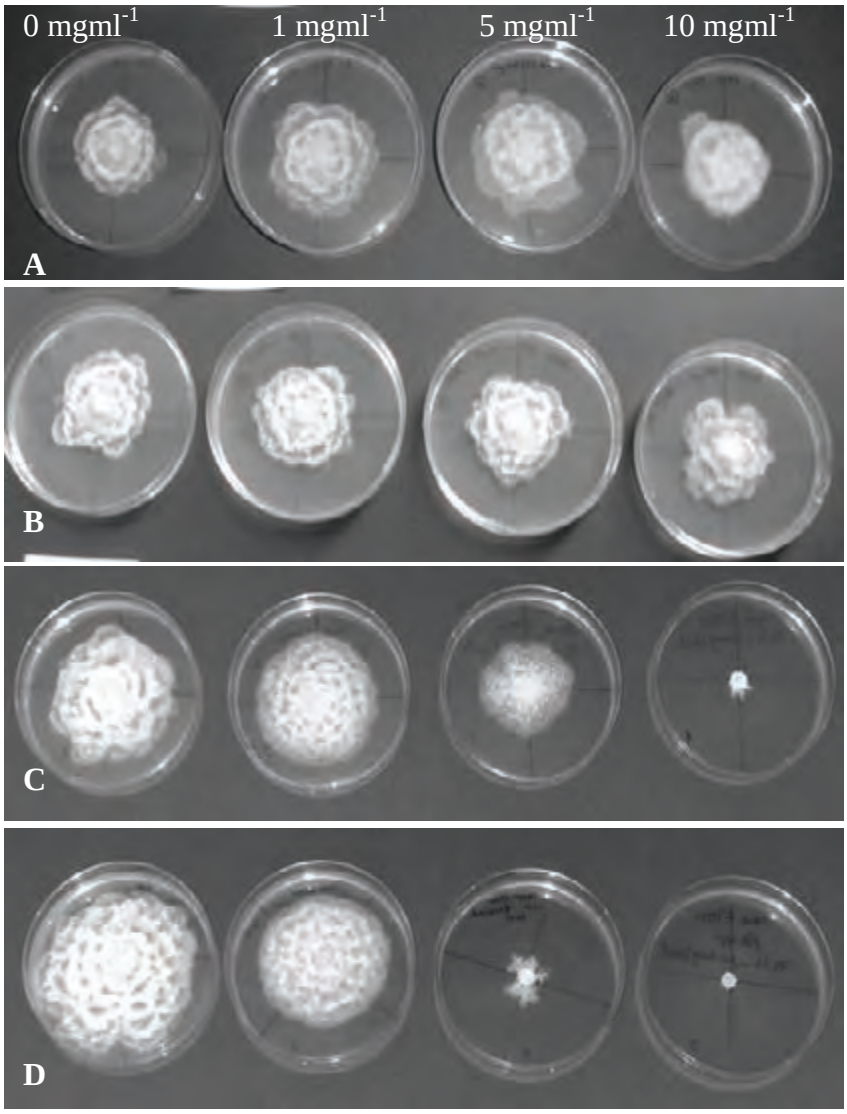

Plate 1ð Growth of *Phytophthora cinnamomi* (**A** and **C**: PA37; **B** and **D**: PA45 isolates) on Potato Dextrose Agar (PDA) medium with 0ï 10 mgml^{-1} *Helichrysum stoechas* root (**A** and **B**) extract after 5 days growth and leaves and flowers (**C** and **D**) extract after 10 days growth.

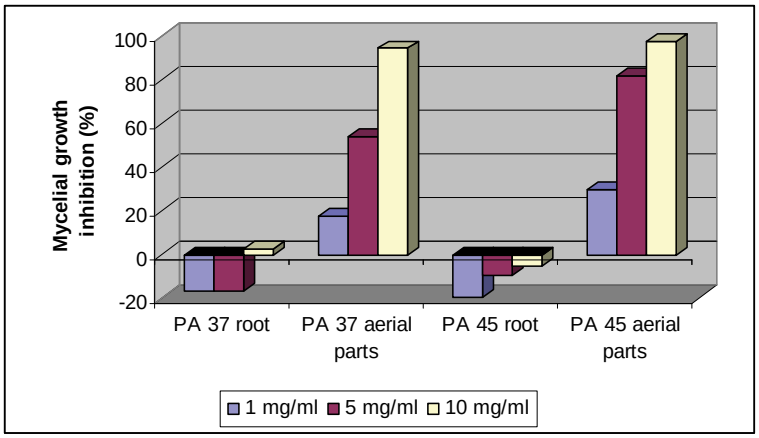

Figure 1—Growth inhibition percentage of mycelial *Phytophthora cinnamomi* (isolates PA37 and PA45), on Potato Dextrose Agar (PDA) supplemented with 1, 5 and 10 mgml^{-1} of *Helichrysum stoechas* ethanol root and aerial parts extract.

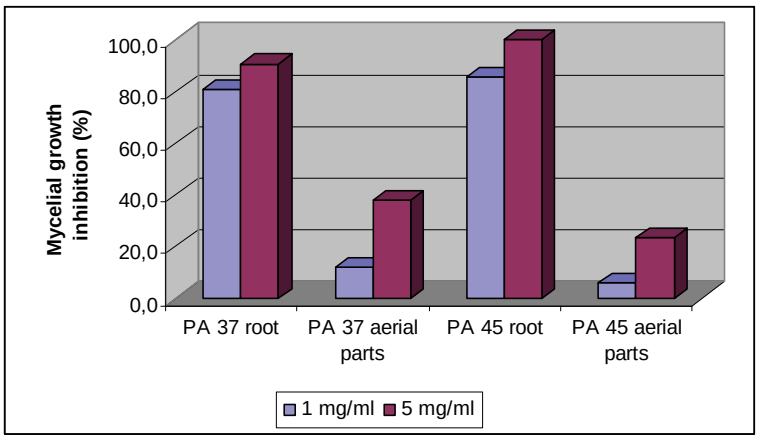

Figure 2—Growth inhibition percentage of mycelial *Phytophthora cinnamomi* (isolates PA37 and PA45), on Potato Dextrose Agar (PDA) supplemented with 1 and 5 mgml^{-1} of *Phlomis purpurea* ethanol root and aerial parts extract.

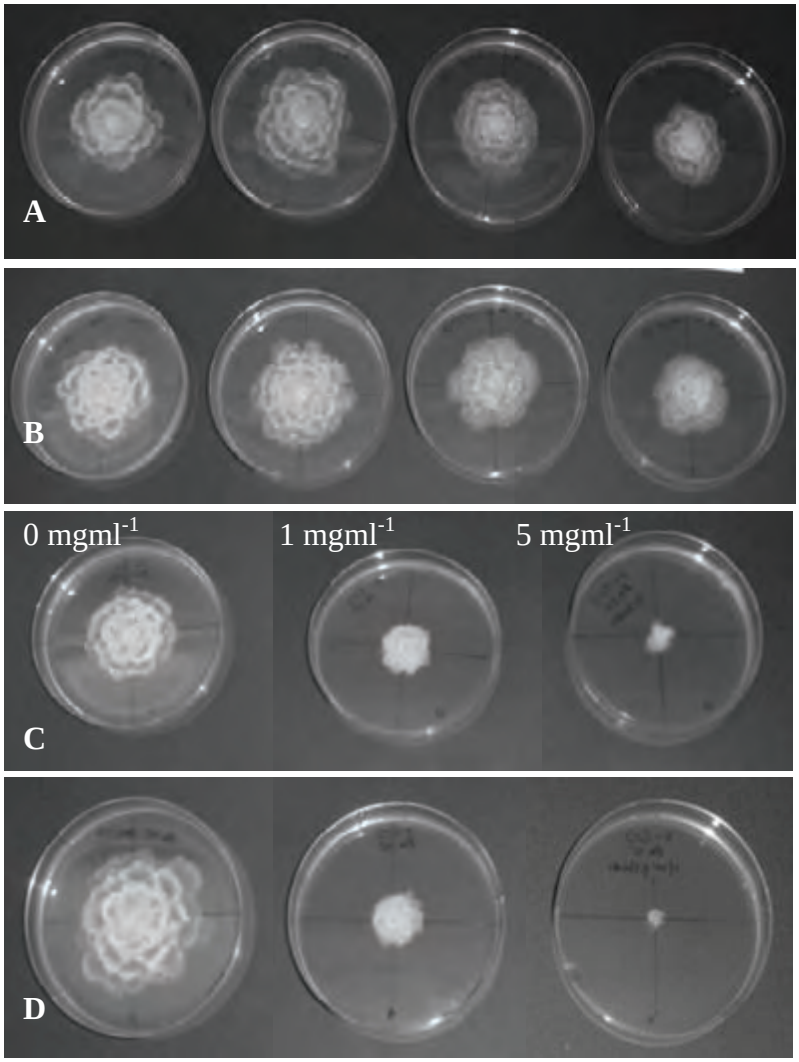

Plate 2—Growth of *Phytophthora cinnamomi* (**A** and **C**: PA37; **B** and **D**: PA45 isolates) on Potato Dextrose Agar (PDA) medium with 0-10 $mgml^{-1}$ *Phlomis purpurea* leaf (**A** and **B**) and 0-5 $mgml^{-1}$ root (**C** and **D**) extracts after 5 days growth. For the root extract assay the 10 $mgml^{-1}$ plates are not present due to insufficient root extract to perform the experiment.

Pathogenicity tests

Phytophthora cinnamomi was recovered from *A. unedo* root fragments of plants infested with the isolate PA37 after 24 h but not after 48, 72 and 96 h, while recovery was always observed from roots of infested with isolate PA45. *Phytophthora cinnamomi* was recovered neither from any of *H. stoechas* or *P. purpurea* roots infested with this pathogen nor from the control root fragments at 24 h, 48 h, 72 h and 96 h. After 96 hours all agar plugs were viable and *P. cinnamomi* recovered from the root's first centimetre.

The plants did not show major foliar symptoms except *Q. ilex* and to a lesser extend *Q. suber*. However the severity of root symptoms was greater than control in all plants, except for *H. stoechas* and *P. purpurea*.

The general comparison of means of severity of foliar and root symptoms by isolates indicates that isolate PA37 is not significantly different from PA45. When comparing means of root symptoms by species, *Q. suber and Q. ilex* are not significantly different from each other, show necrosis of feeder roots and the amount of roots is reduced, but are significantly different from *A. unedo* and *H. stoechas*. There is no significant difference between *H. stoechas* infested with either PA37 or PA45 isolate and the control. *Phlomis purpurea* was not included in the general statistical analysis because there was only a small number of replicates (3) for each isolate; therefore, a separate one-way ANOVA test was performed which revealed that there is no significant difference between infestation with PA37, PA45 and the control.

Phytophthora cinnamomi was re-isolated from roots of *Q. suber*, *Q. ilex* and *A. unedo* but not from the roots of *H. stoechas* and *P. purpurea*, confirming precedent observations by Moreira-Marcelino (2001).

At the end of the pathogenicity trials the plants did not show major foliar symptoms, except for an *A. unedo* plant that died. In a trial in which *Q. suber* was not included the general comparison of means of severity of foliar and root symptoms by species showed that, *A. unedo* and *Q. ilex* are not significantly different from each other, but both are significantly different from *H. stoechas* and *P. purpurea*. Concerning root symptoms, *P. purpurea* presented the least average value, significantly different from *H. stoechas*. *Arbutus unedo* and *Q. ilex* infested with *P. cinnamomi* showed higher root severity symptoms when compared with the control plants. *Phytophthora cinnamomi* was re-isolated from roots of *Q. ilex* and *A. unedo* that were grown in soil infested with *P. cinnamomi* but not from the roots of *H. stoechas* and *P. purpurea*.

Although there was a significant difference in root symptoms between plants grown in infested soil and control plants (except for *P. purpurea*), the same did not occur for the aerial/foliar symptoms. It is, usually, necessary to have a very extensive root lesion to observe the aerial symptoms (Erwin and Ribeiro 1996). However, this oomycete was not re-isolated from infested roots of *H. stoechas* and *P. purpurea* in any of the conducted experiments.

Plants were submitted to waterlogging twice per week. This condition favours the pathogen growth and has a negative effect on plant health (Burgess and others 1999; Davison 1988; Sánchez and others 1998, 2002, 2005; Stolzy and Sojka 1984;

Zentmyer 1980), by a reduction in O_2 supply and in the nutrient uptake. The low level of root symptoms showed by the control compared with the infested plants, in all experiments, confirms that flooding was not responsible for the symptoms showed by infested plants as previously demonstrated by Sánchez and others (2005).

In situ *Protection Trial*

At the end of the protection trial carried out *in situ* neither *Q. ilex* nor *P. purpurea* aerial parts showed symptoms of disease. Roots of infested *Q. ilex* seedlings presented high severity symptoms significantly different from those of infested seedling surrounded by two infested *P. purpurea* plants. The association of *P. purpurea* with *Q. ilex* plants significantly reduced the root symptom severity on the susceptible *Q. ilex*. *P. purpurea* showed no significant difference either in aerial or root symptoms between control and infested plants.
Phytophthora cinnamomi was re-isolated from 100 percent of *Q. ilex* infested roots, but only from 36.8 percent of *Q. ilex* infested roots that have grown surrounded by *P. purpurea* plants, meaning that the association of *P. purpurea* to *Q. ilex* growing in a close environment reduced the infection of *Q. ilex* roots. Like in previous experiments *P. cinnamomi* was not re-isolated from *P. purpurea* roots.

Phytophthora cinnamomi was not recovered from soil associated with *P. purpurea*. The inoculum potential was significantly reduced in soil where *P. purpurea* plants grew next to *Q. ilex* seedlings when compared with soil associated to *Q. ilex* grown alone.

These results confirm the high potential of *P. purpurea* to control this pathogen. *Q. ilex* was firstly chosen because this species is, apparently, more susceptible to *P. cinnamomi* than *Q. suber* and consequently presented symptoms quicker, so glasshouse results could be achieved sooner. A trial with *Q. suber* is presently being prepared.

Studies by D'Souza and others (2004, 2005) revealed that *Acacia pulchella* and *A. extensa* have a high potential for biological control of *P. cinnamomi.* However, in Portugal the genus *Acacia* is considered invasive and a programme to reduce or even eliminate some *Acacia* species is underway (Marchante and others 1999, Invader Project 2002). This problem is not unique, and a biological control must be carefully designed and performed with endemic species to avoid an imbalance in the ecosystem.

In our experiments necrosis was never seen in *P. purpurea* roots. In fact, *P. purpurea* roots were abundantly infested with *P. cinnamomi* mycelium and sections (4 µm thickness) observed by light microscopy—no *P. cinnamomi* was observed in the internal root tissues of *P. purpurea* (Medeira and Maia, pers. comm., 2007). Rarely, the oomycete colonised some epidermal cells and in this case signs of degradation of this pathogen were visible (*Ibidem*). These observations confirm that *P. purpurea* is resistant to *P. cinnamomi* infection. A similar behaviour was observed in *A. pulchella* when inoculated with 10-20 zoospores: *hyphae* associated with necrotic cortical cells were lysed, but some penetrated the stele 16 h after inoculation. However, root necrosis and wilting were absent (Weste and Cahill, 1982).

Bais and others (2002) identified rosmarinic acid (RA) in the root exudates of hairy root cultures of *Ocimum basilicum* (sweet basil) elicited using cell wall extracts

(CWE) from *P. cinnamomi*. Upon elicitation, the production of RA was enhanced 2.67-fold compared with the untreated control. RA showed to have activity against a wide range of soil born microorganisms, including *P. drechsleri*, *P. megasperma* and *P. parasitica*. In the same study, 250 µM of RA provoked clumping and breakage of *P. drechsleri* hyphae. Widmer and Laurent (2006) stated that plants containing caffeic acid and RA, inhibit zoospore germination of the three *Phytophthora* spp. studied, namely *P. palmivora*, *P. megakarya* and *P. capsici*. Phenolic acids, like RA, exist constitutively in members of the Lamiaceae and Boraginaceae families. Both *Ocimum* and *Phlomis* are genera of the Lamiaceae family which comprises more than 7000 species. It will be interesting to find out whether *P. purpurea* roots also exude RA. Phenolic acids have known antimicrobial properties, so it has been hypothesized that these compounds may play the role of phytoantecipins (Dixon 2001; VanEtten and others 1994), and phytoalexins as referred to above (Bais and others 2002). Studies on the action of *P. purpurea* extracts on *P. cinnamomi* sporangia formation, zoospore release and germination and chlamidospore formation are presently underway.

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Investigating the Potential of Disease Control Practices for Integrated Management of *Phytophthora kernoviae* on Mature Magnolias in the United Kingdom[1]

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Abstract

Phytophthora kernoviae (Pk) a newly invasive *Phytophthora* species was first discovered in native woodlands and heritage gardens in Cornwall, U.K., in November 2003. Essentially it is an extremely aggressive pathogen of woodland *Rhododendron ponticum* causing leaf necrosis, shoot tip dieback and defoliation of infected plants. It also but it also attacks inner bark of European beech (*Fagus sylvatica*) causing lethal bleeding cankers. The pathogen forms inoculum on infected rhododendron foliage from where it infects susceptible tree stems in close proximity. However, in heritage gardens a range of exotic plant species are foliage hosts for Pk: *Drimys*, *Magnolia*, *Michelia* and *Pieris*, as well as rhododendron.

Loss of exotic plant species in heritage gardens has negative socio-economic effects for local communities. Not only are the gardens an important source of tourist revenue in south-west England, but cultural and horticulturally valuable plants are housed there. For example part of the 'National Magnolia Collection' occurs on sites in Cornwall, as do a number of 'Champion trees'. Some of these specimens are susceptible to attack by *P. kernoviae*; disease reduces aesthetic value but can also result either in death or removal of infected plants as directed by plant health regulation orders. Many of these plants are well established and loosing them not only changes the structure and composition of gardens, but some are irreplaceable because they are unique genotypes that are rare or even extinct in their natural habitats. Gardeners are finding it difficult to maintain the traditional constitution and ambience of the gardens and are concerned that the impacts of the disease may ultimately be felt in decreased numbers of visitors.

Management to reduce and possibly eliminate disease on culturally valuable specimens in gardens is reliant on scientific data. A key aspect of integrated management is removal of primary sources of inoculum, *i.e*. rhododendron, without which other aspects of an integrated approach cannot be considered. Chemical intervention to reduce sporulation and prevent disease advance onto new tissue is a management tool worth investigating on deciduous trees. However, prior to this it is essential to establish if the pathogen survives and overwinters on infected shoots, and whether new infections are initiated from these overwintering infections, in order to select appropriate chemicals and optimise treatments. This study monitored the spread and intensity of disease and determined whether the pathogen survived in first and second year shoots of Magnolia naturally infected with Pk. To do this symptom expression was monitored and recorded over 20 mo and isolation used to confirm the presence of Pk. A bimodal pattern of symptom expression occurred with peaks in spring (May-June) and autumn (October-November). The pathogen overwintered on the fury outer bud scales, in buds, and in leaf scars only in the current year's tissue, which was infected in autumn. Leaf debris also harboured *P. kernoviae* over winter. In spring infection was first evident in

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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blossom and new foliage developing from buds. Further investigation of sanitation practices and chemical applications for disease management are merited on valuable deciduous trees in gardens.

Population Genetic Analyses of Four *Phytophthora* Species Recently Described in California Forests Reveal They Are All Introduced[1]

M. Garbelotto,[2] R. Linzer,[2] W. Monahan,[2] and S. Bergemann[2]

Abstract

Four *Phytophthora* species have recently been described in California natural ecosystems: *Phytophthora ramorum, P. ilicis, P. pseudosyringae,* and *P. cinnamomi*. The first three species are of recent description, while the last one is a well-known pathogen associated with agricultural crops in the Western USA and in many areas world-wide. *P. ramorum* and *P. cinnamomi* are both causing significant mortality in native California hosts, while the other two species are associated with limited mortality of affected plant hosts. We have used both AFLP and microsatellite analyses to unravel the population genetic structure of all four species. All display a population structure typical of introduced species, independent of the symptoms they are causing. This finding indicates that as long as an ecological niche is available, aggressive and non- aggressive microbes can be introduced in novel environments. While the origin of *P. ilicis* remains a mystery, a perfect match was found between *P. ramorum* and *P. cinnamomi* isolates from the wild with isolates from agricultural situations. In the case of *P. pseudosyringae*, California isolates were nested within European isolates suggesting that the California population originated from Europe. It should be noted that these analyses evidentiate possible pathways of introduction, but not the actual origin of these exotic microbes. An analyses of hundreds of California bay laurel and tanbark oak leaves collected during a time-span of over one hundred years and deposited in the Jepson's herbarium at UC-Berkeley indicated that the symptoms caused by *P. ramorum*, *P. ilicis*, and *P. pseudosyrinage* were absent in historical collections, also supporting the exotic origin of these three pathogens.

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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IUFRO Fourth Meeting of Working Party 7.02.09, Phytophthoras in Forest and Natural Ecosystems: Meeting Summary[1]

Susan J. Frankel[2]

On behalf of the Fourth Phytophthoras in Forest and Natural Ecosystems local organizing committee and co-chairs Everett Hansen, Clive Brasier, and Giles Hardy, I thank you for your contributions to this Working Party meeting. The past week has stimulated much thought and discussion, thanks to the 100 participants from 15 countries, 48 papers and 31 posters, two field trips, and two evening overview sessions.

The following questions, explored throughout the week, highlight some of the critical issues raised.

1. What is the relationship between Phytophthoras in nurseries and forest Phytophthora diseases? What are the implications of the detection of *P. kernoviae*, *P. pseudosyringe, P. nemorosa*, and other forest Phytophthora species in nurseries and orchards? Many forest Phytophthoras have been detected on rhododendron nursery plants. Which species could use rhododendron nursery plants as a pathway into forests? How can we identify the potentially damaging species before they escape?

2. How can *Phytophthora* species be detected earlier in forests? Can we detect them before they reach outbreak levels, while they are still eradicable?

3. How do we define *Phytophthora* species? How important is the division of Phytophthoras into species? Rather than focusing on differences between species, could we learn more by focusing on the common biology of most Phytopthora species?

4. *Phytophthora europaea* has been recovered from oak forests in Europe, isolated from California bay laurel leaves and streams in California, and from Christmas tree plantation soils in Michigan (Balci and others 2007). Can other Phytophthora species adapt to such varied niches in different ecosystems?

5. Ioos' revision of the phylogeny of *Phytophthora alni* based on his genetic analysis (this volume) raises many questions. How well can Phytopthora species hybridize? Are there other "species clusters" that we are not yet aware of? If not, why not?

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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6. How can we integrate *Phytophthora* genetic investigations with natural history or management studies? The gap between molecular biologists and field ecologists seems to be growing. How can we bring these specializations together?

7. How important is designation and support of a forest *Phytophthora* model species? Which forest Phytophthora species would make the ideal model species? What selection criteria should be used?

8. What roles do chlamydospores and sporangia play in *Phytophthora* survival and dispersal? Could chlamydospores be more important in dispersal than previously thought?

9. In laboratory inoculations, Vettraino and Denman each report (this volume) an inverse relationship between *P. ramorum* lesion size and sporulation, with smaller lesions generating greater spore production. What are the implications of this finding for regulatory inspections? Could inconspicuous lesions yield significant inoculum for new infections?

10. Removing infected trees and creating a buffer zone around the infested area is currently the only method used to attempt *Phytophthora* eradication from forest environments. In areas where tree removal is undesireable for environmental or social reasons, what other treatments could be used?

11. Phytophthora species seem ubiquitous in water courses. Are water courses Phytophthora species' primary habitat? What roles do *Phytophthora* species play in rivers and streams? Genetic analysis confirms that the genus Phytophthora is most closely related to brown algae and diatoms, and is evolutionarily distant from Fungi. Does this classification have implications for our understanding or management of *Phytophthora* species?

12. Ramsfield and others (this volume) report that *P. kernoviae* may be native to the Southern Hemisphere. What actions should a country take when they determine a damaging Phytophthora is resident in their soil?

13. How do we engage scientists in other disciplines, the public, policy-makers, politicians, and others to raise awareness and funding to prevent further adverse impacts caused by forest *Phytophthora* species?

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Posters

White Oak Decline and Mortality in Southern Ohio[1]

Yilmaz Balci,[2] Robert P. Long,[3] Selin Balci,[2] Daniel Balser,[4] and William L. MacDonald[3]

Abstract

Extensive white oak (WO = *Quercus alba* L.) decline and mortality has been observed since 2002 in oak forests across southern Ohio. The mortality was especially severe at the 3800 ha Scioto Trail State Forest in southern Ross County. A series of stressors including drought (1998 and 1999) and insect defoliators (forest tent caterpillar, *Malacosoma disstria*; common oak moth, *Phoberia atomaris*; half-wing geometer, *Phigalia titea*) adversely affected tree health through 2004. WO crown dieback and mortality initially developed as single trees or small groups of trees on lower slope and bottomland sites. In 2006, WO mortality was assessed in seven severely affected bottomland stands using 3 to 7 800 m2 plots at each site (total n=34). Standing dead WO basal area ranged from 9 - 18 m2 ha-1, about 57 percent to 84 percent of total WO basal area. Sapling regeneration (stems <10 cm dbh and > 1.4 m in height) is dominated by maples (*Acer saccharum*, *A. rubrum*) that are poised to fill canopy gaps. Random plots (n=103) were established in declining (n= 59) or non-declining (n=44) sites to evaluate soils and roots for the presence of *Phytophthora* spp. Among the species, *P. cinnamomi* was frequently isolated in 69 percent (41 sample trees) of soils from declining trees and 57 percent (25 sample trees) of soils from non-declining trees. However, a significant association was not detected between the presence of *Phytophthora* and deteriorating crown status. The average dry weight of WO roots (< 5 mm diameter) at lower slopes was significantly less than those located on upper slopes. The inoculum level was significantly higher in lower elevations than on higher elevations. Excess moisture in 2003 and 2004 may have favored development of *Phytophthora* spp. or other root pathogens and may have contributed to the WO decline in lower slopes.

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Detection and Diagnosis of *Phytophthora ramorum* With Specific Hybridization Real Time PCR Probes[1]

Lassaad Belbahri,[2] Gautier Calmin,[2] Stephan Wagner,[3] Eduardo Moralejo,[4] Steve Woodward,[5] Tomasz Oszako,[6] and François Lefort[2]

Abstract

Sudden oak death, caused by *Phytophthora ramorum*, poses a serious threat to native American oaks, and is also present in Europe where it has been discovered in numerous European ornamental plant nurseries. Its proven aggressiveness against plants in the Fagaceae and Ericaceae and the damage it has caused in North America lead to assign it a quarantine pathogen. It has also been listed in Europe in the biosecurity group 3, usually mostly devoted to severe pathogens for humans and animals. The timely and accurate detection of *P. ramorum* is a critical aid in the study of the epidemiology and biology of this pathogen. As a regulated organism, the availability of a sensitive and reliable assay is essential when attempting to achieve early detection of the pathogen. In this work, new specific hybridisation probes for a real time PCR amplification method were found to be rapid, robust, and laboursaving, and proved suitable for routine use in a molecular diagnostic laboratory.

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Occurrence of Novel *Phytophthora* Taxa in Hungarian Alder Forests[1]

Lassaad Belbahri,[2] József Bakonyi,[3] Zoltán Nagy,[3] Gautier Calmin,[2] András Koltay,[4] Steven Woodward,[5] and François Lefort[2]

Abstract

Numerous formally or informally designated new *Phytophthora* taxa have been recently described from natural and semi-natural ecosystems using the advantage of molecular techniques. Of the novel taxa, *P. alni* has been identified in Hungary. However, during surveys for the incidence of this pathogen, we also obtained three additional *Phytophthora* isolates from alder root and soil. They could not be identified accurately based solely on morphological characteristics. Therefore, our aim was to carry out a complex morphological, physiological and phylogenetic analysis to reveal their correct taxonomical and phylogenetic positions. The phylogenetic work included sequence analyses of the rDNA ITS, the nuclear a and β-tubulin, translation elongation factor 1 alpha and actin coding genes as well as the mitochondrial NADH dehydrogenase subunit 1 and cytochrome c oxidase 1 and 2 genes. The results indicated that the three studied isolates represented two different taxa, designated as *Phytophthora* sp. 1 and *Phytophthora* sp. 2, each belonging to Waterhouse's group V. Morphological differences between them were observed in the dimension of oogonia, oospores and sporangia as well as the colony pattern on carrot agar. Multigene sequence analysis revealed that *Phytophthora* sp. 1 possessed an internal transcribed spacer identical to that of the informally named *P. taxon* Forestsoil, whereas *Phytophthora* sp. 2 represented another novel taxon that has not been identified elsewhere before. A thorough morphological and physiological characterisation as well as a detailed phylogenetic analysis in relation to other *Phytophthora* spp. will be presented.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Phytophthora polonica sp. *novo* Isolated From Polish Alder Stands in Decline[1]

Lassaad Belbahri,[2] Tomasz Oszako,[3] Gautier Calmin,[2] Eduardo Moralejo,[4] J.A Garcia,[4] E. Descals,[4] and François Lefort[2]

Abstract

In a survey of *Phytophthora* associated with alder decline in Poland, several isolates of a homothallic *Phytophthora* sp., which could not be assigned to other taxa including *P. alni* subspecies, were consistently recovered from rhizosphere soil samples. Their morphology and pathogenicity, as well as sequence data for three nuclear regions (ITS rDNA, EF-1α and β-tub) and a coding mitochondrial DNA region (nadh1), were examined. The new *Phytophthora* species is characterized by moderate to slow growth on carrot agar at 20 ˚C, high optimal (ca. 30 ˚C) and maximum (ca. 38 ˚C) growth temperatures. It forms catenulate, often lateral, hyphal swellings, large chlamydospores in agar media and in soil extract, persistent. Sporangia are ovoid to ellipsoid and non-papillate. Large oogonia with paragynous and sometimes amphigynous antheridia were observed. From pathogenicity tests on alder twigs and on a few Iberian trees, *P. polonica* seems to be a poor inner bark colonizer. Despite being pathogenic to fruits in wound inoculations, no plant diseases attributable to *P. polonica* have appeared in Poland. In a phylogenetic analysis using either Bayesian inference or Maximum Likelihood methods *P. polonica* falls within clade10 "sensu Cooke and others (2000)", together with *P. insolita* and in clade 8 "sensu Kroon and others (2004)" of the *Phytophthora* genus. This new species was named *Phytophthora polonica* Belbahri L, Moralejo E & *Lefort F.* sp. nov.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Production of Viable Oospores by *Phytophthora ramorum*[1]

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Abstract

A method was developed to improve the production of gametangia by *Phytophthora ramorum in vitro*. The agar type, the nutrient source and the genotype of the strains involved in the pairing are important factors in the pathogen's potential for sexual reproduction. Oospore viability was assessed with a coloration method using tetrazolium bromide. Most of the oospores were stained in purple, corresponding to spores in dormancy. A few germinated oospores were subcultured for further characterization. Our results suggest that new strains could be generated through breeding. This observation should be considered during pest risk analysis.

Introduction

Phytophthora ramorum (Werres, De Cock, Man in't Veld) is a heterothallic species. Initial pairing studies revealed that all European isolates were of A1 type while all American isolates were of A2 type (Werres and others, 2001). In 2003, a Belgian isolate was identified as a putative European A2 (Werres & De Merlier, 2003) while some A1 isolates were reported in American nurseries (Hansen and others, 2003), therefore suggesting some possible crossing between both mating types. However, attempts to produce oospores *in vitro* with classical methods were difficult compared to other heterothallic species, therefore suggesting a weak functionality of the sexual system in this *Phytophthora* species (Brasier & Kirk, 2004). The objectives of this study were to evaluate the role of different parameters on the production of oospores *in vitro*, and to assess their viability.

Methods and Materials

The isolates used in this study are listed in Table 1. The Belgian isolates of *P. ramorum* were collected in the framework of the survey carried out by the Belgian Plant Protection Service.

The production of gametangia by *Phytophthora ramorum* as well as by other *Phytophthora* species used as references was compared on media differing in nutrient source (carrot / tomato) and gelling agent (technical agar, bacto-agar, noble agar and agarose) using the method developed by Brasier and Kirk (2004). The amount of gametangia was scored on a scale of 0-4 based on the observation of the Petri dishes under the microscope (125x): 0=no gametangia, 1=less than 10 gametangia per

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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pairing, 2 = up to 5 gametangia per field, 3 = 5-25 gametangia per field and 4 = more than 25 gametangia per field.

Oospores of *P. ramorum* and other *Phytophthora* used as references were extracted after 60 days. To this end, agar blocks containing oospores were comminuted, filtrated and incubated in a Glucanex solution during 24 h. The viability of oospores was evaluated by a staining method using tetrazolium bromide (MTT) developed by Sutherland and Cohen (1983).

Table 1—Isolates of *Phytophthora ramorum* and other *Phytophthora* species used in this study.

Strain Nr	Species	Original Host	Collection	Mating Type	Origin*
2299	*P. ramorum*	*Viburnum sp.*	CBS[1] 101330	A1	EU
3237	*P. ramorum*	*Viburnum bodnantense*	Belgium[2]	A2	EU
2387	*P. ramorum*	*Rhododendron sp.*	Belgium[2]	A1	EU
3206	*P. ramorum*	*Rhododendron sp.*	Belgium[2]	A1	EU
3528	*P. ramorum*	*Quercus sp.*	USA[3] (014)	A2	US
3229	*P. cambivora*	na	BBA[4] 21/95-K2	A1	-
3230	*P. cambivora*	na	BBA[4] 20/95-2b3	A2	-
3231	*P. cinnamomi*	na	BBA[4] 69094	A1	-
3232	*P. cinnamomi*	na	BBA[4] 62660	A2	-
3233	*P. cryptogea*	na	BBA[4] 65909	A1	-
3234	*P. cryptogea*	na	BBA[4] 63651	A2	-
2542	*P. cactorum*	*Fragaria ananassa*	Belgium[2]	Homothallic	-

1: CBS Collection, Utrecht, The Netherlands
2: Our collection
3: Plant Protection service (PD) Geertjesweg 15, Wageningen, The Netherlands (Dr De Gruyter)
4: BBA, Messeweg 11/12 , Braunschweig, Germany (Dr Werres)
*origin of the *Phytopthora ramorum* isolate (EU or US) determined by PCR-RFLP according to Kroon *et al* (2004).
na: not available

Results and Discussion

Optimization of the production of oospores in vitro

The quality of the gelling agent and the genotype of the strain were evaluated in pairings between mating partners. By comparing different agar sources on a carrot based medium, a delay or a failure in the production of oospores was observed in pairings carried out on media supplemented with technical agar. In contrast, oospores were produced on other agar types, the production on media supplemented with agarose being slightly higher (Table 2).These differences in oospores production were not observed with other heterothallic *Phytophthora* species used as reference material, except for *P. cryptogea* on Carrot Agar.

Table 2—Production of gametangia from four heterothallic and one homothallic *Phytophthora* species on different pairing media (CBA=Carrot Bacto-Agar, CA= Carrot Agar, CAN=Carrot Noble Agar, Caose= Carrot Agarose). Amount of gametangia according to a 10-4 scale.

	Pairing	Pairing media			
Species	A1 x A2	CBA	CA	CAN	Caose
P. ramorum	2299 x 3528	4	2	4	4
P. ramorum	2299 x 3237	4	2	4	4
P. ramorum	2387 x 3528	2	0	2	3
P. ramorum	2387 x 3237	2	1	2	3
P. ramorum	3206 x 3528	2	1	2	2
P. ramorum	3206 x 3237	3	2	2	2
P. cambivora	3229 x 3230	4	4	4	4
P. cinnamomi	3231 x 3232	4	4	4	4
P. cryptogea	3233 x 3234	4	0	4	4
P. cactorum	542 (homothal	4	4	4	4

Besides the gelling agent, the formation of gametangia was also influenced by the genotype of the strains involved in the pairing. A European A1 strain producing very few chlamydospores was found to be a better mating partner than other A1 strains. Moreover, media containing carrot nutrient were more suitable than media containing tomato (data not shown).

Viability of the oospores

Oospores from intraspecific pairings between European strains were extracted after 60 days and coloured with the MTT method. As shown on figure 1, the distribution of oospores of *P. ramorum* in the different classes of coloration (purple, blue, black and colourless) was similar to that found in other *Phytophthora* species, *i.e.* a vast majority of oospores displayed a purple coloration corresponding to dormant structures. Blue coloration seemed to indicate an activated state as germinated asexual spores were stained in blue (data not shown). Unstained and black oospores were considered as non viable oospores. However, a high variation of color intensity was observed between oospores and the distinction between dark blue and black oospores was sometimes questionable, leading to potential misinterpretation. Germinated oospores were observed in several cases. Some of them died rapidly. The surviving progenies were subcultured in order to be characterised.

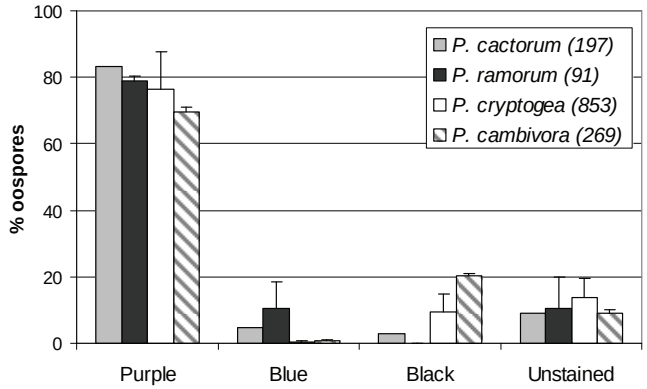

Figure 1—Distribution of 60 day-old oospores in different color classes after incubation in MTT solution for *P. ramorum* and three other *Phytophthora* species (in brackets= number of oospores)

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Phytophthora cinnamomi Populations on *Quercus* Forests From Spain and Portugal[1]

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Abstract

Molecular characterization of *P. cinnamomi* isolates obtained from soil or root samples from declining holm and cork oak trees from different locations of the southwest region of the Iberian Peninsula (Spain and Portugal), was performed by AFLP (amplified fragment length polymorphism). *Phytophthora cinnamomi* isolates from *Quercus* spp. structured in six populations according to their geographical origin, and were separated into two clusters based upon their AFLP genotypic profiles, showing only 20 percent of similarity between them. Cluster 1 comprises the populations coming from Portugal and southwestern Spain from both *Q. rotundifolia* and *Q. suber*. Cluster 2 comprises populations from the eastern Spanish part of our area of study and also comprises isolates coming from *Q. rotundifolia* and *Q. suber*. Both populations of *P. cinnamomi* are in good agreement with previous results based upon morphology and temperature-growth relationships of the isolates.

Introduction

In recent years, oak forests have suffered several episodes of decline all around Europe. In the Iberian Peninsula, symptoms of declining trees are unspecific, including dieback of branches and part of the crown, and yellowing and wilting of leaves (Brasier 1996). In southern Spain and Portugal, a strong association was shown between *P. cinnamomi* root infection and decline and mortality of cork (*Quercus suber*) and holm oak (*Quercus rotundifolia*) (Sánchez and others 2002).

Attempts to distinguish isolates of *P. cinnamomi* infecting *Quercus* spp. through molecular tools has not been performed in the past. Molecular analysis is important to understand the genetic diversity and structure of *Phytophthora* populations in order to help our understanding of disease development. The aim of the present study was to fingerprint *P. cinnamomi* isolates from *Quercus* spp., exploring the intraspecific variation to try to correlate the genetic profiles with their plant hosts, geographical location, mycelial morphology and temperature-growth relationships.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Materials and Methods

Fungal isolates

Isolates of *P. cinnamomi* (46) were obtained from soil or root samples taken from declining holm and cork oak trees from different locations of the southwest region of the Iberian Peninsula (Spain and Portugal) (Table 1), the geographical area more severely affected by Mediterranean oak decline (Sánchez and others 2002).

Table 1—Characteristics of *Phytophthora cinnamomi* isolates from *Quercus*

Group	Clade	Code	Host	Geographic origin			Source
				Country	Province	Location	
A	1	PA001	*Quercus suber*	Portugal	Algarve	S. Brás Alportel	Root
		PA003	*Quercus suber*	Portugal	Algarve	Loulé	Soil
		PA006	*Quercus suber*	Portugal	Algarve	Lagos	Soil
		PA007	*Quercus suber*	Portugal	Algarve	Lagos	Root
		PA008	*Quercus suber*	Portugal	Alentejo	Grândola	Root
		PA011	*Quercus suber*	Portugal	Alentejo	Sines	Soil
		PA012	*Quercus suber*	Portugal	Alentejo	Sines	Root
		PA013	*Quercus suber*	Portugal	Algarve	S. Brás Alportel	Soil
		PA014	*Quercus suber*	Portugal	Algarve	S. Brás Alportel	Root
		PA019	*Quercus suber*	Portugal	Algarve	S. Brás Alportel	Soil
		PA020	*Quercus suber*	Portugal	Algarve	S. Brás Alportel	Soil
		PA021	*Quercus suber*	Portugal	Algarve	S. Brás Alportel	Root
		PA043	*Quercus suber*	Portugal	Alentejo	Alcácer do Sal	Soil
		PA044	*Quercus suber*	Portugal	Alentejo	Grândola	Soil
		PE008	*Quercus rotundifolia*	Spain	Huelva	El Andévalo	Root
		PE010	*Quercus rotundifolia*	Spain	Huelva	El Andévalo	Soil
		PE011	*Quercus rotundifolia*	Spain	Huelva	El Andévalo	Soil
		PE013	*Quercus rotundifolia*	Spain	Huelva	El Andévalo	Soil
		PE054	*Quercus rotundifolia*	Spain	Huelva	Srra Aracena	Root
		PE055	*Quercus rotundifolia*	Spain	Huelva	Srra Aracena	Soil
		PE056	*Quercus rotundifolia*	Spain	Huelva	Srra Aracena	Soil
		PE058	*Quercus rotundifolia*	Spain	Huelva	El Andévalo	Root
		PE059	*Quercus rotundifolia*	Spain	Huelva	El Andévalo	Soil
		PE060	*Quercus rotundifolia*	Spain	Huelva	El Andévalo	Soil
		PE061	*Quercus rotundifolia*	Spain	Huelva	El Andévalo	Soil
		PE062	*Quercus rotundifolia*	Spain	Huelva	El Andévalo	Soil
		PE085	*Quercus rotundifolia*	Spain	Huelva	Calañas	Soil
		PE086	*Quercus rotundifolia*	Spain	Huelva	El Andévalo	Soil
		PE095	*Quercus rotundifolia*	Portugal	Algarve	S. Brás Alportel	Root
		PE097	*Quercus rotundifolia*	Portugal	Algarve	Tavira	Root
B	2	PA025	*Quercus suber*	Spain	Cádiz	Los Alcornocales	Soil
	2	PA030	*Quercus suber*	Spain	Cádiz	Los Alcornocales	Soil
	2	PA031	*Quercus suber*	Spain	Cádiz	Los Alcornocales	Soil
	2	PA032	*Quercus suber*	Spain	Cádiz	Los Alcornocales	Soil
	2	PA033	*Quercus suber*	Spain	Cádiz	Los Alcornocales	Soil
	2	PA035	*Quercus suber*	Spain	Cádiz	Los Alcornocales	Soil
	2	PA037	*Quercus suber*	Spain	Cádiz	Los Alcornocales	Root
	1	PA040	*Quercus suber*	Portugal	Ribatejo	Ponte de Sôr	Root
	1	PA041	*Quercus suber*	Portugal	Ribatejo	Ponte de Sôr	Soil
	1	PA042	*Quercus suber*	Portugal	Ribatejo	Ponte de Sôr	Root
	2	PA118	*Quercus suber*	Spain	Cádiz	Los Alcornocales	Soil
	2	PE078	*Quercus rotundifolia*	Spain	Cáceres	Herreruela	Soil
	2	PE079	*Quercus rotundifolia*	Spain	Cáceres	Herreruela	Soil
	2	PE080	*Quercus rotundifolia*	Spain	Cáceres	Herreruela	Root
	2	PE082	*Quercus rotundifolia*	Spain	Badajoz	Azagala	Root
	2	PE094	*Quercus rotundifolia*	Spain	Córdoba	Pozoblanco	Soil

In molecular analysis, some additional isolates were used as controls: two isolates of *P. cinnamomi* from *Castanea sativa*, two *P. megasperma* and two *P. inundata* isolates from *Olea europaea*, and two *P. drechsleri* and two *P. cryptogea* isolates from *Q. rotundifolia* seedlings (Sánchez and others 2001, 2005).

Hyphal morphology and growth-rate

To determine mycelial growth, CA plugs of 7-mm diameter from edges of *P. cinnamomi* colonies in active growth were placed in the centre of CA Petri dishes (9-cm diameter, 20-ml media/plate) and incubated at different temperatures in darkness. Temperatures tested were 5, 10, 15, 20, 25, 30 and 35 °C. Three replicates per isolate and temperature were prepared. Two colony diameters were measured after 2, 4, 6, 10 and 15 days.

Growth-rates per day were calculated for the different temperatures and average data of the three replicates were adjusted to a regression curve. Adjustment was made using Statistix (Statistix for Windows. Analytical Software, Tallahassee, Florida, 2000). The best polynomial model for all the isolates was chosen from several combinations of terms, based on the significance of the estimated parameters ($P \leq 0.05$): coefficients of determination (R2), coefficients of determination adjusted for degrees of freedom (Ra2), and pattern of residuals (Draper and Smith, 1981). The optimum growth temperature was estimated in the adjusted model.

Colony morphology was assessed immediately after measurement at both 4 and 6 days of growth at 20º C. Production of hyphal swellings and chlamydospores was assessed after removal of small portions of mycelium to a glass microscope slide and staining with acid fuchsin in lactophenol.

Molecular analysis

Petri dishes containing 20 ml of carrot broth and one agar plug cut from the growing margin of fresh cultures of *P. cinnamomi* and other *Phytophthoras* growing on clarified V8 juice agar were prepared. After 5 days of incubation at 25 °C, the resulting mycelium was washed in sterile distilled water. Clean mycelia were separately frozen in liquid nitrogen and ground into a fine powder with a mortar and pestle. DNA extraction was performed with the DNeasy Plant Mini kit following manufacturer instructions.

AFLP analysis was performed using the AFLP Analysis System (Gibco BRL, Grand Island, NY) with some modifications of the manufacturer's recommended protocol (Vos and others 1995).

Oomycete genomic DNA (300 ng) was digested for 2 h at 37 °C with EcoRI (2.5 U) and MseI (2.5 U) restriction enzymes. Two oligonucleotide adaptors, complementary to the restricted sites of the DNA fragments, were ligated. A pre-amplification reaction was performed with primers annealing to the EcoRI and MseI adaptors with an additional selective 3' nucleotide (A or T). Selective PCR reactions were performed using five primer combinations. The EcoRI primer was radioactively

labelled and the amplification products were separated on 6 percent polyacrylamide gels. The DNA fragments were visualised after exposition of the gel to X-ray film.

The reproducible bands were scored manually as present (1) or absent (0) for each DNA sample. One hundred and forty nine bands were scored, resulting in a binary matrix of 56 × 149 bands.

A similarity matrix was constructed based upon the Jaccard coefficient using the SIMQUAL program in the NTSYS-pc software package (Rohlf, 1998). Cluster analysis of the matrix values was performed using the SAHN program in NTSYS-pc 2.0 and a dendrogram was built.

Populations were defined according to their geographic origin and plant host, and the binary matrix was analysed using the POPGENE 32 software. The corresponding dendrogram was constructed based on a matrix of genetic distances using unweighted pair-group mean analysis (UPGMA) through the same software.

The assessment of the genetic diversity within *P. cinnamomi* species and population genetic structure was evaluated using AFLP-SURV 1.0 (Vekemans, 2002). Analysis of molecular variance (AMOVA; Excoffier and others 1992), based upon Euclidian distances between AFLP multilocus phenotypes, was conducted with ARLEQUIN 3.01.

Results

Hyphal morphology and growth rates

Colony morphology assessment at 20 ºC in CA medium did not clearly let us to separate the isolates into groups. Each isolate produced flat to slight petaloid colonies after 4 days growth, with abundant, cottony aerial mycelium after 6 days of growth. However, two different groups could be well distinguished based on hyphal morphology: *Phytophthora cinnamomi* Group A produces abundant, subspherical hyphal swellings, mainly in terminal clusters. In contrast, Group B isolates show hyphal swellings irregularly shaped, in a botryose branching pattern.

After the evaluation of different models, the growth rate data were adjusted to a third degree polynomial model: y = a*t + b*t2 + c*t3, in which y = the growth rate (mm/day), t = temperature, and a, b, c are the regression constants. Analysis of variance was performed and Fisher's test showed significant differences in average growth rate at the different temperatures tested. Two different growth patterns were detected in good agreement with the morphological groups A and B (fig. 1). According to the model selected, the estimated optimal temperature for growth was 30.1 °C for Group A and 26.9 °C for Group B. Average maximum growth rates recorded at the temperatures tested are in table 2.

Figure 1. Growth patterns of *P. cinnamomi* morphological Groups A and B. Arrows indicate the estimated optimum growth temperature (30.1 and 26.9º C respectively).

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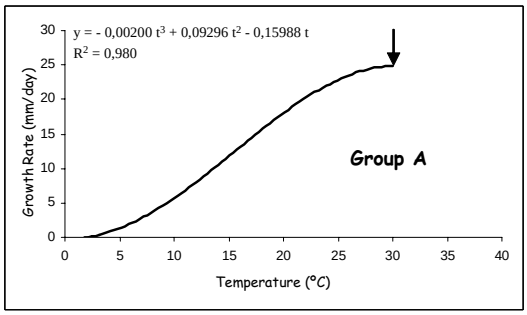

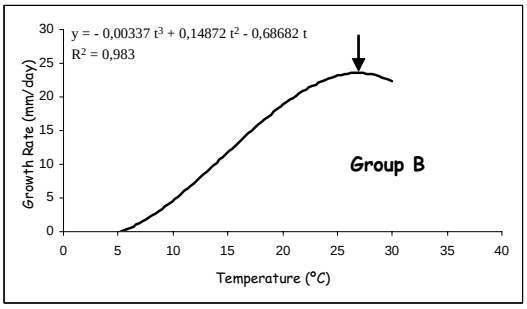

Table 2—Growth rate, minimum and optimal temperatures for growth in CA medium of the morphological Groups of *P. cinnamomi*

Group	Growth model	R^2	Minimum Temperature Estimated (ÁC)	Optimal Temperature Estimated (ÁC)	Average Maximum Growth Rate (mm/day)
A	$y = -0.002t^3 + 0.09296t^2 - 0.15988t$	0.980	1.8 (0-4.4)	30.1 (28.3-32.4)	24.9 (19.4-29.8)
B	$y = -0.00337t^3 + 0.14872t^2 + 0.68682t$	0.983	5.2 (3.9-6.8)	26.9 (25.2-28.0)	23.5 (22.6-24.6)

Molecular analysis

Isolates of *P. cinnamomi* obtained from *Q. suber and Q. rotundifolia* in Portugal (Algarve, Alentejo) and Spain (Cádiz, Badajoz, Cáceres, Córdoba, Huelva) were analysed by AFLP generating a total of 149 DNA fragments. One hundred and one (67.8 percent) out of 149 amplified fragments were polymorphic within or among populations. In single populations the percentage of polymorphic fragments ranged from 41.6 percent to 54.4 percent. All the individuals tested displayed unique AFLP band patterns, suggesting a high level of genetic variability. The results of the analysis of genetic diversity were 0.2267 for Ht (total diversity); 0.2070 for Hw (average diversity within populations); 0.0198 for Hb (average diversity among populations), and 0.0868 for Fst (differentiation between populations). High levels of gene diversity within populations (Hw = 0.2070) were observed. AMOVA revealed that 88.59 percent of the total genetic variation was attributed to differences among isolates within populations, while 11.41 percent was attributable to differences

among populations. Of the total variance only 5.09 could be attributed to geographic structure.

Phytophthora cinnamomi isolates from *Quercus* spp. structured in six populations according to their geographical origin and host provenance and were separated into two groups based upon the AFLP genotipic profiles, showing only 20 percent of similarity between them. The dendrogram calculated from Nei´s unbiased genetic distances among populations by the UPGMA method, showed two distinct clusters (fig 2). Cluster 1 comprises the populations coming from Portugal and Huelva (southwestern Spain) from *Q. rotundifolia* or *Q. suber*. Cluster 2 comprises populations from the eastern Spanish part of our area of study (province of, Badajoz –single isolates from Córdoba and Cáceres included- and Cádiz), and also comprises isolates coming from *Q. rotundifolia* and *Q. suber*.

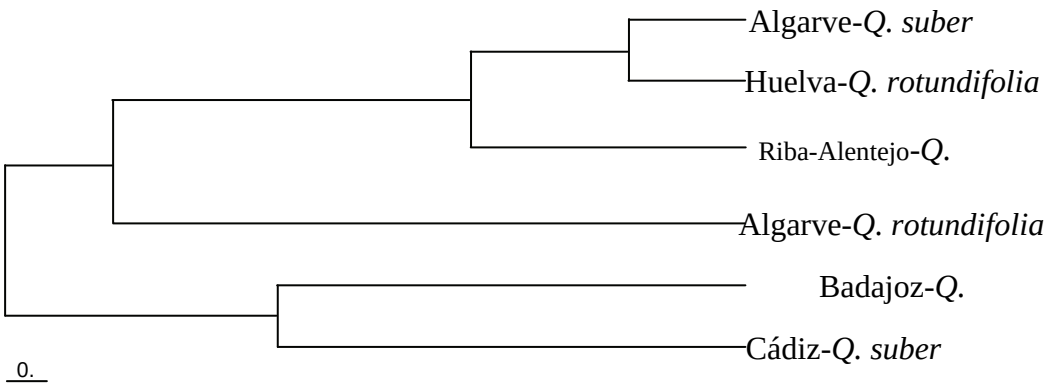

Figure 2—Unweighted pair group mean analysis dendrogram illustrating Nei´s genetic distance based on amplified fragment length polymorphism from geographical origin and host populations of *Phytophthora cinnamomi* from *Quercus* spp.

Both Clusters 1 and 2 showed a good correspondence with the morphological Groups A and B respectively (Table 1). Only three isolates (PA040, PA041 and PA042) coming from *Q. suber* Ribatejo (Portugal) belong to the morphological Group B but their AFLP profiles are in better agreement with the rest of isolates on Group A, belonging to the Cluster 1 (Table 1).

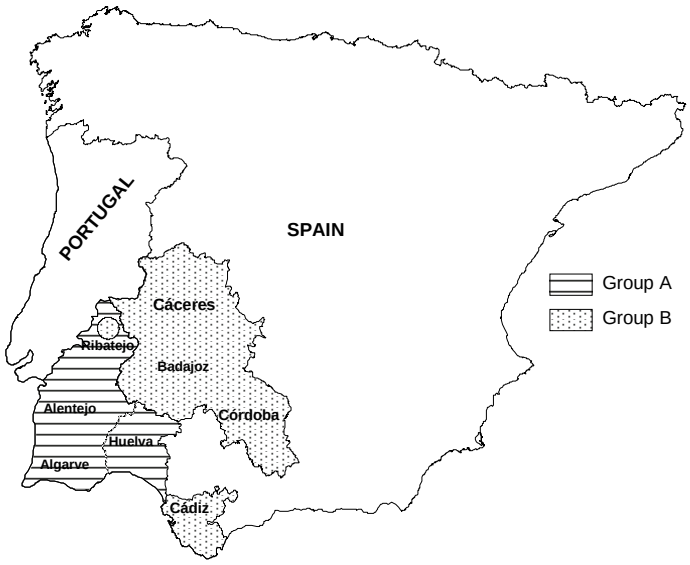

Figure 3-Geographical origin of *P. cinnamomi* isolates belonging to the morphological Group A and B. Isolates coming from Ribatejo (Portugal) (encircled) belong to the Group B but their AFLP profile correspond to the Cluster 1.

Discussion

Two different populations have been detected among *P. cinnamomi* isolates infecting Mediterranean *Quercus* species in southern Spain and Portugal by using molecular tools as AFLP fingerprintings. In previous studies, dealing with the morphological characterisation of *P. cinnamomi* isolates coming from *Quercus* roots located in this geographical area (southwest of the Iberian Peninsula), two different hyphal patterns were detected (Sánchez and others 2003a). A first group, coming from the province of Cádiz (eastern part of our area of study) showed straight hyphae, poorly branched and with abundant spherical hyphal swellings. The second group, coming from *Quercus* roots located in the west area of southern Spain, was characterised by the presence of a botryose-coralloid mycelium clearly different from the first one (Sánchez and others 2003a). As the first group of isolates came from *Q. suber* samples and the second one came from *Q. rotundifolia*, it was hypothesized that these differences could be related with the host origin (Sánchez and others 2003a, 2006). Analysing a higher number of isolates in a wider range of locations, it seems that these morphological differences former detected were better associated with the geographical origin of the isolates. The differences among both morphological groups of *P. cinnamomi* were also noticed when their cardinal temperatures of growth were studied. Isolates coming from the western part of southern Iberia (Group A) showed a higher optimal growth temperature (30.1º C) in CA medium than the isolates coming from the east (Group B, optimum temperature 26.9º C). In contrast,

minimal temperatures for growth were lower in Group A than in Group B, suggesting a higher adaptability of Group A to survive in a wider range of temperatures.

Previous studies carried out in the region of Andalucía (southern Spain) showed that the oak decline was strongly associated with *P. cinnamomi* root rot in the province of Huelva, where found the Cluster 1-GroupA of the pathogen (Sánchez and others 2002, 2003a, 2006, Navarro and others 2004). In this province, *P. cinnamomi* appear near the only factor associated whith decline, as also noticed in south Portugal (Moreira and Martins, 2005). In contrast, in the oaklands affected by decline in the province of Córdoba, the root rot caused by *P. cinnamomi* is the main biotic factor involved, but not the only one (Sánchez and others 2003a, 2006, Navarro and others 2004). In the south (province of Cádiz), the main disease associated with oak decline is the trunk canker caused by *Botryosphaeria corticola* (Sánchez and others 2003a; 2003b, Navarro and others 2004), remaining the Phytophthora root rot as secondary importance (Sánchez and others 2003a, Navarro and others 2004). In both provinces *P. cinnamomi* population belong to the Cluster 2-Group B. Nevertheless, despite the fact that both populations seem to play different roles in oak decline development in southern Iberia, differences on pathological ability between them seem to be inexistent when tested by artificial inoculations (data not published).

The present results suggest that there are two different populations of *P. cinnamomi* causing root rot on *Quercus* species in southern Iberia, one very aggressive, coming from Portugal and also colonizing the southwestern part of Spain (Huelva) causing root disease, and a second Spanish population of *P. cinnamomi* acting as decline factor in the eastern part of the geographical area affected by decline.

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Genetic Transformation of *Phytophthora ramorum* With the Jellyfish GFP Gene[1]

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Abstract

The important quarantine organism *Phytophthora ramorum* has been dramatically increasing its host range in the past years and most of the studies concerning *P. ramorum* focus on these issues. Very little is known about the latency period. For sampling and analyzing potentially infected plant material, detailed information on the latency of the disease is of high importance. In order to provide such a tool for the infection process follow-up, we established a reliable method for the stable genetic transformation of *P. ramorum* isolates. The transferred genes were the marker gene nptII for resistance to geniticin and the target gene GFP. The first and most important step in this protocol was to develop a stable system to produce protoplasts from *P. ramorum* tissue. The next step was the transformation of protoplasts with plasmids containing marker gene and target gene, following an improved polyethylene glycol (PEG)-mediated protocol of protoplasts transformation. After transformation, protoplasts were cultivated on a selective medium and allowed to regenerate mycelium. The selected transformants were checked for integration and expression of transferred genes by PCR amplification and the use of anti-GFP antibodies.
About forty-three different transformed isolates were produced and then tested for GFP fluorescence, GFP expression and GFP gene integration. They were further tested for their infection potential on Rhododendron plants.

Introduction

The oomycete *Phytophthora ramorum* Werres, De Cock & Man in't Veld sp. nov. is a ubiquitous plant pathogen with a wide host range. One of the major diseases caused by *P. ramorum* is sudden oak death (SOD), a serious threat to native American oaks and European relatives where it has been discovered in numerous ornamental plant nurseries.

We are interested in the molecular, cellular and physiological processes underlying infection pathways and tissue colonization. Using visible genetic markers has been proved to be an useful tool for the analysis of plant-pathogen interactions.

Here, we analyse the suitability of the Green Fluorescent Protein (GFP) as a reporter gene in *P.ramorum*. A $CaCl_2$ and polyethylene-glycol-based DNA transformation

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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protocol was developed and transformants expressing GFP were evaluated for usefulness in future studies.

Material and Methods

Transformation

The transformation vector used in the present study was p34GFN (Si-Ammour and others 2003) which contained the selectable marker nptII and the visible marker gfp expressed under the control of the ham34 promoter and terminator sequences. Four isolates of *P. ramorum* (BBA 9/95, BBA 26/02, BBA MSOD03-002 and PR01) were used as recipients for transformation according to the protocol of Judelson and others (1991) with some modifications.

Immunocytochemistry

The GFP expression was verified by immunocytochemical detection with Alexa Flour 488-labeled anti-GFP antibodies (Molecular Probes).

Microscopical analysis

For microscopical observation the Leica TCS SP2 confocal laser scanning microscope (CLSM) was used. GFP and anti-GFP antibody excitation was done with a 488 nm Argon Krypton laser at power levels from 10 to 50 percent. GFP expression of transgenic *P. ramorum* structures was measured by generating emission spectra profiles (lambda scan) from 500 to 580nm. Images and lambda scans were analysed with the Leica LCS software.

Results

About forty-three different transformed isolates were produced and then tested for GFP gene integration and GFP expression. Integration of GFP genes was approved with Real Time PCR and sequencing. GFP expression could be observed in all *P. ramorum* propagules obtainable from in vitro cultures like: germinating cysts, hyphae, zoosporangia and chlamydospores (Fig. 1).

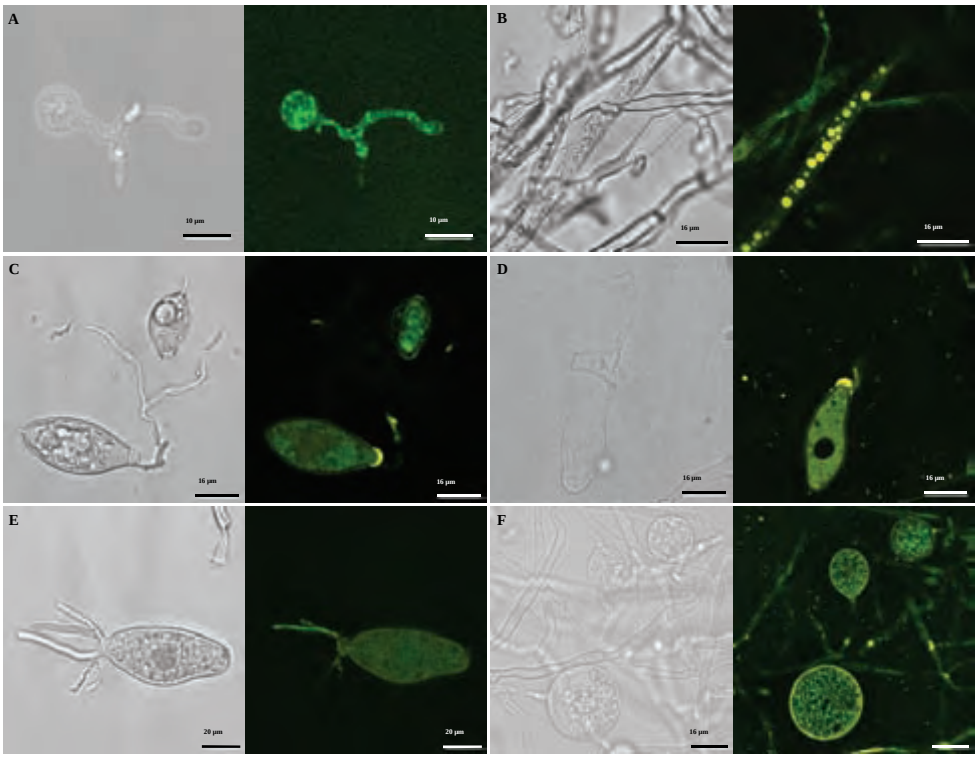

Figure 1-CLSM micrographs indicating GFP expression of transgenic *P. ramorum* strains BBA26/02_4 (**A** and **C**) and BBA9/95_6G (**B, D, E** and **F**). **A:** germinating cyst, **B**: hyphae grown in vitro, **C, D** and **E**: zoosporangia releasing zoospores and germinating respectively, **F**: chlamydospores grown in vitro. **Each left image:** transmission images. **Each right image:** CLSM signals with green signal indicating GFP expression in protoplasm and yellow signals showing natural *P. ramorum* autofluorescence in wall and papilla of zoosporangia and wall of chlamydospores.

Acknowledgements:

We would like to thank USDA-Forest Service, Pacific Southwest Research Station for funding the project.

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Posters

Phytophthora pseudosyringae Found on European Beech and Hornbeam Trees in the United Kingdom[1]

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There is major concern about the spread and impact of *Phytophthora ramorum* and *P. kernoviae* on trees and plants in the U.K. Annual national surveys monitoring their presence on *Rhododendron ponticum* are carried out but intensive spot surveys are also done on sites of particular concern. At two such sites in Wales further inspections identified a few trees with bleeding cankers potentially caused by *P. ramorum*. Three very mature trees (>150 yr-old) were affected: two European beech (*Fagus sylvatica*) and a hornbeam (*Carpinus betulus*), all were in close proximity to rhododendrons infected with *P. ramorum*.

Two bark panels were excised from each tree and isolations made from the dead live junctions of necrotic inner bark tissue onto a Phytophthora selective medium (SMA). Tissue from the hornbeam was also washed several times before *Phytophthora* could be isolated. Colonies did not show typical morphological characteristics of *P. ramorum*, so they were sub-cultured on fresh CA and sexual structures were examined after 10 d. Plugs taken from the margins of actively growing colonies were also placed in pond water and incubated at 20°C on the laboratory bench to stimulate sporangial formation.

Detailed examination of the cultures and sporangia revealed a homothallic *Phytophthora* species with semi-papillate, caducous sporangia and catenulate hyphal swellings formed in liquid culture. The morphological features correspond to those described for *P. pseudosyringae*. Sequences of the ITS1 and ITS2 rDNA matched those of *P. pseudosyringae* in GenBank (>99 percent).

Pathogenicity tests conducted on logs 1.2m long, *ca*. 40cm diam. of beech, included a reference isolate of *P. pseudosyringae* obtained from infected oak trees in Bavaria. *P. pseudosyringae* caused lesions on the beech logs, but isolates originally obtained from beech caused significantly larger lesions (15–20 cm^2) than isolates from either hornbeam or oak (2 cm^2). All isolates used in inoculations were re-isolated.

This is the first time that *P. pseudosyringae* has been reported from the U.K. and also the first record of this pathogen on hornbeam. The infected trees at both sites were very close to pathways running through the woodlands with a source of water close by. In Italy, this pathogen has also been recorded from mature beech with bleeding cankers, situated close to pathways. This study raises questions about host specificity and aggressiveness of isolates of *P. pseudosyringae* and the possibility of anthropogenic introduction to the U.K. through recreational activities.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Introduction

There is major concern about the spread and impact of *Phytophthora ramorum* and *P. kernoviae* on trees and plants in the U.K. With *Rhododendron ponticum* being the main foliar host on which inoculum is produced in Britain, annual national surveys monitoring the presence of these two pathogens on wild rhododendron are carried out but intensive spot surveys are also done on sites of particular concern. At two such sites in Wales further inspections identified two very mature European beech (*Fagus sylvatica*) and a hornbeam (*Carpinus betulus*) with bleeding cankers potentially caused by *P. ramorum*. These trees were investigated to determine the cause of the bleeding lesions.

Symptoms and tree characteristics

On the beech trees bleeding lesions were present on root flares extending up the trunk to between 1.5m and 2.5m respectively. On one of the two trees the lesions extended from the soil-surface interface upwards. The bleeds were a purplish-brown colour and towards the end of the growing season when the bleeding had slowed down, dark blobs of ooze congealed on some of the necrotic lesions (fig. 1). Underneath the outer bark the inner bark was stained a pale smoked-salmon orange colour. Numerous additional stresses including damage to bark (graffiti engraving, lightning, mammal damage), loss of limbs and colonisation by wood rot fungi and *Armillaria* further compromised tree health. The canopy of one of the beech trees was very thin, the other was less affected.

The hornbeam had similar situation to the beech trees. It was also a very mature tree, coppiced with six stems. Bleeds were present on most stems but extended up to 6m. Overall health of the tree was also compromised evidenced by a broken out limb, decay fungi present, and *Armillaria* on the roots. The crown however, was in good condition.

All trees were mature with ages estimated between 150−180 years-old.

Environment

Both sites are managed woodland-parkland areas that have networks of foot and cycle paths lacing the area and are highly visited by the public for recreational purposes. All the trees were close to water sources (10-15 m) and pathways (2−3 m) about midway up a slope. Both sites were at a low elevation (70-75 m above sea level). At the site with the infected beech the soil was brown earth but was stony with a pH of 6.5. The two infected trees were about 20 m apart and the setting was similar to parkland with an open undercanopy and rhododendron along the

watercourse. The site with the hornbeam by comparison, was more akin to woodland having brown earth that had a gleyed layer and a pH of 6.0. The hornbeam site was generally wetter than the beech site.

Methods

Isolation and identification

Panels of diseased bark (3x5 cm^2) were excised from the trees over a dead-live junction, so that necrotic inner bark and a fresh margin of active lesion were obtained. Samples were washed under running tap water for 1 to 24 hours and pieces of tissue from the dead-live margins of the inner bark plated on *Phytophthora* selective medium (SMA) (Brasier and Jung 2001). Isolates that developed on SMA were sub-cultured on carrot agar (CA) (Brasier 1969) for morphological studies. Pieces of the colonies were cut from the actively growing margins, placed in to pond water and exposed to natural light for 48 h (Jung and others 2003) to induce sporangia formation; oospores were formed on CA after 10 days. Morphological features were examined using the microscope (x40 magnification). Measurements of 10 sporangia, antheridia and oogonia per isolate were made and compared with those described by Jung and others (2003). Daily radial growth rate on CA at 20ºC were also compared with the description given in Jung and others (2003). Molecular diagnosis was used to confirm identification. DNA was extracted using and the ITS regions amplified using primers ITS1 and ITS4 (White and others 1990). Sequences were compared with those in GenBank using BLAST.

Inoculations

Log inoculation tests were carried out using 1 cm diameter mycelial plugs on wounded beech logs (*ca*. 20 cm diam.) according to the method described by Brasier and Jung (2001). Four isolates of *P. pseudosyringae* were tested on beech and two control *Phytophthora* species - *P. cambivora* as an aggressive positive control, and *P. alni* as a non-pathogenic negative control were included (Table 1).

Table 1—*Phytophthora* species and isolates used in pathogenicity tests and details of original host and country isolates were obtained from

Species	Isolate number	Host of origin	Country of origin
P. alni subsp *alni*	P772	*Alnus glutinosa*	England, U.K.
P. cambivora	P1832	*Fagus sylvatica*	England, U.K.
P. pseudosyringae	PSEU 6*	*Quercus robur*	Germany
P. pseudosyringae	TLB 14	*Fagus sylvatica*	Wales, U.K.
P. pseudosyringae	TLB 13	*Fagus sylvatica*	Wales, U.K.
P. pseudosyringae	TLB 105	*Carpinus betula*	Wales, U.K.

* Isolate kindly supplied by T. Jung.

Inoculated logs were incubated at 20°C for 5 wk (Brasier and Jung 2001). Paring away the outer bark exposed lesions (fig. 2) which were traced, the tracings cut out and weighed and disease area determined (Brasier and Jung 2001).

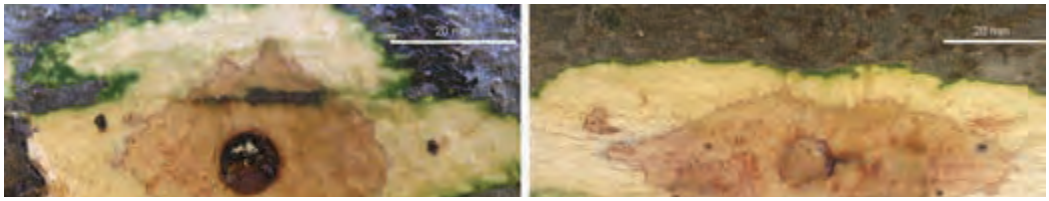

Figure 2—Exposed, inner bark lesions after 5 wk on wound inoculated European beech, caused by *P. cambivora* (left) and *P. pseudosyringae* (TLB 14A) (right).

Results

Cultural and morphological features were in close agreement with those described for *P. pseudosyringae* (Jung and others 2003) (fig. 3) and the ITS BLAST searches showed >99 percent homology to sequences of *P. pseudosyringae*, confirming the morphological identification.

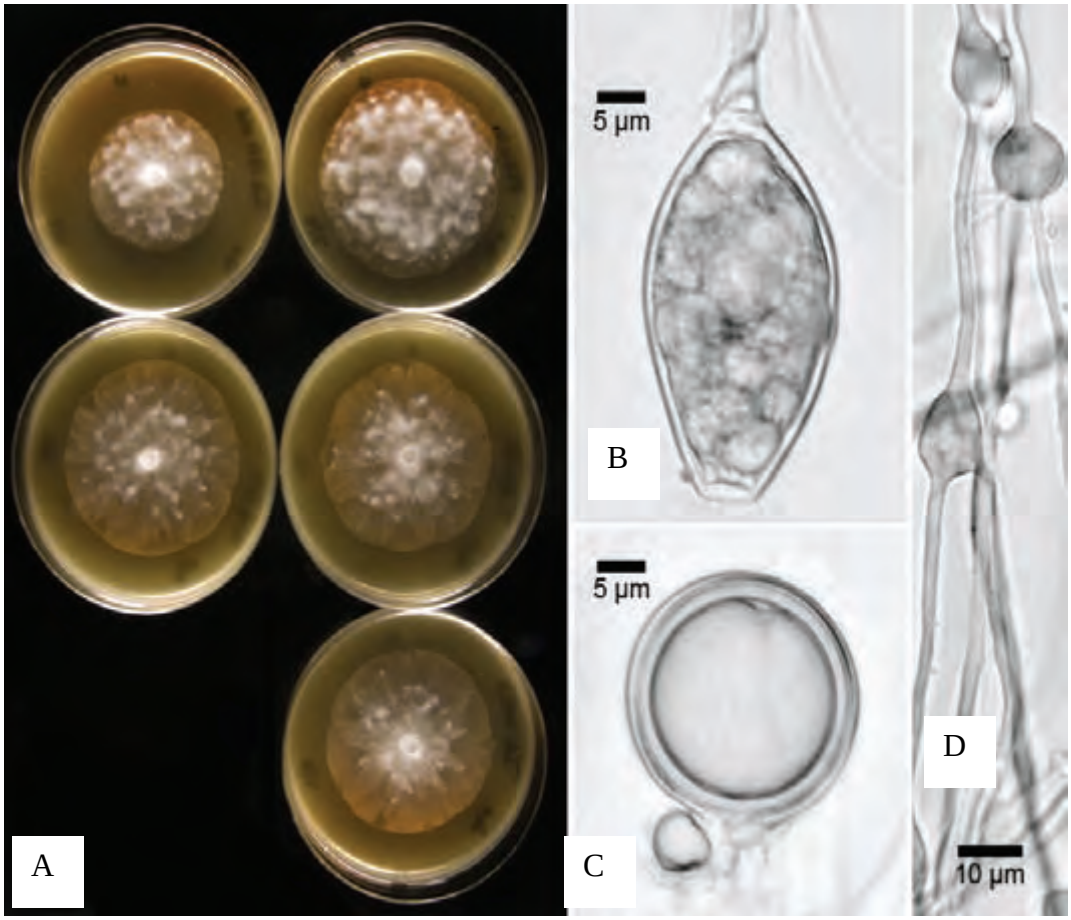

Figure 3—Morphological features of U.K. isolates of *P. pseudosyringae* – (a) Colony morphology similar to German isolate: (top, left to right) PSEU 6; TLB 105C; TLB 105D (bottom, left to right) TLB 13A; MAR 13A; (b) Sporangium: Attached (although some caducous in colony), terminal, semi-papillate; (c) Oogonium with attached single celled, paragynous antheridium, and golden oospore; (d) Catenulate hypal swellings.

The inoculation studies confirmed that *P. pseudosyringae* was pathogenic on beech but there was much variation in aggressiveness amongst the isolates. One isolate was highly aggressive, one moderately so and two were benign (fig. 4), the more aggressive two originating from beech while the two benign isolates originated from other hosts (oak and hornbeam, fig. 4; Table 1).

P. cambivora was the most aggressive *Phytophthora* species tested while *P. alni* subsp. *alni* was non-pathogenic.

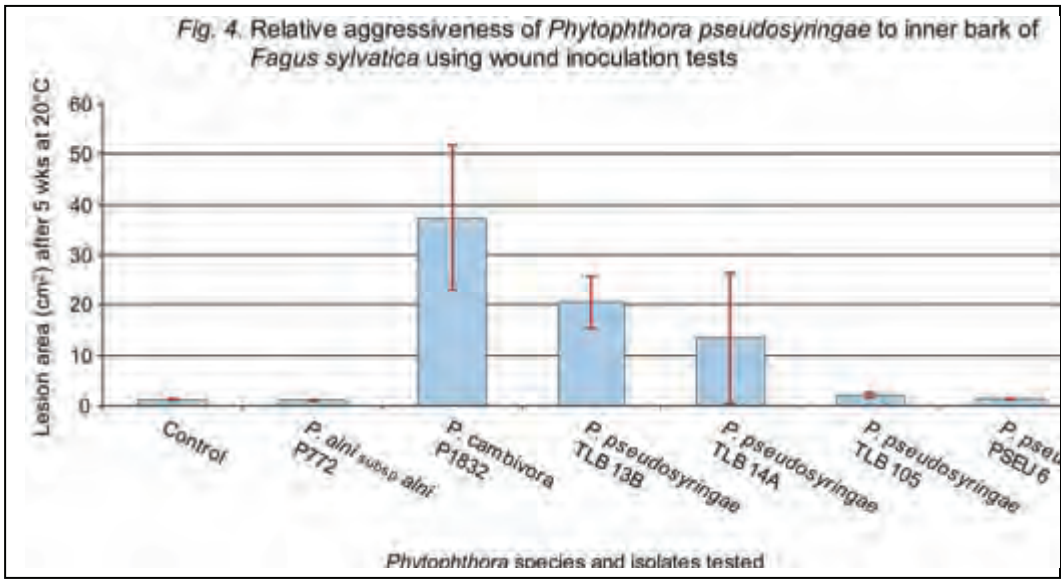

Figure 4—Relative aggressiveness of P*hytophthora pseudopsyringae* to inner bark of *Fagus sylvatica* using wound inoculation tests.

Discussion

This is a first record of *P. pseudosyringae* in the U.K. and hornbeam is a new host for this pathogen. Although our isolates closely matched previous descriptions (Jung and others 2003), since *P. pseudosyringae* is a recently described species and very similar morphologically to *P. syringae*, it is possible that it has been misidentified previously in the U.K. (Jung and others 2003). Koch's postulates need to be carried out on hornbeam to confirm pathogenicity on this host.

Initial pathogenicity tests on beech indicate that *P. pseudosyringae* is much less aggressive than *P. cambivora* corroborating previous work (Brasier and Kirk 2001, Fleischmann and others 2004, Jung and others 2003), but the large range in aggressiveness of isolates suggests that there may be a host specificity isolate aggressiveness relationship which has also been mentioned by others (Brasier and Kirk 2001, Jung and others 2003, Martin and Tooley 2003). Cross-pathogenicity tests need to confirm this hypothesis. Fleishmann and others (2004) show that *P. pseudosyringae* has a different pathogenic effect on beech seedlings than some of the more aggressive *Phytophthora* species such as *P. cambivora* or *P. citricola.*

Much remains unknown about the ecological role and behaviour of *P. pseudosyringae*. In Europe it is regarded as a pathogen causing fine root rot and bleeding stem cankers of native tree species. However, in the USA it is isolated from necrotic leaves and twig cankers in tree canopies specifically

bay laurel (*Umbellularia californica*), with only a few records of it being associated with oak bleeding stem cankers (Murphy and Rizzo 2005, Wickland and Rizzo 2005). Another interesting ecological aspect of *P. pseudosyringae* is that other *Phytophthora* species are frequently isolated with it whether from infected roots and stems (Diana and others 2006, Fleishmann and others 2004, Jung and others 2003, and also in this study) or from leaves where it is reported to co-exist with other *Phytophthora* species in tree canopies (Murphy and Rizzo 2005, Wickland and Rizzo 2005).

The site details reveal that the diseased trees in Wales were close to pathways and a source of water. First reports of this pathogen in Italy also emphasise this observation (Diana and others 2006). This could suggest disease development either through introduction of a foreign pathogen connected with human recreational activity; or to stress imposed on the tree by compaction and altered drainage caused by the pathways as well as activity around its roots thus altering their susceptibility to this particular *Phytophthora*.

Acknowledgement

We thank Dai Evans, FC Technical Support Unit, Talybont, Wales for field assistance.

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Root Associations of *Phytophthora ramorum* and *Phytophthora kernoviae* in U.K. Woodlands[1]

Elizabeth J. Fichtner,[2] David M. Rizzo,[2] Susan A. Kirk,[3] Alistair Whybrow,[3] and Joan Webber[3]

Abstract

Phytophthora kernoviae and *Phytophthora ramorum*, two pathogens recently introduced to the U.K., incite foliar lesions, shoot necrosis, and death of *Rhododendron ponticum*, an invasive weed pervading U.K. woodlands. In infested woodlands, *R. ponticum* serves as an epidemiologically important host, supporting sporulation of both pathogens. Bleeding cankers on trunks of European beech (*Fagus sylvatica*) caused by either *P. ramorum* or *P. kernoviae* are often associated with neighboring infected *R. ponticum*.

Rhododendron ponticum has been removed from several woodlands as an inoculum management strategy, but the long-term efficacy of plant removal is unknown, in part due to lack of knowledge of pathogen persistence in roots and in emerging seedlings.

The potential for *P. ramorum* and *P. kernoviae* to infect roots of *R. ponticum* in U.K. woodlands is unknown. To assess pathogen association with rhododendron roots, roots initiated from natural layering were excavated from two sites infested with *P. kernoviae* and one site infested with *P. ramorum*. At each site, four sets of layered roots were sampled, in addition to the associated leaf litter, rhizosphere soil, and symptomatic leaves. In the laboratory, soil and leaf litter were individually baited using leaf disks of *Rhododendron catawbiense* 'Cunninghams White.' Tissue from symptomatic leaves was embedded in SMA agar for isolation of *Phytophthora* spp. Neither pathogen was baited from rhizosphere soil, but both were routinely recovered from leaf litter. *Phytophthora ramorum* was baited from one set of layered roots; *P. kernoviae* was baited from three sets of roots at one site and from two sets at another site.

A second objective focused on investigating the potential for infection of *R. ponticum* seedlings in a woodland cleared of *R. ponticum* in 2005 for management of *P. kernoviae*. Nineteen seedlings were excavated from the woodland and all foliar lesions were sampled for pathogen isolation. Rhizosphere soil and roots were independently baited with rhododendron leaf disks. *Phytophthora kernoviae* was recovered from foliar tissue on 2 seedlings, from roots of 5 seedlings, and from two samples of rhizosphere soil.

The results suggest that both *P. ramorum* and *P. kernoviae* are associated with *R. ponticum* roots in infested U.K. woodlands. Furthermore, the presence of inoculum in litter but rarely in soil suggests that the pathogens may infect the roots, rather than simply persist on the rhizoplane. Further research is needed to assess the frequency of root associations and to histologically visualize root infections of thesetwo pathogens. These preliminary data suggest that the potential persistence of these pathogens in roots and litter should be considered when managing the diseases in infested woodlands.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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"Mal del Ciprés" Disease: Analysis of the Association Between Aerial Symptoms and Vitality of Trees[1]

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Abstract

Austrocedrus chilensis is an indigenous Cupressaceae of the Patagonian Andes forests in southern Argentina and Chile that suffers a disease called "mal del ciprés". *Phytophthora austrocedrae*, a new *Phytophthora* species, was recently shown to be the main cause of the disease. The progressive withering and subsequent defoliation of the tree, which finally dies, has been traditionally considered the main symptom. Other symptoms are basal resinous exudates and red-brown necrotic lesions in the inner bark extending up the bole from killed roots. Brown cubic rots in roots and sapwood are many times associated with dead or dying trees.

Although the symptomatology of the disease has been described as a slow process of defoliation that culminates with the death of the tree, dying and recently dead trees with abundant foliage are frequently observed. This shows that sudden death with little or no previous defoliation also happens. On the other hand, trees with crowns looking almost healthy but with many big lesions at the root collar, and defoliated trees with few or no lesions at the root collar have been observed several times.

Traditionally, it was thought that crown transparency is a suitable measure of the vitality of a tree. Nevertheless, the contradictory observations previously mentioned make such a supposition doubtful and make it difficult to assess the extent of disease in a tree or a stand. Since an accurate method for evaluating the vitality of affected trees is essential for establishing management and conservation strategies in affected forests, an evaluation of symptomatology and its association with tree vitality was done. The aims of this work were to analyze the association between aerial symptoms and vitality and to adjust the method used for determining vitality of trees affected by "mal del ciprés".

Sixty trees, in three different vitality classes, of a stand affected by "mal del ciprés" were evaluated. Percentage of defoliation was estimated visually, with an accuracy of 5 percent, using two different methods. The pattern of defoliation, the presence of yellow or red leaves in the crown, cankers and resinous exudates in the stem, and other features that could be related to vitality were also recorded. Bark was removed to expose inner bark and the vitality at the collar root was determined as the percentage of the perimeter with dead tissues. Then the root system was excavated to expose roots in a perimeter of 1m from the base of the tree. Bark of main roots was removed and vitality of each root was evaluated as percentage of tissues dead/affected. Vitality of root system was estimated as the total percentage of affected tissues of main roots. The association between aerial symptoms and tissue vitality was evaluated through a correlation analysis between each of the three variables (defoliation,

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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vitality at root collar and vitality of roots). The association between root collar and root vitality and the other recorded symptoms was also evaluated through a correlation analysis. This work presents the results of the study and a discussion on the reliability of aerial symptoms for estimating vitality of trees affected by the “mal del ciprés”.

The *Phytophthora* Species Known as "Pg chlamydo"[1]

Everett Hansen,[2] Paul Reeser,[2] and Wendy Sutton[2]

Abstract

Phytophthora taxon Pg chlamydo is perhaps the second most abundant *Phytophthora* species in the world, after *P. gonapodyides*, although it is commonly misidentified. Pg chlamydo is frequently encountered in streams and rivers in western North America, Argentina, China, and Europe. It has occasionally been recovered from forest soil and was once isolated from a bole canker on a tanoak tree; it was pathogenic to tanoak in artificial inoculation.

Pg chlamydo resembles *P. gonapodyides* in culture, and is related to that species. It is apparently sterile, not itself forming sexual structures even when paired with tester isolates of known mating type. Sporangia are non-descript nonpapillate, similar to other species in the *P. megasperma/P. gonapodyides* ITS clade. It is distinguished from *P. gonapodyides* by the formation of chlamydospores in culture. It has also been misidentified as *P. lateralis*, *P. drechsleri or P. cryptogea*, although the latter species are heterothallic, and readily distinguished with a mating test.

ITS-DNA sequences of isolates from Oregon, California, Argentina, and France were identical, but at least three mitochondrial genotypes were distinguished.

Introduction

Phytophthora taxon "Pg chlamydo" is perhaps the second most abundant *Phytophthora* species in the world, after *P. gonapodyides*, although it is commonly misidentified. It resembles *P. gonapodyides* in culture, but is distinguished by the formation of chlamydospores and chains of hyphal swellings. Like P. *gonapodyides*, it is a sterile species, but has also been misidentified as *P. lateralis*, *P. drechsleri or P. cryptogea*. "Pg chlamydo" is frequently encountered in streams and rivers in western North America, Argentina, and Europe. It has occasionally been recovered from forest soil and was once isolated from a bole canker on a tanoak tree; it is occasionally encountered as a foliar pathogen of woody plants in ornamental nurseries (Blomquist unpublished). It was associated with root rot of Port-Orford-cedar in German nurseries, where it was initially misidentified as *P. lateralis* (Hansen and others 1999), and with root rot and stem cankers of *Abies* species in nurseries and Christmas tree plantations, where it was misidentified as *P. drechsleri* (Brasier and others 1993).

Objectives

1. To raise general awareness of this common but poorly understood *Phytophthora*.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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2. To explore the population genetics of this sexually sterile and very widespread species.

Methods

Isolates recovered from water, soil and plant tissues originating from Europe (France and Germany), Argentina, and western United States (California and Oregon) were compared. All isolates from Argentina were baited from different locations on a single stream near Corcovado. Water isolates from western USA were all from different streams in SW Oregon and northern California.

Isolates were grown up on CMA and DNA was extracted from colonized agar plugs using a CTAB buffer protocol. DNA was PCR amplified with each of the five primer sets listed below (Table 1) and sequenced. These sequences were then edited and aligned.

Table 1—DNA regions analyzed.

Gene or DNA Region	Primers	Length Amplified	Citation
ITS rDNA	ITS 4 and ITS5	~820bp	White,T.J,.and others 1990
Mitochondrial COX spacer	FMPH8 and FMPH10	~340bp	Martin,F.N. and Tooley,P.W. 2003
60S ribosomal protein L10	60SL10F and 60SL10R	~420bp	http://www.phytophthoradb.org/
B tubuln	BTubF1 and BTubR1	~990bp	Kroon, L.P.N.M., and others 2004
Elongation Factor	EF1AF and EF1AR	~865bp	Kroon, L.P.N.M., and others 2004

Morphology

Morphology and growth were similar for all isolates examined regardless of source.

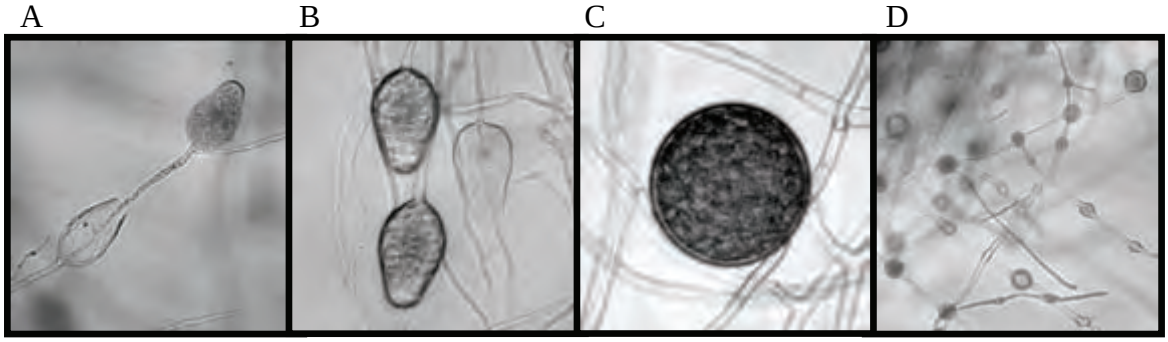

Figure 1—Sporangia of “Pg chlamydo” are non-papillate and proliferate both internally and externally (A and B). Chlamydospores (C) and hyphal swellings (D) characteristic of “Pg chlamydo”. Photos C. Delatour

Population Structure

Although isolates identified as “Pg chlamydo” are monophyletic within ITS clade 6, they exhibit remarkable intra-specific molecular variation in all genetic regions examined. “Double peaks,” commonly interpreted as evidence for past hybridization events, are unusually common in the DNA sequences. Only the mitochondrial COX spacer region lacked double peaks.

All isolates shared a basic ITS DNA sequence, but four ITS groups were distinguished based on the presence or absence of double peaks at four loci.
The mitochondrial COX spacer DNA region also had four genotypes among the 24 isolates fully characterized, although two genotypes were represented by single isolates. The 60S L 10 gene (RL10) encodes a ribosomal protein. Most isolates exhibited double peaks at 4-5 loci. Four related genotypes were identified among the isolates.
Elongation factor and β-tubulin gene sequences have not yet been not fully aligned, but many isolates exhibit double peaks at multiple loci.

Multilocus DNA Phenotype

With four genotypes recognized within each of the three DNA sequences fully analyzed, a total of 64 multi-locus genotypes are possible. Only five of these were identified in the 24 isolates fully characterized (Table 2).

Five isolates formed multi-locus Group A. There were no double peaks scored for Group A in any of the three DNA regions. These isolates were from streams and soil in Oregon, and water in France.

All of the isolates from Argentina were identical, and formed multi-locus Group B, distinguished by double peaks at four RL 10 loci, and a single ITS locus.
Multi-locus Group E had a distinctive COX spacer sequence and unique patterns of double peaks in the RL10 and ITS sequences. The ITS variation within Group E resulted from differences in relative peak strengths at the same double peak loci.
The remaining multi-locus Groups, Group C and Group D, were represented by single isolates.

Conclusions

Phytophthora isolates identified as P.sp.”Pg chlamydo” from Europe, North America, and Argentina are indistinguishable morphologically and closely related phylogenetically.

Isolates could be grouped in three main multi-locus DNA clades. Isolates from Oregon, California, and Europe were mixed in two clades, and all isolates from Argentina formed the third main clade.

Isolates in clade A (Oregon and France) exhibited no double peaks in the sequences analyzed. The other clades were distinguished by patterns of multiple double peaks.
P.sp.”Pg chlamydo” evidently has a complex evolutionary history.

Table 2—Groupings according to DNA alignments of three DNA regions.

Isolate	Source	Substrate	Collector	FMPH GROUP	60SL10 GROUP	ITS GROUP	MULTI-LOCUS GROUP
9364	Oregon	Tanoak canker	Hansen lab	I	c	2	A
33-2-2-0603	Oregon	Soil	Hansen lab	I	c	2	A
Haye3b	Europe	Water	Hansen & Delatour	I	c	2	A
WA42.2-062204	Oregon	Water	Hansen lab	I	c	2	A
WA49-031907	Oregon	Water	Hansen lab	I	c	2	A
AG27	Argentina	Water	Greslebin	I	d	4	B
AG29	Argentina	Water	Greslebin	I	d	4	B
AG30	Argentina	Water	Greslebin	I	d	4	B
AG48	Argentina	Water	Greslebin	I	d	4	B
AG50	Argentina	Water	Greslebin	I	d	4	B
WCK2.7.21	California	Water	Rizzo lab	II	e	1	C
WA46.3-100404	Oregon	Water	Hansen lab	III	d	2	D
107	Europe	POC soil	Hansen	IV	a	3.1	E1
62689	Europe	POC soil	Hansen	IV	a	3.1	E1
Haye3.1	Europe	Water	Hansen & Delatour	IV	a	3.1	E1
RIZZO-BUL 1-2.7.8	California	Water	Rizzo lab	IV	a	3.1	E1
WA20.1-041904	Oregon	Water	Hansen lab	IV	a	3.1	E1
WA38.1-083104	Oregon	Water	Hansen lab	IV	a	3.1	E1
WA39-100404	Oregon	Water	Hansen lab	IV	a	3.1	E1
WA6-011304	Oregon	Water	Hansen lab	IV	a	3.1	E1
H1	Europe	Water	Hansen & Delatour	IV	a	3.2	E2
WA48.2-070404	Oregon	Water	Hansen lab	IV	a	3.2	E2
RIZZO-RCK1.9.2	California	Water	Rizzo lab	IV	a	3.3	E3
WA5.1-072003	Oregon	Water	Hansen lab	IV	a	3.3	E3

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Virulence Behaviour and Gene Expression Patterns of a *Phytophthora cinnamomi*-Cinnamomin Silenced Transformant[1]

Marília Horta,[2] Paula Caetano,[2] Ana C. Coelho,[3] Dina Neves,[4] Nelson Sousa,[2] and Alfredo Cravador[2]

Abstract

Phytophthora cinnamomi is an oomycete parasite of *Quercus* suber roots. This pathogen secretes abundantly cinnamomins, proteins from the elicitin family. It was demonstrated that these proteins interact with several lipidic molecules, namely sterols but their biological role remains unknown.

The silencing of the gene coding for β-cinnamomin was induced by genetic transformation of protoplasts by chemical methods (liposomes and CaCl2/PEG) with an antisense sequence of the β-*cin* gene (our recent work). Selection of transformants was achieved through co-transformation with a gene conferring resistance to hygromicin B. The presence of the transgenes was certified by PCR. The absence of the protein in the culture media was confirmed through western blotting using monoclonal antibodies anti-β-cinnamomin, and the absence of the coding mRNA was proven by means of real time RT-PCR quantification. The geneticexpression of the genes coding to the other elicitins was shown to decrease as well.

Greenhouse pathogenicity tests were now carried out in parallel in *Q. suber* plants with the only stable cotransformant obtained (antibiotic resistant and with the β-*cin* gene silenced), with a simple transformant (antibiotic resistant) and with the wild type isolates. The results revealed that the β-*cin* silenced isolate has a decreased virulence; and that this impaired virulence could not be caused solely by the presence of the resistance gene.

Genetic expression profiles obtained by cDNA-AFLP disclosed marked differences between the cotransformant and the wild isolate. Sequencing of some fragments differentially expressed showed that these are potentially associated with the expression of the β-cin gene; among them, a gene associated with the fatty acids metabolism is particularly important.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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The Spatial and Temporal Dynamics of *Phytophthora lateralis*-Related Mortality on *Chamaecyparis lawsoniana* After Ten-Plus Years on Two Field Sites[1]

Scott E. Kolpak,[2] Everett M. Hansen,[3] Richard A. Sniezko,[2] and Wendy Sutton[3]

Abstract

Chamaecyparis lawsoniana (Port-Orford cedar, POC) is distributed throughout southwest Oregon and Northern California, located predominantly along river drainages. A non-native root pathogen, *Phytophthora lateralis*, was first detected in a Seattle Nursery in 1923 and is responsible for extensive mortality across the range of POC. The spatial structure of infection was examined in the Page Mountain area (Siskiyou Mountains, Oregon/California, USA) using dendrochronology methods and the Smith River National Recreation Area (California, USA) using GIS methods. There is no published data on the effects of site and micro-site variation on the patterns of *P. lateralis* infection and its relative virulence using a uniform population background.

Two replicated field trials (Quosatana and Flannigan sites) were established in 1993 in the Siskiyou National Forest planted with seedlings from 28 open-pollinated families from a subset of 200 phenotypic resistant selections chosen in the late 80s. A single seedling from 28 open-pollinated families was planted in each plot. The trees were planted in a circular plot consisting of an outer ring and an inner ring centered around a dead POC to provide a consistent and uniform inoculum hazard. A group of four to eight plots were planted within five meters of a neighboring plot and groups were separated by 35 to 65m. The trees were monitored for mortality (*P. lateralis* and other factors) over ten-plus years; *P. lateralis* isolation/confirmation was attempted in the first two years after planting establishment and again ten years later.

There are significant differences among families in the level of mortality over 10 years, with family 510015 showing greater survivorship than the most susceptible families. However the survivorship functions are not different among families because survival curves have a similar shape but plateau at different mortality levels. Site by family interactions and site effects are not significant but the group (clusters of plots) effects are significant in the ANOVA. Survivorship analysis found significant difference across sites, groups, and plots. The Quosatana site has higher mortality (88 percent) than Flannigan site (74 percent) after 10 years and the survivorship functions are different. The absolute level of mortality is different between groups and plots with plot mortality ranging between zero and 100 percent. The data suggests that the density *P. lateralis* in the early years of the planting was not uniform within or between sites reflected by the differences in mortality. There are some weak associations of group and plot mortality with the surrounding vegetation composition. *Phytophthora lateralis* was isolated from most plots in the first couple years after planting and ten years later the

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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pathogen is detectable in less the half of the plots indicating the density of *P. lateralis* spores are significantly reduced or now absent in some areas of the plantings.

Adaptability and Susceptibility of Oaks to *Phytophthora quercina* Under Drought and Nitrogen Stress[1]

Sindy Leonhard,[2] Jörg Schumacher,[2] and Alfred Wulf[2]

Abstract

The effects of global climate change are nowadays a frequently discussed problem. Among them especially temperature increase and drought stress during the growing season can predispose plants to diseases. Therefore a project of the Deutsche Bundesstiftung Umwelt (DBU) was started in 2005 to investigate the influence of changing environmental factors to autochthonic tree species as well as their adapted and potential pathogens. The purpose of the present study is to examine the effects of controlled stress factors, particularly drought and nitrogen stress, on the susceptibility and adaptability of two to three years old *Quercus petraea* plants to infection by *Phytophthora quercina*. The host-pathogen interactions of two different German proveniences of *Q. petraea* under controlled greenhouse conditions have been studying since 2005. First results about the differences in the response of the species to drought and nitrogen stress are compared and discussed.

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Prevalence and Development of Disease on Coast Redwood Seedlings Caused by *Phytophthora ramorum*[1]

Sunny Lucas,[2] Jennifer Parke,[2] and Yana Valachovic[3]

Abstract

Coast redwood *(Sequoia sempervirens)* is a host for *Phytophthora ramorum* but it is unclear if the pathogen represents a significant disease risk to this tree species. In an on-going field experiment, we are examining the prevalence of infection and the development of symptoms on coast redwood seedlings in naturally infested sites in southern Humboldt Co., California. In November 2006, healthy redwood seedlings were placed amid tanoak and bay trees naturally infected with *P. ramorum.* Disease incidence and symptom development are being observed monthly, and every two months, a subset of redwood seedlings is destructively sampled to investigate the location and extent of tissue colonization by *P. ramorum.* In order to correlate inoculum levels across field sites with disease development, rainwater is being collected and baited with rhododendron leaves to determine presence and frequency of positive *P. ramorum* rain traps. Results of this study will help evaluate the risk of *P. ramorum* to coast redwood seedlings and inform land managers of the potential for reforestation of infested sites with this species.

Introduction

Coast redwood (*Sequoia sempervirens*) is a host for *Phytophthora ramorum* but it is unclear if the pathogen represents a significant disease risk to this species. *P. ramorum* was first isolated from saplings in Sonoma and Santa Cruz Counties, California in 2002 and Koch's Postulates were completed (Maloney and others 2002). Symptoms include needle lesions, cankers on small branches, and dieback of epicormic sprouts (Davidson and others 2003). Researchers also observed discoloration in sapling xylem up to 90 cm from stem inoculation (Maloney and others 2002). Detection from mature redwood tissue has been reported, but conclusive disease causality was not determined (Garbelotto and others 2003). In a comparison of artificial inoculation methods, whole plant dips led to symptoms most similar to those found in nature (Hansen and others 2005).

The objectives of this study were to evaluate the risk of disease to coast redwood seedlings caused by *P. ramorum*. To do this, we attempted to determine disease incidence in relation to time of year, weather and inoculum. We also wanted to examine disease development on redwood seedlings in association with a specific overstory species, California bay (*Umbellularia californica*).

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Methods

Experimental Sites

Experimental sites are located in southern Humboldt County, the site of the northernmost California SOD infestation (Table 1). Conditions in this area are potentially conducive for sporulation because of mild temperatures, heavy precipitation, and presence of hosts. Infestation around the Avenue of the Giants is unsettling due to the valuable ecosystem and the amount of human traffic in this area.

All sites are located along the Eel River on alluvial flats. The overstory is dominated by coast redwood. Symptomatic tanoak (*Lithocarpus densiflorus)* is present at all sites. California bay is present at two of the sites (Table 1).

Table 1—Location of experimental sites.

Site	Location	Prevalence of disease symptoms on tanoak	Presence of California bay
O'Meara Grove	Redway, CA Humboldt Redwood State Park (HRSP)	High	No
Tooby Park	Garberville, CA Garberville Community Park	High	Yes
Chandler Grove	8 km north of Meyers Flat, CA HRSP	Low	No
Chandler Grove	8 km north of Meyers Flat, CA HRSP	Low	Yes

Seasonality of Disease Incidence

Using a complete random design, 20 healthy, potted seedlings were placed at Tooby Park for a two-month period beginning in December, 2006. A new group of seedlings was placed at Tooby Park every two months through June, 2007.

Presence of *P. ramorum* was determined by isolation and PCR from selected stems, needles, and buds of each seedling. No seedlings were found positive for *P. ramorum* from any time period. Therefore, we were unable to determine when redwood seedlings are most susceptible to infection.

Symptom Development

Thirty healthy seedlings were planted at each of the four research sites in December, 2006. Symptom development was monitored monthly through June, 2007. A count of necrotic needles, cankers and length of stem dieback per seedling was measured monthly. Growth of seedlings over time was used as an indicator of overall vigor.

No symptoms were detected on seedlings at any of the sites. Therefore, no relationship between symptom development and inoculum levels or presence of California bay could be determined.

Detecting Inoculum

Rainwater was collected and attempts made to quantify inoculum (fig 1). Initially fifteen raintraps were established at each of the sites. The first method used, from December, 2006 through April, 2007, followed the protocol set forth by the Rizzo lab at UC Davis (Davidson and others 2005). Water was collected, and then filtered. The filter was placed onto pimaricin-ampicillin-rifampicin selective media (PAR), and the plates examined for colony forming units (CFU) at OSU.

Due to erratic detection, methodology was altered at the end of April. The second method followed a detection protocol similar to that used in Curry County, Oregon. Rhododendron leaf baits were placed in each of 60 raintraps, collected semi-monthly, and plated onto PAR to determine presence or absence of inoculum.

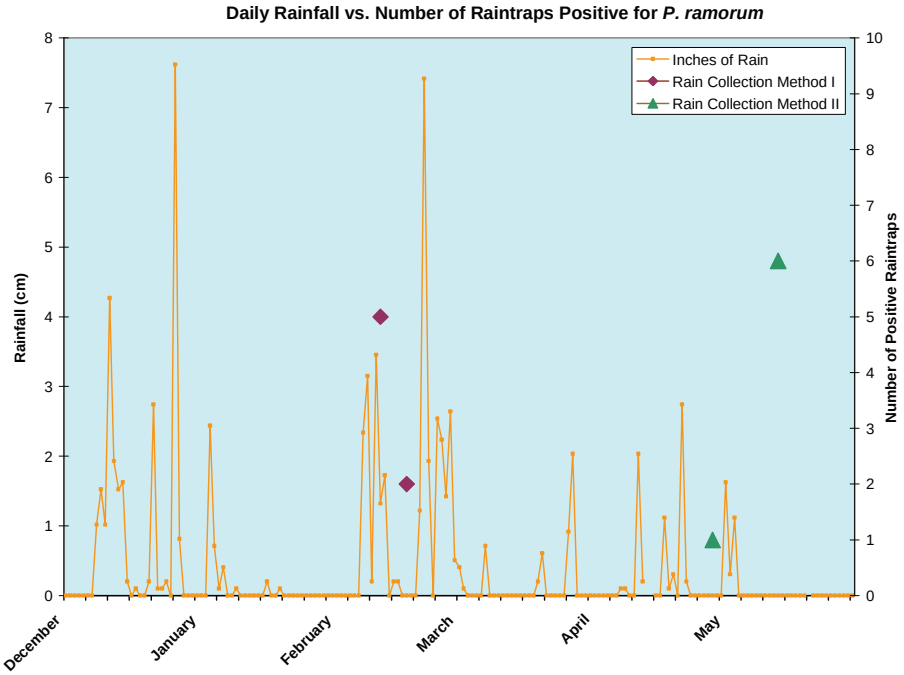

Figure 1-Rainfall was measured at the Eel River Conservation Camp in Redway, CA (approx. 8 km from the O'Meara Grove field site). Rain collection method I (♦) was discontinued at the end of April. Rain collection method II (▲) was begun at this time.

Discussion

P. ramorum was rarely detected from rainwater in Humboldt County during the first half of 2007. In contrast, Curry County, Oregon had consistent detections from rainwater during this same period, but also received considerably more rainfall (179.7 cm vs. 76.5 cm). Lack of symptoms on redwood seedlings may be due to lower than average rainfall (76.5 cm from Dec.-May vs. 132.6 cm average). An additional season is necessary to assess the potential threat to coast redwood by *P. ramorum* in the field. In future studies on incidence and symptom development, a susceptible host, such as rhododendron seedlings, should be included as a positive control.

Controlled laboratory experiments are currently underway to determine the relationship between inoculum dose and disease incidence (or symptom development), examine distance of spread and tissues colonized within inoculated seedlings, and assess the level of sporulation on redwood seedlings.

Acknowledgements

California Department of Parks & Recreation, North Coast Redwoods District
Tooby Park Board of Directors
Plum Creek Nursery

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Vegetation Dynamics and Impacts by *Phytophthora ramorum* in Redwood-Tanoak Forests in California[1]

Shannon C. Lynch,[2] Richard C. Cobb,[2,3] and David M. Rizzo[2]

Introduction

Pathogen epidemiology, community ecology of host species, and land use history all play major roles in the current distribution of *Phytophthora ramorum* and the extent of damage this pathogen has caused in California forests (Rizzo and others 2005, Meentemeyer and others 2008b). Research on the community ecology and the interaction of community composition and disease intensity will help enable synthesis of management actions at the stand level and reduce rates of infection, mortality, and fuel accumulation. Disease and mortality of tanoak (*Lithocarpus densiflorus*) are patchy across California landscapes, and causative mechanisms for these patterns likely operate at different spatial and temporal scales. Theoretical and empirical models that reflect the interdependences of vegetation and pathogen ecology are important to identify high-risk stands and developing management practices to reduce disease impacts.

In this study, we assess the ecological characteristics of redwood forests to understand impacts by *P. ramorum* on redwood-tanoak forests in California. Since 2002, we have monitored the survival of 5769 trees in the central and southern parts of the distribution of redwood forests (Maloney and others 2005). Using this dataset, we have asked the following questions: How does variation of host community structure affect the frequency of infection and mortality due to *P. ramorum*? What are the ecological relationships which structure these vegetation types? We address these questions by examining relationships between species biomass and edaphic factors. We then use preexisting host community structure to explore extent of *P. ramorum* infection and resulting mortality across the main area of infestation.

Methods

The study was conducted within a network of 120 500m^2 circular plots covering redwood forests within Sonoma, Marin, Santa Cruz, and Monterey counties (Maloney and others 2005). A total of 5,769 trees ≥ 1cm DBH were surveyed in 2002 and 2007. Every tree species within a plot was measured for diameter at breast height and given a crown vigor rating. Basal area for each species was calculated at the plot level and reported in square meters per 1/20 ha. Susceptible host trees included coast redwood (*Sequoia sempervirens*), tanoak, California bay laurel (*Umbellularia*

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26–31, 2007, Monterey, California.

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californica), Douglas-fir (*Pseudotsuga menziesii*), big leaf maple (*Acer macrophyllum*), Pacific madrone (*Arbutus menziesii*), coast live oak (*Quercus agrifolia)*, and California black oak (*Quercus kelloggii*). Tissue symptomatic of *P. ramorum* infection was collected from each tree; in the lab, pieces of collected leaf and bark tissue were placed in Petri dishes containing pimaricin-ampicillin-rifampicin-Pentachloronitrobenzene agar (PARP), a selective medium for *Phytophthora* spp. (Davidson and others 2003). In 2003, one soil sample was collected within each plot to evaluate soil texture and chemical properties. Soil analysis was conducted with standard methods for soil analysis at the University of California Davis ANR Analytical Laboratory. Plot location was recorded using a global positioning system (GPS) device (GARMIN, Olathe, KS, USA); plot locations were used to derive edaphic variables including topographic moisture index (TMI), elevation, and percent maximum solar radiation input. Principal component analysis (PCA) was conducted to assess multivariate correlation patterns of tree distributions with environmental variables.

Sites were divided into central (Sonoma and Marin counties) and southern (Santa Cruz and Monterey counties) redwood forest sub regions (Sawyer and others 2000). Principle components for vegetation were calculated using correlations of plot level basal area. Three tree species including redwood, tanoak, and bay laurel accounted for 90 percent (±14 percent standard deviation) of the total basal area within the plot network. Host community structure was analyzed by including estimated basal area of tanoak, bay laurel, and redwood with all other species collapsed into a general category of other species (including black oak, coast live oak, Douglas-fir, maple, and madrone). These categories reflect a general continuum of susceptibility weighted by the biomass of individual species.

Two PCA analyses were conducted. The first used only vegetation structure (four categories) to examine multivariate associations in host community structure. A second PCA analysis included edaphic variables and vegetation structure in order to elucidate environmental controls on host community composition. Eigenvalues for the first five principal components were used to examine associations between vegetation and edaphic factors. Effects of host community composition on intensity of sudden oak death was examined with multiple linear regression (MLR) using vegetation structure as independent variables and amount of tanoak and bay laurel, and tanoak mortality as dependent variables (Maloney and others 2005). Independent and dependent variables were square root transformed prior to analysis.

Results

Community composition and disease impacts

Results from PCA analysis indicate strong negative correlations among bay laurel, tanoak, and redwood when considered at the redwood forest community level (fig 1). The first and second principle components accounted for 62 percent of the variation in vegetation structure and suggest segregation of host species within redwood forest types. Redwood and bay laurel basal area were negatively correlated and both species showed less pronounced negative correlation with tanoak. All other species were positively correlated with tanoak basal area suggesting that stands with greater tanoak dominance have higher overstory tree diversity compared to redwood stands

dominated by redwood or bay laurel at the plot level. The large amount of spread in the data and lack of obvious plot clustering suggests that segregation of host species occurs along gradients within redwood forests.

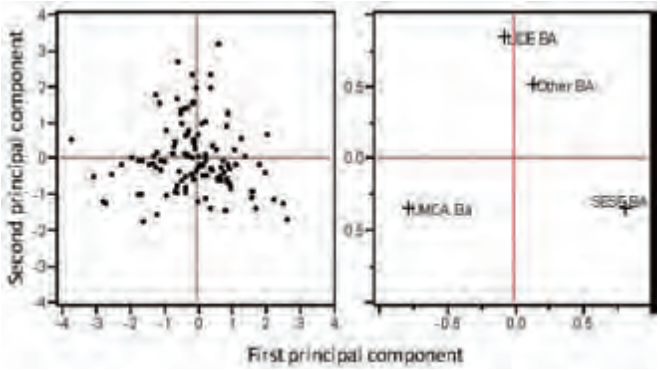

Figure 1—Score (left) and loadings (right) from a PCA analysis on correlations among tanoak (LIDE), bay laurel (UMCA), redwood (SESE), and all other species (Other) basal area from 120 plots in redwood forests. This analysis illustrates variation in host community structure and suggests that edaphicand/or competitive interactions are important in structure host populations.

Results from our second PCA analysis suggest that stand level vegetation structure in redwood forests is related to edaphic factors but the relative importance of edaphic drivers differ between sub regions (figs. 2A and B). Overall, edaphic factors and competition were important in determining species biomass at the plot level in both regions. For the central redwood forest sub-region, all species out-compete coast redwood at relatively higher elevation sites with edaphic factors determining the abundance of alternative dominant species. Bay laurel and other species occurred at greater basal area in sites with higher soil clay content. At these sites, tanoak was more abundant where TMI values were lower suggesting that tanoak may out-compete bay laurel in relatively drier high elevation sites. Low resource sites are known to support high species richness in Coastal scrub ecosystems (Moody and Meentemeyer 2001). Figure 1 shows strong tanoak association with all other overstory species that commonly occur in our plots. This pattern may reflect resource poor growing conditions in these plots and suggests that tanoak is adapted to more resource poor conditions compared to redwood and bay laurel. Tanoak and other species were associated with fine texture soils at middle elevation and moderate moisture sites. Within these middle elevation areas, in which solar radiation output was high, tanoak and bay dominated over redwood and other species where there was relatively more clay in the soil (fig. 2A). In the southern redwood forest sub region bay laurel was dominant at low elevation, high solar radiation sites that were relatively wet and comprised of fine textured soils. At moderate elevations, redwood dominated bay laurel on fine textured soils. Redwood was dominant over other species at lower elevation sites that had soils with less clay content. In this subregion, bay laurel and tanoak were more dominant over other species in sites with relatively lower solar radiation and high soil clay content (fig. 2B).

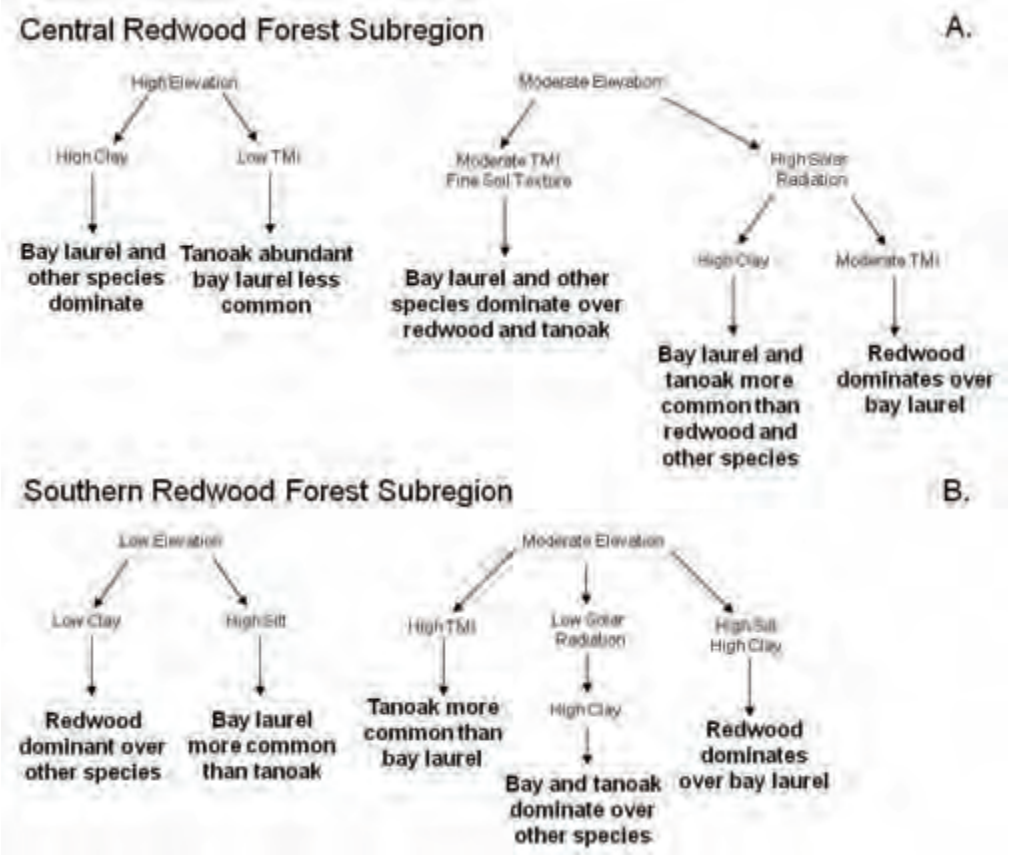

Figure 2—Edaphic dependencies of species dominance in central and southern redwood study sites. Edaphic-species relationships are based on dominant eigenvalues from a PCA analysis of vegetation abundances and edaphic factors including soil texture, elevation, and topographic moisture index (TMI).

Host community structure was an important factor influencing the intensity of disease impacts in coastal California redwood forests. Amount of bay laurel infected basal area was best described as a simple linear function of bay laurel basal area (table 1). Very little residual variation occurred in this model (r^2adj = 0.82) suggesting that additional factors such as host and pathogen genetic structure did not dominate bay laurel infection dynamics within our study sites. Tanoak infection levels were linear functions of tanoak and bay laurel basal area (table 1) but had considerably more residual variation compared to bay laurel. This difference in model performance may reflect greater sensitivity to historical, host genetic, and pathogen genetic effects but the overall slower rate of tanoak infection compared to bay laurel likely accentuates these effects. Consequently, our understanding of *P. ramorum* infection will be improved with better understanding of pathogen invasion dynamics including the effects of tanoak and pathogen genetics. Tanoak mortality was also a linear function of tanoak and bay laurel basal area (table 1). The overall dependency of tanoak infection and mortality on tanoak and bay laurel basal area reflects transmission dynamics of *P. ramorum*. Bay laurel produces substantially higher inoculum levels

Table 1—Results from multiple regression modeling of vegetation structure on infection and mortality caused by *P. ramorum*.

Dependent variable	Bay laurel infection	Tanoak infection	Tanoak mortality
Independent variables			
LIDE BA	NS	0.73	0.75
UMCA BA	0.95	0.18	0.29
SESE BA	NS	NS	NS
Other species BA	NS	NS	NS
DF	119	119	119
Model r^2_{adj}	0.88	0.51	0.60

Standardized regression coefficients are reported when the effects of a vegetation factor were significant at the p <0.05 level. Degrees of freedom for calculation of statistical effects (DF) and adjusted r square values are listed at the bottom of the table for each an alysis.

compared to tanoak; therefore, higher levels of infected bay laurel basal area should increase tanoak infection levels at the stand level (Davidson and others 2008, Rizzo and others 2005). High inoculum is likely to increase rate of secondary infection and increase the frequency of tanoak mortality (Gilligan 2006). Inoculum loads have been recovered from rainwater in significantly larger proportions under bay laurel canopies compared to mixed canopies at Jack London State Park (Davidson and others 2008). Assuming a linear relationship between infected basal area of inoculum supporting species and stand level inoculums load, we would expect to find the overall linear relationships between amount of infection and mortality present in our dataset (Table 1). In our system of study plots, highest inoculum pressures likely occur in stands with high bay laurel basal area; however, sudden oak death impacts are likely highest in stands at the edaphic and competitive interface between bay laurel and tanoak as these stands will have the highest inoculums loads on tanoak and the most frequent opportunities for secondary infection.

Discussion

Implications for stand level management

Understanding the biology and ecology of *Phytophthora ramorum* as well as the ecology of the forests it invades are necessary for managing sudden oak death on a landscape level (Rizzo and others 2005). This study assesses host distribution and community structure of redwood forests invaded by *P. ramorum* from a comprehensive data set comprising of nearly six thousand trees. Edaphic factors in conjunction with historical disturbance are likely major drivers of current species distribution and abundance in these forests (Foster 1988, Meentemeyer and others 2008b). Compounded with the fact that host community composition is

heterogeneous within the central and southern distributions of the redwood forest type in coastal California, management actions may be targeted differently depending on forest composition.

The finding that *P. ramorum* most readily establishes in stands with more tanoak and laurel bay basal area follows predictions based on ecological niche modeling and community level investigations of pathogen establishment (Davison and others 2003, Maloney and others 2005, Rizzo and others 2005, Meentemeyer and others 2008a). These data also demonstrate that community level vegetation manipulation may be risky at sites where edaphic factors enhance the dominance of sporulation supporting species. For example, results from PCA suggest that bay laurel removal efforts would be most successful in sites where tanoak has a competitive advantage over bay: drier sites at higher elevations in the central sub region and wetter sites at moderate elevations within forests of the southern redwood sub region. These efforts should be avoided in the south where soil silt content is relatively high at lower elevations and in the central region at mid-elevations where soils are fine textured at moderate moisture levels because of likely reestablishment of bay laurel and consequently *P. ramorum* over a decadal time scale. Areas in which bay and tanoak coexist and dominate over redwood and other species may require further study to identify the outcome of stand manipulation. Pruning lower branches of bay laurel and thinning of understory tanoak may be effective in reducing infection due to an overall higher susceptibility of these individuals. Pruning may be most effective at moderate elevation sites where the soil clay content is high for both regions and solar radiation is low in the southern region. Because tanoak supports pathogen sporulation, thinning of tanoak in the understory would be prudent in any case to decrease P. ramorum dispersal. Future research efforts will use pre-disease vegetation structure to make predictions of future forest structure and improve disease and forest management strategies.

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Epidemiology of the Alder *Phytophthora*[1]

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Abstract

Since the beginning of the nineties, *Phytophthora alni*, a new hybrid species, has been inducing a major decline of alder trees along European watercourses. To evaluate the long-term impact of the disease on the alder population, we set up a permanent survey area in 2002 along the Sarre River (Moselle). All alder over 1.3m high were inventoried and mapped along approximately four kilometers of river, and their health status and growth was annually determined. Each year, new trees reaching 1.3m high were recruited in the sample.

In this study, we used the collected data to document the speed of disease development on the trees, based on survival analysis. Additionally, we proposed an estimation of local tree infection probability based on kernel estimators. The disease is characterised by presence of trunk cankers and decline of the crown. The trunk cankers might better reflect the present infection activity while the crown decline might more reflect past infection, being slower to appear. We thus based local tree infection probability on the likelihood of new canker cases.

We then checked whether or not it is important to take into account the lag between infection and decline of the crown. For that, we estimated the likelihood that a tree with a healthy crown would decline, given trunk dbh and the local likelihood of previous year's new canker cases. The lag depends on the tree size, with seedlings showing very quick crown decline in area with high likelihood of new canker cases while large trees decline more slowly. We used the fitted function to identify trees that appeared healthy, but nevertheless had a high likelihood of being already infected. These trees were compared to healthy trees with little risk of being already infected according to our model, slightly declining trees, severely declining trees and dead trees. *Phytophthora alni* presence in the soil was estimated by baiting followed by PCR for each of the studied trees in order to determine which are major inoculum producers.

The results show that *P. alni* inoculum is detected in larger amounts with healthy trees that have a high probability of being already infected and moderately declining trees than with severely declining trees. By contrast, *P. alni* is seldom detected in association with healthy trees that our model predict to be uninfected or with dead trees. We conclude that it is very important for accurately modelling disease evolution to take into account asymptotic uninfected trees as they can potentially be major inoculum producers.

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Monitoring *Phytophthora ramorum* Distribution in Streams Within Coastal California Watersheds[1]

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Abstract

One hundred eighty-seven sites were established in perennial watercourses and sampled for one to four years between 2004 and 2007 to monitor for the presence of *Phytophthora ramorum* throughout coastal central and northern California watersheds as well as portions of the Sierra Nevada mountain range. In 2007, 132 sites were monitored, including 65 new sites. The majority of the monitored watersheds have limited or no *Pr* at this time, but are near the epidemic range of *P. ramorum* and/or are considered high-risk for invasion by *P. ramorum.* Three currently infested watersheds in Sonoma, Humboldt, and Monterey Counties were included as a baseline for successful recovery of *Pr*. *Rhododendron* leaves were placed in mesh bags and secured in watercourses for one- to three-week intervals to bait for *Phytophthora* species. Recovered symptomatic leaves were plated on *Phytophthora*-selective medium (PARP-H) and monitored microscopically.

Phytophthora ramorum has been detected at 37 total stream monitoring sites, including all sites with a priori knowledge of nearby forest infestation. *Phytophthora ramorum* was detected at 23 streams sites without prior knowledge of adjacent forest infestation in Humboldt, Contra Costa, Mendocino, Monterey, and Santa Cruz counties. Forest infestations have thus far been confirmed at only nine of these sites; surveys are underway to identify the source(s) of inoculum for the other sites. Additionally, *P. ramorum* was recovered as far as 25km downstream from known forest infestations. This year (2007) was an unusually dry year in California which impacted our recovery of *P. ramorum* from watercourses. *Phytophthora ramorum* was detected in only 15 streams this year; seven of those were new sites for 2007. We recovered no *P. ramorum* from four streams that were positive for *P. ramorum* in 2006. At the most heavily infested sites, the frequency and quantity of recovery of *P. ramorum* was greatly reduced in 2007.

Stream monitoring has extended the southern range of *Pr* in Monterey County and the northern range in Humboldt County. All sites in the Sierra Nevada remain negative for *Pr*. With culturing and molecular sequencing we have identified several other *Phytophthora* species within these watersheds; *P. gonapodyides* is the most commonly detected species and was isolated from at least 60 sites. Streams were monitored year-round in 2004 and 2005 and revealed a distinct seasonality associated with *P. ramorum* recovery. Therefore, in 2006 and 2007 watersheds were monitored monthly, February through June, during the peak seasonal period.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Real Time PCR Protocols for Environmental Monitoring of *Phytophthora alni* and Its Three Subspecies[1]

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Abstract

Appearing in the U.K. at the end of the 1980's, *Phytophthora alni* has severely reduced alder populations in Western Europe and has been emerging as a major threat to alder stands in Poland in the past years. For addressing *Phytophthora* epidemics, which spread in the wild, environmental monitoring must be made easier, quicker and cheaper than classical description or PCR methods. The objective of the work presented here was to provide with a set of real time PCR assays for environmental monitoring of *Phytophthora alni*, fulfilling the desired requirements. We present here the development and evaluation of a set of real-time PCR assays for the LightCycler real time hot air thermocycler (Roche Diagnostics), using primers that target the Sequence Characterised Amplified Regions (SCARs) previously developed for *P. alni*. The real time PCR format allowed to perform a melting curve analysis of the PCR products once amplification was completed and to offer an additional characterization of the targeted SCARs. The availability of rapid and specific assays for the detection and characterization of *P. alni*, such as provided in the present study, should facilitate the detection, control of outbreaks and the occurrence and distribution of the different subspecies of this pathogen in Poland. However, care should be taken when conducting environmental studies using these oligos since we show a clear cross contamination with at least *P. gonapodyides* and *P. quercina*, two species frequently encountered in alder stands.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Stand Structure and Local Distribution of *Phytophthora ramorum* in Oregon Forests[1]

Ebba Peterson,[2] Molly Botts,[2] Everett Hansen[2]

Introduction

The *Phytophthora ramorum* eradication program in effect in Oregon has allowed for the rapid detection of new infection foci, typically before they develop within each stand and expand into adjacent sites. Yet despite gallant efforts, new locations that previously harbored no apparent infection have been identified each year since the original detection in 2001. Multiple factors may influence why and where each new infection occurs including, but not limited to, stand characteristics such as dominant understory and upper canopy vegetation, age and stem density (which influence microclimate variations within the stand), the distribution of host and non-host species, and the various possible dispersal mechanisms of the pathogen (Shaw 2004). Previous studies in California have found that landscape patterns of disease severity are poorly explained by variation at small scales (Condeso and Meentemeyer 2007); however detailed work on infestations at their origin, prior to local intensification, has not yet been performed. This study examined the stand and canopy characteristics and the local distribution of *P.ramorum* around the first trees detected, if not the first trees infected, in a remote location. We further set out to document and characterize a site with infestation that has developed to an extent previously unseen in Oregon.

Methods

The two sites of this study, site 2748 and North Bean Creek (NBC), were located within a mile of each other on land privately owned by South Coast Lumber Company near Brookings, OR. Both sites were composed of mixed Douglas fir (*Pseudotsuga menziesii*), alder (*Alnus rubra*), and tanoak (*Lithocarpus densiflorus*), and bordered fir plantations. The topography of the sites is similar; both are uphill from the nearest road and occupying east facing slopes with similar pitches (35-45 degrees), elevations (approximately 320-365 m above sea level), and locations relative to the top of their respective ridges. Both sites were first detected and confirmed positive for *P.ramorum* in summer 2007.

To assess the distribution of understory vegetation likely to contribute to the lateral spread of *P.ramorum*, notably *Rhododendron macrophyllum* and *Vaccinium ovatum*, a grid was established either around positive trees (site 2748; 30m by 40m) or through the area of worst infestation (NBC; 25m by 60m). For each $5m^2$ block within the grid presence or absence of foliar hosts was noted (*R.macrophyllum, V.ovatum, L.densiflorus* sprouts, and *Umbellularia californica* only). Symptomatic tissue

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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samples were taken from every block and *P.ramorum* presence was confirmed through culture on cornmeal, ampicillin, rifamycin, pimaricin (CARP) medium. Stand composition and canopy structure was assessed by running a belt transect (10m x 50m for NBC and 10m x 40m for site 2748) nested within the vegetation plot. For all tree species, tree height and length of crown were measured, as well as distance traveled along the length and width of the transect. Distances were later corrected for changes in elevation for depiction. Disease severity on tanoak was rated by estimating crown condition (percent browned to the nearest 10 percent), and recording presence or absence of symptoms associated with *P.ramorum* (bleeding, frass, symptomatic basal or stem sprouts). The stands were surveyed between July 25th and August 9th, 2007.

Results

Stems were denser at site 2748 though individual trees were much smaller, especially amongst tanoak (Table 1). Tanoak comprised a greater proportion of the upper canopy and total basal area in NBC (Table 1). In contrast, at site 2748 there was a marked stratification between the emergent Douglas fir, which were distributed throughout the transect, and the codominant tanoaks. While many of these stems were dominant, nearly all tanoak crowns were adjacent to taller Douglas fir crowns; a notable exception is the few known positive trees. At site 2748, of the 91 tanoaks measured there were only two symptomatic, brown-canopied trees and, directly adjacent to these, three bleeding stems. In contrast, within NBC 35 percent of the 78 tanoaks surveyed were bleeding, though 85 percent had some symptoms associated with *P.ramorum*, predominantly symptomatic, succulent basal sprouts.

Table 1ð Stand characteristics for the three tree species measured at North Bean Creek (NBC) and site 2748.

	No. live trees counted [a]		Tree density (stems/ha)		Total basal area (m^2/ha)		Mean tree height (m)	
Species	NBC	site 2748	NBC	site 2748	NBC	site 2748	NBC	site 2748
L.densiflorus	78	91	1520	2275	35.6	16.7	13.8	7.6
A.rubra	8	0	160	0	7.1	0	20.5	0
P.menziesii	3	23	60	575	5.7	19.1	24.0	14.8
Total for all species	89	114	1740	2850	48.4	35.8		

[a] NBC: 500m^2 plot, all trees greater than 7cm DBH; site 2748: 400m^2 plot, all trees greater than 5cm DBH

Both sites had a continuous understory of host vegetation (Table 2). *Umbellularia californica* was present only at NBC, though only as small understory saplings in four blocks. In previous studies in California recovery of the pathogen from attached leaves declined dramatically over the summer months (Fitchner and others 2007), however *P.ramorum* was readily recovered from the highly infested site in Oregon. *P.ramorum* was isolated from leaf tissue in all but two 5m^2 blocks within NBC (96

percent of 69 samples taken, Table 2). In contrast, at site 2748 despite extensive sampling within the grid *P.ramorum* was only recovered within 5m of bleeding trees (4 percent of 80 samples taken, Table 2).

Table 2ð Prevalence of host vegetation within the vegetation grid, number of samples taken, and proportion of samples positive for *P.ramorum* at site 2748 (2a; low incidence) and North Bean Creek (2b; high incidence).

	Proportion of blocks with species present [a]		# vegetation samples tested		Proportion positive samples	
Species	NBC	site 2748	NBC	site 2748	NBC	site 2748
L.densiflorus	1	1	55	26	0.98	0.08
R.macrophyllum	0.93	0.96	11	45	0.91	0.02
V.ovatum	0.9	1	1	9	0	0
U.californica	0.07	0	2	-	1	-
Total for all species:			69	80	0.96	0.04

[a] 500m^2 (NBC) or 400m^2 (site 2748) plot divided into 5m^2 blocks

Discussion

Our results are consistent with previous observations that the development of disease in Oregon is dependant upon the infection of mature tanoak. Site 2748 is a typical location: highly restricted, local infection centered around isolated trees whose disease developed from inoculum produced at an unknown source. While other studies have focused upon movement of infested soil to explain the original introduction of inoculum into remote areas (Davidson and others 2005), the location of this site is not consistent with movement by either people or vehicles. Other possibilities include movement by animals, either from below or above, or the aerial dispersal of inoculum. The crown exposure of the initially infected trees, as well as the lack of any observable infection on surrounding vegetation is suggestive of aerial dispersal. Further studies are needed to determine whether the observed pattern reflects the dispersal method of the pathogen or microclimate variation that favors initial establishment (or both).

It is likely not for the lack of host continuity or topographical differences that the severity of disease in either site differs so drastically, and which other factors may best explain this remain unclear. Nevertheless, this study highlights the potential for the diseases caused by *P.ramorum* to reach epidemic proportions in Oregon.

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Physiological Changes and Gene Expression on European Beech (*Fagus sylvatica*) Infected With *Phytophthora citricola*[1]

Roberto L. Portz,[2] Julia Koehl,[2] Frank Fleischmann,[2] and Wolfgang Oßwald[2]

Abstract

In the last decade *Phytophthora citricola* Saw. was described as an important and aggressive pathogen on European beech (*Fagus sylvatica* L.). Inoculated beech seedlings, grown under controlled conditions, showed a significant decrease of photosynthesis, transpiration and water potential after three days. Typical wilt symptoms on leaves were observed only after a consistent reduction of these parameters. Sugar contents of roots of infected plants were significantly lower in infected plants as compared to control seedlings. A negative correlation between photosynthesis, transpiration and water potential and the increase of *P. citricola* in roots was demonstrated. In addition synthesis of ethylene of infected plants was increased two days after inoculation compared to controls. Expression of genes of the primary and secondary metabolism, as well as of defense-related genes, is ongoing in order to get more detailed information of the studied host-parasite interaction.

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Characterizing the Community of *Phytophthora* Species in an Oregon Forest Stream[1]

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Introduction

Phytophthora species are best known as pathogens of agricultural crops, or invasive pathogens destroying forests. Little is known about indigenous species, especially in wild ecosystems. Previous work showed that *Phytophthora* species are relatively abundant in natural streams in forests, but the species present are poorly characterized, and their ecology is essentially unknown. The aim of this work was to compare methods for the collection, identification, and enumeration of *Phytophthora* spp. in streams. Three methods of isolate collection were compared (leaf baits, pear baits, and filtration), and species identification was carried out using morphological and growth characters, and by DNA sequencing two regions of the genome (ITS rDNA, mitochondrial COX spacer region). Stream sampling was conducted at five locations in the Oak Creek drainage near Corvallis Oregon.

Oak Creek

Oak Creek starts in forest, then runs through an agricultural valley and an urban area, where it joins Marys River and then the Willamette River. Sampling Site 1 was at the beginning of the stream, in forest. Site 2 was downstream at the edge of the forest, before the first habitations. Site 3 was located in the plain with scattered housing, a few orchards, meadows and crop fields. Site 4 was in Marys River, just after Oak Creek flows into it. Site 5 was in the Willamette River, which flows through a fertile valley with many orchards, fields and forests. Tree species present at all sites included Douglas-fir, grand fir, big leaf maple, and red alder.

Methods

- Isolations were made on CARP + (CMA with 25ppm hymexazol (99 percent), 20ppm delvocid (50 percent natamycin salt), 200ppm ampicillin sodium salt, 10ppm rifampicin SV sodium salt and 30ppm benlate (benomyl 50WP)) then transferred to carrot agar.
- Isolates were collected every other week for 12 weeks, by filtration, leaf baiting, and pear baiting. 700 ml of stream water, in 50, 100, and 200ml

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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portions, was passed through 5 μm nitrocellulose filters using a portable vacuum filtration unit. Filters were plated on CARP +.

- Two *Rhododendron* leaves, one tanoak leaf and three small pieces of Port-Orford-cedar foliage were placed in a mesh bag and immersed in the stream. After two weeks, leaves were removed, washed, and plated on CARP +. One d'Anjou Pear was also immersed in the stream. After three days, pears were removed, washed and tissues with lesions were plated on CARP +. All isolates were grown up on CMA and DNA was extracted from colonized agar plugs using a CTAB buffer protocol. The COX gene spacer region was PCR amplified using primers FMPH8 and 10, and sequenced in one direction. These single strands were aligned with other known (reference) sequences for initial species identification. Isolates not matching a reference isolate were then amplified and sequenced using ITS4 and 5, and blasted against the GenBank database.

Results

A total of 514 isolates were recovered from baits or filters. About 57 of these proved to be *Pythium* or zygomycetes. Another 20 isolates were not characterized because of sequencing problems or other difficulties. 437 *Phytophthora* isolates were analyzed. An average of 13 *Phytophthora* colonies were grown from each liter of stream water filtered.

Phytophthora Diversity

ITS and COX spacer DNA analysis distinguished at least 18 groups of isolates. These 18 groups formed nine clusters.

- Four of the clusters matched known species (*P. citricola, P. gonapodyides*, *P. pseudosyringae* and *P. syringae*).
- Three clusters contain groups of isolates that match and/or whose sequence is very similar to a known species (*P. cambivora*, *P. megasperma and P. sp. "Pg chlamydo"*).
- Two clusters contain groups of isolates that are similar but not identical to a known or unnamed species (*P. europaea* and Clade 6 "Kalamazoo").
- More than 90 percent of all isolates were in ITS clade 6: *P. gonapodyides, P. megasperma*, *P.sp. "Pg chlamydo"*, and unnamed related groups ("Kalamazoo" types).

Capture Method

Water filtration yielded more different species groups than either leaf baits or pears baits. *P. megasperma* and related isolates were only recovered from filters. On the other hand, *P. cambivora* and variant group and *P. syringae* were only recovered from baits (Table 1).

Table 1ð Recovery of *Phytophthora* species groups/clusters on filters, leaf baits, and pears.

	Filter	Leaf	Pear	Total
P. cambivora cluster	0	3	6	9
P. citricola	7	11	5	23
Clade 6 "Kalamazoo" cluster	41	15	26	82
P. europaea cluster	2	0	1	3
P. gonapodyides	92	74	20	186
P. megasperma cluster	7	0	0	7
P. sp. "*Pg chlamydo"* cluster	48	66	8	122
P. pseudosyringae	0	0	1	1
P. syringae	0	1	3	4
All Phytophthoras	197	170	70	437

Sample Time and Location

Phytophthora species varied by sample time and location within the watershed (Tables 2 and 3). *Phytophthora syringae* was recovered from 3 different locations but only at one June sample time. *P. cambivora* and cluster isolates and *P. europaea* cluster isolates came only from sample sites 1 and 2. In contrast, *P. citricola* was recovered only from downstream sites, late in the season, while *P. megasperma* and cluster isolates came from downstream sites early in the sampling period. The clade 6 groups (*P. gonapodyides*, *P.sp. "Pg chlamydo"*, and the cluster of variants similar to P. "Kalamazoo") tended to dominate at all locations and sample times.

Table 2ð Recovery of *Phytophthora* species group/clusters by sampling time, Juneï August 2006.

	Site1	Site2	Site3	Site4	Site5	Total
P. cambivora cluster	7	2	0	0	0	9
P. citricola	0	0	4	17	2	23
Clade 6 "Kalamazoo" cluster	1	9	30	26	16	82
P. europaea cluster	3	0	0	0	0	3
P. gonapodyides	37	55	52	32	10	186
P. megasperma cluster	0	2	2	2	1	7
P. sp. "*Pg chlamydo*" cluster	4	58	25	30	5	12
P. pseudosyringae	0	1	0	0	0	1
P. syringae	0	1	1	2	0	4
All Phytophthoras	52	128	114	109	34	437

Table 3ð Recovery of *Phytophthora* species group/clusters by sampling location in Oak Creek and downstream sites.

	Time1	Time2	Time3	Time4	Time5	Time6	Total
P. cambivora cluster	0	0	0	1	6	2	9
P. citricola	0	0	1	1	7	14	23
Clade 6 “Kalamazoo” cluster	6	2	7	22	14	31	82
P. europaea cluster	0	0	0	2	0	1	3
P. gonapodyides	49	33	29	21	16	38	186
P. megasperma cluster	4	2	1	0	0	0	7
P. sp. “*Pg chlamydo*” cluster	10	16	22	21	23	30	122
P. pseudosyringae	0	0	1	0	0	0	1
P. syringae	0	4	0	0	0	0	4
All Phytophthoras	69	57	61	68	66	116	437

Conclusions:

- Water filtration provided more isolates and more species than baiting and also enabled quantification of propagules.
- 12 to 20 *Phytophthora* propagules/liter of water were usually detected.
- At least 18 different groups of isolates were distinguished.
- 12 groups did not match named species.
- *P. gonapodyides* was most numerous, followed by *P.sp. “Pg chlamydo”* and its variants (cluster), and another unidentified group of taxa from ITS clade 6.
- The *Phytophthora* community changed through the season, and differed at different locations in the watershed.

Acknowledgements

We would like to thank Frank Martin for suggesting the use of COX spacer single strand alignments as a species screening tool.

Phytophthora ramorum Tissue Colonization Studied With Fluorescence Microscopy[1]

Marko Riedel,[2] Stefan Wagner,[2] Monika Götz,[2] Lassaad Belbahri,[3] François Lefort,[3] and Sabine Werres[2]

Abstract

The proceeding worldwide spread and the expanding host spectrum of *P. ramorum* has become a serious threat to natural plant communities. To encounter this threat detailed knowledge about infection pathways and tissue colonization is essential. To analyze these issues, histological studies of infected tissue with epifluorescence microscopy have been started. For first infection tests *Rhododendron* has been taken as a model host. Root infection of potted *Rhododendron* cuttings with the *P. ramorum* isolate BBA 9/95 (ex-type strain) were started. Inoculation of the non injured plants was done by application of a zoospore suspension onto the surface of the pot soil. The plants were then incubated with 16 hour light at 20°C in a quarantine chamber. Samples of healthy looking plants and plants with typical symptoms were taken and fixed. Unstained hand-cuttings were analyzed with fluorescence microcopy. The development of zoosporangia on the leafsurface was analyzed using the vital stain FUN®1 (Molecular Probes). Natural autofluorescence of *P. ramorum* and plant tissue is enhanced using the fixing fluid FAA (formaline-aceto-alcohol). Epifluorescence images showing *P. ramorum* structures in different tissues and in different stages of disease development are presented. An overview of the development of *P.ramorum* in *Rhododendron* root, twig and leaf tissue is offered for discussion.

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Phytophthora Species in Finland[1]

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Abstract

Potato late-blight epidemics caused by *Phytophthora infestans* have been reported in Finnish newspapers since 1847. The pathogen has caused considerable losses in potato fields in southern Finland. Before the late 1970's only the A1 mating type of the heterothallic pathogen was present outside Mexico. At the early 1980's a new potato late blight population migrated from Mexico to Europe and replaced the old asexual population (Fry eand others 1993). The first indication of the change was the appearance of A2 mating type of *P. infestans* in Europe (Hohl and Iselin 1984). The monitoring of the occurrence of A2 mating type in Finland was started in 1992 when mating type A2 proved to be present in a very low proportion in the potato late blight population. Unfortunately there were no older isolates of the pathogen available and the exact time of migration of A2 remains unsolved (Hermansen and others 2000). In the Northern climate the cold winters limit the survival of the asexual stage of potato late blight fungus in infected tubers in frozen soil. Therefore the build up of blight epidemics derived from asexual sources of primary inoculum usually was delayed until the end of August in Finland (Mäkelä, 1966). The oospores formed in a sexually reproducing population provide a new serious overwintering source of primary inoculum independent of the survival of the tubers during cold winters in Finland.

Phytopthora cactorum was first time isolated in Finland in the early 1990's from strawberries suffering from leather rot (Parikka 1991). Since then the pathogen has caused losses in strawberry production as an agent of crown rot as well as causing stem lesions on silver birch in forest nurseries. Fosetyl-Al used to control the pathogen on both host plants. The genetic variation on *P. cactorum* in Finland has been studied by Random Amplified Microsatellite (RAMS) and Random Amplified Polymophic DNA (RAPD) analyses. The Finnish isolates from birch have been shown to contain considerable genetic variation, in contrast to the strawberry crown rot isolates, which are found by fingerprinting analyses to be largely clonal in Europe (Lilja and others 1998, Hantula and others 1997, 2000, Eikemo 2004). As well as separating genetically in fingerprinting analyses, the strains causing crown rot on strawberry and stem lesions on birch in Finland have also shown to be pathogenically separate. *P. cactorum* isolates from strawberry have been able to infect birch, but strawberry seedlings have not been affected by isolates from birch in pathogenity tests (Hantula and others 2000). We have also baited *P. cactorum* from a natural pond from which a forest nursery is taking its water for irrigation. The pathogen was present in the pond every year during the 3-year study. The *P. cactorum* strains in the pond were tested by RAMS analysis to be genetically similar to those causing symptoms on birch in the nursery.

Phytopthora ramorum was described in 2001 in Germany (Werres and others) causing twig blight on *Rhododendron* and *Viburnum*, and it has later been found to be the cause of sudden oak death (SOD) disease in *Quercus* and *Lithocarpus* spp. in California (Rizzo and others 2002). The pathogen was recognized worldwide as a threat both to the nurseries and wild

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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plant populations, and many countries created quarantine programs in order to limit its spread. *Phytopthora ramorum* is a regulated pathogen also in Finland. In the spring 2004 *P. ramorum* was isolated from marketed *Rhododendron* plants imported from other EU countries (Lilja and others 2007). In August 2004 the pathogen was also found in one Finnish nursery on German *Rhododendron catawbiense* plants and on several Finnish *Rhododendron* spp. cultivars. *P. ramorum* was detected by species specific PCR and isolated. *Phytopthora ramorum* has been found every year since 2004 on plant material transported to Finland from other EU countries.

In surveys for *P. ramorum* on Finnish *Rhododendron* cultivars, another *Phytophthora* sp. was isolated in 2004 and 2005 from *Rhododendron* spp. It was confirmed as *Phytopthora inflata* by morphological characteristics and beta tubulin gene sequencing, where one identical sequence was found in the GenBank. It is likely that our *P. inflata* strain has been imported with the ornamental plant trade, since the reference strain has been isolated from *Syringa* sp. in the U.K. in 1990 (Hall and others 1992).

The pathogenicity of *P. inflata* and *P. ramorum* isolates to the most common Finnish tree species and ornamentals was tested by stem-wound inoculation tests. Both *P. inflata* and *P. ramorum* caused necrotic lesions on *Alnus glutinosa*, *A. incana* and *Betula pendula*, but *P. ramorum* was less pathogenic than *P. inflata*. *P. inflata* was also able to infect *Picea abies*, *Vaccinium myrtillus, V. uliginosum, V. vitis-idaea, V. angustifolium* and *Fragaria* x *ananassa*. *Viburnum lentago* was the only plant tested showing susceptibility to *P. ramorum* while being resistant to *P. inflata*. *Pinus sylvestris* was resistant to both *Phytophthora* species. Survival at low temperatures was tested by inoculating apples with *Phytophthora* isolates and incubating them at -5 ºC for two weeks. The growth was evaluated after plating on MA. *P. ramorum* isolates remained viable after two weeks at -5 ºC in laboratory conditions, but isolates of *P. inflata* did not. However, we have been able to isolate viable *P. inflata* from host plants (*Rhododendron* 'Cunningham's White' and *Vaccinium angustifolium*) in a private garden one year after first detection, which indicates that *P. inflata* is able to survive in Finnish winter conditions.

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Screening Wild Cherry (*Prunus avium*) Micropropagated Clones for Resistance to Four *Phytophthora* Species[1]

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Abstract

Prunus avium L., wild cherry, is a valuable component of diversity in mixed coniferous forests and temperate broadleaf forest ecosystems in Europe. Wild cherry is also one of the most prized species for wood production, and as a result it has been increasingly planted both in artificial stands and in natural forests. A number of *Phytophthora* species have been associated with root rot of wild and cultivated cultivars of cherry trees under different environmental conditions. *In vitro Phytophthora* pathogenicity assays are commonly used in order to avoid soil contamination and to speed up the selection procedure to obtain resistant plants. For these assays the cited Authors used callus tissue cultures, however, the symptom assessment could lead to questionable results far from the natural host-pathogen interactions. Thus, the purpose of this paper was to determine *in vitro* the pathogenicity of four *Phytophthora* isolates of several species (*P. alni* ssp. *uniformis*, *P. megasperma* var. *megasperma*, *P. citrophthora*, and *P. cinnamomi*) on micropropagated wild cherry genotypes, previously selected for having superior phenotypic characters and adapted to different Italian environments, by using plantlets 10 cm high cultivated in sterile environment. Host susceptibility was evaluated in accordance with a disease score scale, taking into account the percent of yellowing/wilting. All the wild cherry clones resulted highly susceptible to *P. citrophthora*. The *P. cinnamomi* virulence varied according to the challenged clone, while *P. alni* ssp. *uniformis* and *P. megasperma* var. *megasperma* were able to cause only modest symptoms. Three of the clones showed resistance to the last three pathogens. The results are consistent with what reported in literature. The method is functional and quick, moreover any contamination risk of the environment by the pathogens spores is avoided. For these reasons it could be considered for early resistance screening tests. On the other hand, the need of a specific protocol for regeneration and multiplication of each clone acted as a limiting factor in terms of number of challenged clones and replicates of each clone.

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Involvement of *Phytophthora* Species in the Decline of Beech (*Fagus sylvatica*) in the Southern Part of Belgium[1]

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Abstract

During the last decade, typical symptoms of *Phytophthora* diseases were observed in beech stands of several European countries. The main symptoms were the presence of bleeding cankers on the stem, a low crown density as well as the yellowing of foliage and the small size of leaves. Several species of *Phytophthora,* such as *Phytophthora citricola*, *P. cambivora* and *P. cactorum*, were reported as the causal agents.

In order to evaluate the implication of the different *Phytophthora* species in beech decline in the southern part of Belgium, a monitoring was undertaken with the help of managers of public and private forests. *Phytophthora* strains isolated from beech of different stands as well as from soil were characterized through morphological and molecular analyses (PCR-RFLP of ITS). All the isolated strains were identified as *P. cambivora* which is considered as one of the most aggressive *Phytophthora* species involved in beech decline in Europe.

Molecular analysis was also directly applied to necrosed tissues of bleeding beeches and enabled the detection of additional cases. All positive cases exhibited a profile characteristic of the *P. cambivora* species, except for one of the sampled trees showing a different RFLP profile. Identification of the involved species is ongoing.

Regarding *P. cambivora*, both mating types (A1 and A2) were identified, sometimes in the same sampling site. Ornamented oogonia characteristic of the *P. cambivora* species were produced by pairing A1 and A2 strains isolated from the same site. Oospores from intraspecific pairing were characterized in terms of viability and germinability.

Introduction

During the last decade, typical symptoms of *Phytophthora* diseases were observed in beech stands of several European countries (Jung and others 2005). The main symptoms are the presence of bleeding cankers on the stem and typical necroses of inner bark tissues. A low crown density, as well as yellowing of foliage dectreased leaf size, are also observed. Several species of *Phytophthora*, such as *Phytophthora citricola, P. cambivora* and *P. cactorum,* were reported as the causal agents. In order to evaluate the involvement of the different *Phytophthora* species in beech decline in the southern part of Belgium (Wallonia), trees were sampled on the basis of notification from the managers of public and private forests. Inner bark tissues displaying necroses were collected at the margin between infected and healthy areas.

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Soil samples were also collected at the foot of some of the bleeding beeches using an auger.

Methods

Identification

Microbiological and molecular methods were used to determine the occurrence of a *Phytophthora* disease.

Nineteen *Phytophthora* strains were isolated from necroses of the inner bark of bleeding beeches. Isolations from rhododendron leaves used as baits enabled the recovery of one additional strain from a soil sample. Among these twenty strains, nineteen showed identical morphological characteristics: uniform colonies on V8 juice agar; no chlamydospore; nonpapillate, nondeciduous, ovoid sporangia; no oogonium in pure culture. They were identified as *P. cambivora*. Only one strain presented different morphological characteristics, notably corresponding to *P. gonapodyides*: petaloid colony on V8 juice agar; no chlamydospore; nonpapillate, nondeciduous, ovoid sporangia; nesting of sporangia; no oogonium in pure culture or after pairing with tester strains.

Molecular analysis was used to confirm the specific identification of the isolated strains but also to detect additional positive cases from beech necrotic tissues. Total DNA was extracted from the mycelium of the isolated strains or from necrotic tissues using the HighPure PCR Template Preparation kit (Roche Diagnostic). PCR-RFLP was carried out on total DNA using the test developed by Cooke and others (2000) targeting the ITS rDNA and the test of Martin and Tooley (2004) on the mtDNA. With both methods, the identity of *P. cambivora* was confirmed for the nineteen collected isolates. Six additional cases of infection by this *Phytophthora* species were detected from necrotic tissues. The identity of the *P. gonapodyides* strain was established by sequencing the ITS rDNA. Sequence similarity searching was achieved using the FASTA program. One additional case of infection caused by *P. gonapodyides* was revealed by PCR-RFLP of the ITS rDNA from necrotic tissues.

Geographic Distribution

A total of 83 beeches presenting bleeding cankers on the trunk were sampled in 49 different sites in the southern part of Belgium. Isolation proved the infection of nineteen beeches by a *Phytophthora:* eighteen by *P. cambivora* and one by *P. gonapodyides*. PCR-RFLP from necrotic tissues enabled the detection of a *Phytophthora* on seven additional beeches: six infected by *P. cambivora* and one by *P. gonapodyides.* These 26 positive cases were identified in nineteen different sites mainly distributed around the French border (fig 1). However, the non-uniform character of sampling makes interpretation difficult.

No preferential orientation of necroses was observed among affected trees. Dimensions of trees infected by a *Phytophthora* were highly variable but the smallest tree had a circumference of 80 cm. For half of the affected trees, bleeding cankers appeared from collar and reached 6 to 10m high in 27 percent of the cases.

Figure 1ð Geographic distribution of the sampling sites in Wallonia: ○ *Phytophthora*-free beeches; ● beeches from which a *Phytophthora* was isolated; ▲ beeches from which a *Phytophthora* was detected by PCR-RFLP

Characteristics of the Isolated Strains

Mating Type and Viability of Oospores

Proportions of each mating type (A1 and A2) were evaluated among *P. cambivora* isolates by pairing with reference strains of known mating type (Dr Werres – BBA, Germany). It was established that, among the nineteen *P. cambivora* strains, seventeen were from A2 type and two were from A1 type. Both mating types were identified in the site of Aublain. Mating between A1 and A2 strains of this site produced ornamented oogonia. So far, attempts to obtain germinated oospores from mating between Belgian isolates of *P. cambivora* were unsuccessful but their viability was evaluated using the staining method of Sutherland and Cohen (1983). Most of the oospores (76 percent) presented a purple coloration corresponding to dormant structures. Unstained oospores (non viable) were also frequently encountered (22 percent). Blue staining associated with active structures was observed for 1 percent of the oospores.

Pathogenicity Tests

One isolate of each mating type of *P. cambivora* was tested for pathogenicity on woody twigs of three forest species (*Fagus sylvatica, Castanea sativa* and *Quercus petraea)*. Inoculations (six replicates) were performed by inserting agar plugs into bark flaps at the middle of the twig. Necroses were observed on the three forest species regardless of the strain inoculated. No symptom developed on negative controls. Moreover, it was noticed that necroses on *Fagus sylvatica* were particularly scattered while a continuous necrosis of the whole twig was observed for *Castanea sativa* and *Quercus petraea*.

Conclusions

The study demonstrates the involvement of the genus *Phytophthora* in the decline of beech stands in Wallonia. A sampling was conducted on 83 bleeding beeches and *P. cambivora* – known to be the most aggressive species among the different *Phytophthora* involved in the decline of beech in Europe (Jung and others 2005), was detected 24 times in 18 different sites by combined use of morphological and molecular analyses. Moreover two affected trees proved to be infected by *P. gonapodyides.* Only five sites show multiple infections of beeches by a *Phytophthora* (from two to five affected trees). In the other sites, single infections were observed and affected trees were generally surrounded by many beeches without symptom. Both mating types of *P. cambivora* are present in Wallonia, sometimes in the same site, and pairing between A1 and A2 isolates produced oospores *in vitro*.

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Phosphite Application as an Explorative Tool for Understanding and Controlling *Eucalyptus gomphocephala* (Tuart) Decline in Southwest Western Australia[1]

Peter M. Scott,[2,3] Harry T. Eslick,[3] Paul A. Barber,[2,3] Michael C. Calver,[3] Giles E.St.J. Hardy,[2,3] and Bryan L. Shearer[4]

Abstract

Eucalyptus gomphocephala is a Mediterranean forest canopy species endemic to a narrow (5-10 km wide) coastal strip approximately 300 km in length in south-west Western Australia. *Eucalyptus gomphocephala* is undergoing a significant decline that was first identified as a spot decline in 1994 and now occurs throughout large sections of its remnant distribution within Yalgorup National Park, in some areas resulting in 100 percent mortality. The reduction of this keystone species represents a significant modification to the associated ecosystem. Modifications to hydrology, fire regimes, entomological pressures, and fungal and Pythiaceous soil pathogens have been identified as possibly contributing to the decline syndrome. The potential of phosphite (phosphonate), nutrient and insecticide treatments to reverse the decline in tree health was assessed as (a) a method for controlling the decline and (b) a method for diagnosing possible causal agents. Phosphite has been successfully used to control *Phytophthora* and Pythiaceous soil pathogens by inducing a host defense response within the plant. Stem injection of declining *Eucalyptus gomphocephala* in the present study has resulted in improved canopy health and vigor, indicating that *Phytophthora* and/or other Pythiaceous microorganisms may be playing a role in the decline. The impact of phosphite application on nutrient uptake and fine feeder root concentration was also assessed.

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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An Evaluation of Stream Monitoring Techniques for Surveys for *Phytophthora* Species in Victoria, Australia[1]

Benjamin Smith,[2] Ian W. Smith,[2] Rodney H. Jones,[3] and James Cunnington[3]

Abstract

In Australia, surveys for *Phytophthora* species generally rely on either soil or plant testing to confirm their presence in an area. Stream monitoring has become an important part of early detection surveillance systems for *Phytophthora ramorum* in the USA. In the present study, water monitoring methods used for the detection of *Phytophthora* species in streams in the USA were evaluated in surveys in June 2006 and March 2007 at 8 sites and 4 locations to the east of Melbourne, Australia.

At each site, five leaves from four different bait types were floated in mesh bags in the streams for periods varying from one to three weeks depending on the bait type (total 160 baits). In the first survey in June, bait types included *Rhododendron* leaves (undamaged and cut in a herringbone pattern), mature *Eucalyptus regnans* leaves and *E. sieberi* cotyledons, a bait used often for isolation of *Phytophthora cinnamomi* from soil in Victoria. In the March survey, *Pittosporum undulatum* leaves were added as baits and only undamaged *Rhododendron* leaves used, along with the other eucalypt baits. One litre of water was collected from each site at the end of each baiting period and filtered in the laboratory using 0.2µm Metricel membrane filters. Lesions that developed on the bait samples, and the complete filters, were plated directly onto P5ARP(H) agar. DNA sequencing of the rDNA ITS region was carried out on purified cultures of *Phytophthora* species isolated from the plates.

Phytophthora species were isolated from all sites, bait types and filters used in the study. Isolated species included *Phytophthora cinnamomi*, *P. citricola*, *P. gonapodyides*, undescribed *Phytophthora* species from clades 6 and 7, and an unknown, potentially new clade. Species isolated varied with season, bait and filter used. This highlights the need to use a variety of parameters for stream monitoring when targeting a wide range of Phytophthora species.

Stream baiting has potential as a valuable method to determine the presence of a range of *Phytophthora* species in catchments across Australia. It also provides a valuable tool for early detection of *Phytophthora* species, and needs to be included in Australia's network of quarantine surveillance activities. The method may also be potentially applicable to the monitoring of stream and dam water for use in irrigation and fire fighting activities, and of water runoff from gravel pits and nurseries so as to identify the presence of the pathogens, and reduce their spread across Australia.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Molecular Testing Uncovers New *Phytophthora* Taxa From Natural Ecosystems in Western Australia[1]

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Abstract

Verification of mapping of the extent of *Phytophthora* dieback disease, based on shadowless colour aerial photography, involves the routine testing of soil and root samples collected from beneath dying, *Phytophthora*-sensitive native plant "indicator species" for the presence of the pathogen. In addition to *P. cinnamomi*, other isolates have been recovered on selective agar following the baiting of soil, or the direct plating of plant tissue, during these operations. These have been identified, using morphological characters, as *P. citricola*, *P. megasperma*, *P. cryptogea*, *P. drechsleri*, *P. nicotianae*, and *P. boehmeriae*.

The recent advent and availability of DNA sequencing techniques for the identification of *Phytophthora* species has enabled the testing of new isolates that were difficult to identify from their morphology, as well as a range of historical isolates dating back to the 1980s from the Department of Environment and Conservation culture collection.

DNA was extracted from pure cultures grown on cornmeal agar and the Internal Transcribed Spacer (ITS) regions of the rRNA were amplified using primers ITS6 and ITS4. BLASTn searches of sequence data were conducted in GenBank to determine the most closely related *Phytophthora* spp. Sequences were then aligned and parsimony and distance analyses conducted in PAUP. Based on phylogenetic analysis, seven potentially new and undescribed taxa of *Phytophthora* can be distinguished. Several of these are morphologically indistinguishable from known species (eg *P. citricola*, *P. megasperma*, and *P. cryptogea*). In some cases the new taxa are indeed most closely related to the known species (eg *P. citricola*), but in others their DNA sequences show that they are not closely related to the morphologically similar species (eg *P. megasperma*).

Phytophthora inundata, described in Europe in 2003, has been identified based on phylogenetic analysis from several locations in the south-west where it has been associated with dying native plants. Some of these isolates have been stored since the 1980s. One of the new species, with morphology similar to *P. citricola*, but most closely related phylogenetically to *P. bisheria* and *P. multivesiculata*, has been isolated from dying 1- to 2-year-old jarrah (*Eucalyptus marginata*) seedlings in rehabilitated open-cut bauxite mine pits.

Further work is planned to describe the new taxa and their relationships, and to test their pathogenicity, so that an estimate of the level of threat they pose to native vegetation can be made.

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Long-Term Storage of *Phytophthora* Cultures in Water[1]

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Abstract

Long-term storage of cultures of *Phytophthora* species is a challenge for any lab managing a working collection of isolates. Storage in liquid nitrogen is generally considered to be optimal for archival storage, and successful recovery of most species is regularly achieved after many years. Nitrogen storage has its drawbacks, however, especially for a working collection. It requires species specific freezing conditions, and thawing must be done carefully. Equipment is bulky, and regular addition of liquid nitrogen is necessary. Storage in vials in water at room temperature is an efficient, effective alternative for many collections. Isolates are grown on agar, and plugs are removed from the colony margin and placed in sterile water in 1.5 ml plastic tubes, with or without pieces of sterilized hemp seed. Tubes are kept in coded racks at room temperature, in the dark. Agar plugs can be removed one at a time as needed and plated on selective agar to resume growth. In a recent test, we had nearly 100 percent recovery of several *Phytophthora* species, including *P. ramorum*, after 7 years in water storage.

Introduction

Long-term storage of cultures of *Phytophthora* species is a challenge for any lab managing a working collection of isolates. Storage in liquid nitrogen is generally considered to be optimal for archival storage, and successful recovery of most species is regularly achieved after many years. Nitrogen storage has its drawbacks, however, especially for a working collection. It requires species-specific freezing conditions, and thawing must be done carefully. Equipment is bulky, and regular addition of liquid nitrogen is necessary.

In 1976 Boesewinkel described a new storage method for storing *Phytophthora* using colonized agar blocks in bottles (90 x 26 mm) of sterilized distilled water. This method eliminated the space and cost issues associated with nitrogen storage. We have adapted the method, using smaller vessels and adding hemp seeds.

Methods

Culture Storage

Water vials are 1.5 ml of de-ionized water placed in 1.5 ml cryogenic vials. Hemp seed/water vials are one and a half ml of de-ionized water and one hemp seed placed in 1.5 ml cryogenic vials. The vials are sealed, sterilized by autoclaving and cooled to room temperature.

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[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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Isolates are grown on agar (usually corn meal agar amended with 20 ppm β sitosterol) for five to ten days. Each isolate is stored in a pair of vials, one 5mm plug placed in a hemp seed/water vial and three to five plugs in a water vial. The vials are placed in CryoBoxes and stored in the dark at room temperature.

Culture selection

Species selection for this test was based on availability in long term storage and interest to the larger community. Cultures that have not been identified to species were not included in this test. When possible, five isolates per species were included.

Water Tube Culture Retrieval

A single plug was retrieved from a water vial and plated on the selective medium CARP (17 g BBL Corn Meal Agar, 1 L de-ionized water, 20ppm Delvocid (50 percent natamycin salt), 200ppm Ampicillin, sodium salt and 10ppm Rifamycin SV sodium salt). At 14 days plates were examined for growth. If new mycelium had grown into the agar it was scored a success. If no new mycelium was present the water storage vial was re-sampled, this time plating all remaining plugs. Again if there was new growth in 14 days it was scored as a success and if there was no growth it was considered a failure.

Hemp/water tube culture retrieval

Both the hemp seed and plug were plated on CARP. Frequently in the hemp tubes a mass of mycelium forms and this was plated as well. At 14 days the plates were examined for new growth. New mycelium emerging from the plug, hemp seed or mycelial mass was scored a success.

Results

Cultures were successfully retrieved from 72 of the 78 water vials after three to eight years in storage (Table 1). Of the six failures, the four 7-year-old *P. lateralis* and one 5-year-old *P. ilicis* vials had only one remaining plug and so couldn't be re-sampled. The 8-year-old *P. megasperma* had five non-viable plugs.

Table 1ð Water vial culture success ratios (number of successful retrievals/number of vials sampled) by years stored.

	YEARS IN STORAGE						
SPECIES	8	7	6	5	4	3	Total
P. cactorum	1/1	5/5					6/6
P. cambivora			2/2	2/2			4/4
P. cinnamomi				3/3			3/3
P. citricola	2/2		1/1	1/1			4/4
P. cryptogea				2/2		2/2	4/4
P. sp. *"*DF1*"*	5/5						5/5
P. europaea			1/1				1/1
P. gonapodyides				2/2	5/5		7/7
P. ilicis				0/1	1/1		1/2
P. lateralis		0/4	3/3				3/7
P. megasperma BHR	7/8						7/8
P. nemorosa			5/5				5/5
P. sp. *"Pg chlamydo"*	5/5						5/5
P. pseudosyringae				4/4			4/4
P. pseudotsugae	1/1						1/1
P. ramorum			7/7				7/7
P. siskiyouensis					5/5		5/5
Totals	21/22	5/9	19/19	14/15	11/11	2/2	72/78

Cultures were successfully retrieved from 34 of the 38 hemp/water vials after three to six years in storage (Table 2). Of the four failures, two had bacterial contamination. Of the successes, in all but one case, both the hemp/water plug and the hemp seed/mycelium mass were viable.

Table 2ð Hemp seed/water vial culture success ratios (number of successful retrievals/number of vials sampled) by years stored.

	YEARS IN STORAGE				
SPECIES	6	5	4	3	Total
P. cactorum					
P. cambivora	5/5	2/2			7/7
P. cinnamomi		3/3			3/3
P. citricola	1/1	1/1			2/2
P. cryptogea		2/2		2/2	4/4
P. sp. *"DF1"*					
P. europaea					
P. gonapodyides		2/2	4/5		6/7
P. ilicis		0/1			0/1
P. lateralis					
P. megasperma BHR					
P. nemorosa					
P. sp. *"Pg chlamydo"*					
P. pseudosyringae		3/4			3/4
P. pseudotsugae					
P. ramorum	2/2	3/3			5/5
P. siskiyouensis			4/5		4/5
Totals	8/8	16/18	8/10	2/2	34/38

Comparing the success of the two methods was possible in 31 cases, where isolates had been stored in both water and hemp/water vials at the same time (Table 3).

Table 3—Culture success comparing water vial to hemp/water vial.

	Water Vial	
Hemp/Water Vial	Success	Failure
Success	27	0
Failure	3	1

Discussion

There are many advantages to this type of culture storage, cost, ease and high success rate to name a few. The ability to remove one plug at a time is more efficient than thawing and plating an entire vial. However, there are a few cautions. Repeated visits to the same tube increase the odds of contamination. Our "room temperature" ranges from about 18-24^{o}C, but some species may need special conditions, especially cooler or more controlled temperatures. After retrieval of a culture it's a good idea to restore anything that's been in storage more than two years.

We have been using water storage exclusively for at least 12 years. Recently, *P. lateralis* was successfully cultured from 4 hemp vials stored in 1995. While there is no way of knowing which isolates are still viable until they are retrieved, over the course of the 12 years our success rate has been high.

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Occurrence and Impact of *Phytophthora* Species in Forest Trees in Hungary[1]

Ilona Szabó[2] and Ferenc Lakatos[2]

Abstract

Occurrence of *Phytophthora* species and their phytopathological role have been investigated in forest stands in Hungary since 1999. Decline symptoms, specific stem lesions and unspecific top drying signs were surveyed in forest stands of different tree species.

The isolation of *Phytophthora* was carried out from soil samples taken from around the diseased trees by baiting with *Prunus laurocerasus* leaves on selective medium PARPNH. The isolates were identified by morphological and molecular way. The morphological characters were observed in cultures growing on carrot agar medium. The formation of sporangia was induced by flooding the cultures with soil extract. The molecular identification was performed by sequencing the ITS regions of rDNA and comparing the sequences with the known *Phytophthora* sequences accessible in GenBank database. The pathogenicity of the isolates was tested by wound inoculation in the stem of seedlings and by root infections.

Phytophthora species were found in *Alnus glutinosa* with bleeding stem lesions and crown drying symptoms, in *Juglans nigra, Quercus petraea* and *Q. cerris* with crown drying symptoms. The morphological and molecular identification resulted in 8 *Phytophthora* species in *Alnus* (*P. alni, P. citricola, P. gonapodyides, P. inundata, P. megasperma, P*. sp.1, *P*. sp. 2., *P*. sp. 3.), 4 in *Juglans* (*P. cactorum, P. cirticola, P. hedraiandra, P*. sp.1) and 2 in *Quercus* (*P. citricola, P. gonapodyides*). The artificial inoculations caused well-delimited bark necrosis in the stem of the seedlings, the largest by *P. alni* in alder and *P. citricola* in black walnut, but generally not exceeding 3-4 cm length. Root infections resulted in lesion and reduction of the fine roots, most pronounced by *P. citricola* in black walnut. The impact of *Phytophthora* species on the healthy condition of the forest trees proved to be most important in planted *Alnus glutinosa* and *Juglans nigra* stands situated in wet sites and flood areas respectively. A community of *Phytophthora* species occurs in the rhizosphaera of these trees causing root and collar rot and bleeding stem lesions in alder and fine root reduction manifesting by crown drying in black walnut. The appreciation of the role of *Phytophthora*s in oak decline needs further research because of their fewer occurrences in the oak drying cases. The identified *Phytophthora* species, except of *P. cactorum*, were recorded first time in Hungary during this research work.

Introduction

Phytophthora root and collar rot of alder was found first time in Hungary in 1999 in some planted alder forests of medium age situated in Northwestern area of the country (Szabó et al. 2000). Typical bleeding stem lesions and crown drying symptoms also were common. The rate of symptomatic trees varied up to 20.6

[1] A version of this paper was presented at the Fourth Meeting of IUFRO Working Party S07.02.09, Phytophthoras in Forests and Natural Ecosystems, August 26-31, 2007, Monterey, California.

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percent and the mortality reached 10.7 percent in five forest subcompartments evaluated in 2000. The annual ring analysis showed that the disease has begun a few years earlier, and up to 20.7 percent dead trees were already removed during the precedent years. This first record of a destructive Phytophthora disease promoted the research of this group of pathogens in Hungary. Since 1999 long time investigations have been started in order to detect the occurrence of *Phytophthora* species and to clarify their phytopathological role in forest decline processes.

Materials and Methods

Decline symptoms, specific stem lesions and unspecific crown drying signs were surveyed in forest stands of different tree species all over the country. The *Phytophthora*s were isolated from soil samples taken from around the diseased trees, by baiting method using *Prunus laurocerasus* leaves as baits. The isolations were performed on selective medium, than the colonies were passed on PDA in tubes for long time storage. The isolates were identified by morphological and molecular way. The morphological characters were observed in cultures growing on carrot agar medium (Kröber 1985). The colony patterns and growth rate were recorded (Erwin and Ribeiro 1996). Formation of sporangia was induced by flooding the cultures with soil extract (Nagy and others 2000) after that the microscopical structures were observed and measured. The molecular identification was performed by sequencing the ITS1 and ITS2 regions of rDNA and comparing with the known *Phytophthora* sequences accessible in GenBank. The DNA was extracted from pure culture using REDExtract-N-Amp kit from Sigma, PCR was performed with specific primers ITS6 and ITS4 (Cooke and Duncan 1997). The pathogenicity of the isolates was tested by wound inoculation with mycelium in the stem of seedlings and by root infections.

Results

Decline symptoms occurred in alder, oaks, beech, poplars and black walnut stands, as well as in silver birch, chestnut and horse chestnut individual trees. *Phytophthora* species were found in *Alnus glutinosa* showing bleeding stem lesions and crown drying symptoms, in *Juglans nigra, Quercus petraea* and *Q. cerris* with crown drying symptoms. Between 1999 and 2005 a number of 376 isolates were collected and investigated, 98 of them were identified as *Pythium* by their morphology. The morphological and molecular identification resulted in eight *Phytophthora* species in *Alnus* (*P. alni, P. citricola, P. gonapodyides, P. inundata, P. megasperma, P*. sp.1, *P*. sp. 2, *P*. sp. 3), four in *Juglans* (*P. cactorum, P. cirticola, P. hedraiandra, P*. sp.1) and two in *Quercus* (*P. citricola, P. gonapodyides*).

Alder stands were investigated in five different areas of Hungary. The host specific *Phytophthora alni* was isolated in stands of two areas. This species was found less frequently and only in the active disease processes of typical symptoms of fresh exudates at the stem basis of the trees. *Phytophthora inundata, P. gonapodyides,* and *P.* sp.1 were found the most common wide-spread species in alder stands. *Phytophthora citricola, P. megasperma, P*. sp.2 and *P*. sp. 3 occurred more rarely in alder stands, they are represented by only a few isolates.

Black walnut is cultivated in Hungary mostly in flood areas of Danube and some other wet lowland sites. Decline symptoms as yellowing the leaves, crown drying then death of the trees have been observed by the foresters some years ago. The investigation of the role of *Phytophthora* species started in 2004 in a heavily affected stand where the rate of the symptomatic and dead trees reached 42 percent. Bleeding lesions were not observed, unspecific crown drying occurred only. *P. citricola* was common in the soil around these trees, and *P. cactorum* also was less frequently detected. *P. hedraiandra* and *P*. sp. 1 are represented by only one isolate each from walnut.

Phytophthora species were not found in the most of studied oak decline processes in Hungary. *P. citricola* and rarely *P. gonapodyides* were isolated in one site only, in a mixed forest near to Sopron, in soil samples taken from declining *Q. petraea* and *Q. cerris* trees.

Pathogenicity tests were conducted in alder and black walnut seedlings using isolates of different *Phytophthora* species. The artificial inoculations caused well-delimited bark necrosis in the stem of seedlings, the largest by *P. alni* in alder and *P. citricola* in black walnut. The lesions generally not exceeded 3-4 cm length, but some inoculations with *P. citricola* in black walnut resulted in large diffuse cankers growing along the stem and destroying the seedlings. Root infections resulted in lesion and reduction of fine roots, most pronounced by *P. citricola* in black walnut.

Conclusions

The impact of *Phytophthora* species on the healthy condition of forest trees proved to be most important in planted *Alnus glutinosa* and *Juglans nigra* stands situated in lowland sites and flood areas. A community of *Phytophthora* species occurs in the rhizosphaera of these trees causing root and collar rot and bleeding stem lesions in alder and fine root reduction manifesting by crown drying in black walnut. The appreciation of the role of *Phytophthora*s in oak decline needs further research because of their fewer occurrences in the oak drying cases. The identified *Phytophthora* species, except of *P. cactorum*, were recorded first time in Hungary during this research work (Szabó et al. 2000, Szabó 2003, 2005, Szabó and Lakatos 2007).

Acknowledgement

The research was supported by the Hungarian Research Found, grants OTKA T 037352 and OTKA T 049077.

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