Detection of Possible *Phytophthora pinifolia* Infection in *Pinus radiata* Green Sawn Timber Produced in Chile[1]

R. Ahumada,[2] C. Díaz,[2] M. Peredo,[2] C. Barría,[2] P. González,[3] and G. Cuevas[3]

Abstract

A new needle blight disease was observed on *Pinus radiata* in Chile during 2004. The disease, known in Chile as Daño Foliar del Pino (DFP), stretches southward from the Arauco to Valdivia Provinces, and was present over an area of about 60 000 ha in 2006, with different levels of intensity. The disease is typified by needle infections and exudation of resin at the bases of the needle brachyblasts. Only *P. radiata* trees have been affected by DFP. Other *Pinus* species in the area, such as *P. pinaster*, remain healthy. Isolations from infected needles on selective media have consistently yielded a *Phytophthora* sp. DNA sequence comparisons for the ITS rDNA and *cox* II gene regions, and morphological observation, showed that this oomycete represents a previously undescribed species, which has been named *Phytophthora pinifolia* (Durán, Gryzenh, and M.J. Wingf). Research is underway to fully elucidate the life cycle of *P. pinifolia* and to develop appropriate management strategies on Chilean pine plantations.

Despite being an aggressive pathogen and an aerial *Phytophthora*, *P. pinifolia* is phylogenetically closely related to other *Phytophthora* spp. that are mildly pathogenic and normally associated with soil and roots. Pathogenicity trials with *P. pinifolia* have clearly shown that it is pathogenic to *P. radiata* and causes rapid death of the succulent apical parts of young plants. *P. pinifolia* is the first *Phytophthora* sp. known to infect needles of a *Pinus* sp. and its aerial habit is well-matched with the occurrence and symptoms of DFP in Chile.

To understand the behavior of *P. pinifolia* in green timber, a study was conducted to determine the possible presence of *P. pinifolia* in green sawn timber produced from trees that had been exposed to infection by the pathogen for at least 4 years. Green timber from the infected trees, and green wood samples exposed to *P. pinifolia* inoculum, were analyzed by making extensive isolations on *Phytophthora*-selective media. In addition, fluorescence microscopy was used to observe the possible presence of structures of the organism and PCR was conducted using species-specific primers developed for *P. pinifolia*.

Results of the study showed that the green sawn timber taken from trees infected by *P. pinifolia*, or even green timber exposed to contaminated pine plantations, showed no evidence that the pathogen can survive or develop in green wood. These results provide strong evidence that green sawn timber produced from *P. radiata* trees infected with *P. pinifolia* is free of the pathogen and that it can be exported safely without any specific treatment against *P. pinifolia*.

[1] A version of this paper was presented at the Fourth Sudden Oak Death Science Symposium, June 15-18, 2009, Santa Cruz, California.

[2] Bioforest S.A., Concepción, Chile.
[3] Servicio Agrícola y Ganadero, Chile.
Corresponding author: rahumada@arauco.cl.

Introduction

Daño Foliar del Pino (DFP) was observed for the first time during the year 2004 affecting *Pinus radiata* plantations located on the coast of the Bio-Bio Region in Chile. The disease, caused by *Phytophthora pinifolia*, corresponds to a new species of pathogen recently isolated from needles from *P. radiata* plants and trees (Durán and others 2008, R. Ahumada, Bioforest S.A., personal communication).

The disease is characterized by the appearance (in late autumn and early winter) of translucent bands seen at first glance as black bands on pine needles, and later turning into a generalized discoloration of foliage, resulting in a greyish aspect of the crown, which turns reddish brown at the end of spring as a consequence of the necrosis of the affected foliage. In addition to the damage of the foliage, damage occurs in the succulent tissue of branches and stems of young trees. In 1-year-old plantations, the attack normally causes the death of plants, whereas in 2- to 6-year-old plantations, needle damage is observed without the occurrence of mortality. In adult plantations (above 6 years), DFP is mostly seen in the foliage. No clear evidence of an effect on stems or branches of attacked trees has been detected to date.

Currently, the symptoms may be observed in plantations of all ages which grow in coastal zones of the Province of Arauco, Region of Bio-Bio and the Regions of La Araucania and Los Rios, as well as in some young plantations from the Region of Maule.

Objectives

This study was conducted over the last 4 years in order to determine if *P. pinifolia* is present in green sawn lumber from *P. radiata* trees with high levels of damage by DFP, and to evaluate if *P. pinifolia* may contaminate and develop on the sawn wood when exposed to pathogen inoculum, and thus be a source of disease spread.

Materials and Methods

The study was developed in two stages. The first consisted of evaluating and monitoring for traceability specimens of green wood obtained from trees in plantations severely affected by DFP for the last 4 years. The second stage consisted of the artificial inoculation of specimens of green wood with *P. pinifolia* under laboratory and field conditions. Additionally, a laboratory analysis was made of samples of green sawn lumber for export coming from plantations located in the area of DFP occurrence. The samples were taken in ports from the Bio-Bio Region in order to determine the occurrence of *P. pinifolia*.

The establishment of the occurrence of *P. pinifolia* in samples of green sawn pine lumber, foliage of trees, and bark and wood disks was carried out through cultures in selective medium for species of the genus *Phytophthora*, identification of morphological characteristics (described by Durán and others 2008), and by direct molecular analysis of wood and foliage using *P. pinifolia*-specific primers (developed by Durán and others, in press). The presence of structures of the pathogen was also verified through observations with a fluorescence microscope.

Evaluation of the Occurrence of *P. pinifolia* in Green Sawn Lumber Obtained From Trees Affected by DFP

Selection of Samples

The selection of farms was made based on the historical damage and age of stands. A total of three stands were chosen at Llico, Trana-Trauco, and Quebrada de Rumena Farms. These plantations had damage at least over the last 4 years. In each of these plantations, a total of 15 trees were selected and sampled on the basis of the presence of typical symptoms and signs (black bands on needles or resin tapping on the stem). Prior to harvest, a characterization was made of the DFP degree in foliage, diameter at breast height (DBH), total height, and live crown height, corresponding to the 2008 period. In addition, samples from foliage, and from bark and wood disks were taken to validate the occurrence of *P. pinifolia* in the laboratory.

The number of trees selected was determined according to the standard NCh 1208 (50 percent associated with the average diameter, 25 percent of the upper diameter class, and 25 percent of the lower diameter class). The trees were felled and cut into operational saw logs (3.6 m in length to a minimum diameter of 14 cm) and pulp timber (variable length, diameter 14 to 8 cm). The saw logs were gathered in the forest for 10 days, simulating a harvest operational process of *P. radiata* plantations in Chile.

Sawing Process

Saw logs were transported to a sawmill (Horcones II) yard located in the county of Arauco (Annexe 1), where they were processed. The sawing schemes used were defined depending on the diameter class of the logs, maximizing the obtaining of sideboards in millimetre thickness (side 25 mm and central 38 mm, per variable width).

A total of 40 samples for laboratory analysis were obtained, according to the provisions in the Chilean standard NCh 1208 EOf.76 (rigorous inspection mode). The selection of samples was aimed at logs with visible resin tapping, identified from the forest, with location on the stem (log one, two, three or four) and type of wood (30 percent of samples associated with central wood and 70 percent associated with lateral wood). The samples were characterized to maintain traceability during the whole study process (Annexe 4). The size of the samples obtained were 15 cm long, 25 and 38 mm thick, and variable in width depending on the type of wood obtained after the first 10 cm from one of the ends of the boards.

Once the samples were obtained and sent to the laboratory, the remaining lumber continued with the normal procedure of sawn wood production, being subject to a bath with anti-stain solution made up of a fungicide mixture. After the anti-sapstain treatment, the lumber was stored in the sawmill yard (Aserradero Horcones II).

Laboratory Analysis of Sawn Wood Specimens

The specimens obtained from the sawing process were analyzed through culture and PCR as described below:

Culture: From each specimen obtained, 10 wood pieces were selected, which were cultured in plates with selective medium (CARP). The plates were maintained

between 18 and 22 °C for 30 days until their evaluation. The culture validation was made through molecular biology using *P. pinifolia*-specific primers.
PCR: A chip sample was taken from each specimen. Each sample was evaluated through molecular biology directly from wood, directed to zones with evidence of any kind of stain. The DNA extraction was carried out using CTAB buffer, whereas PCR was made through *P. pinifolia*-specific primers.

Evaluation of the Capability of *P. pinifolia* to Contaminate and Develop on Green Sawn Lumber of *P. radiata*

Specimen Inoculation in the Laboratory

The inoculation of *P. pinifolia* was performed through the application of 250 ul of suspension of 50,000 zoospores per ml on the specimen surface (ASTM D 4445-03, 2003, standard) and with an 8 mm diameter mycelium plug placed in perforations of the same diameter on the specimens. The treatments corresponded to specimens with and without anti-sapstain treatment, whereas the control included specimens inoculated with water and agar plugs without mycelium, respectively (table 1). Inoculum viability was validated using rhododendron bait and later culture of the bait. All treatments were maintained at ± 22 °C in a humidity chamber for 30 days until their evaluation.

Table 1—Treatments of laboratory tests

Treatment Code	Inoculum, Mycelial Plug	Inoculum, Zoospore Suspension	Anti Sapstain Treatment	No. Specimens
T0	No		Yes	10
T1	No		No	10
T2	Yes		Yes	10
T3	Yes		No	10
T4		No	Yes	10
T5		No	No	10
T6		Yes	Yes	10
T7		Yes	No	10

Every week an evaluation was performed through a visual review during the period in which the specimens were kept in humidity chamber in order to observe the occurrence or development of *P. pinifolia*. At day 30, the specimens were evaluated by culturing, PCR directly from wood, and fluorescence microscopy, as described below:
Culture: From each specimen (80 in total), five pieces were selected and cultured in a plate of selective medium (CARP), for a total of 350 cultures. The plates were incubated between 18 and 22 °C for 30 days until their evaluation.
PCR: A chip sample was taken from each specimen. Each sample was evaluated through molecular biology directly from wood. The DNA extraction was carried out using CTAB buffer (Annexe 4), whereas PCR was made through *P. pinifolia*-specific primers.
Fluorescence Microscopy: A section of the inoculated surface was selected from each sample. The samples were immersed in calcofluor for 10 seconds and observed under a fluorescence microscope (Olympus CX31).

On-Site Specimen Inoculation

A total of 128 specimens of *P. radiata* green wood were placed in a stand on the Llico Farm (plantation 2002) with a high incidence of DFP. Under the canopy (foliage) of this plantation, 96 wood specimens were placed on a tray with trap plants to monitor the occurrence of DFP. Additionally, 12 specimens with and without anti-stain treatment were placed in contact with soil, as well as 20 specimens artificially-inoculated with *P. pinifolia*, tied to *P. radiata* trees, which were also inoculated (table 2).

Table 2—Treatments placed in field

Treatment	Specimen Location	Anti-Sapstain Application	No. Specimens
T0	Tray with trap plants	Yes	48
T1	Tray with trap plants	No	48
T2	Soil in contact with trays	Yes	6
T3	Soil in contact with trays	No	6
T4	Branches inoculated with *P. pinifolia*	Yes	5
T5	Branches inoculated with *P. pinifolia*	No	5
T6	Branches non-inoculated	Yes	5
T7	Branches non-inoculated	No	5

The 128 specimens were maintained in the field for 30 days and later taken to the laboratory for their evaluation. The evaluation of the specimens was conducted with the same methodology described above.

Lumber Sampling at Ports

In order to support the management of Agriculture and Livestock Service (SAG) in monitoring green sawn lumber for export, sampling of specimens was performed in Lirquen and Coronel ports to evaluate the occurrence of *P. pinifolia* during August, October, November, and December 2008.

Twenty lumber packages (17 of green sawn lumber and three of dry sawn lumber) were sampled, obtaining specimens especially from boards with bark. The samples were analyzed in laboratory through operational protocol for molecular biology using *P. pinifolia*-specific primers.

Results and Discussion

Occurrence of *P. pinifolia* in Green Sawn Lumber Obtained from Trees Affected by DFP

The evaluation of specimens of green sawn lumber, conducted through molecular biology directly from wood and isolates in selective medium, did not show the occurrence of *P. pinifolia* (table 3).

Table 3—Evaluation of specimens of green sawn wood

Type of Sample	No. Samples	Analysis		*P. pinifolia* Identification	
		PCR	Culture	PCR	Culture
DFP Farm Specimens	40	80	400	0	0
Control Specimens	20	40	200	0	0

Capability of *P. pinifolia* to Contaminate, Colonize, and Survive on Green Sawn Lumber

Specimen Inoculation in the Laboratory

The inoculated specimens with and without anti-sapstain treatment did not show growth of *P. pinifolia* after incubation for 4 weeks (table 4). In specimens treated with anti-stain bath, no growth from any kind of fungi was observed, whereas in those without bath, structures of staining fungi typically seen in *P. radiata* sawn wood developed. This validates that the incubation conditions of the specimens were proper for the growth of fungi.

Table 4—Evaluation of specimens inoculated in laboratory

Type of Sample	No. Samples	No. Analyzed		*P. pinifolia* Identification	
		PCR	Culture	PCR	Culture
Specimens inoculated with zoospores	20	40	100	0	0
Specimens inoculated with mycelium	20	40	100	0	0
Control specimens	40	80	200	0	0

The presence of sporangia or other reproduction structures that may validate the occurrence of *P. pinifolia* was not detected in the evaluations made through fluorescence microscopy. The molecular analysis through PCR was negative for the presence of *P. pinifolia*.

Specimen Inoculation in the Field

The evaluation of the 128 specimens did not show the occurrence of *P. pinifolia* in culture, PCR directly from wood, or humidity chamber (table 5). In specimens without anti-sapstain treatment, some evidence of staining fungus development were found, which suggests that the conditions under which the specimens were maintained were suitable for the growth of fungi and that the anti-stain bath is effective against that type of agent.

Table 5—Evaluation of specimens in field

Type of Sample	Analysis			*P. pinifolia* Identification	
	No. Samples	PCR	Culture	PCR	Culture
Specimens under canopy	96	192	480	0	0
Specimens on soil	12	24	60	0	0
Specimens of inoculated branches	20	40	100	0	0

Lumber Sampling at Ports

The occurrence of *P. pinifolia* was not detected in the evaluations performed on samples obtained at the ports of Lirquén and Coronel from August to December.

Background information to determine contamination of wood by *Phytophthora* species genetically related to *P. pinifolia* was not available; however, other species such as *P. ramorum, P. nemorosa, P.pseudosyringae, P. kernoviae* are able to cause damage on the xylem and produce cankers (Wickland and others 2008, Brown and Brasier 2007).

Conclusions

The results obtained in this study allow us to conclude that:
1. *P. pinifolia* is not present in samples of green sawn lumber obtained from plantations with high incidence of symptoms and damages caused by DFP over the last 4 years, even though the sample selection was directed to conditions more favorable for the presence of the pathogen.
2. When exposed under the canopy of a plantation highly affected by DFP, *P. pinifolia* is not able to colonize and survive on green sawn wood artificially or naturally inoculated with zoospores or mycelium.
3. Green sawn lumber produced from plantations of *P. radiata* infested with *P. pinifolia* are free of the pathogen and may be exported without needing to use treatments against *P. pinifolia*.

Selected References

American Society for Testing Materials (ASTM). 2003. Standard test method for fungicides for controlling sapstain and mold on unseasoned lumber (laboratory method). D 4445-03, Annual Book of Standards, Section 4, Vol. 04.10., 2004 . pp: 531–534.

Australian Quarantine Inspection Service (AQIS). 2008. New import requirements for imports of sawn timber and logs from Chile. http://www.daff.gov.au/data/assers/pdf.

Brasier, C.M.; Hamm, P.B.; and Hansen, E.M. 1993. Cultural characters, protein patterns and unusual mating behavior of *Phytophthora gonapodyides* isolates from Britain and North America. Mycological Research. 97(11): 1287–1298.

Brown, A.V. and Brasier, C.M. 2007. Colonization of tree xylem by *Phytophthora ramorum*, *P. kernoviae* and other *Phytophthora* species. Plant Pathology. 56(2): 227–241.

Durán, A.; Gryzenhout, M.; Slippers, B.; Ahumada, R.; Rotella, A.; Flores, F.; Wingfield, B.D.; and Wingfield, M.J. 2008. *Phytophthora pinifolia sp. nov.* associated with a serious needle disease of *Pinus radiata* in Chile. Plant Pathology. 57(4): 715–727.

Durán, A.; Slippers, B.; Gryzenhout, M.; Ahumada, R.; Drenth, A.; Wingfield, B.D.; and Wingfield, M.J. [In press.] DNA-based method for rapid identification the pine pathogen, *Phytophthora pinifolia*. FEMS Microbiology Letters.

Erwin, D.C. and Ribeiro, O.K. 1996. *Phytophthora* diseases worldwide. The American Phytopathological Society, St. Paul, MN: The American Phytopathological Society Press. 562 p.

Instituto Nacional de Normalización, INN – Chile. 2000. Control de Calidad –Inspección por Variables – Tablas y procedimientos de muestreo, NCh 1208.EOf76. 166 p.

National Plant Quarantine Service (NPQS, Korea). 2008. Import restricted plants: seeds, seedlings, logs, and sawn wood of *Pinus radiata.* [Comunicación Oficial].

Rizzo, D.M.; Garbelotto, M.; Davidson, J.M.; Slaughter, G.W.; and Koike, S.T. 2002. *Phytophthora ramorum* as the cause of extensive mortality of *Quercus* spp. and *Lithocarpus densiflorus* in California. Plant Disease. 86(3): 205–214.

Stange, C.; Prehn, D.; and Arcer-Johnson. P. 1998. Isolation of *Pinus radiata* genomic DNA suitable for RAPD analysis. Plant Molecular Biology Report 16: 1–8.

Wickland, A.C.; Jensen, C.E.; and Rizzo, D.M. 2008. Geographic distribution, disease symptoms and pathogenicity of *P. nemorosa* and *P. pseudosyringae* in California, USA. Forest Pathology. 38(4): 288–298.