

Potential of *Phytophthora pinifolia* to spread via sawn green lumber: A preliminary investigation

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Abstract

Phytophthora pinifolia causes the needle and shoot disease of *Pinus radiata* in Chile known as Daño Foliar del Pino (DFP). Although *P. pinifolia* is primarily a needle pathogen, there are concerns that it might be spread to new environments via the export of contaminated timber. In order to determine whether *P. pinifolia* can enter or persist in green sawn lumber, its presence in lumber produced from trees exposed to the pathogen for at least four years was examined. Green lumber produced from the infected trees, and green wood samples artificially exposed to *P. pinifolia* inoculum, were analyzed by making extensive isolations on *Phytophthora* selective media. In addition, PCR was conducted using species-specific primers developed for *P. pinifolia*. Results of the study showed that the green sawn lumber taken from trees infected by *P. pinifolia*, or green lumber exposed in infected pine plantations, displayed no evidence of the pathogen surviving in this material.

Keywords: *Phytophthora pinifolia*, green sawn lumber, green wood blocks, wood discs, artificial and natural inoculation.

Introduction

Pinus radiata D. Don is one of the most important species utilized in plantation forestry world-wide. The species has been established extensively in the southern hemisphere with the largest plantation areas found in Australia, Chile, New Zealand and South Africa (Rogers 2004), one third of which are in Chile (Guerrero and Bustamante 2007).

Disease and insect pest problems have presented challenges for the Chilean *P. radiata* in the past (Lanfranco 2000, Ahumada 2003, Barnes et al. 2004, Jacobs et al. 2007), although these have not substantially affected productivity. However, the new foliar disease known as Daño Foliar del Pino (DFP), recently emerged on *P. radiata* in Chile and caused by *Phytophthora pinifolia* (Durán et al. 2008), has caused concern to the local industry. The disease is known only in Chile and was observed for the first time during 2004, affecting *P. radiata* plantations located on the coast of the Biobío region (Durán et al. 2008). Currently, the symptoms can be observed in plantations of all ages, growing in coastal zones of the Arauco gulf in the Biobío region. It can also be found in the Los Ríos regions (Valdivia province: 39°46'58"S, 72°31'13"W) and in some young plantations in the Maule region (Constitución locality: 35°25'36"S, 71°39'78"W).

The most characteristic symptom of DFP is the resinous bands, which can appear black in colour, on the infected pine needles. Symptoms develop to include a general discoloration of the needles and a grayish appearance of the

tree crowns, which turn brown at the end of spring due to necrosis of the affected foliage. In addition to the foliar damage, branches and stems of young trees (less than six-years-old) can develop lesions due to the movement of the infections from the needles to the cambium in the succulent green tissue. In one and two-year-old plantations, heavy infection frequently causes the death of trees, whereas in three to six-year-old plantations, needle damage is observed without the occurrence of mortality. In adult plantations (older than six years), DFP is seen in the foliage and in the succulent tissue of young branches (Durán et al. 2008). There is no evidence to suggest that the pathogen penetrates the wood of branches or stems in these older trees.

Species of *Phytophthora* have the capacity to survive in the plant tissue of the hosts that they infect. This can be in the foliage, stems or roots (Oh and Hansen 2007, Fry 2008, Moralejo et al. 2009). Most of the *Phytophthora* species that cause disease in conifers are root rot pathogens such as *P. lateralis* and *P. cinnamomi* on Port-Orford-cedar (*Chamaecypariss lawsoniana*), *P. cinnamomi* and *P. cambivora* on Noble fir (*Abies procera*), *P. megasperma* on Douglas fir (*Pseudotsuga menziesii*) and *P. austrocedrae* on *Austrocedrus chilensis* (Hamm and Hansen 1987; Hansen et al. 1989;; Greslebin and Hansen 2010). *Phytophthora ramorum* has been found associated with needle necrosis or tip wilting on redwood (*Sequoia sempervirens*), Douglas fir and Grand fir (*Abies grandis*) (Garbelotto et al. 2003; Riley et al. 2011).

Some species of *Phytophthora*, like *P. austrocedrae*, *P. cambivora*, *P. cinnamomi* and *P. ramorum* among others can be found in the phloem, normally

associated with stem cankers (Hamm and Hansen 1987, Rizzo et al. 2002, Davidson et al. 2003, Garbelotto et al. 2003, Brasier et al. 2005, Brown and Brasier 2007, Parke et al. 2007, Moralejo et al. 2009, Greslebin and Hansen 2010). There are also a few *Phytophthora* species that have been detected in the xylem tissue. For example, Davison et al. (1994) showed that *P. cinnamomi* can be isolated from *P. radiata* wood, up to three months after an artificial inoculation. Brown and Brasier (2007) showed that *P. citricola*, *P. ramorum* and *P. kernoviae* among other *Phytophthora* species, were present in the xylem several centimetres ahead of the phloem lesions on several broadleaved tree species. Such isolations of *Phytophthora* spp. from woody tissue are usually from discolored wood or areas surrounding this discoloration. Because discoloration of the *P. radiata* wood has not been found in trees infected by *P. pinifolia*, it is thought that this organism does not infect the xylem (R. Ahumada, unpublished).

An aim of this study was to evaluate whether *P. pinifolia* is present in green sawn lumber (lumber that has not been dried or treated) from *P. radiata* trees affected by DFP. In addition we evaluated whether *P. pinifolia* can contaminate and develop on freshly sawn lumber when it is directly exposed to inoculum of the pathogen.

Materials and Methods

Inoculum production

To produce *P. pinifolia* inoculum, three isolates (CMW33983, CMW33986 and CMW34012) recently collected from resinous bands (black bands) on *P. radiata*

needles, were grown in V8 agar at 22 °C for two weeks. Five discs (7 mm diameter) were cut from the edges of actively growing cultures of each of the isolates, transferred to 60 mm Petri dishes containing 25 ml of 10% V8 broth and incubated for 24 h at 22 °C (Erwin and Ribeiro 1996).

The agar discs were washed twice with autoclaved cold distilled water, and then immersed in filtered pond water for 48 h at 22 °C under continuous cool white fluorescent light (6 600 lux). Isolates were checked for the presence of sporangia and chilled at 4 °C for 2 h to induce the release of zoospores.

The zoospore solution was poured from plates of all three isolates, mixed in a sterile beaker and maintained at 4 °C between 1 to 2 hours prior to inoculation (Parke et al. 2002, Denman et al. 2005, Hansen et al. 2005). Three aliquots of 10 µL were taken and the zoospore concentration was measured using a hemacytometer. The final zoospore suspension was prepared by adding autoclaved distilled water to adjust the concentration to approximately 5×10^4 zoospores ml⁻¹. This was then transported to the field for inoculation on the same day. In order to determine the inoculum viability, three aliquots of 30 µL of the zoospore suspension were transferred to CARP medium (0.01 g benomyl, 0.01 g pimaricin, 0.2 g ampicillin, 0.01 g rifampicin and 17 g corn meal agar per liter of water) subsequent to completing the inoculations and then incubated at 18–22 °C for 10 days (Erwin and Ribeiro 1996). The number of colony-forming units was counted and the zoospore viability recorded.

Survival and development of P. pinifolia on green sawn lumber

Two independent laboratory inoculation trials were carried out using green wood block samples (standardized pieces of 7×20×70 mm) provided by a sawmill located near Arauco town. All the blocks were taken using the standard methods for producing green lumber for exportation and that are derived from both heartwood and sapwood of the logs. For each of the inoculation trials, 40 green wood blocks were used and 20 of these were treated in the sawmill with a commercial solution of anti-sapstain (Chlorothalonil 0.5%, Carbendazim 0.2% and Copper 8-quinolinol 3.7%), which is normally applied to green wood before exportation. The remaining twenty wood blocks were not treated with anti-sapstain chemicals. An additional twenty wood blocks were used as controls. Ten wood blocks with anti-sapstain solution and ten untreated blocks were inoculated and the same numbers were used as controls.

A first inoculation trial was with a zoospore suspension of *P. pinifolia* [250 µl (5 × 10⁴ zoospores ml⁻¹) produced as described above] applied with micropipette to the upper surface of the wood, using a similar methodology as that described by the American Society for Testing Materials (2003). This method was designed to test fungicides against conidiogenous wood-infecting fungi, but was applied in the present study to test whether green lumber could be a substrate for *P. pinifolia* growth. In the 20 wood blocks used as controls, distilled water was applied in the same manner as for the inoculations with *P. pinifolia*.

A second inoculation trial was carried out using an 8 mm diameter mycelial plug of *P. pinifolia* rather than zoospores. In this case, the mycelial disc was placed

in a cavity of the same size in the wood block (Figure 1a). The control treatment was exactly the same, but utilised plugs of sterile agar.

All wood blocks used in both inoculation trials were maintained at ± 22 °C in a moist chamber. To ensure the viability of the inoculum, ten Petri dishes with CARP medium were incubated with the same *P. pinifolia* sources of inoculum (zoospore suspension and mycelium plugs) used in the studies described above.

Evaluation of results included in a visual examination of the all wood blocks every week for evidence of *P. pinifolia* mycelial growth. After 30 days, the wood blocks were evaluated for the presence of *P. pinifolia* by taking a total of 200 samples per trial, including five wood pieces (2-3 mm²) per wood block, from the surface of samples, focusing specifically on visual signs of possible infection. The wood pieces were plated onto CARP medium in Petri dishes. Resultant colonies were transferred to Carrot Agar (CA) and maintained between 18–22 °C for 20 days after which they were identified based on mycelial characteristics. Identifications were confirmed using species-specific PCR with the DNA extracted from the mycelia (Durán et al. 2009).

In order to evaluate the ability of *P. pinifolia* to infect freshly (green) sawn wood in the field under natural conditions, 96 green wood blocks (7×20×70 mm) were placed under the canopy of trees in a seven-year-old plantation in the Llico farm (37°22'45"S; 73°58'W) in the Arauco province. This was a plantation where a high incidence of DFP has been observed since 2004. The wood blocks were

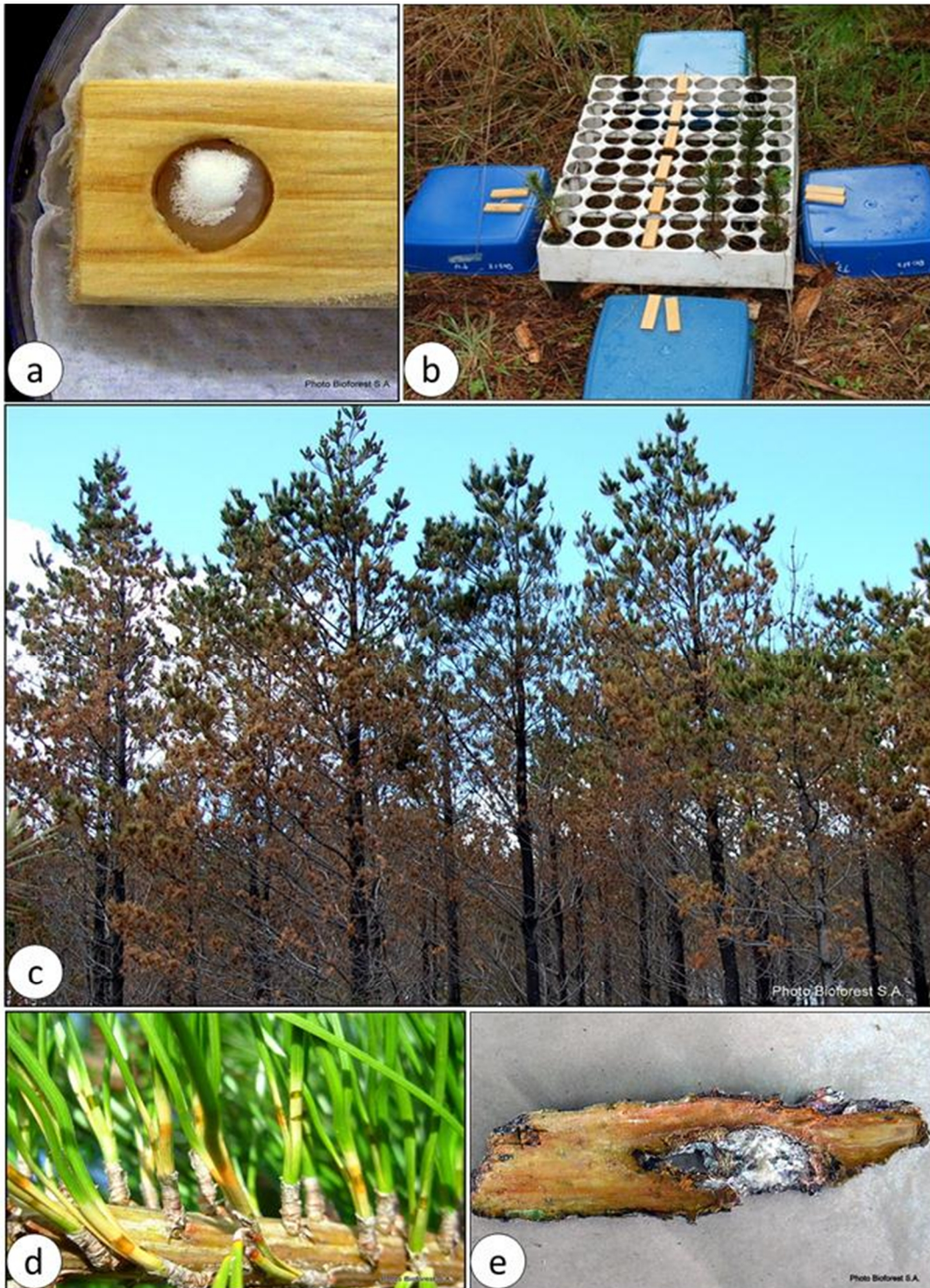


Figure 1: Symptoms of *P. pinifolia* infection and material used in isolations. (a) Punched sample in the wood with a disc of colonized agar. (b) Wood blocks on plastic trays exposed to natural inoculum. (c). Discoloration of the foliage of an infected *P. radiata* plantation selected for sampling timber. (d) Branch with infected needles showing black bands typical of infection (e) Inner part of a bark sample showing a typical lesion in the cambium.

placed on the surface of plastic trays, 10 cm above the ground, to prevent flooding due to rainfall (Figure 1b). Forty eight of the wood blocks were treated before the inoculation with anti-sapstain chemicals (described above) and 48 were left untreated.

All the wood block samples were maintained in the field for 30 days and then taken to the laboratory for evaluation, using the same methodology described above. From the 96 samples taken from the wood blocks placed under the canopy of infected trees, 5 pieces of 2-3 mm² of wood per sample (480 isolations) were plated onto CARP medium in Petri dishes for culturing and 2 pieces of wood, of similar size as described above, were taken per sample (192 subsamples) and subjected to baiting with rhododendron leaves (Sutton et al, 2009).

The three cultures used for the inoculation trials in this study, are maintained in the culture collection of Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria (CMW).

Evaluation of lumber from trees in naturally infected plantations

Three stands of *P. radiata* (14, 15 and 18-years-old) were chosen for evaluation, because they are located in the area having the highest incidence of DFP since 2004. In each of these plantations, a total of 15 trees were selected based on the presence of characteristic DFP symptoms (Figure 1c).

Selected trees were felled and cut into operational saw logs (3.6 m in length to a minimum diameter of 14 cm) and pulp lumber (variable length, diameter 14 to 8 cm; Table 1). Samples including 165 symptomatic foliage samples (needles with resinous bands; Figure 1d), 75 pieces of bark approximately 10 cm², each including resinous cankers or evidence of a potential infection (Figure 1e) and 36 wood discs (a horizontal cross section cut through the of a tree) approximately 15 cm long, taken along the trunk especially where there was evidence of damage, cankers or any suspected infections. All samples (n=276) were evaluated in the laboratory for the presence of *P. pinifolia*. Asymptomatic tissue was not considered in the analyses as this was considered less likely to contain *P. pinifolia* than samples showing signs of possible infection.

Table 1: Characteristics of trees selected for evaluation and log type used in the laboratory analyses.

Farm	Year of planting	No of trees sampled	LOG section sampled No (%)		
			Total	For Timber	For Pulp
Llico	1994	15	93	24 (26%)	69 (74%)
Trana	1993	15	146	69 (47%)	77 (53%)
Quebrada & Rumena	1990	15	73	30 (41%)	43 (59%)

The needles were analyzed using isolations on CARP medium and the identity of developing cultures as *P. pinifolia* was confirmed by species-specific PCR (Durán et al. 2009). From each bark sample, five pieces (n=375; 2-3 mm²) were selected randomly from the lesions. The wood discs were analyzed by taking five wood pieces (2-3 mm²) per disc specifically from areas with signs of possible infection. The evaluation was made using three different methods. Ten percent of these pieces were used in baiting with rhododendron leaves, as was described above, and after 10 days small pieces of rhododendron leaves were

transferred to CARP medium. An additional 10% of the samples were immersed in a solution of Calcofluor Fluorescent Brightener 28 (Sigma-Aldrich, St. Louis) at 0.001% in 0.05 M Tris-HCl (pH 8.0) for 30 sec., to evaluate the presence of sporangia or mycelium on the wood surface. The remaining 80% of the samples were subjected to direct isolations on CARP following the standard methods described before. The possible presence of *P. pinifolia* amongst the cultures obtained using baiting with rhododendron leaves or direct isolation was confirmed using species-specific PCR (Durán et al. 2009).

Each of the saw logs was identified with a unique number and left in the forest for approximately 10 days, simulating a normal harvesting operation for *P. radiata* in Chile. Saw logs were transported to a sawmill (Arauco town) and cut following normal operational procedures linked to the diameter class of the logs and maximizing the number of sideboards that can be obtained from a log.

Logs were selected with visible resin exudation. Of these samples, 30% were from the central wood (heartwood) and 70% associated with lateral wood (sapwood), thus providing an enhanced opportunity to detect damage or contamination by *P. pinifolia*. The samples were labeled in such a way that they could be traced throughout the entire study. From the resulting green lumber, a total of 40 samples were randomly selected for laboratory analysis. The samples were 15 cm long and 2.5 cm thick, and variable in width, depending on the sawing process.

Once the samples had been obtained and dispatched to the laboratory, the remaining lumber continued through the normal procedure of saw wood production, being subject to a bath with anti-stain solution. All 40 lumber samples were kept at ± 22 °C in a moist chamber for 30 days. These were visually examined every week for evidence of *P. pinifolia* mycelial growth. The lumber samples were further evaluated for the presence of *P. pinifolia* by collecting five randomly selected wood pieces (2-3 mm²) per sample. The pieces were plated onto CARP medium. Resultant colonies were evaluated for the presence of *P. pinifolia* in the same way as for preceding tests.

Results

Survival and development of P. pinifolia on green sawn lumber

None of the wood blocks inoculated, under laboratory conditions, using zoospores (n=20) or mycelial plugs (n=20; Figure 1a) displayed growth of *P. pinifolia* after incubation for 30 days. In the case of the wood blocks treated with anti-sapstain chemicals, no fungal growth was observed, whereas those without anti-sapstain treatment had clear evidence of mold fungi.

No colonies emerged from any of the isolations made (n=100) on CARP from the green sawn lumber samples. None of the 100 isolations from the control wood blocks (without inoculation) was positive for the presence of *P. pinifolia*. All of the ten Petri dishes used as positive controls showed the presence of *P. pinifolia* colonies.

None of the samples placed under naturally infected trees showed evidence of the presence of *P. pinifolia*. Of the 116 samples from which isolations were made, no *P. pinifolia* was detected. Likewise, PCR analyses from the 192 samples showed no evidence of *P. pinifolia*. However, all the needles analyzed (10 per branch) were positive for *P. pinifolia* using isolations on selective medium and with species specific PCR, confirming that the trees on which the wood blocks had been placed were infected.

The samples without anti-sapstain treatment, showed evidence of stain fungi developing. In contrast, no fungi were found on samples collected from wood treated with anti-sapstain chemicals.

Evaluation of lumber from trees in naturally infected plantations

All of the 45 trees chosen for this portion of the study showed clear symptoms of DFP on the foliage. However, *P. pinifolia* could be isolated only from the foliage on 70% (462 from 660) of the trees. *Phytophthora pinifolia* was detected from 6 of 375 of the green bark samples tested and all of these were from the sections of the logs classified for pulping. None of the wood pieces cultured on CARP or those baited with rhododendron leaves showed any evidence of the presence of *P. pinifolia*. Furthermore, fluorescence microscopy on the samples also failed to show any evidence of the pathogen.

The 40 samples of lumber selected for laboratory analyses were free of any signs of *P. pinifolia* based both on isolation and then PCR tests. The samples used as controls were also free of the pathogen.

Discussion

The wood taken from trees growing in areas with a high incidence of DFP, as well as wood artificially inoculated with *P. pinifolia*, were free of the pathogen based on rigorous isolations from a large number of samples. This is in contrast to the fact that some other *Phytophthora* spp. have been isolated from wood of infected trees using similar techniques (Brasier et al. 2005, Hansen et al. 2005, Jung et al. 2005, Greslebin et al. 2007, Greslebin and Hansen 2010). Some of these *Phytophthora* spp. are also able to produce cankers and cause damage to the xylem and this is linked to survival in wood for certain periods of time (Rizzo et al. 2002, Brown and Brasier 2007, Parke et al. 2007, Wickland et al. 2008). In the case of *P. pinifolia*, despite field observations showing stem damage due to the death of the cambial cells infected by *P. pinifolia* (R. Ahumada 2009, unpublished report), there is no evidence that the pathogen can survive in the xylem tissue, and the results of this investigation support that view.

Green lumber is apparently not contaminated by *P. pinifolia*, even when it comes from trees that have been infected for several years. In contrast to the high isolation frequency of *Phytophthora* spp. from xylem and bark of different broadleaved tree species (Brown and Brasier, 2007), the presence of the *P. pinifolia* on *P. radiata* bark samples was minimal (1.4%). The positive isolations were likely linked to the fact that bark samples with symptoms or damage came from the upper parts of the trees, where the bark is thin and has needle remnants that are an important source of contamination of the cambial tissue. It

is important that the upper part of the trees (between 6 and 14 cm of diameter) is used for pulp production and not to produce solid wood products such as green lumber for exportation.

Phytophthora pinifolia is apparently not able to survive on sawn green *P. radiata* wood exposed to the aerial inoculum. In this study, the artificial application of zoospores to freshly cut timber did not show any subsequent presence of the pathogen, which evidently died after application.

Although it was not an objective of this study to assess the effect of anti-sapstain treatment on cut wood, this process is routine for all the green lumber exported from Chile. Isolations from lumber treated in this way yielded no fungi and *P. pinifolia* was also not present. While the results of this study showed that freshly cut *P. radiata* timber is free from *P. pinifolia*, and that it does not become contaminated by this organism, it is likely that anti-sapstain treatments would further reduce the chance of such contamination occurring.

Conclusions

Results of this study provide the first and preliminary evidence that *P. pinifolia* does not occur in the wood of naturally infected trees or on wood that has been artificially inoculated with the pathogen under the tested conditions. Even when wood was exposed to natural inoculum from trees heavily infected with *P. pinifolia*, this inoculum was apparently unable to infect or contaminate wood samples. The absence of *P. pinifolia* from wood samples in this study confirms

that it is primarily a pathogen of the foliage and succulent or green tissues of *P. radiata*. Damage to the stems is normally found when the trees have green needles and where infections pass down the needles into young and succulent cambial tissue.

This is the first study of the relationship between the infection on foliage of *P. radiata* plantations and potential contamination of the wood of trees. The results suggest that the transport of sawn timber from Chile does not represent a likely pathway of movement of *P. pinifolia*. This investigation is an important first step for research that will provide the best options to avoid the pathogen being moved via the movement of *P. radiata* products. Ongoing research considering the epidemiology and biology of *P. pinifolia* will also help to improve the understanding of the mechanism of survival of the pathogen.

Acknowledgements - We thank Bioforest S.A. and Bosques Arauco S.A., subsidiaries of Arauco Group for their financial and technical support. We also thank to Mr R. Nova, Miss C. Díaz, Mr R. Quezada, Mr Boris Palma and Mr C. Barría from Bioforest S.A., Mr R. Gómez from Bosques Arauco and Mr P. González, Mrs R. Canales, Miss G. Cuevas, Mr M. Beeche and Mr C. Barrientos from Servicio Agrícola y Ganadero for their assistance with field and laboratory work. We further thank Mr E. Rodríguez and Dr M. Peredo for useful discussions that supported the completion of this study.

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