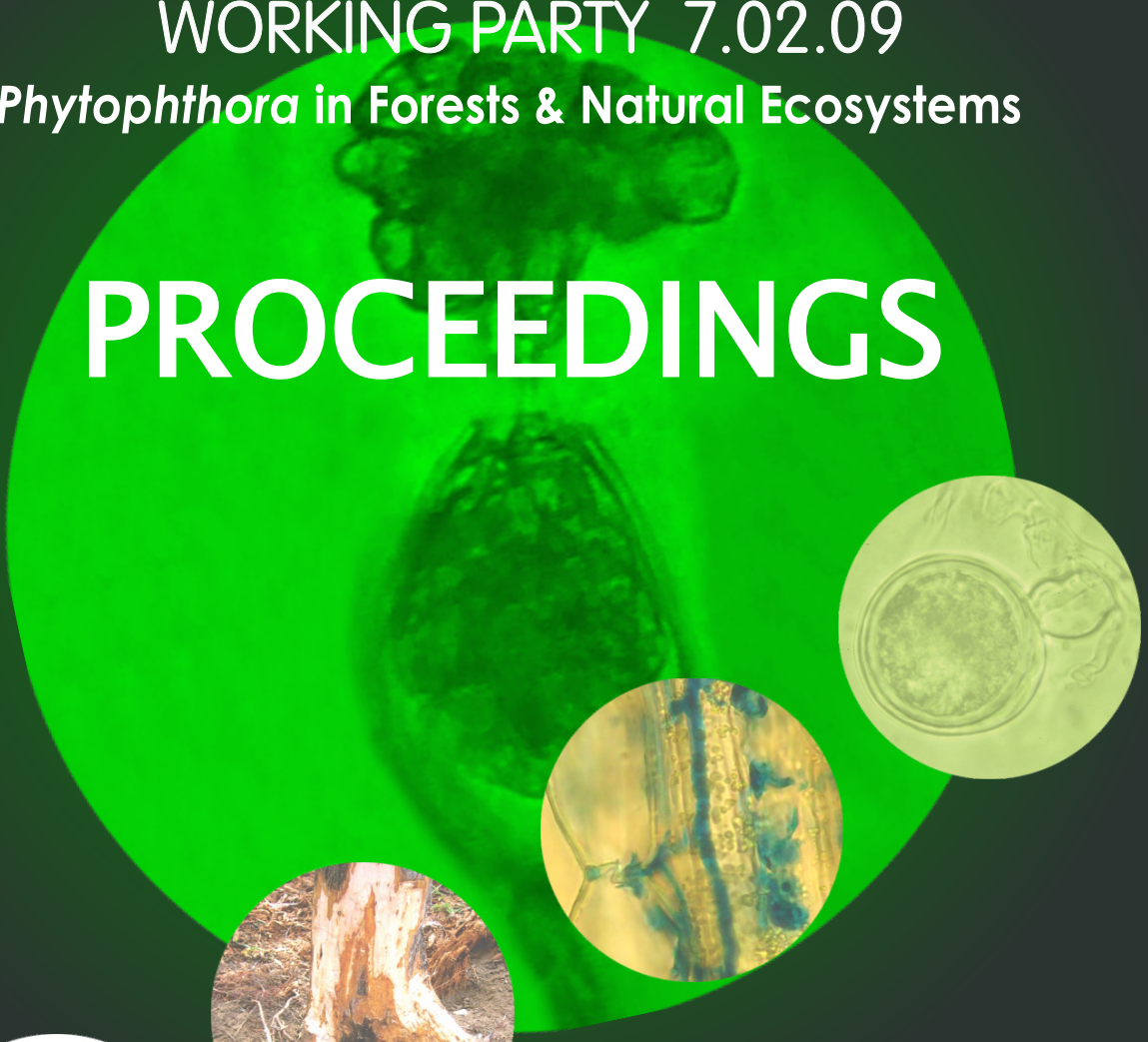
# 7th meeting IUFRO

## WORKING PARTY 7.02.09

***Phytophthora* in Forests & Natural Ecosystems**

# PROCEEDINGS



10th · 14th November 2014
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# Phytophthora in Forests & Natural Ecosystems

9th – 14th November 2014

Esquel Argentina

**The 7th Meeting of the International Union of Forest Research Organizations**
**IUFRO Working Party 7.02.09**
**Phytophthora in Forests & Natural Ecosystems**

Wendy Sutton, Paul W. Reeser and Everett M. Hansen,
Technical Coordinators

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Phytophthora in Forests & Natural Ecosystems

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# Table of Contents

**Taxonomy**

1 Six new *Phytophthora* species from ITS Clade 7a including two sexually functional heterothallic hybrid species detected in natural ecosystems in Taiwan
T. Jung, M. Horta Jung, B. Scanu, A. Pérez-Sierra, T. Chang, P. Abad-Campos, M. Léon, G. M. Kovács, C. Husson and J. Bakonyi

2 The Taxonomy of *Phytophthora*: What is done and what is needed for the correct identification and diagnostics of species in the Genus
Z. G. Abad

3 A new multi-locus phylogeny for the genus *Phytophthora*
X. Yang , B. M. Tyler and C. Hong

4 What is a species? The challenge of *Phytophthora*
E. Hansen

**Role of *Phytophthora* species in emergent diseases**

8 Pathogenicity of *Phytophthora* species isolated from declining European blackberry (*Rubus anglocandicans*) in natural ecosystems of South Western Australia
S. Aghighi , T. I. Burgess , J. K. Scott , M. Calver and G. E. St. J. Hardy

9 The comparative pathogenicity of two *Phytophthora ramorum* lineages, EU1 and EU2, on a range of hosts
A. McCracken, L. M. Quinn, M. A. Wilson and J. F. Webber

10 Involvement of *Phytophthora* species in beech decline in Lower Austria
T. L. Cech , T. Jung, T. Corcobado Sanchez and A. Daxer

11 *Phytophthora pluvialis* on Douglas-fir in Oregon, USA
P. Reeser , W. Sutton , J. Laine and E. Hansen

14 Identification and pathogenicity of oomycetes causing root disease on wild olives
M. González , M. E. Sánchez , A. Pérez-Sierra and M. S. Serrano

17 Unexpected discovery of *Phytophthora siskiyouensis* in the UK
A. Perez-Sierra, S. Sancisi-Frey, M. Kalantarzadeh and C. Brasier

18 Mycelial growth and pathogenicity of *Phytophthora cinnamomi* Rands
E. A. Gutierrez Rodriguez, R. C. Panizzi, A. B. G. Martins and R. A. Andrade

19 The role of *Phytophthora* in the decline of *Corymbia calophylla* (marri), a dominant and widespread tree species in southwest Western Australia
T. Paap, L. Croeser, T. I. Burgess and G. E. St. J. Hardy

20 *Phytophthora austrocedri* emerges as a serious threat to juniper (*Juniperus communis*) in Britain
S. Green, M. Elliot, A. Armstrong and S. J. Hendry

21 Evolutionary epidemiology of *Phytophthora austrocedri* on juniper in Great Britain and the Northern Hemisphere
J. Assmann , S. Green and P. Sharp

22 *Phytophthora pseudosyringae* associated to the mortality of *Nothofagus* forests of central southern Chile
S. N. Fajardo , A. Figueredo , S. Valenzuela and E. Sanfuentes

23 *Phytophthora kernoviae* detection in *Drimys winteri* (Winter's Bark) forest of southern Chile
E. A. Sanfuentes , S. N. Fajardo , M. A. Sabag , E. Hansen and M. G. González
24 Pathogenicity tests of *Phytophthora* species on *Agathis australis*
I. J. Horner and E. G. Hough
25 Aerial stem cankers associated with *Phytophthora syringae* on *Fraxinus*: how and why?
J. Webber , B. Wylder , A. Harris and C. Brasier
26 Multiple *Phytophthoras* associated with larch (*Larix*) in Britain
S. Sancisi-Frey and J. Webber
27 Aerial dieback on *Thuja* caused by *Phytophthora lateralis*
A. Schlenzig, S. Clark and R. Campbell
28 First report of *Phytophthora* sp. on *Epipremnum aureum* in Mexico
M. Díaz-Celaya , A. L. Mora-Dañino , S. P. Fernández-Pavía , G. Rodríguez-Alvarado and K. Lamour

**Tools for *Phytophthora* surveys**

29 Fishing for *Phytophthora* 2.0
S. Català, A. Puértolas, S. Larregla, A. Pérez-Sierra and P. Abad- Campos
30 Exploring hidden *Phytophthora* via amplicon Pyrosequencing using eDNA from soil and water
S. Català, R. Arenas, P. Abad-Campos and A. Pérez-Sierra
31 Next Generation Sequencing reveals unexplored *Phytophthora* diversity in Australian soils
T. Burgess, S. Català, D. White and G. Hardy
32 Molecular Tools for the PCR detection of *Phytophthora austrocedri*
Z. G. Abad, K. J. Owens, J. C. Bienapfl, S. Green and M. K. Nakhla
33 Tools for rapid characterization of *Phytophthora infestans* and *Phytophthora ramorum* using real-time PCR and microsatellites from genomic resources
G. J. Bilodeau, M. Gagnon, C. A. Lévesque, L. Kawchuk, C. P. Wijekoon, N. Feau, M. Bergeron, N. J. Grünwald, C. M. Brasier, J. F. Webber and R. C. Hamelin
34 Whither the species? *Phytophthora* taxa, MOTUs and barcodes in the world of metabarcoding
D. E. L. Cooke, M. I. Prigigallo, A. Abdelfattah, L. Schena, E. Randall and J. N. Squires

**Surveys and new records**

35 *Phytophthora* spp. invasions in post-communist economies – the example of the Czech Republic
K. Černý, M. Hejná, Z. Haňáčkova and M. Mrázková
38 Survey of Oomycetes found in western Washington streams
M. Elliott, G. Chastagner, K. Coats, G. Dermott and L. Rollins
39 Diversity of *Phytophthora* species in forests, forest nurseries and riparian ecosystems of Portugal
M. Horta Jung, A. Cravador, C. Maia and T. Jung
40 Diversity of *Phytophthora* species in the oak forests of Southwest China
W. Huai, E. M. Hansen, W. Zhao, G. Tian, Y. Yao and X. Cheng

41 *Phytophthora* species associated to Holm oak decline in western Spain
B. Mora-Sala, R. Moliner, T. Corcobado, A. Solla and P. Abad-Campos

42 Multiple new and invasive alien *Phytophthora* taxa from Mediterranean maquis ecosystems in Italy
B. Scanu, B. T. Linaldeddu, A. Deidda, L. Maddau, A. Franceschini and T. Jung

43 *Phytophthora* detections in native plant nurseries and restorations sites in California
S. Rooney Latham, C. Blomquist, T. Swiecki, E. Bernhardt, E. Natesan and S. J. Frankel

45 *Phytophthora* - an emerging threat to plantation forestry in Vietnam
T. Q. Pham, D. N. Quynh, T. Burgess and B. Dell

46 Diversity and impact of *Phytophthora* spp. in natural ecosystems of Taiwan
T. Jung, T. Chang, A. Pérez-Sierra, K. Hsueh, C. Fu, P. Abad-Campos, M. Léon and M. Horta Jung

**Biology and genetics**

47 Visualisation of early infection by *Phytophthora* "taxon Agathis" in the roots of two-year old kauri plants
M. Padamsee, S. E. Bellgard, S. E. Williams, C. Probst , N. Anand and T. Lebel

49 *Phytophthora ramorum*: Study of the lineage EU2 / EU1 in Ireland
L. de la Mata Saez, C. Fleming and A. McCracken

50 Basic and applied research into *Phytophthora ramorum* in Ireland: the PHYTOFOR project
R. O'Hanlon , J. Choiseul , H. Grogan and J. M. Brennan

53 Lineage, phenotype and environment factors influencing the *Phytophthora ramorum* epidemic on larch
J. Webber, A. Harris and C. Brasier

54 Investigation of the tree pathogen, *Phytophthora lateralis*, newly discovered in Northern Ireland
L. M. Quinn, A. R. McCracken, L. R. Cooke, D. J. Studholme and M. J. Larkin

55 Phenotypic, genotypic, genetic, genomic, analyses of plant pathogens and their application in plant pathology
M. Garbelotto

**Ecology**

56 Landscape heterogeneity and features are associated to the impact of Ink disease in chestnut orchards in Italy
A. Vannini, G. Natili and A. M. Vettraino

57 Ecology and pathology of *Phytophthora nemorosa, P. pseudosyringae*, and other ITS clade 3 species in forests in western Oregon
W. Sutton, P. Reeser and E. Hansen

58 Maternal effects mediate the resistance of *Quercus ilex* to *Phytophthora cinnamomi*
A.Solla, J. Hernández, T. Corcobado and E. Cubera

59 Long term impact of *Phytophthora alni* on an alder riparian stand
B.Marçais, C. Husson and Z. Nagy

60 The interplay among human, biotic and abiotic factors explains quick *Phytophthora cinnamomi* spreading and tree decline in a Mediterranean Biosphere Reserve
L. V. García , P. De Vita , M. S. Serrano , C. Ramo , J. S. Cara , M. R. Écija and M. E. Sánchez

**Ecophysiology and Physiopathogenicity**

62 Screening *Quercus ilex* for tolerance to water stress and *Phytophthora cinnamomi*
T. Corcobado, E. Pérez, B. Krajnc, A. Martos, A. Pérez, E. Cubera, L. Nuñez, M. Horta Jung, A. M. Vettraino and A. Solla
63 The spatial and temporal spread of *Phytophthora alni* subsp. *alni* in alder bark tissue – an ecophysiological study
H. Pfanz, J. Mombour, C. Wittmann, F. Fleischmann and W. Oßwald
64 Diterpene resin profile of *Austrocedrus chilensis* affected by *Phytophthora austrocedri*
V. Olate , M. L. Vélez , A. G. Greslebin and G. Schmeda-Hirschmann
68 Effect of cinnamomins on *Phytophthora cinnamomi* biomass growth and on the oxidative burst in infected *Quercus suber* roots
G. Ebadzad , J. Martins and A. Cravador
73 De Novo Assembly of *Phlomis purpurea* Transcriptome challenged with *Phytophthora cinnamomi*
A. Baldé, A. Cravador, D. Neves and M. S. Pais
77 Screening of Asian oak species for potential resistance to *Phytophthora cinnamomi*
M. Horta Jung, C. Maia, T. Chang, K. Hsueh and T. Jung
78 Recent advances in understanding *Phytophthora*-woody plant interactions
F. Fleischmann

**Management and Control**

79 Challenges associated with the management of *Phytophthora* diseases in Australia and the importance of community engagement for success
G. Hardy, B. Dunstan, T. Paap and T. Burgess
80 Continued Monitoring of Sudden Oak Death Treatments in Oregon Tanoak Forests
E. M. Goheen , A. Kanaskie , E. Hansen , P. Reeser and W. Sutton
81 Sudden Oak Death in Oregon forests: recent disease intensification and spread, and changes to the management program
A. Kanaskie, E. M. Goheen, E. Hansen, P. Reeser, W. Sutton, N. Grunwald, R. Rhatigan, R. Wiese, J. Laine,
89 Approaching 15 years of research on SOD control
M. Garbelotto, D. Schmidt, S. Schechter, P. Croucher and C. Hayden
90 Eradication of *Phytophthora cinnamomi* from infested *Eucalyptus marginata* (jarrah) forest during large scale mining operations
B. Dunstan, J. Gyeltshen, A. Vettraino, V. Stokes, T. Burgess and G. Hardy

91 Potential impacts of the revised APHIS *Phytophthora ramorum* domestic quarantine regulatory requirements on the spread of this exotic pathogen within Washington state
G. Chastagner and M.Elliott
92 Enabling technologies to combat *Phytophthora* diseases
N. M. Williams, R. L. McDougal, P. Scott, E. Telfer, L.J. MacDonald, N. Graham and A. Wagner
93 Searching for *Phlomis purpurea* metabolites with anti-*Phytophthora cinnamomi* activity
D. Neves, C. Maia, S. Durães, M. Horta, O. Holdenrieder and A. Cravador
96 Screening of biofumigants against *Phytophthora cinnamomi* root disease
P. Ríos, M. S. Serrano, A. Pérez-Sierra, A. de Haro and M. E. Sánchez

**Field trip presentations**

100 A brief historic account of "Mal del Cipres"
M. Rajchenberg
107 Other species threatened by *Phytophthora austrocedri*
M.L. Velez and A.G. Greslebin

**Poster session**

108 Surveillance and management of Kauri Dieback in New Zealand
T. Beauchamp and N. Waipara
109 What is a species? – you be the judge
C. Brasier , N. Grunwald , E. Hansen , P. Reeser , L. Sims and W. Sutton
112 Determining an optimal sequence identity threshold value for *Phytophthora* spp. retrieval from environmental data
S. Català, A. Puértolas, A. Pérez-Sierra and P. Abad-Campos
113 Development of new Real-Time specific assays for the detection of *Phytophthora* species in Holm Oak calcareous forests
S. Català, M. Berbegal, A. Pérez-Sierra and P. Abad-Campos
114 Discovering *Phytophthora* species in the laurel forest in Tenerife and La Gomera islands (Canary Islands, Spain)
S.Català, A. Pérez-Sierra, C. Rodríguez Padrón , F. Siverio de la Rosa and P. Abad-Campos
115 Climate change can affect the impact of *Phytophthora alni* subsp. *alni*
K. Černý, N. Filipová and V. Strnadová
118 Economical losses caused by *Phytophthora alni* in riparian stands. Typological study of Vltava River basin (Czech Republic)
K. Černý, V. Strnadová, L. Fedusiv, Š. Gabrielova , Z. Haňáčkova, L. Havrdová, M. Hejná, M. Mrázková, K. Novotná, V. Pešková, P. Štochlová and D. Romportl
121 Factors affecting *Phytophthora alni* distribution in State Forests of the Czech Republic
K. Černý, V. Strnadová, D. Romportl, M. Mrázková, L. Havrdová , M. Hrabětová, R. Modlinger and V. Pešková

124 Invasive pathogens in Austrian forests: preliminary planning within the European project "Responses of European forests and society to invasive pathogens (RESIPATH)"
T. Corcobado Sanchez, T. L. Cech, C. Huettler, M. Brandstetter, A. Daxer and T. Majek
125 Spectral measurements for detecting *Phytophthora*-related stress in *Corymbia calophylla* (marri)
L. Croeser, T. Burgess, G. Hardy, T. Paap and M. Andrew
126 Next generation sequencing of *Phytophthora ramorum*: differences in the gene expression during infection in the lineages EU1/EU2
L. de la Mata Saez, A. McCracken, J. Jia, F. Doohan and C. Fleming
128 The detection and quantification of four *Phytophthora* species in soil in the UK
M. Elliot, S. Green
129 RNAseq reveals different defense responses of Quercus robur microcuttings against Phytophthora quercina during root and shoot flush
F. Fleischmann, O. Angay, S. Recht, L. Feldhahn, M. Tarkka, S. Hermann and T. Grams
130 Alternatives for detection of *Phytophthora cinnamomi* in commercial subtrate
E. A. Gutierrez Rodriguez, M. Panizzi Penariol, M. C. Ohya, R. C. Panizzi and R. A. Andrade
132 Influence of multiple stress sources on cork oak (*Quercus suber* L.) seedling susceptibility to *Phytophthora cinnamomi*
O. Gutiérrez-Hernández, L. V. García Fernández, P. de Vita, M. S. Serrano, C. Ramo, E. Gutiérrez, P. Ríos, I. Pérez-Ramos, L. Gómez- Aparicio and M. E. Sánchez
134 Species of *Phytophthora* on rhododendrons in Argentina
P. E. Grijalba and H. E. Palmucci
135 Comparative fitness of European lineages of *Phytophthora ramorum*
A. Harris, B. Scanu and J. Webber
136 Oomycetes survey in Northern Norway
M. L. Herrero, L. Sundheim, A. M. Brevik, M. Tojo and M. B. Brurberg
137 Biology of *Phytophthora* species in aquatic ecosystems
C. Hong , P. Kong , P. A. Richardson , X. Yang, H.B. Zhang, S. R. Ghimire , W.E. Copes, G. W. Moorman and J. D. Lea-Cox
139 Phosphite for control of kauri dieback: forest efficacy trials
I. J. Horner and E. G. Hough
140 *Phytophthora* species in forest streams in Nyingchi, Tibet Autonomous Region and Ganzi, Sichuan Province, China
W. Huai, E. M. Hansen, W. Zhao, G. Tian, Y.Yao and X. Cheng
150 Wooden vectors of *Phytophthora ramorum*: Are Douglas-fir logs a risk?
J. M. Hulbert, J. J. Morrell and E. M. Hansen
152 Introduction and spread of *Phytophthora ramorum* in Northern Ireland, UK
A. McCracken , J. Finlay, M. Wilson, L. Quinn, B. Searle, R. Barron and S. Morwood
163 Community structures of root-rotting *Phytophthora* species affecting *Abies* in U.S. christmas tree farms & screening true fir for resistance to *Phytophthora* root rot
K. M. McKeever and G. Chastagner
164 Citizen science helps predict risk of emerging infectious disease
R. K. Meentemeyer, J. B. Vogler, and M. Garbelotto

165 Detection of *Phytophthora* species on different woody species in nurseries
D. Migliorini, E. Tondini, N. Luchi, L. Ghelardini, P. Capretti and A. Santini
166 Evaluation of Illumina MiSeq as a new tool for the detection of *Phytophthora* species
C. Morales-Rodríguez, W. Oßwald and F. Fleischmann
167 In vitro control of *Phytophthora cinnamomi* with *Brassica* pellet (*BioFence*)
C. Morales-Rodríguez , A. Vannini and A.M. Vettraino
169 Maps of *Austrocedrus chilensis* forests affected by dieback
C. I. Núñez, A. Pérez and C. Raponi
170 Status of the genus *Phytophthora* in Argentina
H. E. Palmucci and S. M. Wolcan
171 Genetic, morphological and physiological characters of the plant pathogen *Phytophthora cactorum* and its hybrids
M. Pánek and M. Tomšovský
172 Metagenomic analysis of *Phytophthora* diversity in nurseries of potted ornamental species
M. L. Prigigallo, A. Abdelfattah, S. O. Cacciola, D. E. L. Cooke and L. Schena
173 Molecular and morphological data shows two consistent lineages in *Phytophthora plurivora* strains isolated from streams in northern Spain
A. Puértolas, S. Català, A. Pérez-Sierra and P. Abad-Campos
174 Distribution and impact of *Phytophthora* species on alder (*Alnus spp*.) in Southern Sweden
M. A. Redondo, J. Boberg, C. Olsson and J. Oliva
175 Chemicals for management of red needle cast in *Pinus radiata* plantations in New Zealand: efficacy and persistence of phosphite and other fungicides
C. Rolando, N. Williams and M. Bader
177 Age-related susceptibility of *Eucalyptus* species to *Phytophthora boodjera* nom. prov.
A. Simamora, M. Stukely, G. Hardy and T.Burgess
178 Epidemiology of *Phytophthora boodjera* nom. prov., a damping-off pathogen in tree production nurseries in Western Australia
A. Simamora , T. Paap , M. Stukely , T. Burgess and G. Hardy
179 Phytophthora disease on alder (Alnus sp.) in Norway
G. M. Strømeng , M. B. Brurberg , M. L. Herrero , W. Couanon , A. Stensvand , I. Børja and V. Talgø
180 Phytophthora pseudosyringae found on bilberries in Norway
V. Talgø, M. L .Herrero, M. B. Brurberg, L. Kitchingman, K. H. Telfer and G. M. Strømeng
181 *Phytophthora* survey in a beech forest in Norway
K. H. Telfer, V. Talgø, M. Herrero, M. B. Brurberg and A. Stensvand
182 Polyacrylamide and movement of *Phytophthora ramorum* in irrigation water
S. Tjosvold, D. Chambers, S. Koike and M. Cahn
183 Identification *of Phytophthora alni* subspecies in riparian stands in the Czech Republic
M. Tomšovský, P. Štěpánková, V. Strnadová, P. Hanáček and K. Černý
184 Histopathology of *Phytophthora austrocedri* in *Austrocedrus chilensis*
O. Troncoso, A. G. Greslebin and M. L. Vélez

185 Genetic variation in *Phytophthora lateralis* lineages by analysis of microsatellite profiles
A. Vannini , C. M. Brasier , E. M. Hansen , S. Green , C. Robin , J. F. Webber , A. Tomassini , N.Bruni and A. M. Vettraino
186 Genotypic variability of *Phytophthora cinnamomi* mating type A1 in native forests of Taiwan
A. Vannini , C. M. Brasier , A. Tomassini , V. Forlenza , N. Bruni and A. M. Vettraino
187 Assessing the risk of chestnut ink disease spreading using TOPMODEL
A. M. Vettraino , T. Mazzetto , N. Bruni , A. Tomassini , A. Petroselli and A. Vannini

188 List of Participants

192 Authors Index

# Taxonomy

# Six new *Phytophthora* species from ITS Clade 7a including two sexually functional heterothallic hybrid species detected in natural ecosystems in Taiwan

Thomas Jung[1,2], Marília Horta Jung[2], Bruno Scanu[3], Ana Pérez-Sierra[4], Tun-Tschu Chang[5], Paloma Abad-Campos[6], Maela Léon[6], Gábor M. Kovács[7,8], Claude Husson[9], József Bakonyi[7]

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During a survey of *Phytophthora* diversity in natural ecosystems in Taiwan a swarm of six new species was detected. Multigene phylogeny demonstrated that they belong to ITS Clade 7a with *P. alni*, *P. cambivora*, *P. europaea*, *P. fragariae* and *P. rubi* being their closest relatives. Despite sharing general morphological characters all six new species differed from related species and from each other by a unique combination of characters including the breeding system, oogonial abortion rates, the proportion of ornamented oogonia, ratios of paragynous and amphigynous antheridia, size of sporangia and oogonia, cardinal temperatures and growth rates. Four homothallic species, *P. attenuata* prov. nom., *P. formosa* prov. nom., *P. intricata* prov. nom. and *P. flexuosa* prov. nom., were isolated from rhizosphere soil of *Fagus hayatae*, *Quercus glandulifera*, *Q. tarokoensis*, *Castanopsis carlesii*, *Chamaecyparis formosensis* and *Araucaria cunninghamii* in different forest stands using leaves of *Quercus variabilis* and *Castanopsis indica* as baits. The other two species, *P. heterohybrida* prov. nom. and *P. incrassata* prov. nom., which were exclusively detected in three rivers using leaves of *Citrus sinensis* and *Q. variabilis* as *in-situ* baits, were heterothallic. All *P. incrassata* isolates belonged to the A2 mating type while isolates of *P. heterohybrida* represented both mating types. Abortion rates of oogonia from extensive mating tests were consistently according to Mendelian ratios (4-33%) which was unexpected as the highly polymorphic sequences of the nuclear ß-tubulin, HSP and ITS genes demonstrate that the two heterothallic species are hybrids. Phylogenetic analyses of ß-tubulin, HSP, and ITS clones and of cox1 and NADH gene sequences showed that (1) both hybrids arose from sexual recombination, (2) the six putative parental species are unknown and (3 the hybrids have not been involved in the hybridizations that created *P.* x *multiformis* and *P.* x *alni*. Pathogenicity trials on *Quercus suber* seedlings indicate that all six new species might be a potential threat to European forests.

# The Taxonomy of *Phytophthora*:

# What is done and what is needed for the correct identification and diagnostics of species in the Genus

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The genus *Phytophthora* contains 141 spp., many of which cause significant economic impact to crops, ornamentals, and forests. Many species are globally recognized of regulatory concern including 29 spp. at the USA (Schwartzburg et al, 2009). As of 1999, 59 spp. were described based on morphology, and at present 82 additional spp. have been described based on morphological/molecular characters of nuclear and mitochondrial genes. A great number of species are expected to be described in the near future thanks to the availability of powerful molecular tools and extensive international surveys associated to *Phytophthora* spp. of concern (i.e. *P. ramorum* and *P. kernoviae*). Although **c**onsiderable progress has been made in identification, phylogenies, and diagnostics, the work is still challenging due to omissions of the types in taxonomic publications and culture collections (i.e. Herb. IMI) and numerous misidentifications submitted to NCBI. The problems are exacerbated especially when dealing with species "complexes." The importance of the rules for nomenclature stated at the "International Code of Nomenclature for algae, fungi and plants" is many times overlooked and the *Nomen invalidum* (i.e. *P. asparagi*, *P. hydropathica*, and *P. katsurae*) are not known in many instances. Many of the types of the species described in the early 1900"s have been lost and there is a need to create neotypes or epitypes. CPHST-BL is pioneering the "Revision of the Taxonomy of *Phytophthora*" and the implementation of the "Online Identification Tools of *Phytophthora*: Lucid Key, Tabular Key and Sequencing Analysis" based on the type isolates with the intention to provide correct information for identification of species in the genus. Establishing proper nomenclature and making correct identification of species in the genus will enhance diagnostic systems significantly and will facilitate the understanding and management of the pathogens of concern.

# A new multi-locus phylogeny for the genus *Phytophthora*

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The genus *Phytophthora* includes many destructive pathogens that attack a huge number of agriculturally and ecologically important plants. Investigation into its phylogeny is critical to understanding of the evolutionary history and formulating sustainable disease management strategies. The current 10-clade framework was established in 2007 based on seven nuclear genetic markers, covering approximately 80 species [1]. Thereafter, a number of new species have been isolated and named with many from forests and natural ecosystems. The total number of species in this genus now is about 130. Among the new species is *P. stricta* that does not belong to any known ITS clade [2].

In this study, seven phylogenetically-informative genes were sequenced for *P. stricta* and a vast majority of other recently-described species. These sequencing included many ex-type and authentic cultures from domestic and international Phytophthora research and extension communities. An array of phylogenetic analysis methods is being employed to develop a new phylogeny for this genus. This study helps understand the evolutionary relationships among and within groups while providing the signature sequences of known species to assist their identification in this rapidly-expanding genus.

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# What is a species? The challenge of *Phytophthora*

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What is a species? This is really two related questions. First, there is the fundamental evolutionary question: How do we interpret the biological species concept for organisms like *Phytophthora*, that live “alternative lifestyles?” And second, the very practical question: How do we recognize species of *Phytophthora* in the everyday world of diagnostic laboratories and quarantine regulations?

The biological species concept is rooted in two key concepts of Darwinian evolution: Individuals don’t evolve, populations do, and reproductive isolation of populations, based on geographic or genetic barriers to gene flow. Paraphrasing Ernst Mayr: ‘Biological species are interbreeding populations reproductively isolated from other such populations.’

This definition makes sense in a zoological world of big animals, with orderly, genetically enforced mating habits. But it is problematic in some other groups, notably for us, in *Phytophthora*. Our species are anything but bisexual. Even in heterothallic species selfing is often induced by other species, and many species are homothallic, or even sterile. Certainly not neatly “interbreeding” as Mayr would have it. To make matters worse, even the sexual species reproduce most frequently and abundantly asexually, via sporangia and zoospores.

The key to a biological species concept for *Phytophthora* I think, is to stay clear of the mechanics of sex, and focus on the evolutionary principles- population based, and barriers to gene flow. *Phytophthora* species are: populations that share a common evolutionary lineage and have maintained genetic similarity in morphology, physiology, and ecological behavior. A species of *Phytophthora* is a population of individuals, or a group of populations, with the same evolutionary trajectory. They have a "recent" common ancestor, and they share the same adaptations to their environment.

But- Evolution is an ongoing process. It should be no surprise that at any point in time some species have fuzzy edges. But the human demands for regulatory certainty and orderly measures of biodiversity ask for clean identifications. So how do we distinguish and identify *Phytophthora* species?

Classically, we relied on morphology- the distinguishing features of oospores, chlamydospores, and sporangia we can see in culture. Today, with 3 or 4 times the number of described species as in Waterhouse’s day, it is conventional wisdom that morphology is hopeless. New technology and DNA sequence-based identification rule the diagnostic labs.

Lost in GenBank, however, is the reality that with very few exceptions, sequence and morphology are congruent. With experience, and a relevant culture collection, and by taking advantage of the context from which isolates come- host, geography, behavior- nearly all named species can be distinguished without DNA sequence. Perhaps we can say all good species can be distinguished without molecular characters. But it does take time and experience. My lab often has the experience, but we rely on sequencing for many of our routine identifications.

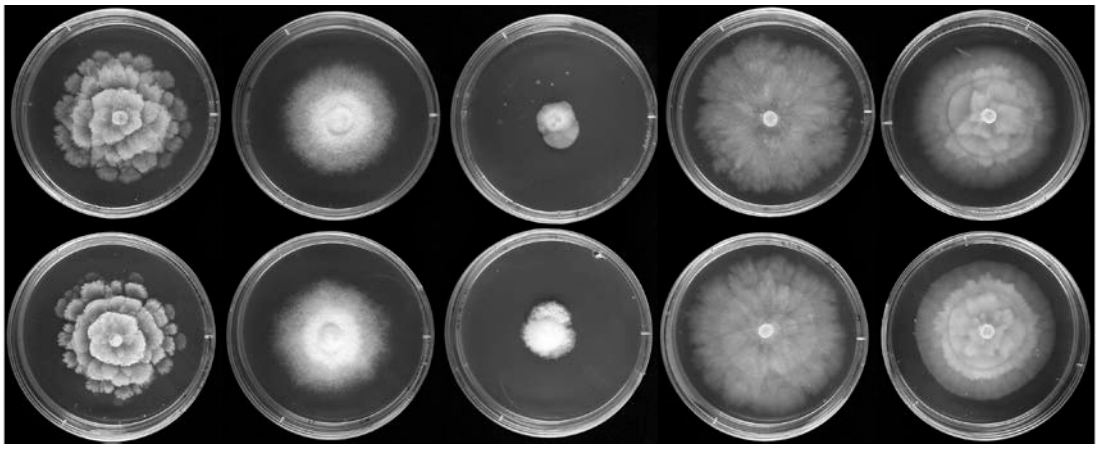
Reliance on DNA sequence differences commonly stirs its own questions: “How different must they be to consider as different species? How many base pairs different? How many DNA regions must be compared?”

My answers: ITS is the gold standard. Additional genes seldom contradict the ITS identification, although they may well be necessary for phylogenetic determination of close relationships. One base difference in ITS sequence may be enough to indicate 2 species, although more often such small differences indicate variation within a population. Nearly all recognized *Phytophthora* species have 5 or more ITS base differences from closely related species, however. Warning! With 7 bases different in a 700 base sequence, the isolates are 99% similar!

Table 1. ITS sequence similarity within and among species of Clade 3.

| | P.pluvialis | P.pseudosyringae | P.psychrophila | P.ilicis | P.nemorosa | P.quercina |
|---|---|---|---|---|---|---|
| **P.pluvialis** | **100** | | | | | |
| **P.pseudosyringae** | **99** | **99.8** | | | | |
| **P.psychrophila** | **99.1** | **98.8** | **99.5** | | | |
| **P.ilicis** | **99** | **99.3** | **98.7** | **99.6** | | |
| **P.nemorosa** | **98.7** | **99.5** | **99** | **99.3** | **100** | |
| **P.quercina** | **92.4** | **92.1** | **92.8** | **92.1** | **92.3** | **98.8** |

Figure 1. Appearance of closely related Clade 3 species grown 24 days on carrot agar medium. From left to right, two isolates each of *P. ilicis, P. psychrophila, P. pseudosyringae, P. nemorosa and P. pluvailis*.



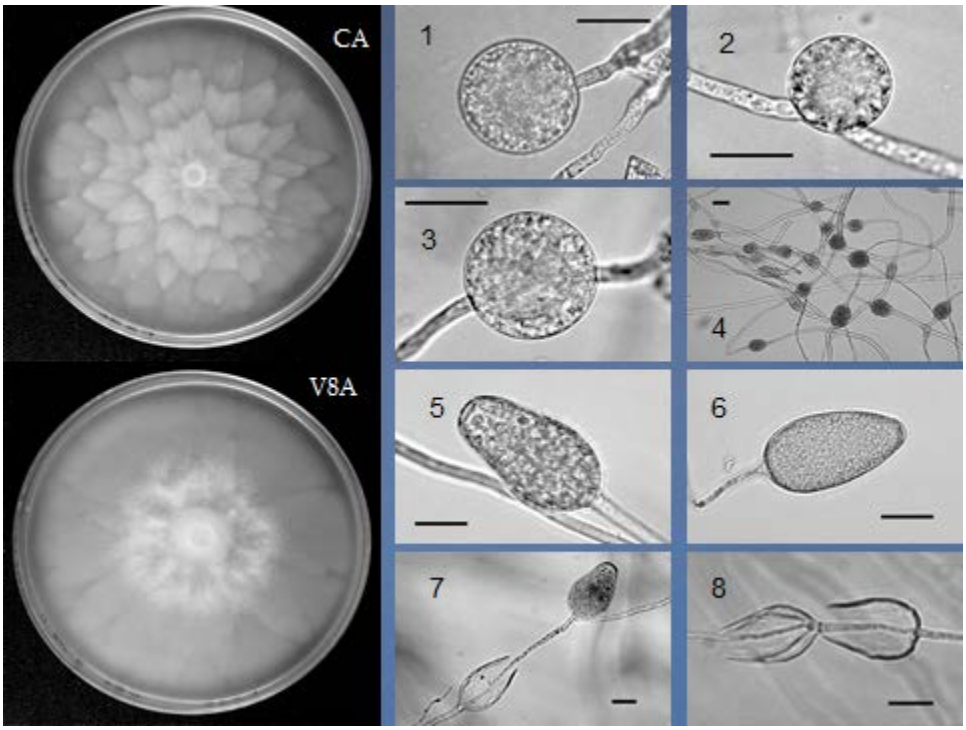
Consider ITS clade 3. As recognized in our lab, these are 5 very closely related, but ecologically distinct, species. Three of them include intraspecific ITS variants. The lowest interspecies similarities are 98.7% (Table 1). Yet grown side-by-side, they are readily distinguished (Figure 1).

Our biological species definition for *Phytophthora* combines morphology as it reflects behavior, and phylogeny (sequence). What do we do when they aren't congruent? Consider *P. bilorbang* (Abhighi et al. 2012) and *P*. taxon Oaksoil (Brasier et al. 2003). The former was described properly, as a homothallic species from aquatic habitats in Western Australia. P. taxon Oaksoil is described informally as a sterile aquatic species from Europe and North America (Sims et al. 2015). Presence or absence of oospores in single culture would seem to be a very fundamental morphological difference between two populations, certainly a species level character in the Waterhouse sense, and we would suppose, indicative of important differences in population genetics and even epidemiology. Yet these two taxa are nearly indistinguishable in their nuclear genes. ITS sequences differ by polymorphisms (double peaks) at 3 loci. β-tubulin is identical. The mitochondrial COX 1 sequence is more variable, especially within *P*. taxon oaksoil.

What to do? Is *P*. taxon oaksoil an undescribed species, needing a proper name? Or is it best called *P. bilorbang*? Is a compromise, *P. bilorbang* ssp *oaksoil*, a biologically reasonable solution?
The oaksoil case raises another conumbrum- how do we deal with "double peaks"? Do they indicate a hybrid history? If so, how do we recognize hybridization within our nomenclature? The challenge of *P. chlamydospora* (*P*. taxon pgchlamydo) highlights the issue (Figure 2). "Double peaks" are especially common in clade 6 species, including many isolates of pgchlamydo. We deliberately avoided the issue when submitting the formal species description (Hansen et al. 2015); the type we chose has the most common COX sequence, and no double peaks at the sequenced nuclear loci. It is typical of many isolates from around the world, but there are also many isolates with different combinations of COX sequence and with numerous double peaks. All share the morphology and behavior of the type. Colleagues in Australia and South Africa (Nagel et al. 2013) cloned some of the putatively hybrid isolates and identified candidate parents. They named hybrid species. My question: "are these stable new populations, starting a new evolutionary trajectory, or are they points in a fluid genetic mix that collectively comprises *P. chlamydospora?"*

We make (and change) the taxonomic rules. Ultimately, a species description is a hypothesis, to be tested, accepted, or rejected by our colleagues. There are rules for *Phytophthora* nomenclature, but they deal with priority and documentation and grammar, not "what is a species?" That question requires research into the population genetics and evolutionary processes of the genus. There is no short answer.

Figure 2. *Phytophthora chlamydospora*. Left: colony morphology. Frames 1 – 3: chlamydospores in agar. Frame 4: hyphal swellings in water. Frames 5,6: non-papillate sporangia. Frame 7,8: internal proliferation of sporangiophore. Bar 20µ.




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10th · 14th November 2014
Esquel, Chubut. Patagonia Argentina

# Role of *Phytophthora* species in emergent diseases

# Pathogenicity of *Phytophthora* species isolated from declining European blackberry (*Rubus anglocandicans*) in the natural ecosystems of South Western Australia

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## Abstract

European blackberry is a species complex within the *Rubus fruticosus* L. aggregate [1] and is one of the 20 Weeds of National Significance in Australia. Blackberry is the most widespread and abundant *Rubus* species in Western Australia (WA). A disease recorded as 'blackberry decline' has been observed in some blackberry sites in WA since 2006. In order to isolate and identify root-associated pathogen(s), a disease survey was conducted in the Manjimup-Pemberton region along the Warren and Donnelly river catchments in WA between 2010 and 2012 [2]. *Phytophthora amnicola*, *P. bilorbang* [3], *P. cryptogea, P. inundata, P. litoralis, P. multivora, P.* taxon personii*, P. thermophila, P. thermophila-amnicola* hybrid were recovered from decline, adjacent decline-free sites, streams and rivers. *P. cinnamomi* was only isolated from two non-decline sites. Of these ten species, *P. bilorbang* and *P. cryptogea* appeared to be more pathogenic than others in underbark inoculations using excised stems (primocanes) and *in planta* primocane inoculations in blackberry growing wild in native forest stands. In glasshouse trials, *P. bilorbang* and *P. cryptogea* were both confirmed to be pathogens of blackberry, and when co-inoculated disease impact was more severe, indicating a synergistic response. It was concluded that blackberry decline is a complex syndrome and *Phytophthora* species and in particular *P. bilorbang* and *P. cryptogea* together with temporary inundation are major biotic and abiotic factors, respectively contributing to blackberry decline [2].

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# The Comparative Pathogenicity of Two *Phytophthora ramorum* Lineages, EU1 and EU2, on a Range of Hosts

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*Phytophthora ramorum*, the causative agent of Sudden Oak Death (SOD), is a lethal pathogen of oak and Bay laurel in North America. However, in Europe its primary hosts include rhododendron, larch and vaccinium (bilberry). The pathogen is known to have four distinct evolutionary lineages; NA1, NA2, EU1 and EU2. The North American *P. ramorum* population is predominantly comprised of NA1 and NA2 lineages, whilst EU1 and EU2 lineages occur within Europe. EU2 is the most recent of the four lineages to be identified and is found exclusively within a relatively small area, in south-western Scotland and in the east of Northern Ireland.

To understand the implications of the occurrence of this newly identified *P. ramorum* lineage, EU2, we compared the relative pathogenicity of EU1 and EU2 on a range of hosts. Detached rhododendron and laurel leaves were inoculated with six EU1 and six EU2 isolates and after both 7 days and 14 days, EU1 produced larger lesions compared with EU2. However, wound-inoculations of the bark of 11 tree species, including Japanese, European and Hybrid Larch, sessile oak, beech and noble fir, with the same 12 isolates revealed that EU2 isolates produced larger lesions on all three larch species, compared with EU1. This could indicate that EU2 is specifically adapted to the colonisation of larch, which has implications for the management of *P. ramorum* in areas where EU2 is present.

# Involvement of *Phytophthora* species in beech decline in Lower Austria

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In Lower Austria, studies were conducted in 43 forest stands and 6 urban sites to analyse the relations between root pathogens, crown condition and stem cankers of European beech (*Fagus sylvatica*). 24 forest stands and 3 urban sites were found infested by these pathogens. Among the six species found *P. cambivora* and *P. plurivora* were most common. From one beech stand a yet undescribed *Phytophthora* taxon was isolated which is closely related to *P. quercina*. The quarantine species *P. ramorum* and *P. kernoviae* were not recorded. The symptoms caused by *Phytophthora* on beech comprise extensive fine root losses, death of large parts of the root system, lesions on above-ground parts of the roots and the stem, and as a consequence thinning and dieback of the crowns. Disease symptoms were found in all stand types and all regions of Lower Austria with presence of beech. Although fine root damage and mycorrhization are correlated to the crown thinning the whole phenomenon is not exclusively related to *Phytophthora*-infestation of the soil. The *Phytophthora*-lesions on superficial roots and stems are colonized by several secondary fungal species, which commonly cause rot and consequently a destabilisation of the trees. Among those, honey fungus (*Armillaria* sp.) and Carbon cushion (*Hypoxylon deustum*) were most frequent. Damaged trees were concentrated on sites subject to water logging and also in the vicinity of forest roads which, especially if having been paved with soil material from urban areas, most likely acted as pathway for the introduction of *Phytophthora*. Since the precipitation showed an increase in intensity and duration during the preceding years, an increase in inoculum of *Phytophthora* in the soil can be assumed reaching thresholds able to cause decline of stands; in particular if the fine root losses interact with succeeding periods of drought stress.

# *Phytophthora pluvialis* on Douglas-fir in Oregon, USA.

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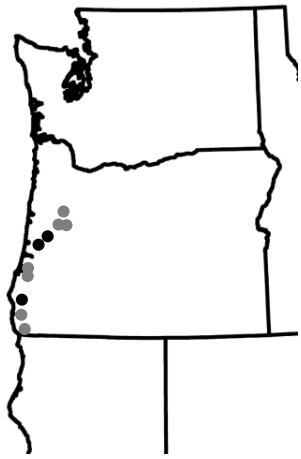
**INTRODUCTION:** *Phytophthora pluvialis* was originally isolated at very low frequency from baited soil, streams, raintraps, and tanoak stem lesions in Oregon as early as 2002. It was later associated with needle casts of radiata pine (*Pinus radiata*) and Douglas-fir (*Pseudotsuga menziesii*) in New Zealand (1). Subsequently, we began studies to explore the relationship between *P. pluvialis* and Douglas-fir in Oregon.

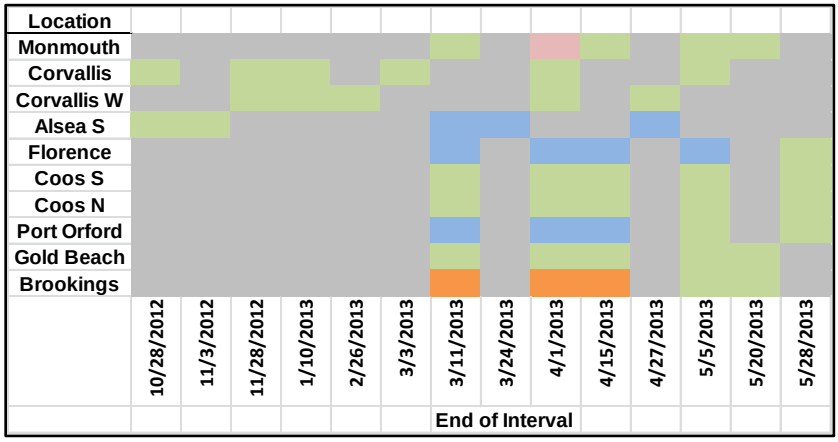
**MATERIALS AND METHODS:** Detached Douglas-fir twigs were inoculated by immersing in a suspension of *P. pluvialis* zoospores (ca. $2 \times 10^4$/ml) for 24 h at 20 C, and incubating in a moist chamber for 2 wk at 18 C, 12 h photoperiod. Sporangia were collected by washing in de-ionized water on a rotary shaker, deposited on a 12 µm polycarbonate membrane, and counted at 400 X.

Rhododendron leaf baits were exposed in raintraps for 2 wk intervals beneath ca. 30 y old Douglas-fir plantations at 10 locations in Oregon during winter and spring in 2013 (Fig.1). *Phytophthora* isolates were recovered by plating bait pieces on semi-selective agar, and identified by sequencing the mitochondrial *cox* spacer region. In 2014 Douglas-fir seedlings were exposed to natural inoculum in stands where raintraps yielded *P. pluvialis* in 2013 (Fig 1). The seedlings were returned to the laboratory when co-located raintraps became positive for *P. pluvialis*. Ten symptomatic and ten non-symptomatic needles, and pieces from stem lesions when they developed, were collected from each seedling, surface disinfested, and plated in CARP. After needle casting had occurred, seedlings were rated (10% increments) for retention of 1 and 2 yr old needles.

Pathogenicity was confirmed by inoculating Douglas-fir seedlings with sporangia (200-300/ml) or zoospores ($5 \times 10^4$/ml), both applied at 20 ml/seedling, from an isolate of *P. pluvialis* collected from infected seedlings, and re-isolating the same from symptomatic needles and stem lesions. Inoculum was applied with an air brush sprayer to all the foliage on seedlings in a growth chamber and incubated at ca. 18 C, 12 h photoperiod.

**RESULTS:** *P. pluvialis* was found to sporulate on detached Douglas-fir needles two weeks after inoculation. We observed around 330 sporangia per twig for the 15 twigs tested. We detected *P. pluvialis* in baited raintraps at two coastal sites and in one central Coast Range site during March and April of 2013 (Fig. 2). We obtained a similar result at these same sites in 2014.






Left: Figure 1. Location of raintraps in 2013. Black dots show raintraps yielding *P. pluvialis* in 2013, and location of study sites in 2014. Right: Figure 2. Influence of season and location on recovery of *P. pluvialis* in raintraps set beneath Douglas-fir stands in 2013. Green, no *Phytophthora* recovered. Blue, *P. pluvialis* recovered. Pink, *P. syringae* recovered, Orange, *P. ramorum* recovered. Gray, no raintrap baits during this interval.

Naturally infected Douglas-fir trap plants showed needles with yellow-green mottling. Fifty-three percent of exposed trap plants were infected. *P. pluvialis* was re-isolated from both symptomatic and asymptomatic surface-disinfested needles, making it difficult to establish an association of infection with specific needle symptoms. Of 820 symptomatic needles, 120 (14.6 %) were positive for *P. pluvialis*. Of 803 non-symptomatic needles, 30 (3.7 %) were positive for *P. pluvialis*.

Some naturally infected Douglas-fir trap plants also developed stem cankers and wilted emerging shoots (Fig. 3). Of 98 stem cankers plated, 36 (37 %) were positive for *P. pluvialis*. Of 67 wilted shoots plated, 5 (7.5 %) were positive for *P. pluvialis*.

Artificially infected Douglas-fir seedlings produced symptoms which reflected those observed in naturally infected trap plants (Figure 3). *P. pluvialis* was recovered from infected needles and stem lesions.



Figure 3. Symptomatic needles (left) and twig (middle) from artificially inoculated Douglas-fir seedlings. Red circles indicate regions of *P. pluvialis* recovery. Twig cankers (right) on Douglas-fir seedlings exposed to natural inoculum. Yellow circles indicate regions of *P. pluvialis* recovery

Increasing needle infection rate was correlated with decreasing needle retention in both 1 yr (Fig. 4) and 2 yr age classes.

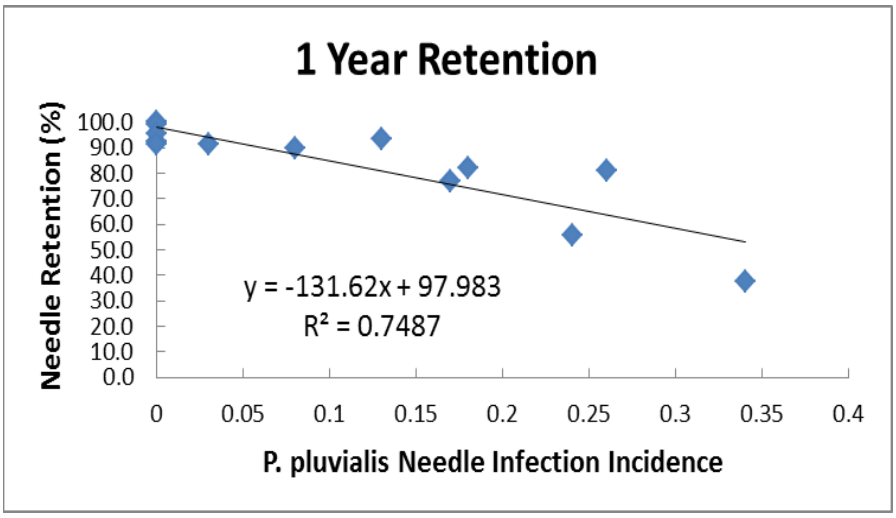



Figure 4. Relationship between infection incidence in needles and retention of 1 y old needles in Douglas-fir trap plants exposed to natural inoculum of *P. pluvialis*. The result for 2 y old needles was similar, and is not shown.

**DISCUSSION:** We consider *Phytophthora pluvialis* to be indigenous to Oregon. However it causes significant needle casts of radiata pine (*Pinus radiata*) and Douglas-fir (*Pseudotsuga menziesii*) in New Zealand (1). Although we can observe symptoms on, and re-isolated the pathogen from, naturally infected Douglas-fir seedlings in Oregon, we do not know if this pathogen is causing significant disease in Oregon forests.

Future collaborative studies will explore the epidemiology and physiological impact of *P. pluvialis* on Douglas-fir in New Zealand and Oregon forests. Studies will investigate the susceptibility of Douglas-fir stands and established breeding lines to infection and needle cast caused by *P. pluvialis* in New Zealand. Other studies will test models of disease establishment regionally and temporally across New Zealand and Oregon forest plantations.

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# Identification and Pathogenicity of Oomycetes Causing Root Disease on Wild Olives

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From the beginning of the 90's, wilting and death of cultivated olive trees (*Olea europaea*) caused by *Phytophthora* spp., are common in southern Spain. Recently, a similar root rot was detected in a protected wild-olive woodland of high ecological value (Dehesa de Abajo, Seville, Spain), located near Doñana National Park. This wild olive forest spreads around a natural pond and seasonal soil flooding occurs depending on rainfall. The root disease was first detected in 2009, and *P. megasperma* and *P. inundata* were identified associated with necrotic roots in 2011(**1**).

Symptomatic (necrotic) feeder roots and rhizosphere soil from symptomatic trees were sampled from a total of 25 wild-olives distributed in three different foci: one big focus uphill (17 symptomatic trees), and two small foci (four symptomatic trees each one) downhill, nearby the pond. The first sampling trial was performed in spring 2013 and a second one was conducted in autumn 2013. For each sample, feeder root segments were washed and directly plated on NARPH (Nistatine, Ampiciline, Rifamicin, PCNB, Hymexazol-CMA medium). Olive leaves were floated on soil-water suspensions and plated on NARPH medium for isolation from soil samples.

Isolates obtained were purified and morphologically identified as *Pythium spiculum*, *Phytophthora cryptogea* A1 and *P. megasperma* based on oogonia, antheridia and oospores characteristics observed under the microscope. To obtain *P. cryptogea* gametangia dual cultures were prepared with both mating type testers *of P. cryptogea*, *P. dreschleri* and *P. cinnamomi* A2. Morphological identification was confirmed by amplification and sequencing of their ITS regions and later comparison with sequences published on GenBank.

Isolation frequencies obtained in spring or autumn are in Table 1. *Pythium spiculum*, known as weak pathogen of *Quercus ilex* and *Q. suber* (**2**,**3**), was consistently isolated from necrotic roots, with increased frequency from spring (48%) to autumn (76%), as expected for a soilborne oomycete well adapted to infect roots in dry summer soils by direct germination of sporangia (**4**). The opposite situation occurred to *P. cryptogea*, species depending of zoospore production in wet soils for root infections. *Phytophthora megasperma*, known pathogen for cultivated olives (**5**) and the main species associated with wild olive root disease in 2011 (**1**) were only isolated in autumn 2013 at low frequency and *P. inundata* was not isolated.

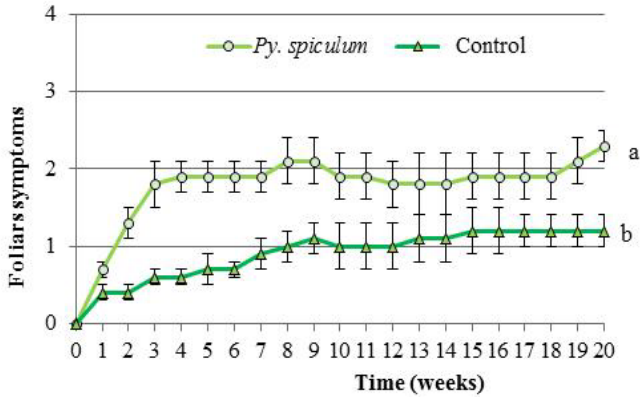
**Table 1** Number of wild olives with positive isolation of oomycetes and total isolation frequencies (percentage of positive trees)

| Focus | Spring 2013 | | | | Autumn 2013 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ***Py. spiculum*** | | ***P. cryptogea*** | | ***Py. spiculum*** | | ***P. cryptogea*** | | ***P. megasperma*** | |
| | Roots | Soil | Roots | Soil | Roots | Soil | Roots | Soil | Roots | Soil |
| 1 (17 trees) | 8 | 13 | 11 | 10 | 11 | 14 | 5 | 5 | 1 | 0 |
| 2 (4 trees) | 2 | 4 | 4 | 4 | 4 | 4 | 3 | 2 | 0 | 1 |
| 3 (4 trees) | 2 | 4 | 4 | 4 | 4 | 4 | 3 | 2 | 1 | 0 |
| Total (25 trees) | 48% | 84% | 76% | 72% | 76% | 88% | 44% | 36% | 8% | 4% |

For pathogenicity tests, isolates from each species were grown or in dual culture (*P. cryptogea* A1 × A2 tester) for 21 days in Petri dishes containing carrot broth. After this time, the mycelium obtained was washed, added to sterile water, shaken and adjusted to $2.2\times10^4$ oospores $ml^{-1}$. One year-old wild olives were inoculated by adding to the root ball 50 ml of oospore suspensions. Ten plants (replicates) per oomycete species were inoculated and transferred to plastic pots with 2 L of fertilized peat plus 10 non-inoculated (control) plants. All the plants were incubated in an acclimatized greenhouse and submitted to soil flooding 2 days per week (**2**). Foliar symptoms were weekly evaluated on a 0-4 scale (0 = 0-10% symptomatic leaves, 4 = total wilt). Root symptoms were assessed as percentage of root necrosis at the end of the experiments (14 weeks for Phytophthoras, 20 weeks for *Py. spiculum*). Results are in Figure 1 and 2.

**Figure 1**. Foliar symptoms recorded for wild olives inoculated with *Py. spiculum* (a) *P. cryptogea* or *P. megasperma* (b). Vertical lines are the SE of 10 replicates. At the end of experiments, different letters indicate significant differences (ANOVA) according with the Tukey test for P<0.05

a)




b)

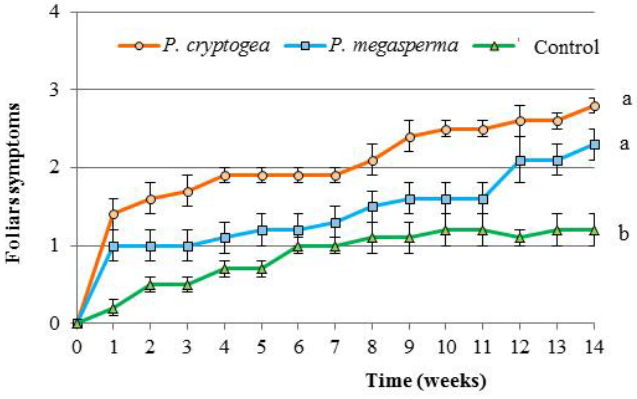
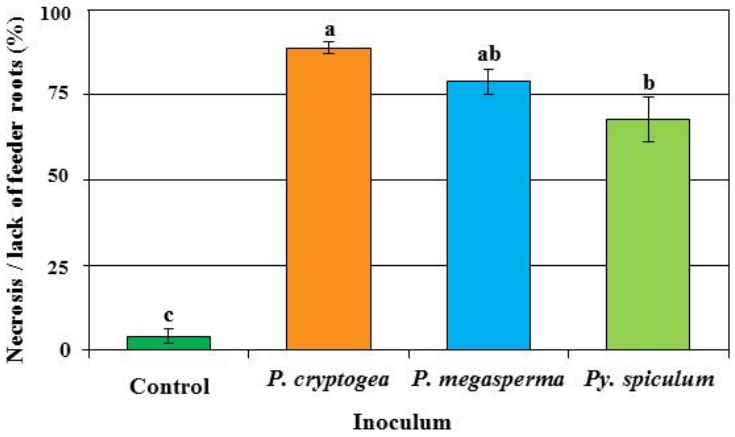


**Figure 2**. Root symptoms recorded for wild olives inoculated with *P. cryptogea*, *P. megasperma* or *Py. spiculum* at the end of experiments. Vertical lines are the SE of 10 replicates. Different letters indicate significant differences (ANOVA) according with the Tukey test for P<0.05




All the three oomycetes were consistently reisolated from symptomatic roots and their pathogenicity on wild-olives demonstrated. All of them caused root necrosis in comparison with uninoculated control plants. However, *Phytophthora* species were more virulent for wild olives than *Py. spiculum*, resulting in higher levels of foliar and root symptoms, with no significant differences between *P. cryptogea and P. megasperma*. *Phytophthora megasperma-P. inundata* were first associated with the disease in 2011 but now their frequency is low or even non-existent. For this reason we hypothesize that *P. cryptogea-Py. spiculum* could be better adapted to the decreasing rainfall rates in the region and therefore replacing the previously detected *Phytopththora* species in olive trees in southern Spain.

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# Unexpected discovery of *Phytophthora siskiyouensis* in the UK

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Dieback of alder caused by *Phytophthora alni* was first recorded in the UK 20 years ago. It is now widespread over many river systems and is routinely isolated from symptomatic native *Alnus glutinosa*. During a recent visit to a mixed alder and ash site in the county of Dorset in south west England typical symptoms of 'alder Phytophthora' were apparent on a number of the alders, including bleeding basal lesions and some crown dieback. The area was planted in the late 1990s with a mixture of ash, introduced European *Alnus incana* and oak. Alder symptoms were first noticed 2 years ago and assumed to be due to *P. alni*. However, following isolations from basal lesions in November 2013 the morphological features and ITS sequences of the isolates identified them as *Phytophthora siskiyouensis*. Subsequently *P. siskiyouensis* was obtained from alder bark, root and soil samples collected from the site. Previously *P. siskiyouensis* has been recorded from urban *A. glutinosa* in Melbourne eastern Australia, native streams and forest trees (*Umbellularia californica* and *Notholithocarpus densiflorus*) in south west Oregon and urban *A. cordata* and *A. rhombifolia* in southern California. This is apparently the first time the pathogen has been found affecting *A. incana* and the first record from Europe. It seems likely that *P. siskiyouensis* was introduced onto the site with the planting stock. Many trees are affected. Although the affected trees had been planted 15-20 years ago alder planting continued on the site until 2000. Some of the younger trees appear to have been killed by the infection, others survive with active symptoms. Kochs Postulates were confirmed and work is underway to compare the risk posed by *P. siskiyouensis* to some other European and North American alder species.

# Mycelial growth and pathogenicity of *Phytophthora cinnamomi* Rands

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Production of Avocado (*Persea americana*), among other fruits, is considered an alternative to the establishment of agroforestry systems, such as ecosystems conservation strategy. However, incidence of *Phytophthora cinnamomi* (Oomycete) has been a constant limitation in avocado crop production worldwide, due to the limited availability of tolerant rootstocks and the high cost of clonal seedlings. In a preliminary study of seedling tolerance, seeds pollinated to colonization by *P. cinnamomi*, two isolates were tested LRS 21/88 and LRS 22/93, donated by the *Agencia Paulista de Tecnologia Agropecuaria* - APTA (Brazil), and two methods of inoculation in seedlings of the susceptible selection "Ouro verde". Both evaluated the mycelial growth of the three isolates in four culture mediums: lima bean agar (ML), carrot agar (CA), V8 (Cambell) and potato dextrose agar (PDA). The three isolates were tested in both light conditions (10 $\mu m.m^2. s^{-1}$) and in the dark. Results of pathogenicity among the isolates tested after fifteen days suggest that the LRS 21/88 was more pathogenic, developing lesions that averaged 2,33 $cm^2$ from an initial injury method, and averaging 0,50 $cm^2$ without initial injury method, prior to inoculation. Already in mycelial growth after four days of testing, an interaction was found between the test variables; the effect of the medium depends on lighting and isolation. Independent of the test factors, the ML medium, in general, induced higher development of mycelium. However, the media V8 and CA, resulted in lower observed growth and greater formation of hyphal swelling, typical in *P. cinnamomi*. These are partial results and other studies are being developed in parallel regarding protein expression in avocado seedlings in response to *P. cinnamomi* and photosynthetic behavior of the fluorescence ratio and chlorophyll in plants inoculated.

# The role of *Phytophthora* in the decline of *Corymbia calophylla* (marri), a dominant and widespread tree species in southwest Western Australia

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*Corymbia calophylla* (marri), a keystone tree species in the majority of woodlands and forests in the southwest of Western Australia, is suffering a major decline syndrome associated with the canker fungal pathogen *Quambalaria coyrecup.* Evidence suggests *Q. coyrecup* is endemic, however, mortality attributed to the canker pathogen has increased since the 1970s with disease incidence and severity much greater in anthropogenically disturbed areas, suggesting there are additional biotic and abiotic predisposing factors. The current study investigated the role of *Phytophthora* species in marri decline. An extensive survey was undertaken across the marri range, an area of approximately 70 000 $km^2$. Within this region, 62 sites were assessed for canker disease presence, and soil samples collected for *Phytophthora* detection. *Phytophthora* species were recovered from more than half the sites, with up to three species present at a single location. A total of six *Phytophthora* species, including *P. boodjera* prov. nom., *P. cinnamomi, P. cryptogea, P. elongata, P. multivora* and the previously undescribed *Phytophthora* sp. *calophyllaphile* prov. nom. (a species closely related to *P*. *quercina*), were isolated from the roots and rhizosphere of healthy and diseased marri. The pathogenicity of these species towards marri seedlings was tested in glasshouse experiments, with isolates of *P. cinnamomi* and *P. multivora* significantly reducing root health and mass. The results of these experiments and their implications for marri health will be discussed in detail.

# *Phytophthora austrocedri* emerges as a serious threat to juniper (*Juniperus communis*) in Britain

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**Abstract**
From 2011-2013, *Phytophthora austrocedri* was isolated from diseased *Juniperus communis* exhibiting dieback and mortality at eight geographically separate sites in Scotland and northern England. The pathogen was also confirmed present either by standard PCR and sequencing of the ITS locus or by real-time PCR on symptomatic *J. communis* at a further eleven sites in northern Britain. Out of 167 *J. communis* sampled across the nineteen sites, 154 had foliage dieback over all or part of the crown as a result of basal lesions originating in the root system and extending up the stem, killing phloem and cambial tissues. Thirteen sampled trees had aerial branch lesions or discrete stem lesions with no apparent connection to the base of the tree. At thirteen sites, dieback was concentrated in areas of poor drainage and /or alongside streams and other watercourses. In artificial inoculation experiments, *P. austrocedri* caused rapidly extending stem and root lesions on *J. communis* and was re-isolated from these lesions. Lesions also developed on *Chamaecyparis lawsoniana* and *Chamaecyparis nootkatensis* but the pathogen was not re-isolated. All *P. austrocedri* isolates obtained from *J. communis* in Britain shared 100% identity across the ITS locus but were distinct at one position on the alignment from *P. austrocedri* isolates collected in Argentina from diseased *Austrocedrus chilensis*. This study provides clear evidence that *P. austrocedri* is a primary pathogen of *J. communis* and now presents a significant threat to this species in Britain. Pathways for the emergence of *P. austrocedri* in Britain, and the possible ways in which the pathogen may have spread within the country, will be discussed.

# Evolutionary epidemiology of *Phytophthora austrocedri* on juniper in Great Britain and the Northern Hemisphere

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The oomycete forest pathogen *Phytophthora austrocedri* is causing large-scale dieback of *Austrocedrus chilensis* in Argentina, where it was first described in 2007. It was not known to occur anywhere else until 2011, when it was found in Great Britain causing mortality in the common juniper (*Juniperus communis*). Genetic differences between British and Argentinian isolates have been observed, making an introduction of *P. austrocedri* from Argentina unlikely. So far, little is known about the pathogen and its ecology in the Northern Hemisphere and a three and a half year PhD project has begun in order to shed light on its evolution and epidemiology. *J. communis* populations will be surveyed in Europe and in North America, to determine the wider distribution of the pathogen within the broad boreo-temperate host range. Samples will be collected from soil and trees for isolation and molecular analysis. The ecology of *P. austrocedri* will be studied via surveys of asymptomatic and symptomatic juniper sites with regard to environmental factors, such as climatic conditions and distribution of waterways. *In vitro* experiments will be carried out to determine the growth conditions (temperature and pH) required by *P. austrocedri* for the completion of its life cycle. Finally, the population genetics and evolutionary history of the pathogen will be studied using microsatellite markers and phylogenetic analyses based on multiple loci (ITS, coxI, coxII among others) to elucidate the intriguing distribution of the pathogen.

# *Phytophthora pseudosyringae* associated to the mortality of *Nothofagus* forests of central-southern Chile

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In recent years, mortality has been observed in several species of *Nothofagus* in Chile, especially in the Los Andes mountains. In 2009, partial defoliation and bleeding cankers were detected on *Nothofagus obliqua* trees located in Nahuelbuta coast ranges of southern central Chile.

In the United States and Europe, native trees related to the *Nothofagus* genus (*Quercus* spp. and *Fagus* spp.) have been affected by species of *Phytophthora*. These pathogens cause stem cankers, defoliations, root rots and the death of the tree. Currently there are no official reports of disease or mortality cases related with *Phytophthora* spp. in Chilean *Nothofagus* spp. forests.

The objective was to determine the association between *Phytophthora* species and mortality, cankers, defoliation and wilting symptoms occurring in *N. procera* and *N. obliqua* trees.

Stem cankers and litter samples were collected around symptomatic *N. obliqua* trees and processed in PAR-CMA and by apple baits. The obtained isolates were identified with the use of morphological keys and the ITS 1 and 4 DNA sequences.

*N. obliqua* and *N. procera* plants were inoculated at the stem, using mycelial plugs and the roots, with a soil infestation method that consisted in a mix with vermiculite, carrot juice and inoculum mix of the collected *Phytophthora* isolates.

The isolates were identified as *P. pseudosyringae*. All inoculated *N. obliqua* plants at the stem resulted in the formation of cankers. Results obtained in the root inoculations, for both species, caused a significant reduction in the root dry weight compared with controls. All inoculations assays were confirmed positive with the re-isolation of *P. pseudosyringae*.

This is the first official report in front of a scientific committee of the association of *Nothofagus* spp. and *P. pseudosyringae* in South America.

**Keywords:** *Phytophthora*, *Nothofagus,* forest diseases.

# *Phytophthora kernoviae* detection in *Drimys winteri* (Winter's Bark) forest of southern Chile

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Partial defoliation and necrotic leaves were found in winter's bark (*Drimys winteri*) forest located in southern Chile.

Soil and litter collected from symptomatic trees were baited. The isolates obtained were identified with the use of morphological and molecular techniques. Pathogenicity tests were carried out inoculating detached *D. winteri* leaves and plants using collected isolates, under controlled conditions.

Morphological characteristics and the Ypt1 DNA sequence of the isolates, led to the identification of the species *Phytophthora kernoviae*. The pathogenicity of the isolates was confirmed on *D. winteri* leaves and plants, causing similar foliar symptoms observed on trees.

This is the first detection of *P. kernoviae* in South American native forest.

# Pathogenicity tests of *Phytophthora* species on *Agathis australis*

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*Phytophthora* taxon Agathis (PTA) is a devastating pathogen of iconic kauri (*Agathis australis*) in New Zealand. Soil surveys to detect PTA, targeting sites with kauri trees showing disease symptoms, detected a number of other *Phytophthora* species (Waipara et al. 2013). *P. cinnamomi*, *P. multivora* and *P. cryptogea* were particularly common. *In vitro* and glasshouse studies were carried out to determine the relative pathogenicity of these four species, prior to investigating potential interactions among these species in the field. When excised leaves were inoculated with colonized agar plugs, all four *Phytophthora* species produced lesions. Lesion advance was significantly slower with *P. cinnamomi*, *P. multivora* and *P. cryptogea* (<1 mm/day) than with PTA (>3 mm/day). Similar results were obtained with inoculated excised twigs, with average lesion advance of 0.04, 0.49, 0.17 and 4.0 mm/day for *P. cinnamomi*, *P. multivora*, *P. cryptogea* and PTA, respectively. The growth rate of PTA through live kauri twig tissue was similar to that on V8 agar. Potted 2-year-old kauri seedlings were trunk-inoculated. Small lesions (mostly <10 mm over 4 months) appeared with *Phytophthora cinnamomi*, *P. multivora* or *P. cryptogea*, no trees died, and plant growth was suppressed only slightly. When PTA-inoculated, lesions spread rapidly, trunks were girdled, and all trees died within 4-6 weeks. All kauri seedlings died within 10 weeks when soil was inoculated with PTA. Feeder root damage occurred following soil inoculation with *P. cinnamomi*, *P. multivora* or *P. cryptogea*, and the respective *Phytophthora* species were readily isolated from root lesions, but there were no plant deaths. Results suggest that PTA is a highly aggressive pathogen on kauri while relatively, the other three species are weaker pathogens.

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# Aerial stem cankers associated with *Phytophthora syringae* on *Fraxinus*: how and why?

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*Phytophthora syringae* is known for causing foliar and twig blights of shrubs such as *Syringa* and *Kalmia* and lesions on fruits such as apple and *Pyracantha,* but not as a pathogen of forest trees. Curiously for an aerial pathogen it has non-caducous sporangia. Recently reports of dieback in young plantation European ash, *Fraxinus excelsior,* came from several sites in southern England. At one mixed plantation of <1 hectare in Dorset, over100 trees exhibited sunken bleeding aerial stem lesions, bark cracking and crown dieback. The trees were 15-20 years old with a diameter of 10-15 cm. The stem lesions were mostly 2-3 m above ground level, lenticular and several cm long. Isolations from the lesion margins readily yielded *Phytophthora syringae* as identified by morphological criteria including semi papillate non-caducous sporangia, supported by ITS sequence. At another similar site leaflet necroses were also observed and again yielded *P. syringae.* However in Dorset regular summer rainwater samples were negative for *P. syringae* in PCR testing i.e. no evidence was obtained for that canopy sporulation the source of inoculum for the stem lesions. This appears to be a new host record (Kochs postulates not yet completed) and the first record of *P. syringae* associated with aerial lesions on a forest tree. It is notable that both *Fraxinus* and *Syringa* belong to the Oleaceae. Early descriptions of *P. syringae* on lilac emphasized its role as a foliar pathogen, with sporangia forming on the surfaces of infected leaves. Regarding *P. syringae* on ash, outstanding questions include (i) How does foliar infection occur if sporangia are non caducous? (ii) How does infection of ash stems occur? (iii) Why has *P. syringae* infection of ash not been observed previously in UK or Europe? (iv) Is this another new UK tree disease outbreak resulting from imported plants?

# Multiple Phytophthoras associated with larch (*Larix)* in Britain

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Since the first findings in 2009 of *Phytophthora ramorum* killing plantation grown larch in the UK (mainly Japanese larch – *Larix kaempferi*), thousands of larch samples have come into Forest Research laboratories for diagnosis. If *P. ramorum* is confirmed, woodland owners must fell affected trees as part of the management of *P. ramorum* as a quarantine plant pathogen. Therefore, correct identification is critical. Occasionally larch samples with resinous lesions and bark cankers consistent with those of *P. ramorum* give a strong positive with a *Phytophthora* Lateral Flow Device (LFD) test (Pocket Diagnostics®) but *P. ramorum* cannot be confirmed with the real-time PCR assay developed to detect it in larch samples. This raises the possibility that other *Phytophthora* species regularly infect larch bark but have not been detected previously. This may be due in part, to the difficulty of isolating *Phytophthora* from infected larch tissue. Also, the symptoms caused by other Phytophthoras may be relatively localised and easy to overlook in mature trees. In 2010-11, attempts were made to isolate from~200 samples of Japanese larch bark which had fresh, necrotic phloem lesions and gave *Phytophthora* positive LFD tests. Of those, 28% yielded *P. ramorum* but ~3% produced cultures of two other *Phytophthora* spp – *P. pseudosyringae* and *P. gonapodyides.* Inoculation of all three species into Japanese and European larch bark demonstrated they were able to attack and colonise healthy phloem tissue, thereby satisfying Koch"s Postulates. The lesions caused by *P. pseudosyringae* and *P. gonapodyides* were significantly smaller than those incited by *P. ramorum*. Under natural conditions the lesions caused by *P. pseudosyringae* and *P. gonapodyides* usually occur on branches at least 2-3 m above ground level and are discrete aerial infections. This raises the possibility that they also infect and sporulate on larch needles (as *P. ramorum* does) thereby providing the inoculum for the aerial bark infections.

# Aerial dieback on *Thuja* caused by *Phytophthora lateralis*

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In February 2011, Scottish plant biosecurity inspectors found three potted *Thuja occidentalis* cv. 'Emeraude' infected with *Phytophthora lateralis* in a nursery, originally imported from France [1]. The pathogen was isolated from the foliage; no symptoms were observed on root collar or roots. This was the first record of *P. lateralis* infecting *Thuja.* During garden surveys in January 2014 the inspectors discovered a more than 50 year old *Thuja plicata* as part of a hedge row with aerial dieback, and again *P. lateralis* was isolated from the foliage and no symptoms were present on bark or root collar.
Both obtained isolates were tested for their pathogenicity on their respective hosts and re-isolated, completing Koch's postulates. The ITS and COXII sequences revealed that both isolates belonged to the American 'Pacific Northwest' lineage of the pathogen [2].

The foliage of six different genera of conifers including nine species and 16 different cultivars was tested for its susceptibility against two isolates of *P. lateralis.* Detached green shoot tips were floated in zoospore suspension and the lesion size was assessed after 7 days. *xCupressocyparis leylandii, Juniperus media* and *Cedrus deodara* were the most resistant hosts whereas *Chamaecyparis obtusa* was most susceptible. *Thuja plicata* and *occidentalis*, *Chamaecyparis pisifera* and *lawsoniana, Taxus baccata* and *Cupressus macrocarpa* were of medium susceptibility.

For comparison a small subset of four hosts was also tested for stem susceptibility. The stems of whole potted plants were inoculated with the same two isolates as used for the leaf susceptibility tests. *C. lawsoniana* was highly susceptible whereas the stems of *C. leylandii*, *T. baccata* and *T. plicata* were all resistant. No correlation between foliage and stem susceptibility was observed.

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# First report of *Phytophthora sp.* on *Epipremnum aureum* in Mexico

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The state of Michoacan has an important nursery industry that is actively growing. Wilting plants of *Epipremnum aureum* (commonly known as golden pothos) were observed in nurseries located in Michoacan, Mexico. Diseased tissue was plated out on NARPH selective media. The oomycete *Phytophthora* was consistently isolated. Morphology, mating type, sensitivity to mefenoxam, growth at 35$^{o}$C and sequences of ITS, 60S and Cox I and II, were determined in one isolate. Sporangia were spherical, broadly ellipsoid or obovoid with one papilla (occasionally two papillae), and deciduous with a long pedicel. The isolates were heterothallic, and oogonia with amphigynous antheridia were observed in pairings with A1 and A2 isolates of *P. capsici*. The isolate was A2, sensitive to mefenoxam and grew at 35$^{o}$C. The ITS and 60S sequences showed 99% and 100% similarity with *P*. *capsici* respectively. Cox II sequence showed 100% similiraty with *P*. *tropicalis* therefore, it appears to be intermediate between these species. No amplification was obtained with the Cox I primers. Pathogenicity tests were carried out with 90 days old rooted plants, placed in glass containers with 40 mL of distilled sterile water. The plants were inoculated with a suspension of 10,000 zoospores per mL and maintained at 25$^{o}$C in the laboratory. Symptoms were observed after 7 days with 100% mortality at 10 days. The isolate was none virulent on inoculated pepper plants in tests performed in a greenhouse. To our knowledge, this is the first report of *Phytophthora* sp. on *Epipremnum aureum* in Mexico.

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# Tools for *Phytophthora* surveys

# Fishing for *Phytophthora* 2.0

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Isolation of *Phytophthora* species from water sources is common and worldwide known. Usually, the most common methods used are baiting *in situ* and filtering of water. Both methods are successful for the isolation of aquatic species included in clade 6 like *P. gonapodyides*, *P. megasperma*, *P.* taxon *PgChlamydo* or *P. lacustris.* However, only a few species from other clades are isolated using these methods due to the relative low inoculum available. Therefore, a study was performed to compare the number of *Phytophthora* species isolated using baits and the number of *Phytophthora* species detected using pyrosequencing technology. A total of 16 forest streams from northern Spain were selected for the study. Water samples were filtered *in situ* and the filters were used for isolation and for pyrosequencing studies. Additionally, baits (rhododendron leaves and carnations petals) were placed in the same streams and collected after one week. Isolation from baits were performed using selective media and amplicon pyrosequencing was performed from each sample. Results compared data among culturing of *Phytophthora* species from filters and baits, and those species detected by pyrosequencing.

# Exploring hidden *Phytophthora* via amplicon Pyrosequencing using eDNA from soil and water

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*Phytophthora* is one of the most important and aggressive plant pathogens in agriculture and forestry. Early detection and identification of its pathways are of high importance to minimize the threat that they pose to natural ecosystems. Therefore, a new improved method for its detection in environmental samples is proposed. eDNA was extracted from soil and water from rivers and streams from *Fagus sylvatica* and *Abies alba* forests, and *Chamaecyparis lawsoniana* and *Pseudotsuga menziesii* plantations in the north of Spain (Irati Forest and Villanua). A *Phytophthora*-specific amplicon pyrosequening based on the barcoding target ITS1 was applied. Different score coverage threshold values were tested for optimal *Phytophthora* species separation. Clustering at 99 % was the best criteria to separate most of the *Phytophthora* species. Of the total of 37 *Phytophthora* species detected in the environmental samples, 24 were known to science (*P. lacustris*, *P. gonapodyides*, *P. syringae*, *P. hedraiandra*, *P. cambivora*, *P*. taxon *PgChlamydo*, *P. alni* subsp. *uniformis*, *P. cactorum*, *P. pseudosyringae*, *P. porri*, *P. gallica*, *P. asparagi*, *P. megasperma*, *P. cryptogea*, *P. gregata/gibbosa*, *P. europaea*, *P. lactucae*, *P. niederhauserii*, *P. drechsleri*, *P. inundata*, *P. psychrophila*, *P. plurivora*, *P. trifolii* and *P. quercina*) and 14 were unknown to science. Thirteen of the unknown species were detected in rivers and streams revealing that water environments could represent important pathways and a potential source for pathogen discovery. Pyrosequencing of soil samples revealed low *Phytophthora* diversity (14 species) in comparison with the 35 species detected in water samples, representing the 95 % of the total *Phytophthora* community. Water eDNA pyrosequencing proved to be a valuable method for the detection of *Phytophthora* species in natural ecosystems.

# Next Generation Sequencing reveals unexplored *Phytophthora* diversity in Australian soils

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The Vegetation Health Survey (VHS) at the Department of the Environment, Western Australia has a *Phytophthora* collection extending back to 1979. Isolates in this collection have been recovered during routine monitoring on natural ecosystems in Western Australia for the presence of *Phytophthora cinnamomi.* Through molecular re- evaluation of this collection we have subsequently described 11 new Phytophthora species and the diseases associated with them and additional descriptions are underway. Elsewhere in Australia, however, there is extremely limited information on *Phytophthora* diversity within natural ecosystems. Using modern molecular techniques such as Next Generation Sequencing, it is possible to determine *Phytophthora* species diversity from environmental soil samples. In this study, DNA was extracted from soils obtained from 700 locations around Australia. ITS1 amplicons were generated using *Phytophthora* specific primers (Scibetta et al. 2012) adapted for NGS by Santi Català and sequenced on a Roche Junior GS platform. For 50 samples roots and rhizosphere soil were extracted separately. Results reveal an astonishing diversity, several new species and very different species profiles when comparing roots and rhizosphere soil from the same location. Species described and known only from Western Australia have an Australia-wide distribution raising intriguing questions in regards to origin and movement of species.

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# Molecular Tools for the PCR detection of *Phytophthora austrocedri*

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*Phytophthora austrocedri* (*Phytophthora austrocedrae***,** orthographic variant) was described in 2007 in Argentina causing dieback on Chilean incense cedar in Patagonia. The pathogen was found in 2010 in UK causing a serious decline of native juniper and in Scotland in 2011 on Lawson"s cypress and Nootka cypress. Recent surveys of juniper woodland in northern England and Scotland conducted by CFCC since November are showing that the pathogen is pretty widespread. There is indication that the pathogen was present in Germany around 2001 according to Julius Kühn-Institute Datasheet for the pathogen. Further concern for this pathogen is due to its potential to be disseminated through shipments of contaminated nursery stock. *P. austrocedri* is ranked # 21 in the list of 29 *Phytophthora* spp. of concern for the USA published by CPHST- Plant Epidemiology and Risk Analysis in 2009. Due to the importance of the pathogen denoted by the recent discoveries and its inclusion in the Cooperative Agricultural Pest Survey List (2014), we consider *P. austrocedri* as a priority for USDA regulatory programs. In order to facilitate the efforts in pest detection of *P. austrocedri,* a multiplex conventional PCR was developed using species-specific primers for the 60S Ribosomal protein L10 60SL10_for/60S gene (L10) and paired with the plant gene NADH dehydrogenase subunit 5 mRNA (nad5) as the internal control. In addition a real-time PCR assay that targets the Internal Transcribed Spacer rDNA region (ITS) published by the CFCC in Scotland was validated and paired with the cytochrome oxidase gene (COX) to detect host plant DNA in a multiplex assay. Both methods have been tested with DNA from cultures and environmental samples from the UK provided by the CFCC. In addition, both methods are under evaluation for cross-reactivity with other *Phytophthora* species in clade 8d, as well as other Oomycetes and fungal pathogens.

# Tools for rapid characterization of *Phytophthora infestans* and *Phytophthora ramorum* using real-time PCR and microsatellites from genomic resources

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Oomycete pathogens such as *Phytophthora infestans* (Mont.) de Bary and *Phytophthora ramorum* Werres De Cock & Man in"t Veld cause a devastating impacts worldwide. More DNA-based tools are needed to identify and characterize these species, where some genotypes/lineages may be more problematic than others. For example, some *P. infestans* strains are more resistant to fungicides and have preferred hosts. Mining the full genome sequences of these two organisms allowed development of markers for intraspecific genotyping. Our first objective was the development of Allele Specific Oligonucleotide-PCR (ASO-PCR) assays using real-time PCR to differentiate Canadian strains of *P. infestans* and the four lineages of *P. ramorum* (NA1, NA2, EU1 and EU2). The *P. infestans* genome revealed several regions containing SNPs within genes and in flanking sequences of microsatellite loci. Nine ASO-PCR assays were developed from these SNPs, allowing the unambiguous identification of the five dominant *P. infestans* Canadian genotypes from the most recent outbreaks. Two new ASO-PCR assays were developed in a gene coding for cellulose binding elicitor lectin (CBEL). Combined with two existing assays within the same gene region, it is now possible to identify all four lineages of *P. ramorum*, including the recently discovered EU2 lineage. Our second objective was to develop microsatellite markers to evaluate *P. ramorum* genetic diversity mostly within the NA2 lineage, where fewer markers are currently available. Analysis of the genome of the NA2 *P. ramorum* lineage revealed microsatellite loci that reveal polymorphism within this lineage. Previous markers were biased toward NA1 and did not reveal the level of polymorphism discovered using these new microsatellites. These DNA-based tools will contribute to the available genomic toolbox to assess the genetic diversity of these oomycete pathogens, from the species to the intra-lineage level.

# Whither the species? *Phytophthora* taxa, MOTUs and barcodes in the world of metabarcoding

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The study of ecosystem diversity is being transformed by high throughput sequencing technology that allows an unparalleled depth of sampling of DNA barcode sequences. The opportunities are great at this fascinating interface of pathology, ecology, taxonomy and molecular biology but there are pitfalls. It is important to consider the potential for bias at all steps in the process from sampling through to data analysis. Increasingly *Phytophthora* diversity is being examined by metabarcoding of the PCR-amplified rDNA ITS regions from soil, water or plant sample DNA. We have applied baiting and isolation, cloning and Sanger sequencing and Illumina MiSeq analysis to a time-series of filtered water samples from several Scottish streams within a UK-wide sampling network. These data, the literature and other presentations in this session will be explored in a critical analysis of the field. A specific focus will be placed on exploring the range of species and their boundaries, the potential for species quantification and possible benefits of the technology for plant health legislation.

# Surveys and New Records

# *Phytophthora* spp. invasions in post-communist economies – the example of the Czech Republic

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Invasion of alien pathogens of woody plants including *Phytophthora* spp. poses an important risks for sustainable forestry, rural economy and many other branches. Despite of enormous importance, this field is still highly underestimated in many countries – for instance in post-communist countries in Central and Eastern Europe. This work describes some trends in development of diversity of alien and cryptogenic pathogens in forest woody plants in the Czech Republic (Černý et al. 2014) in comparison with European dataset (Santini et al. 2013). The relation between growth of GDP and increase in introductions was made with use of data from Bolt and Zanden (2014) and Eurostat (Anonymus 2014). Moreover, the in-depth analysis of *Phytophthora* spp. introductions in the area was made and the simple scheme of *Phytophthora* invasions was constructed with use of the data about distribution and hosts of particular species from the Czech Collection of Phytopathogenic Oomycetes.

It was found out, that the development of cumulative number of alien pathogens highly differed in Europe and in the Czech Republic. Firstly the course of cumulative number was linear in Europe and also in the Czech Republic. However, a great difference in the development of new introductions was identified since 1940s. Where the slow linear growth was continuing in the Czech Republic until 2000, in Europe an accelerated exponential growth was characteristic. This exponential growth was primarily given by findings from old EU countries. The dramatic increase in introductions was also identified in the Czech Republic since millennium (fig. 1). This specific development of introductions should be at least partially ascribed to former political and economical isolation of the country during 2nd world war and membership in Eastern bloc (linear part of the curve) and to consequent opening the economy and joining European Union (exponential development) after coup d´état in 1989.

The relation of number of introductions to GDP in the country was identified (fig. 2). However, no new introductions were identified in some periods of the 20th century probably because of poor attention paid to these organisms. In decades when an introduction was identified (plant pathologists apparently studied this problem), the number of introductions was correlated to GDP (Spearman correlation index $r = 0.78$, $p < 0.05$). During last century the two periods of high increase in *Phytophthora* pathogens were identified and both of them agreed with periods of economical growth – the postwar period of economical recovery and the recent period after transformation of economy (fig. 2). During the first period, *Phytophthora* pathogens were imported among others with fruits from Middle East and Central America. The second period was characteristic by increase of pathogen imports with infected ornamentals from Western Europe and by quick increase of forest diseases. A simple scheme of *Phytophthora* spp. invasion was constructed for conditions of the Czech Republic. Five barriers which have

to be overcome by pathogens on their way into natural stands were recognized (fig. 3): 1) geographic, 2) phytosanitary in points of introductions, 3) environmental in artificial habitats (greenhouses, gardening centres etc.), 4) environmental in anthropogenic stands (urban greenery etc.), and 5) environmental in natural stands. These barriers correspond with following particular stages of *Phytophthora* invasion: 1) first stage of casual invasion (for instance *P. ramorum*), 2) following stage of establishment in fully artificial habitats (usually termophilic species as *P. cinnamomi*, *P. citrophthora*, etc.), 3) establishment in anthropogenic stands (cold-tolerant *P. cactorum*, *P. megasperma*, etc.), and full naturalization (*P. plurivora*, *P. alni*, *P. gonapodyides*, *P. multivora* and *P. cambivora*).

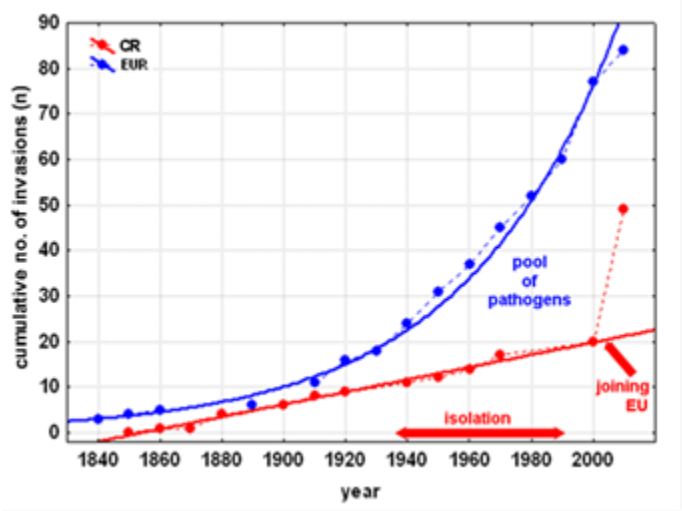



Fig. 1. The development of introductions of alien patogens to Europe and the Czech Republic

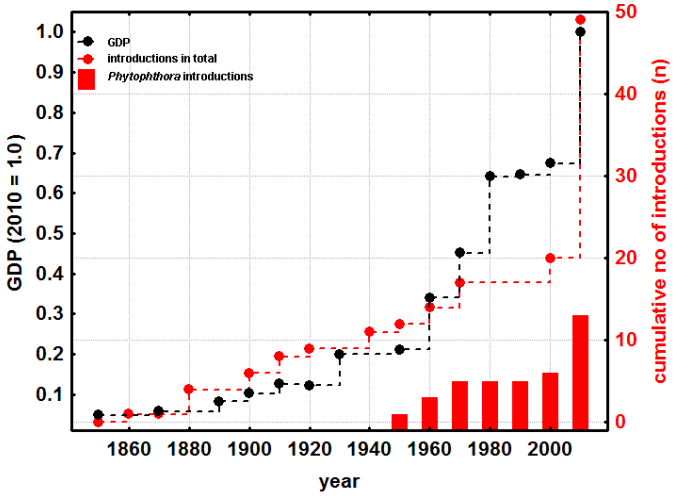



Fig. 2. The development of GDP and cumulative number of introductions in the Czech Republic

There were described 24 *Phytophthora* taxa pathogenic to forest trees in the area and 19 taxa (79,2 %) of them were found to be alien or cryptogenic. Alien species more or less regularly established only in artificial habitats shared in *Phytophthora* spp. diversity by 37,5 %. Species distributed in anthropogenic stands shared by 20,8 %, and alien naturalized species by 20,8 % in the mycoflora of *Phytophthora* spp. in woody plants.

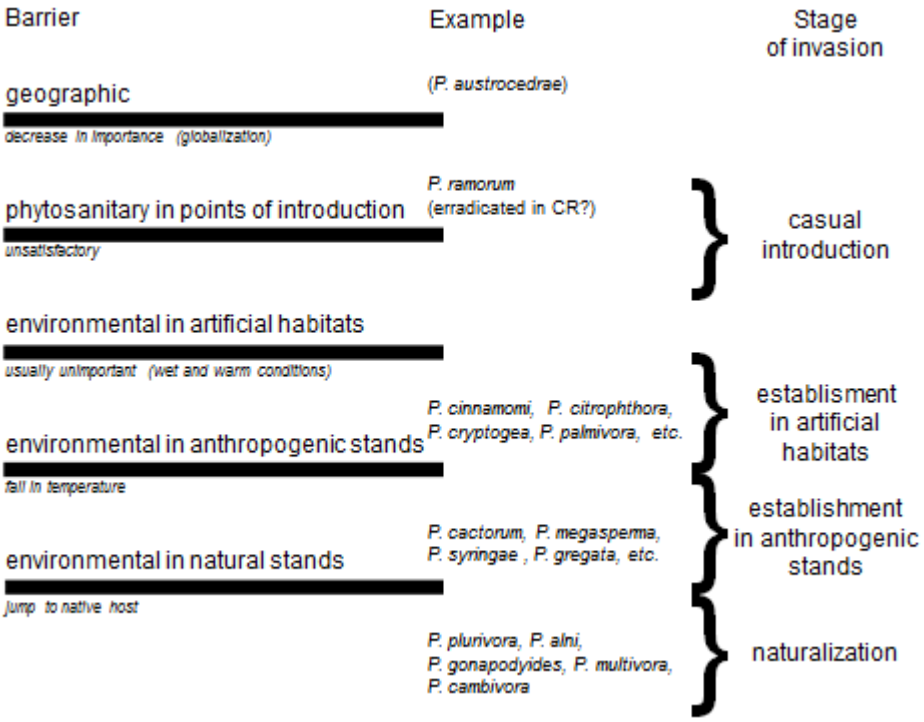



Fig. 3. Scheme of *Phytophthora* spp. invasion in the Czech Republic

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# Survey of Oomycetes found in western Washington streams

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Information on what Oomycete pathogens are present in a waterway is of interest to growers of horticultural, forest, and food crops, especially if a quarantine organism such as *Phytophthora ramorum* is found. Since 2003 *P. ramorum* has been detected in over 50 ornamental plant nurseries in Washington State. Stream monitoring by state agencies has resulted in the detection of this exotic pathogen in nine streams, three ditches, and two rivers outside of nurseries since 2006. In all cases, streams have tested positive for *P. ramorum* in subsequent years after the first detection. Stream monitoring using baiting and culturing methods designed to detect *P. ramorum* was carried out in spring 2011 in the Puget Sound region of western Washington. Ten streams representing a variety of habitats were sampled for six two-week baiting intervals. Much of the sample collection and isolation of Oomycetes was done by volunteers and students as part of an outreach program. Oomycetes were isolated and characterized by morphological methods and by DNA sequence analysis of the ITS region of the rDNA. 276 isolates of *Phytophthora, Pythium, Halophytophthora*, and other Oomycete genera were examined. Some putative new species of *Phytophthora* and *Pythium* were identified for further study. Several Oomycetes having a worldwide distribution were found in Washington streams. The ecology and importance of these species with relation to *P. ramorum* is discussed. In addition to providing preliminary information about the Oomycete species present in western Washington streams, this study was an opportunity to educate the public about the importance of these organisms and their effects on ecosystems. This was done by enlisting the aid of students and volunteers from the community.

# Diversity of *Phytophthora* species in forests, forest nurseries and riparian ecosystems of Portugal

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In Portugal, the involvement of *Phytophthora cinnamomi* in the decline of *Quercus ilex* and *Q. suber* is notorious but the dimension of the risk posed by other *Phytophthora* spp. to both natural ecosystems and forest nurseries is unknown.

In the framework of the European BiodivERsA Project RESIPATH (Responses of European Forests and Society to Invasive Pathogens) a national-wide, two-years *Phytophthora* survey in natural ecosystems and forest nurseries in Portugal is being conducted in close cooperation with the Instituto da Conservação da Natureza e das Florestas (national authority responsible to propose, monitor and ensure the implementation of policies on nature conservation and forestry).

This survey aims to study the diversity of both known and as yet unknown *Phytophthora* species in Portugal and clarify whether they are endemic or of exotic origin. For potentially non-native *Phytophthora* species morphological and physiological studies and pathogenicity to common European tree species will be tested to evaluate their invasive potential. In addition, in cooperation with the Direção Geral de Alimentação e Veterinária (national authority responsible for the design, implementation and evaluation of policies for plant protection and plant health), freshly arrived consignments of plants-for-planting from overseas will be tested for *Phytophthora* infestations at ports of entry to verify this potentially important pathway into Europe.

First results will be presented and discussed in terms of the actual or potential threat to the natural biodiversity of the Portuguese ecosystems.

# Diversity of *Phytophthora* species in the oak forests of Southwest China

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**Abstract**

*Phytophthora* species are best known as destructive pathogens of agricultural crops or invasive pathogens destroying forests and natural ecosystems. Prior to 2005, little was known about the presence of *Phytophthora* in forests in China. From 2005 to 2011, surveys of *Phytophthora* spp. in the oak forests of southwest China were conducted to obtain an overview of the species that inhabit these forests. Twelve stands plus 12 associated streams in four regions were surveyed. A total of 421 isolates of *Phytophthora* spp. were recovered from soils and streams baited with leaves using standard baiting techniques, and 11 taxa including eight known species (*P. borealis*, *P. cryptogea, P. gonapodyides*, *P. gregata*, *P. lacustris*, *P. megasperma*, *P. plurivora*, *P. syringae*), the known but as yet unnamed *P.* taxon PgChlamydo, and two previously unrecognized species, *Phytophthora* sp.1 and *P.* sp.2., were identified based on morphological features and ITS and *cox* I sequence data. *Phytophthora* species residing in ITS Clade 6 were most abundant overall, but only *P.* taxon PgChlamydo was found in all four regions. The abundant *P. gonapodyides* and *P. borealis* were present in three regions, and the three rare species, *P. gregata*, *P. lacustris* and *P. megasperma* were recovered only in one region. Phylogenetic analysis distinguished two novel heterothallic species . The new taxa appear to have limited distribution and definite pathogenicity. *P. syringae* was isolated for the first time from stream water and soil in two regions. The occurrence of *Phytophthora* species in forests in southwest China and their pathogenicity toward some plants highlights once again the urgent need to investigate the potential risk and impact of both native and previously unknown introduced forest Phytophthoras.

# *Phytophthora* species associated to Holm oak decline in western Spain

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The oak-rangeland „dehesa" ecosystem plays an important economic, ecological and social role in south-western Europe. The decline of cork and holm oak trees and the absence of natural regeneration are the main concern of this valuable ecosystem. The decline syndrome can develop in a few months or several years and it has been explained by several concomitant factors of abiotic and biotic nature. Among these factors, *Phytophthora cinnamomi* is considered the main cause of decline because its aggressive behaviour on the feeder roots of the oaks. However, its presence has not been always confirmed in the affected stands. To overcome traditional *Phytophthora* isolation difficulties, the aim of this work was to apply molecular methods to study *Phytophthora* diversity. Surveys were conducted in Extremadura region (western of Spain) in five adult holm oak stands and thirteen regeneration zones. In each sampling site, soil and root samples were collected from declining and non-declining holm oaks (*Quercus ilex*). The study compares three different approaches: (i) traditional isolation methods consisting of roots in contact with selective media, and soil isolation using apples and leaf baits, (ii) 454-pyrosequencing analysis of root and soil samples with tagged amplicons specific for *Phytophthora*, and (iii) TaqMan real-time PCR of root and soil samples using a *P. cinnamomi* specific probe.

# Multiple new and invasive alien *Phytophthora* taxa from Mediterranean maquis ecosystems in Italy

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The Mediterranean basin is recognized as a global biodiversity hotspot accounting for more than 25,000 plant species that represent almost 10% of the world‟s vascular flora. In particular, the maquis vegetation on Mediterranean islands and islets constitutes an important resource of the Mediterranean plant diversity due to its high rate of endemism accounting for 4.3% of all plant species worldwide. Since 2009, a severe and widespread dieback and mortality of *Quercus ilex* trees and several other plant species of the Mediterranean maquis has been observed in the National Park of La Maddalena archipelago (northeast Sardinia, Italy). Infected plants showed severe decline symptoms and a significant reduction of natural regeneration. First studies revealed the involvement of the highly invasive *Phytophthora cinnamomi* and several other fungal pathogens. Subsequent detailed research led to a better understanding of these epidemic showing that the aetiology is more complex than initially assumed and that multiple other *Phytophthora* spp. are also involved, some of them unknown to science. A total of 13 *Phytophthora* species were isolated from roots and soil samples collected from symptomatic trees and shrubs such as *Arbutus unedo*, *Asparagus albus*, *Juniperus phoenicea*, *J. oxycedrus*, *Pistacia lentiscus* and *Q. ilex*. Based on morphological characters, growth–temperature relations and sequence analysis of the ITS and *cox*1 gene regions, the isolates were identified as: *P. asparagi*, *P. bilorbang*, *P. cinnamomi*, *P. cryptogea*, *P. gonapodyides*, *P. melonis*, *P. nicotianae*, *P. parvispora*, *P. psychrophila*, *P. quercina*, *P. syringae* and two informally designated taxa, *P. aplerotica* prov. nom. and *P. ornamentata* prov. nom., both within ITS Clade 6. Studies are currently underway to formally describe the new species in conjunction with large scale pathogenicity trials to confirm Koch‟s postulates for the new host/*Phytophthora* associations.

# *Phytophthora* detections in native plant nurseries and restoration sites in California

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*Phytophthora tentaculata* was recovered from sticky monkey flower (*Mimulus aurantiacus*) at a California native plant nursery in 2012, which was the first detection of *P. tentaculata* in the USA (Rooney-Latham and Blomquist 2014). *Phytophthora tentaculata* is listed as a threat to nurseries and forests in United States federal New Pest Response Guidelines (APHIS 2010). In the first half of 2014, the pathogen was detected in both sticky monkey flower and coffeeberry (*Frangula californica*) at additional native plant nurseries in different counties that have no reported connection to the nursery with the initial detection. In addition, *P. tentaculata* was recovered from outplanted toyon (*Heteromeles arbutifolia)* and sticky monkey flower at native plant restoration sites. In the latter species, *P. tentaculata* was recovered from declining plants growing at the site for over a year.

The detection of *P. tentaculata* and *P. cactorum* in nursery stock planted at restoration sites in Alameda County prompted additional investigations into both symptomatic and asymptomatic plant material at several source nurseries and multiple recently-planted restoration sites. Numerous *Phytophthora* species were recovered, some from rushes and sedges, plant species typically not considered to be *Phytophthora* hosts. While some of the *Phytophthora* species detected are common in California in nurseries and cultivated landscapes (e.g., *P. cactorum, P. cambivora, P. cryptogea, P. megasperma*) other species are relatively uncommon or not previously documented in California (e.g., *P. tentaculata, P. quercetorum, P. inundata, P. plurivora*). The wide diversity of *Phytophthora* species recovered from plants grown at these native plant nurseries and their relative abundance raises concern that native plant nurseries serve as a source of *Phytophthora* introductions in restoration sites in California.

These recent *Phytophthora* detections will be examined as a case study of the potential for the spread of *Phytophthora* species in native plant nurseries and restoration plantings.

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# *Phytophthora* - an emerging threat to plantation forestry in Vietnam

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The impact of diseases caused by a number of *Phytophthora* species has been well documented in Vietnam but the focus until now has been exclusively on horticultural plants and some annual crops, including pepper, fruit trees, taro and potato. In 2012, *Phytophthora* was isolated for the first time from the rhizosphere soil of severely declining *Acacia mangium* plantations in Tuyen Quang province. Initial isolates were identified as *P. cinnamomi* and these isolates were shown to cause root rot and stem lesions in *A. mangium* seedlings. Since then, a program was initiated to assess whether *Phytophthora* was present and causing damage in plantations, hedge orchards and nurseries. So far, a range of highly pathogenic *Phytophthora* and *Phytopythium* isolates have been obtained. *Phytophthora cinnamomi, P. parvispora* and a new species most closely related to *P. elongata* were the most frequently isolated species. The distribution and potential threat from *Phytophthora* spp. to plantations and other forest species in Vietnam will be discussed. Management strategies to manage disease outbreaks and to reduce the spread of *Phytophthora* will be considered.

# Diversity and impact of *Phytophthora* spp. in natural ecosystems of Taiwan

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In spring and autumn 2013 a survey of Phytophthora diversity was performed in 22 natural forest stands and 25 rivers in subtropical and tropical regions of Taiwan with altitudes ranging from 6 to 2287 m asl. In total 144 soil samples were taken from the rhizosphere of 40 tree species. Using leaves of *Q. variabilis*, *C. indica*, *Citrus sinensis* and other species as baits 12 known species, four designated taxa and 17 unknown species of *Phytophthora* have been isolated: *P. cinnamomi* (Pc), *P. citrophthora, P. cryptogea, P. heveae, P. katsurae, P. palmivora, P. parvispora, P. plurivora*, *P.* t. 'PgChlamydo', *P.* t. 'Kunnunara', 5 new species related to *P. botryosa* and *P. meadii*, and 4 new species from ITS Clade 7a from 97 soil samples (67.4%) of 33 tree species (82.5%); *P. capensis, P. citrophthora, P. insolita, P. parvispora, P. tropicalis,* 3 new species from ITS Clade 7a, *P.* t. 'PgChlamydo', *P.* t. 'Ceanothus', 1 new species related to *P. botryosa* and *P. meadii*, 3 new species related to *P. insolita*, *P.* t. 'Kunnunara', *P.* t. 'Peru 4', *P.* t. 'BFR', 6 new species in the *P.* t. 'Kunnunara' cluster, *P.* t. 'forestsoil-like', and another 2 new Clade 9 species from 20 rivers (80%). Most Phytophthora species were not associated with disease symptoms. The A1 mating type of Pc was widespread in most mountain and lowland forests (12 stands, 17 tree species) and was rarely associated with disease symptoms. In contrast, the distribution of the A2 mating type was restricted to subtropical and tropical lowland rainforests (8 stands, 13 tree species) and always associated with often severe decline of different forest types. In one declining rainforest A1, A2, homothallic A2 and sterile Pc isolates were found. It is suggested that both mating types of Pc got geographically separated during the pleistocene and that the A1 mating type is native to Taiwan whereas the A2 mating type is a recently introduced invasive pathogen.

# Biology and Genetics

# Visualisation of early infection by *Phytophthora* "taxon Agathis" in the roots of two-year old kauri plants

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**Introduction:**
*Phytophthora* "taxon Agathis" (PTA) is the causal agent of a root- and collar-rot of kauri (*Agathis australis*, Araucariaceae) in the northern forests of New Zealand. To-date, the host range of this pathogen is restricted to *A. australis*, and it is considered that early infection is facilitated through the fine roots. This study aimed to document the infection biology of PTA in the roots of two-year kauri plants five-, ten-, sixteen-, and twenty-days after inoculation (d.a.i.). The investigation was conducted using light microscopy (LM) of plant material that had been cleared and stained (Crone et al. 2013), to ascertain the pattern of infection over a 20-day time course. A sub-sample of the plant material was also examined via scanning electron microscopy (SEM) to confirm details recorded in the light micrographs. A PTA-specific FISH assay was used to identify the presence of PTA as the causal agent (after the methodology provided by Li et al. 2014).

**Results:**
Cysts were observed on the epidermal cells. Haustoria were mostly produced by five d.a.i. in the cortical cells of *A. australis* fine roots. As the infection progressed (20 d.a.i). the haustoria also developed a thickened, haustorial matrix, and "digitate" morphology. Survival propagules, in the form of thin-walled, putative chlamydospores, and stromata-like aggregations were also observed in these artificially infected plants. These structures have not been observed in pure axenic cultures of PTA on artificial growth media. This study has increased our understanding of the infection process of PTA in young kauri plants. The ability of the pathogen to grow as an endophytic biotroph is indicated by the formation of haustoria. Chlamydospores and stromata produced in roots could serve as long-term survival propagules in infested sites, if these structures are produced in field-infections (e.g., Jung et al. 2013).

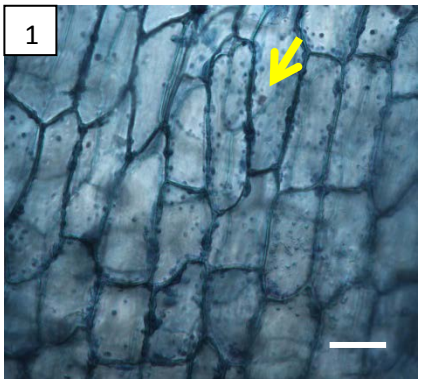
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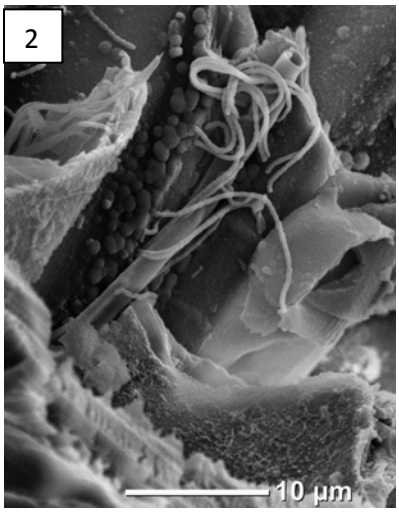
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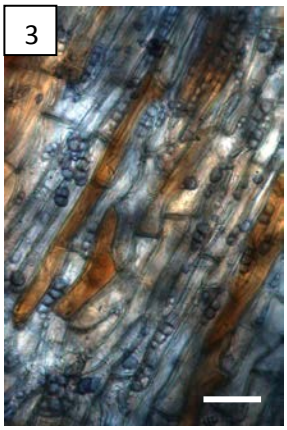
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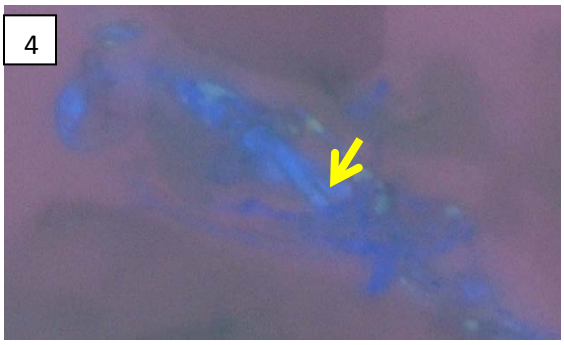
**Figure Legends:**

1) Haustoria of PTA (yellow arrow) five d.a.i. (LM) (scale bar = 20 µm).
2) Hyphae of PTA five d.a.i. (SEM) (scale bar = 10 µm).
3) Stromata of PTA in cortical cells (scale bar = 60 µm).
4) Nuclei (yellow arrow) in hyphae labelled with PTA-specific FISH probe (scale bar = 35 µm).



# *Phytophthora ramorum*: Study of the lineage EU2 / EU1 in Ireland

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*Phytophthora ramorum* is a very pathogenic Oomycete which has caused a great impact in the ecosystem and economy in the last two decades. It was first reported affecting larch in the UK in 2003. This pathogen can infect a wide range of hosts including larch, beech and rhododendron in Europe. Four different lineages have been described to date: NA1 and NA2 in North America and EU1 and EU2 in Europe. The aim of this project is to study the Irish population of *P. ramorum*, focusing on the lineage EU2, which seems to be specifically located in Northern Ireland and the south-west coast of Scotland. Over 300 isolates were studied using RFLP and microsatellite markers (SSR). The result of the study showed that the majority of the Northern Irish population is EU2 (89%), EU1 was observed in small areas where infected plants had been imported from other nurseries. The population of the Republic of Ireland was 100% EU1, as in the rest of Europe. The results of the SSR analyses were processed with software specialized in Population Genetics, resulting in Phylogenetic trees that group the population according the genetic variation.

# Basic and applied research into *Phytophthora ramorum* in Ireland: the PHYTOFOR project

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**Abstract:** We have conducted in-vitro experiments to characterize the four lineages of *P. ramorum*. Between the EU1 and EU2 lineages we have found no difference in either growth rate or pathogenicity in detached rhododendron leaves. Field trials in previously infected larch forests have been established and are being monitored since 10/13. Collectively, our field results indicate that in Japanese larch forests in Ireland, the pathogen is mainly spread via aerial dissemination of spores with limited movement in soil or water-courses.

*Phytophthora ramorum* is an invasive pathogen affecting woody plant and tree species in the wild in Ireland and Britain. In the Republic of Ireland, only the EU1 lineage of *P. ramorum* is present, while Northern Ireland has both the EU1 and EU2 lineages present. As part of the PHYTOFOR project, we have carried out experiments to phenotypically characterize the Irish *P. ramorum* populations in respect to those world-wide (including all four lineages: EU1, EU2, NA1, NA2).

Significant differences have been found in the radial growth rate of all four lineages of *P. ramorum* across five tested temperatures. At 20°C, the NA2 lineage grew significantly faster than the other three lineages, there was no difference between the EU1 and EU2 lineages, and the NA1 lineage grew significantly slower than the other three lineages. There was no difference between EU1 and EU2 in terms of their pathogenicity on detached Rhododendron leaves at 20°C (Fig. 1).

While our experiments found no difference in the pathogenicity of the EU1 and EU2 lineages on detached rhododendron, results from another study did find significant differences (McCracken et al. 2014 this issue). Here, we carried out new experiments using both our experimental design (zoospores and petri-dish container) and that of McCracken et al (mycelial plug and lunch-box container) to investigate if the differences could be due to experimental set-up. We used 5 EU1 and 5 EU2 isolates from the set of isolates used by McCracken et al. (2014)(Table 1). While there was no significant difference between the lineages when using the petri dish method, the analysis of the results from the lunch-box method found marginal significantly different pathogenicities (Mann-Whitney U=1659, n1:36; n2:29; P=0.08). When both the box and petri sets of experiments were analyzed together, a General Linear Model indicated that isolate identity ($F_{10, 79}$ = 11.03, P<0.001) and the container type ($F_{1, 79}$ = 5.93, P<0.05) were the only significant factors in explaining the variation in lesion size. Neither lineage nor inoculation method were significant.

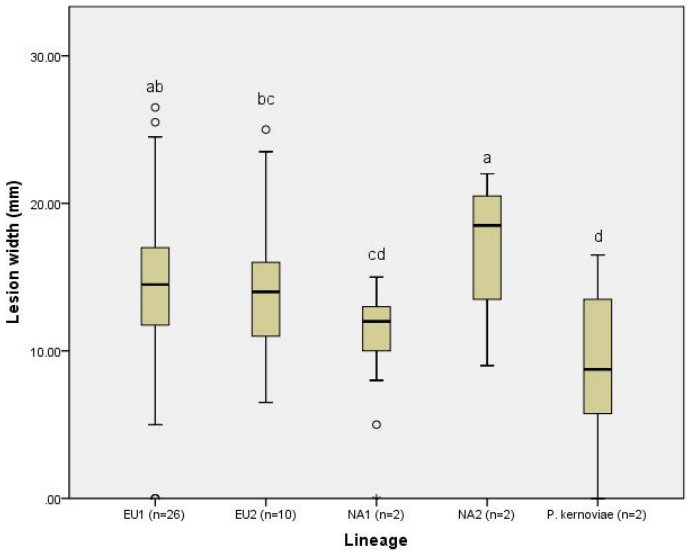



**Figure 1.** Mean pathogenicities of the four *P. ramorum* lineages on detached rhododendron leaves . Different letters denote significantly different means according to One-way ANOVA with Tukey-B posthoc test.

The PHYTOFOR project also investigates the survival and spread of the disease in previously infected, and since felled Japanese larch forests in Ireland. Monthly site visits since August 2013 to rain water traps (1 mt high and ground level), rhododendron bait plants, water baits (rhododendron leaf) and soil samples have found pathogen presence (based on plating onto selective media PARP) in all sites (Table 2).

The Wicklow site still has standing Japanese larch trees, and this plot has had positive rain water traps in 10/13, 11/13, 01/14, 02/14, 03/14, 10/14,12/14 and 01/15. This site has also had almost year round positive findings from a baited puddle (02/14, 03/14, 04/14, 06/14, 07/14, 08/14, 09/14 10/14, 12/14, 01/15).

Rhododendron baiting of randomly selected soil samples from all sites has rarely been positive. Of the 244 tested by baiting with rhododendron, 5 were found to be positive. All of these 5 positives came from close to the infested puddle area described above. Retesting of 86 of the soil samples after (i) drying at room temperature and (ii) storage at 5°C found the samples were still negative. Furthermore, since 12/14 soil samples have been baited using both rhododendron leaves and granny Smith apples. Of the 24 samples tested using both methods, 2 have been positive based on rhododendron baiting and 0 positive based on apple baiting thus indicating the suitability of the rhododendron bait test to our aims.

Stream baiting has been carried out on 29 occasions, and only 3 have been positive (03/14, 07/14, 12/14). The wash-off from footwear used on the site visits has been collected and baited with rhododendron on 25 occasions. Only once has footwear been positive, and this was after walking through the infested puddle area described above.

Taken together, our field results indicate that in Japanese larch forests in Ireland, *P. ramorum* is mainly an aerially spread pathogen. Survival in the soil is minimal, and spread in soil is probably not as epidemiologically important as spread in wind and rain.

**Table 1**. Mean pathogenicities of the EU lineages according to experimental set-up.

| Container type | Lineage | Treatment | Mean pathogenicity (SD) |
|---|---|---|---|
| Petri-dish | EU1 (n=5) | Plug | 7.7 (4.97) |
| Petri-dish | EU2 (n=5) | Plug | 12.37 (6.88) |
| Petri-dish | EU1 (n=5) | Spore | 10.2 (2.29) |
| Petri-dish | EU2 (n=5) | Spore | 13.5 (2.43) |
| Lunch box | EU1 (n=7) | Plug | 8.95 (6.9) |
| Lunch box | EU2 (n=5) | Plug | 8.76 (8.67) |
| Lunch box | EU1 (n=7) | Spore | 9.47 (5.37) |
| Lunch box | EU2 (n=5) | Spore | 4.87 (6.47) |

**Table 2**. Field monitoring results from previously infested Irish Japanese larch sites.

| | Tipperary | Kilkenny | Wicklow |
|---|---|---|---|
| Total samples tested | 320 | 284 | 461 |
| Total samples positive | 4 | 2 | 48 |
| High level traps positive | | | 5 |
| Low level traps positive | | 1 | 17 |
| Soil samples positive | | | 5 |
| Plant material positive | 4 | | 2 |
| Bait Plants positive | | | 7 |
| Footwash positive | | | 1 |
| Water baits positive | | 1 | 11 |

# Lineage, phenotype and environment factors influencing the *Phytophthora ramorum* epidemic on larch

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Between 2003 and 2008 in woodlands in the UK, *Phytophthora ramorum* mainly affected understorey rhododendron. The number of reported tree infections remained low at <100 and largely comprised native beech (*Fagus sylvatica*) and non-native oak species such as *Quercus cerris*. In 2009, *P. ramorum* was unexpectedly found to have spread to plantation grown Japanese larch (*Larix kaempferi*), causing increasingly heavy mortality, and endangering other plant and trees species due to the prolific sporulation on infected larch needles. Rain trap data indicates that with naturally infected larch, sporulation levels peak in October just before and during needle loss, although lab data suggest that sporulation may also occur on larch foliage in spring and summer. Between 2010 and the end of 2013, the combined area of affected larch in England, Scotland and Wales had risen from 2,000 ha to more than 10,000 ha. Many millions of trees have been felled to curtail sporulation and limit pathogen spread. Heavy crown symptoms in 2013 have been partly ascribed to the cool, wet summer and autumn conditions of 2012 considered conducive to sporulation and dispersal of *P. ramorum*. The behaviour of the recently characterised EU2 lineage may also account for exceptionally enhanced disease levels in Scotland, as it is a much more effective coloniser of Japanese larch bark than the EU1 lineage. However, there may be fitness trade-offs between growth rates, sporulation potential and the ability to colonise bark. This is under investigation. It also appears that the more aggressive colonising ability of the EU2, compared with the EU1, may be specific to larch and not replicated in other hosts. Currently, evidence suggests that the ranges of the EU1 and EU2 lineages do not overlap but they are rapidly converging in south west Scotland. This raises the likelihood of mixed lineage populations of *P. ramorum* affecting larch forests with further potential consequences.

# Investigation of the tree pathogen, *Phytophthora lateralis*, newly discovered in Northern Ireland

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*Phytophthora* lateralis is genetically most closely related to *Phytophthora ramorum*, however, it has a limited host range, lethally infecting Lawson cypress [Port-Orford-cedar, Chamaecyparis lawsoniana (A. Murr.) Parl]. *P. lateralis* has been prevalent in Pacific North-Western USA since the 1920s and was undetected in Europe until 1996 when it was isolated from Lawson cypress in France. *P. lateralis* was first detected in the UK, in Scotland in 2011 and was subsequently found in Northern Ireland in the same year. The pathogen is invasive and is spread by the dissemination of motile zoospores throughout waterways and by the movement of infested soil and plant material.

Characterisation of isolates from throughout Northern Ireland has revealed a largely clonal population. Nevertheless, genetic sequencing of three isolates from two geographically distinct sites has revealed single nucleotide polymorphisms, with fewer SNPs present in isolates from the same site. Subtle differences in phenotype, such as sporulation capacity, have also been observed. It is hoped that this information will provide some indication of the extent of new introductions of the pathogen into Northern Ireland and may also enhance the understanding of the epidemiology of emerging plant pathogens within the British Isles.

# Phenotypic, genotypic, genetic, genomic, analyses of plant pathogens and their application in plant pathology

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Time, reproductive isolation, drift and adaptation are the main forces shaping all living species including Phytophthoras. It is well understood that individual histories at the long-term scale shape species diversity, however even short-term histories may have evolutionary implications. In fact, it has been recently discovered that different hosts with their different chemical environments may trigger substantial and permanent changes in the structure of genomes leading to phenotypic changes. The broader concept of phenotypic differentiation includes traits such as virulence levels, mating type, growth rates at different temperatures, sporulation and transmission rates and thus it is key to the understanding of diseases. In this session, a variety of papers address the issue of diversity emphasizing that genetically and phenotypically different groups, even if morphologically undistinguishable, may each represent substantially different threat. The distribution of distinct groups may also provide insights on the spread pathways of microbes, and possibly help us identify their area of origin. Bayesian theory and alternative model testing now provide a much more robust analytical approach to understand spread patterns both at the geographic and at the topographical level. Population genetics help us understand the reproductive mode, the evolutionary potential, and the migration potential of microbes whose life stories are for obvious reasons hard to monitor by direct observation. Genetic epidemiology, based on repeated population genetics analyses in time has surfaced as the best tool to reconstruct the actual epidemiology of a disease. Equally powerful is the range of -omic approaches through which we may understand how pathogens respond to defense mechanisms of the host, to changing climate and to new environments, and to control strategies. In my talk I will cover the studies presented using genetic markers to identify diversity within species, and the experiments aimed at defining the phenotypes that may be associated with diverse groups. I will then present a synthesis of genetic epidemiology studies performed in California, and finally will briefly introduce a couple of transcriptomic and genomic projects with great potential.

# Ecology

# Landscape heterogeneity and features are associated to the impact of Ink disease in chestnut orchards in Italy

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Spread of forest Phytophthoras diseases has been frequently associated to landscape heterogeneity and presence of natural water drainages and forest roads. Aim of the present study was to investigate the interaction between the nets of forest roads and natural water drainages with the spread of ink disease over a large chestnut area in Central Italy. To achieve such objective, remote sensing techniques and GIS applications have been integrated. Presence of ink disease foci have been highlighted through the visual interpretation of high resolution spectral images. A confusion matrix has been elaborated to validate the data; accuracy in ink disease foci identification was 86.1%, while no specific decline was identified with 98.1% accuracy. Informative layers for roads and natural water drainages have been overlapped with the ink disease foci map. A significant association have been found between the presence of ink disease foci and roads (Spearman $r = 0.69$ $P \leq 0{,}0001$), and water drainages density (Spearman $r = 0.63$ $P < 0{,}0001$). Number of intersections between roads and water drainages was also significantly associated to the presence of infection foci (Pearson $r = 0.68$ $P \leq 0{,}0001$). These results support the results of studies carried out in smaller areas in Central Italy and are in accordance with other experiences considering other forest Phytophthoras epidemics.

# Ecology and pathology of *Phytophthora nemorosa, P. pseudosyringae,* and other ITS clade 3 species in forests in western Oregon

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We explore the population structure, pathology, and epidemiology of a group of closely related species: *Phytophthora nemorosa, P. pseudosyringae, P. pluvialis, P. psychrophila*, and to a lesser extent *P. ilicis*. All are in ITS clade 3. These species form a tight phylogenetic cluster and are readily separated from each other by small but consistent differences in ITS and COXI sequences. All are homothallic. They have slow to moderate growth at cool to moderate temperatures. All produce more or less caducous semi-papillate sporangia. They are pathogenic, with apparent canopy infection. The species appear to have distinct host "preferences" despite holding several hosts in common. They appear to be reproductively isolated even though their host ranges overlap and they are sympatric in Oregon. The wide distribution of *P. nemorosa, P. pseudosyringe,* and *P. pluvialis* in western Oregon, their scattered and generally low incidence, and their relatively non-aggressive pathogenic behavior suggest that they are indigenous here. The species diversity of *Phytophthora* in coastal Oregon and California is striking. Thirty or so species have been tallied, in part the result of the concentration of "phytophtherologists" in the region and the extensive sampling that has taken place, but for ecological and geographic reasons as well.

# Maternal effects mediate the resistance of *Quercus ilex* to *Phytophthora cinnamomi*

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The resistance of trees to diseases is increasingly recognised as being impacted by maternal effects, given that environmental conditions experienced by parent (mother) trees affect stress tolerance in offspring. We hypothesised that environmental maternal effects may mediate the resistance of *Quercus ilex* seedlings to *Phytophthora cinnamomi*. In December 2010, acorns from 15 declining *Q. ilex* trees infected with *P. cinnamomi* and from 15 non-declining trees free of infection were collected and weighted (40 acorns per tree). Pots (0.3 l vol) containing peat and sand were used and distributed in a randomized block design (N = 1200). In September 2011 seedlings were inoculated with *P. cinnamomi*. Acorns from non-declining trees were heavier than acorns from declining trees (4.4 and 4.2 g, respectively), and germination rates and dates did not differ significantly between acorns from declining and non-declining trees. Mortality rates, however, were lower and slower in seedlings from declining-infected trees in comparison to seedlings from healthy trees. The more resistant seedlings to *P. cinnamomi* were those collected from the more declining stand. Results indicate a transgenerational induction of resistance, possibly through epigenetic mechanisms non-dependant to acorn weight.

# Long term impact of *Phytophthora alni* on an alder riparian stand

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The evolution of riparian alder stands was monitored for 12 years on the Sarre river in eastern France between 2 villages (about 3000 alders on 4km of river). The recruitment of new seedlings, crown status, mortality and diameter growth of the trees were recorder annually. In 2010, the possibility of natural selection for resistance to P. alni in the stands was investigated. 39 healthy alders were cloned for further characterization, half of them present in 2002 as seedlings and having been under *P. alni* selective pressure since and half of them just recruited in the study in 2010. Ramets of these genotypes were inoculated with *P. alni* in 2013 in greenhouse conditions. The disease was very active in the stands with very high seedlings mortality rate, but much more progressive decline for larger trees. However, despite that, the total alder basal area increased slightly over the study period. The evolution of seedling recruitment showed a dramatic decrease over the 12 years that could not be explained by a canopy closure and increased competition. Finally, the alder genotype which had been exposed to *P. alni* selective pressure showed less infection after inoculation in greenhouse compared to those not exposed indicting that natural selection for resistance is occurring in the stands. The results are discussed for future of alder riparian stands in the area.

# The Interplay Among Human, Biotic and Abiotic Factors Explains Quick *Phytophthora cinnamomi* Spreading and Tree Decline in a Mediterranean Biosphere Reserve

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Alien invasion is one of the main threats to biodiversity and ecosystem integrity. In this work we analyze a case study in a Biosphere Reserve (Doñana National Park), located at SW Spain, which includes both aquatic and terrestrial endangered ecosystems (1, 2). We checked soil for *Phytophthora cinnamomi* presence under both symptomatic and asymptomatic centenarian oaks along 7 years (2008-2014).

Before 2008 there were no reports about the pathogen. In 2008/09 several infected trees were detected in an area recently afforested with seedlings grown in uncertified nurseries (3). An unusual climate event occurred in 2010: late winter/early spring rainfall rates exceeded all previous records, extending the period with flooding/high soil moisture towards warm late spring months. Soil infestation increased from ~20% to >70% of the surveyed trees. In 2014, the isolation frequency in soil raised to 99%. Spore densities in the infested soils quickly increased and, in 2013, it exceeded on average the infection threshold experimentally determined.

On the other hand, pathogen spreading and root infection have complex interactions with soil chemistry (4). Soil changes caused by tree nesting wading bird drops affected to different stages of the pathogen cycle in a different way. As long as nesting birds occupy a significant fraction of the oaks, they will probably have a significant effect on pathogen progression.

Unfortunately, neither individual (trunk injection based) treatments for the big centenarian oaks, nor prevention of afforestation with uncertified seedlings have been implemented by park managers, despite the recommendations of an international board of experts. Therefore, the inaction of forest protection authorities also results an important factor of the ecological consequences derived from *P. cinnamomi* extension.

**Acknowledgements**

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Esquel, Chubut. Patagonia Argentina

# Ecophysiology and Physiopathogenicity

# Screening *Quercus ilex* for tolerance to water stress and *Phytophthora cinnamomi*

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The health of forests in south-western Europe is conditioned by global change through direct rise in average temperature and variability of climate, with potential to increasethe occurrence of severe droughts. In consequence, programs focused on breeding treesfor resistance to pathogens should screen to drought stress too. The tolerance of 16 populations of *Quercus ilex* from five Mediterranean countries (France, Italy, Morocco, Portugal and Spain) to water stress and *Phytophthora cinnamomi* was assessed. The experiment included seedlings grown under greenhouse conditions and following a full randomized block design (12 mother trees per population; 20 seedlings per mother tree). During their first vegetative period, half of seedlings were submitted to a severe water stress treatment. During the second vegetative period, all plants were inoculated with *P. cinnamomi*. Plant mortality after the water stress treatment ranged from 0 to 40% and varied significantly between populations. Mortality after inoculations was about 10% higher in water stressed plants in comparison to non-water stressed plants. Survival time of seedlings varied significantly between populations and mother trees, being generally more resistant the populations from humid areas than the populations from semiarid and suhumid areas. Seed mass did not influence the tolerance of seedlings to water stress and *P. cinnamomi*. From about 3.300 *Q. ilex* seedlings tested, the most vigorous 100 plants were selected for further assessments. Breeding for adaptation to new climatic environments will be discussed.

# The spatial and temporal spread of *Phytophthora alni* subsp*. alni* in alder bark tissue – an ecophysiological study

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The impact of alder *Phytophthora (P. alni subsp. alni*) on corticular photosynthetic metabolism via measurements of chlorophyll fluorescence was explored. Ten weeks after stem-base inoculation the pathogen induced a sharp reduction of maximum (Fv/Fm ) and effective quantum yield of PSII (ΔF/Fm‟) within the visually detectable stem lesion. Observations of the axial as well as radial spread of the pathogen revealed that near to the point of inoculation and in the whole center of the tongue- shaped stem lesion Fv/Fm and ΔF/Fm‟ of the cortex chlorenchym decreased to almost zero, indicating tissue necrosis. Thereby, low values of Fv/Fm and ΔF/Fm‟ was also found in some pre-symptomatic regions beyond the visibly stem lesion. On the opposite substantial photosynthetic activity was found in uninvaded parts of the inoculated trees and in the control. These stem parts showed a marked light-adapted quantum efficiency of PSII as well as marked electron transport rates (ETR) in there bark tissues. Thus, corticular photosynthesis stayed unaffected in these stem parts supporting stem carbon balance. Additional chlorophyll fluorescence measurements in the field further illustrated that stem infection with *Phytophthora alni subsp. alni* and the effect on the bark tissues is not only highly heterogeneous but also underlies very quick temporal changes, due to a rapid destruction of the photosynthetic apparatus by the pathogen.

# Diterpene resin profile of *Austrocedrus chilensis* affected by *Phytophthora austrocedri*

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## Introduction

The exudation of resins, a mixture of monoterpenes, sesquiterpenes, and diterpene resin acids, is a major part of the constitutive defense mechanisms in conifers. It has been reported that the resins protect against pathogenic agents and insects among other types of damage (Phillips *&* Croteau, 1999; Franceschi et al. 2005). Resin can be also part of the inducible defense mechanisms, as in the case of traumatic resin ducts that are created *de novo* in response to wounds, insect damage or pathogenic fungi. The resin formed by traumatic ducts can be different from the constitutive resin (Martin *et al*., 2002; Miller *et al*., 2005, Nagy *et al*., 2000). The diterpene composition of the conifer *Austrocedrus chilensis* was recently reported (Olate et al., 2011), but there is not information about the variability on diterpene composition in this species under biotic or abiotic stress.

In the last decades, the Cupressaceae *Austrocedrus chilensis* (known as “ciprés de la cordillera”) has been suffering a devastating disease commonly referred as “mal del ciprés” or, recently, as “*Austrocedrus chilensis* root disease*”*. This disease is characterized by chlorosis, withering of the foliage, progressive defoliation, necrotic lesions in the phloem of roots and stem, and the death of the tree. It is caused by the pathogen *Phytophthora austrocedri* (Greslebin et al., 2007; Greslebin & Hansen, 2010).

There are few studies on antifungal activity of resins against *Phytophthora* spp. in spite of is a frequent response of trees to *Phytophthora* infection (Brasier & Webber 2010, Duran et al. 2008). *A. chilensis* profusely produces resin associated to *Phytophthora* lesions in phloem. Resin-pockets are usually developed below bark in the advance of the *Phytophthora* lesion (Greslebin & Hansen 2010). Whether this resin is a specific response to the pathogen infection or simply a structural unspecific response is still unknown. To elucidate this aspect the compounds of *A. chilensis* resin from healthy and diseased trees, and their antifungal activity was investigated.

## Materials and Methods

**Plant material.** Resin from *A. chilensis* trees, including healthy and *P. austrocedri*-diseased trees, was collected in Los Alerces National Park and in the Río Grande Valley. To further confirm possible changes in resin composition associated with the pathogen infection, resin from artificially inoculated *P. austrocedri* individuals (controlled conditions) were also included in the study

**TLC analysis and isolation of the resin constituents, GC-MS and NMR analysis.** The composition of the different resin samples was first assessed by analytical TLC using silica gel as stationary phase and PE:EtOAc 80:20 (v/v) as eluent. After TLC comparison, representative samples were selected for GC-MS and NMR studies. The resin constituent profile by GC-MS after derivatization and $^{1}$H-NMR analysis of underivatized samples were compared to establish differences between groups.

**Antifungal activity assays.** Fractions (1 mg) obtained by preparative TLC were dissolved in ethanol and added to sterile dishes containing the culture medium. From the nine fractions obtained (Z1 to Z9) five were selected to test the antifungal activity. A 5 mm plug from the margin of an actively growing colony of *Phytophthora austrocedri* was then added to the central part of the dish and the radial growth of the pathogen was measured after 14 days.

## Results and discussion

The chemical composition of resins from healthy *A. chilensis* trees showed a clear different profile compared to that of the *P. austrocedri*-infected individuals, including naturally infected and artificially inoculated trees (**figure 1**). The resins derived from the infected trees showed a diagnostic peak at Rt 14.17 min (**figure 1**). The mass fragmentation pattern of the compound is in agreement with the diterpene manool.

From the five fractions tested for the antifungal activity (Z1, Z2, Z3, Z4 and Z6), only Z4 and Z6 showed a statistical reduction in the mycelial growth of the pathogen (**figure 2**).

Manool was detected in the Z3 fraction of the resin, which showed a slight inhibitory effect on *P. austrocedri* growth (**figure 2**). The role of this compound in the defense strategy of *A. chilensis* to *P. austrocedri* infection deserves further studies. Even the low inhibition rate of Z3 fraction, the detrimental effect of manool could not be discarded since this compound is not the main representative diterpene present in the fraction and thus, a matter of concentration of the component could have an effect.

The main component of the most active resin fraction Z6 (**figure 2**), is an oxidized product of manool, 18-hydroxymanool. This compound can be further oxidized to the aldehyde (torulosa**l**) and to a carboxylic acid which was found to be the major constituent of the Z4 fraction.

The results suggest that different oxidation products from the diterpene manool play a role in the antifungal effect of the resin. Further studies should be carried out to disclose if the oxidation of manool is a host response or if the derivatives are formed via microbial oxidation of the parent diterpene.

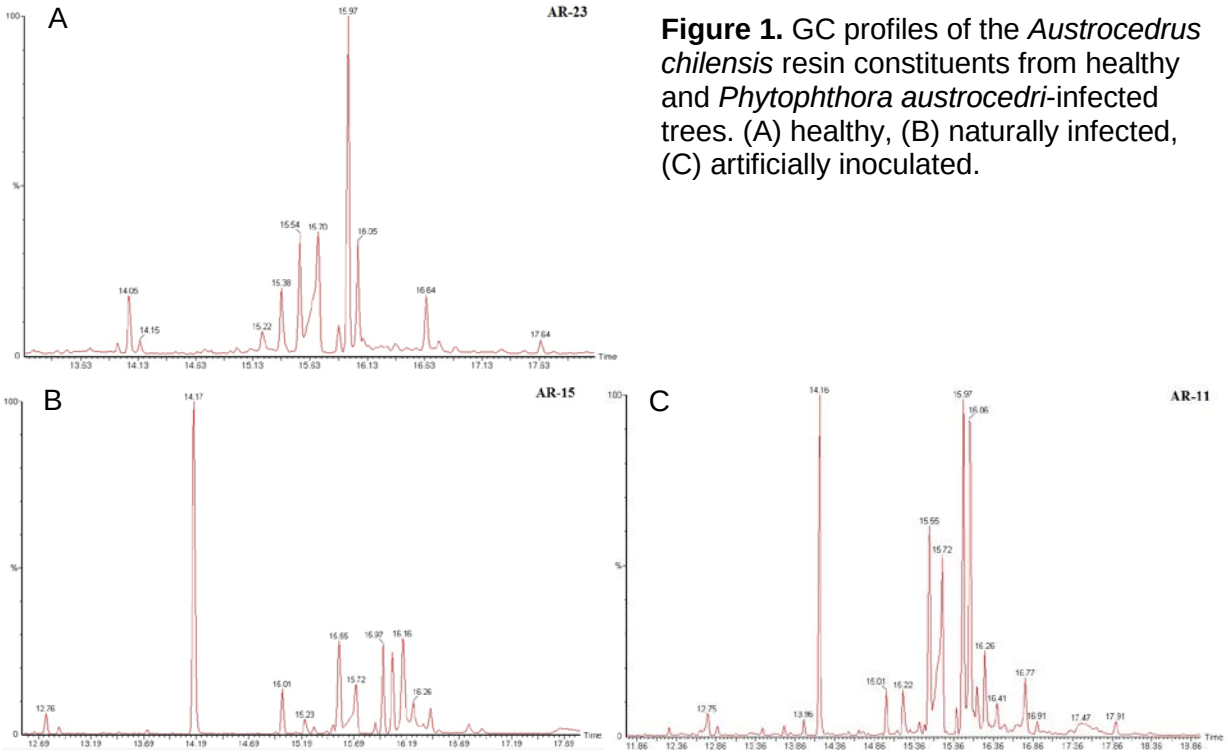



**Figure 1.** GC profiles of the *Austrocedrus chilensis* resin constituents from healthy and *Phytophthora austrocedri*-infected trees. (A) healthy, (B) naturally infected, (C) artificially inoculated.

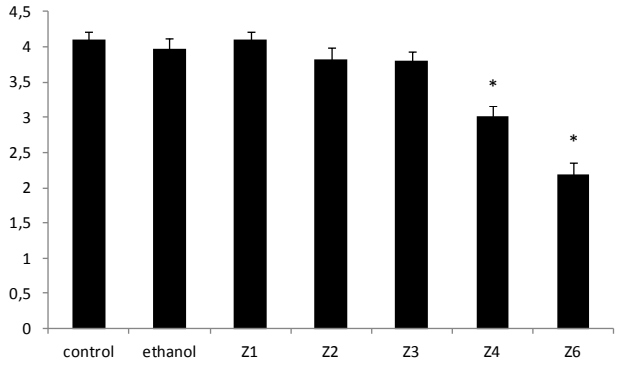



**Figure 2.** Antifungal effect of *Austrocedrus chilensis* resin fractions. 1 mg of each fraction obtained by preparative TLC were dissolved in ethanol and added to sterile dishes containing the culture medium. A 5mm plug from *Phytophthora austrocedri* colony was added and the radial growth of the pathogen was measured after 14 days. (*p<0,001 vs etanol, Tukey test).

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Phillips, M.A., and Croteau R.B. 1999. Resin-based defences in conifers. Trends in Plant Science 4:184-190**.**

# Effect of cinnamomins on *Phytophthora cinnamomi* biomass growth and on the oxidative burst in infected *Quercus suber* roots

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In previous work we have evaluated the effect of elicitins [cryptogein, capsicein, α-cinnamomin (α-CIN) and β-cinnamomin (β-CIN)] on the infection of *Fagaceae (Quercus suber, Q.ilex, Castanea sativa*) by *Phytophthora cinnamomi* (Medeira *et al.* 2012a; Medeira *et al.* 2012b, Horta *et al*. 2010) and shown, namely through histological and ultra-structural studies, assessment of changes in fatty acid composition of roots and leaves and of gas exchanges and fluorescence analysis, that they trigger defence reactions against the pathogen, contradicting the belief that with the exception of Nicotiana, most plant species lack the capacity to respond to elicitins (Kamoun *et al.* 1993; Grant *et al.* 1996; Ponchet *et al*. 1999). Their action is not genus- or cultivar-specific and not only elicitins from *Phytophthora* but also from *Pythium*, like oligandrin play this role. And this, without detriment to other demonstrated roles such as being essential players in the colonization process and being sterol transporters.

In this study, we applied two other approaches to evaluate the effect of cinnamomins, on the infection process of cork oak roots by *P. cinnamomi*: quantification of pathogen biomass and measurement of ROS and antioxidant enzymes during initial responses of *Q. suber* roots.

**Quantification of pathogen colonization**

A quantitative real-time PCR (QPCR) assay following a method described by Eshraghi *et al.* (2011) was performed to study the effect of α-CIN and β-CIN on the colonization of *Q. suber* roots by *P. cinnamomi.* Normalization of the pathogen DNA was done based on a purified plasmid DNA, containing the myelocytomatosis oncogene (Myc) of mouse as an internal control rather than host DNA to avoid any bias introduced by the variation of host DNA during *Q. suber-P. cinnamomi* interaction due to necrosis and degradation. Plasmid and *P. cinnamomi* primers were found to be specific for their target DNA templates, Myc and PDN gene, respectively.

DNA levels of the pathogen in infected *Q. suber* roots relative to the internal plasmid were evaluated for 24 h through standard curves using QPCR. Root colonization by *P. cinnamomi* started 6 h after infection and gradually increased up to 24 h. Observations of distinct levels of *P. cinnamomi* infection in the cork oak roots during the infection period was showed to be reproducible. The major increase in *P. cinnamomi*/plasmid DNA ratio occurred between 18 hpi and 24 hpi (Fig. 1).

The assay that was implemented for the quantitative measurement of *P. cinnamomi* DNA in *Q. suber* roots was applied to roots pretreated with α-CIN and β-CIN in order to assess the elicitor effect of these cinnamomins in this pathosystem. As shown in Fig. 2 a significant reduction of *P. cinnamomi* infection in roots treated with 0.5 µM and 10 µM solutions of these cinnamomins was observed.

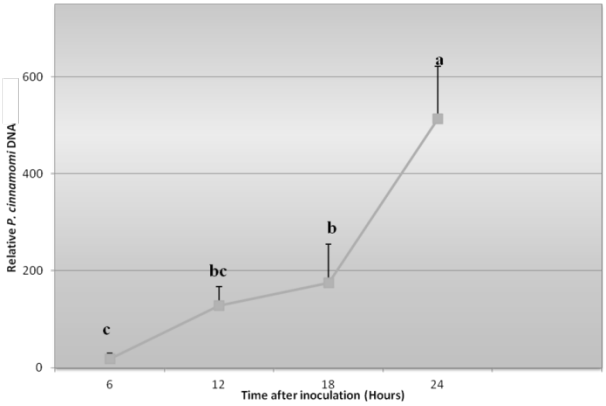



**Fig. 1.** Temporal colonization profile of *Phytophthora cinnamomi* in cork oak roots. The progression of pathogen colonization is expressed as the variation with time of the ratio between the amount of *P. cinnamomi* DNA and the amount of plasmid DNA. Error bars represent the standard deviation of four biological replicates. The different letters indicate a significant difference at 0.05 level.

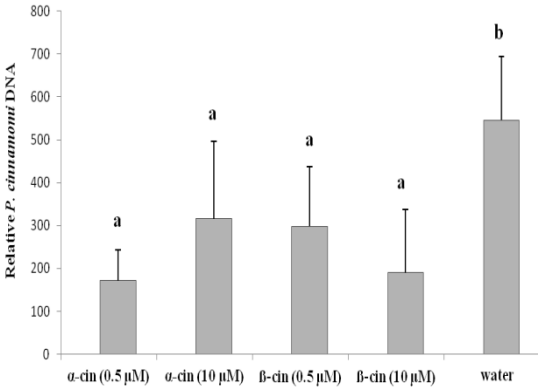



**Fig. 2.** Relative qPCR quantification of *P. cinnamomi* biomass in elicitin-treated and non-elicitin-treated *Quercus suber* roots infected during 24 h at the shown concentrations. Different letters (a, b) indicate significant differences between treatments. Error bars represent the standard deviation of four biological replicates. The mean difference is significant at the 0.05 level.

## Quantification of ROS and activity of POD and ROS-scavenging enzymes

The production of reactive oxygen species (ROS) $H_2O_2$ and $O_2^{\bullet-}$ was measured at 6, 12, 18, 24, 36 and 48 h and POD, SOD and CAT activities were determined during the time course of infection with *P. cinnamomi* mycelia in 2-month-old roots before and after α-CIN treatment (Figs. 3 and 4). $H_2O_2$ peaked at 18 and 24 h in the infected roots in close agreement with the observed temporal colonization profile of *P. cinnamomi.* The pathogen induced the production of radical superoxide anion as well, with the first significant increase observed at 24 h, but it was followed by further accumulations at 36 and 48 h after infection. Pretreatment with α-CIN resulted in a significant reduction of ROS accumulation in infected roots, during all the time course of infection for $O_2^{\bullet-}$ and namely at times 18 and 24 hpi for $H_2O_2$, when its production peaked. Therefore, lower level of ROS production after α-CIN treatment suggests the elicitin exerts a protective effect through a reduction of host cell damage.

The SOD activity in infected cork oak roots increased strongly when compared with the control suggesting an important role of this enzyme in coping with ROS produced by cork oak roots during infection. SOD activity increased in roots pretreated with α-CIN before inoculation when compared with the corresponding control, following the same

time course pattern. The CAT activity was stimulated significantly upon pretreatment with α-CIN when compared with the control group assuring a more effective contribution to the reduction of $H_2O_2$ content. The activation of CAT is an important contribution to the protection of host plant roots against overproduction of $H_2O_2$. POD activity was found to be higher in infected plants in comparison with the healthy ones in agreement with observations in avocado roots infected with *P. cinnamomi* (García-Pineda *et al.* 2010). In α-CIN pretreated roots the activity of POD underwent a further remarkable increase as compared with controls. POD is believed to play a significant role in conferring resistance to pathogens through its action on lignification. Cinnamyl alcohol dehydrogenase (CAD) genes have been shown to be up-regulated in *Q. suber* root seedlings infected with *P. cinnamomi* (Coelho *et al.* 2006, Ebadzad and Cravador 2014) suggesting that the observed POD activity increase is related with the participation of this enzyme in the oxidation of the CADs.

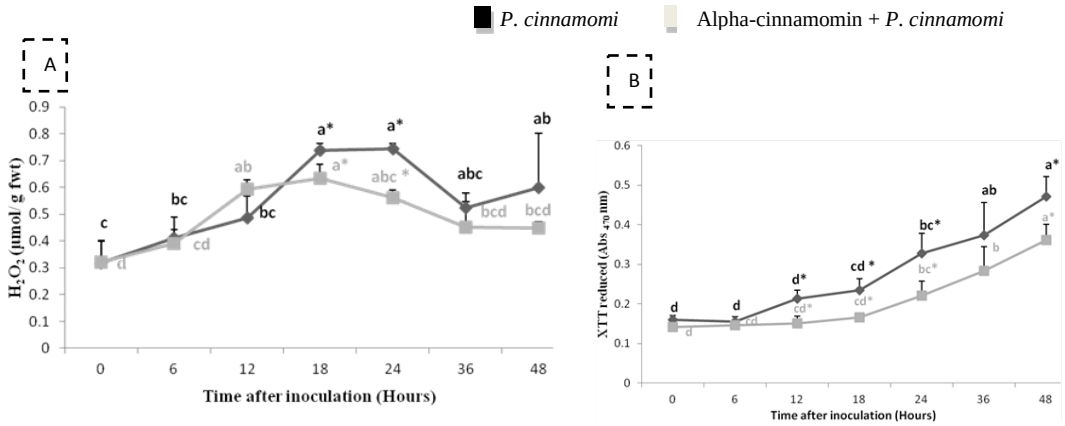



**Fig. 3** Kinetics of accumulation of reactive oxygen species (ROS) induced in cork oak roots submitted to two treatments: inoculated with *Phytophthora cinnamomi* without or with a pretreatment with α-CIN for 24 h. Non-inoculated plants in each treatment were used as a control. **A** $H_2O_2$ quantification. **B** $O_2^{\bullet -}$ accumulation measured by XTT [sodium 3′ -[1-[phenylamino-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate] reduction. Statistically significant differences between infected and control plants for each treatment are indicated by different letters while asterisk shows significant differences between α-CIN treated and non-treated samples at each time point with $P<0.05$. Error bars represent the standard deviation for three biological replicates.

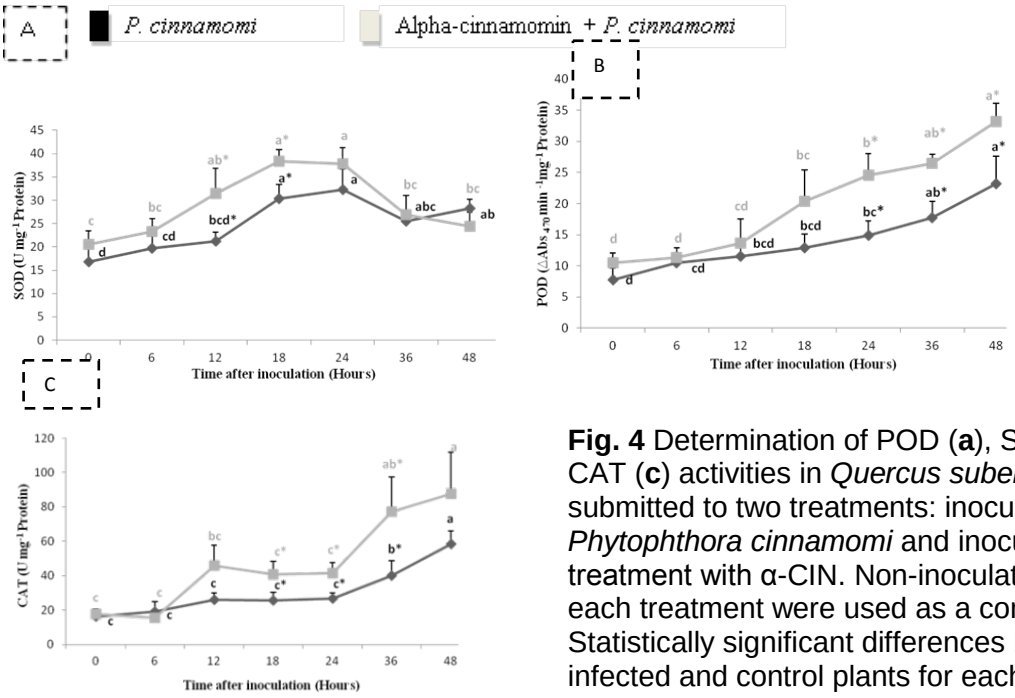



**Fig. 4** Determination of POD (**a**), SOD (**b**) and CAT (**c**) activities in *Quercus suber* roots submitted to two treatments: inoculated with *Phytophthora cinnamomi* and inoculated after pre-treatment with α-CIN. Non-inoculated plants in each treatment were used as a control. Statistically significant differences between infected and control plants for each treatment are indicated with different letters while asterisk shows significant differences between α-CIN treated and non-treated roots at each time point with $P<0.05$. Error bars represent the standard deviation for three biological replicates.


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# *De Novo* Assembly of *Phlomis purpurea* Transcriptome challenged with *Phytophthora cinnamomi*

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*Phlomis purpurea* is a perennial evergreen shrub that grows spontaneously in Mediterranean ecosystems of south Iberian Peninsula. It can be found in Algarve, Portugal, in *Quercus suber* (cork oak) and *Quercus ilex* (holm oak) stand habitats severely infested by *Phytophthora cinnamomi.* It is resistant to *P. cinnamomi*, since this pathogen did not cause any visible symptoms and was never isolated from infested roots. The *P. purpurea* crude root extracts have shown activity against *P. cinnamomi* and to protect susceptible hosts against infection by the pathogen *in planta* (Neves *et al*. 2014).

The molecular mechanisms underlying defence responses are unknown in the *Phlomis* genus. Characterizing changes triggered by the pathogen in gene expression in the resistant plant would provide insights into, when and where each gene is expressed and would offer a glimpse at the strategy used by *Phlomis* to oppose *Phytophthora*.

We used high-throughput deep sequencing technology to profile the *P. purpurea* transcriptome using the Illumina HiSeq™ 2000 platform. A total of 1,272,600,000 reads from 18 libraries were merged with FLASH and *de novo* assembly with Velvet Oases. For the subsequent differential expression analysis, BWA was used to map each sample to the assembled reference. The assembly produced 215,739 unique transcripts with a mean length of approximately 200 bp. Sequence similarity search against Nr NCBI database identified a total of 124,386 unique annotated transcripts (57.7%) with significant hits. Functional annotation identified 83,550 out of 124,386 unique transcripts, which were mapped to 141 pathways. The majority of these annotated unigenes (48,711out of 83,550) displayed the highest homology to genes from plants (Fig. 1). The assembly revealed many transcripts that were previously unknown. Following their Nr annotations, the unigenes were mapped into the records of the GO (gene ontology) database and GO annotations retrieved for 83,550 of them. These unigenes were assigned GO terms from the three main categories, including 27,053 with terms from “Biological processes”, 23,541 with terms from “Cellular component”, and 32,956 with terms from “Molecular function” (Fig. 2). Among them, 31,624 unigenes had an assignment in the three categories. The remaining unigenes [61.3% (215,739-83,550)] failed to obtain a GO term, largely due to their uninformative (e.g. unknown, putative, or hypothetical) protein description. Within the ‘Biological process’ category, the two most abundantly represented functions correspond to metabolic and cellular processes. A

large number of genes are involved in primary metabolic processes, cellular processes and response to stimulus, Most of unigenes of the “Cellular component” category have functions located in the cell membrane and cytoplasmic components: intracellular organelle membrane-bounded. In the “Molecular function” category, the genes covering binding, catalytic and transporter functions are predominantly represented. The comparison of differential gene expression profiles was conducted between 18 cDNA libraries from *P. purpurea* plants challenged with *P. cinnamomi* at six post inoculation time points (hpi: 0, 6, 12, 24, 48 and 72 h). Out of 48,711 total annotated genes displaying the highest homology to genes from plants, 1,558 were down regulated and 3,755 were up regulated. The differential expression patterns among libraries revealed that the largest differences in expression occurred in the interval between 6 and 24 h (Fig. 3). Between 24 h and 48 hpi no significant difference expression was found, but between 48 and 72 h a slight difference in expression occurred. Moreover, a large number of specifically plant fungi interaction transcripts, were differentially expressed.

To assess the reliability of our sequencing-based approach in identifying *Phytophthora*-responsive genes, we monitored the expression of candidate differential expressed genes and performed qPCR for 10 candidates (wall-associated receptor kinase 5-like, cinnamyl alcohol dehydrogenase, hydroxyproline-rich glyco protein, cyanidin-3-O-glucoside 2-O-glucuronosyltransferase-like, resistance protein rgc2, subtilisin-like protease, calcium-binding mitochondrial carrier protein aralar1-like, serine-threonine protein plant, acetyl-coenzyme A synthetase, phosphate-repressible phosphate). The expression profiles of eight candidates were in agreement with the predictions from the IIlumina sequencing results.

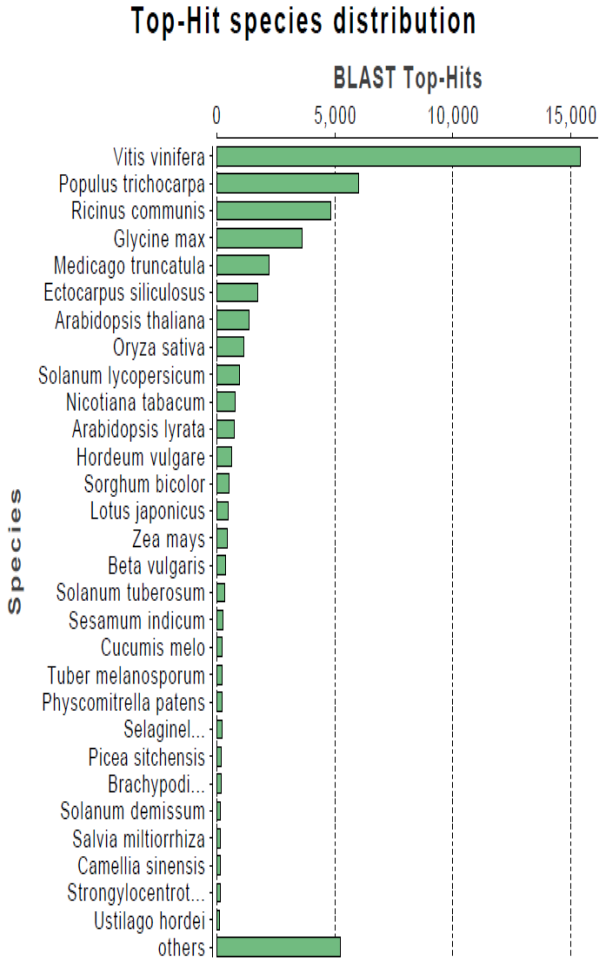



Fig 1. Homology of annotated *Phlomis purpurea* unigenes to genes from plants.

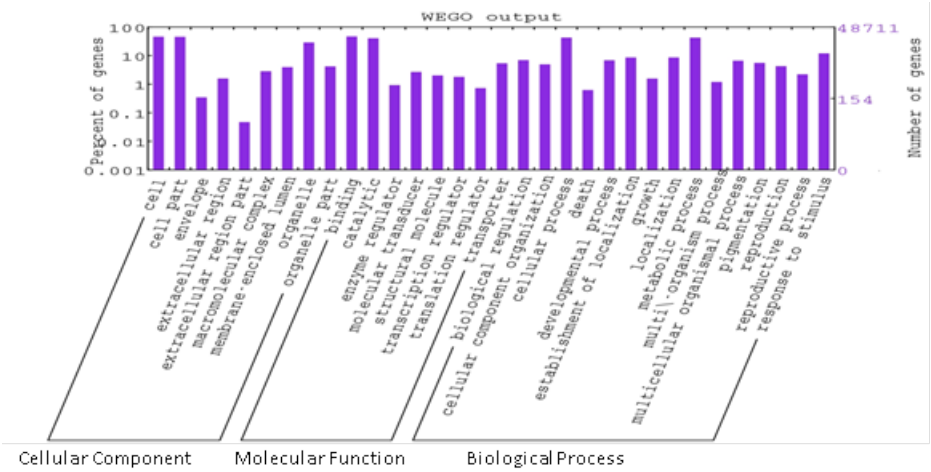
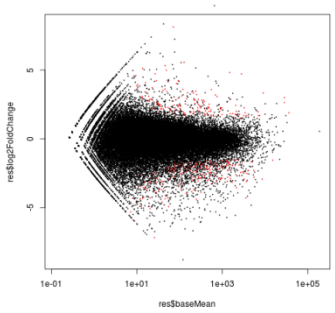
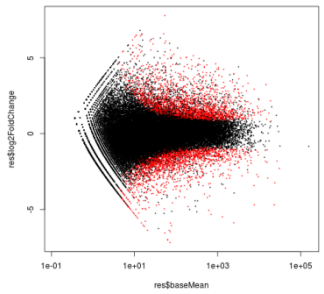


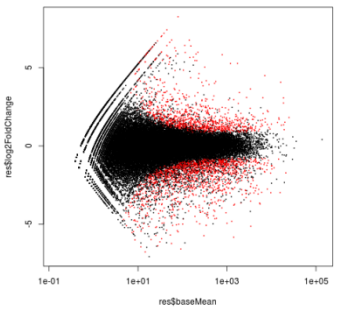

Fig. 2. Gene ontology Slim assignments for Phlomis purpurea transcripts. Proportion of annotated unigenes from Phlomis purpurea ESTs that matched various gene ontology (GO) categories.



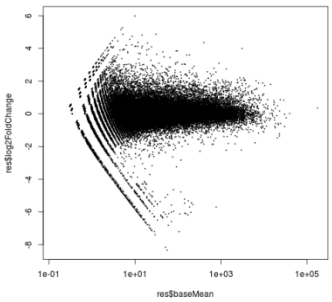

**A**. Normalized mean between zero and 6 hours



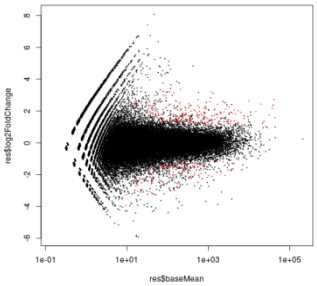

**B.** Normalized mean between 6 and 12 hours




**C.** Normalized mean between 12 and 24 hours




**D**. Normalized mean between 24 and 48 hours




**E.** Normalized mean between 48 and 72 hours

Fig. 3 Plot of normalized mean versus log2 fold change for the contrast between *Phytophthora cinnamomi*-untreated versus *P. cinnamomi*-challenged *Phlomis purpurea*, coloring in red those genes that are significant at 10% Red dots mark contigs detected as being significantly differentially expressed at a 10% false discovery rate with Benjamini–Hochberg multiple testing adjustments ($P < 0.01$).

The unique transcripts derived from this work will provide in the near future information that can be further used in gene expression, biological function, genomics and functional genomics studies.

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# Screening of Asian oak species for potential resistance to *Phytophthora cinnamomi*

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In the past 20 years various studies have demonstrated the involvement of *Phytophthora cinnamomi* and, to a lesser extent, other *Phytophthora* species in the widespread complex declines of *Quercus* suber and *Q. ilex* in Portugal, Spain and the southern parts of France and Italy. The progressive fine root losses by *P. cinnamomi* interact with droughts causing a slow chronic decline. Prolonged droughts and collar infections by *P. cinnamomi* after heavy unseasonal rain can cause rapid and dramatic mortality. Given the modelling projections of a warming climate with an increasing frequency of heavy rain events and prolonged droughts a further intensification of *Phytophthora* activity and root losses is most likely which in turn will enhance the vulnerability of the affected ecosystems to the climatic extremes. On the long-term, increasing the genetic resistance to *P. cinnamomi* in *Q. suber* and *Q. ilex* seems to be the most promising management approach for stabilising the Mediterranean oak ecosystems against the interaction between *Phytophthora* and climatic extremes.

Several evidences point to the hypothesis of Southeast Asia as the center of origin of both mating types of *P. cinnamomi*. High levels of sympatric resistance to *P. cinnamomi* might be expected in co-evolved *Quercus* spp. from Southeast Asia as compared to the high susceptibility of the European *Q. suber and Q. ilex*. In an ongoing research project Asian oak species are being screened for potential resistance to *Phytophthora cinnamomi*. Soil infestation trials with six oak species from Taiwan and the analysis of the transcriptome of infected and healthy roots of the two most promising oak species are in progress. The results obtained in these experiments will be presented and discussed. The finding of resistant oak species could be the basis for future breeding programmes, the development of molecular markers for screening European oak populations for resistance to *P. cinnamomi* and for developing resistant varieties of susceptible species by genetic engineering.

# Recent advances in understanding *Phytophthora*-woody plant interactions

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Within the last decade the so called "-*omics*"-approaches– such as gen*omics*, transcript*omics*, prote*omics* and metabol*omics* – found their way into life sciences, due to newly developed techniques allowing high throughput of samples and data. With some offset in time, these approaches are now also used for research on *Phytophthora*-plant interactions. First comparative genome analyses uncovered an unexpected large arsenal of effector genes in *P. ramorum* (Tyler et al, 2006). Right now, genome sequencing of up to 150 *Phytophthora* isolates is ongoing, including many species pathogenic on woody hosts. The rapid development of microarrays and of *next generation sequencing* technologies enabled the study of a broad spectrum of differentially expressed genes during pathogenesis, both of the host and the pathogen, deepening our understanding of these interactions on the molecular level. The development of modern mass spectrometric techniques allows the rapid identification of proteins and metabolites, the real players in plant-pathogen interactions. All these techniques generate a so far unknown amount of data, challenging the field of bioinformatics to reliably identify relevant information. The recent literature on these "-*omics*"-approaches on *Phytophthora*-woody plant interactions will be summarized and their possible implications on the control of *Phytophthora* diseases will be discussed.

**References**

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10th · 14th November 2014
Esquel, Chubut. Patagonia Argentina

# Management and Control

# Challenges associated with the management of *Phytophthora* diseases in Australia and the importance of community engagement for success

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*Phytophthora cinnamomi* is listed as a "Key Threatening Process to Australia's Biodiversity" by the Commonwealth Government, consequently there is a national threat abatement plan (TAP) in place. The TAP establishes a national framework to guide and coordinate Australia's response to *P. cinnamomi*. It sets out the actions necessary to abate impacts of this key threatening process, and identifies the research, management and other actions needed in Australia's response to this pathogen. The success of this TAP depends on a high level of cooperation between all key stakeholders. We will discuss how different stakeholders have met the challenges of identifying and mapping the pathogen across the landscape, taking into account other *Phytophthora* species, global change, other environmental priorities, the need for prioritizing areas that are „protectable" over the next 50-100 years and the importance for societal engagement to ensure uptake. A number of case studies from the Commonwealth, State, non-government organizations, "friends of groups" and industry will be provided to show how different stakeholders have engaged in attempts to meet the objectives of the TAP. We will discuss how research can help guide and invigorate different stakeholders in the process of managing and containing the spread and impacts of this pathogen across different landscapes. Lastly, examples of the importance of working with community to ensure uptake of processes and procedures will be highlighted, together with the associated challenges.

# Continued Monitoring of Sudden Oak Death Treatments in Oregon Tanoak Forests

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*Phytophthora ramorum*, the cause of sudden oak death, was first identified in tanoak forests in coastal Southwest Oregon in 2001. Since that time, treatments using a combination of herbicides, cutting, and burning affected and exposed vegetation have been done to initially, eradicate the pathogen, and more recently, slow the spread of the disease. Monitoring done in 2010 of treatments completed through 2008 showed that 63 percent of treatment plots were negative for *P. ramorum* in sampled soil and vegetation, 25 percent of plots were positive for *P. ramorum* in sampled soil only, 7 percent were positive in both soil and vegetation, and five percent of monitored treatment plots were positive for the pathogen in vegetation only. *P. ramorum* hosts persisted, at some level, on all treatment plots. Our current monitoring effort is focused on two questions: Does *P. ramorum* recur or persist in sprouting and seedling vegetation?, and Does *P. ramorum* spread plant to plant on sites where it recurs? These concepts are of particular interest in that we have treated sites within a larger geographic area where treatment no longer occurs. Work is ongoing to revisit monitoring plots previously assessed, tally vegetation cover, and sample symptomatic vegetation for *P. ramorum*.

# Sudden Oak Death in Oregon Forests: Recent Disease Intensification and Spread, and Changes to the Management Program

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**Abstract:**
Sudden Oak Death, caused by *Phytophthora ramorum,* is lethal to tanoak (*Notholithocarpus densiflorus*) and threatens the species throughout its range in Oregon. Since July 2001, an interagency team has been attempting to eradicate and slow spread of disease through a program of early detection and destruction of infected and nearby host plants. Because of increasing disease, high eradication costs and limited funding, all infested sites cannot be treated equally. Highest priority for treatment are sites located at or beyond the leading edge of the infestation or near the quarantine boundary. Within a 145 km$^2$ Generally Infested Area near the center of the quarantine area most sites have not been treated and the disease has been allowed to intensify and spread. Where eradication treatments have stopped, canopy tanoak mortality increased from nearly zero to 50 percent during the 2012-2014 period. Changes to the sudden oak death management program are discussed.

## Background

Sudden Oak Death is caused by *Phytophthora ramorum,* a non-native pathogen that has become established in a coastal California and a very small part of southwestern Oregon, and nowhere else in North America. It poses a great risk to tanoak (*Notholithocarpus densiflorus*) ecosystems in Oregon and California, and to forest ecosystems elsewhere in the U.S. and abroad. Quarantine regulations and loss of markets (due to perceived risk) impact both the nursery and forest industries.
The disease was first discovered in coastal southwest Oregon forests in July 2001 (Hansen et al 2008). Mandatory eradication of *P. ramorum* by cutting and burning infected and nearby host plants began in the autumn of 2001 under the statutory authority of the Oregon Department of Agriculture (ODA). During the next several years an interagency team attempted to eradicate the pathogen through an aggressive program of early detection and destruction of infected and nearby host plants. By 2010 it was clear that compete eradication was not feasible, and the goal shifted to slowing spread (Kanaskie et al 2011). In 2013 the quarantine area was increased to 660 km$^2$ and a Generally Infested Area (GIA, 145 km$^2$) was established in which *P. ramorum* eradication is no longer required by the State. Trees have been dying at an alarming rate on private land inside the GIA, increasing the risk of wildfire and damage from falling trees.

Post-eradication monitoring has shown that cutting and burning host plants eliminated the pathogen from approximately 50 percent of infested sites. Cutting and burning new

infestations before the disease can intensify also slows spread of disease across the landscape. Large treatment areas (100 to 200 m buffer around infected trees) are more effective at slowing disease spread than smaller treatment areas. Because of the eradication program, the rate of disease spread in Oregon is slower than in similar areas in California where there is no comprehensive control program.

Only the NA1 lineage of *P. ramorum* has been found in Oregon forests. Genetic evidence suggests two separate introductions to Curry County; one near the infestations found in 2001 near Brookings, and the other approximately 28 km to the north, near Cape Sebastian. The most likely source was nursery stock from California.

From 2001 to 2014 the Oregon sudden oak death program in forests has cost more than $16 million (State, $3 million; Federal, $13 million). Economic analyses of the impact of *P. ramorum* on nursery and forest industry conducted by Entrix, Inc. and Oregon State University in 2009 (Hall 2009) concluded that every dollar spent on control or eradication results in a benefit of $2.70 to $19.67 to the industries. The benefit was due to delayed costs associated with compliance with quarantine regulations. The analyses did not consider ecological impacts, risk of wildfire, loss of markets, or costs associated with widespread tree mortality.

**Disease Spread and Intensification**

From the original infestations of 2001, sudden oak death has been found 32 km to the north and 13 km to the east, although the farthest of these infestations have been cut and burned and the pathogen may not have become established. The area in which the disease is established (the GIA) is approximately 22 km north-south and 10 km east-west (figure 1). Maximum distance of natural spread (no evidence of human assistance) in any given year appears to be 5 to 7 km.

Large areas of tanoak mortality now exist in areas where the disease was not treated. The high level of inoculum in these areas increases the probability of long-distance spread naturally and by people. In contrast, when all tanoak trees were cut and burned promptly within 100 to 200 m of infected trees, disease did not intensify and in many cases was eradicated.

High-resolution aerial imagery taken in 2012, 2013, and 2014 was used to estimate mortality of dominant and co-dominant tanoaks on private land in the western part of the GIA where eradication treatments were stopped several years ago (figure 1). In these areas tanoak mortality increased from nearly zero to 50 percent in the 2012-2014 period (figure 2). Elsewhere inside the GIA, on USDI-Bureau of Land Management (BLM) land where eradication has been ongoing, disease intensification has been negligible (but unmeasurable because all infestations were cut and burned).




Figure 1. Mortality of dominant and co-dominant tanoaks killed by sudden oak death in a one-ha sample block inside the Generally Infested Area near Brookings, Oregon. Hi-resolution (30 cm) digital images taken in August, 2012 (left) and July, 2014 (right).

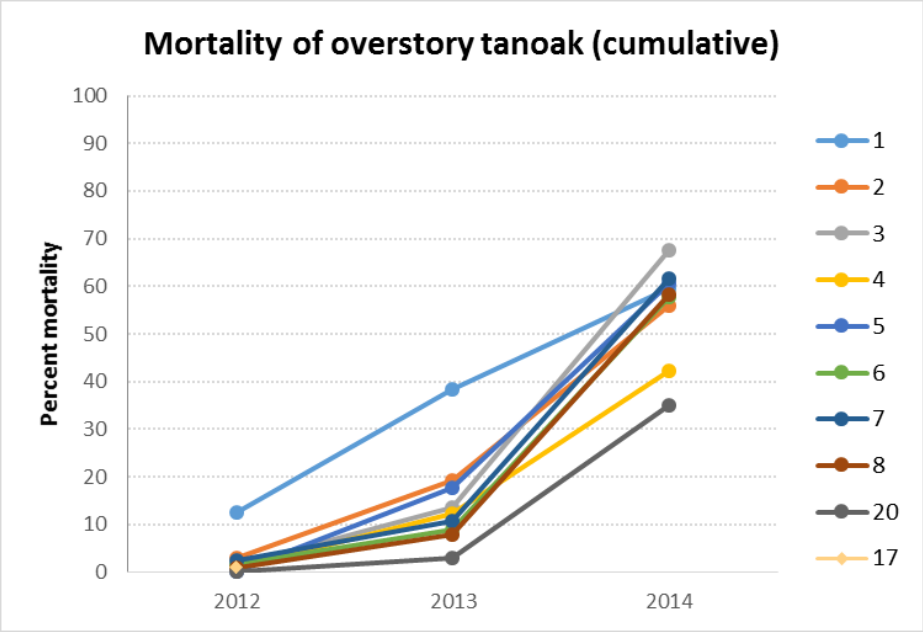



Figure 2. Percentage of dominant and co-dominant tanoaks killed by sudden oak death in 10 one-ha sample blocks located in infested areas that have not received eradication treatments. Each line represents one sample block. Mortality was estimated from digital hi-resolution (30 cm) color aerial imagery collected in July/August each year.

The 2014 aerial and ground survey results showed considerable expansion of the disease. Two large infestations were found at the northern edge of the GIA and 12 other infestations were widely distributed, mostly to the north. Several of these were 5 to 6 km distant from any previously known infestations.  Only one new infestation was found on USDA-Forest Service (USFS) land. It was located along the Chetco River one km inside the eastern boundary of the quarantine area (figure 3).

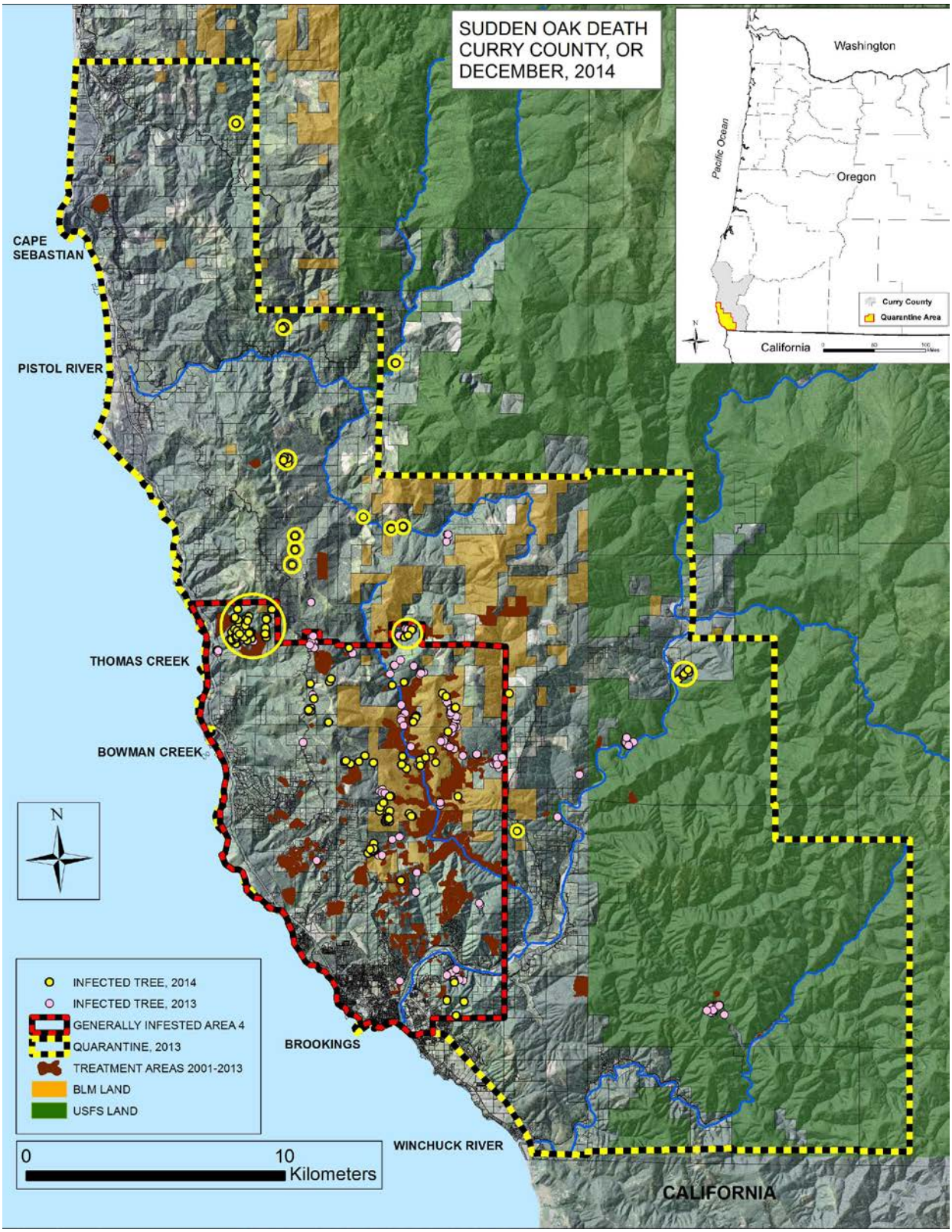



Figure 3. Location of sites infested with *Phytophthora ramorum* in southwest Oregon discovered in 2013 and 2014. Yellow circles indicate high priority for eradication treatments. Sites enlarged for visibility.

## Eradication Treatments in 2014

The BLM and USFS continue to cut and burn all infestations on their ownerships. Due to declining funds for eradication treatments on private land (figure 4), treating the large infestations at the northern edge of the GIA was not possible, so the GIA was expanded to include them. The remaining 12 infestations outside of the GIA are receiving modified treatments to stay within budget. The northernmost infestation received the full cut and burn treatment within a 100 m radius of infected trees. For the next two infestations to the south, the central 15 m radius was cut and burned and the remainder of the site was cut and lopped with no burning. All other sites will be treated by cutting and burning tanoak within 5-15 m of infected trees. The rationale for cutting smaller buffers, as opposed to doing nothing, is that cutting infected and nearby trees reduces the amount of pathogen inoculum available for intensification and spread.

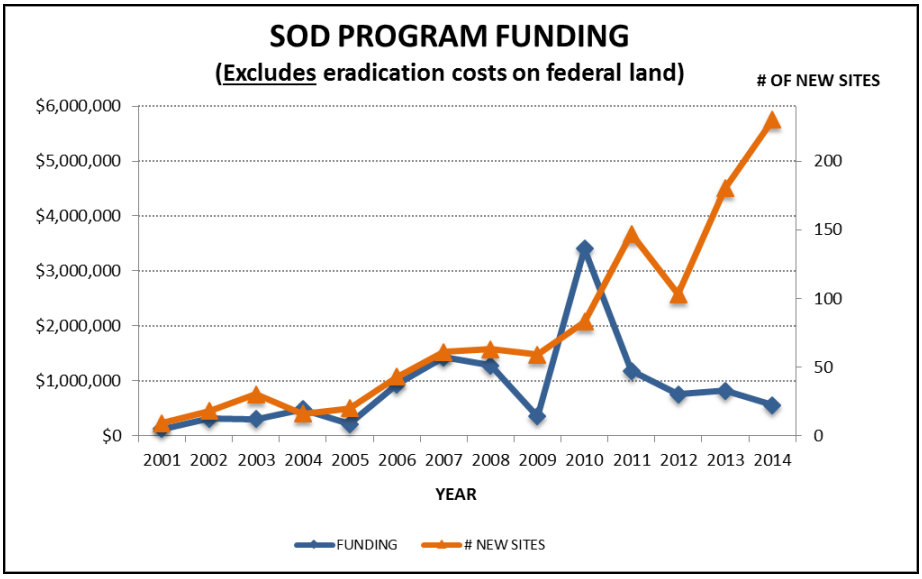



Figure 4. Total funding for sudden oak death program on non-federal land in Oregon (detection, monitoring, administration, and eradication treatments) compared to amount of disease. Blue line is funding, orange line is number of new infested sites. Excludes eradication expenditures on federal lands (BLM and USFS).

## Structure and Cost of Current Program

The current program slows disease spread using early detection and localized eradication to reduce natural spread, and quarantine regulations and landowner education to prevent human-assisted spread. On federal land, The BLM and USFS continue to treat new infestations by cutting and burning host plants within 100 m of infected trees. On private land, where most new infestations have occurred, limited

funding requires reducing the size of the treatment areas and other modifications. Treatments range from cutting and burning an infected tree and its nearest neighbors (0.04 ha) to cutting and burning all host plants within 100 m of infected trees. The GIA is expanded when large infestations are found near its edge and there are insufficient funds to treat them.

Program functions are shared among 5 organizations: Oregon Department of Forestry (detection and monitoring lead, eradication on non-federal land, landowner assistance); USFS (eradication on USFS, detection and monitoring, assistance to federal agencies); Oregon State University (OSU, laboratory diagnostics and support for all surveys); BLM (eradication and related work on BLM land), and; ODA (regulations, quarantine, monitoring).

ODF operations are managed by the forest pathologist in Salem, plus two foresters located in Brookings. Brookings staff conducts surveys, administers contracts, and assists landowners. USFS operations are managed by the zone pathologist in Medford, plus a forester in Gold Beach. BLM operations are managed by foresters in their Coos Bay office. OSU laboratory support is provided primarily by the Everett Hansen laboratory, while the Nik Grunwald laboratory determines genetic lineage and conducts genotype analysis.

The annual cost in 2014 for the entire program of detection and eradication (all agencies combined) was $1.7 million. Average cost to cut and burn infested sites in 2014 ranged from $6,000-$12,000 / ha.

**The Future**

Sudden oak death is now firmly established in the tanoak forests of southwest Oregon and will be a long term forest health problem. The current disease management program structure and funding schemes are a continuation of the emergency response that began in 2001, but they may not be appropriate from this point forward.

The consequences of continuing the current program at current funding levels are becoming clear. In areas where treatments have stopped, disease intensifies rapidly and kills most of the tanoaks in just a few years. As more inoculum is produced in the areas of uncontrolled disease, the leading edge of the main infestation expands northward and eastward, and the probability of human-assisted spread increases. Each year outlier infestations become more numerous and occur farther from the leading edge. Scaling treatment area size to importance of an infestation allows the most important infestations to be cut and burned, which slows disease spread relative to no treatment. Under this scenario, it is likely that the disease will spread northward by as much as 5 to 7 km per year, but eradication may delay establishment at those distances. The quarantine area will expand incrementally to maintain a 5 to 10 km buffer between infested sites and the quarantine boundary.

If the current slow-the spread program is stopped, sudden oak death would become one of many forest health issues handled by agencies. Federal funding likely would decrease and agencies would reduce detection effort and stop supporting cut and burn

projects. Disease intensity and rate of spread would increase. The quarantine area would expand immediately to the entirety of Curry County. Agencies would provide technical assistance to landowners who want to know why their trees are dying and what they can do about it, give advice on how to reduce hazards from fire and tree fall, enforce quarantine regulations, and promote best management practices to make the most of a bad situation. Citizen science programs could help detect and track spread of the disease, providing data to researchers and graduate students. In short, we would rely on educating people to mitigate the effects of the disease and prevent spread to other susceptible forests. It would be much like what has happened in California.

The ecological and economic consequences of abandoning the sudden oak death program are disturbing. Tanoak is rapidly being eliminated from infested areas in California and in part of Curry County. Oregon likely will lose tanoak in at least the western portion of its range. Birds, mammals, insects and fungi dependent on tanoak will migrate or die. Loss of tanoak will impact Native American culture. The quarantine regulations eventually will expand to Coos and Douglas counties, causing all sorts of domestic and international trade problems with species on the *P. ramorum* host list such as Douglas-fir, western hemlock, grand fir, and others. Forest, nursery, Christmas tree and other forest product operations that intend to ship material will need inspections and disease-free certifications, probably on a fee-for-service basis.

We presently are considering altering the sudden oak death program to one that focuses on preventing the disease from entering the adjacent Coos and Douglas-counties (to avoid the economic impact of quarantine regulations there), protecting selected important tanoak ecosystems, searching for disease tolerance/resistance in the tanoak population, and providing long term conservation and adaptation of tanoak genes.

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Kanaskie, A**.**, Hansen, E., Goheen, E.M., Osterbauer, N., McWilliams, M., Laine, J., Thompson, M., Savona, S., Timeus, H., Woosley, W., Sutton, W., Reeser, P., Schultz, R., and Hilburn, D. 2011. Progress of the *Phytophthora ramorum* eradication programme in south-western Oregon forests, 2001-2009. New Zealand Journal of Forest Science. 41S, S169-S175.

# Approaching 15 years of research on SOD control

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In California, SOD is mostly spread by infectious tanoaks and bay laurels, while high mortality is observed in tanoak and coast live oak stands. Intensive surveys and repeated measures analyses indicate that density of bays and tanoak will determine final mortality rates. While thinning of both species is an obvious disease control approach, we suggest that knowledge of natural disease tolerance and infectivity of individual trees during thinning operations is key in maximizing outcomes. We also show that phosphonate treatments increase disease tolerance in oaks and tanoaks. However, the genetic and physiological mechanisms of action of phosphonates and mechanisms leading to natural disease tolerance are still imperfectly known. We used a transcriptomic approach to compare these two tolerance mechanisms: results identified several genic pathways that are up- and down-regulated in both types of tolerance, and show that the two mechanisms, although both quantitative and resulting in a comparable tolerant phenotype, are almost completely not overlapping.

# Eradication of *Phytophthora cinnamomi* from infested *Eucalyptus marginata* (jarrah) forest during large scale mining operations

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*Phytophthora cinnamomi* is widespread throughout the *Eucalyptus marginata* (jarrah) forest in the south-west of Western Australia. Alcoa of Australia Ltd. mine bauxite in both infested and uninfested forest and rehabilitates 350-400 ha of mined forest annually. *P. cinnamomi* is unevenly distributed throughout the areas to be mined, with infested areas adjacent to non-infested areas. In order to minimize the spread of *P. cinnamomi* from infested to non-infested areas, detailed mapping of infestations is used to design detailed hygiene plans. As a result of eradication trials reported at previous IUFRO meetings, we now believe it is feasible to return infested mine pits to pathogen-free post mining. In addition, it will allow strict hygiene practices to be relaxed along haul roads, as these can be made pathogen-free post-mining. This will result in substantial savings in transport costs as *Phytophthora* free road building material will not need to be sourced. As *P. cinnamomi* is a poor saprotroph eradication methods are based on ensuring haul roads, topsoil, overburden (soil horizons not suitable for bauxite extraction) stockpiles and sumps (drainage points designed to collect water running off roads) are kept plant-free for 2-3 years. Where necessary metham sodium and other fumigants will be used. We will report on (1) the fallow, herbicide, fumigation methods being used to eradicate the pathogen, (2) the traditional and molecular genetic approaches being used to monitor the effectiveness of treatments to kill all survival stages (chlamydospores, zoospores, oospores, and stromata), and (3) approaches to containment that will ensure the pathogen is not inadvertently spread during the 2-3 year fallow period prior to revegetation. Successful outcomes will allow many hundreds of hectares of previously *Phytophthora* infested forest to be returned to a pathogen-free forest post-mining.

# Potential Impacts of the Revised APHIS *Phytophthora ramorum* Domestic Quarantine Regulatory Requirements on the Spread of this Exotic Pathogen within Washington State

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Since 2003, *Phytophthora ramorum* have been detected in over 50 ornamental plant nurseries in Washington State. Stream monitoring by state agencies has resulted in the detection of this exotic pathogen in about a dozen waterways in six western Washington counties since 2006. Genotype analysis indicates that the NA1, NA2, and EU1 clonal lineages of *P. ramorum* are present in nursery and waterways. In all cases, streams have tested positive for *P. ramorum* in subsequent years after the first detection. Although *P. ramorum* has not been detected in a forest landscape in Washington State, in the spring of 2009, infested ditch water resulted in the infection of salal (*Gaultheria shallon*) plants by the NA2 lineage along the perimeter of a positive nursery in Pierce County. Composite soil samples collected from along the ditch were also positive in 2010. In addition, positive soil has also been detected at 3 trace forward sites where infected plants from a nursery in Thurston County had been planted in urban landscape sites. Effective March 31, 2014, the USDA Animal and Plant Health Inspection Service (APHIS) revised regulatory requirements relating to the interstate movement of host nursery stock from nurseries located in *P. ramorum* regulated and quarantine areas in California, Oregon, and Washington went into effect. The impact of these revisions on the number of nurseries being inspected in Washington and its potential impact on the spread of *P. ramorum* within the state will be discussed.

# Enabling technologies to combat *Phytophthora* diseases

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New Zealand's conservation, forestry and horticultural tree estates are all impacted by *Phytophthora* species. Of particular note are *Phytophthora pluvialis*, causal agent of red needle cast in *Pinus radiata*, *Phytophthora* taxon Agathis which is causing severe disease in a native tree species, *Agathis australis* (kauri), and *Phytophthora cactorum* which has a long-standing record causing collar rot in apple. These three host trees are central in a Scion led, six-year collaborative research programme with significant support from sector groups, to address the biosecurity threat of *Phytophthora* species to New Zealand's forestry, agriculture and natural ecosystems. The genetic, metabolomic and histological host-pathogen interactions will be investigated between each of these host species and eight species of *Phytophthora* with biosecurity relevance to New Zealand (*P. cactorum, P. cinnamomi, P. kernoviae, P. multivora, P. pinifolia, P. pluvialis* and *P. ramorum*). Through this multi-host-pathogen model we are assessing the potential for utilizing genetic, gene expression and/or metabolite signatures for tree breeding, improving disease management and advancing current knowledge of *Phytophthora*-tree interactions. Initial work has focused on screening established breeding lines of *Pinus radiata* for RNC resistance, assessing cross resistance to the other species of *Phytophthora,* and contrasting the timing of infection by each species of *Phytophthora*. The project model and results from the first year of this six year program will be presented and discussed.

# Searching for *Phlomis purpurea* metabolites with anti-*Phytophthora cinnamomi* activity

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We recently reported that *Phlomis purpurea* root extracts (PRE) inhibit *Phytophthora cinnamomi* mycelial growth and chlamydospore and zoospore germination, and protect susceptible hosts from infection. The plant reduces the inoculum potential of *P. cinnamomi* in the soil, suggesting it has the potential to reduce disease spread (1). These findings prompted us to search for metabolites responsible for this activity or/and produced upon challenge with the pathogen. HPLC analysis of PRE allowed the identification of a fraction with anti-*P. cinnamomi* activity (Figure 1). Fractionation by preparative chromatography resulted in the isolation of a compound accounting for this activity that was further crystallized and structurally characterized by ESI/MS/MS, IR, $^{1}H$ and $^{13}C$ NMR (DEPT, correlation NMR spectrometry−COSY, TOCSY, HMBC and Multiplicity Edited HSQC), and by X-ray diffraction revealing a novel triterpenoid structure: Phlomispurpentaolone ($C_{29}H_{46}O_6$) (Figure 2).

*Phlomis purpurea* metabolites produced constitutively and upon challenge with *P. cinnamomi* were quantified using a LC-MS system, according to established standard workflows. Root exudates were analysed by GC-MS after derivatization. Two and half-month-old *P. purpurea* seedlings were challenged with *P. cinnamomi* zoospores. The samples for analysis consisted of roots of 10 plants inoculated with the pathogen at 6 time points (0 h, 6 h, 12 h, 18 h, 24 h and 72 h), controls and root exudates of the same plants at the same time points. Five replicates were performed. The material from the plants at each time point was pooled for each independent replicate (11 pools x 5 replicates). Roots and leaves were extracted with MeOH. Lipids and slightly polar metabolites were separated using reversed phase chromatography. The exudates were also collected and filter sterilised at each time point, immediately submersed in liquid nitrogen and kept at -80ºC. Data analysis was performed with the software Metaboanalist 2.5 (www.metaboanalyst.ca). The analysis of roots revealed that there was no significant difference between the metabolites of control and infested *P. purpurea* at all-time points. The analysis of the aerial parts showed that there were two metabolites differentially produced, at time 6 h by infested roots (m/z 1494.048 and m/z 742.4841, $P<0.001$) (Figure 3), one at 48 hpi (m/z 414.7606 $P=0.01$) and two at 72 hpi (m/z 920.2796, $P=0.002$ and m/z 845.3147, $P=0.01$). Only results at tme 6 h (Figure 3) is shown as an example.

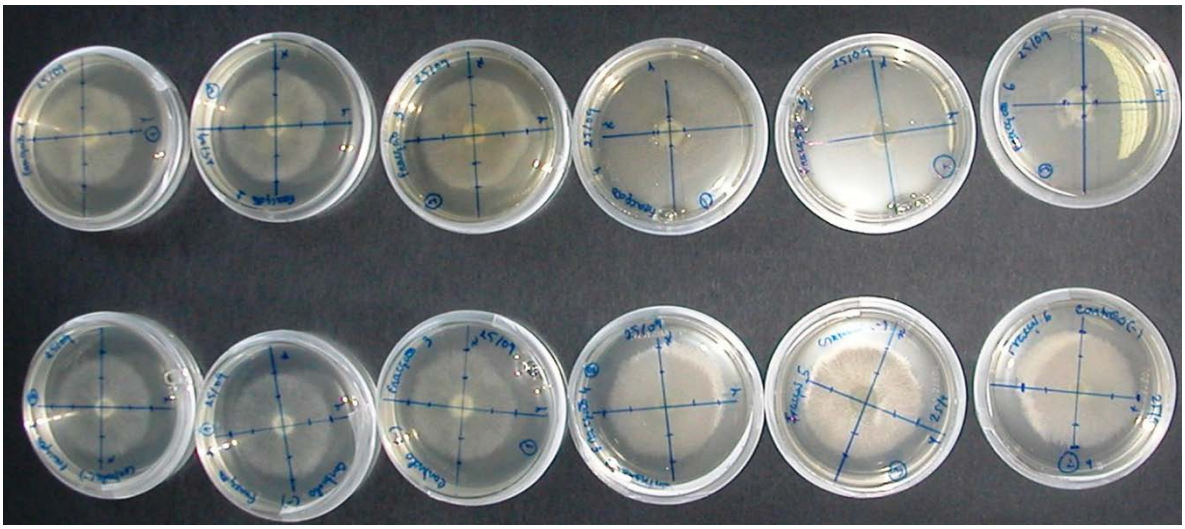
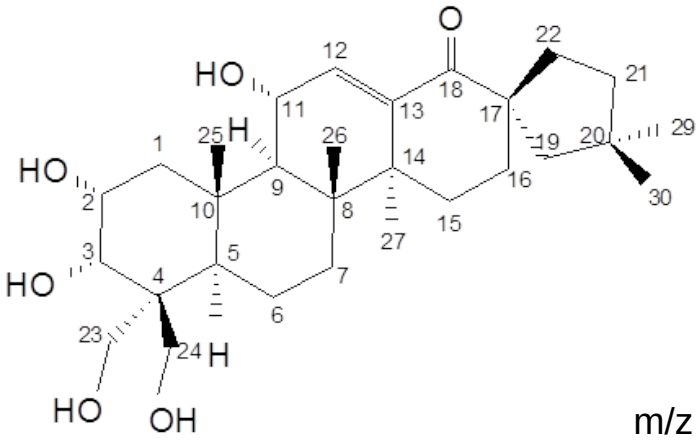


**Figure 1.** Bioassays using 6 fractions of *Phlomis purpurea* root extracts (PRE). The first row shows the HPLC fractions from 1 to 6 (left to the right) and the second row the controls. Fraction 5 caused 100% inhibition of *Phytophthora cinnamomi* mycelial growth.




$C_{29}H_{46}O_6$

m/z $[M+H]^+$ = 491.4

**Figure 2.** Structure and molecular formula of (17*R*)-2*α*,3*α*,11*α*,23,24-pentahydroxy-19(18→17)-*abeo*-28-norolean-12-ene-18-one (phlomispurpentaolone).

Phlomispurpentaolone (m/z $[M-H]^+$= 491.4) is produced constitutively and at 0.1 $mgml^{-1}$ inhibits *P. cinnamomi* mycelial growth by 75.7%.

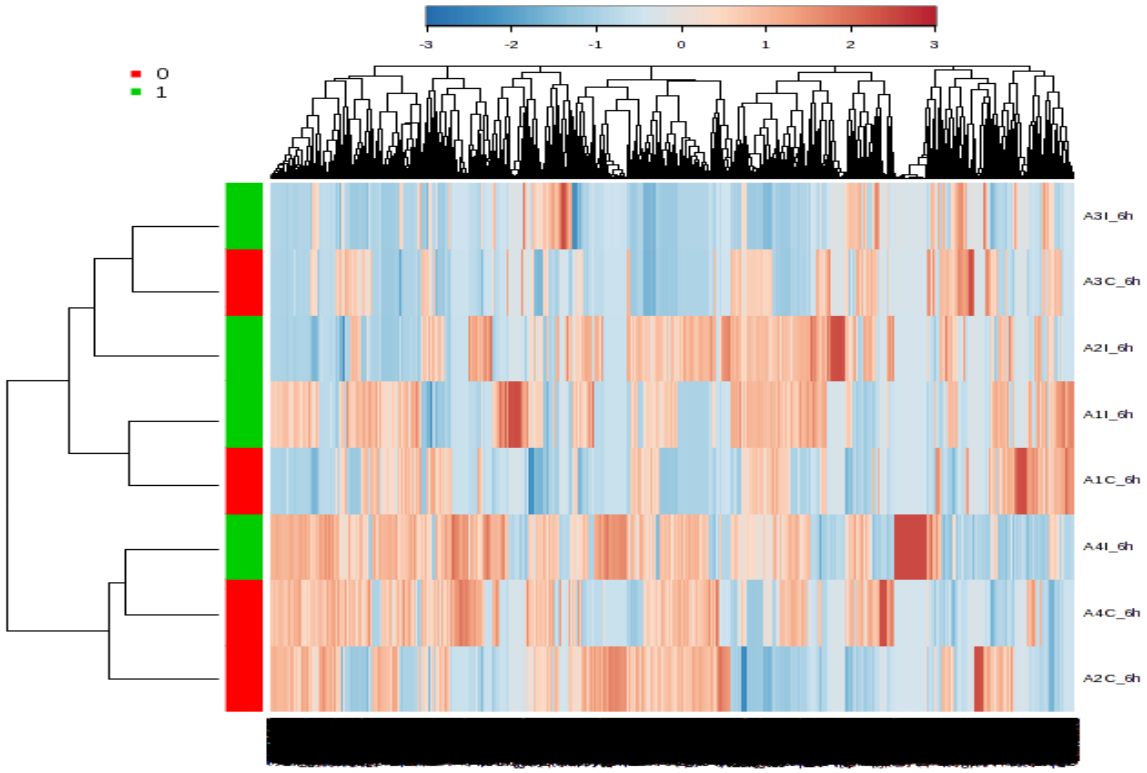



**Figure 3.** Clustering result shown as heatmap (distance measure using pearson, and clustering algorithm using average) of the aerial parts showing that there were two metabolites differentially produced at time 6 h by infested *Phlomis purpurea* with *Phytophthora cinnamomi* (m/z 1494.048 and m/z 742.4841, *P*<0.001).

Additionally it was shown, using histological techniques that the anatomy of *P. purpurea* root can act, per se, as a physical barrier against *P. cinnamomi*.

The results suggest that *P. purpurea* has the potential to reduce disease spread and that their roots produce substances such as phlomispurpentaolone that can control the important pathogen, *P*. *cinnamomi*.

1. Neves, D.; Caetano, P.; Oliveira, J.; Maia, C.; Horta, M.; Sousa, N.; Salgado, M.; Dionísio, L., Magan, N., .Cravador, A. (2014). Anti-*Phytophthora cinnamomi* activity *of Phlomis purpurea* plant and root extracts. European Journal of Plant Pathology, 138:835–846.

# Screening of Biofumigants against *Phytophthora cinnamomi* Root Disease

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Root rot caused by *Phytophthora cinnamomi* is one of the most destructive diseases affecting many woody hosts and specially *Quercus* spp. in rangeland ecosystems in Southern Iberia. Biofumigation is a potential control method suitable to be used in these seminatural ecosystems. Biofumigation is based on the use of biocidal compounds released through the hydrolysis of glucosinolates (GSLs) (**1)**, secondary metabolites produced by plants belonging to the order *Capparales* and especially in the genus *Brassica* (**2**). GSLs are not bioactive until they have been enzymatically hydrolysed into various bioactive breakdown products (isothiocyanates, nitriles, thiocyanates, epithionitriles, oxazolidine-2-thiones, and epithionitriles) by the endogenous plant enzyme Myrosinase (Thioglucoside glucohydrolase, E.C.3.2.1.147) (**1**, **2**).

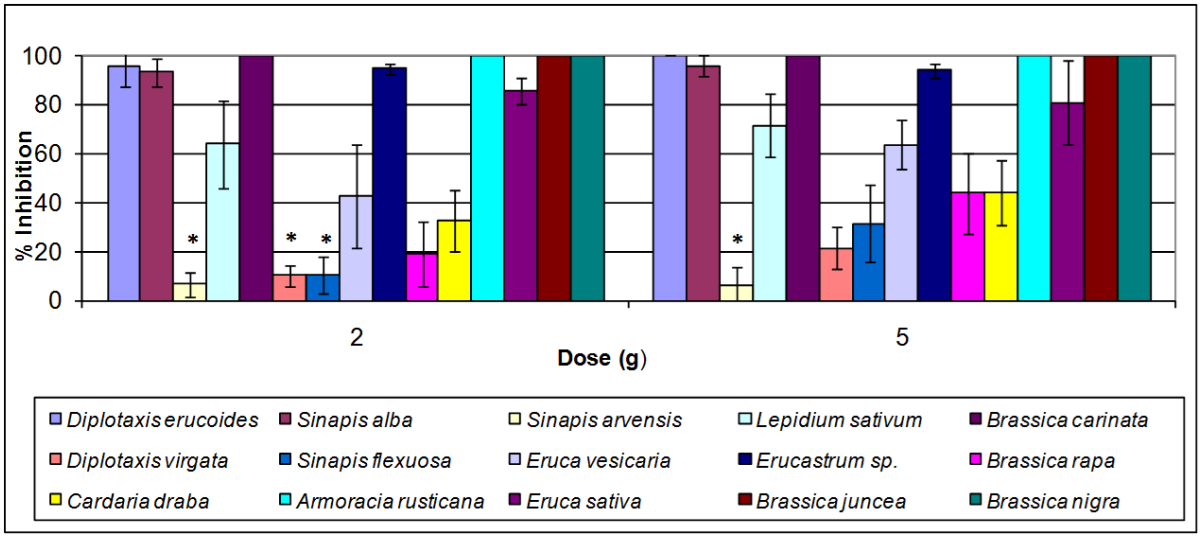
Fifteen plant species of potential biofumigant activity belonging to the family *Cruciferae* were selected based on their ability to grow in the dehesa ecosystems (Table 1). The above-ground parts (leaves, stems and flowers) of 10 plants from each species were harvested when their developmental stages were between first flowers open and full flowering, corresponding to codes 60 to 65 of the BBCH (Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie) scale (**3**). Plant material was washed, disinfested by soaking in aqueous 10% sodium hypochlorite for 10 s, lyophilized and grounded to a fine powder. GSL composition for each plant species was determined by High Performance Liquid Chromatography (HPLC) (Table 1). Based on the chemical nature of their main GSLs, plants were divided into two groups: Group A (red box in Table 1) with high content in aromatic GSLs and Group B (violet, Table 1) with a high content in aliphatic GSLs. In turn, Group B was subdivided into two subgroups, B1 including plants rich in Sinigrin (yellow box, Table 1) and B2, containing plants rich in aliphatic GSLs different from Sinigrin (blue and green boxes, Table 1).Inhibition of mycelial growth by volatiles was tested by plating agar plugs cut from the edges of *P. cinnamomi* colonies actively growing in carrot-agar (CA 2%) plates and immediately placed face down as lidson plastic beakers containing lyophilized and rehydrated plant material. The equivalent to 2 or 5 g of fresh material and a dose 0 (control) were tested. Four replicates were prepared for each species and dose, including dose 0. Beakers were incubated at 25º C in the dark and radial growth of the colonies measured daily until the control colonies covered the whole surface of the agar medium (4 days) (Figure 1). All the plants in the B1 group reached a 100% of inhibition even at the minimum dose tested (2 g). In all these cases, after the exposure to the biofumigant, *P. cinnamomi* was not able to grow again, showing their fungicidal action. At the maximum dose tested, other plant species such as *Sinapis alba, Lepidium sativum, Erucastrum sativa*, *Eruca*

*vesicaria* or *E. virgatum*, also reached a significant level of inhibition of mycelial growth (Tukey´s HSD test for P<0.05). However, after exposure to the biofumigant, *P. cinnamomi* colonies were able to continue growing until agar plates were filled, demonstrating their fungistatic action. The rest of the plant species tested did not show any significant decrease of mycelial growth

**Table 1.** Glucosinolate profiles and concentrations (μmol×g$^{-1}$dw) in the above-ground parts of potential biofumigants against *P. cinnamomi*. [a]RO: Progoitrin; SIN:Sinigrin; GRA: Glucoraphanin, GNA: Gluconapin; GSAT: Glucosativin; GBN: Glucobrassicanapin; GER: Glucoerucin; GTL: Glucotropaeolin; SBN: Sinalbin; 4-OMGBS: 4-Methoxyglucobrassicin; NGBS: Neoglucobrassicin.

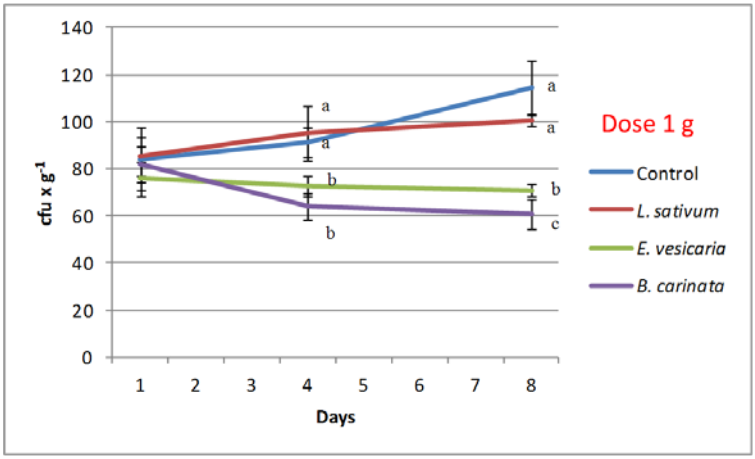
| Biofumigant species | Total GSLs | Aliphatic Glucosinolates | | | | | | | Aromatic Glucosinolates | | Indolic Glucosinolates | | OTHERS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | PRO[a] | SIN | GRA | GNA | GSAT | GBN | GER | GTL | SBN | 4-OMGBS | NGBS | |
| *Sinapis arvensis* | 11.53 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 3.35 | 1.49 | 2.17 | 4.52 |
| *Sinapis flexuosa* | 28.66 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 27.98 | 0.00 | 0.00 | 0.68 |
| *Sinapis alba* | 29.96 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 11.99 | 15.34 | 0.00 | 0.00 | 2.64 |
| *Lepidium sativum* | 35.53 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 35.22 | 0.00 | 0.00 | 0.00 | 0.31 |
| *Diplotaxis virgata* | 36.43 | 2.06 | 0.00 | 0.00 | 31.74 | 0.00 | 1.80 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.84 |
| *Brassica rapa* | 32.81 | 8.11 | 0.00 | 0.00 | 18.95 | 0.00 | 1.87 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 3.87 |
| *Cardaria draba* | 50.86 | 0.00 | 0.00 | 34.40 | 0.00 | 0.00 | 0.00 | 6.30 | 0.00 | 8.44 | 0.00 | 0.00 | 1.72 |
| *Eruca vesicaria* | 35.06 | 0.00 | 0.00 | 19.62 | 0.00 | 12.62 | 0.00 | 1.62 | 0.00 | 0.00 | 0.00 | 0.00 | 0.28 |
| *Eruca sativa* | 43.48 | 0.00 | 0.00 | 22.29 | 0.00 | 12.02 | 0.00 | 6.96 | 0.00 | 0.00 | 0.00 | 0.00 | 0.28 |
| *Erucastrum* sp. | 58.93 | 0.00 | 0.00 | 28.68 | 0.00 | 16.65 | 0.00 | 11.10 | 0.00 | 0.00 | 0.00 | 0.00 | 0.28 |
| *Diplotaxis erucoides* | 35.93 | 0.00 | 35.61 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.32 |
| *Armoracia rusticana* | 49.91 | 0.00 | 49.58 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.33 |
| *Brassica carinata* | 15.06 | 0.00 | 14.19 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.87 |
| *Brassica juncea* | 25.79 | 0.00 | 25.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.78 |
| *Brassica nigra* | 19.30 | 0.00 | 17.79 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.51 |

**Figure 1**. Average maximum radial growth (mm) and standard error of *P. cinnamomi* colonies exposed to volatiles released by biofumigants for 4 days.



Furthermore, we studied the effect on the viability of *P. cinnamomi* chlamydospores in the soil and the ability to cause root disease. Volatiles derived from *Lepidium sativum* (Group A), *Brassica carinata* (Group B1) and *E. vesicaria* (Group B2) were tested on dehesa soil artificially infested with water suspensions of *P. cinnamomi* chlamydospores (final concentration 650 cfu×g$^{-1}$). Lyophilized plant material was placed at the bottom of 250 ml containers and rehydrated before the infested soil was added. All containers were closed in order to avoid loss of volatiles. Chlamydospore viability was evaluated after 1, 4 or 8 days of incubation (22º C day - 18º C night) by counting the number of colony forming units (cfu = chlamydospores) growing in selective NARPH medium.

**Figure 2**. Average number and standard error of viable *P. cinnamomi* chlamydospores (cfu×g$^{-1}$ of dry soil) after exposure of infested soil to volatiles released by biofumigants. For each time, values with different letters differ significantly according to the Tukey´s HSD test (P<0.05)




Results are shown in Figure 2. *Brassica carinata*, rich in the aliphatic GSL Sinigrin and *E. vesicaria*, rich in other aliphatic GSLs (Glucoraphanin, Glucosativin and Glucoerucin, Table 1) significantly decreased the viability of *P. cinnamomi* chlamydospores in the soil, despite the fungistatic action of the second one. This work provides a criterion for the selection of potential biofumigant plants to be used in rangeland ecosystems, based on their richness in aliphatic GSLs, mainly 2-propenyl glucosinolate (Sinigrin), with fungicidal action. Although biofumigation does not appear to be sufficient to reduce *P. cinnamomi* densities in soil below the minimum levels for oak root infections (<1 chlamydospore×g$^{-1}$) (**4**), this tool should be considered as a good choice to decrease inoculum densities in already infested soils.


## Acknowledgements

With acknowledgement to the Andalusian Government (Research Project P10-AGR6501) and the EU (Project LIFE11 BIO/ES/000726) for financial support.

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# Field Trip Presentations



Isla Victoria
Photo by Bruno Scanu



Los Alerces National Park
Photo by Bruno Scanu



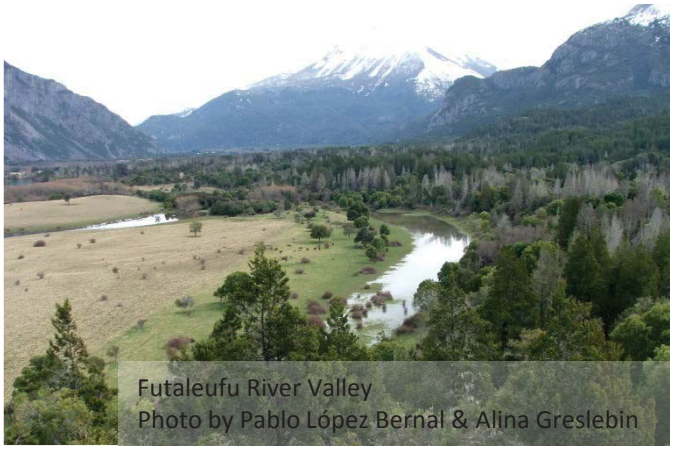
Isla Victoria - Nahuel Huapi National Park
Photo by Everett Hansen



Asado
Photo by Bruno Scanu



*Phytophthora austrocedri* lesion
Photo by Bruno Scanu



Futaleufu River Valley
Photo by Pablo López Bernal & Alina Greslebin



La Trochita
Photo by Bruno Scanu



Torres del Paine National Park Chile
Photo by Steve Tjosvold

# A brief historic account of ‘Mal del Ciprés’

Mario Rajchenberg

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“Mal del Cipres’ was first noted and reported by European foresters, immigrants to Patagonia after World War II, in the late 1940’s‑early 1950’s (fig. 1). In the 1960’s phytopathologists from several national institutions including IFN (Instituto Forestal Nacional), INTA (National Institute of Agricultural Technology), and UBA (University of Buenos Aires) visited these areas but no diagnosis was made. The first scientific report of the disease was published in 1975 by Varsavsky et al, describing a brown rot in the sap wood but not establishing its etiology. In 1979 an international committee of FAO (Food and Agriculture Organization of the United Nations) scientists visited the area but again no diagnosis was made.




Havrylenko et al. published the first systematic analysis of symptoms associated with ‘Mal del Cipres’ in 1989. They described external symptoms, established an association between trees and pointed out the involvement of a biological agent.

In the 1990’s two different groups, Universidad Nacional del Comahue in Bariloche and Centro Forestal CIEFAP in Esquel began systematic studies of ‘Mal del Cipres’.These efforts triggered numerous hypotheses.

Figure 1. First report of ‘Mal del Cipres’ in 1948 at A)Victoria Island Nahuel Huapi National Park followed by B) Epuyen Lake in 1953.

The first published hypothesis was that the forest condition was a normal forest process due to Cypress establishment in inappropriate soils (Colmet-Daage, 1992)(fig.2).

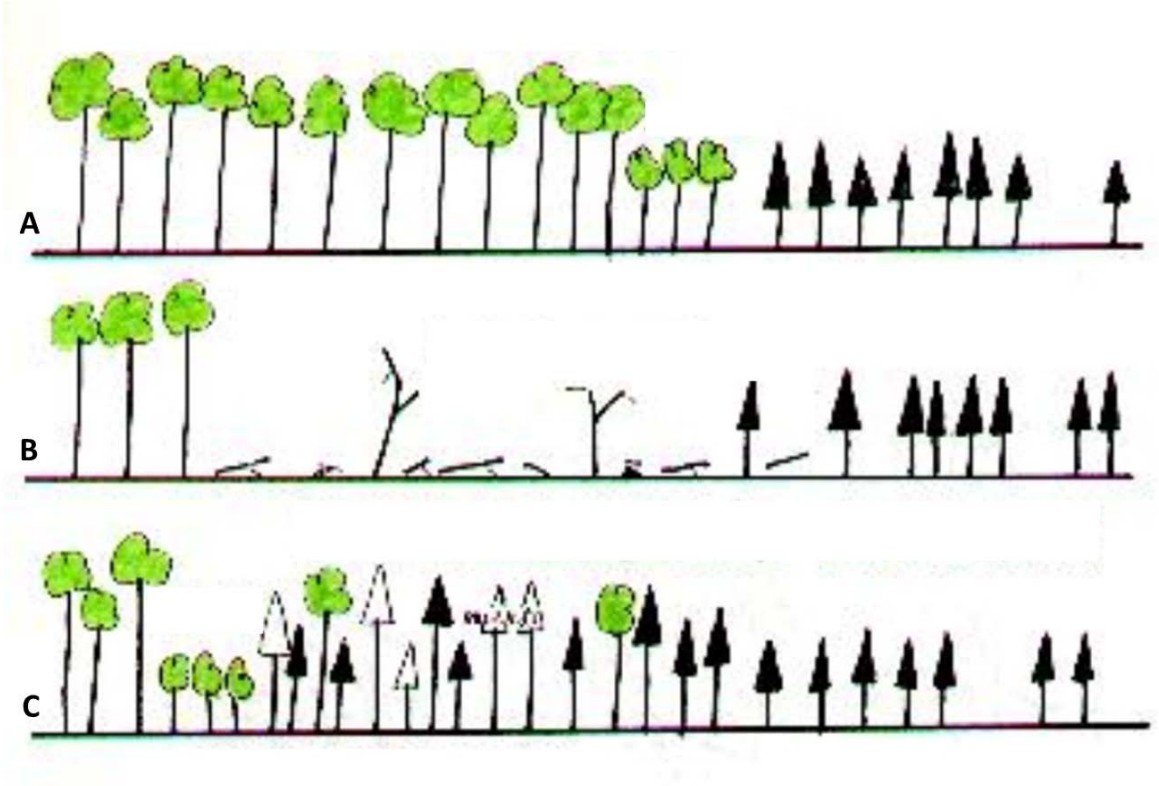



Figure 2. A *Austrocedrus* and a hardwood, perhaps *Nothofagus*, optimally established on different soil types. B An unrelated disturbance destroys a portion of the hardwood stand. C The disturbed área is recolonized largely by *Austrocedrus*, now growing on the "wrong" soil type.

The second hypothesis based on dendrochronological studies was that this was a typical forest decline disease. They looked for a relationship with the climatic (a sequence of several cold and humid springs and summers) or geologic events (earthquakes) data. Several papers were published discussing this work (Cali 1996, Loguercio 1997, Loguercio & Rajchenberg 1998)(Fig. 3).

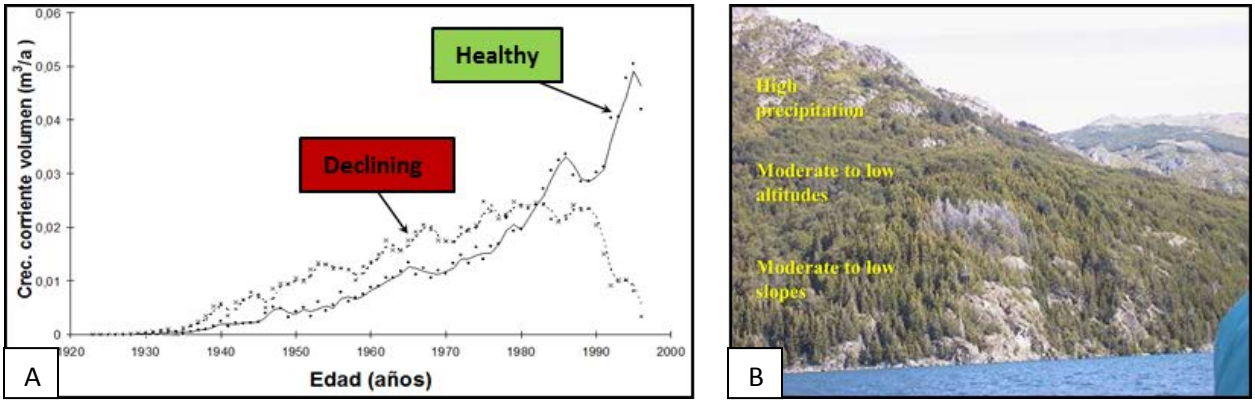



Figure 3. A-Radial growth of declining and healthy trees. B-Large scale ecologic conditions.

Other factors considered were large scale ecological conditions such as precipitation, altitude and slope (Baccalá et al 1998) and soil features as predisposing factors such as a clayish horizon, redoximorphic features close to surface, non- allophanized soils of fine structure, proximity to streams and drainage impediment (La Manna & Rajchenberg 2004) (Fig. 4).




Figure 4. Soil profiles from *Austrocedrus* stands exhibiting Mal del cipres.

A third hypothesis was a wood-rotting fungus destroying the sapwood was responsible for the decline. The main wood-rotting species of sapwood in standing, declining *Austrocedrus chilensis* was *Postia dissecta* (Polyporales, Basidiomycota)(Fig 5A) but also isolated from *Nothofagus* and other broad-leaf species in Patagonia. In the mid 1990's there was an intensive search for a primary pathogenic fungus (Fig 5B). These efforts failed to produce a pathogen.

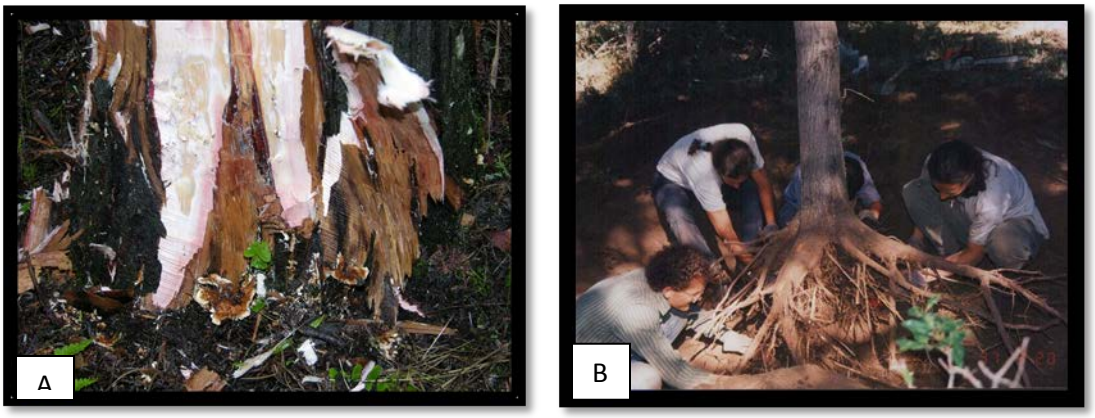



Figure 5. A- *Postia dissecta* (Polyporales, Basidiomycota) on *Austrocedrus chilensis.* B- root excavation in search of a primary pathogenic fungus.

Finally in 2005 an undescribed *Phytophthora* was isolated and *Phytophthora austrocedri* was named in 2007. Much research has followed including inoculation trials, testing for physiological alterations triggered by *P. austrocedri* and the toxic effects of culture filtrates on *Austrocedrus chilensis* (Vélez et al. 2012)(Fig. 6).




Figure 6. A- tree inoculations and B- testing for physiological alterations.

Population genetics studies demonstrate that the pathogen is most likely introduced (Vélez et al 2014)(Fig 7).

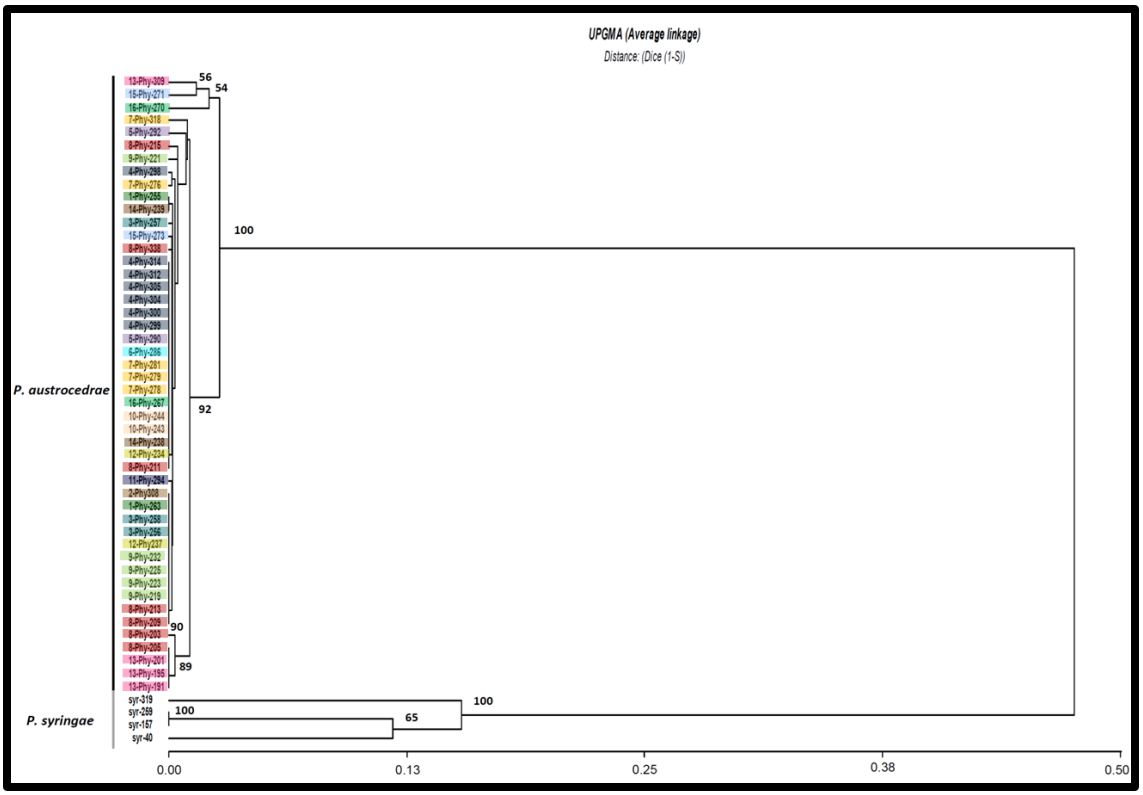



Figure 7 Phylogenetic tree of over 45 *P. austrocedri* isolates aligned with it's nearest neighbor *P.syringae*.

Current topics of research are investigating pathogen control with chemicals and anatomical disruption of the host by *P. austricedri* (Figs. 8, 9 and 10).

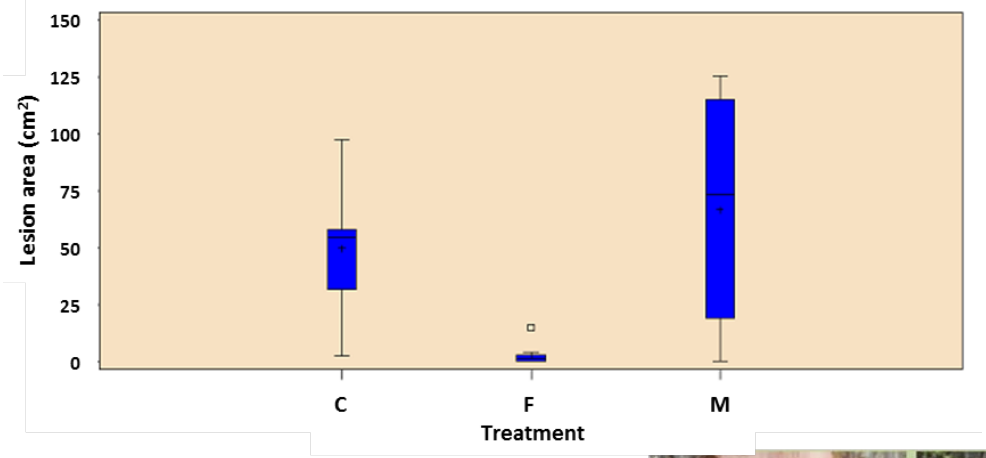



Figure 8. Comparison of lesion area in each treatment. Mean (+), median (line) and extreme values. C: control, F:Fosetyl-Al, M:Metalaxyl.

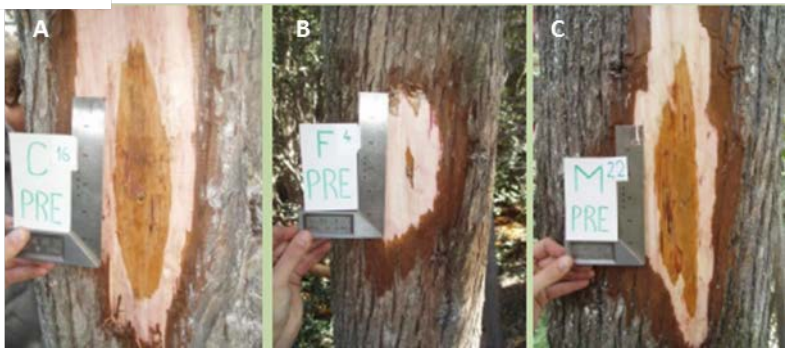



Figure 9. Representative lesions of each treatment. A: control, B:Fosetyl-Al, C:Metalaxyl.

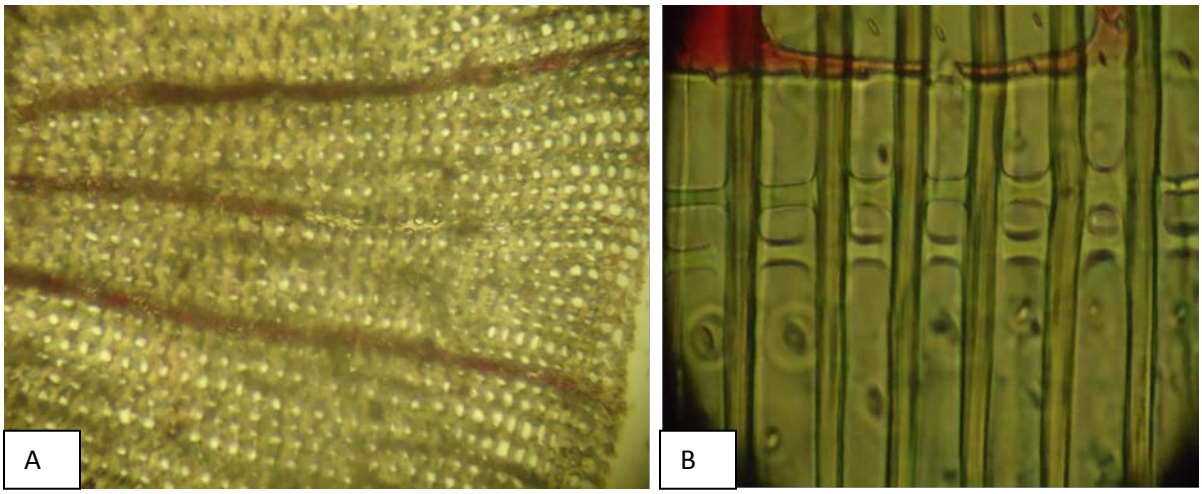



Figure 10. A - radial parenchyma filled with resin-like substances. B - abnormal trabecules formation.

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# Other species threatened by *Phytophthora austrocedri*

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*Phytophthora austrocedri* is a soil pathogen that causes the devastating disease commonly referred as 'mal del ciprés' in *Austrocedrus chilensis*. Affected trees present necrotic lesions on the roots and stems that can affect the entire breadth of the phloem. Recent studies showed that the pathogen was introduced into Patagonia; however the certain origin of the pathogen remains unknown. During 2011-2012 *P. austrocedri* was detected causing *Juniperus communis* mortality in United Kingdom. The species might be also introduced in this case. The susceptibility of species phylogenetically related to *A. chilensis* remains unknown. This information is crucial to prevent new emergent diseases. The susceptibility to *P. austrocedri* of species from the *Cupressaceae* family, native (*Fitzroya cupressoides, Pilgerodendron uviferum*) and exotic *(J. communis, J. virginiana, Thuja occidentalis, T. plicata, Calocedrus decurrens, Chamaecyparis lawsoniana, Sequoiadendron gigantum*), was evaluated by pathogenic assays in plants (native) and branches (exotic). As positive controls, plants and branches from *A. chilensis* were inoculated with the pathogen. The presence and the extent of the necrotic lesion in the phloem, and the confirmation of the presence of the pathogen (isolates, ELISA test) were evaluated. From the exotic species, only *J. communis* and *J. virginiana* showed necrotic lesions (20% of the cases), while all individuals of the native species showed necrotic lesions of similar size to the positive controls. The results evidence a high susceptibility of the native conifer species to *P. austrocedri*. These species are found in areas surrounding *A. chilensis* forests affected by the disease, which involves the design of measures to prevent and control the spread of the disease to protect these species from the pathogen. The possible pathogenicity of *P. austrocedri* in other species is an aspect to continue studying in the case of probable introduction of the pathogen in regions where the species are endemic.

# Poster Presentations

# Surveillance and management of kauri dieback in New Zealand


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## Introduction

Kauri (*Agathis australis*) is a conifer endemic to northern New Zealand. In recent years, kauri trees have been found to be suffering from a disease given the common name of 'kauri dieback'. The causative organism of kauri dieback is *Phytophthora* taxon "Agathis" (PTA), which affects the feeder and structural roots and kills trees of all ages. Kauri dieback has been identified as a significant threat to kauri-dominated ecosystems (Beever et al. 2009).

In 2009, three regional councils (Northland, Auckland and Waikato), central government (Ministry of Primary Industries, Department of Conservation (DOC)) and representatives of local Māori set up a joint agency which has managed a substantial programme of science, public engagement and surveillance to determine the extent of the problem and to mitigate transport of PTA to other areas (see www.kauridieback.co.nz).

Table 1. Distribution and area (acres) of kauri plantings sourced from Waipoua nursery in the 1950s and the resulting locations of transported PTA contamination.

| Forest name | Forest, PTA status (+ve or –ve) | PTA status of nearby kauri (+ve or –ve) | Year plantation established and area planted (acres) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1949 | 1950 | 1951 | 1952 | 1953 | 1954 | 1955 | 1956 | 1957 | 1958 | 1959 |
| **Great Barrier Island** | | | | | | | | | | | | | |
| | +ve | +ve | 1 | | | | 1.8 | 2.8 | | 2.4 +ve | | | |
| **Coromandel** | | | | | | | | | | | | | |
| Katikati | Plantation failed | –ve | | | | | | | | | 8 | | |
| Kauaeranga | –ve | –ve | 5 | 17 | | | | | | | | | |
| Whangapoua | Sick, destroyed | +ve | 5 | 7 | | | | | | | | | |
| Tairua | –ve | –ve | | | | | | | | | | | |
| **Northland** | | | | | | | | | | | | | |
| Raetea | +ve | n/a | | | | | | | 0.8 | 8.5 +ve | | | 2.5 +ve |
| Waipoua | +ve/–ve | +ve | | | | 2.5 | | | 5 | 5.7 +ve | 2.5 | 2.7 | |
| Omahuta# | Plantation failed +ve/–ve | –ve | | 66 | 32 | 37 | | 10 | | | | | |
| Puketi | Plantations failed | –ve | | | | | | 19 | 28 | | | | |
| Waitangi | Plantations failed | –ve | 3 | | | | | | | | | | |
| Russell | +ve | +ve | | 8 | | | | | | | | | |
| Puhipuhi | Plantations failed | n/a | 1.3 | 10 | | | | | | | | | |
| Glenbervie | +ve | –ve | 6 +ve | 6 +ve | | | | | 13 +ve | | | | |
| Riverhead | –ve | –ve | 2 | 31.5 | | | | | | | | | |

# Amenity planting of unknown date.
RED text Indicates a transfer of infection between plantations.
YELLOW shading indicates the consignment of plants most likely to have been contaminated with PTA.
GREEN shading indicates areas that may have been contaminated through forest management activities and/or pigs and cattle.



Figure 5. The originally contaminated (1956) part of the Raetea plantation is in the middle of the photo. The kauri on the hills above were planted in 1981–85 and are now also contaminated.



Figure 7. Kauri dieback in farmland, with trees showing the full range of canopy collapse conditions

## Developing detection techniques and surveillance

In 2010, sites identified from genetic analysis as being positive for PTA in the Waitakere Ranges and three other forests were used to refine existing field site collection and soil bioassay methods to improve PTA detection techniques (Beever et al. 2010).

Sites were then selected for surveillance both randomly and based on structured decisions that assessed likely vectors from PTA positive sites. Surveillance targeted former New Zealand Forest Service (NZFS) plantations (Table 1), sites considered to be contaminated only with *P. cinnamomi* (Podger & Newhook 1971), iconic large kauri trees, stands important to Māori, stands 100–1000 years old, large areas of forest, kauri on islands and infected kauri and other sites on farmland.

Two active, ground-based methods and aerial surveillance were used. Auckland Council conducted a detailed assessment of the distribution of kauri dieback throughout its region using symptomology, and one laboratory for testing soil at 'positive' sites. The Joint Agency tested all other areas using a differently structured approach and three laboratories (including that used by Auckland Council). The aim was to consistently improve detection and refine test methods (Table 2).

Data were collected on tree canopy and lesion status (Figs 1 and 2), and soil was collected from near the trunk and drip line (Waipara et al. 2013). Aerial surveillance was used to review the contamination at very infected sites, assess where we need to look in places of high conservation value (Fig. 3), identify at-risk sites and obtain a photographic record of the current status of kauri forests. We are now testing RT-PCR in parallel with the soil bioassay, and improving the guidelines for selecting the soil collection sites for more intensive field sampling (Than et al. 2013, McDougal et al. 2014).

Public engagement and reporting has been substantial, and reports from the public of diseased trees have been followed up.

## Surveillance results

PTA has not been detected in a number of the larger areas of kauri forest (Fig. 4). It appears that it was transported from Waipoua Nursery to three other sites between 1954 and 1956 (Table 1, Fig. 5), probably in consignments of trees that were grown in plots containing forest-collected leaf litter and then shipped in reusable planting tubes. At two sites, the disease has spread to other neighbouring plantings. Cattle and feral pigs are likely to have spread the disease more widely in nearby forests.

In the Waitakere Ranges, sites thought to have been degraded by *P. cinnamomi* in the late 1960s (Podger & Newhook 1971) are infected with PTA, which is now having a substantial impact on ricker (<100-year-old trees) (Fig. 6). PTA has spread on farms between Auckland and the Northern Kaipara Harbour since the 1970s (Fig. 7). It is well distributed on Great Barrier Island and in parts of Russell forest, which were NZFS kauri management areas with substantial canopy release and under-planting activity up until 1985.

## Management

Currently, people, cattle and, possibly, wild pigs are regarded as being the principal vectors of PTA. Both Auckland Council and DOC are carrying out multi-million-dollar programmes to improve walking tracks by removing muddy soil contact areas and restoring the surface hydrology of kauri ecosystems (Figs 8 and 9). Five contaminated areas have been closed to the public through existing legislation or by Māori enacting traditional rahui.

Pigs are being controlled in contaminated forests and their elimination is proposed for some areas. The role of farm activities and livestock movement has emerged as an important issue in the spread and distribution of kauri dieback. Rural communities are being engaged to define the extent of the PTA problem and to implement measures to stop its spread.

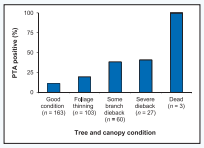



Figure 1. Proportion (%) of trees indentified as PTA positive (n = no. trees assessed) by canopy condition in joint agency surveillance.

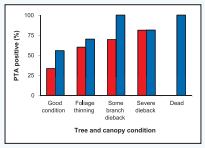



Figure 2. Proportion (%) of PTA positive trees with different canopy conditions that displayed fresh (red) and old (blue) cork cambial lesions.

| PTA positive trees | | | | | | |
|---|---|---|---|---|---|---|
| Lab | Surveillance 1 (*n* = 15) | | Waipoua (*n* = 29) | | Surveillance 2 (*n* = 21) | |
| | Mean | SE | Mean | SE | Mean | SE |
| SCION | 0.27 | 0.13 | 0.49 | 0.10 | 0.60 | 0.16 |
| Landcare | 0.67 | 0.14 | 0.76 | 0.09 | 1.00 | 0.00 |
| Plant & Food | 0.60 | 0.13 | 1.00 | 0.00 | 0.82 | 0.17 |
| **PTA positive sites** | | | | | | |
| Lab | Surveillance 1 (*n* = 24) | | Waipoua (*n* = 34) | | Surveillance 2 (*n* = 26) | |
| | Mean | SE | Mean | SE | Mean | SE |
| SCION | 0.22 | 0.12 | 0.41 | 0.11 | 0.56 | 0.16 |
| Landcare | 0.56 | 0.18 | 0.65 | 0.11 | 1.00 | 0.00 |
| Plant & Food | 0.40 | 0.14 | 1.00 | 0.00 | 0.77 | 0.14 |

Table 2. Probability of detecting PTA positive samples and sites in the three laboratories as surveillance rounds have progressed (Surveillance 1 in 2011, Waipoua 2011,and Surveillance 2 in 2012).



Figure 3. Sites identified and photographed from a helicopter survey on Te Hauturu-o-toi/Little Barrier Island for assessment and possible follow-up soil sampling.



Figure 6. Kauri dieback in regenerating (100-year-old) kauri stands.



Figure 8. Track construction using large-cell perforated plastic moulds at a site that is muddy for part of the year. The cells are filled with mixed bark and gravel and the structure is then capped with gravel to allow water movement and root development in the cells.



Figure 9. Track reconstructions using wooden rafts placed over bags filled with locally collected leaf litter.

## Conclusion

Kauri dieback remains undetected in many large areas of kauri forest. Development of cheaper and more accurate detection tools is needed to fully assess the kauri dieback status of forests and improve monitoring. Risk assessments are being used at each contaminated site/forest to refine management and reduce spread of the disease.


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Stan Bellgard, Ian Horner, Margaret Dick and the staff in the labs at Landcare, Plant and Food and SCION developed the bioassay methods and tested soils. Staff affiliated to the Joint Programme for Kauri Dieback, especially Jeannie Allport, Ross Johnson and John Beachman supervised some surveillance work, Katrina Upperton and the Kerikeri DOC recreation staff constructed the experimental track plots. Terry Conaghan produced the maps.

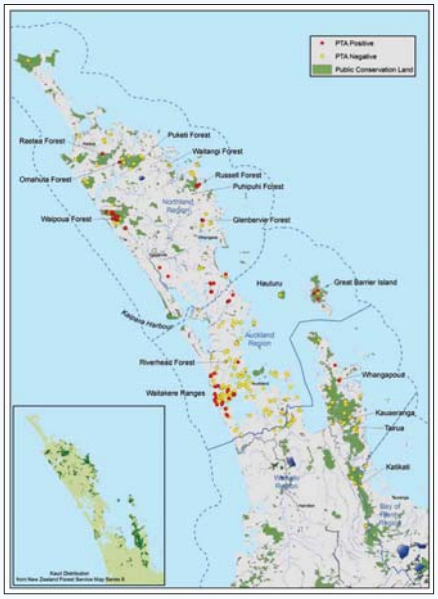




Figure 4. Map showing PTA status of sites/forests assessed during kauri dieback surveillance, along with regional boundaries. The insert map shows the location of the remaining large areas of kauri forest in northern New Zealand in 1954.

TĀNGATA WHENUA | MINISTRY FOR PRIMARY INDUSTRIES | DEPARTMENT OF CONSERVATION | NORTHLAND REGIONAL COUNCIL | AUCKLAND COUNCIL WAIKATO REGIONAL COUNCIL | BAY OF PLENTY REGIONAL COUNCIL

KEEP KAURI STANDING
STOP KAURI DIEBACK DISEASE SPREADING | KIA TŪTŪ NGĀ KAURI

## What is a species? – you be the judge

### *Phytophthora* taxon Pgchlamydo=*P.chlamydospora* or a *Phytophthora* complex?
### *Phytophthora* taxon Oaksoil=*P.obrutafolium* or *P.bilorbang?*
### *Phytophthora* taxon Ceanothus=*P .himalsylva* or *P.* "himalsylva-like"?

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We can define evolutionary species of *Phytophthora* as populations that share a single most recent common ancestor and have maintained genetic similarity in morphology, physiology, and ecological behavior. The theory is straightforward, the practice is not so easy. Three current challenges to our species concept are presented, including the working group's input delivered at the Conference and most recent publications involving these species/groups.

### *Phytophtora* taxon Pgchlamydo

*Phytophtora* taxon Pgchlamydo has been a puzzle for many years. Early isolates were identified as *P. drechsleri* and later thought to be a variant of *P. gonapodyides* that formed chlamydospores. This species was first cited as *P.* taxon Pgchlamydo in 2003 by Brasier et al. It has been found on 6 continents from a variety of hosts/sources. In 2013 Nagel et al. suggested it was a parent species to clade 6 hybrids found in South Africa and Australia.

We have analyzed *Phytophthora* isolates identified as *P.* taxon Pgchlamydo, from Europe, North America, and Argentina. Morphologically they are indistinguishable. Phylogenetically, all isolates are closely related in the COX1 region and show little diversity in the COX spacer region. Except for double peaks they are identical in the ITS region. Yet isolates sequenced for the nuclear beta tubulin locus showed a range from zero to twenty eight double peaks. The working group's opinion on whether this is one species or a complex was mixed. We believe *P.* taxon Pgchlamydo while evidently having a complex evolutionary history, is one species. Hansen et al. recently published a new species paper, selecting an isolate from the Brasier work that does not contain double peaks in ITS as the holotype for *P. chlamydospora*.

### *Phytophtora* taxon Oaksoil

*Phytophtora* taxon Oaksoil has been found in Oregon for over 15 years. Although we have over 350 isolates we are unaware of this species causing any significant damage. It is recovered mostly in environmental/stream samples and occasionally from a plant host. This species was first cited as *P.* taxon Oaksoil in 2003 by Brasier et al. In 2012 Aghighi et al. named a closely related new species associated with diseased blackberries in Australia, *P. bilorbang*. This new species is homothallic and readily

forms oospores. *P.* taxon Oaksoil from Oregon appears to have differences in sexual physiology from *P. bilorbang* because it is sterile. In her 2014 thesis Laura Sims suggested the *P.* taxon Oaksoil populations from Oregon constitute a species that is separate from *P. bilorbang* and should be named *P. obrutafolium*.

Beta tubulin sequences of *P. bilorbang* are a 100% match to those of *P.* taxon Oaksoil. In the COX1 region there is a 99% match. ITS sequences are a 99% match differing only in double peaks. The Oregon isolates fall into ten COX spacer groups, one with a 30 base deletion and another with a 2 repeat three base insert. While we have only examined 3 isolates of *P. bilorbang*, these isolates are identical to each other in the COX spacer and do not match any Oregon haplotypes. US and Australian isolates have been tested for oospore production in both countries and in both cases Australian isolates formed oospores and US isolates did not. The majority of the working group was of the opinion that these data were not compelling enough to warrant two species.

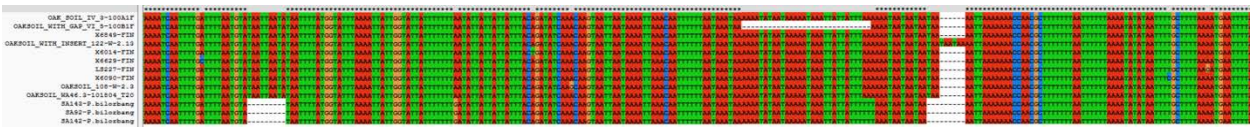


Figure 1. Alignment of the COX spacer region. The first 10 isolates listed are *P*.taxon Oaksoil, the last 3 are *P.bilorbang*.

### ***Phytophthora*** **taxon Ceanothus**

*Phytophthora* taxon Ceanothus is a new discovery in Oregon. Found only in nurseries and recent out-plantings, this species has been found to cause damage on *Ceanothu*s species as well as *Buxus* and *Rhododendron*. ITS sequences are unique, being closely related to *P. himalsilva* (a new species only known in Nepal) and *P. citrophthora* (a citrus host species).

ITS alignments show *P. himalsilva*, *P. citrophtora* and *P*.t axon Ceanothus are all very closely related. Beta tubulin sequences in *P*. taxon Ceanothus matched *P. himalsilva*. COX1 sequences showed variation between the three species, with *P. citrophthora* and *P*. taxon ceanothus appearing to be more closely related. Oogonia and antheridia measurements on *P. himalsilva* and *P*. taxon Ceanothus were similar, as were growth rates on both agar plates and inoculated *Ceanothus sanguineus* plants. Growth patterns appear to differentiate *P*. taxon Ceanothus from the other two species. *P. citrophthora* is described as being heterothallic with amphigynous antheridia while the other 2 are homothallic with mostly paragynous antheridia. Two recent papers have taken very different approaches to this quandary. Man in 't Veld et al. opted for “splitting”: naming their new species *P. occultans*. Considering their *P.* taxon Buxus, Nechwatal et al. concluded the data “do not provide sufficient proof for the existence of a new species.Since *P. himalsilva* was reported to be a highly variable species, a definite decision on the taxonomic status of the isolates described here cannot be made yet.” Sequences of the proposed species *P. occultans,* and *P.* taxon Buxus are both a 100% match to *P*. taxon Ceanothus in COX1 (126 base comparison only), Beta tubulin and ITS. These sequences match exactly to *P. himalsilva* in beta tubulin and are 99% similar in COX1 and ITS. Two thirds of the working party felt *P*. taxon ceanothus was *P. himalsilva*.

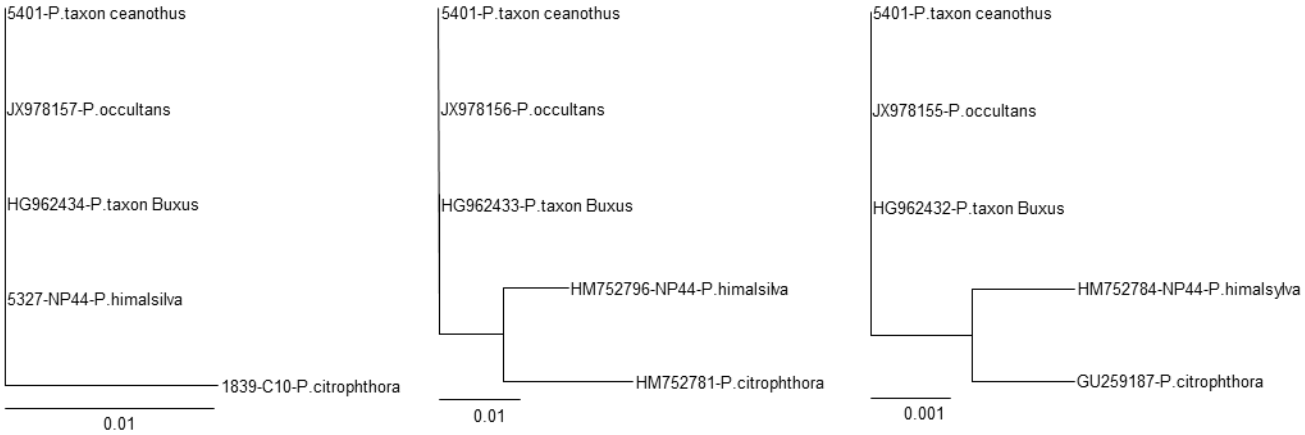


Figure 2. Trees representing alignments using B-tubulin (left), COX1 (center) and ITS (right).

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# Determining an optimal sequence identity threshold value for *Phytophthora* spp. retrieval from environmental data

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Generation of molecular data from environmental samples via DNA or amplicon massive sequencing becomes an easy and fast process in genomics and metagenomics analysis. The increasing number of new technologies allow the easy data generation, but bioinformatics still representing a critical step and a bottleneck in metagenomics analysis. One of the key steps in the data analysis for species identification purposes is the clustering of the reads based on their identity. Clustering parameters will define the species community composition and its reliability by reducing the risk of creating false MOTUs (Molecular Operational Taxonomic Units). An equimolecular mix composed with the DNA of eight pure cultures of *Phytophthora* species was used as control, and pyrosequenced using a nested PCR for library generation. Different identity threshold values were tested for MOTU clustering with 6.698 sequences obtained from the DNA mixture and with a custom-curated database including 146 ITS1 sequences of described and new *Phytophthora* taxa. Clustering at 100% of the reference sequence database, or at 99.5%, separated the higher number of *Phytophthora* species. However, applying this barcoding threshold to control data generated an exponential increase of MOTUs (mainly composed by singletons) due to the presence of sequencing and homopolymer errors. Furthermore it was not possible to separate some species (10%) in the custom-curated database using 100% of score coverage threshold (ITS1 taxonomic limitations). The cut-off value of 99% was the lower value able to separate all the species in the mix. Applying a cut-off value of 99% guarantee an optimal species separation and reduce the risk of false MOTUs, with minimum loss of data.

# Development of new Real-Time specific assays for the detection of *Phytophthora* species in Holm Oak calcareous forests

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Oak decline in non-calcareous soils in south-western Spain has been associated with *Phytophthora cinnamomi* for decades. However, other *Phytophthora* species such as *P. quercina* and *P. psychrophila* have been associated with *Quercus* decline in the eastern part of Spain where calcareous soils are predominant. With the aim of investigating the involvement of *Phytophthora* spp. in oak decline in eastern Spain, two forests in different geographical areas (Alcoi and Vallivana) were selected as sampling sites. Both forests are similar in altitude, soil and vegetation composition and are located 230 km apart. Soil samples were analyzed in parallel by isolation using baiting methods and by amplicon massive sequencing. Results showed that one of the most frequent species detected in both sampling sites was *P. quercina*, although cultures were only obtained from Alcoi"s holm oak forest. Pyrosequencing showed a very similar *Phytophthora* species composition in both areas. Furthermore, an uncultured *Phytophthora* taxa (named provisionally *Phytophthora* taxon *ballota*) was the dominant species, followed by *P. quercina*. Considering the difficulty in the isolation of *Phytophthora* taxon *ballota,* new Real-Time specific assays based in the ITS1 region were developed for the detection of this new taxa and *P. quercina* in environmental samples from oak declined areas. Taqman assays were tested on soil samples and on *Phytophthora* pure cultures. Results revealed the coexistence of both species in most of the samples, with the predominance of *P.* taxon *ballota* in terms of the amount of DNA available. Quantitation assays were high congruent with pyrosequencing results (relative number of reads per species). In order to evaluate the implication of different *Phytophthora* spp. in oak decline in eastern-Spain a new Real-Time specific detection protocol is proposed.

# Discovering *Phytophthora* species in the laurel forest in Tenerife and La Gomera islands (Canary Islands, Spain)

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A survey in the laurel forest was performed in three different sites in Tenerife (Anaga Rural Park, Corona Forestal Park and Teno Rural Park) and in La Gomera (Garajonay National Park) during January 2013. Water and soil samples were collected and DNA was extracted using E.Z.N.A. and Zymo DNA kits respectively. Amplicon library generation was performed using a nested PCR with *Phytophthora*-specific primers. A total of 100.095 sequences were obtained from a single pyrosequencing run and used to assess *Phytophthora* species diversity. In total, 24 *Phytophthora* species were detected, 22 species (*P. gonapodyides*, *P. megasperma*, *P*. taxon *PgChlamydo*, *P. gregata*, *P.* taxon *oaksoil*, *P. lacustris*, *P*. taxon *walnut*, *P. asparagi*, *P. cryptogea*, *P. drechsleri*, *P. syringae*, *P. plurivora*, *P. multivora*, *P. quercetorum*, *P. cactorum* and *Phytophthora* sp1, sp2, sp3, sp4, sp5, sp6 and sp7) were found in Tenerife and 11 species (*P. gonapodyides*, *P. megasperma*, *P.* taxon *PgChlamydo*, *P*. taxon *oaksoil*, *P. hydropathica*, *P. europea*, *P. quercetorum* and *Phytophthora* sp2, sp5, sp6 and sp7) were detected in La Gomera. Seven of these *Phytophthora* species are new to science. *Phytophthora* taxon *walnut* was detected in a water sample from Tenerife (Anaga Rural Park), with a total of 65 sequences, which could represent a new world location of this species. Fifteen of the species identified by pyrosequencing are also commonly detected in mainland Spain. This study showed that Macaronesian Islands could be considered as a hotspot for genus *Phytophthora* research due to the isolation and species evolution with their hosts

# Climate Change Can Affect the Impact of *Phytophthora alni* subsp. *alni*

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The sensitivity of alder pathogen *Phytophthora alni* subsp. *alni* (PAA) to low temperatures should be supposed because of absence of resting structures and from many other reasons (Černý and Strnadová 2012). Therefore the awaited climate change can have fundamental impact on spread of the pathogen, its importance and also on management of black and grey alder stands.

The sensitivity of PAA to deep frost and its survival was investigated in the series of investigations in the field and laboratory. The real survival rate was investigated in the riparian stand of Moravská Dyje River (southern Bohemia) after two climatically different winters (mild winter with avg. temperature 2.54°C in 2006/7 and standard one with avg. temperature –1.96°C in 2008/9). Each winter 115 samples of fresh necroses were taken and 10 segments (ca 5 × 5 × 5 mm) from each necrose were cultivated 14 days at 20°C on PARPNH and then the PAA survival rate was counted. The impact of temperature on altitudinal gradient on necroses dimension was investigated in riparian stands of Blanice River (southern Bohemia) highly affected by PAA. PAA necroses of 20 affected alders in each locality (approx. January temperature: –2°C, –3°C and –4°C and altitude: 400, 600 and 800 m a.s.l.) were measured and compared (PAA was consistently isolated from investigated stands. The effect of low temperature and frost duration on PAA survival was investigated ) with help of in vitro test,. Ten selected PAA isolates were incubated at 20°C on V8A for two weeks. Agar segments (diam. 0.5 cm) with mycelium and well developed oospores from margin of 2-week-old colonies were cut out, plated by mycelium downward on V8A plates and cultivated at different temperatures (from –0.1 to –10.0°C) and frost durations (0–28 days) in ten measurements per treatment (temperature × period). The survival rate and critical temperature was identified by following 2-week cultivation of plates at 20°C. The additional incubation test was used to verify the importance of bark thickness in pathogen survival. Black alder trunk segments differing in bark thickness were cut out in several distances from collar to top, infected with PAA and incubated 14 days at room temperature and then 3 days at critical temperature –7.5°C. Then 10 segments from each necrosis (five repetitions) were cultivated at 20°C on PARPNH and the survival rate was counted after two weeks.

The following outcomes were found out during investigation in the field: the PAA survival in trees differed according to winter temperature course. Its survival was highly limited (2.70%) after standard winter (avg. temperature –1.96°C), whereas after the extremely mild winter (2.54°C) without deep frosts the survival was ca 10× (25.52%) more successful. The thickness of covering tissues and exposure to the most heated (SW) quadrant of stem girth significantly enhanced the survival. The length of stem necroses of alders caused by PAA in the field depends on temperature and altitude (fig. 1).

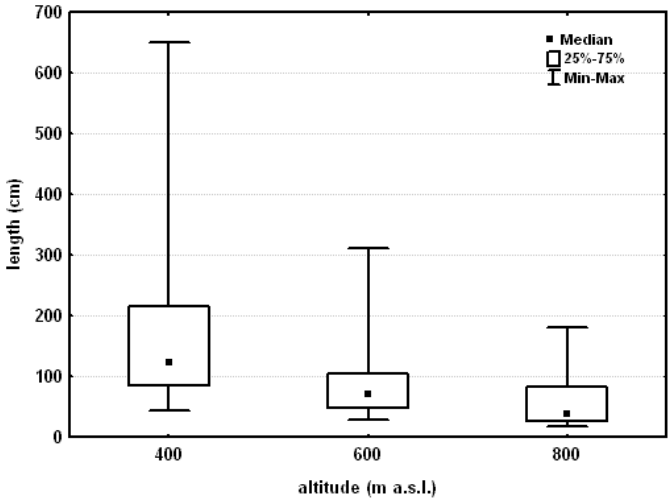



Fig. 1. The length of stem necroses caused by PAA on alders on altitudinal gradient

The effect of bark thickness was also verified by in vitro test. PAA under thin tissues (upto 8 mm) died within 3 days at –7.5 °C, whereas under thicker tissues survived at different rate (0.1–0.8). The significant regression (r=0.76) of PAA survival on thickness of covering tissues was identified (fig. 2). The failure time analysis showed that PAA survival significantly decreased after 4-days-incubation at –7.5 °C and completely died after 2 days at –10.0 °C (figs. 3,4). The tests revealed that PAA is capable to survive (in low frequency) in host tissues at least 28 days at –5.0 °C.

The results proved PAA to be very sensitive to heavy frost. The supposed climate change characterized by the increase in the lowest winter temperatures (IPCC 2007) poses a significant risk for alder population in Europe. On the other hand, it was also found out, that PAA is unexpectedly cold-tolerant and it is apparently capable to survive in roots in non-frozen water and soil at zero for a long time. The outcomes were partially published (Černý and Strnadová 2012, Černý et al. 2012) and verified by modeling (Aquayo et al. 2014).

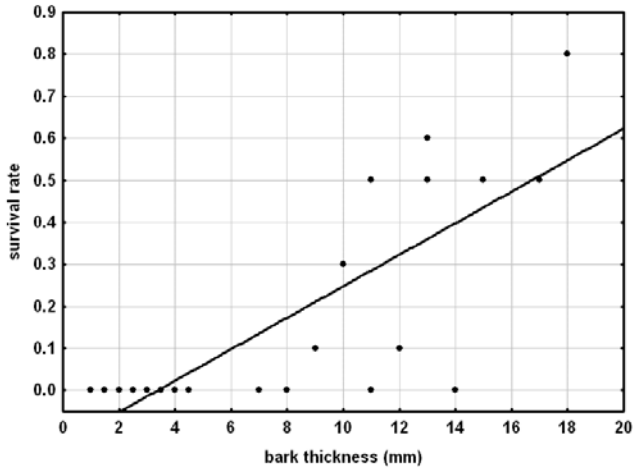



Fig. 2. The effect of bark thickness on PAA survival

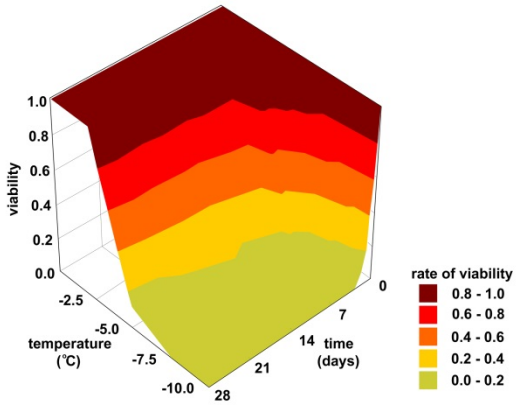



Fig. 3. The effect of temperature and frost duration on PAA survival

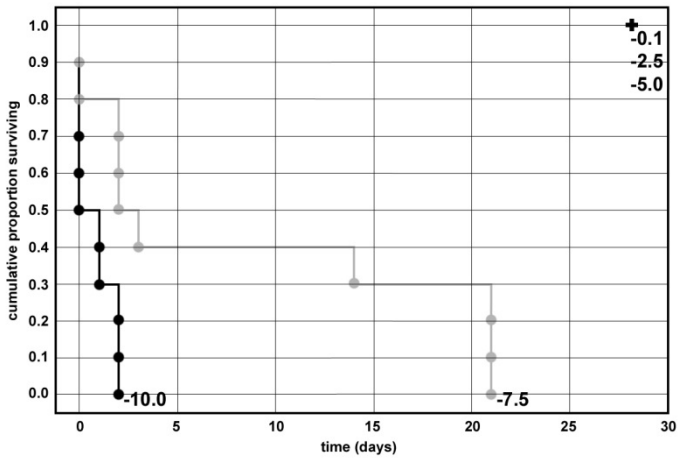



Fig. 4. The Kaplan-Meyer graph of PAA survival at different temperatures

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# Economical losses caused by *Phytophthora alni* in riparian stands. Typological study of Vltava River basin (Czech Republic)

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In recent years *Phytophthora alni* (PA) causes heavy but heterogeneously distributed losses in European alder riparian stands. It is necessary for river authorities to have basic information on economical losses caused by PA in different landscape types.

The information about pathogen distribution and ecology was summarized and the most important environmental factors influencing the pathogen distribution and disease impact (density of river system, density of forest alder plantings, vertical heterogeneity and temperature) were selected and used in a statistical model. The Vltava River basin (VRb) and served as a model area. Vrb is located in Central Europe (fig. 1), covers about 29 000 $km^2$ and its riparian stands are highly affected by the pathogen. The area of VRb was divided by rectangular grid (2.5 × 2.5 km) and the average values of variables were computed for all quadrates using GIS. The quadrates were clustered into 6 groups according to their environmental similarity (fig. 2). Detection of PA distribution and evaluation of losses started in randomly selected quadrates in 2013. The economical losses in alder stands were computed according to applicable regulations evaluating the price of trees (Anonymous 2008) and the cost of necessary works in affected alder stands – removal of dead and highly diseased trees and planting and protection of more resistant substitute species (Anonymous 2014).

The following should be stated after the first year of investigation. The pathogen was identified in ca 70% of investigated squares. The average economical losses exceeded 1700 €/100 m of affected alder riparian stand. The most affected landscape types were flat landscapes in middle altitudes and (South Bohemian) pond basins (fig. 3). The valleys of broad rivers in low altitudes and varied uplands with relatively sparse water systems were damaged in less extent. The mountain landscape with high vertical heterogeneity (and usually with cold climate) and vice versa dry ad warm landscape with low frequency of alder plantations in low altitudes were the least affected landscape types. The study is ongoing.

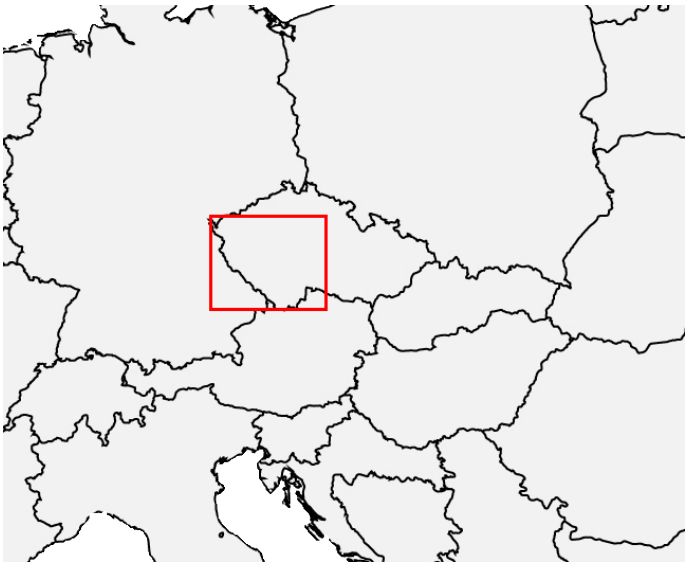


Fig. 1. Vltava River basin. Situation

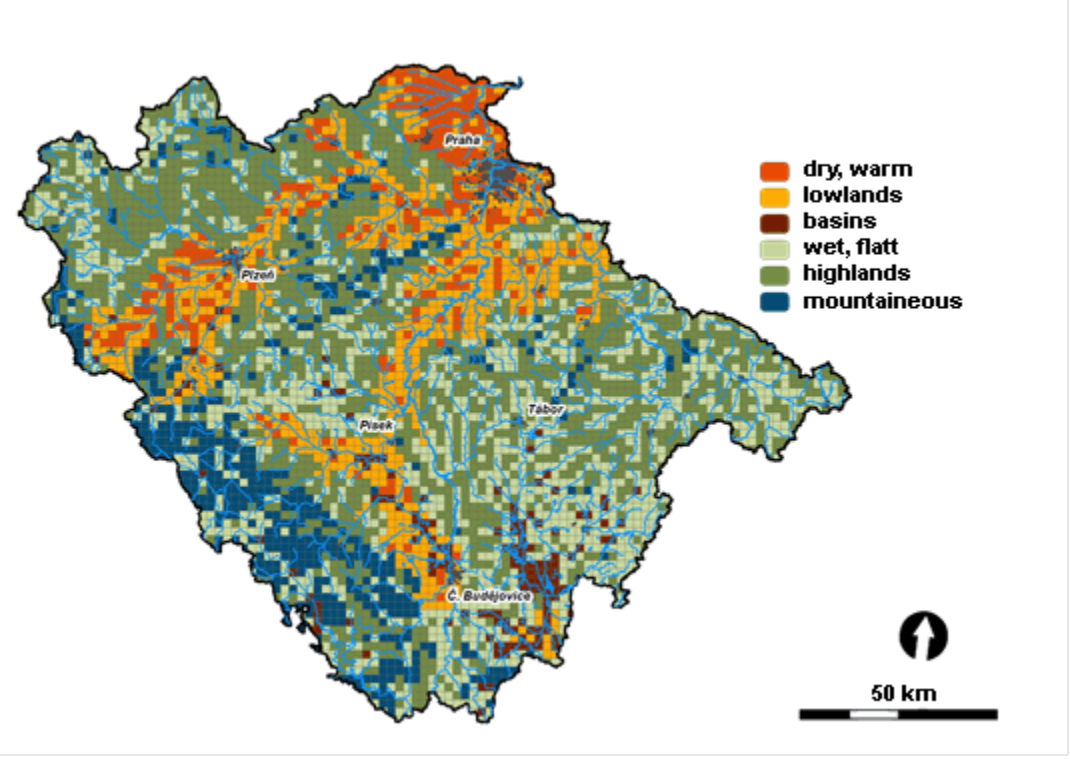



Fig. 2. Landscape types in VRb base on suitability of environment to *P. alni*

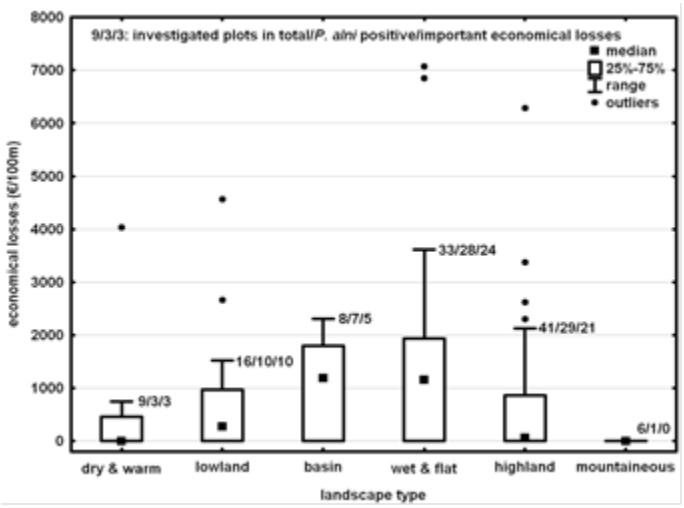



Fig. 3. Economical losses caused by *P. alni* in different landscape types

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Anonymous. 2014. Catalogue of descriptions and guide prices of construction works, URS, Prague.

# Factors Affecting *Phytophthora alni* Distribution in State Forests of the Czech Republic

Karel Černý[1], Veronika Strnadová[1], Dušan Romportl[1], Marcela Mrázková[1], Ludmila Havrdová[1]1, Markéta Hrabětová[1], Roman Modlinger[2], Vítězslava Pešková[2]

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*Phytophthora alni* (PA) is the most important pathogen of European alders. In addition to riparian stands, the pathogen causes important losses in forests where it can be introduced with saplings from nurseries (Jung and Blaschke 2004). It is very important for State Forests of the Czech Republic to know real distribution of the pathogen in forests, its importance and possible ways of introduction.

The distribution of PA in forest stands was investigated with cooperation with State Forests in 2013. The information on distribution of phytophthora root and collar rot of alder was collected from more than 840 stands covering the whole area of the Czech Republic. The data were evaluated in Statistica 8.0 with use of GLM. The determination of primary sources of infection in landscape was carried out in highly forested area (covering ca 420 $km^2$) on the border between Central and Southern Bohemia during field investigation in 2009 – 2011. The presence of *Phytophthora* and *Pythium* pathogens was investigated in 13 forest nurseries covering the whole area of the Czech Republic in 2014. Ten plants from each nursery were repeatedly flooded and maintained at 20 ˚C. Segments of tissues of plants showing necroses were incubated as decribed in Jung and Blaschke (2004). Acquired isolates were determined by standard methods (Jung and Blaschke 2004, Štěpánková et al. 2013).

Phytophthora alder disease was identified in 53% stands. The presence of the disease was positively influenced by presence of watercourse in the stand or in its margin and volume of alder biomass (standing timber stock). Presence of the disease was negatively influenced by altitude ($p < 0.01$). Watercourses were identified in 78% of diseased stands. Significant differences in damage according to width of the watercourse was identified (fig. 1) and positive regression of disease impact to watercourse width was also identified ($p < 0.05$). Median of age of affected stands was 59 years and the difference in age of infected and healthy stands was not found out (fig. 2). There were 34 hot spots of infection identified in the investigated area: 32 fishfarming ponds and 2 forest stands. Presence of PA in forest nurseries was very low – the pathogen was identified only in two nurseries from 13 sampled (15 %, table 1)

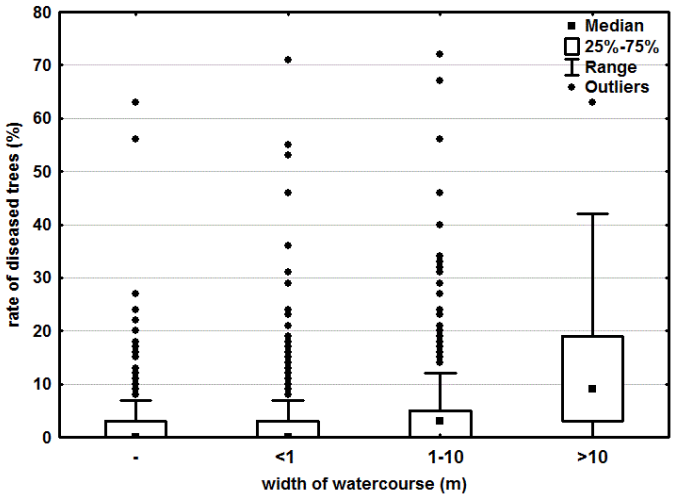



Fig. 1. The dependence of the disease intensity on presence and width of the watercourse.

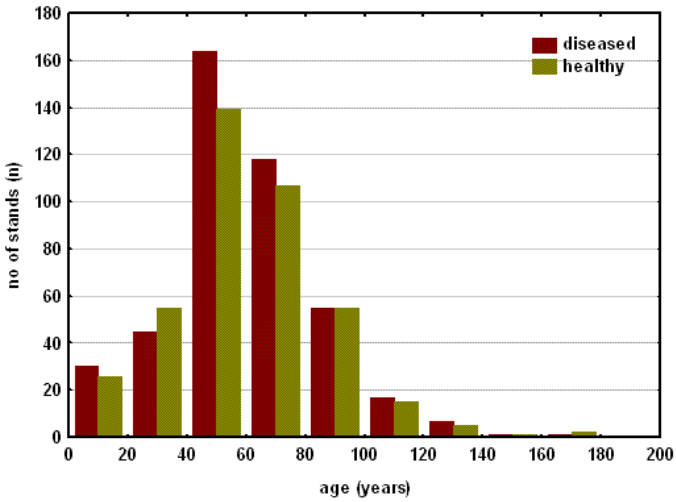



Fig. 2. The number of healthy and diseased forest stands in particular age classes

Very likely, natural spread of the pathogen throughout water system predominates over the spread via infected seedlings from nurseries in the area because of: 1) strong relation of pathogen presence in forests to watercourses, 2) usually high age and biomass volume of infected stands, 3) fishfarming ponds as dominating hot spots in landscape and 4) low frequency of PA in nurseries. Moreover, there is still another unknown important way of pathogen introduction into new areas and catchments. The pathogen could possibly spread in water with fry or with machinery and equipment of fishermen.

Table 1. Isolated oomycetes from alder sapling from forest nurseries

| Nursery no. | Isolated oomycetes |
|---|---|
| 1 | PAA, PAU, *P. plurivora, Py. chamaihyphon, Py.* cf. *litorale* |
| 2 | PAA |
| 3 | *P. cryptogea, Py. chamaihyphon, Py.* cf. *litorale* |
| 4 | *Py*. cf. *heterothallicum* |
| 5 | *Py. mercuriale, Py*. cf. *undulatum* |
| 6 | *Py. chamaihyphon, Py. litorale* |
| 7 | *Py. vexans, Py. cf. echinulatum* |
| 8 | *Py. cf. litorale* |
| 9 | *Py. ultimum* |
| 10 | - |
| 11 | - |
| 12 | - |
| 13 | - |

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# Invasive pathogens in Austrian forests: preliminary planning within the European project “Responses of European forests and society to invasive pathogens (RESIPATH)”

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The ongoing European project “Responses of European forests and society to invasive pathogens (RESIPATH)” within the BiodivERsa network includes the collaboration of 14 countries and comprises five work packages: (WP1) long term sustainability of tree species affected by invasive pathogens and framework for impact assessment; (WP2) understanding the mechanisms involved in adaptation of forest tree populations to new pathogens;(WP3) mechanisms of hybridisation in Europe; (WP4) detection and early warning of fungal and oomycete pathogens and WP5: public perception on impact of invasive pathogens. The Austrian project part aims to study the impact of both host and pathogen population demographics and evolution, to develop detection systems and to better understand the public perception. The Austrian project part will focus on the following pathogens that threaten these tree species: *Ophiostoma novo-ulmi* in elms (*Ulmus* spp.); *Chalara fraxinea* in ash (*Fraxinus* spp.); *Phytophthora alni* in alder (*Alnus* spp.) and *Erysiphe alphitoides* in oak (*Quercus* spp). To accomplish these aims, initial activities included a survey throughout Austria in late spring and summer 2014 to study the presence of *Phytophthora* spp. in alder riparian forests. Additionally, detection of *Phytophthora* was carried out in nine rivers and their tributaries. Monitoring of decline status and pathogen presence in five alder plots, previously established, was also performed. Preliminary results will be shown.

# Spectral measurements for detecting *Phytophthora*-related stress in *Corymbia calophylla* (marri)

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*3)School of Veterinary and Life Sciences, Murdoch University, Perth, Western Australia*
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*Corymbia calophylla* (marri) is a keystone species in the forests and woodlands of south-west Western Australia. Since the 1970's widespread marri mortality has been reported and various factors have been cited as the cause of this decline. In this study we investigated the pathogenic effect of *Phytophthora* root infection to evaluate its potential role in marri decline.

Field surveys were conducted to determine the extent of *Phytophthora* infection on marri. Soil and root samples from the rhizosphere of declining marri, from both remnant and natural sites, were collected and baited to recover *Phytophthora* species. The recovered *Phytophthora* species were used in pathogenicity trials. Hyperspectral remote sensing measurements, sensitive to leaf chemical and functional traits (especially related to foliar pigment and water content), and stomatal conductance measurements of plant function, in addition to estimates of above- and below-ground biomass, were taken during the trials to develop indicators of *Phytophthora*-related stress in marri.

Five *Phytophthora* species were isolated from marri, *P. cinnamomi, P. cryptogea, P. elongata, P. multivora and P. calophyllaphile* prov. nom. These varied in their pathogenicity to marri. *P. cinnamomi* was the most pathogenic species whilst some isolates of *P. multivora* stimulated root and shoot growth on marri compared to the control plants. Stomatal conductance measurements correlated with the results of the pathogenicity trials, as did a number of spectral indices. The Normalised Difference Vegetation Index (NDVI) and Simple Ratio Index (SRI), general indicators of "greenness" and vegetation condition, and the Vogelman Red Edge Index 3 (VOG3), indicator of auxiliary pigment content were closely related to marri response to *Phytophthora* infection. Conductance and greenness measurements were decreased and pigment content was increased by the *P. cinnamomi* infection. Non-destructive spectral measurements taken regularly throughout the trial reveal time-courses of marri decline and may provide early-warnings of infection. More experimental work is underway, including dual inoculation with *Phytophthora* and *Quambalaria coyrecup*, the cause of marri canker disease.

# Next generation sequencing of *Phytophthora ramorum*: differences in the gene expression during infection in the lineages EU1/EU2

Lourdes de la Mata Saez[1], Alistair McCracken[1], Jianguang Jia[2], Fiona Doohan[2] & Colin Fleming[1].

[1]Agri-Food and Biosciences Institute (AFBI), Belfast, UK
[2]University College Dublin (UCD), Dublin, Ireland

*Phytophthora ramorum* is an exotic fungus-like organism that has been affecting a wide range of woody hosts since it was first reported in North America in 1993 and in Europe in 2001. There are four lineages of *P. ramorum*: NA1, NA2, EU1 and EU2. EU2 seems to be unique as it is only located in Northern Ireland and the south-west coast of Scotland. Japanese and European larch trees were inoculated with two different isolates of the pathogen, which had different pathogenicity as shown in an artificially inoculated trial, and RNA was extracted from the lesson. The transcriptome was sequenced using Illumina 2000 in order to observe the differences in the expression of the genes involved in the process of infection. Preliminary results show differences in pathogenicity between EU1 and EU2 and also differences in defence responses of the hosts were observed at a gene expression level.

# *Phytophthora ramorum*: differences in the gene expression during infection in the lineages EU1/EU2

Lourdes de la Mata[a]*, Alistair McCracken[a], Jiangguang Jia[b], Fiona Doohan[b] & Colin Fleming[a]

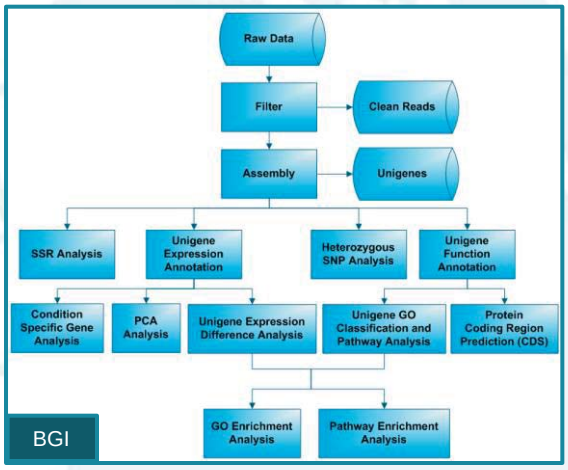
[a]*Agri-Food and Biosciences Institute (AFBI) 18a Newforge lane, BT9 5PX, Belfast, Northern Ireland, UK.*

[b]*School of Biology and Environmental Science, College of Life Sciences, UCD, Belfield, Dublin 4, Ireland.*

Small Japanese and European larch trees were inoculated with one of two EU1 or EU2 isolates of *Phytophthora ramorum*. Differences in genes expression during the first seven days of the infection process were determined.



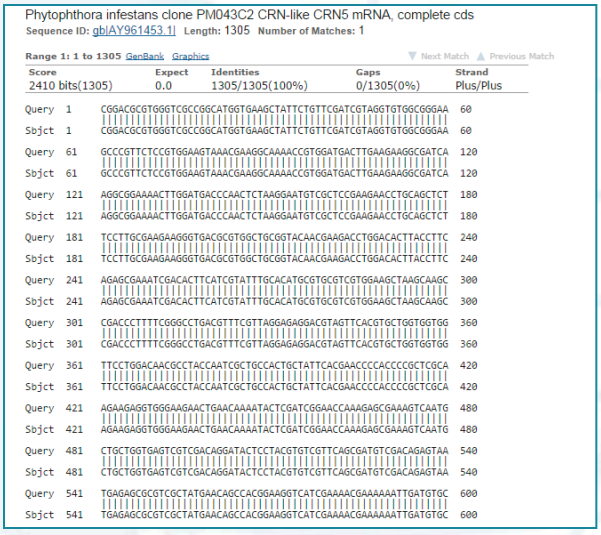

Next generation sequencing with Illumina's HiSeq 2000 system.




Preliminary results EU1 vs EU2.

| | EU1 | EU2 |
|---|---|---|
| **Up-regulated** | 13536 | 12744 |
| **Down-regulated** | 12503 | 11870 |

| Sequence File | NR | NT | Swiss-Prot | KEGG | COG | GO | ALL |
|---|---|---|---|---|---|---|---|
| All-Unigene.fa | 22,441 | 17,062 | 11,948 | 12,229 | 8,438 | 11,771 | 22,686 |



Phytophthora infestans clone PM043C2 CRN-like CRN5 mRNA, complete cds

Sequence ID: gb|AY961453.1| Length: 1305 Number of Matches: 1

Range 1: 1 to 1305 GenBank Graphics

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 2410 bits(1305) | 0.0 | 1305/1305(100%) | 0/1305(0%) | Plus/Plus |

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Query  541  TGAGAGCGCGTCGCTATGAACAGCCACGGAAGGTCATCGAAAACGAAAAAATTGATGTGC  600
Sbjct  541  TGAGAGCGCGTCGCTATGAACAGCCACGGAAGGTCATCGAAAACGAAAAAATTGATGTGC  600
```

CRN-like CRN5, cell death-related nuclease, is up regulated in EU2 and not expressed in EU1.

**Next Steps:**

1) Examine other genes which may have a role in differences in pathogenicity.
2) Identify key pathways which make EU2 more aggressive.
3) Develop SSR and SNPs markers.
4) Design strategies to control *P. ramorum* by silencing key genes involved in the infection?

Aknowledgements: PHYTOFOR (COFORD), Queen's University of Belfast, AFBI Belfast

*Corresponding author: ldelamatasaez@gmail.com

# The detection and quantification of four *Phytophthora* species in soil in the UK

M. Elliot[1], S. Green[1]

[1]*Forest Research, Northern Research Station, Roslin, EH25 9SY*

A number of newly described *Phytophthora* species have been discovered in the UK over the past decade infecting a wide range of host species. Little is known about the role of soil in the epidemiology of these pathogens including the role of humans and animals in the spread of soil both within and between infected sites. We describe new methods for the detection and quantification of four *Phytophthora* species in soil; *Phytophthora ramorum*, *P. kernoviae*, *P. lateralis* and *P. austrocedrae*. These methods will lead to a better understanding of *Phytophthora* disease spread and inoculum persistence in soil.

# RNAseq reveals different defense responses of *Quercus robur* microcuttings against *Phytophthora quercina* during root and shoot flush

F. Fleischmann[1], O. Angay[1,2], S. Recht[3], L. Feldhahn[3], M. Tarkka[3], S. Hermann[3] and T. Grams[2]

*1) Pathology of Woody Plants, Technische Universität München, Freising, Germany;*
*2) Ecophysiology of Plants, Technische Universität München, Freising, Germany;*
*3) Soil Ecology, UFZ-Helmholtz Centre of Environmental Research, Halle (Saale), Germany.*
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Within the joint research project "TrophinOak", we analyze multitrophic interactions of *Quercus robur* micro-cuttings with respect of the rhythmic growth of oak. Our research team focuses on interactions with the root pathogen *Phytophthora quercina*. In addition, we compare the effects of the ectomycorrhizal (EM) fungus *Piloderma croceum*, as an additional interacting partner. It turned out, that infestation of roots with *P. quercina* is positively correlated with the concentration of non-structural carbohydrates (NSC) in oak roots (Angay et al., 2014). NSC concentrations were strongly influenced by flush status of microcuttings, and mycorrhization further accentuated this flush dependent shift without protecting roots against the pathogen. To elucidate the processes in oak roots after infection with *P. quercina* on the molecular level, we performed a transcriptomic approach using RNAseq as described by Tarkka et al. (2013). It turned out that more than 4,000 contigs were differentially expressed in lateral roots, when root and shoot flush, respectively, were compared. However, only a low number of contigs (about 80 in root flush and 40 in shoot flush, respectively) were differentially expressed upon *Phytophthora* infection, with almost no overlap in differential expression patterns between flush stages. Moreover, contigs related to pathogen defense were hardly addressed indicating that *P. quercina* might suppress defense response in oak roots in a similar way as it has been described for *Fagus sylvatica* and *P. plurivora* (Schlink, 2009, 2010).

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Tarkka MT, Herrmann S, Wubet T, et al. 2013. New Phytologist 199(2): 529-540.

Schlink K. 2009. Plant Cell Reports 28(5): 873-882.

Schlink K. 2010. Functional & Integrative Genomics 10(2): 253-264.

# Alternatives for detection of *Phytophthora cinnamomi* in commercial substrate

Edwin Antonio Gutierrez Rodriguez[1], Mauricio Panizzi Penariol[2], Marcia Cristina Ohya [2], Rita de Cassia Panizzi[3], Renata Aparecida de Andrade[4]

[1] *MSc., PhD student in Agronomy, Crops Program;* [2] *Student of Agronomy Engineer.,* [3] *Agronomy Engineer, Assistant Professor Dr. Department of Plant Pathology;* [4]*Agronomy Engineer, Assistant Professor. Dr., Department of Plant Production, Faculdade de Ciências Agrárias e Veterinárias, UNESP – Universidade Estadual Paulista, Câmpus de Jaboticabal.*

*Corresponding author: edunillanos@hotmail.com*

Different materials can be used as bait for diagnosis of the presence of pathogens in different environments such as water, soil and plant tissue. Specifically, to *Phytophthora sp*., several sources have been referenced, including leaf explants of *Camellia Japonica*, *Eucalyptus cinerea, Rhododendron catawbiense, Citrus lemon*, rose petals, fruits, cotyledons, and others. In Brazil, for commercial production of certified fruit plants, substrate among other things, should be free of pathogens . In order to test alternative for baiting of *Phytophthora cinnamomi* 5 materials as bait (carrot, avocado leaves, red rose petals, champagne rose petals and cellophane) were compared under light conditions (35 ± µm.m2 . s-1) and in the dark at constant temperature (± 21 ° C) room for 48 h. In a completely randomized design, the unit with ten replicates consisted of a plastic container with 10 g of substrate previously inoculated with *P. cinnamomi* (LRS 21/88 donated by *Agencia Paulista de Tecnología Agropecuaria* - Brazil - APTA) immersed in 30 mL of deionized water stelilized and a fragment of about 1 $cm^2$ of each material used as bait. This evaluation of the effectiveness of bait to capture the oomycete was taken from a scale (0, 1-5, 6-10, 11-15, 16 sporangia). As a result, the factors tested, both as light as in the dark there was formation of sporangia. In relation to materials used as bait, in cellophane did not find the presence of sporangia, however, the rose petals was observed increased amount of sporangia and avocado leaves sporangia observed in lower density. In the case of carrot that, unlike other materials did not remain on the water surface, there were more elongated sporangiophores. These are partial results and other studies are being developed in parallel with the expression of related proteins in plants and seeds of avocado for their photosynthetic behavior of chlorophyll and chlorophyll content of plants inoculated with *P. cinnamomi.*

# Alternatives for detection of *Phytophthora cinnamomi* in commercial subtrate

Edwin Antonio Gutierrez Rodriguez[1], Mauricio Panizzi Penariol [2], Marcia Cristina Ohya [2], Rita de Cassia Panizzi[3], Renata Aparecida de Andrade[4]

*[2] MSc., PhD student in Agronomy, Crops Program, email: edunillanos@hotmail.com; [2] student of Agronomy Engineer., [3] Agronomy Engineer, Assistant Professor Dr. Department of Plant Pathology; [4]Agronomy Engineer, Assistant Professor Dr. Department of Plant Production, Faculdade de Ciências Agrárias e Veterinárias, UNESP – Universidade Estadual Paulista, Câmpus de Jaboticabal.*

## INTRODUCTION

Different materials can be used as bait for diagnosis of the presence of pathogens in different environments such as water, soil and plant tissue. Specifically, to *Phytophthora* sp., several sources have been referenced, including leaf explants of *Camellia japonica*, *Eucalyptus cinerea*, *Rhododendron catawbiense*, *Citrus lemon*, rose petals, fruits, cotyledons, and others. In Brazil, for commercial production of certified fruit plants, substrate among other things should be free of pathogens.

## MATERIALS AND METHODS

In order to test alternative for baiting of *Phytophthora cinnamomi* 5 materials as bait (carrot, avocado leaves, red rose petals, champagne rose petals and cellophane) were compared under light conditions (± 35 µm.m$^2$.s-$^1$) and in the dark at constant temperature (± 21 ° C) room after 4 days of incubation.

In a completely randomized design, the unit with ten replicates consisted of a plastic container with 10 g of substrate previously inoculated with *P. cinnamomi* (LRS 21/88 donated by *Agencia Paulista de Tecnología Agropecuaria* - Brazil - APTA) in 30 mL of deionized stelilized water and a fragment of approximately 1 cm$^2$ of each material used as bait. This evaluation of the effectiveness of bait to capture the oomycete was taken from a scale (0, I, II, III, IV, and V corresponding to 0, 1-5, 6-10, 11-15, +16 sporangia observed on the microscope with 400x of increase).

## RESULTS

The detection of *P. cinnamomi* on commercial substrate it is a tool to decrease incidence of the disease on agroecosystems for the utilization of plants coming from greenhouses, and the detection efficiency of pathogen is necessary.

On this research, as a result, the factors tested, both as light as in the dark there was formation of sporangia. In relation to materials used as bait, in the cellophane the presence of sporangia was not found, however, in the rose petals were observed increased amount of sporangia and avocado leaves sporangia observed in lower density. In the case of carrot that, unlike other materials did not remain on the water surface, there were more elongated sporangiophores.

Others materials like apples, avocado, lupine bean, Sicilian lemon leaf, are used to detect on water and plant tissue.

Table 1. Analysis of variance of the factors evaluated.

| Source of variation | F | |
|---|---|---|
| Lighting(a) | 8.469 | ** |
| Material (b) | 141.298 | ** |
| Inter axb | 4.238 | ** |

Table 2. Analysis of variance of the factors evaluated.

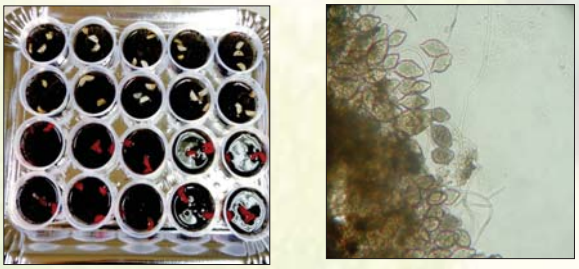
| Lighting | Bait | | | | |
|---|---|---|---|---|---|
| | Celophane | Carrot | Avocado leaf | Champagne Rose petals | Red rose petals |
| Light | 0.707 aE | 1332 aD | 1619 aC | 1856 aB | 2096 aA |
| Dark | 0.707 aD | 1209 aC | 1590 aB | 1867 aA | 1697 bAB |
| CV (%) 12.66 | | | | | |

LSD (row) = 0.2315 (capital letters)

LSD (columns) = 0.1651 (lower letters)

The data was transformed (x+0.5)½ . Averages followed by the same letter are different statistically from each other. Tukey test was applied (P ≥ 0.05)

Figure 1. Typical sporangium (not papilated) of *Phytophthora cinnamomi* on rose petals bait.



## CONCLUSION

Rose petals, independent of color, were more attractive to detection *P. cinnamomi* present in substrate and sporangium formation. However, there was interaction between the factors evaluated.

## ACKNOWLEGMENT

To Science, Technology and Innovation Colombian Departament (COLCIENCIAS) and to *Faculdade de Ciencias Agrarias e Veterinarias-UNESP Jaboticabal* for the resources and the research development.

# Influence of Multiple Stress Sources on Cork Oak (*Quercus suber* L.) Seedling Susceptibility to *Phytophthora cinnamomi*

Oliver Gutiérrez-Hernández[1], Luis V. García[1], Paolo De Vita[2], María S. Serrano[2], Cristina Ramo[3], Eduardo Gutiérrez[1], Pedro Ríos[2], Ignacio Pérez-Ramos[1], Lorena Gómez-Aparicio[1] & Mª Esperanza Sánchez[2]

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Some evergreen species within the genus *Quercus* (namely *Q. ilex* and *Q. suber*) are of paramount socio-economic and ecological importance in Spain and Portugal. Two main threats to their long-term survival are currently recognized. Firstly, invasive soil-borne pathogens, particularly *Phytophthora cinnamomi,* which kill myriads of trees every year. Secondly, the potential long-term response of these key species to extended drought stress derived from climate change. A rise in mean temperatures and a significant loss of annual rainfall drop, together with an increase of extreme rainfall events, is forecast for this area by the end of the XXI$^{th}$ century **(1)**. These environmental changes could enhance the incidence of the pathogen, favored by alternating mild/extreme rainfall events and drought periods (4).

Previous studies showed that abiotic stress may affect both plant and pathogen performance **(2, 3),** which make very difficult (or even impossible) to separate direct effects of abiotic stress on trees from indirect pathogen-mediated effects.

When the combined effects of abiotic stress and pathogens on plants are considered, a critical question is whether increased abiotic stress is able to induce a weakening of trees and facilitate root infections ('host weakening hypothesis'). Alternatively, it can be hypothesized that, as long as the conditions will be favorable for a highly virulent pathogen, the previous stress-history of a susceptible host is not especially relevant for disease spreading ('primary pathogen hypothesis')

We tested these two alternative hypotheses in a greenhouse experiment where *Q. suber* seedlings were submitted to two water regimes (current and a dryer scenario of 1/3 reduced water inputs) and three levels of soluble salts (a source of physiological drought). After 150 days, plant performance was evaluated and transferred to new pots infested with resting spores of *P. cinnamomi.* Pots were subjected to periodical soil flooding and assessed weekly for crown symptoms. After 6 weeks plants were assessed for root necrosis.

We found that both water and salt stresses significantly affected plant performance, but no significant differences in *Phytophthora* symptom severity were found among plants subjected to different intensity of both stresses after they were infected.
We concluded that:

1. Extended exposure of cork oak seedlings to different abiotic stress scenarios did not increase their susceptibility to *Phytophthora cinnamomi.*

2. Our experimental results held the ‘primary pathogen hypothesis’ against the ‘host weakening hypothesis’.

3. Notwithstanding, the direct effect of these harsh environmental scenarios on the pathogen itself may determine different outcomes that need to be experimentally tested.

**Acknowledgements**

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# Species of *Phytophthora* on rhododendrons in Argentina

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Since 2011 affected plants growing in gardens and nurseries near Buenos Aires have been surveyed. Samples of plant tissue with typical oomycetes disease symptoms have been collected and examined. On rhododendrons two *Phytophthora* species were consistently isolated from symptom-bearing leaf tissues and roots on PARBH medium. Sporangia were produced abundantly in non-sterile soil extract. In Isolation 1, most of the sporangia were semi-papillate and ovoid, limoniform, ellipsoid or obpyriform; chlamydospores were not observed. Isolates were homothallic with plerotic oospores, 22.9 ± 1.9 µm and paragynous antheridia. The optimum growth temperature was 25 ± 1°C on V8A and the maximum growth temperature was 32 ± 1°C. The ITS1 and the *B- Tubulin* genes were amplified. Isolation 2: sporangia without papilla, ovoid, ellipsoidal, obpyriform, terminal, 45-25 µm, internal proliferation. The isolate produced spherical, terminal or intercalary chlamydospores, in clusters on short side stalks. This isolate was heterothallic (no sexual structures were formed). Cardinal temperatures were 5 (24-28) > 35 °C. The ITS gene was amplified too. The rDNA sequences obtained from both isolates were compared with sequences deposited at the GeneBank, using the Basic Local Alignment Search Tool (BLAST) program. Both sequences from Isolation 1 proved to be identical to *Phytophthora multivora* ex-type CBS 124.094 (FJ237517). The sequence from Isolation 2 was identified as *Phytophthora cinnamomi,* showed 99,74% homology with NFJ801806, culture type. These species were determined on the basis of their morphological, cultural and molecular characteristics. *Azalea, Viburnum tinus* and *Photinia fraseri* are *Phytophthora multivora* potential hosts because they were infected on artificial inoculations but were not found during this survey on natural infections.

# Comparative fitness of European lineages of *Phytophthora ramorum*

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Until recently the population structure of *Phytophthora ramorum* was known to consist of three largely clonal evolutionary lineages, with only the EU1 present in the UK and wider Europe. However, a fourth evolutionary lineage of *P. ramorum*, the EU2, was discovered in 2012 and can be distinguished from the other lineages (EU1, NA1 and NA2) both genetically and phenotypically. The EU1 and EU2 are both present in the UK, although the EU1 is much more widespread. Both cause mortality to plantation grown larch (mainly Japanese larch – *Larix kaempferi* which accounts for 6% of all forest cover in Britain) and this is now suffering heavy disease levels as a result of *P. ramorum*. To understand if the EU2 could pose an increased threat to forests, and in particular to larch, the ability of both European lineages to attack bark (phloem) tissue of mature larch (*L. kaempferi* and *L. decidua*), oak (*Quercus robur*) and beech (*Fagus sylvatica*) was evaluated. In addition, the susceptibility of Japanese larch and European larch saplings was tested against EU1 and EU2 isolates at two incubation temperatures, 10$^{O}$C and 20$^{O}$C. Out of the four tree species, the ranking of bark susceptibility (from most to least) was Japanese larch> European larch> beech>oak, but on average EU2 isolates produced markedly larger lesions in the bark of Japanese larch and European larch compared with EU1 isolates. With sapling material, the same pattern emerged of increased susceptibility of Japanese larch to the EU2 lineage at 20$^{O}$C, although it was striking that even 10$^{O}$C both lineages were capable of causing significant damage to both larch species after just 7 days incubation. With rhododendron foliage however, there were no consistent differences in the amount of necrosis caused by the two lineages, suggesting that the increased threat posed by the EU2 may be unique to larch.

# Oomycetes survey in northern Norway

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In 2012, we started a survey of oomycetes in natural habits in Norway as part of a national biodiversity project. We sampled in two areas north of the Arctic Circle in continental Norway (Finnmark and Nordland counties) and in the Svalbard archipelago.

We collected mostly mosses in Svalbard and grass and mosses in continental Norway. Furthermore, we baited with grass and rhododendron leaves in streams, lakes and brackish water in Finnmark and Nordland.

The isolates obtained were identified by ITS sequencing. Preliminary results shows that most of the species recovered belong to the the genus *Pythium*, but *Phytophthora*, *Halophytophthora* and *Saprolegnia* were also found, Table 1.



From Barentsburg



From New Ålesund



From Longyearbyen






From Jarfjord in Finnmark



From Neidenelva in Finnmark



From Futelva near Bodø in Nordland

Table 1. Oomycetes found in a survey in northen Norway. Species identified to species level by ITS sequence similarity of 99% or more to isolates in GenBank. Shore: intertidal areas, splash areas or brackish water; Inland: Out of splash area. bg: baiting with grass pieces or pepper seeds; br: baiting with rhododendron leaves; bl: baiting in labatory; dim: direct isolation from moss; dig: direct isolation from grass, dix: direct isolation from plant material.

| *Phytopthora* and *Pythium species* (clades) | Svalbard | Finnmark | | Nordland | |
|---|---|---|---|---|---|
| | | Shore | Inland | Shore | Inland |
| *Phy. gonapodyides* (6) | | | x (br) | | x (br) |
| *Phy. lacustris* (6) | | | x (br) | | |
| *Py. anandrum* (H) | | | | | x (br) |
| *Py. angustatum* (B1) | | | | | x (bg) |
| *Py. attrantheridium* (F) | | | | x (dig) | |
| *Py. dissimile/contiguanum* (B1) | | | | x (dig) | |
| *Py. intermedium* (F) | | | | x (dig) | x (bl) |
| *Py. monospermum* (A) | | | x (dim) | | |
| *Py. pachycaule* (B2) | x (dim) | | | x (dig, bg) | |
| *Py. polare* (G) | x (dim) | | | | |
| *Py. pyrilobum* (B) | | | x (dim) | | |
| *Py. undulatum* (H) | | x (bg) | x (bg) | | |
| *Py. volutum* (B1) | | x (dig) | | | |
| *Py. sp. balticum* (F) | | | | x (dig) | |
| *Pythium* B2 (*P. diclinum, P. coloratum, P. dissotocum, P. lutarium, P. marinum*) | | | x(bg) | x (bl) | |
| *Py. pachycaule/oopapilum* (98-99% ITS similarity) | x (dim) | x (bg) | x (bg) | x (dig, bg, br) | |
| *Pythium* new especies (ITS similarity < 95%) | x(dim) | x (dig, bg, dim) | x (dim) | | x (dim, bg) |
| *Saprolegnia australis* | | x (bg) | x | | |
| *S. hypogyna* | | | x (dim) | | |
| *S. ferax* | | | x (bg, dix) | | |
| *S. parasitica* | | x (bg) | x (bg) | | |
| *Halophytophthora* sp. nov | | x (dix) | | | |

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# Biology of *Phytophthora* species in aquatic ecosystems

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*Phytophthora* species were added to the list of water moulds back in 1944 [1]. Over the past 70 years, a total of approximately 60 species have been detected from natural waterways and agricultural irrigation systems [2]. However, little is known about their biology in aquatic ecosystems [3]. This is a major gap in our knowledge about this important genus and its members.

To fill this knowledge gap, we have conducted three lines of studies:

1. Determining the diversity and population dynamics of *Phytophthora* species along water path in an irrigation reservoir with irrigation runoff entrance in one side while exit and pump house locating in the opposite side.
2. Continuously monitoring water quality including temperature, pH, dissolved oxygen, oxidation-reduction potential, electrical conductivity, salinity, total dissolved solids, turbidity, and chlorophyll *a* in the same reservoir.
3. Assessing zoosporic responses to pH, electrical conductivity and dissolved oxygen stresses of selected *Phytophthora* species in a simulated aquatic system.

The data from these studies do not support the conventional wisdom that all *Phytophthora* species are water moulds. Some *Phytophthora* species may be a resident of aquatic ecosystems, while others may be merely a transient or even terrestrial. These data are crucial to developing sustainable management strategies for Phytophthora pathogens in agricultural water reservoirs and crop health locally and plant biosecurity globally. They also help understand the diversity and population dynamics of these species in natural aquatic ecosystems where water quality does not fluctuate as dramatically and frequently as in agricultural reservoirs.

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# Biology of *Phytophthora* Species in Aquatic Ecosystems

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**G. W. Moorman (Pennsylvania State University)**
**J. D. Lea-Cox (University of Maryland)**

## Introduction

The genus *Phytophthora* was added to the list of "water moulds" 70 years ago (1) and nearly half of the 130 plus known species have been found in irrigation reservoirs, natural lakes and waterways (3,6). However, little is known about their aquatic biology. Here we will show three lines of studies undertaken to fill this knowledge gap over the past 15 years. Our latest project has a website at **http://www.irrigation-pathogens.ppws.vt.edu/**.

## Diversity and Population of *Phytophthora* spp. in an Irrigation Reservoir

Table 1. Diversity of known *Phytophthora* spp.

| Major pathogen | Recently named species |
|---|---|
| *P. cactorum* | *P. aquimorbida* |
| *P. citrophthora* | *P. hydrogena* |
| *P. drechsleri* | *P. hydropathica* |
| *P. megasperma* | *P. rosacearum* |
| *P. nicotianae* | *P. polonica* |
| *P. parsiana* | *P. inundata* |
| *P. pini* | *P. irrigata* |
| *P. pseudosyringae* | *P. sansonmeana* |
| *P. syringae* | *P. t. lagoariana* |
| *P. tropicalis* | *P. t. PgChlamydo* |



Fig. 1. Deployment of rhododendron leaf baits at 20-m intervals from a runoff entrance

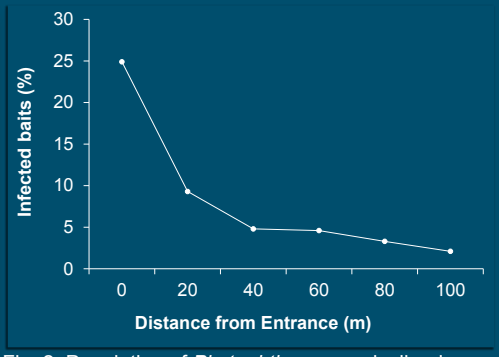



Fig. 2. Population of *Phytophthora* spp. declined with increasing distance from the entrance

## Water Quality Dynamics in Reservoirs

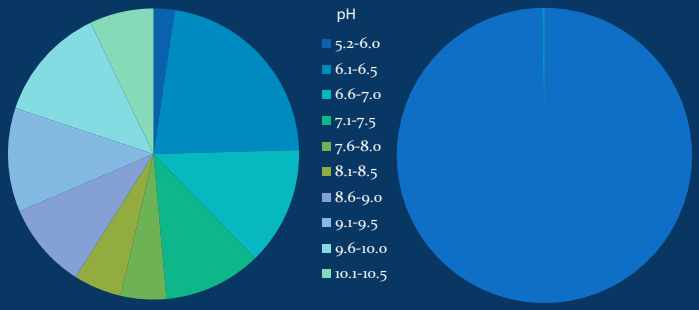



Fig. 3. Distribution of water pH readings (every 15 min) over a 1-year period in an irrigation reservoir (left) and an adjacent creek (right) (2)

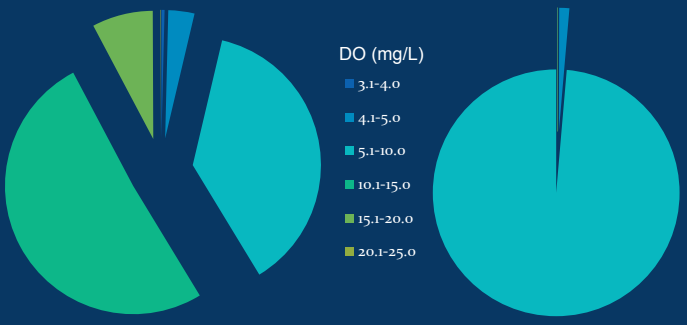



Fig. 4. Distribution of dissolved oxygen (DO) readings over a 1-year period in an irrigation reservoir (left) and an adjacent creek (right)

## Zoosporic Responses to pH and DO stresses

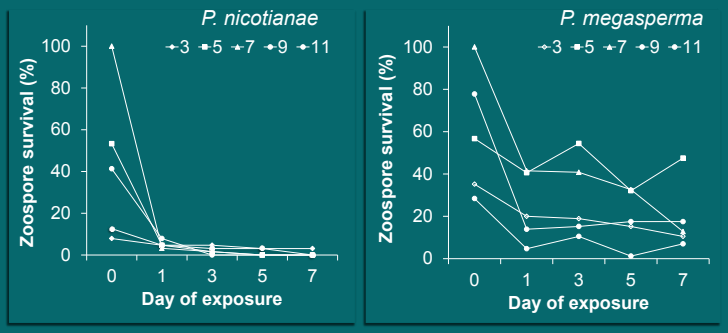



Fig. 5. *Phytophthora nicotianae* and *P. megasperma* under pH stress in a simulated aquatic system (4)

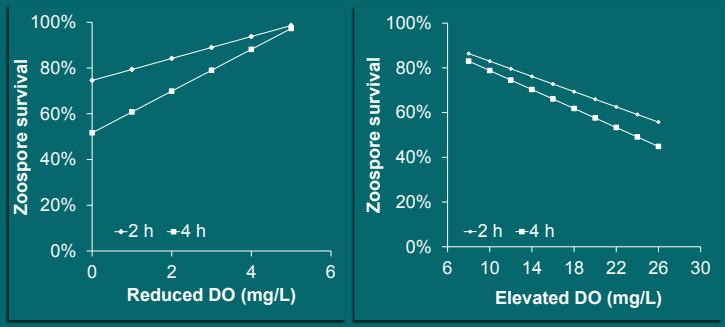



Fig. 6. Zoosporic response to dissolved oxygen (DO) stress in a simulated aquatic system (5)

## Summary

- A great diversity of *Phytophthora* species including some important pathogens are present in individual reservoirs.
- Their distribution within reservoirs is NOT even with the greatest population at runoff entrance and the least at exit/pump inlet.
- This declining pattern along the water path is due in part to zoosporic intolerance of pH, DO and other water quality stresses.
- *Phytophthora* spp.may be grouped in three categories:
  - Resident
  - Transient
  - Terrestrial

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# Phosphite for control of kauri dieback: forest efficacy trials

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Kauri dieback, caused by *Phytophthora* taxon Agathis (PTA) threatens the health and survival of kauri (*Agathis australis*) trees in New Zealand. In January 2012, trials were established in four kauri forest sites severely affected by PTA, to determine the potential of phosphite (phosphorous acid) as a control tool. Trial trees ranged from 40 to 120 cm girth and all 160 trees showed symptoms of PTA infection (canopy thinning/dieback and/or lower trunk lesions) at the start of the trial. Photographs for future comparisons were taken of all canopies and at cardinal points around the base of each trunk. Baseline assessments of each tree included canopy disease rating, trunk lesion dimensions and lesion activity (recent bleeding/oozing). To ensure a balance of disease severities across treatments, trees were grouped into disease severity classes and then randomly assigned to the various treatments. Phosphite (Agrifos®600) at concentrations of either 7.5% or 20% was injected (20 ml at 20-cm intervals around the trunk) using Chemjet® stem injectors. Control trees were left untreated. After one year, half the previously injected trees were re-injected, in all cases with 7.5% phosphite. Phytotoxicity symptoms (leaf yellowing, browning or leaf/twig abscission) were noted in some PA-injected trees, particularly where the 20% concentration was used. In assessments made 2 years after initial treatment, canopy health and vigour was similar to or slightly worse than that noted initially in most trial trees, with no obvious differences between treatments. However, treatment differences were detected in the activity of trunk lesions. Averaged across sites, many more lesions remained active (expressing ooze, continued expansion) in untreated trees (46%) than in phosphite-treated trees (1.5%). Average lesion expansion after two years was 6.5 cm in untreated and 0.4 cm in PA-treated trees. There were no obvious differences in lesion activity/expansion among the different phosphite rates or regimes.

# *Phytophthora* species in forest streams in Nyingchi, Tibet Autonomous Region and Ganzi, Sichuan Province, China

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**Abstract**
*Phytophthora* species were surveyed by placing bait leaves in selected streams at six sites during June to October in the year 2006, 2010 and 2011 in rhododendron-oak forests in south-east Tibet and west Sichuan Province, China. A total of 202 isolates of *Phytophthora* spp. were recovered from 120 baited leaf samples. Five *Phytophthora* species were identified by observation of morphological features and ITS1-5.8S-ITS2 rDNA sequence analysis. The five taxa included one well-known species *P. gonapodyides*, three recently described species *P. borealis*, *P. lacustris* and *P. plurivora*, and one named but as yet undescribed taxon, *P.* taxon PgChlamydo. The most numerous species, *P. gonapodyides*, the second most abundant species, *P. borealis*, and the third most numerous species, *P.* taxon PgChlamydo, were all recovered at four sites, while the other two species were found only at the same one site in Sichuan Province. Phylogenetic analysis showed that the isolates belonged to two ITS clades, one species including 17 isolates in clade 2 and four species including 185 isolates in clade 6. The relatively richness of *Phytophthora* species and genetic diversity in the species based on the ITS gene were examined, and interpreted in light of the various environments from which they were isolated.

**Keyword**: *Phytophthora* spp., stream baiting, ITS rDNA sequence, phylogenetic analysis

## Introduction

*Phytophthora* species are plant pathogens with world-wide distribution, and many of them are notorious pathogens. With the increased attention given to the genus *Phytophthora* in the last decade in response to the ecological and economic impact of several invasive species (such as *P. ramorum*, *P. cinnamomi*), there has been a significant increase in the number of surveys in many forest ecosystems, which have demonstrated the existence of various *Phytophthora* species (Balci & Halmschlager, 2003a, 2003b; Balci et al. 2007; Brasier & Jung, 2003; Jung & Nechwatal 2008; Burgess et al. 2009).

The sclerophyllous broad-leaved evergreen forests with oaks as the dominant species are widely distributed in southwestern China, and are especially characteristic of western

Sichuan, NW and NE Yunnan, and southeastern Tibet (Jin and Ou 1981). Since there are many potential host plants in these oak forests, and the climate in this area is best suited to *P. ramorum*, it was believed to be one of the most likely sources for the origin of the pathogen. During the course of surveys in oak forests conducted since 2004 in Diqing Tibetan Autonomous Prefecture of NW Yunnan, *P. ramorum* was not found, but eight additional *Phytophthora* species were recovered from stream water or soils (Goheen et al. 2006; Huai et al. 2013 ).

Before this, there were 28 described species of *Phytophthora* reported in China, most of which are pathogens of agricultural crops or ornamental plants (Zheng 1997; Yu 1998), and there were few reports of *Phytophthora* diseases of trees or *Phytophthora* surveys in the forests in China. The existence of native or exotic species of *Phytophthora* in soils and streams in southwestern China oak ecosystems is largely unknown. This lack of information and the potential threat of *P. ramorum* to Chinese oak and Rhododendron species provided the main impetus for this study.

Therefore, the primary aims of the present work were not only to find *P. ramorum*, but also to isolate and identify *Phytophthora* species by baiting techniques from streams in the oak forests in southeast of Tibet Autonomous Region and west Sichuan Province, and to evaluate the genetic diversity of *Phytophthora* populations using a combination of morphological and molecular tools.

**Methods and Materials**

Study area, sampling procedure and isolation methods

The study was carried out in the southeast part of Tibet Autonomous Region and western Sichuan Province. There are sclerophyllous forests with evergreen oaks as the dominant species, and usually accompanied by some *Rhododendron* species in both areas. A total of six decline sites and streams in oak forests were surveyed in this area (Fig. 1). The coordinates, altitude, sampling number and date of six bait sites are listed in Table 1.

*Phytophthora* species were surveyed by placing bait leaves in selected streams during May to October in the years 2006, 2010 and 2011 at six sites in oak forests. Three or four pieces of *Rhododendron* leaves were placed in each nylon mesh bag, and then floated in relatively slow-moving water in streams as baits, which were removed after seven to ten days when lesions developed. Pieces about 2 mm square were cut from the margin of the brown spots of baited leaf samples and plated in VARP+. Possible *Phytophthora* colonies growing from plated baits were transferred to VARP to confirm purity and then to CMA for characterization and storage.

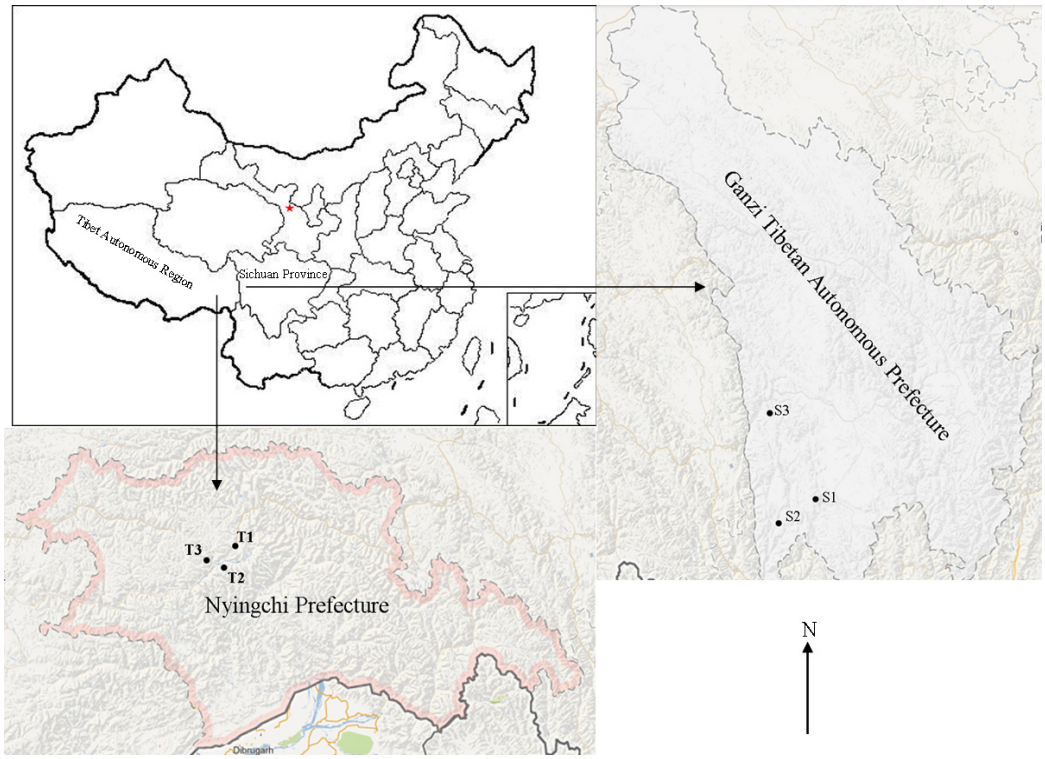



Fig. 1 Outline map of Nyingchi Prefecture in the south-eastern part of Tibet Autonomous Region and Ganzi.

Tibetan Autonomous Prefecture in the western part of Sichuan Province, showing locations of the sampling and baiting sites.

Table1 The coordinates, altitude, sampling number and date of six bait sites

| Localities | No. | Site | Coordinates | Alt.(m) | Sampling number | Sampling date |
|---|---|---|---|---|---|---|
| Ganzi, Sichuan | S1 | Xiangcheng | 29°01' N, 99°45'E | 3417 | 30 | 7-9/2006, 8-10/2011 |
| | S2 | Derong | 28°51' N, 99°14'E | 3382 | 30 | 7-9/2006, 8-10/2011 |
| Nyingchi, Tibet | S3 | Cuopugou | 30°25' N, 99°25'E | 3711 | 15 | 8-10/2011 |
| | T1 | Lulang | 29°45' N, 94°43'E | 3406 | 15 | 5-7/2010 |
| | T2 | Kangzha | 29°34' N, 94°33'E | 3377 | 15 | 5-7/2010 |
| | T3 | Tangdi | 29°41' N, 94°23'E | 3191 | 15 | 5-7/2010 |

Morphological and molecular characterization

Isolates were grouped by colony growth patterns and identified to species by comparing morphological features of sporangia, oogonia, antheridia, chlamydospores and hyphal swellings with authenticated isolates and species descriptions (Erwin and Ribeiro 1996). Heterothallic isolates were crossed with reference cultures of known mating types of *P. cinnamomi* and *P. cambivora* from the laboratory of Everett Hansen, Oregon State University. DNA was extracted from pure cultures of *Phytophthora* grown on CMA. The species identifications were confirmed by analysis of ITS DNA sequences and comparison with published *Phytophthora* sequence in GenBank using BLAST searches.

Phylogenetic analysis

The sequence data of *Phytophthora* isolates were initially assembled using Staden Package 1.6.0. The phylogenetic tree was constructed by the neighbor-joining algorithm using MEGA 4.0, and bootstrap analysis was performed with 1000 trials (Tamura et al. 2007).

## Results

During May to October in the years 2006, 2010 and 2011, a total of 120 baited leaf samples were collected from six sites in oak forests in Nyingchi, Tibet Autonomous Region and Ganzi, Sichuan Province. In total, 238 *Phytophthora* or *Pythium* colonies were recovered. Through selective medium and subculturing, the *Pythium* spp. were readily separated from *Phytophthora* spp.(Jeffers and Martin 1986), and consequently 202 pure isolates of *Phytophthora* spp. were obtained that qualified for further

examination and sequence analysis for this study. The five taxa included one well-known species *P. gonapodyides*, three recently described species *P. borealis*, *P. lacustris* and *P. plurivora*, and one named but as yet undescribed taxon, *P.* taxon PgChlamydo (Table 2).

Table 2 Number and distribution of isolates of each *Phytophthora* species recovered from streams in rhododendron-oak forests, Nyingchi, Tibet Autonomous Region and Ganzi, Sichuan Province

| *Phytophthora* species | Positive isolations | Site | Year |
|---|---|---|---|
| *P. borealis* | 47 | S1, S2, S3, T2 | 2006, 2010, 2011 |
| *P. gonapodyides* | 94 | S1, S3, T1, T3 | 2010, 2011 |
| *P. taxon PgChlamydo* | 33 | S1, S3, T1, T3 | 2006, 2010, 2011 |
| *P. plurivora* | 17 | S1 | 2011 |
| *P. lacustris* | 11 | S1 | 2011 |

Phylogenetic analysis

Phylogenetic analysis showed that the isolates belonged to two ITS clades (Cooke et al. 2000), one species including 17 isolates in clade 2 and four species including 185 isolates in clade 6.

The 17 isolates in ITS clade 2 recovered in this study were phylogenetically very close to GenBank sequences of *P. plurivora* and were closely related to *P. pini* and *P. citricola* (Fig. 2). Among them, eight isolates were clustered with the type isolate of *P. plurivora*, while the other nine isolates grouped into a distinct but closely related cluster from the other isolates in the phylogenic tree, suggesting they are different strains of *P. plurivora*.

Four *Phytophthora* species were identified in ITS clade 6 (Fig. 3). *P. gonapodyides* was the most frequently identified species overall. All ninety-four new isolates of *P. gonapodyides* grouped into one consensual cluster with several reference isolates (AF541887, AF541888 and AF541889). Forty-seven isolates formed a well supported clade with *P. borealis* (HM004232, JQ626597and JQ626601). Eleven isolates were identified as *P. lacustris*, a recently described species in ITS clade 6 with 98% bootstrap support. The remaining 33 isolates corresponded to *Phytophthora* taxon PgChlamydo, an undescribed taxon in ITS clade 6 with 90% bootstrap support.

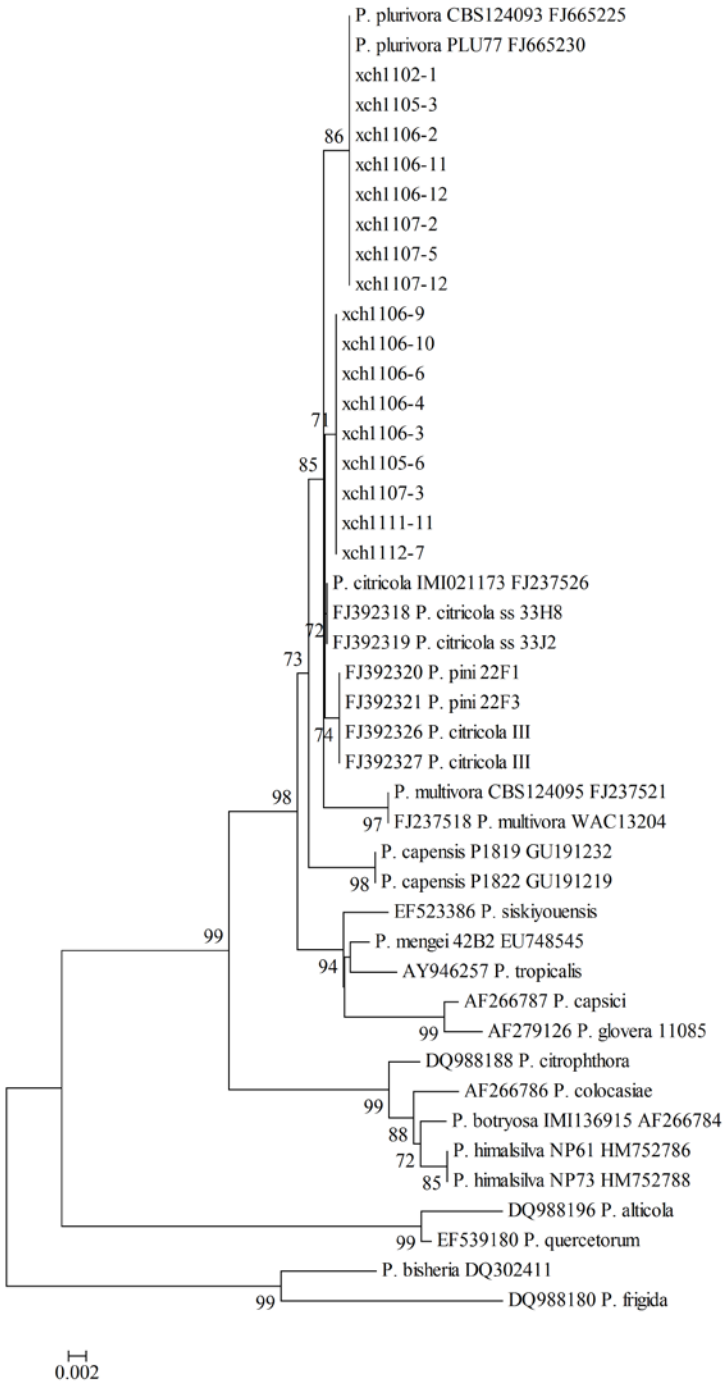



Fig. 2 Phylogenetic tree showing the relationship of *P. plurivora* within *Phytophthora* Clade 2 based on rDNA ITS sequence

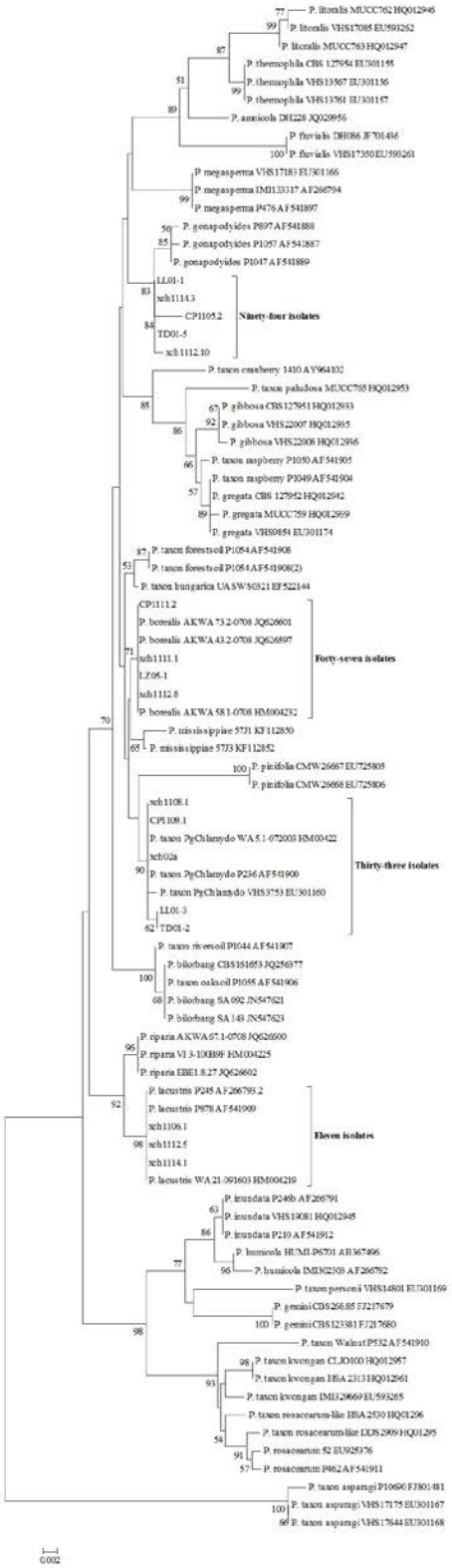



Fig. 3 Phylogenetic tree showing the relationship of *Phytophthora* species within Clade 6 based on rDNA ITS sequence

Distribution and diversity of *Phytophthora* species

Isolates in ITS Clade 6 were most frequently recovered from streams at all six sites (91.6% of all isolates). Of five *Phytophthora* species detected in this study, the most numerous species, *P. gonapodyides* (46.5% of all isolates), the second most abundant species, *P. borealis* (23.3% of all isolates), and the third most numerous species, *P.* taxon PgChlamydo (16.3% of all isolates), were all recovered at four sites, while the other two species (*P. plurivora and P. lacustris*) were found only at the same one site in Sichuan Province.

The five *Phytophthora* species were all recovered from site S1, while three species were detected from site S3, and three species were obtained from site T1 and T3. Only one species, *P. borealis,* were recovered from Site T2 and S2.

## Discussion

Five *Phytophthora* taxa were isolated from stream water in oak forests in southeast of Tibet Autonomous Region and west Sichuan Province. Four of the taxa were of the known species, *P. gonapodyides*, *P. borealis*, *P. lacustris* and *P. plurivora*, and one matched previously informally designated taxon, *P.* taxon PgChlamydo. *Phytophthora ramorum*, the pathogen of sudden oak death disease, was not detected in this area. The *Phytophthora* taxa recovered in this study were fewer than the previous study in Diqing Tibetan Autonomous Prefecture of NW Yunnan (Huai et al. 2013). It is likely that the use of additional isolation methods and repeated samplings in different seasons would increase the percentage of positive isolations and perhaps reveal the presence of more *Phytophthora* species.

Seventeen isolates with semi-papillate sporangia were recovered from stream water at site Xiangcheng (S1), which were placed in Waterhouse group as the "*P. citricola* complex," and were finally identified as the recently described species, *Phytophthora plurivora* (Jung and Burgess 2009) confirmed with ITS sequence. The occurrence and the role of these *Phytophthora* species in oak forests in south-eastern China and their pathogenicity toward some plants highlights the urgent need to investigate the potential risk and impact of both native and previously unknown introduced forest *Phytophthoras*.

## Acknowledgements

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# Wooden Vectors of *Phytophthora ramorum:* Are Douglas-fir Logs a Risk?

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The potential to disseminate *Phytophthora ramorum* through the trade of Douglas-fir logs was investigated. Three methods of inoculation were attempted to infer if *P. ramorum* could colonize Douglas-fir log material using three isolates. Collectively, the studies suggest there is potential for accidentally transporting *P. ramorum* propagules through the transport of Douglas-fir sapwood material. However, the results were not consistent between methods, and in some cases, could not be replicated between trials. The most effective method for *P. ramorum* inoculation involved soaking sapwood material for 24-48 hours in a zoospore suspension of the 7904 isolate. The results of this study demonstrate the importance of using multiple methods to evaluate the risk of spreading microorganisms on woody material before inferring risk.

# Wooden Vectors of *Phytophthora ramorum*: Are Douglas-fir logs a risk?

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## Introduction

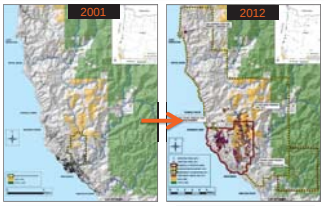
Sudden Oak Death is a disease of oaks caused by an introduced pathogen, *Phytophthora ramorum*.



As of 2007, *P. ramorum* was recognized as a pest by ninety countries shown in orange (Sansford *et al* 2009).



Quarantined areas continue to expand to include more commercial lands.




Douglas-fir (*Pseudotsuga menziesii*), which accounted for 60% of the logs exported from Oregon and Washington, is a host for *P. ramorum (APHIS 2012; Xiaoping & Warren 2012).*



Because of the commercial importance of Douglas-fir, its presence in infected areas, and the trade restrictions placed on infected material, we investigated whether moving Douglas-fir logs presents a risk of spreading *P. ramorum*.

## Objectives

Determine if *P. ramorum* could colonize woody tissues of Douglas-fir.

Test multiple methods of inoculation and determine which method was most effective.

Determine which tissues were susceptible

Investigate whether zoospores and actively growing mycelia could colonize Douglas-fir sapwood tissues

## Methods

### Method 1: Zoospore inoculation and surface sterilization of Douglas-fir sapwood wafers

Four trials were completed. 1-2 mL of zoospore suspensions from three isolates were added to well plates containing 1 x 0.5 x 3 cm Douglas-fir sapwood wafers.





Wafers were removed from zoospore suspensions after 24 hours (Trails 1 and 2 only), incubated, surface sterilized with a household bleach rinse, split, and plated into *Phytophthora* spp. selective CARP media. Wafers were not removed from zoospore suspensions prior to incubation in Trials 3 & 4.

### Method 2: Zoospore inoculation of Douglas-fir sapwood blocks containing bark

Douglas-fir blocks containing bark, cambial and sapwood tissues were inoculated by either dipping (Trials 1 & 2) or soaking for 24 hours (Trial 3) in zoospore suspensions.

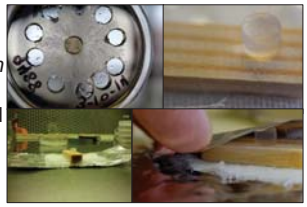




Blocks were incubated, split into separate tissue types, and plated onto *Phytophthora* spp. selective CARP media.

### Method 3: Microscopy of Douglas-fir sapwood wafers inoculated with agar plugs

Agar plugs from actively growing edges of *P. ramorum* cultures were removed and placed directly on Douglas-fir sapwood wafers



Wafers with agar plugs were wrapped in moist cheesecloth and foil, and incubated for several months



After incubation, slivers were cut from unexposed surfaces and evaluated using light and scanning electron microscopy.

## Results

### Method 1: Zoospore inoculation and surface sterilization of Douglas-fir sapwood wafers

Percent recovery after surface sterilization

| Zoospore Suspension Isolate | Wafer Trial 1 (n=20-25 per isolate) | Wafer Trial 2 (n=20-22 per isolate) | Wafer Trial 3 (n=6 per isolate) | Wafer Trial 4 (n=6 per isolate) |
|---|---|---|---|---|
| NA1-7904 | 80-100% | 70-96% | 0% | 0-17% |
| NA1-9488 | 0% | Not Tested | 0% | 0% |
| NA1-4313 | 0% | Not Tested | 0% | 0% |
| Control (DI water) | 0% | 0% | 0% | 0% |

### Method 2: Zoospore inoculation of Douglas-fir sapwood blocks containing bark

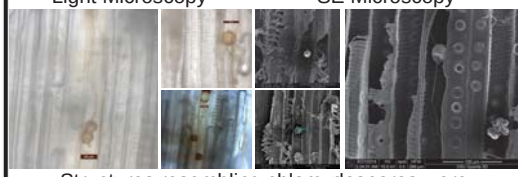
Percent recovery

| Zoospore Suspension Isolate | Block Trial 1 (n=10 per isolate) | Block Trial 2 (n=10 per isolate) | Block Trial 3 (n=12-13 per isolate) |
|---|---|---|---|
| NA1-7904 | 20% | 0% | 15% |
| NA1-9488 | 0% | 0% | 8% |
| NA1-4313 | 0% | 0% | 25% |
| Control (DI water) | 0% | 0% | 0% |

*P. ramorum was* recovered from: 0 bark sections, 1 cambial section, and 7 sapwood sections.

### Method 3: Microscopy of Douglas-fir sapwood wafers inoculated with agar plugs

Light Microscopy SE Microscopy



Structures resembling chlamydospores were frequently observed in tracheids of inoculated wafers.

## Discussion

The most effective method for *P. ramorum* inoculation involved soaking the material for 24 hours in a zoospore suspension of the 7904 isolate. Collectively, the studies suggest there is a limited risk of accidentally transporting *P. ramorum* propagules through the transport of Douglas-fir sapwood material. However, the results were not consistent across all of the studies, and in some cases, could not be replicated.

Protocols and disease control efforts aimed at preventing the global spread of *Phytophthora* spp. should consider xylem tissues as a potential pathway.

## Acknowledgments

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# Introduction and Spread of *Phytophthora ramorum* in Northern Ireland, UK

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## Abstract

*Phytophthora ramorum* was detected in Northern Ireland on plants in trade, almost exclusively *Rhododendron* spp., from 2002 – 2007. In August 2007 it was found on Rhododendron plants in private gardens. The first record of *P. ramorum* on Japanese larch (*Larix kaempferi)* in N. Ireland was in July 2010 on a mature larch stand on the Antrim Plateau in the east. Almost all of the *P. ramorum* isolates from larch, rhododendron and other hosts in N. Ireland belong to the EU2 lineage. Infected trees, plus non-symptomatic larch trees in a buffer zone of up to and in some cases exceeding 250 m, were felled immediately and processed under strict biosecurity measures. Since 2011 aerial surveys have been carried out in spring and late summer, symptomatic trees identified and after confirmative diagnosis, infected, symptomatic and buffer trees were felled. To date around 1,000 ha (out of approximately 5,500 ha) have been required to be felled due to the disease. In spite of the eradication and containment policy the pathogen has continued to spread north, south and west. It is probable that when trees are felled in response to symptom development, that this is happening behind the disease front. Aerial surveys can identify stands with small numbers of infected trees and this would appear to be the pattern in areas of low infection. On other occasions widespread symptom development occurs very rapidly. At Castlewellan Forest no symptoms were observed in September 2012, however when surveyed again in spring 2013, over 90 ha of Japanese larch was severely damaged.

## Introduction

*Phytophthora ramorum* (Werres, de Cock & Man in't Veld) was first recorded in the UK infecting *Rhododendron* spp. in the wider environment in 2003, and subsequently on beech, oak and horse chestnut (5). The pathogen had almost certainly been introduced into Europe on infected plants in trade although, its source is still uncertain. *Rhododendron* sp. is a sporulating host and was assumed to be the primary source of inoculum for dissemination of the pathogen into the wider environment. It had also been observed that where broad-leafed trees e.g. common beech or red oak, were infected the diseased trees were always associated with infected *Rhododendron* sp. These broad-leafed trees were regarded as terminal hosts as the pathogen did not appear to infect or sporulate on foliage. It was therefore something of a surprise when, in 2010, *P.*

*ramorum* was reported as causing severe infection and death of mature Japanese larch (*Larix kaempferi* (Chamb.) Carr.) in south-west England (6). Infections of Japanese larch stands did not appear to be always associated with infected *Rhododendron* sp. and the pathogen was observed sporulating freely on needles, especially in early spring when they emerged and late autumn when they started to senesce

The deciduous sporangia formed on the needles near the top of the trees are locally splash-dispersed or spread over long distances on wind-driven rain. Motile zoospores are released, cause infection and repeat the asexual cycle resulting in severe epidemics (7; 8). Since it was first identified on larch in south-west England symptoms have subsequently been observed in Wales, western England and western Scotland. Up until 2011 there were only three, largely clonal, genetic lineages of the pathogen recognised (11,12) called NA1, NA2 and EU1 after their initial outbreak sites (9). Lineage NA1 was found both in forests in western USA and in nurseries, while NA2 seemed to be only present in nurseries. The European lineage EU1 was found in forests, woodlands, estates, gardens and nurseries (9). All of the isolates in England and Wales on all hosts were and remain to be lineage EU1 (14).

In Northern Ireland (NI) the first record of *P. ramorum* was in 2002 on *Rhododendron* spp. in trade, which had mainly been brought from continental Europe. For the following four years *P. ramorum* was only found on *Rhododendron* spp. and *Viburnum* spp plants in trade. In the summer of 2007 the pathogen was diagnosed causing death of ornamental *Rhododendron* spp. plants in private gardens. In almost all of these cases the outbreaks could be traced back to recently introduced ornamental plants. There was one more significant outbreak in 2007, at a site where the disease was more fairly widespread in distribution over a larger area and infecting a number of *Rhododendron* spp. and other plant genera. Subsequently, Plant Health Inspectors from the Department of Agriculture and Rural Development (DARD) ordered an eradication programme in line with plant health policy and over the following seven years infected plants and those in close proximity were removed and burned or deep buried.

In September 2010 *P. ramorum* was diagnosed causing significant symptoms and tree death on Japanese larch in a forest on the Antrim Plateau in the eastern part of NI. Isolates of the pathogen from this, and subsequently other sites in NI were shown to belong to a fourth evolutionary lineage, EU2. (19). It has since been shown that almost 90% of all isolates found in NI are EU2 and that the only other region of the world where EU2 is found is in south-west Scotland which is less than 20 km from the NI coast-line (16). These authors concluded that there had been a single introduction of the pathogen into NI, although again the original source is unknown. All of the isolates of *P. ramorum* obtained from the Republic of Ireland were EU1.

**Surveillance**

With the detection of *P. ramorum* in larch, the Plant Health authorities in NI increased surveillance and commenced an extended surveillance plan which included the use of a helicopter aerial survey. All of the main larch stands within the public held forests were surveyed along with any significant areas of privately owned larch forests. The survey

was accompanied by a programme of eradication in forests where the pathogen was diagnosed. Strict biosecuriy measures were also put in place. Ireland, north and south is one of the least forested regions in Europe with less than 10% tree cover compared to, for example, > 65% in Sweden and Finland, and > 50% in Estonia and Latvia. Almost all of the 111,000 ha (>90%) of the NI forests are plantations with around 5,500 ha of larch (Japanese, hybrid and European) in NI, present in many forests, often in combination with other tree species. (Fig. 1).

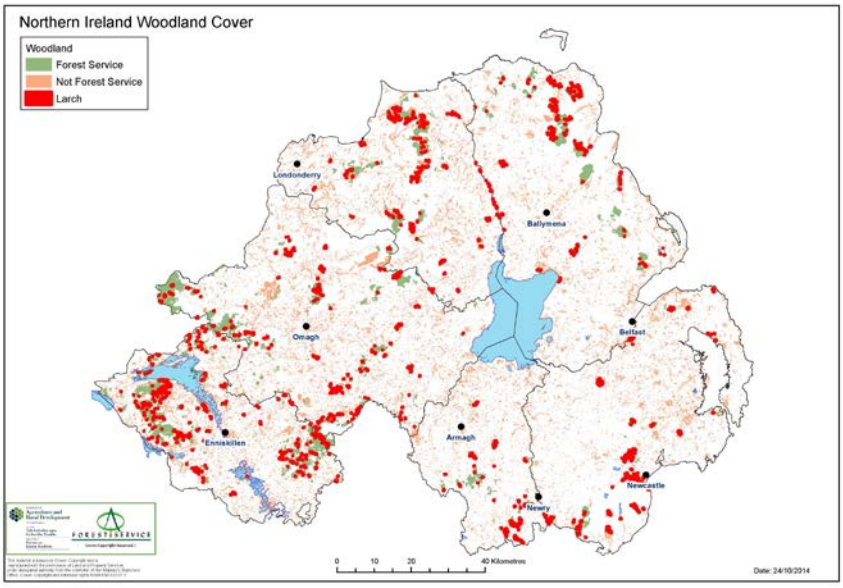



Fig.1. Northern Ireland woodland cover: Forests containing larch (Japanese, hybrid and European) shown in red. *Larix* spp. represents only 5% of the total forest cover but is widely planted in association with other conifers such as *Picea sitchensis*.

## Eradication

*P. ramorum* is subject to EU phytosanitary measures and action is required in order to contain the disease. The Plant Health Order (Northern Ireland) 2006 gives DARD inspectors authority to destroy any plant material infected or suspected of being infected with a non-indigenous pest or pathogen. The policy followed by DARD was to attempt to eradicate *P. ramorum* from the region. Hence, when the pathogen was diagnosed in larch trees in a forest, the symptomatic trees, plus all larch trees in a buffer zone of up to 250 m were felled. If trees were of sufficient size to have commercial value they were transported under licence to recognised sawmills. Biosecurity measures were put in place, including maintenance of forest roads to a high standard, to reduce the risk of disease spread from soil and plant debris on wheels of vehicles and machinery leaving infected sites. All bark was required to be burned and larch could only be processed on its own, with careful clearance of debris after each run. Since 2010 1,150 ha of mature larch have been clear-felled and salvaged by the commercial timber market (Table 1).

From 2011 larch stands have been surveyed, using a helicopter, in June, after flushing and again in late September. High resolution pictures of trees showing typical die-back symptoms (Fig. 2 and 3) were obtained and suspect sites were followed up by a ground survey. Where necessary, samples were taken and submitted to the AFBI laboratories for diagnosis. Experience had shown that the most consistent results were obtained when fresh samples of bleeding cankers on the stem or branches were taken from close to the top of the tree, which frequently involved felling suspect trees in order to get the best samples.





Figs 2 & 3. Aerial photographs of larch stands with *P. ramorum* infected trees (brown foliage), taken in June 2011.

Table 1. Number of larch infected sites (2010 – 2014) and the area of trees felled

| Year | No. Infected sites | Area of larch felled |
|---|---|---|
| 2010 | 10 | 283 |
| 2011 | 16 | 71 |
| 2012 | 15 | 150 |
| 2013 | 28 | 495 |
| 2014 | 17 | 45 |
| Total | 92 | 1,046 |

Once trees are felled sites quickly become un-infective. Several studies carried out by AFBI failed to isolate the pathogen from soil or plant debris a few months after felling. In a replanting trial, Japanese larch and other species including both hosts and non-hosts were planted in a recently clear-felled site where there had been a high degree of infection. Only a very small number of the highly susceptible Japanese larch showed signs of infection one year after planting.

In contrast to DARD, the Scottish Government took a different approach. In June 2013 aerial surveys showed an area of 4,000 – 6,000 ha in the Dumfries and Galloway area, in South West Scotland, likely to have been infected with *P. ramorum*. It is thought that this may have been related to particularly wet and windy conditions in the region in 2012. A limited number of samples from this region have been shown to be EU2 (16, 14), so that it is known that the EU2 lineage is present. However, there are no data to indicate whether or not it is the only or dominant lineage present. It has been reported

that EU1 has been found less than 10 km away from an EU2 infection (14). Eradication of *P. ramorum* on larch in that part of Scotland was no longer considered to be achievable and so the Dumfries and Galloway region was designated a "Ramorum Management Zone" and legislation to this effect was passed in June 2014. As a result Statutory Plant Health Notices within the Management Zone and Statutory Controls on the movement of all roundwood, larch timber and associated, potentially infectious, products will be applied to such material only if it leaves this area (4). It remains to be seen how successful this management strategy will be. There has been no evidence of spread of EU2 into England and Wales (14), or into the Republic of Ireland (16) where only EU1 has been found.

**Spread and Impact of Felling Diseased Trees on the Pathogen**

In spite of the extensive felling of trees accompanied by strict biosecurity around infected sites the pathogen has continued to spread within N. Ireland with many new cases each year (Table 1). The general direction of spread has been northwards into north Co. Antrim, south west into south Co. Down and Co. Armagh and west towards Co. Fermanagh (Figs 5 – 8).

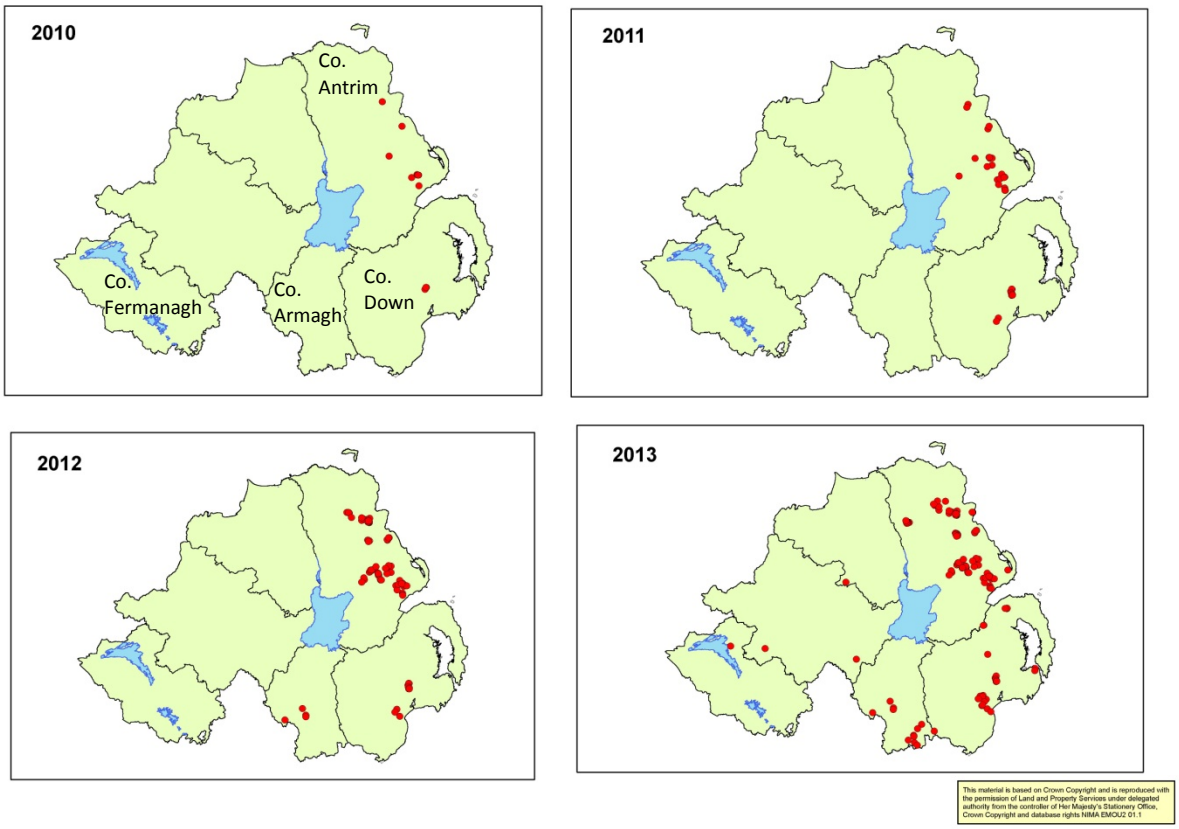



Fig 5 – 8. Larch forests where *P. ramorum* was diagnosed in 2010 (Co. Antrim in the east); 2011 (further north in Co. Antrim and south Co. Down); 2012 (south Armagh); and 2013 (far west of N. Ireland into Co. Fermanagh).

When a new, non-indigenous plant pathogen is diagnosed in a region it is important that the local Plant Health Authorities attempt to eradicate and contain the organism before it has the opportunity to become established and / or spread. Hence, the actions taken by DARD in N. Ireland, following similar approaches in Great Britain (England, Scotland and Wales) and in the Republic of Ireland were in line with best practice. Felling relatively large areas, 1,000+ ha (~ 20% of the total stands) of larch probably had a slowing down impact on the disease, most obviously in the reduction in number of sites and area infected between 2010 and 2011. However in subsequent years there has been a resurgence of cases indicating that the pathogen is not only still present but, is actively spreading. Would a different approach have been more effective?

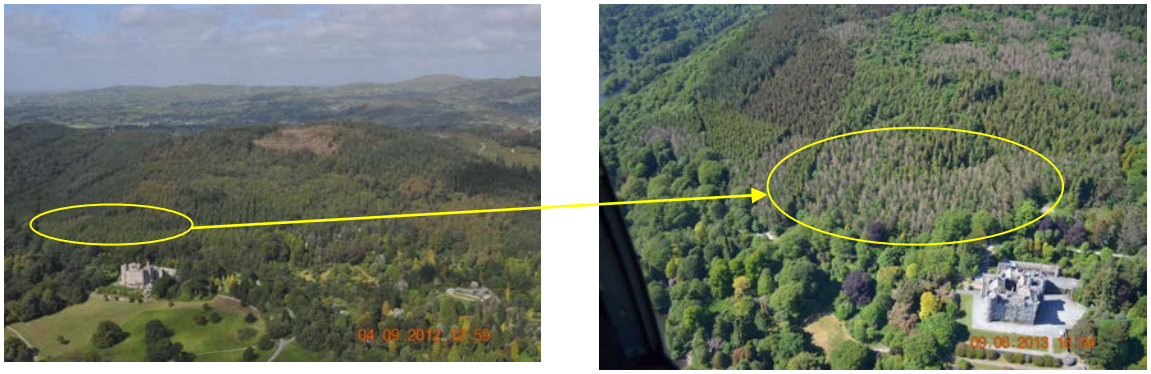
Early diagnosis is vital. The aerial surveys were effective in picking out dead and symptomatic trees. However, dead trees have almost certainly ceased to sporulate and so their removal will have limited impact on the pathogen inoculum. Dead trees, symptomatic trees and all larch trees within a buffer of up to and in some cases exceeding 250 m were felled. Perhaps these buffer zones were insufficient. Data have shown that viable *P. ramorum* inoculum is able to disseminate in the air for up to a few, possibly 4 km (8; 10). Furthermore, apparently symptomless trees may actively produce spores. Therefore more effective eradication may involve removal of "healthy" trees within a wider *cordon sanitaire*. Although good plant pathology practice, it could create problems for implementation, as land owners are more likely to challenge removal of "healthy" trees on the basis of possible infection.

Since all of the outbreaks on larch in NI have been EU2, a lineage which is not known elsewhere, we can be reasonably certain that they are all associated with a single, or at least a very limited number of introductions. Some of the outbreaks in the south and in the extreme west of NI are many kilometres away from the nearest infected trees. This may suggest that the pathogen can move much greater distances in the air than first anticipated, and / or that there are small 'stepping-stone' outbreaks, not identified, which has enabled the organism to move effectively. Other mechanisms are possible, for example several bird species are associated with the long distance spread of chestnut blight caused by *Cryphonectria parasitica* (1), thread blight disease of certain broad leafed trees caused by *Cylindrobasidium argenteum* (15) and other pathogens (20). Although there does not appear to be any references to birds being involved in the dissemination of *Phytophthora* spp., this cannot be ruled out.

It is difficult to determine the impact of infected soil movement between sites. Viable *P. ramorum* propagules, probably chlamydospores, can be found in soil which therefore must be considered as a potential route for dissemination. NI forests are widely and extensively used by walkers, mountain bikers, families and other visitors and the risk of movement of infected soil from a contaminated to a clean forest is high. However, in Oregon, USA, there seemed to be no relationship between the roads network and outbreaks of *P. ramorum* in tanoaks (18). *P. ramorum* behaves differently from *P. lateralis,* for example, where dispersal of soil-borne inoculum by foot or hoof contributed to relatively short ranged spread within a site (13). There are also differences between the hosts, larch and tanoak. Larch is a major sporulating host with numerous spores being produced high in the canopy, in contrast to tanoak. Air borne inoculum of *P.*

*ramorum* in larch is clearly the most important mechanism of spread within a forest. Spores produced on the foliage of a single or small number of trees can easily infect neighbouring trees. Aerial spread, however does not seem to fully explain long distance dissemination. This would suggest that infected soil movement on vehicles, feet or hooves may explain significant new infections. However, the evidence from other regions has not found a close correlation between soil movement and new infections (17, 18). Nevertheless the biosecurity recommendations for all forest users would seem to be a minimum requirement to reduce spread.

A further complication is the speed at which symptoms of infection can appear. On several occasions sites which have displayed no symptoms of infection during an aerial survey have extreme levels of tree death by the next time the survey is conducted (Figs. 9 – 10). One example of this is Castlewellan Forest Park, approximately 50 km south of Belfast. This is a large forest park with significant areas of larch (50+ ha), mainly Japanese larch around 50 years old. There were sites in the same geographic region where *P. ramorum* had been diagnosed in previous years, and was therefore deemed to be at relatively high risk. The forest was a focus for the bi-annual (June and September) aerial surveys. When the forest was inspected on 4th September 2012 there were no obvious signs of infection and no evidence of dead trees (Fig 9). However when surveyed again on 9th June 2013, soon after flushing there were large areas of dead trees (Fig. 10).



Figs. 9 – 10. Aerial survey photographs of Castlewellan Forest park on 4th September 2012 (no evidence of infections) and 9th June 2013 (large numbers of dead trees (circled area shows larch trees)).

## Conclusions

The attempted eradication of an aerial borne plant pathogen, with a wide host range, is always going to be a difficult challenge. Until *P. ramorum* was diagnosed on Japanese larch, extensive surveillance in NI followed by prompt removal and destruction of infected plants appeared to be at least partially effective in slowing down the spread and establishment of the disease in the region. However, when in 2010 the organism was found infecting and sporulating on the needles of larch the challenge became even greater. There were a number of compounding factors which have made the eradication and containment of *P. ramorum* especially difficult.

- Sporulation is occurring at some height above the ground. When inoculum is produced high in the air then there is the potential for long distance spread in wind-driven rain. There is still some uncertainty surrounding how far *P. ramorum* sporangia can be disseminated. One of the first ideas about the introduction of *P. ramorum* into NI was as wind borne inocululm from England which is less than 150 km. However the N. Irish *P. ramorum* population is EU2 compared to the English population which is EU1. There was possibly infection between NI and Scotland, although there is no definitive evidence to suggest in which direction. Movement of infected plants remains a strong possible means of spread.

- It is not clear what role other sporulating hosts, in particular *R. ponticum* plays. *R. ponticum* is a common wild plant often, but not always, associated with forests. On occasions there has been infected *R. ponticum* near diseased *L. kaempferi*, although it may well be that the larch has infected the *Rhododendron* and not vice versa. When infected larch are removed, where possible rhododendron are also cleared.

- Infected, heavily sporulating trees may not be displaying obvious symptoms. When trees are dead and fail to flush in the spring they are almost certainly not producing any significant level of inoculum. Hence their removal, while it may have other economic or cultural benefits, does not significantly reduce inoculum. In order to achieve this it would be necessary to remove 'healthy' trees in a large *cordon sanitaire*, well in advance of the disease front. This would be difficult to implement and enforce, as landowners are more likely to challenge this action unless the pathogen had been shown to be present. It may be possible to develop methods of early detection. Molecular methods are very sensitive, but it would be almost impossible to know when and where to obtain samples. There have been attempts to trap spores of *P. ramorum*, using a number of different methods, but at best the results are inconsistent.

- *Phytopthora* spp. including *P. ramorum* have the ability to be dispersed in waterways or on soil / plant debris. Around every infected site in N. Ireland strict, but practical, biosecurity protocols were put in place. Peterson *et al.* (17; 18) suggested that disease patterns of *P. ramorum* infection in Oregon tanoak forests were not consistent with a dominant soil or waterway mediated dispersal mechanism. They did on the other hand present evidence supporting the dispersal of inoculum in blowing fog or rain which makes eradication virtually impossible.

**Effectiveness of eradication?**

There have been few successes in eradicating invasive pests or pathogens anywhere in the world. The effective response to a newly identified invasive plant pathogen depends on reliable early detection. Since tree infections are not always apparent, by the time the symptoms are observed the pathogen is well on its way to becoming established. However horizon scanning for impending threats, development of rapid and sensitive molecular detection technologies and careful surveillance, especially at the points of plant importation will reduce the risk of introduction and also give early warning for

further action. It is also essential to understand mechanisms of spread. With a recently identified organism, its biology and life cycle are unknown and it is necessary to base action on the knowledge of similar related organisms. However, *P. ramorum* behaved differently from most other woody infecting *Phytophthora* spp., in particular its ability to sporulate heavily in the foliage so that aerial dispersal is the most common means of spread. In many ways this is more similar to the potato blight pathogen *P. infestans.* To achieve a rapid and meaningful response to a new invasive organism it is essential to have access to pathologists and entomologists with experience, training and knowledge to advise on the best approaches. The European and Mediterranean Plant Protect Organisation (EPPO) developed standards for eradication and containment (3) which are set out in the EPPO International Standards for Phytosanitary Measures (IPSM No. 9) (2).

The adoption of strategies to attempt to eliminate an invasive pathogen will depend on a many factors including a realistic assessment of their effectiveness; the cost; the trade, economic, and environmental benefits; responsibilities within the European Union; and resources available. A vital element of success is early engagement and ongoing support of stakeholders, and respect for their situation, faced with a problem they could not have anticipated or otherwise protected themselves from. Unless they are fully engaged, well informed and supportive of the measures being taken, even limited success is unlikely. The introduction of *P. ramorum* EU2 into NI was a single introduction, becoming established and spreading rapidly, when larch became infected. DARD policy has been effective in slowing the spread but not eradicating the pathogen from the region.

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# Community structures of root-rotting *Phytophthora* species affecting *Abies* in U.S. christmas tree farms & screening true fir for resistance to *Phytophthora* root rot

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True fir trees in the genus *Abies* are common hosts of various root–rotting *Phytophthora* species. Losses due to root disease can significantly affect bareroot conifer nurseries and Christmas tree plantations. There are limited methods available to control *Phytophthora*, but practicality may vary depending on field topography, crop maturity, and available capital. For these reasons, investigation of a marker-assisted selection system for identifying fir trees that can resist *Phytophthora* infection is justified to help alleviate current losses. Our research is intended to provide information on the host-parasite interactions between true fir and *Phytophthora* species, with the ultimate goal of facilitating the development and implementation of molecular markers associated with resistance. Main objectives include construction of an isolate collection of *Phytophthora* species affecting fir roots, assessment of relative virulences among isolates from different geographic regions, and screening of true fir seedlings for resistance to *Phytophthora*. The compiling of *Phytophthora* isolates from fir roots has provided information on the community structures and habitats of soilborne *Phytophthoras* from widely differing geographical regions within the U.S. A subset of isolates will comprise the inoculum that will be used in a subsequent phenotype screening for resistance in fir. Prior to utilization as inoculum for the screening project, virulence testing will facilitate the selection of the three most virulent genotypes from each of the four most commonly-occurring *Phytophthora* species from the U.S. collection. Resistance phenotype screening of seven true fir species will be performed to provide information about which species of fir can generally be regarded as resistant to PRR and whether there are individual genotypes *within* the different fir species that are more resistant than others. This information will be used to facilitate a future genomics project to identify molecular marker patterns that are common among resistant fir species and/or genotypes within species.

# Citizen Science Helps Predict Risk of Emerging Infectious Disease

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Citizen science holds great potential for advancing spatial prediction of biological invasions by providing inexpensive location-based, time series data of unprecedented quantity and distribution. In 2008, we developed a citizen science program to detect the spread of the emerging forest disease Sudden Oak Death (SOD) across the metropolitan region of the San Francisco Bay Area in California, including under-sampled habitat within urban areas and along the wildland-urban interface. Each year, our "SOD Blitz" program used crowdsourcing methods to encourage citizens to collect leaf tissue symptomatic of this disease and submit it to our lab for molecular diagnosis. Results are made public through the internet each year, they are added to the disease distribution database known as SODmap (available also through the App SODmap mobile) and they helped scientists identify critical elements correlated to disease spread, generating the strongest predictive model yet known.

# Detection of *Phytophthora* species on different woody species in nurseries

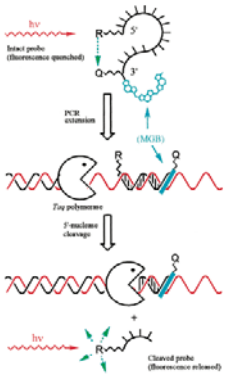
**D. Migliorini[1, 2], E. Tondini [1], N. Luchi [1], L. Ghelardini [1], P. Capretti[2], A. Santini[1]**

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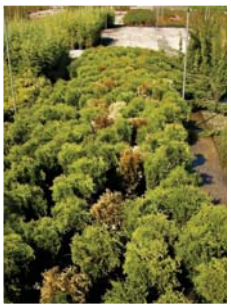
Among the diseases affecting plants in nurseries, root rots are one of the most serious, especially those caused by *Phytophthora* species. Since this class of pathogens are difficult to isolate, and they may remain alive in the soil for long time before causing any symptom, the availability of an early detection tool would be crucial to reduce the risk of spread of these pathogens. Their high capacity of surviving in potted soil and the wide trade of nursery products are strong factors in the *Phytophthora* spreading around the globe. Against this hazard, an efficient evaluation of pathogen diffusion inside the nursery by estimating its amount in potted soil has a primary role.

**Our work is aimed to evaluate the efficacy of Taqman MGB probe as a quick tool for Phytophthora detection and quantification of the inoculums inside nursery material**.




TaqMan MGB probe working principle (Kutyavin *et al.*, 2000)

The study was carried out in two anonymous and large retail nurseries in Europe. Seventy-two pot plants belonging to 17 woody ornamental species (4-6 per species), compost and irrigation water were collected . The plant material was divided into plants with visible crown's damage (wilting, yellowing of the leaves, fading/greyishing in the colour of the foliage on conifers, defoliation, or dieback of leaves or shoots) (symptomatic) and plants with healthy crown (asymptomatic). The two classes had a balanced number of plants. Roots of asymptomatic plants were carefully examined for the presence of symptoms (rotten fine and feeder roots; major roots softer than normal or easy to break, showing reddish-brown lesions, internal brown or black tissues, or evidence of decay).













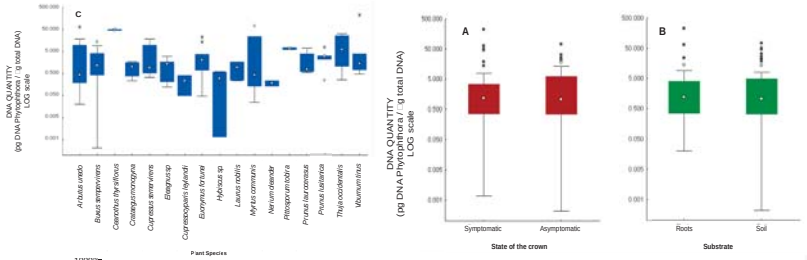


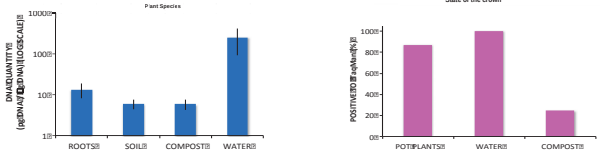
In order to get a picture of the range of *Phytophthora* species present in our sample pure cultures of *Phytophthora* have been obtained by isolation from plant root tissues carried out by direct platting on selective media (PARPN) (Erwin and Ribeiro, 1996), from soil and water (baiting) by apples and leaves baits (Moralejo et al., 2009). Cultures DNA was extracted by using EZNA Plant DNA kit (Omega) and molecular analysis by amplification and sequencing of ITS region was performed in order to screen the diffusion of *Phytophthora* taxons in the sampled material. Soil and root DNA extraction for qPCR essay was carried out by using EZNA Soil DNA kit (Omega)

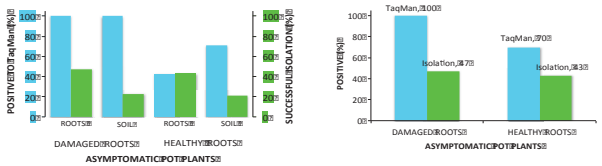
**Real time PCR validation (TaqMan MGB Probe)**

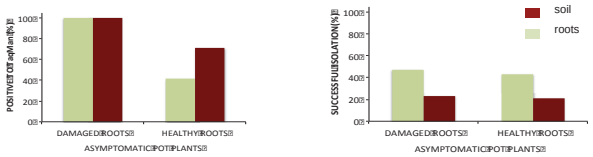
According to the recent revision of the *Phytophthora* genus (Kroon et al., 2012) **TaqMan® MGB probe** and primers for **the quantitation of *Phytophthora* spp.** were designed using Primer Express® Software 3.0 (Applied Biosystems, Forster City, CA, USA) and the ITS2 (Internal Transcribed Spacer) from *Phytophthora palmivora* DNA (isolate Ph32SA culture collection IPSP-CNR, Italy).
Oligonucleotide sequences were
PhyF 5'TCGGCTGTGAGTCCTTTGAA-3' (Forward primer),
PhyR 5' GCCACGCTTTTGGAGCAA-3'
(Reverse primer),
PhyPr: 5'- FAM ACTGAACTGTACTTCTC-MGB-3'
(TaqMan® MGB probe; MGB: Minor Groove Binder, Applied Biosystems).
In the TaqMan MGB Probe system the probe is a 13-18 nucleotides long non fluorescent quencher that increase the Tm of a probe because of It's minor grove binding ability. During amplification, the probe binds to its target sequence and is cleaved by the 5' nuclease activity of Taq DNA polymerase when the enzyme extends from an upstream primer into the region of the probe. The cleavage separates the quencher from the reporter dye and restores fluorescence. This dependence on polymerization ensures that cleavage of the probe occurs only if the target sequence is being amplified (Schena et al., 2004).
Study of the homology of the amplicon sequence with other species was performed by the Standard nucleotide-nucleotide BLAST (blast n) of the NCBI .
In order to assess specificity, the qPCR assay was tested on DNA from axenic cultures of *Phytophthora* species isolated during this survey (in results). Moreover, the qPCR assay was also tested in order to evaluate the possible cross-reaction with DNA of closely related (*Phytium* spp.) and unrelated (*Fusarium* sp, *Mortariella* sp) species, commonly isolates from soil and roots in nurseries.

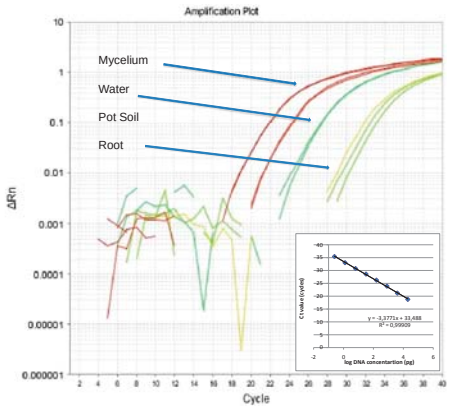
After isolation on selective medium, nearly 50% of the pot plants were positive to *Phytophthora* Species detected: ***P. cinnamomi*, *P. palmivora*, *P. nicotianae*, *P. citrophthora*, *P. cactorum*, *P. citricola*, *P. cryptogea* and *P. syringae***




As European measures against entry and spread of alien soil-borne pathogens through trade of pot plants are based on only visual inspections of aerial and/or subterranean plant organs, molecular tools for the detection of pathogens in container mixes at the time of arrival are a key element for management of soil born microorganisms. Phytophthora species specific TaqMan MGB probe developed in this work fulfils this requirement as qPCR assay has detected Phytophthora in tissues and substrates of cultivation of all plant species at any symptomatic stages with an high resolution grade.

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# Evaluation of Illumina MiSeq as a new tool for the detection of *Phytophthora* species

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Next-generation sequencing (NGS) applied to metagenomic offers the opportunity to obviate most of the limitations of biological detection. Recent papers have been published on the application of pyrosequencing assay to detect and identify fungal and Oomycetes communities in forest ecosystems. Different NGS platforms have been developed which are available for the metagenomic analysis of natural communities of microorganisms. The 454 GS-FLX amplicon pyrosequencing method has been employed successfully for describing Phytophthoras in environmental chestnut soil samples (Vannini *et al.*, 2013).

The MiSeq (Illumina) platform based on the existing Solexa, presents the highest throughput per run and the lowest error rates compared with 454 GS (Loman *et al.*, 2012). Moreover the Miseq workflow has the fewest manual steps as template amplification is done directly on the instrument without manual intervention; consequently the difficulty of workflow and the running cost are lower. However, the Miseq delivered shorter read lengths than the 454 GS (Loman *et al.*, 2012).

**This poster present the preliminary steps done to evaluate the accuracy of MiSeq in describing a *Pythiaceae* community.**

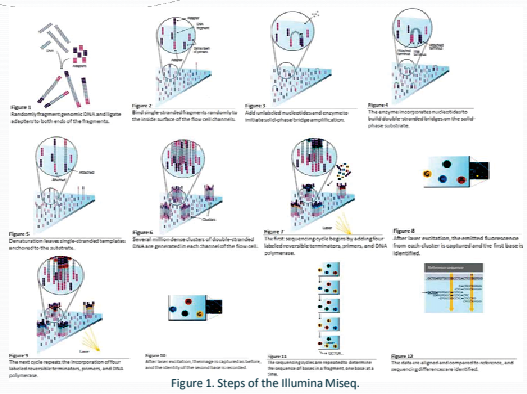


Figure 1. Steps of the Illumina Miseq.

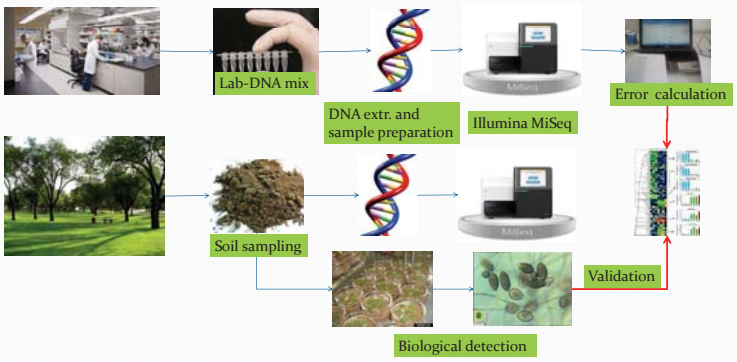
## Material and Methods

The variable Internal Transcribed Spacer 1(ITS1) was chosen as the template sequence. DNA of each sample was amplified with primer set ITS6/7 (Cook *et al.*, 2000) with the ITS6 tagged with a sequence of 8 nucleotides (TagX) to mark each sample. Two different experiments were carried out:

**Laboratory control samples:** the DNA from 10 *Pythiaceae* was pooled at different ratios (Fig.2) in a single mix and then amplified. The amplification was replicated 3 times by using 3 different ITS6-TagX .

**Soil samples:** 3 soil samples were collected from a park located in Bamberg (Germany) where *Phytophthora* spp. Have been previously recorded. DNA from each sample was extracted and the amplified using a different ITS6-Tag**X** for each sample.

The PCR was carried out at the conditions described by Vannini *et al.* (2013). Amplicons were purified using the MagJET NGS Cleanup (Thermo scientific,USA) and quantified with the Qubit Quantitation kit (Invitrogen, USA). The samples were processed by GATC-biotech (Constance, Germany) which carried out the mass sequencing, preliminary trimming and assemblage of sequences. The sequences (reads) were analysed using CLC Genomic Workbench (Qiagen, Netherlands) and compared with a *Phytiaceae* non-redundant custom-curated data base (CCD) (DIBAF-University of Tuscia).




## Results and Discussion

| Species | ng (%) | nºreads | % |
|---|---|---|---|
| *Py. sylvaticum* | 2 (20%) | 297277 | 46,90 |
| *P. gonapodyides* | 1 (10%) | 126265 | 19,92 |
| *P. cambivora* | 1 (10%) | 81064 | 12,79 |
| *P. cactorum* | 0,5 (5%) | 44504 | 7,02 |
| *P. pseudosyringae* | 0,2 (2%) | 24932 | 3,93 |
| *P. megasperma* | 0,1 (1%) | 15970 | 2,52 |
| *Py. attrantheridium* | | 15703 | 2,48 |
| *P. cinnamomi* | 0,1 (1%) | 11974 | 1,89 |
| *Py. intermedium* | | 5930 | 0,94 |
| *P. quercina* | 0,1 (1%) | 3951 | 0,62 |
| *P. alni* | | 1219 | 0,19 |
| *P. ilicis* | | 961 | 0,15 |
| *P. fragariae* | | 734 | 0,12 |
| *P. europaea* | | 610 | 0,10 |
| *Py. undulatum* | 3 (30%) | 534 | 0,08 |
| *P. nicotianae* | | 442 | 0,07 |
| *Py. macrosporum* | | 416 | 0,07 |
| *P. plurivora* | 2 (20%) | 303 | 0,05 |
| *P. hedraiandra* | | 273 | 0,04 |
| *P. syringae* | | 184 | 0,03 |
| *Py. heterothallicum* | | 143 | 0,02 |
| *Py. rastratifingens* | | 138 | 0,02 |
| *Py. violae* | | 110 | 0,02 |
| *Py. paroecandrum* | | 85 | 0,01 |
| *Py. minus* | | 47 | 0,01 |
| *P. nemorosa* | | 16 | 0,00 |
| *P. idaei* | | 5 | 0,00 |
| *P. pseudotsugae* | | 5 | 0,00 |
| *P. psychrophila* | | 2 | 0,00 |

Figure 2. Species used in the control (highlighted) and false MOTUs originated by comparison with custom-curated database with the percentage respect to the total of reads.

❖ A total of 2.009.191 reads were obtained after assemblage and quality filtering

❖ Lab-control samples reads accounted for 282.472 (R1), 222.127 (R2) and 286.294 (R3). No significant differences were found among the lab-control replicates, therefore, reads were combined. After internal trimming 695.036 reads left that were compared with the CCD choosing an identity barcoding threshold of 98% (Vettraino *et al.*, 2012) (Fig.2). All the species included in the DNA mix were detected. A correlation was found between the amount of template DNA in the mix and the numbers of reads for 8 species over 10. The number of reads for *P. plurivora* (0.05%) and *P. undulatum* (0.08%) was largely lower than expected. Up to 20 false MOTUs (Molecular Operational Taxonomic Units) were obtained, eighteen of which were below 0.2% in presence. Most of then derived from one or few bases sequences error on the DNA in the mix.

❖ Soil samples reads accounted for 252.640 (S1), 479.593 (S2) and 371.950 (S3). After internal trimming 222.085 (S1), 418.479 (S2), 325.813 (s3) reads were left for further analysis. As is showed in the Fig,3, again *P. plurivora* and *Py. undulatum* were under-represented compared to the other MOTUs despite they were among the most frequently isolated species from soil with traditional baiting (data not showed) .

❖Based on results obtained from both Lab-control and Soil samples for *P. plurivora* and *Py. undulatum*, it was decided to check the quality of sequences assemblage as provided by the company. The set of sequences for each of the 2 primers (forward and reverse) before the assemblage were compared with the CCD resulting in a number of reads addressing the two species in the proportions expected.

| Soil 1 | | | Soil2 | | | Soil 3 | | |
|---|---|---|---|---|---|---|---|---|
| Species | nº reads | % | Species | nº reads | % | Species | nº reads | % |
| *Py. sylvaticum* | 92617 | 56,43 | *Py.sylvaticum* | 155573 | 53,53 | *Py. sylvaticum* | 179040 | 68,26 |
| *Py. attrantheridium* | 39261 | 23,92 | *Py.attrantheridium* | 78489 | 27,01 | *Py.attrantheridium* | 53736 | 20,49 |
| *Py. intermedium* | 11303 | 6,89 | *Py.intermedium* | 44374 | 15,27 | *Py.intermedium* | 13985 | 5,33 |
| *P. cactorum* | 10496 | 6,39 | *P. gonapodyides* | 2793 | 0,96 | *P. quercina* | 7362 | 2,81 |
| *Py. macrosporum* | 2934 | 1,79 | *P. pseudosyringae* | 2007 | 0,69 | *P. gonapodyides* | 2067 | 0,79 |
| *P. pseudosyringae* | 1775 | 1,08 | *P. cambivora* | 1825 | 0,63 | *P. cambivora* | 1353 | 0,52 |
| *P. gonapodyides* | 1546 | 0,94 | *P. cactorum* | 1363 | 0,47 | *Py.heterothallicum* | 1008 | 0,38 |
| *P. syringae* | 1092 | 0,67 | *Py.rastratifingens* | 1207 | 0,42 | *P. cactorum* | 994 | 0,38 |
| *P. cambivora* | 825 | 0,50 | *Py.heterothallicum* | 676 | 0,23 | *P. pseudosyringae* | 633 | 0,24 |
| *P. cinnamomi* | 672 | 0,41 | *P. quercina* | 544 | 0,19 | *Py.paroecandrum* | 624 | 0,24 |
| *Py.violae* | 528 | 0,32 | *P. megasperma* | 531 | 0,18 | *Py.minus* | 540 | 0,21 |
| *P. quercina* | 337 | 0,21 | *P. cinnammomi* | 415 | 0,14 | *P. megasperma* | 261 | 0,10 |
| *P. megasperma* | 327 | 0,20 | *P. syringae* | 405 | 0,14 | *P. cinnammomi* | 251 | 0,10 |
| *P. nicotianae* | 139 | 0,08 | *Py.macrosporum* | 139 | 0,05 | *Py.violae* | 137 | 0,05 |
| *Py.heterothallicum* | 86 | 0,05 | *P. ilicis* | 74 | 0,03 | *Py.macrosporum* | 88 | 0,03 |
| *P. ilicis* | 76 | 0,05 | *Py.violae* | 42 | 0,01 | *Py.rastratifingens* | 63 | 0,02 |
| *Py. rastratifingens* | 32 | 0,02 | *Py.paroecandrum* | 38 | 0,01 | *P. syringae* | 30 | 0,01 |
| *Py. paroecandrum* | 29 | 0,02 | *P. alni* | 33 | 0,01 | *P. alni* | 26 | 0,01 |
| *Py. undulatum* | 13 | 0,01 | *Py.minus* | 26 | 0,01 | *P. ilicis* | 24 | 0,01 |
| *P. europaea* | 11 | 0,01 | *P. fragariae* | 22 | 0,01 | *P. fragariae* | 14 | 0,01 |
| *P. alni* | 10 | 0,01 | *P. undulatum* | 18 | 0,01 | *Py.undulatum* | 14 | 0,01 |
| *P. minus* | 10 | 0,01 | *P. nicotianae* | 16 | 0,01 | *P. europaea* | 13 | 0,00 |
| *P. hedraiandra* | 7 | 0,00 | *P. europaea* | 15 | 0,01 | *P. nicotianae* | 13 | 0,00 |
| *P. fragariae* | 6 | 0,00 | *P. plurivora* | 6 | 0,00 | *Py.erinaceum* | 11 | 0,00 |
| *P. idaei* | 3 | 0,00 | *P. hedraiandra* | 4 | 0,00 | *P. plurivora* | 4 | 0,00 |
| *P. nemorosa* | 2 | 0,00 | *P. nemorosa* | 4 | 0,00 | *P. hedraiandra* | 3 | 0,00 |
| *P. plurivora* | 2 | 0,00 | *Py.erinaceum* | 2 | 0,00 | *P. citricola* | 2 | 0,00 |

Figure 3. Analysis from the soil samples. MOTUs and false MOTUs originated by comparison with custom-curated database with the percentage respect to the total of reads.

Max sequencing with Illumina Miseq platform is powerful techniques to describe *Pythiaceae* communities in environmental samples. However, accuracy should be put in raw data handling and analyses before comparison with available database. In particular, when adopting a new NGS technique, before to analyses any environmental samples, it is mandatory to run laboratory DNA mix samples in order to calculate the error rate derived from the double amplification and the mass sequencing steps. Furthermore, it is recommended to carry out the analyses of the own raw data in order to fully control the assembling steps and decide the protocol for quality filtering of the sequences. This procedure would facilitate the identification of false MOTUs and maximize the efficacy of the technique even for the quantification of the different taxa. The data of the present study are currently undergoing re-analysis on the basis of the results here illustrated.

**References:** **Cook *et al.*,2000**,Fungal Gent. Biol, 30:17-20. **Loman *et al.*, 2012,Nature** Biotechnology 30: 434–439. **Vannini *et al.*, 2013**,FEMS Microbiol Ecol 85: 433–442. **Vettraino *et al.*, 2012**,Lett . Appl. Microbiol.,55:390-396.



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Bayerische Landesanstalt für Wald und Forstwirtschaft

# *In vitro* control of *Phytophthora cinnamomi* with *Brassica* pellet

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*Phytophthora cinnamomi* is the causal agent of serious epidemic in forest and natural ecosystems. Its control in the open field can only be done through integrated pest management protocols, often expensive and difficult to implement. The biofumigation, widely used in horticulture, could be a viable and effective alternative to the use of chemicals in agroforestry systems, given its easy use and low cost. In this work we report the results of *in vitro* analysis of the inhibiting effect of the pellet of *Brassica carinata* (BioFence ®, Triumph Italy) on the growth of *P. cinnamomi* and the "in vivo" effect in a greenhouse trial. The effect of treatments has been evaluated under different "in vitro" experimental conditions. Four biofumigant concentrations (5, 10, 20 and 40 mg; 40% humidity) at four different temperatures (15°C, 20°C, 25°C and 30°C) were tested vs an isolate of *P. cinnamomi* from *Castanea sativa* (Allumiere, Italy). The efficacy of treatment is strongly influenced by the ambient temperature; even at the temperature favourable to the development of the disease, 25°C. The *Brassica carinata* pellet, when was applied at a dose of 40mg showed a fungicidal effect. In the greenhouse trial, a treatment consisted in *P. cinnamomi* infested soil with and without Brassica pellets (BioFence ®) (3g BioF/l). After 3 months treated soils with BioFence ® resulted in significant lower values of inoculum density of *P. cinnamomi.*

*B. carinata* pellets (BioFence ®) present a good opportunity to control *P. cinnamomi*. The potential of biofumigation as a component of the integrated management of soil pest and pathogens in combination with other practices to trim down disease incidence (e.g. phosphite or application of antagonists) will serve as an important part of disease management in forest and agroforestry systems.

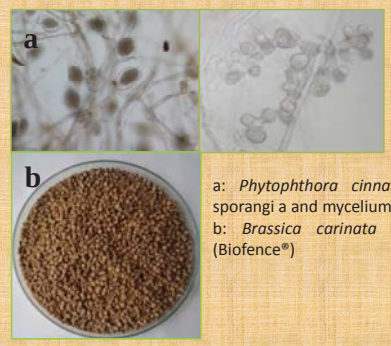
# *In vitro* control of *Phytophthora cinnamomi* with *Brassica* pellet *(BioFence®)*

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## Introduction

*Phytophthora cinnamomi* is the causal agent of serious epidemic in forest and natural ecosystems. Its control in the open field can only be done through integrated pest management protocols, often expensive and difficult to implement. Increasing restrictions on the use of chemicals, owing to their adverse effects on human and environmental health (Du Fretay et al. 2010), have prompted the interest on non-chemical methods for the control of soilborne pathogens. The biofumigation, widely used in horticulture, could be a viable and effective alternative to the use of chemicals in agroforestry systems, given its easy use and low cost.




a: *Phytophthora cinnamomi* sporangi a and mycelium
b: *Brassica carinata pellet* (Biofence®)

The purposes of this study was to evaluate a *Brassica carinata* commercial pellet (BioFence®) for its ability to inhibit *P. cinnamomi in vitro* and in greenhouse trials.

## Materials and Methods

***Mycelial growth rate***. Five biofumigant concentrations (0, 5, 10, 20 and 40 mg/plate; 40% UR) were compared at four temperatures (15, 20, 25 and 30°C). As described by Morales-Rodríguez et al. (2014), mycelial plugs were placed in the centre of the plate of V8 juice agar after 24h of incubation at 25°C, biofumigant material was placed on the covers. The plates were incubated inverted at the different temperatures. The radial growth of the pathogen (two perpendicular diameters) was measured after 6-day incubation at each temperature.

***Greenhouse trial.*** Treatments consisted in *P. cinnamomi* infested soil with and without *Brassica* pellets (BioFence®). Colonized millet grains (Vettraino et al. 2001) were mixed with the potting soil at a concentration of about 4%. A week after inoculation and biofumigation (3g BioF/l), *Quercus cerris* seedlings were transplanted and incubated in greenhouse for 3 months. Soil samples of each pot were collected to estimate germinable propagule density of *P. cinnamomi* as described by Jeffers and Martin (1986).

## Results and Discussion

The biofumigation with *B. carinata* pellet suppressed *in vitro* the growth of *P. cinnamomi.* The growth rate resulted significantly different between temperatures and doses (temperatures: d.f=3, F-ratio =10.84; P<0.05; doses: d.f=4, F-ratio =27.79, P<0.05). Tuckey's test of growth showed the dose to be inversely correlated with the colony growth rate at different temperatures (Fig 1). The dose of 40mg/plate totally inhibited the growth at any temperature. The inhibition was high related to temperature in order that as the temperature increases higher doses of product are needed to inhibit the pathogen significantly. As show in the figure 2, EC50 increase with the increase of the temperature. After 3 months treated soils with BioFence® resulted in significant lower values of inoculum density of *P. cinnamomi* (T-test-Mann Whitney test, P< 0.05) (Fig.3).

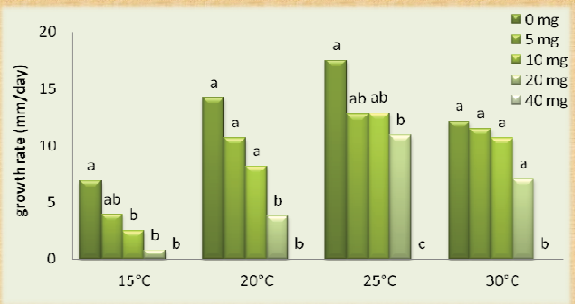



Fig 1. Mycelium growth rate at different doses (mg/plate) and temperature. For each temperature columns with the same letter are not significantly different according to Tukey's test (P≥0.05)

| Temperature (°C) | EC50 (mg) |
|---|---|
| 15 | 1,92 |
| 20 | 8,38 |
| 25 | 11,73 |
| 30 | 13,75 |

**Fig. 2.** Effective concentration (EC50) for each temperature.

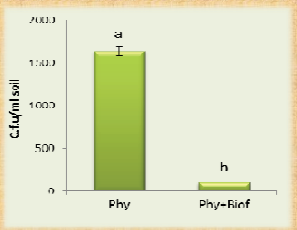



**Fig. 3.** Inoculum density (CFU/ml soil) of *P. cinnamomi* in absence (PHY) and presence (PHYBIO) of BioFence®.

*B. carinata* pellets (BioFence®) present good opportunity to control *P. cinnamomi*. The potential of biofumigation as a component of the integrated management of soil pest and pathogens in combination with other practices to trim down disease incidence (e.g. phosphite or application of antagonists) will serve as an important part of disease management programmes in forest and agroforestry systems.

Du Fretay, G. et al., 2010. Acta Hort. 883: 113-120.
Jeffers, S.N.; Martin, S.B., 1986.Plant Dis. 70: 1038-1043.
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Vettraino, A. M. et al., 2001. Plant Pathol. 50, 90–96.

# Maps of *Austrocedrus chilensis* forests affected by dieback

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Forests of the monotypic native conifer *Austrocedrus chilensis* are affected with the dieback caused by *Phytophthora austrocedrae*. However, no regional maps were available, even when these are fundamental to understand the dynamics of the disease. Therefore, we collected information of the spatial distribution of affected populations under the jurisdiction of the National Parks Administration. Using existing maps of *Austrocedrus chilensis* distribution (1:250.000, INTA-APN, 2005) information collected in the field was set in a grid of 2x2 km (according to geographical coordinates) indicating areas with or without symptoms of dieback, or with mortality of *Austrocedrus chilensis*, in the four National Parks where the species is present: Lanin, Nahuel Huapi, Lago Puelo and Los Alerces. Sectors with proven pathogen presence are also shown (tests by CIEFAP laboratory). To gather information from such a vast area we involved key informants (rangers, researchers, technicians, etc.) by developing a method to standardize the sampling and further monitoring. The presence of dieback signs is discontinuous and isolated, ranging from a few individuals to large clumps. In Lanin NP the percentage of forest area with dieback was *ca*. 20%, while in Nahuel Huapi NP was *ca*. 57%, being eastern forests (lower rainfall) less affected. Lago Puelo NP had the lowest percentage of affected forest (11%), however, most of the species distribution is outside the Park and not included in this survey. The higher percentage of affected forest area was found in Los Alerces NP, where *ca*. 87% of the forest showed symptoms of decay or large clumps of dead trees. As the methodology induces to overestimate percentages, further monitoring, with more detail, is required. This work provided a regional sight of the dieback presence and allowed establishing bases for regional monitoring and diagnosis, which helps to make decisions related to the conservation and management of *Austrocedrus chilensis.*

# Status of the genus *Phytophthora* in Argentina

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The genus *Phytophthora* includes important pathogens affecting a wide range of hosts causing severe losses on the crops. The knowledge of these species allows a better management of the diseases. In the past century Frezzi studied and summarized the information about *Phytophthora* in Argentina. Since then no update of this matter was performed. In order to have a more comprehensive vision of this genus, a review and an updated report of recent progress in this matter were carried out. Information was taken from printed and on line resources. As a result, the information was analyzed and categorized, thus updating the number of species of *Phytophthora*, its geographical distribution, the affected hosts and symptoms, percentage of host-pathogen relation, distribution maps, citing the first report on each localities and the first references of molecular studies. According to this searching the first species reported by Spegazzini was *Phytophthora infestans* (Mont.) de Bary in 1901, infecting *Solanum tuberosum* in Buenos Aires province. Until 1977, 17 species were identified. Since then, between 2005 -2012, 5 species of were found affecting important crops, nursery crops and trees growing in natural ecosystems. To date 22 *Phytophthora* spp have been reported on 222 hosts-pathogen relations and 5 species were isolated from soil or water*. P citrophthora* and *P nicotianae* are the species that affect the greatest number of hosts. The main species affecting tress are *P. cinnamomi* and *P. citrophthora*. The most recently identified species were *P. austrocedrae* associated with *Austrocedrus chilensis* mortality in Patagonia; *P. lacustris* on *Pyrus communis* as a postharvest fruit pathogen in Northern Patagonia, *P.* taxon *kelmania* on *Gerbera jamesonii* and *P. multivora* on *Rhododendron* sp. in surroundings Buenos Aires city. The review provides information that allows interpreting more clearly the current and future status of investigations concerning the genus *Phytophthora* in Argentina.

# Genetic, morphological and physiological characters of the plant pathogen *Phytophthora cactorum* and its hybrids

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*Phytophthora cactorum* is homothalic species placed in Cooks *Phytophthora* group I. Its host spectrum includes more than 200 plant species growing worldwide in the temperate climatic zone. *P. cactorum* can form two hybrids: *P.* × *serendipita (P. cactorum* × *P. hedraiandra)* and *P.* × *pelgrandis (P. cactorum* × *P. nicotianae)*. Hybridization events are possible way how to extend or change the host spectrum and improve pathogenicity of *Phytophthora*. During this work *P. cactorum* and both its hybrids were examined to compare morphological, physiological and genetic differences. The isolates included in the study were isolated from 27 different host species in twelve European countries. The characters of isolates were determined using i) light microscopy for morphological characters, ii) cardinal temperatures measurement iii) the RAMS PCR fingerprinting method to determine genetic differences, iv) the sequencing analysis of selected isolates using ITS, *cox*I and *Pheca* DNA region. According to RAMS results, all isolates were divided in three main lineages, one includes exclusivelly *P.* ×*pelgrandis*, and the other ones grouped both *P. cactorum* and *P.* ×*serendipita* grouped together according to their geographical origin. Very similar results were obtained also in cardinal temperatures measurement, again without differences between *P. cactorum* and *P. serendipita*. According to morphological characters, only *P. pelgrandis* was distinguishable from the other species. The sequence analysis showed different levels of heterozygosity for ITS and *Pheca* nuclear gene regions in *P.* ×*serendipita* and *P.* ×*pelgrandis,* those regions included heterozygous sites inherited from both parent species. The sequence analysis of mitochondrial *cox* I gene revealed that in *P.* ×*serendipita* this locus can be inherited from either parental species, *P. cactorum* or *P. hedraiandra*. Currently we continue in identificaion of the hybrids and population structure of *P. cactorum* and its hybrids in Europe using AFLP method.

# Metagenomic analysis of *Phytophthora* diversity in nurseries of potted ornamental species

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The genetic diversity of *Phytophthora* was investigated in soil and root samples of potted ornamental and fruit tree plants collected in nurseries located in Apulia and Calabria (Southern Italy) using metagenomic approaches based on *Phytophthora* genus specific primers. PCR amplicons containing the ITS1 region of the rDNA were sequenced using both a conventional cloning and Sanger sequencing approach and a 454 pyrosequencing protocol. All sequences were accurately analyzed with an appropriate software and used as barcode for species identification utilizing a validated ITS database. The cloning/Sanger sequencing approach enabled the identification of nine different *Phytophthora taxa* (*P. nicotianae, P. citrophthora, P. meadii, P. cinnamomi, P. parvispora, P. cambivora, P. niederhauserii, P. taxon Pgchlamido,* and *P. lateralis*), 3 phylotypes associated to "species complexes" (*P. citricola*, *P. cryptogea* and *P. pseudosyringae*) and three other phylotypes considered as unknown or non well identified *Phytophthora taxa*. The 454 pyrosequencing confirmed above results and provided a higher levels of accuracy enabling the detection of four additional species (*P. cactorum*, *P. psycrophila*, *P. palmivora* and *P. ramorum*) and a general higher level of diversity (number of detected genotypes) within analyzed samples. Data of the present study indicate the use of genus specific primers combined with next generation sequencing approaches as valuable tools to investigate *Phytophthora* diversity in different environments and pathosystems. Furthermore, the large number of genotypes and *Phytophthora taxa* detected in a limited geographic area confirms a primary role of nurseries in favoring the diffusion and the evolution of *Phytophthora* species.

# Molecular and morphological data shows two consistent lineages in *Phytophthora plurivora* strains isolated from streams in northern Spain

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*Phytophthora plurivora,* included in the *P. citricola* complex, was recently described as new species based on its morphology, physiological and molecular characters. Recently, isolated strains from natural waterways in northern Spain showed a high morphological colony variability in pure cultures at PDA medium. The isolates were subjected to temperature assays, morphological studies and phylogenetic analysis based on five different molecular markers: ITS, HSP90, Elongation Factor 1-α and the mitochondrial genes COX II and NADH. The result of these assays revealed differences between isolates of *P. plurivora*. Temperature assays showed two distinct patterns of growth at the optimal temperature. Statistical analyses of sporangia and oogonia measurements showed significant differences (p-value 0.5 and 0.1). Phylogenetic analysis based on Maximum Likelihood showed two consistent lineages, which corresponded with the same groups obtained in the temperature assays, revealing *P. plurivora* as a possible species complex.

# Distribution and impact of *Phytophthora* species on alder (*Alnus* spp.) in Southern Sweden

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Invasive pathogens of the genus *Phytophthora* threaten forest ecosystems globally. One of the most significant examples is the damage associated to the hybrid *Phytophthora alni,* an alien pathogen infecting and causing dieback of alders *(Alnus* spp*)*. In Sweden, damages attributed to alder *Phytophthora* were first identified in the 90s, but since then, scarce research has been carried out to determine the extent of the spread and damages caused by this pathogen. This study aimed to determine the distribution and impact of *Phytophthora* species infecting alders (*Alnus glutinosa* and *Alnus incana*) in Sweden. A systematic survey was performed on the sixteen major river systems of southern Sweden and *Phytophthora* colonies were isolated from alder tissue. The recovered isolates were typed according to morphological features and using molecular markers. In each plot, an estimation of the damages was obtained by measuring defoliation, dieback or chrorosis symptoms on ten dominant trees. *Phytophthora*-infected alders were recorded in 28% of the 176 assessed plots. We isolated two subspecies of *P. alni: P. alni* subsp. *uniformis* (Pau) and *P. alni* subsp. *alni* (Paa). In addition, *Phytopthora plurivora* were also isolated from alder tissue. In total, 115 *Phytophthora* isolates were recovered. Estimation of damages showed that the percentage of trees with healthy crowns in plots with *P. alni* presence was lower than in plots without the pathogen. *Phytophthora alni* appeared to be widespread in southern Sweden and preliminary results showed that the distribution of both subspecies seems to be correlated with winter temperature. Pau was present across the whole range of temperatures of the host while Paa was located in areas with milder winter temperatures. The presence of both *P. alni* and *P. plurivora* in Southern Sweden could threaten other riverbank ecosystems thus further monitoring is required.

# Chemicals for management of red needle cast in *Pinus radiata* plantations in New Zealand: efficacy and persistence of phosphite and other fungicides.

Carol Rolando, Nari Williams and Martin Bader

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Red needle cast (RNC), a foliar disease of *Pinus radiata* D. Don caused by *Phytophthora pluvial* has the potential to cause up to 38% annual growth loss in severely infected mature *P. radiata* plantations in New Zealand (Dick et al., 2014). A cost-effective, chemical control strategy is needed to provide a short-term management option for control of severe outbreaks of RNC in existing *P. radiata* forests. Phosphite, a fungicide known to be effective against diseases caused by *Phytophthora's*, is the primary active ingredient currently being investigated for its potential to manage RNC in *P. radiata* plantations in New Zealand. However, the efficacy of other fungicides is also being investigated.

The results of controlled trials to determine the efficacy and persistence of phosphite, copper, metalaxyl-M and two disinfectants will be discussed together with the progress that needs to be made before an operational control strategy for control of RNC can be deployed.

Dick, M, Williams, NM, Hood, IA, Bader, M. (2014). Pathogenicity of *Phytophthora pluvialis* on *Pinus radiata* and its relation with red needle cast disease in New Zealand. *New Zealand Journal of Forestry Science, in press (to be updated on publication).*



# Chemicals for management of red needle cast in *Pinus radiata* plantations in New Zealand

**Carol Rolando, Nari Williams and Martin Bader**
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## Introduction

Red needle cast (RNC), a foliar disease of *Pinus radiata* caused by *Phytophthora pluvialis* has the potential to cause up to 38% annual growth loss in severely infected mature *P. radiata* plantations in New Zealand (Dick et al., 2014). A cost-effective, chemical control strategy is needed to provide a short-term management option for control of severe outbreaks of RNC in maturing *P. radiata* forests.

## Materials and Methods

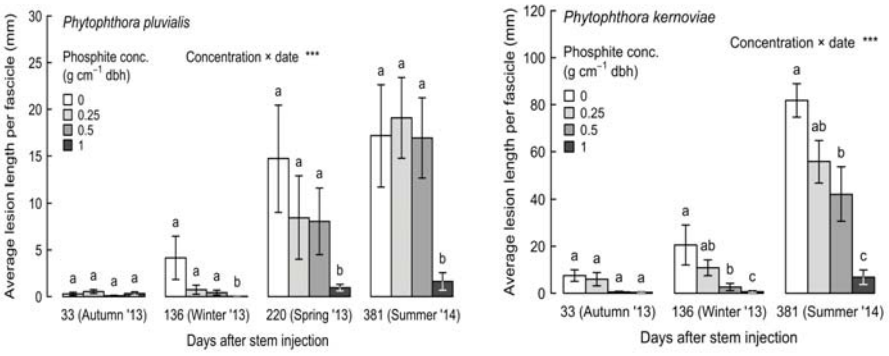
**Detached Needle Assay:** The base of complete mature fascicles of *P. pluvialis* were incubated overnight in zoospore suspensions of *P. pluvialis* and *P. kernoviae*. Needles were incubated in damp chambers at 17 ºC for 10 days when lesions were measured.

**Phosphite Dose Response:** Phosphite was applied by stem injection (0, 0.25, 0.5 and 1.00 g a.i. $cm^{-1}$ dbh) to six-year-old *Pinus radiata*. The efficacy and persistence of these treatments was assessed 33, 126, 220 and 381 days after phosphite application.

**Alternate Chemical Control:** Agrifos® 600, AGPRO Copper Oxychloride 800 WP® and Ridomil® Gold SL were applied at four concentrations (0, 0.5, 1 and 2 x label rate) to two clones of *P. radiata*. The clones differed in their susceptibility to red needle cast. The efficacy and persistence of treatment was assessed 1 week and 3 months after treatment application.

## Results and Discussion

Needles sampled from trees injected with phosphite at 1 g a.i. cm-1 dbh had consistently smaller lesions than needles sampled from control trees for up to one year after injection for both species of *Phytophthora* tested. Persistence and efficacy was lower for the intermediate concentrations tested, particularly for *P. pluvialis*. Persistence of phosphite is dose dependent.

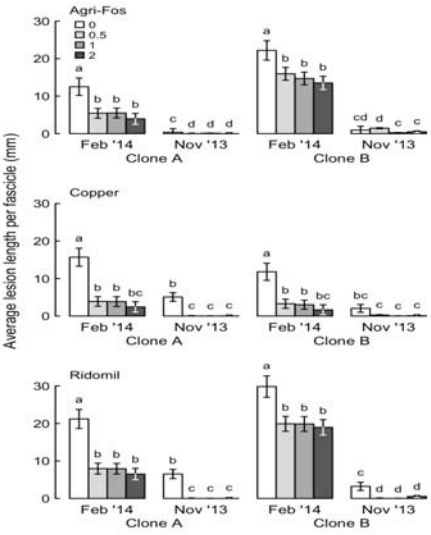



**Stem Injection:** Average lesion length on *Pinus radiata* needles inoculated with (A) *P. pluvialis.* (B) *P. kernoviae*

**References:** Dick, M. A. et al.,(2014). NZ Journal of Forestry Science, 44(6), 1-12.

**Acknowledgements:** This work was co-funded by the New Zealand Forest Owners Association and Scion's Core Purpose Funding. This poster was presented at IUFRO Phytophthora in Forests and Natural Ecosystems, Esquel Argentina November 2014

Three months after application AGPRO Copper Oxychloride 800 WP®, Ridomil® Gold SL and Agrifos® 600 showed efficacy against *P. pluvialis* and *P. kernoviae*.




**Alternate Chemical Control:** Average lesion length per fascicle in ramets from two *Pinus radiata* clones inoculated with *Phytophthora pluvialis.*

## Conclusions

Phosphite reduced *Phytophthora* infection of *P. radiata* needles for up to 12 months after injection. A chemical management programme for red needle cast will need to incorporate host susceptibility. Further testing is required to confirm the potential of the three most promising products, especially copper as it is already widely used by the forest industry. Known cases of agricultural *Phytophthora* diseases developing resistance to Ridomil Gold® SL make it a less attractive long-term option. Copper based products and salts of phosphorous acid, like Agrifos 600®, are low cost fungicides presenting a cost effective options for RNC control.

# Age-related susceptibility of *Eucalyptus* species to *Phytophthora boodjera* prov. nom

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Since 2011 damping-off and mortality of *Eucalyptus* seedlings in Western Australian (WA) nurseries has been observed. The casual agent of this disease was identified as *Phytophthora boodjera* prov. nom based on a combination of morphology and a multi-gene phylogeny.

This study evaluated the age-related susceptibility of five species of *Eucalyptus* (*E*. *polybractea*, *E*. *kochii* subsp. *plenissima*, *E*. *kochii* subsp. *borealis*, *E*. *loxophleba* subsp. *lissophloia*, and two seedlots of *E*. *loxophleba* subsp. *gratiae*) to six isolates of *P*. *boodjera* and three isolates of *P*. *arenaria* in sterilised washed river sand-infestation pot trials. *P*. *cinnamomi* was included for comparison. *Eucalyptus* spp. were inoculated with all *Phytophthora* isolates at 0, 2, 4, 12 and 88 weeks post-germination. The following measurements were included in data sets where applicable: number of seedlings germinated, height of seedlings, root length and dry root weight.

Pre-emergent mortality was almost 100%. Post-emergent mortality was 50-100% depending on isolate. Mortality was also high for 1-month old seedlings (46 to 68%) and root length of surviving seedlings was severely reduced. Death from root infection was not observed for seedlings inoculated at 12 and 88 weeks, but this resulted in root necrosis and reduced root dry weight compared to non-inoculated controls. *P*. *boodjera* is a pre- and post-emergent pathogen of mallee eucalypts. These eucalypts are susceptible to *P. boodjera* at all life stages tested, but the mortality rates declined with seedling age. The events leading to its recent appearance in the nurseries remain unknown and further investigations are underway to determine if this is an introduced or endemic pathogen.

# Epidemiology of *Phytophthora boodjera* prov. nom., a damping-off pathogen in tree production nurseries in Western Australia

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The recently emerged plant pathogen *P*. *boodjera* prov. nom. is responsible for damping-off and mortality of *Eucalyptus* seedlings in Western Australian (WA) tree production nurseries. It was first isolated in early 2012, in a nursery producing mostly eucalypt seedlings for restoration purposes in agricultural land. Symptoms included mortality of newly-germinated seedlings, or stunting of growth that was often not observed until seedlings reached the 4-6 true leaf stage.

Extensive sampling on-site detected *P*. *boodjera* under benches, in drainage outlets, in used seedling trays, in the nursery lawn, and in nearby eucalypt tree shelterbelts. Prior to seeding in November 2012, stringent hygiene was applied and to prevent contamination of potting mix, trays were boiled and machinery separated. Testing of potting mix and trays returned negative results for *P*. *boodjera,* and yet it reappeared in seedling trays in January 2013. Additionally, *P*. *boodjera* was found in other nurseries after an up-regulation of the sampling regime. Studies are currently being undertaken to determine the epidemiology of this pathogen.

Recent epidemiological studies have shown that: (a) *P. boodjera* can be reisolated from used seedling trays but not from used trays that have been sterilised or pasteurised, (b) when used seedling trays were seeded with various hosts only the eucalypts become infected and died, (c) even though increased mortality of seedlings was observed along the drip lines on benches, the infection process does not require excess water, and (d) *P. boodjera* was not present in seed, fungal gnats or dust collected from the site.

In the 2013 seeding season, all trays containing potting mix were pasteurised and no symptoms developed in any seedlings although the pathogen is known to be persisting on site. This suggests that good hygiene coupled with pasteurisation prevented disease development. It also implies that the potting mix itself, or on-site contamination of the potting mix, are the most likely sources of *P. boodjera* inoculum in the trays.

# *Phytophthora* disease on alder (*Alnus* sp.) in Norway

Gunn Mari Strømeng[1], May Bente Brurberg[1], Maria-Luz Herrero[1], Willy Couanon[1], Arne Stensvand[1], Isabella Børja[2] & Venche Talgø[1]

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1

**In 2012, we detected and identified the plant pathogen *Phytophthora alni* from discolored lesions on trunks of grey alder (*Alnus incana*) trees at Årungen lake in Ås, Akershus county in Southern Norway. During the last few decades this pathogen has killed numerous alder trees in many European countries. There are several subspecies of *P. alni* which may differ in aggressiveness, but at present it is not clear if we have one or more subspecies in Norway.**


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## Symptoms

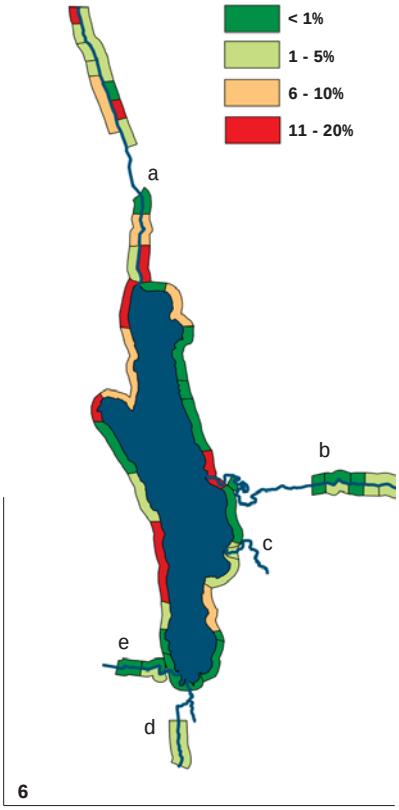
The trunks of infected trees have dark colored, bleeding lesions 1, mainly close to the base. The wood underneath the damaged bark varies in color from reddish brown to brown, and often with a marbled or streaked appearance in areas where the fungus is active 2. Other symptoms are small leaves, short internodes (distances between buds), and crown dieback (foliage and shoots) resulting in dead branches 3.


4


5

## Survey on grey alder around lake Årungen

In 2012, a survey was carried out to clarify how widespread the disease was in the grey alder stands growing along the banks of Årungen lake 4,6 and small rivers/streams both downstream (6-a) and upstream of the lake (6-b,c,d,e). Close to 200 out of around 6000 trees, showed typical *Phytophthora* symptoms on the trunks. The symptomatic trees were not evenly distributed; in many sections along the water there were less than 1% trees with symptoms (6 – dark green sections), while in some sections close to 20% of the trees had symptoms (6 – red sections). Many of the trees were dead or dying 5.



6

## Acknowledgements

We thank Trude Slørstad and Grete Lund at Bioforsk for technical assistance.



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# *Phytophthora pseudosyringae* found on bilberries in Norway

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Norwegian Institute for Agricultural and Environmental Research

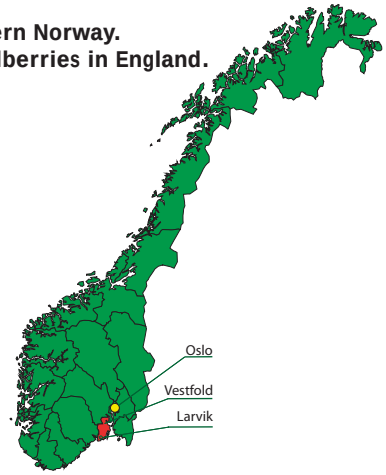
**In August 2012, dead bilberry plants (*Vaccinium myrtillus*) were found in southeastern Norway. *Phytophthora pseudosyringae* was isolated, a pathogen which is also reported on bilberries in England.**



1

## Symptoms

The diseased plants were found in a natural environment on a peninsula in the lake Farris 1, which is situated close to the city of Larvik in Vestfold county 2. In two patches, diameters 2-3 meters, most of the plants were completely dead 3, 4. Plants along the edges had chlorotic foliage. On the shoots of diseased plants there were dark sections 5, a typical symptom when bilberries are attacked by *Phytophthora* spp. *P. pseudosyringae* was isolated from the leading edges on such symptomatic shoots.




2



3

## Damage potential

Bilberry is a very common plant in Norwegian forests, and is highly appreciated by both humans and animals. Especially deer and moose are to a large extent depending on bilberry plants for feeding. Over a large area in southeastern England a number of *Phytophthora* spp. are threatening to eradicate bilberries; *P. ramorum, P. kernoviae*, and *P. pseudosyringae*. Since *P. ramorum* also has been found on bilberries in Norway (Herrero *et al*. 2011), we fear the same situation as in England may occur in Norway, especially in the mild and humid climate in coastal areas in southwestern Norway.

A survey is urgently needed to enable implementation of proper management strategies.



4

## Reference

Herrero, M.L., Toppe, B. & Brurberg, M.B. 2011. First report of *Phytophthora ramorum* causing shoot dieback on bilberry (*Vaccinium myrtillus*) in Norway. Plant Disease 95:355.



5



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# *Phytophthora* survey in a beech forest in Norway

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Norwegian Institute for Agricultural and Environmental Research

**In 2011, *Phytophthora* canker was observed in a beech tree (*Fagus sylvatica*) forest in the city of Larvik in Vestfold county. A survey in 2012, revealed that nearly 5 % of the trees had symptoms in the most affected part of the forest.**

## Symptoms

Diseased trees showed tar-coloured spots («bleeding cankers») on the trunk 1, 2. Underneath the bark there was a distinct line between healthy (whitish) and diseased (brownish) tissue, the so-called leading edge 3. Crown dieback, including yellow foliage and dead branches was also observed, a typical *Phytophthora* symptom 4.

## Survey and sampling

During 2012, trees with a circumference above 20 cm were surveyed for *Phytophthora* symptoms. Coordinates (GPS) were recorded for all symptomatic trees. Samples from the leading edge on selected diseased trees where collected and isolations were carried out on an artificial *Phytophthora* specific media (PARPH). To search for *Phytophthora* spp. in the water in the forest, rhododendron leaves were used as bait in open ditches. Isolates were identified by DNA analysis (ITS sequencing of the rDNA).

## Results and discussion

A total of 49 beech trees had *Phytophthora* symptoms. Two localities had denser concentration of diseased trees than the rest of the forest, with 16 of 329 trees (4,9 %) and 12 of 680 trees (1,8 %) showing *Phytophthora* symptoms 5.The remaining diseased trees were mainly situated close to frequently used paths.

DNA analysis of isolates resulted in *P. cambivora* from examined trees and *P. plurivora and P. gonapodyides* from ditch water. Both *P. cambivora* and *P. plurivora* are well known pathogens on beech in European forests.


1


2


3

## Management

- Avoid traffic outside paths, especially in wet areas. Infected soil on footwear, dog paws, bicycle wheels etcetera may spread the disease.
- Do not throw garden waste in the forest. Dead plants from the garden may contain *Phytophthora* spp.
- Avoid using wood chips from diseased trees on paths.
- Drain moist areas in the forest.
- Fell dead trees when the ground is frozen and preferably covered with snow to prevent spread of infected soil.
- Avoid movement of potentially infected soil.


4



5

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# Polyacrylamide and Movement of *Phytophthora ramorum* in Irrigation Water

**Steve Tjosvold, David Chambers, Steve Koike and Michael Cahn**

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## Introduction and Purpose

Water molds, including *Phytophthora ramorum* are high risk pathogens in the nursery industry. They affect the viability of the nursery industry, create serious quarantine concerns and, in many cases, threaten the health of susceptible natural ecosystems. Linear anionic polyacrylamide (PAM) is used in mitigating sediment runoff and other water quality issues in field crops (Lentz et al. 1992), and was demonstrated to reduce transport of microorganisms associated with animal waste over and through soil (Entry and Sojka 2000). The goal of this research was to test a novel method of applying PAM directly in potting soils to determine its impact on the movement and viability of *P. ramorum* propagules in leachate from pots. This method may limit plant disease and reduce propagules in water from entering recycling ponds and moving off nurseries into natural water bodies.

## Methods

**Determine the effect of PAM solutions on the production of zoospores and their movement.** One agar plug (from a #2 cork borer) colonized with *Phytophthora ramorum* was incubated in a 35 x 10 mm Petri plate containing ml of either 0.0, 0.5, or 1.0 g/L PAM solution (PAM: SoilFloc 100D, Hydrosorb Inc., Orange, CA.). DI water in these solutions contained 1 meq $CaCl_2$ to activate the PAM. These PAM concentrations were representative of those that could be previously found in PAM treated pots and leachate. After 2 days incubation at 20 C in the dark, the resulting sporangia were induced to produce zoospores by chilling at 5 C for one hour and then warmed to room temperature. The cultures were examined for zoospore release and mobility, and documented with images and video. After all zoospores were released (1 hr), the 5 ml zoospore solution was diluted with DI water containing 1 meq $CaCl_2$ to make up a 500 ml solution. From this solution, 1 ml samples were uniformly spread over five 100 x 15 mm Petri plates containing semi-selective media (PARP). The number of resulting viable colonies (CFU) were counted 48 and 96 hours after plating. The data was presented as the mean CFU per ml produced at incubation for each PAM rate. There were 5 replications of each PAM rate treatment. This evaluation was repeated (Experiments A and B).

**Determine the effect of PAM treated soils on propagules in soil leachate.** PAM treated potting soil (50, 100, 400 g/m3 and untreated check) in 1 gallon nursery pots were irrigated two times, a day apart, with 500 ml of DI water containing 1 meq/L $CaCl_2$ to settle soil and activate the PAM. A zoospore concentrate was produced and diluted with 1 meq/L $CaCl_2$ as described above and the final concentration was determined with a hemocytometer. On subsequent irrigations, either 500 ml of the diluted zoospore solution or DI water was irrigated into the pot (Fig 1 below). The soil leachate (water draining from the bottom of the pot) from each pot was collected at each irrigation. Three 1 ml samples were taken from each leachate sample and each spread uniformly on a 100 x 15 mm Petri plate containing semi-selective media (PARP), and the number of resulting CFU were counted.



**Fig 1. Soil in nursery pots is treated with various rates of PAM and irrigated with a zoospore solution or just DI water. Leachate is collected below the pot and the propagules are counted.**

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**Entry, J. A., and R. E. Sojka. 2000.** The efficacy of polyacrylamide and related compounds to remove microorganisms and nutrients from animal wastewater. Journal of Environmental Quality 29: 1905-1914.

**Lentz, R. D., I. Shainberg, R. E. Sojka, and D. L. Carter. 1992.** Preventing irrigation furrow erosion with small applications of polymers. Soil Science Society of America Journal 56: 1926-1932.

University of California
Agriculture and Natural Resources

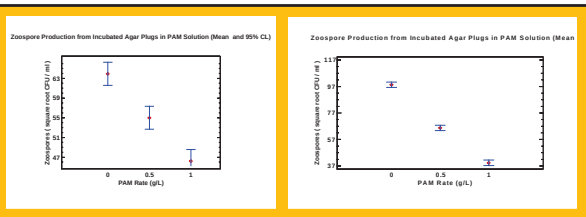
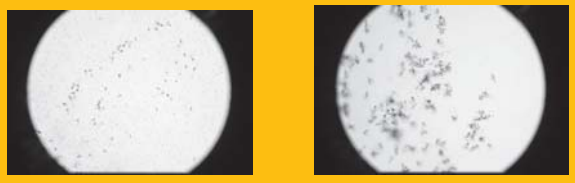



**Fig 2. A and B. Effect of PAM concentrations on the production of zoospores during agar plug incubation of *P. ramorum*. Experiment A (left) and repeated Experiment B (right).**

**Fig 3. A and B. Effect of PAM on mobility of zoospores during agar plug incubation of *P. ramorum*. No PAM (left) and 1 g / L PAM (right). Note that zoospores encysted soon after they were released from sporangia.**



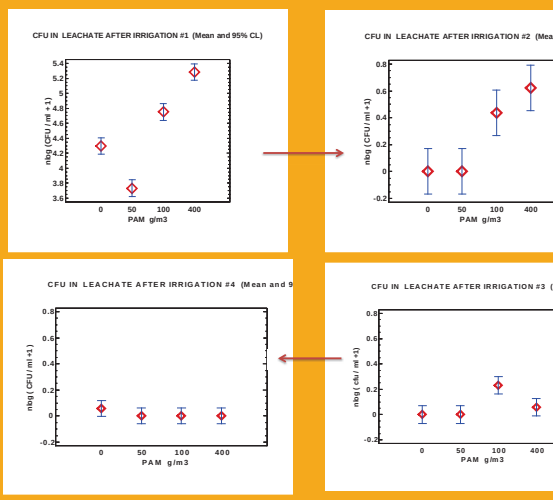



**Fig 4 A, B, C, D (clockwise). Experiment 1. Effect of PAM treated soil on propagules detected in leachate. A. First irrigation with 500 ml with 7.3 X $10^3$ / ml zoospores. B, C, and D: Each of 3 subsequent 500 ml irrigations contained no zoospores. (Note concentration scale in A is different than B,C and D).**

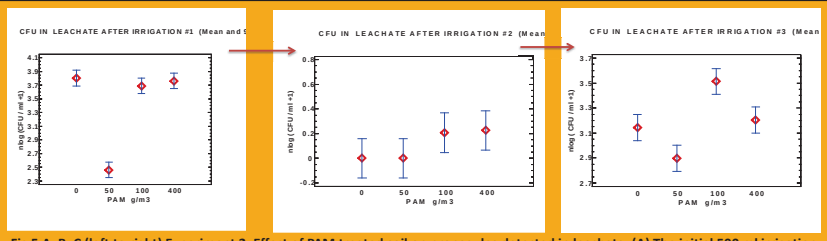



**Fig 5 A, B, C (left to right) Experiment 2. Effect of PAM treated soil on propagules detected in leachate. (A) The initial 500 ml irrigation with 2.5 X $10^3$ /ml zoospores. (B) Second 500 ml irrigation was applied with no zoospores, and (C) third 500 ml irrigation with 4.5 X $10^3$ / ml zoospores.**

## Results and Discussion

**Production of zoospores and movement:** There was a significant linear reduction of the number of viable propagules produced from *P. ramorum* agar plugs incubated with increasing PAM concentrations (Fig 2). Zoospore mobility was greatly slowed with increasing PAM concentrations, and the 1 g/L concentration caused the zoospores to almost immediately encyst upon their release from the sporangia (Fig 3). PAM in solution is noticeably viscous; with the highest concentration (1 g/L) almost like the consistency of pancake syrup. It is understandable that the tiny, vulnerable swimming zoospores are slowed down, or with the highest concentration, are entrapped and encyst quickly.

**Propagules in soil leachate:** There was a significant reduction of propagules detected in the soil leachate with the 50 g/$m^3$ PAM treatment when pots were irrigated with inoculum (Figs 4A and 5A, C). However, there were significantly more propagules with the 100 and 400 g/$m^3$ PAM treatments. After the first inoculated irrigation, subsequent irrigations, without the zoospores, produced very few zoospores to none being detected in the leachate (Figs 4 B,C,D and 5B). In experiment 2 (Fig 5), only 5 % of the zoospores in the irrigation water were found in the leachate. So even though the 50 g/L PAM treatment significantly reduced propagules in the leachate, the total numbers of propagules affected were relatively small compared with the filtering that was taking place by the soil itself.

**Several questions arise:** If the PAM reduces zoospore motility and viability as this evaluation suggests, could it reduce infectivity of *P. ramorum* (or other soil inhabiting Phytophthora species) on roots in those pots? Could PAM solution reduce viability of *P. ramorum* propagules in leachate/runoff water as the water drains away from nursery beds? Will PAM solution enhance the sequestration of propagules in leachate/runoff water near the gravel surface where ultraviolet light and drying would have a deleterious effect on viability of zoospores?

# Identification of *Phytophthora alni* subspecies in riparian stands in the Czech Republic

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In the Czech Republic, *Phytophthora alni* was first confirmed in 2001 and the pathogen has been quickly spreading and occupying almost the whole area of the country. The pathogen attacks *Alnus glutinosa* or *A. incana* to a lesser extent and causes considerable losses of alder trees along hundreds of kilometres of riverbanks. The aim of our work was to perform the identification of *P. alni* isolates at the subspecific level using PCR and to determine the frequencies and distribution of particular subspecies. The allele-specific PCR primers focused on allele diversity of orthologs of *ASF*-like, *TRP*1, *RAS*-*Ypt*, and *GPA*1 genes were selected for identification. Eighty-eight per cent of the 59 analysed isolates belonged to *P. alni* ssp. *alni* while 12% were *P. alni* ssp. *uniformis. P. alni* ssp. *multiformis* has not been recorded in the country till now (Štěpánková et al. 2013). The two subspecies differed in distribution. *P. alni* ssp. *alni* dominated in riparian stands along broader rivers in lowlands and the results confirmed the more effective spreading of *P. alni* ssp. *alni* based on its higher aggressiveness and ecological advantage. *P. alni* ssp. *uniformis* was acquired rather from riparian stands of small watercourses at relativelly higher altitudes. The insular distribution of *P. alni* ssp. *uniformis* may represent the remains of its former occurrence. Therefore, *P. alni* ssp. *uniformis* may be an previously introduced subspecies suppressed by the more aggressive related taxon.

Reference:
Štěpánková, P., Černý, K., Strnadová, V., Hanáček, P., and Tomšovský M. 2013. Identification of *Phytophthora alni* subspecies in riparian stands in the Czech Republic. Plant Prot. Sci. 49 (Special Issue): S3–S10.

# Histopathology of *Phytophthora austrocedri* in *Austrocedrus chilensis*

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*Austrocedrus chilensis* (D. Don) Pic. Ser. & Bizzarri (mountain cypress), Cupressaceae, suffers a disease caused by *Phytophthora austrocedi* that leads the trees to death. It has been speculated that trees are killed by extensive death of bark and cambium tissues and by the disruption of phloem transport but the pathogen mechanisms are not totally elucidated yet. The histopathology of *A. chilensis* disease is being studied to understand the pathogenic mechanisms of *P. austrocedri* as well as the tree defense mechanisms. Necrotic and healthy tissues, of naturally and artificially infected adult trees, were studied in order to assess the effects of the pathogen on the phloem and xylem. Portions of tissues from the advancing, medium and old areas of necrotic lesions, as well as from healthy areas at least 60-80 cm above lesion, were sliced into transverse, tangential and radial sections of 15 µm using a microtome and observed in a light microscope. Oospores of *P. austrocedri* were observed in affected phloem, especially in resin pockets. Hyphae were observed in phloem and xylem. In the xylem hyphae grow through rays, and pass from rays to tracheids through the cross-field pitting, and from one tracheid to another through the pits. Crossing hyphae filled the torus of the pit completely and consequently blocked the pit. Affected xylem showed the formation of trabeculae, single or double and frequently aligned, that were absent in healthy xylem. The trabeculae might appear as a response of the tree against the presence of the pathogen and could also contribute to the decrease of hydraulic conductivity observed in affected trees. Formation of traumatic resin ducts in the phloem associated to necrotic lesions was also observed. The resin ducts were much more abundant and bigger than the normal resin ducts of healthy phloem and can fuse to form resin pockets. Thus, these structures are related to the profuse resination produced associated to the advancing zone of the lesion that is assumed as a defense mechanisms of the tree.

# Genetic variation in *Phytophthora lateralis* lineages by analysis of microsatellite profiles

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*Phytophthora lateralis* is one of the most destructive of introduced Phytophthoras. First isolated from roots and collar of dying ornamental cedars (*Chamaecyparis* spp.) in North America, it has been recently discovered in Europe. In 2007 *P. lateralis* were recovered from soil beneath an old growth *Chamaecyparis obtuse* cloud forest in Taiwan in the absence of visible host symptoms: Taiwan probably lay within the natural range of this pathogen (Brasier *et al.*, 2010). During an expedition in Taiwan in 2010 a larger number of isolates were collected from necrotic foliage and soil samples (Webber *et al.,* 2012). The comparison of phenotypic and genotypic traits of Taiwan population with provenances from Pacific Northwest (PNW), France, United Kingdom, Netherlands and Northern Ireland evidenced for four phenotypically and genotypically distinct lineages in *P. lateralis* (Brasier *et al.,* 2012). Furthermore, sequencing of five polymorphic loci among representative isolates demonstrated that these populations were well-supported phylogenetic units. To strengthen the phylogenetic data this study aims to analyze microsatellite (SSR) profiles of representative isolates belonging to the lineages defined in previous studies. A total of 29 primers pairs, including newly developed in this work, have been tested. The clustering of *P. lateralis* isolates analyzed based on the only polymorphic primer pairs is described and discussed.

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# Genotypic variability of *Phytophthora cinnamomi* mating type A1 in native forests of Taiwan

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*Phytophthora cinnamomi* is an important plant pathogen with a wide host range and worldwide distribution. Its presence in the native forests of geographical areas distant from each other poses doubts about the real center of origin of this species, and there are studies that lead us to collocate the origin in some Asian regions, especially Papua New Guinea. In this area, in fact, *P. cinnamomi* is located in the natural environment without specific impact to native flora and showing high genetic variability in the population of the dominant mating type A1. The island of Taiwan has been indicated as an alternative center of origin of this species. *P. cinnamomi* is also widely present in the natural forests of the island mainly with the A1 mating type and without visible impact to native vegetation. No data are however available on the genotypic diversity of the resident populations. In this study, isolates of *P. cinnamomi* identified between 2008 and 2010 in two forest areas of the island of Taiwan (Chilan and TaipingSan) were compared with the Australian population A1 in terms of morphological, molecular, and of the optimum growth, in order to assess the genetic and phenotypic variability. The presence of microsatellite markers (SSR) was examined using 12 pairs of primers, 4 of which developed within this work. Among tested primers, 4 primer pairs were polymorphic and used to examine the level of genotypic diversity. Isolates from TaipinShan and Australia showed a similar genetic structure, confirming the possibility of the introduction of *P. cinnamomi* in Australia from Taiwan. The mitochondrial loci at a DNA sequence level have been also evaluated as marker for their ability to differentiate mitochondrial haplotypes of *P. cinnamomi*. Comparison of genetic and biometric data of the micro and macro structures is reported.

# Assessing the risk of chestnut ink disease spreading using TOPMODEL

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Ink disease is one of the most destructive diseases of sweet chestnut (*Castanea sativaI,* Mill.). This is currently under strong recrudescence and, for some areas in Italy, is responsible of environmental emergencies. The causal agents of the disease are *Phytophthora cambivora* and, the less present in Italy, *P. cinnamomi*, which spread through flowing water during mild winters. The gradual increase of temperatures and the different distribution and intensity of rainfall, due to climate changes, affect the distribution areas of pathogens furthering the more aggressive and polyphagous *P. cinnamomi*. The containment of the disease in the forest is mainly based on integrated pest management systems, under which the prevention and the rapid identification of areas at risk play a crucial role. For this scope, in this study we applied the TOPMODEL hydrogeological model for identifying the areas affected by chestnut Ink disease in an area in the municipality of Allumiere (Rome, Italy). During a 2 years period (2009-2010), *P. cinnamomi*, in association with other species such as *P. cactorum* and *P. plurivora*, has been consistently isolated in that area. In this study we report on the results of the spatial and temporal evolution of the disease in relation to hydrogeomorphological variables, such as the potential saturation of the soil, that is spatially affected by the topography, and the depth of the ground water respect to the soil surface, that is influenced by the precipitation time series.

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# Author Index

Abad, Z.G. 2, 32
Abad-Campos, P. 1, 29, 30, 41, 46, 112, 113, 114, 173
Abdelfattah, A. 34, 172
Aghighi, S. 8
Anand, N 47
Andrade, R.A. 18, 130
Andrew, M. 125
Angay, O. 129
Arenas, R. 30
Armstrong, A. 20
Assmann, J. 21
Bader, M. 175
Bakonyi, J. 1
Baldé, A. 73
Barron, R. 152
Beauchamp, A. 108
Bellgard, S.E. 47
Berbegal, M. 113
Bergeron, M-J. 33
Bernhardt, E. 43
Bienapfl, J. C. 32
Bilodeau, G.J. 33
Blomquist, C. 43
Boberg, J. 174
Børja, I. 179
Brandstetter, M. 124
Brasier, C. M. 17, 25, 33, 53, 109, 185, 186
Brennan, J. 50
Brevik, A.M. 136
Bruni, N. 185, 186, 187
Brurberg, M. B. 136, 179, 180, 181
Burgess, T. I. 8, 19, 31, 45, 79, 90, 125, 177, 178
Cacciola, S.O. 172
Cahn, M. 182
Calver, M. 8
Campbell, R. 27
Capretti, P. 165
Cara, J.S. 60
Català, S. 29, 30, 31, 112, 113, 114, 173
Cech, T.L. 10, 124
Černý, K. 35, 115, 118, 121, 183
Chambers, D. 182
Chang, T. 1, 46, 77
Chastagner, G 38, 91, 163
Cheng, X. 40, 140
Choiseul, J. 50
Clark, S. 27
Coats, K. 38
Cooke, D.E.L. 34, 172
Cooke, L.R. 54
Copes, W.E. 137
Corcobado Sánchez, T. 10, 41, 58, 62, 124
Couanon, W. 179
Cravador, A. 39, 68, 73, 93
Croeser, L. 19, 125
Croucher, P. 89
Cubera, E. 58, 62
Daxer, A. 10, 124
de Haro, A. 96
de la Mata Saez, L. 49, 126
De Vita, P. 60, 132
Deidda, A. 42
Dell, B. 45
Dermott, G. 38
Diaz-Celaya, M. 28
Doohan, F. 126
Dunstan, B. 79, 90
Durães, S. 93
Ebadzad, G. 68
Écija, M.R. 60
Elliot, Mattew 20, 128
Elliott, Marianne 38, 91
Fajardo, S. N. 22, 23
Feau, N. 33
Fedusiv, L. 118
Feldhahn, L. 129
Fernández-Pavía, S.P. 28
Figueredo, A. 22
Filipová, N. 115
Finlay, J. 152
Fleischmann, F. 63, 78, 129, 166

Fleming, C. 49, 126
Forlenza, V. 186
Franceschini, A. 42
Frankel, S. 43
Fu, C.-H. 46
Gabrielová, S. 118
Gagnon, M-C. 33
Garbelotto, M. 55, 89, 164
García Fernández, L.V. 60, 132
Ghelardini, L. 165
Ghimire, S.R. 137
Goheen, E.M. 80, 81
Gómez-Aparicio, L. 132
González, M. 14
González, M. G. 23
Graham, N. 92
Grams, T. 129
Green, S. 20, 21, 32, 128, 185
Greslebin, A. 64, 107, 184
Grijalba, P.E. 134
Grogan, H. 50
Grünwald, N.J. 33, 81, 109
Gutiérrez Rodríguez, E.A. 18, 130
Gutierrez, E. 132
Gutiérrez- Hernández, O. 132
Gyeltshen, J. 90
Hamelin, R.C. 33
Hanáček, P. 183
Haňáčková, Z. 35, 118
Hansen, E. M. 4, 11, 23, 40, 57, 80, 81, 109, 140, 150, 185
Hardy, G. 8, 19, 31, 79, 90, 125, 177, 178
Harris, A. 25, 53, 135
Havrdová, L. 118, 121
Hayden, C. 89
Hejná, M. 35, 118
Hendry, S.J. 20
Hermann, S. 129
Hernández, J. 58
Herrero, M. L. 136, 179, 180, 181
Holdenrieder, O. 93
Hong, C. 3, 137
Horner, I.J. 24, 139
Horta Jung, M. 1, 39, 46, 62, 77, 93
Hough, E.G. 24, 139
Hrabetova, M. 121
Hsueh, K-L 46, 77
Huai, W. 40, 140
Huettler, C. 124
Hulbert, J.M. 150
Husson, C. 1, 59
Jia, J. 126
Jung, T. 1, 10, 39, 42, 46, 77
Kalantarzadeh, M. 17
Kanaskie, A. 80, 81
Kawchuk, L. 33
Kitchingman, L. 180
Koike, S. 182
Kong, P. 137
Kovács, G.M. 1
Krajnc, B. 62
Laine, J. 11, 81
Lamour, K. 28
Larkin, M.J. 54
Larregla, S. 29
Lea-Cox, J.D. 137
Lebel, T. 47
Léon, M. 1, 46
Lévesque, C.A. 33
Linaldeddu, B.T. 42
Luchi, N. 165
MacDonald, L. J. 92
Maddau, L. 42
Maia, C. 39, 77, 93
Majek, T. 124
Marçais, B. 59
Martins, A.B.G. 18
Martins, J. 68
Martos, A. 62
Mazzetto, T. 187
McCracken, A.R. 9, 49, 54, 126, 152
McDougal, R. L. 92
McKeever, K.M. 163
Meentemeyer, R.K. 164

Migliorini, D. 165
Modlinger, R. 121
Moliner, R. 41
Mombour, J. 63
Moorman, G.W. 137
Mora-Dañino, A.L. 28
Morales-Rodríguez, C. 166, 167
Mora-Sala, B. 41
Morrell, J. J. 150
Morwood, S. 152
Mrázková, M. 35, 118, 121
Nagy, Z. 59
Nakhla, M.K. 32
Natesan, E. 43
Natili, G. 56
Neves, D. 73, 93
Novotná, K. 118
Nuñez, C.I. 169
Nuñez, L. 62
O'Hanlon, R. 50
Ohya, M.C. 130
Olate, V. 64
Oliva, J. 174
Olsson, C. 174
Oßwald, W. 63, 166
Owens, K.J. 32
Paap, T. 19, 79, 125, 178
Padamsee, M. 47
Pais, M.S. 73
Palmucci, H.E 134, 170
Pánek, M. 171
Panizzi Penariol, M. 130
Panizzi, R.C. 18, 130
Pérez, Anahí 169
Pérez, Andrea 62
Pérez, E. 62
Pérez-Ramos, I. 132
Pérez-Sierra, A. 1, 14, 17, 29, 30, 46, 96, 112, 113, 114, 173
Pešková, V. 118, 121
Petroselli, A. 187
Pfanz, H. 63
Pham, T.Q. 45
Prigigallo, M.L. 34, 172
Probst, C. 47
Puértolas, A. 29, 112, 173
Quinn, L.M. 9, 54, 152
Quynh, D.N. 45
Rajchenberg, M. 100
Ramo, C. 60, 132
Randall, E 34
Raponi, C. 169
Recht, S. 129
Redondo, M.A. 174
Reeser, P. 11, 57, 80, 81, 109
Rhatigan, R. 81
Richardson, P.A. 137
Ríos, P. 96, 132
Robin, C. 185
Rodríguez Padrón, C. 114
Rodríguez-Alvarado, G. 28
Rolando, C. 175
Rollins, L. 38
Romportl, D. 118, 121
Rooney Latham, S. 43
Sabag, M. A. 23
Sánchez, M.E. 14, 60, 96, 132
Sancisi-Frey, S. 17, 26
Sanfuentes, E. 22, 23
Santini, A. 165
Scanu, B. 1, 42, 135
Schechter, S. 89
Schena, L. 34, 172
Schlenzig, A. 27
Schmeda- Hirschmann, G. 64
Schmidt, D. 89
Scott, J. K. 8
Scott, P. 92
Searle, B. 152
Serrano, M.S. 14, 60, 96, 132
Sharp, P. 21
Simamora, A. 177, 178
Sims, L. 109
Siverio de la Rosa, F. 114
Solla, A. 41, 58, 62

Squires, J.N. 34
Stensvand, A. 179, 181
Štěpánková, P. 183
Štochlová, P. 118
Stokes, V. 90
Strnadová, V. 115, 118, 121, 183
Strømeng, G.M. 179, 180
Studholme, D.J. 54
Stukely, M. 177, 178
Sundheim, L. 136
Sutton, W. 11, 57, 80, 81, 109
Swiecki, T. 43
Talgø, V. 179, 180, 181
Tarkka, M. 129
Telfer, E. 92, 180, 181
Tian, G. 40, 140
Tjosvold, S. 182
Tojo, M. 136
Tomassini, A. 185, 186, 187
Tomšovský, M. 171, 183
Tondini, E. 165
Troncoso, O. 184
Tyler, B.M. 3
Valenzuela, S. 22
Vannini, A. 56, 167, 185, 186, 187
Vélez, M. L. 64, 107, 184
Vettraino, A.M. 56, 62, 90, 167, 185, 186, 187
Vogler, J.B. 164
Wagner, A. 92
Waipara, N. 108
Webber, J.F. 9, 25, 26, 33, 53, 135, 185
White, D. 31
Wiese, R. 81
Wijekoon, C.P. 33
Williams, N.M. 92, 175
Williams, S.E. 47
Wilson, M.A. 9, 152
Wittman, C. 63
Wolcan, S.M. 170
Wylder, B. 25
Yang, X. 3, 137
Yao, Y. 40, 140
Zhang, H.B. 137
Zhao, W-X 40, 140