# Diseases of *Pinus radiata* in Chile

by

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**Declaration**

I, Rodrigo Ahumada declare that the dissertation/thesis, which I hereby submit for the degree *Philosophiae Doctor* at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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# Table of contents

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# Preface

*Pinus radiata* is one of the most important conifer species used in plantation forestry. This species was introduced into Chile from the USA in the 1800´s and is currently the most important plantation forestry species, covering an area of around 1.5 million ha in this country. The forestry industry based on this tree species is the second most important economic activity in Chile after copper mining. The southern-central part of Chile is the main area for commercial forestry due its suitable climatic conditions. Other than *P. radiata*, *Eucalyptus globulus* and *E. nitens* are also widely planted, and together represent about 93 % of the total commercial plantations.

As is true for all exotic plantation species, part of the success of *P. radiata* is due to the fact that it has been separated from its native pests and pathogens. Over the years, these have however accumulated and now pose serious threats to the sustainable growth of these species. Diseases began to have significant negative impacts on *P. radiata* productivity during the late 1960's with *D. septosporum* emerging in Africa, and subsequently in most of the *P. radiata* plantations in the Southern Hemisphere. Another serious pathogen, *Fusarium circinatum*, which is one of the most aggressive pathogens in nurseries and in plantations, emerged more recently and is now distributed in most of the countries that plant *Pinus* spp. These pathogens represent only a few examples of a substantially longer list of threats, and they illustrate a pattern of increasing pressure on *P. radiata* forestry that is set to continue in the future.

In **Chapter one** of this thesis, I review the development and spread of *P. radiata* as a commercial plantation species around the world, providing a particular focus on the major pathogens affecting this important tree species in plantations. Special emphasis is placed on data for *P. radiata* from Chile (often unpublished or only locally available in Chile), and especially focusing on diseases that, with the exception of *P. pinifolia*, have a worldwide distribution. I also consider control methods applied to these diseases, and emphasize the importance of quarantine regulations to prevent the introduction and spread of new pests and pathogens. Despite the strict quarantine system used by the government it is important to develop more capacity to deal with pests and diseases that will be present in the future and provide a strong support to the Chilean forestry sector. The remainder of the thesis represent research chapters that focus on the three main

pathogens that are currently present in nurseries (*Fusarium circinatum*) and plantations (*Neonectria fuckeliana* and *Phytophthora pinifolia*) of *P. radiata* in Chile.

In **Chapter two** of this thesis, I have treated key aspects of the epidemiology of Daño Foliar del Pino (DFP), caused by *P. pinifolia* on *P. radiata* that were unanswered. Firstly, I have sought to confirm that *P. pinifolia* is the causal agent of the CAPS Daño Foliar del Pino (DFP) on *P. radiata* in Chile by testing Koch`s postulates for the first time. Previous studies failed to do this for technical reasons related to the production and use of zoospore/sporangial suspensions for infection. I have also considered the susceptibility of different *Pinus* spp. and hybrids to *P. pinifolia* using traditional inoculation. Finally, I studied the patterns of sporulation and potential for dispersal of sporangia on the pine needles throughout the year.

One of the main concerns expressed by the international community when *P. pinifolia* was first discovered was whether green wood exported from Chile could be a pathway for the pathogen to spread over long distances. In **Chapter three** of this thesis, I address this concern by evaluating whether *P. pinifolia* is present in green sawn lumber from *P. radiata* plantations affected by DFP. I also evaluated whether *P. pinifolia* can contaminate and develop on freshly sawn lumber when it is directly exposed to inoculum of the pathogen.

Much work has gone into developing management options for DFP in Chile since its discovery in 2007. In **Chapter four** I present key aspects of the current work and progress towards such management protocols. This includes selection of *P. radiata* genetic material that is tolerant to infection by *P. pinifolia*. Secondly, I consider the selection of sites with different levels of risk for disease development. Finally, I also consider the use of specific chemicals to reduce the inoculum.

*Fusarium circinatum* is one of the most serious concerns to *P. radiata* production in nurseries in Chile, and has consequently been well studied. One of the remaining questions, however, concerns the diversity and introduction history of this pathogen. In the light of the importance of quarantine and resistance breeding in protecting Chilean *P.*

*radiata* plantations, these are important questions. In **Chapter five**, I used microsatellite markers to determine the genetic diversity and population structure of *F. circinatum* populations in Chile. I considered collections from three geographically isolated regions, representing the breadth of the distribution of the pathogen in the country. I also characterized the diversity and distribution of mating type loci in these populations. I then used this information to select a representative group of isolates for a pathogenicity trial to characterize the aggressiveness of the *F. circinatum* population to *P. radiata* seedlings.

In **Chapter six** I focus on a canker disease of growing concern in recent years, namely neonectria flute canker, for which some basic information is needed. Firstly, I conducted a systematic survey of the incidence and disease severity across the distribution range of the disease, over two years. Using isolations made during these surveys, I aimed to confirm that the disease is caused by *Neonectria fuckeliana*, and compared it to isolates from elsewhere in the world. I used DNA sequence data from the Internal Transcribe Spacer (ITS) rDNA locus. Finally, I use a pathogenicity test to characterize the relative virulence of a selection of isolates.

# Chapter 1

# *Pinus radiata* and its most important diseases, with special reference to Chile

## 1. Introduction

Commercial forest plantations based on non-native tree species have been established in many countries, particularly in the tropics and Southern Hemisphere, dating back to the late 1800's (Richardson et al. 1994; Wingfield et al. 2001a). Among the most common genera used in productive plantations are *Pinus* Linnaeus, *Eucalyptus* L'Heritier and *Acacia* Miller. The trees in these genera are grown for pulp and paper industries, timber for construction, fuel, firewood, and other products for both local consumption and export (Raga 2009). The global forest industry is an important contributor to the economy of many countries and plays an important role in social issues such as employment and protection of the environment (Raga 2009; Mead 2013).

*Pinus radiata* D. Don was introduced into Chile from the USA (California) at the end of the 1800's. This tree is the most important forestry plantation species in Chile, comprising about 1.5 million ha of the approximately 2.3 million ha of commercial forest (INFOR 2011; Mead 2013). Currently, this is the foundation of the forest industry in Chile and the second most important economic activity in the country after copper mining (Morales 2002; Raga 2009). For example, in 2010 the major market for the Chilean forestry industry was Asia with approximately 29 % of the total trade, followed by North America (18 %) and Europe (12 %). Pulp and paper were the principal export products worth about 3 billion USD, followed by finished wood products worth 650 million USD and wood panels and veneers worth approximately 500 million USD (INFOR 2011).

The introduction of exotic forest pests and pathogens represents an important threat to the productivity of the forestry industry in Chile and other countries of the world (Wingfield et al. 2001a). There are a large number of pests and pathogens not present in Chile and to which *P. radiata* is very susceptible. Some of these threatening organisms include species of bark beetles such as *Ips grandicolis* (Eichhoff), *Dendroctonus ponderosae* (Hopkins), the pine wood nematode *Bursaphelenchus xylophilus* (Steiner &

Buhrer) Nickle, root disease fungi such as *Heterobasidion annosum* (Fr.) Bref., and western gall rust caused by *Endocronartium harknessii* (Moore) Hiratsuka (SAG 2013). These would add substantially to the damage already being caused by the pitch canker fungus *Fusarium circinatum* Nirenberg & O'Donnell, flute canker caused by *Neonectria fuckeliana* (C. Booth) Castl. & Rossman, dothistroma needle blight caused by *Dothistroma septosporum* (Dorog.) Morelet, needle and shoot blight caused by *Diplodia pinea* (Desm.) Kickx. and the Daño Foliar del Pino (DFP) disease caused by *Phytophthora pinifolia* Durán, Gryzenh. & M.J. Wingf. (Wingfield et al. 2002; Ahumada 2003).

Given the disease and pest threat to Chilean forestry, quarantine measures to reduce the chance of accidental introductions of pathogens and insects are crucially important. The objectives of the development and implementation of such a plant quarantine strategy is not only to prevent the introduction of new pests and pathogens, but also to retard their later spread. Exclusion of new diseases is one of the most important steps in forest and crop protection and it is crucially important to delay their arrival (Wingfield et al. 2001a). However, it is recognized that it is inevitable that some of these agents will eventually arrive. Thus, the timely discovery of these newly arrived pests and pathogens is essential. The most effective action towards early detection of pests and diseases is based in the implementation of a structure with sufficient numbers of staff appropriately trained in the diagnosis and recognition of new invasive agents (Wingfield et al. 2001a).

There are some good examples of efficient quarantine programs developed in Australia, New Zealand and USA (DAFF 2013; MAF 2003; APHIS 2010). Based on these examples, Chile has been making extraordinary efforts to reinforce its quarantine strategy (SAG 2011). Yet, despite these efforts, new pests and pathogens in commercial plantations have been reported almost every year over the last decades (Figure 1).

The aim of this review is to bring together the most relevant information regarding the planting of *P. radiata* as a non-native plantation species, providing a particular focus on the major pathogens and pests affecting this important forestry crop. Special emphasis is placed on published data for *P. radiata* in Chile, especially relating to pests and diseases that, with the exception of *P. pinifolia*, are widely distributed. Aspects related to the

importance of quarantine regulations to prevent the introduction and spread of new pests and pathogens are also presented.

## 2. *Pinus radiata* – a worldwide overview

*Pinus radiata* is native to the south-western USA, California. Taxonomically it has been placed in different sub-groups in the genus *Pinus*. For example, sub-sections *Pinaster* (Shaw 1914; Duffield 1952), *Oocarpae* (Little & Critchfield 1969), *Patula* (Perry et al. 1991) and finally *Attenuatae* (Price et al. 1998) where the tree is currently classified and is considered to be part of the group of coastal closed-cone pines. Other *Pinus* spp. also accommodated in this sub-section are *P. muricata* D. Don and *P. attenuata* Lemmon (Forde 1964; Millar 1999).

*Pinus radiata* is an evergreen tree of 15 to 35 m in height and 60 to 90 cm in DBH (Munz 1973). The common names for the tree include Monterey pine, insignis pine, radiata pine, Cambria pine, Guadalupe Island pine and Cedros Island pine (Cope 1993). The scientific name "radiata" refers to the characteristic cone flakes and the common name "Monterrey" refers to the peninsula of California where it grows naturally (McDonald & Laacke, 1990). The natural distribution of *P. radiata* is limited to five localities that cover between 5 000 and 8 000 ha (Millar 1998). The main populations are in the central coast of California, specifically in the Monterrey peninsula (Monterey County with 1 800 to 2 800 ha), Cambria (San Luis Obispo county with 930 ha) and Año Nuevo (Santa Cruz county with 600 ha). Another two localities where this tree is found as a native are the northern coast of Mexico in the Guadalupe and Cedros Islands where about 180 ha of trees remain (Dilworth 2004; Millar 1986; 1998).

Currently three varieties are recognized in *P. radiata*. These include "radiata" which has developed into the continental population and has three needles per fascicle. The varieties "binate" and "cedrosensis" corresponding to Guadalupe and Cedros islands respectively have two needles per fascicle. *Pinus radiata* can hybridize with *P. attenuata* (knobcone pine) and *P. muricata* (bishop pine) (McDonald & Laacke 1990). The native populations on the North American continent occur no more than 11 km from the coast

and their longitudinal distribution is about 200 km between 37° and 35° 30’ N (McDonald & Laacke 1990). The climate where these trees grow naturally is typically Mediterranean and the weather conditions include an average rainfall of 380 to 890 mm per year, relative humidity of 60 to 70 %, many foggy days in winter and summer and average annual temperatures of 9 to 11 °C and 16 to 18 °C in the winter and summer seasons, respectively (McDonald & Laacke 1990). Native *P. radiata* trees have been used in California as ornamentals to provide a pleasing environment for recreational activities along the coast (Patrick 2005). In addition, commercial planting of these trees in their areas of origin has been for the production of Christmas trees (McDonald & Laacke 1990).

*Pinus radiata* is one of the most versatile *Pinus* species used for commercial plantation forestry. This is due to its rapid growth, medium-density softwood, and its suitability for a wide range of applications. This accounts for the fact that it is one of the most widely planted conifers with about 4.2 million ha planted in many parts of the world, but particularly in the Southern Hemisphere (McDonald & Laacke 1990; Mead 2013). The countries with the largest areas plantations of *P. radiata* are Chile (1.5 million ha), New Zealand (1.5 million ha), Australia (770 thousand ha), Spain (280 thousand ha), South Africa (80 thousand ha) and Italy (40 thousand ha), (INFOR 2011; Mead 2013). Countries such as Argentina, Turkey, Albania, Ecuador, Kenya, Zimbabwe, Colombia and Peru have also planted this tree, but in relatively small proportions (Lavery 1990; Rogers 2004; INFOR 2011; Mead 2013).

There are several reasons other than wood properties and fast growth, why *P. radiata* has become one of the most extensively planted exotic conifers in the world (Scott 1961; Lavery 1986). These include its exceptional growth in moist temperate climates, the versatility of the wood, the fact that it is suitable for various industrial uses, the ability to collect large quantities of seed and its easy propagation. These characteristics are accentuated by the genetic diversity of the tree in its natural populations, which can provide suitable genotypes for planting in different environments and that contribute to its plasticity to adapt to different conditions without a reduction in growth (Lavery & Mead 1998). Monterey pine is planted for erosion control, barriers to wind and noise and to stabilize soils on steep slopes (McDonald & Laacke 1990; Libby & Rodrigues 1992).

## 3. Some of the most important diseases of *Pinus radiata*

Species planted for commercial purposes in plantations outside of their native range are exposed to many pest and disease threats that are not serious problems, or are unknown in their natural environment (Burgess & Wingfield 2001). Diseases of *P. radiata* started to have a significant impact by the late 1950's with the first report of *D. septosporum* in Kenya, Africa, and subsequently in most of the *P. radiata* plantations in the Southern Hemisphere (Burgess & Wingfield 2001). This pathogen, together with *Cyclaneusma minus* Butin (Bulman et al. 2008) has subsequently become one of the major needle pathogens negatively affecting *P. radiata* in countries with large areas planted to this species. *Fusarium circinatum* is responsible for the disease known as pitch canker and is one of the most aggressive pathogens of *P. radiata* (and various other *Pinus* spp.) where it damages plants in nurseries and in the field in South Africa, Spain and Chile (Roux et al. 2007; Wingfield et al. 2008a; Landeras et al. 2005; González 2007). It has also been reported in Uruguay (2009) and Colombia (2012) on other species of *Pinus* (Alonso & Bettucci 2009; Steenkamp et al. 2012). The pathogen in South American countries is still restricted to nurseries, as was the case in South Africa for more than 15 years until the first outbreak in *P. radiata* plantations (Coutinho et al. 2007).

*Neonectria fuckeliana* represents another "new" pathogen of *P. radiata* in plantations and it is found in New Zealand and Chile, causing a disease known as flute canker (Gadgil et al. 2003; Morales 2009). This pathogen is thought to have originated in the Northern Hemisphere and it is still uncertain how it was spread to *P. radiata* plantations in New Zealand and Chile (Dick & Crane 2009). Another pathogen recently affecting *P. radiata* is *Phytophthora pinifolia*, which was identified in 2007 (Durán et al. 2008). This pathogen is known only in Chile. A new *Phytophthora* sp. has, however, recently appeared on the foliage of *P. radiata* in New Zealand plantations and is awaiting formal description (Bulman 2013 pers. comm.). Some of the major diseases affecting *P. radiata* in plantations are described in more detail in the following section.

### *3.1 Dothistroma septosporum*

Dothistroma needle blight (red-band) caused by *Dothistroma septosporum* has been a problem in plantations of exotic pines in the Southern Hemisphere for many decades and is considered to be one of the most important foliar diseases affecting *Pinus* species worldwide (Gibson 1972; Bradshaw 2004). In the early 1960's, outbreaks of dothistroma needle blight in *P. radiata* plantations were severe in East Africa, New Zealand and Chile (Gibson 1974). In the late 1990's and early 2000's, outbreaks were recorded in British Columbia (Woods et al. 2005) and parts of Europe (Brown 2007). This pathogen is known from more than 45 countries including those in Africa, North and South America, Asia, Europa and Oceania (Ivory 1994; Watt et al. 2009). Its wide geographical distribution implies a relatively wide host range of more than 80 host species, most of them in *Pinus* species, as well as some species of *Abies*, *Picea* and *Larix* (Bednárová et al. 2006; Kirisits & Cech 2006; Groenewald et al. 2007).

The most characteristic symptoms associated with infection are distinct red bands on the needles that first appear on green needles and remain there until the needles become brown or gray (Gibson 1972; Bulman et al. 2004; Sinclair & Lyon 2005). The sexual state of *D. septosporum* has been identified is *Mycosphaerella pini* E. Rostrup (Funk & Parker 1966). This state of the fungus has never been found on *P. radiata* in countries of the Southern Hemisphere such as Australia, New Zealand and Chile (Evans 1984).

*Pinus radiata* has been categorized as highly susceptible to infection by *D. septosporum* (Gilmour 1967a). It is also known to become less-susceptible with age, being more tolerant after the trees reach 15 years of age, depending on climatic conditions (Franich et al. 1982; Bulman et al. 2008). The pathogen apparently entered New Zealand in the late 1950's, but it was not identified there until 1964 (Gilmour 1967b). In Chile its presence was confirmed in 1965 (Dubin 1965) and in Australia it was reported for the first time in 1975 (Edwards & Walker 1978). *Dothistroma septosporum* has been very intensively studied in New Zealand, Australia and Chile where it has been the cause of a significant reduction propagated *P. radiata* (Gibson 1974; Van der Pas 1981).

Estimations of annual economic losses in New Zealand due to dothistroma needle blight ranged from US$ 3.8 million in 1989 to US$ 20.5 million in 2007 and for control measures about US$ 15 million per year (New 1989; Bulman et al. 2004; Watt et al. 2011). The impact on the diameter growth and height of the trees when the defoliation is over 25 %, have been assessed in different studies in Tanzania, New Zealand and Australia (Gibson 1974; Van der Pas 1981). These have shown that disease severity is linearly related to stunting in young trees; growth almost ceased at 75 % defoliation (Bulman et al. 2008). The relationship between volume loss and disease level has also been demonstrated (Van der Pas 1981; Old & Dudzinski 1999). In Kaingaroa, New Zealand, spray trials on 13-year-old *P. radiata* trees showed that trees with 25 % or more infection, sprayed on three occasions, had 30 to 40 $m^3ha^{-1}$ more volume at the end of a rotation, compared to unsprayed trees (Kershaw et al. 1988). Other studies have shown that the height of the trees is affected if the defoliation is greater than 30 to 40 % (Van der Pas 1981). Similarly, the increment of trees can decrease by 50 %, if the infection level is more than 50 % of the active foliage (Whyte 1976; Van der Pas 1981).

The management of dothistroma needle blight has been based on the utilization of three measures to avoid the spread to uninfected areas and to control the disease. These include silvicultural practices to reduce inoculum levels, fungicide applications and the selection of disease resistant *P. radiata*. Silvicultural practices include pruning lower branches to reduce the humidity within the tree canopy, weed control and thinning to increase the ventilation within stands (Bulman et al. 2004; 2008). Chemical control has been used in New Zealand and in Chile at an operational scale for several years. The implementation of this measure has been based on research done since the 1950's in the USA and then in the early 1960's in Africa, where different fungicides were tested in Tanzania and Kenya on *P. radiata* plantations (Thomas & Lindberg 1954; Gibson et al. 1966). Later in 1965, chemical control of dothistroma needle blight started in New Zealand with trials to better understand the use of different copper formulations (Cuprous oxide and copper oxychloride), to select the optimal time of the applications and to determine the number of applications required per season (Gilmour 1967c; Gilmour & Noorderhaven 1973). Chemical control of *D. septosporum* using aerial

applications of copper fungicides is generally effective and is widely practiced in New Zealand where infection levels reach 25 % (Bulman et al. 2008).

The population biology of *D. septosporum* is not well understood and preliminary research using molecular markers showed that the New Zealand population has limited genetic diversity (Hirst et al. 1999). Analyses of mating type distribution later showed that *D. septosporum* populations from Australia, Chile and New Zealand have only one mating type (*MAT2*) and this might explain the absence of the sexual state in those countries (Groenewald et al. 2007). It is thus intriguing that no sexual state has been observed in the pathogen in South Africa where both mating types are present (Groenewald et al. 2007).

### *3.2 Diplodia pinea*

*Diplodia pinea* is an endophytic fungus and pathogen that causes a shoot and tip blight disease, rapid death of shoots and kills crowns and branches of *Pinus* spp. and some other conifers (Punithalingam & Waterson 1970, Swart & Wingfield 1991). This pathogen is known to be an opportunistic organism that infects pines through natural openings when wounded by hail or pruning (Chou & MacKenzie 1988). The pathogen commonly also infects unwounded tissue and can persist in the host for long period of time and can thus be isolated from all parts of asymptomatic established trees (Bihon et al. 2011b). It can also cause disease when the host trees are stressed by factors such as drought and frost damage (Wingfield 1999). Serious disease development on pine trees caused by *D. pinea* after hail has been reported from South Africa (Swart et al. 1987) and Spain (Iturritxa 2001). The importance of the pathogen in South Africa especially has been attributed to a combination of hail storms and planting of the highly susceptible *P. radiata* (Swart & Wingfield 1991). Wounds made by hail or pruning have been noted as important factors influencing infection in New Zealand and Australia (Chou & Mackenzie 1988).

*Diplodia pinea* is globally distributed in temperate and tropical zones (Swart & Wingfield 1991; Stanosz & Carlson 1996) and occurs on many *Pinus* species, and where *P. radiata* is considered as one of the most susceptible species (Swart et al. 1988). This pathogen has

been particularly aggressive in plantations of *P. radiata* in South Africa, New Zealand, Chile and Australia (Chou 1976; Currie & Toes 1978; Swart et al. 1987) causing characteristic symptoms such as shoot blight, die-back, sometimes with deformation of the main stem and stem cankers (Gibson 1979; Swart & Wingfield 1991). *Diplodia pinea* usually kills the current-season buds and shoots and second-year cones that are important because they are sources of inoculum for further infections (Sinclair & Lyon 2005).

The major economic impact of infection by *D. pinea* is related to the growth of the trees and quality of the timber. If the infection occurs in the main stem, deformation occurs and this can result in loss in wood production around of 40 % (Foster & Marks 1968). In young plantations, damage to the terminal shoots can affect either the main stem or the lateral shoots, increasing the amount and the thickness of whorls, which also results in considerable loss in the quality of wood (Foster & Marks 1968; Wright & Marks 1970). The infection can be the cause of losses in nurseries and commercial *P. radiata* plantations due to shoot blight (Swart & Wingfield 1991; Stanosz & Cummings 1996; Stanosz et al. 2005). Further direct losses due to *D. pinea* infection are related to death of leading shoots, defoliation or tree mortality. There can also be indirect losses that are associated with the timber degradation due to blue stain (Eldridge 1957; Farrell et al. 1997). Evaluations made in the 1970's in New Zealand and Australia showed a maximum reduction in timber increment of approximately 40 % (Wright & Marks 1970; Currie & Toes 1978). An estimation made in South Africa in the 1990's showed that compartments prematurely clear-felled due to *D. pinea* infection had a timber volume loss of 28 % and a loss of 55 % of value of potential production (Zwolinski et al. 1990).

The management of disease caused by *D. pinea* has been through silvicultural activities to reduce the chances of infection and also by selecting tolerant planting stock for areas prone to damage by the pathogen (Peterson 1977; Swart & Wingfield 1991). However, to avoid damage, perhaps the most important issue is to correctly choose the sites for planting using a risk analysis approach. In the past century, application of various fungicides, including benomyl, manzate, thiophanate-methyl and tebuconazole, have been tested for *D. pinea* control in nurseries and plantations (Peterson 1977; Palmer et al.

1986). The high costs, operational inconvenience and environmental factors have, however, restricted the use of these products to occasional applications in nurseries (Swart & Wingfield 1991). Timely thinning, especially on poor sites, dry seasons or in heavily stocked stands, as well as avoidance of pruning during summer can reduce the risks of infection. Biological control using dsRNA viruses has also been explored as an environmentally friendly alternative to fungicides, but this needs further research before it can be applied (Preisig et al. 1998; De Wet et al. 2008).

Population genetic studies using microsatellite (SSR) markers have revealed that the fungus has a fairly large amount of diversity across its global distribution. In Central Europe the population of the fungus has low diversity, while in countries like South Africa the diversity is exceptionally high probably due to multiple introductions (Burgess et al. 2004; Bihon et al. 2011a). The low diversity of the pathogen in some countries could be advantageous in disease management where trees are selected for tolerance and where this would be of a durable nature. Low levels of diversity can be ensured by the fact that the fungus is only known to reproduce asexually (Bihon et al. 2011a).

### *3.3 Cyclaneusma minus*

Cyclaneusma needle-cast caused by *Cyclaneusma minus* has been an important foliage disease on *P. radiata* and has thus been the focus of a large amount of research over the last forty years (Bulman et al. 2008). Cyclaneusma needle-cast normally appears in plantations of between 6 and 20 years of age and is most damaging in areas with mild and wet conditions during autumn and winter (Bulman & Gadgil 2001). The pathogen is distributed worldwide and the main hosts are species of *Pinus* including *Pinus caribaea* Morelet, *P. contorta* Dougl. ex Loud., *P. flexilis* James, *P. jeffreyi* Grev. & Balf., *P. Montana* Miller, *P. mugo* Turra, *P. nigra* Arnold, *P. patula* Schl. et Cham, *P. ponderosa* Dougl. ex Laws., *P. radiata*, *P. sabiniana* Dougl., *P. strobus* L., *P. sylvestris* L., *P. uncinata* Ramond, *P. wallichiana* AB Jacks (Butin 1973; Gibson 1979). The new needles are infected and then turn yellow and brown. The fungus is a latent pathogen that appears on senescent needles together with *Lophodermium* spp. (Minter & Millar 1980).

*Cyclaneusma minus* has caused a reduction of approximately 60 % in volume on *P. radiata* plantations in New Zealand when the disease severity reached 80 % on average. Where 50 % of the final crop trees are affected by the disease, losses of 71 $m^3ha^{-1}$ were predicted at the end of the rotation (Bulman 1993). For every 10 % increase in the proportion of diseased trees, a total reduction in volume of 10 - 14 $m^3ha^{-1}$ was observed (Van der Pas et al. 1984; Bulman & Gadgil 2001). Projections made for the whole of New Zealand showed a growth loss of 6.6 % per annum for the *P. radiata* estate aged between 6 to 20 years old (Bulman & Gadgil 2001). In New Zealand the pathogen accounts for approximately $38 million worth of loss per year (Bulman 2009), which makes it one of the more serious disease problems.

Management of cyclaneusma needle blight with aerial fungicide applications is not cost-effective, have been unsuccessful and expensive (Vanner 1986). Although some success has been achieved by injection with systemic fungicides (Hood & Vanner 1984) this control method is not feasible for large scale. Silvicultural treatments also showed that stocking density and pruning had no effect on disease incidence or severity (Bulman & Gadgil 2001). Breeding and selection of resistant planting stock thus is the only option to reduce the impact of the pathogen.

### *3.4 Fusarium circinatum*

*Fusarium circinatum* (sexual state = *Gibberella circinata*) is the causal agent of pitch canker and it is considered as one of the most important pathogens on *P. radiata* plantations worldwide (Gordon et al. 2006; Wingfield et al. 2008a). *Fusarium circinatum* has a wide host range and has been recorded on more than 60 pine species (Hodge & Dvorak 2000) as well as *Pseudotsuga menziesii* (Storer et al. 1997). The name pitch canker stems from the fact there is a characteristic exudation of resin at the site of infection (Hepting & Roth 1946). The disease was first reported in North Carolina, USA affecting *Pinus virginiana* Mill. (Hepting & Roth 1946), and later, in the 1980's the disease was detected in California (McCain et al. 1987). *Fusarium circinatum* has been recorded in countries including Haiti, Japan, South Africa, South Korea, Mexico, Chile, Spain, Italy, Uruguay, Portugal and Colombia (Hepting & Roth 1953; Kobayashi & Muramoto 1989;

Viljoen et al. 1994; Guerra-Santos 1999; Lee et al. 2000; Wingfield et al. 2002; Landeras et al. 2005; Carlucci et al. 2007; Alonso & Bettucci 2009; Bragança et al. 2009; Steenkamp et al. 2012).

*Fusarium circinatum* is known to have a heterothallic mating system with two mating types (Kerényi et al. 1999; Britz et al. 1999), but asexual reproduction apparently predominates in the field and the sexual stage has been seen under laboratory conditions (Nirenberg & O'Donell 1998; Viljoen et al. 1997a). The main symptoms of pitch canker are needle wilting, branch and tip die-back, crown discoloration and resinous cankers on the stems of infected trees (Barrows-Broaddus & Dwinell 1985; Storer et al. 1995; Gordon et al. 2001; Wingfield et al. 2008a). The root collars of hedge plants or production seedlings in nurseries can also be infected (Barrows-Broaddus 1990; Correll et al. 1991; Carey & Kelly 1994; Viljoen et al. 1994). To identify the pitch canker pathogen, morphological characteristics have been used (Nirenberg & O'Donnell 1998; Leslie & Summerell 2006). However, this is time consuming and less accurate than when PCR-RFLP based on the Histone H3 gene (Steenkamp et al. 1999) or PCR-CIRC specific primers (Schweigkofler et al. 2004) are applied.

Losses due to infection of trees by the pitch canker pathogen can reach millions of United States dollars per year (Barrows-Broaddus & Dwinell 1985; Pérez-Sierra et al. 2007). A general estimation made in the USA showed a reduction of volume growth of 20 to 40 % due to the infection on *P. elliottii* Engelm. var. *elliottii* and *P. taeda* L. (Arvanitis et al. 1984) and 5 % mortality of *P. radiata* Christmas trees in California (Storer et al. 1995). In South Africa, it has been estimated that 25 % of all seedlings die in the first year (Crous 2005) and there has been an annual decline in survival of *P. patula* seedlings from about 88 % in 2000 to 64 % in 2007 (Morris 2011). On other hand, a nursery located in the Western Cape Province in South Africa lost between 22 % and 30 % of seedlings over two consecutive years (Van Wyk et al. 2012).

Considerable efforts have been made to develop protocols for *F. circinatum* control. Most of these recommend appropriate nursery sanitation (Wingfield et al. 2008; Van Wyk et al. 2012), chemical and biological control (Barrows-Broaddus & Kerr 1981; Dumroese et al.

1988; Runion & Bruck 1988). However, selection of *Pinus* species and pine hybrids tolerant to infection is the most important option for reducing long-term losses (Hodge & Dvorak 2000; Roux et al. 2007; Mitchell et al. 2012). To prevent potential damage in the future, it is also crucial to ensure an adequate quarantine system, especially in countries where the importation of seed is common. For example, the Ministry of Agriculture of New Zealand has decided to stop all seed imports from countries where pitch canker is known to occur, due to the uncertainty of the effectiveness of treatments against *F. circinatum* (Storer et al. 1998; Wingfield et al. 2008a).

Genetic diversity of *F. circinatum* was first determined using vegetative compatibility groups (VCGs) with samples from the United States (Florida and California), Mexico, South Africa and Japan (Gordon et al. 1996; Viljoen et al. 1997b; Wikler & Gordon 2000). The results showed a high VCG diversity in samples from Mexico, South Africa and the South East USA (Florida) and very low diversity in samples from Japan and California (Viljoen et al. 1997b; Britz et al. 1998; Wikler & Gordon 2000). New protocols have also been developed to study the population genetics using microsatellites markers (Santana et al. 2009) and have been used in a recent study to compare the genetic diversity in Spain with collections from the rest of the world, including from South America from Chile and Uruguay (Berbegal et al. 2013). The studies have shown that *F. circinatum* is diverse in many of the introduced populations, and has been introduced more than once in some of them. It has also shown that reproduction is possibly not exclusively asexual in all regions, such as USA, South Africa and Mexico where the data indicate a cryptic sexual cycle (Berbegal et al. 2013).

Markers to characterize the mating types of the heterothallic isolates have been developed (Steenkamp et al. 2000; Wallace & Covert 2000; Schweigkofler et al. 2004). These markers have shown differences in mating types between countries and localities, for example in Spain, both mating types are present in Asturias, Cantabria and Galicia regions (Pérez-Sierra et al. 2007), while in the Basque region of Spain only the *MAT-2* has been reported (Iturritxa et al. 2011). They have been also used to characterise the mating types of *F. circinatum* isolates from Mexico, Spain, South Africa and the USA and in most of the case, both mating types were found suggesting that the potential for sexual

reproduction exists in all these environments (Britz et al. 1998; Covert el al. 1999; Pérez-Sierra et al. 2007; Iturritxa et al. 2011; Berbegal et al. 2013).

### *3.5* *Armillaria* species

The species in the genus *Armillaria* found in many countries has been historically been referred as *Armillaria mellea* (Vahl: Fr.) Kummer (= *Armillariella mellea* (Vahl: Fr.) Karst. (Watling & Gregory 1982; Harrington & Wingfield 1995). The root rot disease has been considered amongst the most important killers and causes of decay of deciduous and coniferous trees, shrubs, fruit trees and many indigenous trees (Wargo & Shaw 1985; Coetzee et al. 2000). Species of *Armillaria* are generally natives of the region in which they occur and have been described as secondary pathogens of stressed trees, saprophytes on dead trees and also as an aggressive primary pathogens on a wide range of hosts, with a world-wide distribution in temperate, sub-tropical and tropical regions (Shaw & Roth 1978; Gregory et al. 1991; Hood et al. 1991). *Armillaria* species have been reasonably well studied in Europe, North America, Asia, Australia and New Zealand (Kile & Watling 1983; Anderson 1986; Ota et al. 1998; Pegler 2000). During the last 15 years, investigations have been made in African countries to clarify the often arbitrary classification the Armillaria root rot pathogen as *A. mellea*, showing that the species mostly corresponds to *A. heimii* Pegler from, Cameroon, Zimbabwe and Zambia and *A. fuscipes* Petch from South Africa and Ethiopia (Coetzee et al. 2000; Gezahgne et al. 2004). Very little is known about *Armillaria* species in South American countries where most of the investigations have been focused in Brazil, Chile and Argentina (Coetzee et al. 2003; Lima et al. 2008).

Infection by *Armillaria* spp. can be aggressive in some coniferous species such as *Pinus*, *Abies* and *Pseudotsuga,* where the fungus colonizes and kills the healthy tissues (Wargo & Shaw 1985). The fungus can also colonize stumps and roots of cut trees and the root systems can help the spread of the infection (Wargo & Shaw 1985). The main above ground symptoms are crown discoloration with needles turning from yellow to brown, branch and shoot die-back and eventually tree death (Gibson 1979). Similar symptoms can appear in trees affected by bark beetles, rodents, prolonged drought, other root

fungi; the distinguishing symptoms for *Armillaria* in these cases are often the typical white mycelial that develops under the dead bark (normally around the collar of the tree) and the presence of string-like rhizomorphs under the bark and in the soil surrounding the tree (Wargo & Shaw 1985).

Identification of *Armillaria* species has classically been based on conventional morphological characteristics of the basidiocarps, which are not always present or appear in the final stages of the disease. More than 39 species of *Armillaria* have been described, but most of them group into the *A. mellea sensu lato* complex (Wargo & Shaw 1985; Volk & Burdsall 1995). In the last two decades, tests for inter-fertility have been applied to identify species like *A. gallica*, *A. cepistipes* and *A. mellea* and molecular tools have also been developed to facilitate the identification process (Harrington & Wingfield 1995; Coetzee et al. 2001; Gezahgne et al. 2004).

Management of Armillaria root rot is typically through cultural practices removing the source of nutrition of the pathogen. But this is an expensive and time consuming process. Sometimes isolating areas where the infection occurs can help to avoid new contamination, but it is not always practical. Severity of Armillaria root disease is dependent upon host susceptibility, virulence of the *Armillaria* species involved and factors like soil properties (Mallett & Maynard 1998). The impact caused by *Armillaria* is always over estimated due to a startling mortality of groups of trees (Gibson 1979). In northern regions of USA, *Armillaria* has been responsible for the death of many coniferous trees, killing about 2 % of trees during the first years of growth. Mortality of up to 25 % of *Pinus contorta* (lodgepole pine) stands has been recorded in Alberta, Canada (Mallett 1992).

## 4. *Pinus radiata* in Chile

Continental Chile is located to the West of Argentina and to the South of Peru in the Southern Hemisphere, 4 300 km in length and 427 km at its widest point, with a total land area of 75.5 million hectares (INFOR 2011). Forestry resources correspond to 16 million hectares (about 21 %) of the total Chilean land area, of which natural forest comprises

85.5 % (Lowy 1995; INFOR 2011). The southern-central part of Chile is the most important area for commercial forestry due its suitable climatic conditions. Commercial plantation forestry only rose to prominence in Chile after 1974 when a decree law (DL 701) was made that called for the government to stimulate afforestation. The main species used to establish plantations are *P. radiata*, *Eucalyptus globulus* Labill. and *E. nitens* (Deane and Maiden), which together represent 92.5 % of the total commercial plantations (Jayawickrama et al. 1993; INFOR 2011).

*Pinus radiata* was introduced into Chile at the end of the 19$^{th}$ century for ornamental purposes, together with *P. ponderosa* and *P. pinaster* Aiton (Richardson et al. 1994). The first plantations of *P. radiata* were established in 1893 near Concepción and comprised about 10 ha (Contesse 1987). In the early 1900's, the Chilean government started with a program of planting *P. radiata* to control soil erosion (Mead 2013). Government subsidies to private growers started in 1974, after which there was a large increase in new *P. radiata* plantations (Mead 2013). This species has been the preferred species and has shown impressive growth ever since (Toro & Gessel 1999). Currently *P. radiata* constitutes one of the most important products for exportation (Cartwright & Gaston 2002; Raga 2009). By 1943, there were 143 500 ha of pine plantations by 1993 these had reached 1.24 million ha and currently *P. radiata* constitutes the largest surface area of plantations, including about 1.48 million ha (63.7 %) of the approximately 2.32 million ha of commercial plantations in the country (INFOR 2011; Mead 2013).

Almost all the commercial plantations in Chile are planted with industrial objectives, including pulp and paper manufacturing, sawmills, wood panels and particle boards (Lowy 1995; WFI 1999; Morales 2002; INFOR 2011). For example, in 2007 the annual timber harvest was 52 million m$^3$ of which 73 % was used by the industry and the rest used to produce energy (Raga 2009). Ninety-eight percent of the total wood used by the industry comes from local plantations, while 44 % of the wood used to produce energy comes from native forests (Raga 2009). In 2010, the industrial round wood consumption was 98 % from commercial plantations, of which 66 % (22.9 million m$^3$ solid without bark) came from *P. radiata*. Of the *P. radiata* wood used, 33 % was for wood pulp, 51 % for saw timber, 13 % for wood paneling and veneers and 1.3 % for wood chips (INFOR 2011).

The majority of the commercial plantations in Chile belong to private industry. At present, two large forestry groups own approximately 50 % of *P. radiata* forests and 75 % of all pulp production in the country (INFOR 2011). The forestry activity in Chile is the second largest export industry, following copper, generating more than 180 000 jobs and about 5 000 million US$ in exports, which is around 7 % of the national exports (INFOR 2011). The major markets for the Chilean forestry industry in 2010 were Asia (approximately 29 % of the total trade), North America (18 %) and Europe (12 %). Pulp and paper were the principle export products generating about 3 billion US$ (60 % of the total), wooden finished products worth 650 million US$ (13 %) and wood panels and veneers worth an amount of 500 million US$ (10 %) (INFOR 2011).

## 5. Diseases of *P. radiata* in Chile caused by introduced pathogens

There are very few publications available in Chile treating diseases of *P. radiata* plantations. One of the first efforts to improve this situation was made in the 1980's (Mujica & Vergara 1980; Butin & Peredo 1986) who gathered information about the diseases of forest trees in South America, but with a special focus on Chile. Some of the species mentioned in this first treatment were *Trametes versicolor*, *Botrytis cinerea*, *Macrophomina phaseolina* (Tassi) Goid., *Colletotrichum acutatum* Simmds. f. sp. *pinea* Dingley & Gilmour and *F. oxysporum* Snyder & Hansen (Mujica & Vergara 1980; Butin & Peredo 1986). The most important pathogens found on *P. radiata* in Chilean plantations between the 1960's and 1970's were *D. septosporum, Armillaria mellea*, *Diplodia pinea* (reported as *Sphaeropsis sapinea*) and *Cyclaneusma minus* (Mujica & Vergara 1980; Butin & Peredo 1986), but none of these were reported as having significant negative impacts (Figure 2). The most serious sanitary problems to emerge since then on *P. radiata* in Chile have been those caused by *F. circinatum* found in the early 2000's and is confined to nurseries, and *Phytophthora pinifolia,* identified in 2007 as the cause of daño foliar del pino (Wingfield et al. 2002; González 2007; Durán et al. 2008; Ahumada et al. 2013).

Forestry companies and the government have undertaken long-standing surveillance programs in both commercial and ornamental plantations for early detection of pest and diseases. However, during the last 20 years the numbers of insects and pathogens

detected in the country on commercial plantations have increased very rapidly (Figure 1). The most significant impact of these problems has been on the establishment of new plantations in areas with high risk of infection. This increase of new pests and diseases can be attributed to the increase in world trade in unmanufactured wooden articles, as well as an increase in tourist activity (González & Parra 1994; Tkacz 2002).

Currently, the most important diseases in Chile are caused by three pathogens that pose tremendous challenges for forestry since they affect the three main parts of the trees, namely the root systems and collars in the case of *F. circinatum* in nurseries, the stems by *Neonectria fuckeliana* and the foliage by *Phytophthora pinifolia*.

### *5.1 Fusarium circinatum*

*Fusarium circinatum* was detected in 2001 on *P. radiata* hedge plants in nurseries located in the Biobío region (Wingfield et al. 2002). In 2003, the fungus was also isolated from containerized and open root *P. radiata* plants in nurseries distributed from Constitución (35° 20’ S, 72° 25’ W) in El Maule region to Valdivia (39° 48’ S, 73° 14’ W) in Los Ríos region (SAG 2003; González 2007). In the past few years, initiatives between the government and the private sector have been implemented, such that a greater body of knowledge could be gained regarding the distribution, host range, biology, epidemiology, sanitation and options for control of *F. circinatum*. Because of the potential damage that this fungus can cause to commercial forest plantations in Chile, the Agriculture and Livestock Service (SAG), via a suite of formal resolutions, has declared this pathogen under official control (SAG 2011).

The official control program for *F. circinatum* implemented by SAG has helped to reduce its spread to plantations, restricting it to defined nurseries that also apply strict phytosanitary controls. Today all nurseries propagating *P. radiata* require certification provided by SAG to move plants for planting and they also need to have implemented a sanitization procedure that has been standardized by SAG (González 2007; SAG 2011). The economic impact of *F. circinatum* remains uncertain and estimates by foresters suggest that the number of nursery plants destroyed between 2006 and 2012 as part of

the quarantine regulations amounted to about 4.3 million. This represents close to 0.65 % of the total production with an estimated total cost of US$ 540 000 (R. Ahumada, unpublished data).

It is difficult to predict how pitch canker will affect the future of commercial plantations in Chile. One concern is that the pathogen might be able to enter wounds made by insects such as *Rhyacionia buoliana* Schiff*., Hylaster ater* Paykull or *Pissodes castaneus* De Geer. These aspects of the disease in Chile have not been studied and will need to receive careful attention in the future.

## 5.2 *Neonectria fuckeliana*

The flute canker disease caused by *N. fuckeliana* was officially reported in Chile in 2009 in the Araucanía region (Morales, 2009). However, the disease is known to have been present in Chile for at least 10 years where it was observed in a few managed *P. radiata* plantations, but never was officially reported. The pathogen produces characteristic red perithecia that contain ascospores and it also has two anamorph stages, one of which is *Acremonium*-like and the other that is known as *Cylindrocarpon cylindroides var. tenue*. In Chile the most typical signs of *N. fuckeliana* are the red perithecia growing on wounds made by pruning and also abnormal growth of the stems resulting in flute-shaped cankers. The anamorph stages of *N. fuckeliana* are seldom observed on affected trees.

Consistent with the fact that *N. fuckeliana* is a relatively newly discovered pathogen in Chile, very little research has been afforded to it. A project carried out by Universidad Austral, with the support of the three main forestry companies and government funding, has determined some general aspects related to the life cycle and epidemiology of the pathogen (Morales 2013). Additionally, a great deal of effort has been made by Bioforest, the research company of the Arauco forestry group, to define a strategy that can protect the quality of the tree parts of greatest value, which corresponds to the bottom 5.5 meters in pruned plantations. *Neonectria fuckeliana* has now been detected from the southern Biobío region (Lebu) to Los Lagos region (Morales 2013). It is not possible to use

chemical control given the high cost and thus the most likely control strategy is related to the management of pruning periods.

### *5.3 Diplodia pinea*

*Diplodia pinea* was reported on *P. radiata* plantations in the Biobío regions of Chile in 1973 (Osorio 1977). Currently the pathogen is distributed from Valparaíso region to Los Ríos region following the geographical distribution of *P. radiata*, which is one of the most susceptible species. The most important damage caused by *D. pinea* in Chile was recorded in Maule and Biobío regions and where disease expression was associated with water stress (Osorio & Sobarzo 1986).

In Chile, sandy soils and basaltic andesite, with deficiencies in nutrients such as boron and potassium, and areas subjected to water stress favor the development of diseases (Osorio 1977). All of the symptoms described for infection by *D. pinea*, such as die-back, cankers or blue-stain (Gibson 1979; Swart et al. 1985) are found in Chile. It is common to see the fruiting bodies (pycnidia) on the bark of branches and stems. However, death of the trees is infrequent unless conditions for the trees are very unfavorable.

Given the fact that *D. pinea* is an opportunistic pathogen, no single control strategy has been defined for it. When infection occurs in nurseries, the contaminated plant material is eliminated and occasionally preventative fungicide applications are applied on cuttings and seedlings. Control programs have been established in seed orchards to reduce the incidence of cones infected with *D. pinea,* which can affect the embryos used for somatic embryogenesis.

### *5.4 Dothistroma septosporum*

*Dothistroma septosporum* causing the disease known as dothistroma needle blight was first reported in Chile in 1965 and is now found wide-spread in *P. radiata* plantations (Dubin 1965). This pathogen has been one of the most serious organisms affecting Chilean plantations of *P. radiata*, which is considered as one of the most susceptible

species to this disease (Evans 1984; Butin & Peredo 1986). It has thus been the subject of considerable research since it was first found in the country, aimed at understanding the biology, epidemiology, infection mechanisms of the pathogen, as well as to develop effective control systems (Bustamante 2000). Much remains unknown, however, regarding the impact and control of *D. septosporum*.

Studies in Chile have shown a linear relationship between height growth and defoliation due to infection by *D. septosporum*. When defoliation is lower than 25 % of the crown, the effects on growth are unimportant. However, when defoliation exceeds 50 %, the annual growth increment is strongly affected (Pérez 1973; Contreras 1988; Hauer 2000). When defoliation is 80 % or greater, the reduction in growth can be as much as 50 % (Alzamora et al. 2004).

The control strategy for dothistroma needle blight in Chile is similar to that applied in New Zealand, the USA and Kenya in the past (Thomas & Lindberg 1954; Gibson et al. 1966). Aerial application of copper-based fungicides was implemented in the 1990's and early 2000's where thousands of hectares of trees between 3 and 10-years-old were sprayed once or twice per year, depending on infection levels. Particularly in young plantations, the amount of inoculum was drastically reduced due to the use of copper. However, no fungicides have been sprayed by aerial application in the last ten years due to more environmental restrictions and also for an operational decision (MINSAL 2010). In Chile, as was in New Zealand, an intensive silvicultural management program was implemented (pruning and thinning) in the commercial plantations to provide more ventilation inside of the plantations and thus to reduce the humidity and the consequent risk of infection.

Selection of *P. radiata* planting stock resistant to dothistroma needle blight has not been consistently applied in Chile. Thus the country does not have evidence such as that available in New Zealand, Australia and the USA to suggest that different families of *P. radiata* differ in their susceptibility infection by *D. septosporum* (Contreras 1988; Elmudesi 1992).

## *5.5 Phytophthora pinifolia*

*Phytophthora pinifolia* is the most recently discovered pathogen on *P. radiata* in Chile (Durán et al. 2008). The pathogen, not known to occur anywhere else in the world, causes the disease that has been given the name Daño Foliar del Pino (DFP). The disease occurs only on *P. radiata* in plantations mainly in the coastal area of the Biobío and Los Ríos Regions (Ahumada et al. 2013). The first symptoms of the disease were observed on the Arauco coast during the winter 2004 causing a discoloration of the foliage followed by defoliation and death of adult trees. It also causes death of trees in one and two-year-old plantations where the damage is often very dramatic (Durán et al. 2008; Ahumada et al. 2013). The affected areas increased from 3 300 ha in 2004, to 30 000 ha in 2005 and 54 000 ha in 2006. The affected area has, however, subsequently decreased to less than 1 000 ha between 2009 and 2012 (Ahumada et al. 2013). This decrease in infection is clearly linked to climate and to the removal of *P. radiata* from areas of highest risk to infection.

Daño Foliar del Pino is normally observed from early autumn to late spring, which coincides with the rainfall season. Infected needles display typical resinous bands across their laminas. The dead needles can remain attached to the stems until the late spring when wind causes them to fall from the trees and branches. Very little is known regarding the biology of *P. pinifolia* such as the factors that affect sporulation and the infection process. Most of the sporangia are produced on green or dead needles on the trees during the winter, which is also the season when rain occurs in Chile. Needle wetness and temperature are important factors for the development of DFP symptoms. The incidence of new infections has been monitored using trap plants and new infections have been shown to occur throughout the year with the highest incidence during the wet winter and spring months (Ahumada et al. 2013).

Management of DFP in *P. radiata* plantations has included the application of chemicals such as mefenoxam and Phosphorous acid, selection of tolerant clones, selection of sites with low risks of infection and replacement of *P. radiata* with *Eucalyptus globulus* or *E. nitens*, depending on the site conditions. Control using chemicals must occur before the appearance of the first symptoms. On the Arauco coast, applications are made from April

to July in plantations older than one year. To achieve a good plantation establishment, all the plants introduced into a planting area having medium risks of infection, are treated in the nursery before they are planted. They are then treated during the following three months with phosphates to prevent the development of DFP.

Research (R. Ahumada, unpublished data) has shown that some *Pinus* species are tolerant to the infection and also that some clones of *P. radiata* appear to be more tolerant to the infection than the general population. Furthermore, a population diversity study with *P. pinifolia* (Durán et al. 2010) showed that the species involved in outbreaks have a very low genetic diversity, and might be clonal. These preliminary results showing the existence of some levels of host resistance, together with the near-clonal population structure of *P. pinifolia*, suggest that there is an opportunity to strengthen the management of the disease through selection and hybridization.

## 6. Native pathogens and pests adapted to *P. radiata*

Very little is known about native pathogens that have caused diseases on *P. radiata* in Chile. Little research has been conducted in order to identify and understand native fungi, adapted or present in *P. radiata* plantations and there are few native pathogens causing damage in *P. radiata* plantations. One of the most important pathogens has been the *Armillaria* sp. classified as *A. mellea*. Coetzee et al. (2003), however, showed that this species represent two taxa, namely *A. luteobubalina* isolated from *P. radiata* and *A. novae-zelandiae* isolated from a *Nothofagus* species.

There are some examples of native insects that infest *P. radiata* in plantations and sometimes have been cause of damage. These include *Ormiscodes socialis* Feisth. (Lepidoptera: Saturnidae), *Antandrus viridis* Blanchard (Orthoptera: Acrididae) and *Coniungoptera nothofagi* Rentz y Gurney (Orthoptera: Tettigoniidae) (Lewis 1996) (Figure 3).

## 7. Importance of the quarantine systems

Quarantine regulation systems are formulated by governments to prevent the introduction and spread of pathogens that may affect natural forests and exotic plantations (SAG 2013a). Since the 1950's, such quarantine issues have been coordinated by the Food and Agricultural Organization (FAO) (Mathyus & Smith 1984; Schrader & Unger 2003). Quarantine is achieved through prohibition of movement, interception and elimination of pests and diseases at various levels, including pre-entry, entry and post entry points of the countries (Sharma & Thakur 2007). Globalization has given rise to a very substantial increase in trade between countries (and continents), greatly increasing the use of wooden containers to protect machinery and equipment and to store products that are distributed rapidly across large distances. This pattern has increased the risk of introducing new pests and pathogens (Mathyus & Smith 1984; Burgess & Wingfield 2001). Germplasm including plant material and seed represents a serious hazard, and contributes seriously to the movement, dissemination, dispersal and introduction of new and threatening pests and pathogens to forest ecosystems (Wingfield et al. 2001; Sharma & Thakur 2007; Stenlid et al. 2011).

Distance is no longer a significant constraint for trade between countries and this implies greater risks for the introduction of pests and diseases. Many examples can be mentioned in forestry where pests and pathogens have been introduced from neighboring countries. Thus in the case of Chile, *R. buoliana* (1985), *Gonipterus platensis* Marelli (1998), *Sirex noctilio* F. (2001) or lately *P. castaneus* (2013) have moved from Argentina to this country. There are also many examples of other pests and pathogens that have been introduced into Chile (Table 1).

Clearly there has been a dramatic increase in the numbers of exotic pests and pathogens of forestry trees introduced into new areas. New introductions of pests and pathogens also appear to be increasing and this is a trend that will continue in the future. Thus, better coordination of actions against new arrivals will be needed (Santini et al. 2013) and human activity is intensifying fungal disease dispersal by modifying natural environments (Fisher et al. 2012). One example is that of France, where new introductions increased

from 0.5 species on average per year before the 1930's to two species reported an average per annum in the 1970's. A similar situation has been observed in USA (Liebhold 2012), South Africa (Wingfield et al. 2001b; Wingfield et al. 2008b), Chile (SAG 2011) and New Zealand (Turner et al. 2004; L. Bulman 2012, pers. comm.) among many other cases, where new detections of pests and diseases have increased substantially. Thus, between 1980 and 2002, over 52 million hectares of forest in 37 countries were damaged by the introduction of pests and pathogens (FAO 2003).

Chile has a very strict quarantine system that is coordinated by the official governmental agency SAG. To prevent the introduction of new pests and pathogens that may affect forest productivity and cause severe damage to the ecology and economy of the country, controls were established on the borders with Argentina, Peru and Bolivia. Very strict controls have also been implemented in international airports and ports where transport, passenger as well as crew baggage, and commercial loads of forestry and agricultural products are inspected to ensure that they comply with health regulations established (SAG 2011). Quarantine regulations are formulated by governments to reduce the chances of pests being introduced from foreign countries and it is achieved through prohibition, interception and elimination of pests and diseases at pre-entry, entry and post-entry points (Sharma & Thakur 2007). Plant quarantine can be effective with international cooperation and agreements to apply the international standards of pest risk analysis, the requirements for the establishment of pest-free areas, surveillance, pest eradication programs and the introduction and release of exotic biological agents (Schrader & Unger 2003). The quarantine system in Chile will probably not prevent introductions of all of the possible pests and diseases, but it has certainly increased the awareness of the importance of making a major effort to delay entry of more alien forest pathogens.

## 8. Conclusions

*Pinus radiata* is the most widely planted species of *Pinus* in the world and it is valued for rapid growth and desirable lumber and pulp qualities. Most of the research on *P. radiata* has been focused on genetic and silvicultural issues and very little published information

is available regarding pests and diseases affecting this tree. This is true globally, but is especially true in Chile. Clearly the tree is highly threatened by invasive alien pests and pathogens and these have already begun to limit its planting in some areas.

Given the importance of *P. radiata*, it is ironic that so little has been done regarding the pests and diseases threatening this tree in Chile. This situation must surely change as greater numbers of pests and pathogens threaten the long-term sustainability of this tree. There has, in the past, been a very obvious lack of human capacity to deal with these problems, but this is rapidly changing with appropriate education in this field being provided. With time it must be hoped that a point will be reached where Chile can rely on a strong team of well-qualified researchers in the fields of forest pathology and forest entomology. Efforts must also be made to improve the quality of quarantine aimed at protecting the future of the forestry resource.

The research in this thesis has sought to improve this situation and thus to bring to the Chilean forestry sector more effective and robust tools to provide support for the management of the diseases in *P. radiata* plantations. The research in this thesis deals with three major pathogens of *P. radiata* i.e. *Fusarium circinatum*, *Neonectria fuckeliana* and *Phytophthora pinifolia* that are relatively new to Chile. The aim of these studies has been to advance our knowledge of the biology and control of these pathogens and thus to contribute to the long-term sustainably of *P. radiata* forestry in Chile.

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Table 1. Detection of fungal pathogens on *P. radiata* plantations in Chile in chronological order of discovery.

| Agent | Date recorded | Type of damage | Reference to first record |
|---|---|---|---|
| *Trametes versicolor* | 1920 | Wood rot | Lloyd 1920 |
| *Phytophthora sp.* | 1943 | Root rot | Anónymous 1943 |
| *Botrytis cinerea* | 1951 | Gray mold | Vergara 1951 |
| *Mycosphaerella dearnessii* | 1962 | Needle blight | Oehrens 1962 |
| *Dothistroma septosporum* | 1965 | Needle blight | Dubin 1965 |
| *Armillaria mellea* | 1966 | Root rot | Herrera 1966 |
| *Fusarium oxysporum* | 1970 | Damping off | Tollenaar et al. 1970 |
| *Pythium sp.* | 1970 | Root rot | Tollenaar et al. 1970 |
| *Rhizoctonia solani* | 1970 | Root rot | Tollenaar et al. 1970 |
| *Sphaeropsis sapinea* | 1973 | Blue stain | Osorio 1973 |
| *Ophiostoma piliferum* | 1973 | Blue stain | Butin 1973 |
| *Cyclaneusma minus* | 1973 | Needle blight | Butin 1973 |
| *Lophodermiun sp.* | 1981 | Needle blight | Rack 1981 |
| *Colletotrichum acutatum* | 1982 | Needle blight | Santamaría 1982 |
| *Macrophomina phaseolina* | 1985 | Root rot | González & Santelices 1985 |
| *Ophiostoma piceae* | 1986 | Blue stain | Butin & Peredo 1986 |
| *Sporothrix sp.* | 1988 | Blue stain | Peredo & Alonso 1988 |
| *Pesotum sp.* | 1988 | Blue stain | Peredo & Alonso 1988 |
| *Sporothrix curviconia* | 1988 | Blue stain | Peredo & Alonso 1988 |
| *Fusarium circinatum* | 2001 | Cankers/mortality | Wingfield et al. 2002 |
| *Ophiostoma ips* | 2004 | Blue stain | Zhou et al. 2004 |
| *Ophiostoma quercus* | 2004 | Blue stain | Zhou et al. 2004 |
| *Ophiostoma galeiformis* | 2004 | Blue stain | Zhou et al. 2004 |
| *Ophiostoma huntii* | 2004 | Blue stain | Zhou et al. 2004 |
| *Phytophthora pinifolia* | 2007 | Needle blight | Durán et al. 2008 |
| *Neonectria fuckeliana* | 2009 | Stem canker | Morales 2009 |

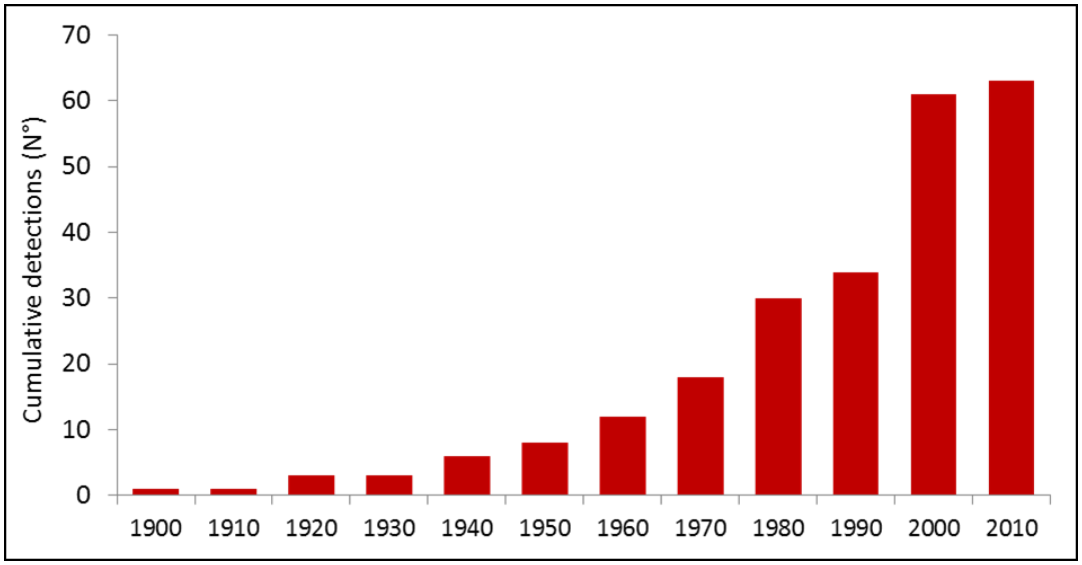


Figure 1. Cumulative detection of pests and pathogens in commercial forestry plantations in Chile over the last 110 years.

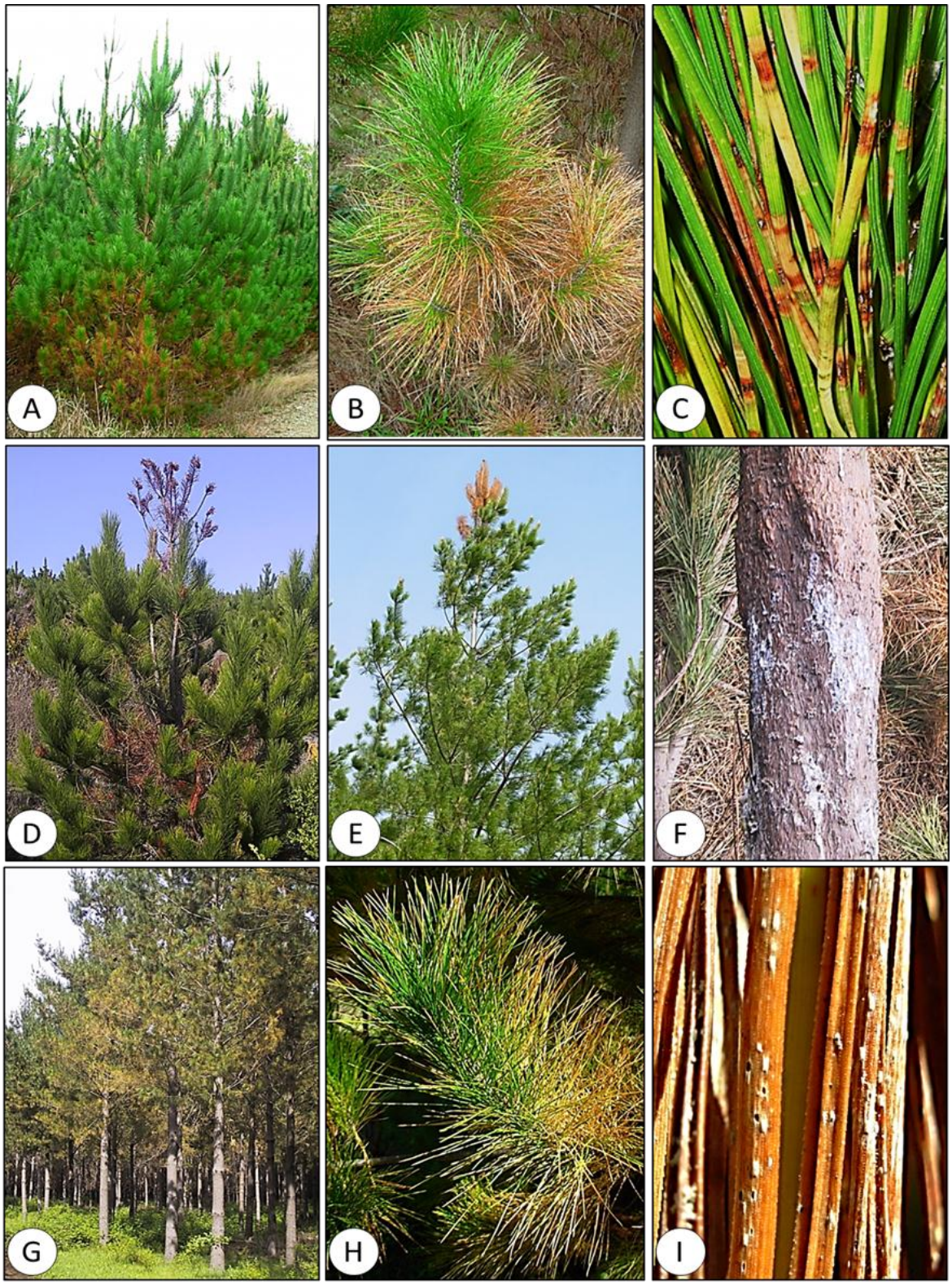


Figure 2. Some of the most important introduced pathogens affecting *P. radiata* plantations in Chile. A-C) *Dothistroma septosporum*, A-B) symptoms in needles in branches at the base of the tree, and C) red band and fruiting bodies on needles. D-F) Dieback, canker and resin resulting from *Diplodia pinea* infection. G-H) *Cyclaneusma minus* symptoms on needles of mature trees, and I) *C. minus* fruiting bodies.

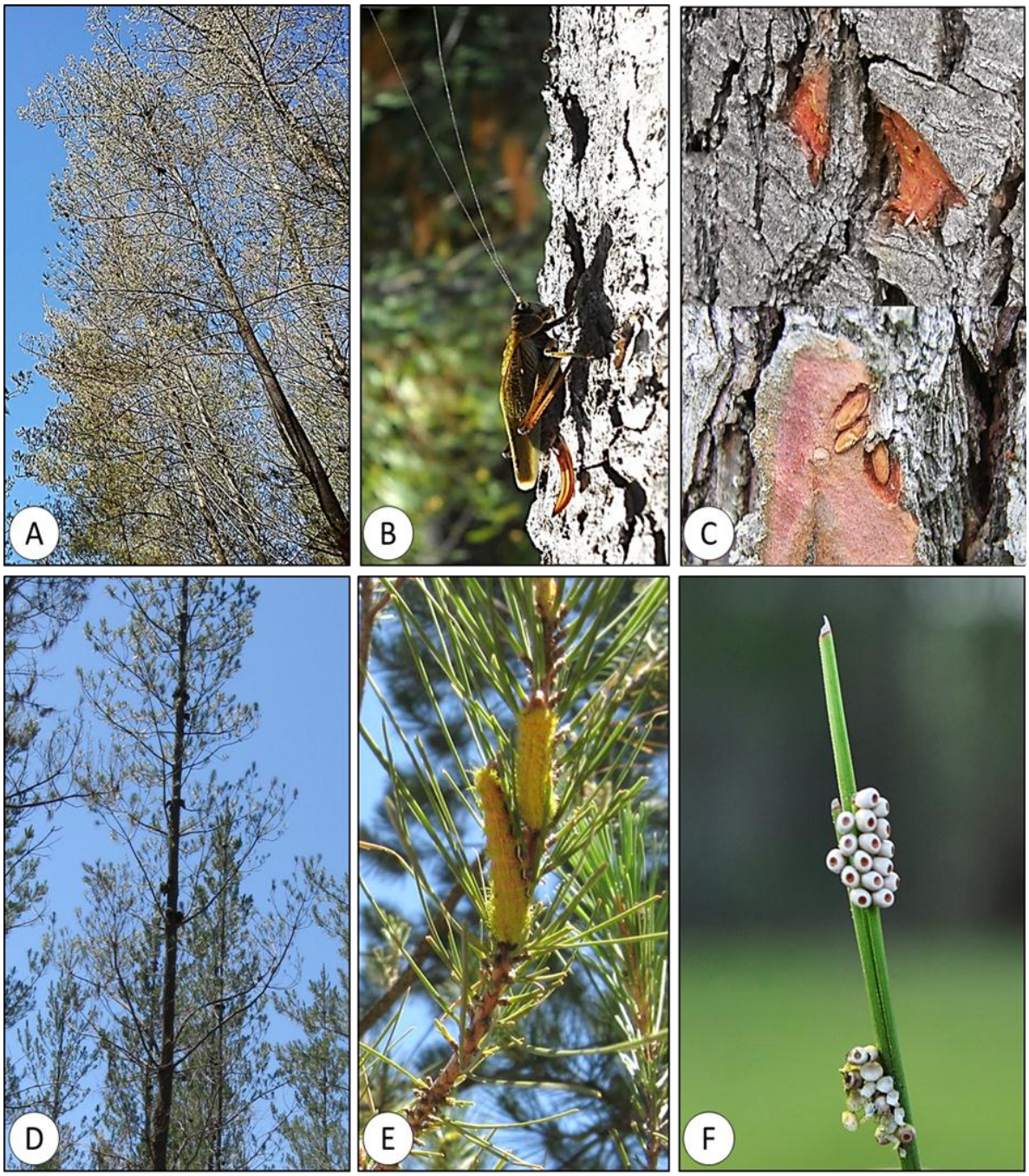


Figure 3. Two of the native insects that cause damage in *P. radiata* plantations. A-C) *Coniungoptera nothofagi*, A) Defoliation, B) adult, and C) eggs laid into the *P. radiata* bark. D-F) *Ormiscodes socialis*, D) Defoliated tree, E) larvae eating needles, and F) a group of eggs laid on a needle.

# Chapter 2

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# Pathogenicity and sporulation of *Phytophthora pinifolia* on *Pinus radiata* in Chile


## Abstract

*Phytophthora pinifolia* causes the needle and shoot disease of *Pinus radiata* known as daño foliar del pino (DFP) in Chile. The first pathogenicity trials with this organism utilized mycelial plugs placed on stem wounds. These resulted in lesions in the tissue, but did not reproduce the resinous bands on the needles, which are the most characteristics symptoms of the disease under natural conditions. In this study, stem inoculations were repeated, but to complete Koch's postulates fully, and to confirm that *P. pinifolia* causes the symptoms observed on naturally infected trees, zoospore/sporangial suspensions were used to inoculate pine foliage. This method produced the same symptoms observed on needles infected naturally. These results confirm that *P. pinifolia* is the causal agent of the Daño Foliar del Pino on *P. radiata* in Chile and successfully completed Koch`s postulates for the first time. Pathogenicity tests on different *Pinus* spp. and hybrids showed a wide range of responses to inoculation with *P. pinifolia* mycelial plugs, from highly susceptible to resistant. Monitoring of sporulation revealed that the sporangia commonly remain on the needles for extended periods of time and their frequency of occurrence and dispersal appear to increase during the rainy season.


## 1. Introduction

*Phytophthora pinifolia* is a recently described pathogen that causes the disease known as daño foliar del pino (DFP) on *Pinus radiata* in Chile (Durán et al. 2008). DFP has caused significant damage to the foliage of *P. radiata* growing in plantations in the coastal area of the Biobío and Los Ríos Regions (Durán et al. 2008). Symptoms of the disease were first observed on the Arauco coast (37° 17' S; 73° 36' W) in July 2004 on trees including a range of age classes. This was followed by mortality of one and two-year-old plantations in October 2004 (Durán et al. 2008). Annual monitoring showed a clear increase in the area affected over time, from 3 300 ha in 2004, to 30 000 ha in 2005 and 54 000 ha in 2006, with varying levels of severity across the region. The level of infection and damage

decreased substantially in the subsequent years to 21 000 ha in 2007, 1 500 ha in 2008 and less than 1 000 ha between 2009 and 2011 (R. Gómez, pers. comm.).

Daño Foliar del Pino is characterized by relatively rapid death of infected needles followed by the defoliation of trees (Durán et al. 2008). Infection is normally observed from autumn to late spring, which is also the main rainfall season. Infected needles typically display distinct resinous bands on their laminas. When the infections reach the needle bases, resin exudes from the points of attachment causing death of the cambial cells surrounding the fascicles and generating small cankers at the contact point between the needles and the stem. The dead needles remain attached to the stems until the late spring when they fall from the trees and branches. While much is known about the development of the disease caused by *P. pinifolia*, there is little knowledge regarding the biology of the pathogen including issues such as its sporulation, dispersal and how it infects trees.

*Pinus radiata* plantations in Chile have in the past been affected by shoot and needle diseases caused by *Dothistroma septosporum*, *Diplodia pinea* and *Cyclaneusma minus* (Toro & Gessel 1999; Ahumada 2003; Barnes et al. 2004). Damage has also been caused by European pine shoot moth, *Rhyacionia buoliana*, which has been the most important constraint to plantation forestry in the past (Lanfranco 2000; Ahumada 2003). However, the increase of areas infested by the woodwasp, *Sirex noctilio* and the emergence of DFP are by far the most important problems currently facing *P. radiata* forestry in Chile.

*Phytophthora* spp. are amongst the most serious and invasive plant pathogens on both agricultural and forestry crops (Fry & Goodwin 1997; Rizzo et al. 2002). In terms of forestry, the recent emergence of species such as *Phytophthora ramorum* and *P. austrocedrae* (Rizzo et al. 2002; Greslebin et al. 2007) has reinforced the fact that these pathogens are a serious threat to forestry worldwide, both in natural ecosystems and plantations (Davidson et al. 2003; Garbelotto et al. 2003; Greslebin & Hansen 2010). The appearance of a needle blight of *Pinus* spp. caused by a *Phytophthora* sp., as has occurred with *P. pinifolia* was, however, unprecedented and unexpected.

The first pathogenicity tests conducted with *P. pinifolia* using mycelial plugs showed clearly that *P. pinifolia* was able to infect and cause disease in the shoots in a period of less than 30 days (Durán et al. 2008). However, the typical DFP symptoms observed in the field have never been artificially reproduced. In this study, we confirm the results from previous mycelial inoculation studies regarding the pathogenicity of *P. pinifolia*, and extend these to additional *Pinus* spp. that are of importance as potential plantation species in Chile and other parts of the world. The primary aim was, however, to produce sporangia and zoospores of *P. pinifolia* and to attempt to reproduce the characteristic resinous bands on the foliage of *P. radiata*, to re-isolate the organism from the infected needles, completing Koch's postulate of proof for the organism. The focus on infection propagules also provided a parallel opportunity to study the means of dispersal of *P. pinifolia,* by monitoring sporangial production and potential correlation with rainfall across different areas.

## 2. Materials and methods

### 2.1 Isolates and inoculum production

The three isolates used throughout the study were selected from a previous investigation where six isolates were inoculated into 180 1-year-old *P. radiata* plants (30 per isolate) and where it was shown that they were all pathogenic and did not differ in their relative levels of aggressiveness (R. Ahumada, unpublished). One isolate (CMW33986) of *P. pinifolia* was used in the first experiment with mycelial plugs and three isolates (CMW33983, CMW33986 and CMW34012) in the second experiment using suspensions of sporangia and zoospores. All the isolates were obtained from needles of *P. radiata* with the resinous bands characteristic of infection by *P. pinifolia*. The three isolates selected for the inoculations were chosen because they were similar in relative aggressiveness in a previous study (R. Ahumada, unpublished).

Isolates were identified based on culture morphology on the selective medium CARP (Hansen & Hamm 1996) and using the species-specific primers developed by Durán et al. (2009). Emerging colonies were transferred to Carrot Agar (CA) (Erwin & Ribeiro 1996)

and maintained between 18–22 °C for 20 days until they could be identified. All three isolates have been maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

For inoculum production, 20 replicates of each isolate were grown in V8 agar at 22 °C for two weeks. From the edges of actively growing cultures, 5 discs (7 mm diam.) were transferred to a 60 mm Petri dish containing 25 ml of 10 % V8 broth and incubated for 24 h at 22 °C (Erwin & 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 examined for the presence of sporangia and those bearing these structures were chilled at 4 °C for 2 h to induce the release of zoospores. The primary zoospore suspension was poured into a sterile beaker and maintained at 4 °C until inoculation. Three aliquots of 10 µl were taken from the beaker and used to determine the zoospore concentration using a hematocytometer. The final zoospore suspension was prepared by adding autoclaved distilled water and adjusting this to approximately 5 x $10^4$ zoospores $ml^{-1}$. The zoospore suspension was maintained at 4 °C and transported to the field for inoculation on the same day. To determine the viability of the zoospores, three aliquots of 30 µl of the suspension were sampled in the field after the inoculation and spread onto CARP, transported to the laboratory at 4 °C, incubated at 22 °C for two weeks and evaluated for growth.

## 2.2 Inoculation experiments

The inoculations were performed in two different experiments, under laboratory conditions and in the field. The laboratory trials were conducted at Bioforest research facilities in Concepción. The screening facility was set to provide a photoperiod of 12 h of artificial light, at 75 % relative humidity, a temperature of between 18–22 °C and the containers were irrigated daily for 1 h.

The field experiment was installed on the Llico farm (37° 22' S; 73° 58' W; Arauco province). A shade house (70 % shade netting) was erected for this inoculation study and

a fogging system was installed and applied for 15 minutes, three times per day (10, 14 and 18 hours) to maintain a high level of humidity over the foliage of the inoculated trees. The shade house was surrounded by a plantation of *Eucalyptus globulus* trees so as to reduce any chance of natural infection by *P. pinifolia*.

### 2.2.1 Inoculation of *Pinus* spp. with *P. pinifolia* mycelium plugs

Twenty plants (15-month-old, an average of 22 cm tall and 0.8 cm average diam. at the substrate level) of each of thirteen *Pinus* spp. including varieties [*P. arizonica* (PAR), *P. durangensis* (PDU), *P. greggii* mix of families (PGR1), *P. greggii* var *australis* (PGR2), *P. greggii* var *greggii* (PGR3), *P. maximinoi* (PMA), *P. muricata* (PMU), *P. patula* mix of families (PPA1), *P. patula* var *longipedunculata* (PPA2), *P. patula* var *patula* (PPA3), *P. pinaster* (PPI), *P. radiata* (PRA) and *P. taeda* (PTA)] were established in 140 cc containers. The plants were acclimatized for two weeks prior to inoculation in the screening facilities using the conditions described above. Small bark discs (4 mm diam.) were removed from succulent tissue of the stems, between 10 to 12 cm from the growing tips and a plug of mycelium (3-week-old culture of *P. pinifolia* grown on CA) of similar size was placed into the wounds. Discs of clean CA were used as negative controls. Fifteen plants of each species and the varieties were inoculated with the isolate CMW33986 of *P. pinifolia* and five plants per species were inoculated as controls. Inoculation wounds were covered with Parafilm to reduce desiccation and contamination. Four weeks after inoculation, the Parafilm and the bark around the inoculation points was removed with a sterile scalpel and the lesion lengths were recorded.

Re–isolation was attempted from the leading edges (top and bottom) of the lesions on all inoculated plants. Small pieces (< 5 $mm^2$) of succulent infected tissue from the leading edges of the lesions were plated onto CARP medium to re–isolate the inoculated organism and ensure that it was associated with the lesions. The identity of 10 % of the resulting cultures that were characteristic of *P. pinifolia* was confirmed using the PCR specific primers (Durán et al. 2009).

Analysis of data was conducted separately for all *Pinus* species, using the linear model of analysis of variance (ANOVA) and means were separated based on LSD (Least Significant Difference) using the software Statistica V9 for Windows (StatSoft 2004).

### 2.2.2 Inoculation of *P. radiata* with *P. pinifolia* zoospores

This experiment was conducted with a suspension of zoospores and sporangia using a mixture of the three isolates as described above. An equivalent volume of zoospore suspension (adjusted to approximately 5 x $10^4$ zoospores $ml^{-1}$) was transferred to forty 2 L plastic bags, in which individual *P. radiata* plants were immersed (75 % of their foliage) overnight (~12 h). After immersion, half of the plants (20) were placed in the screening facility in the laboratory and the other half (20) transported in a cooler box, to the shade house in the field on the Llico farm. Forty plants, with the same characteristics as those inoculated, were used as controls and were dipped into sterile distilled water overnight. Half of the plants were placed in the laboratory in an area separate from the plants inoculated with *P. pinifolia,* to avoid cross contamination and the other half were transported to the field and placed in the shade house together with the inoculated plants. All plants were inspected weekly for the appearance of symptoms.

Four weeks after the inoculation, symptoms were recorded on all inoculated plants. Evaluations included the presence (1) or absence (0) of resinous bands on the needles of both inoculated and control plants. A needle was considered to be infected if it had at least one resinous band. A sample (~10 %) of the bands produced on the needles was used for re–isolation of the inoculated pathogen using the same methodology described above for plants inoculated with mycelial plugs.

A Bayesian approach was used for data analyses. The variable response was assumed to be a binomial random variable and the percent of infection was set to a beta prior distribution. The analyses were separated between the plants in the laboratory and the plants maintained in the field, in order to avoid possible confounding effects in the responses measured. Bayesian credible intervals for each proportion ($\pi$) were computed using Win BUGS (Bayesian Inference Using Gibbs Sampling) software (Lunn et al. 2000).

The Gibbs sampling chain was run for 11,000 iterations with the first 1,000 iterations used as burn-in. The convergence of the Gibbs chains was checked using the Heidelberger and Welch's test (Heidelberger & Welch 1983).

Fluorescence microscopy was used to provide an additional confirmation of the presence of *P. pinifolia* on the foliage. A sub-sample of 10 % of the needles with resinous bands 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. These needles were mounted on glass slides and examined under a fluorescence microscope (Olympus CX31, Olympus America Inc.) for the presence of sporangia or mycelium on the needle surfaces.

### 2.2.3 Monitoring the presence of sporangia in plantations

Six field sites were selected for monitoring the presence of *P. pinifolia* inoculum in a natural environment. This monitoring was carried out between May 2009 and December 2011. The selected sites were located in three 7-year-old *P. radiata* plantations that have been historically infected with *P. pinifolia*. Each monitoring site consisted of a block of approximately 100 $m^2$ (20 m x 5 m) from which 10 trees with typical symptoms of *P. pinifolia* damage were selected. From each tree, 20 needles were sampled twice each month and transported to the laboratory for analysis. In the laboratory, a minimum of 50 needles from the total collection (~200 per site) having clear resinous bands were selected for detailed examination. For each needle, a section of approximately 4 cm was cut around the resinous band. These needle pieces were immersed in a solution of Calcofluor Fluorescent Brightener 28 for 30 sec and then mounted on glass slides for microscopic observations. All observations were made using an Olympus fluorescence microscope as above, to quantify the presence of inoculum (sporangia, zoospores and mycelium) on the needle surface. The sections of every needle examined corresponded to approximately 30 stomata and thus, approximately 18 000 stomata (30 stomata x 50 needles x 6 areas x 2 sampling periods) were examined each month.

The data recorded using fluorescence microscopy were tabulated and then plotted as monthly average of sporangia per needle (or per resinous band abbreviated as RB). The

monthly rainfall was also recorded at the sampling sites during the same period of observation. The average of inoculum (sporangia) per month was plotted together with the rainfall for the entire evaluation period.

## 3. Results

### 3.1 Inoculation experiments

#### 3.1.1 Inoculation of *Pinus* spp. with *P. pinifolia* mycelium plugs

Eleven of the 13 inoculated *Pinus* spp. or varieties developed wilting shoots and lesions around the inoculation points within 30 days (Figure 1). The percentage of plants with symptoms ranged from 13 % in the *P. patula* mix of families to 100 % in both *P. radiata* and *P. muricata* while *P. pinaster* and *P. taeda* seedlings were free of any symptoms when this experiment was terminated. Mean inner lesion lengths ranged from 17.2 mm in the *P. patula* mix of families to 54.6 mm in *P. durangensis*. Other species with large average lesion lengths were *P. radiata* (50 mm), *P. greggii* var *greggii* (49.3 mm) and *P. arizonica* (48.9 mm), while the species with the lowest average lesion lengths were *P. greggii* var *australis* (24.3 mm), *P. greggii* mix of families (28.9 mm) and *P. patula* var *longipedunculata* (31.9 mm). *Pinus patula*, *P. greggii* and its varieties tested showed a wide range of symptoms and lesion lengths.

*Phytophthora pinifolia* was consistently re-isolated from the lesions and the identity of the cultures selected was confirmed in 100 % of the cases using PCR-specific primers. None of the control plants in the all species treated showed any evidence of symptoms and *P. pinifolia* could not be isolated from them.

#### 3.1.2 Inoculation of *P. radiata* with *P. pinifolia* zoospores

After 4 weeks, plants inoculated with the *P. pinifolia* zoospore suspensions showed the characteristic symptoms on the needles but a non-uniform distribution of the resinous bands (Figure 2). No stem necrosis was observed on any of the inoculated *P. radiata*

plants. Seventy seven percent of the inoculated plants in either the laboratory or the field trials showed the presence of these resinous bands characteristic of the DFP (Figure 2). There was no evidence of symptoms on untreated plants either in the laboratory or the field trial (Figure 2).

Eighty-three percent of the inoculated plants maintained in the laboratory developed symptoms, while those maintained in the field showed a lower incidence of symptomatic seedlings (71 %). The Bayesian credible interval, however, showed that these differences were insignificant and the only statistically significant differences were those between the inoculated plants and the controls (Table 1).

More than 95 % of the isolations made from the resinous bands yielded isolates having characteristic *P. pinifolia* morphology and those analysed with PCR specific-primers were all confirmed to be of this organism. It was possible to consistently produce the necessary amount of inoculum for the all experiments and zoospores were shown to have an average of 86 % viability.

### 3.1.3 Monitoring the presence of sporangia in plantations

Monitoring the presence of *P. pinifolia* sporangia and zoospores on infected needles in the field showed that the characteristic resinous bands were present (Figure 3) during the entire assessment period between May 2009 and December 2011. The variation in the incidence of resinous bands on the needles was closely correlated with the number of sporangia present on the needles. The highest monthly value for inoculum was observed in September 2009 with 16.7 sporangia per resinous band (RB), September 2010 with 14.6 sporangia per resinous band and then in July 2011 with 6.3 sporangia per resinous band. There was a clear decreasing trend in the average of inoculum from 2009 to 2011, with an annual average of 11.7, 8.3 and 1.8 sporangia per resinous band, respectively (Figure 3). The average rainfall between May to November (rainy season) showed a similar trend of that observed for the sporangia. The highest annual rainfall between May to December was of 1 489 mm in 2009, decreasing to 1 368 mm in 2010 and then to 1 154 mm in 2011 (Figure 3).

Fluorescence microscopy made it possible to confirm the presence of sporangia that typically emerged from the stomata on the resinous bands and sporangia were found in about 62 % of the needles sampled (Figure). Despite the fact that 84 % of the sporangia were open (Figure 4), zoospores (Figure 4) were seen only infrequently.

## 4. Discussion

While it was been relatively certain that *P. pinifolia* causes the needle disease DFP on *P. radiata* in Chile, it has never been shown experimentally that this organism can cause symptoms identical to those associated with natural infections. Results of this study have resolved this problem and have completed Koch's postulates, showing unequivocally that *P. pinifolia* causes DFP on *P. radiata.*

In this study, where *P. radiata* tissue was immersed in zoospore/sporangial suspensions, infection occurred only on needles and never on the succulent stem tissue. This is an important observation because green stem tissue is commonly seen to be infected under field conditions (Durán et al. 2008). Results of this study also confirm that natural infection occurs mainly via the needles and that *P. pinifolia* moves down the needles to infect the stem tissue. Despite these observations, it is not clear whether the succulent tissue of young stems can be infected directly although this seems not to be the case from the results of the present study.

Pathogenicity tests made by inoculating stems of 13 different *Pinus* spp. and varieties with mycelial plugs, showed a significant variation in the susceptibility of the species tested, from apparently resistant species such as *P. pinaster* and *P. taeda* to highly susceptible species including *P. durangensis*, *P. radiata* and *P. arizonica*. Despite the fact that *P. pinifolia* caused infections on various *Pinus* spp., operational field monitoring carried out since 2006 has never found evidence of infection in other plants such as *Nothofagus* spp., *Eucryphia cordifolia*, *Luma apiculata*, *Chusquea quila*, *Aristotelia chilensis,* which are very common in the area (unpublished data). This is in contrast to many other *Phytophthora* spp., such as *P. ramorum* that has a wide range of hosts (Davidson et al. 2003; Rizzo et al. 2005; Tooley & Browning 2009). The fact that *P. pinifolia*

has been found only in Chile and its low genetic diversity (Durán et al. 2010) suggests strongly that it is a pathogen specific to *Pinus* spp.

Stem inoculation with mycelial plugs gave similar results to those observed in a previous study (Durán et al. 2008). The inoculated seedlings showed a general chlorosis of the needles, but did not produce the characteristic symptoms on the needles. The stem inoculations were, however, substantially more efficient in terms of the time needed to prepare inoculum and the evaluation of symptoms. Lesion lengths were easily measured and showed a clear tendency in terms of susceptibility. This inoculation method has been used many times as a test of the susceptibility of different hosts to *Phytophthora* spp. (Rizzo et al. 2002; Hüberli et al. 2002; Oh et al. 2006; Durán et al. 2008; Greslebin & Hansen 2010) and appears to offer a reliable means to estimate broad differences in susceptibility of *Pinus* spp.

*Phytophthora pinifolia* is unlike other aerial *Phytophthora* spp. where sporangia are caducous and serve as the major dispersal propagules. This is for example the case of *P. ramorum, P. kernoviae, P. cactorum, P. infestans* and *P. capsici*, which typically produce caducous sporangia, as the main propagules for dissemination (Erwin & Ribeiro 1996; Hansen et al. 2003; Brasier et al. 2005; Rizzo et al. 2005). Our results also showed that sporangia are produced prolifically from the stomata of infected needles and specifically from the areas where the typical resinous bands are present. Empty sporangia can be seen suggesting that zoospores are released from the intact sporangia on the needles. The sporangiophores appear to twist and break at some distance from the sporangia, although this was observed only occasionally. The strong coastal winds and the rain-splash during the rainy season on the coast of the Biobío region of Chile would facilitate this form of dispersal. This suggests that these structures can also represent a means of spread for *P. pinifolia* but our observations suggest that this happens infrequently and it is a question that deserves further study.

The zoospore/sporangia inoculation technique used in this study was technically challenging and is unlikely to be suitable in its current form for large scale experimentation and resistance screening. This is in contrast with other systems, such as

those of Hansen et al. (2005) who found that leaf-dip inoculation represented a rapid and reliable method to predict susceptibility to *P. ramorum* infection. In the present study, inoculation with zoospores reproduced the typical symptoms of Daño Foliar del Pino, but it was variable and inconsistent in the level of infection observed. For example, the number of resinous bands produced per plant in the laboratory was different to that seen under field conditions, although these differences were not statistically significant. In contrast with *P. ramorum,* which is very easy to inoculate by spraying of zoospores (P. Resser, pers. comm.), this has not been achieved with *P. pinifolia* thus far. It will thus be important to develop a more complete understanding of the factors influencing infection by *P. pinifolia*, in order to standardize the protocols for resistance screening.

Detailed monitoring of sporangial development highlighted key aspects of the biology and epidemiology of *P. pinifolia*. It was evident that sporangial development was closely linked to rainfall patterns. Not only did sporangial development correlate with the onset and seasonal variation of rainfall, but it was also remarkably closely linked to the amount of rainfall in a particular period of time. The same patterns have also been observed with *P. ramorum* in California and Oregon, which has a Mediterranean climate with a distinct wet and dry season, and sporulation of the pathogen with subsequent plant infection appear to be primarily restricted to the rainy season (Davidson et al. 2005; Rizzo et al. 2005).

This study allowed a deeper understanding of *P. pinifolia* as the cause of the Daño Foliar del Pino on *P. radiata* in Chile, because it was possible to reproduce symptoms identical to those observed in natural infections. The pathogenicity test showed that *P. pinifolia* is not specific to *P. radiata* and was able to infect many of the other *Pinus* spp. and hybrids that were artificially inoculated. It was also shown that two of the 13 tested species (*P. pinaster* and *P. taeda*) appeared to be most resistant. In the case of *P. pinaster,* this confirmed field observations where the species shows no symptoms when grown close to heavily infected *P. radiata* trees. The sporangial monitoring demonstrated that propagules of *P. pinifolia* remain on the foliage throughout the year, but their presence is very closely correlated with climatic conditions, of which rainfall is most important.

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Table 1. Incidence of *P. pinifolia* symptoms on *P. radiata* plants inoculated with zoospores and maintained in the laboratory or under field conditions.

| Treatments | mean | | median | sd | MC error [1] | Credible Interval [1] | |
|---|---|---|---|---|---|---|---|
| | | | | | | 2.5% | 97.5% |
| Control | 0.0238 | a | 0.0166 | 0.02335 | 2.21E-01 | 0.0064 | 0.0879 |
| Laboratory inoculation | 0.8333 | b | 0.8380 | 0.05683 | 6.27E-01 | 0.7092 | 0.9278 |
| Field inoculation | 0.7136 | b | 0.7168 | 0.06964 | 7.43E-01 | 0.5669 | 0.8386 |

[1]. MC error refers to mean square error and Credible intervals proportion ($\pi$) were computed using Gibbs sampling. Values in column of means followed by the same letter are not significantly different according to the Bayesian credible interval.

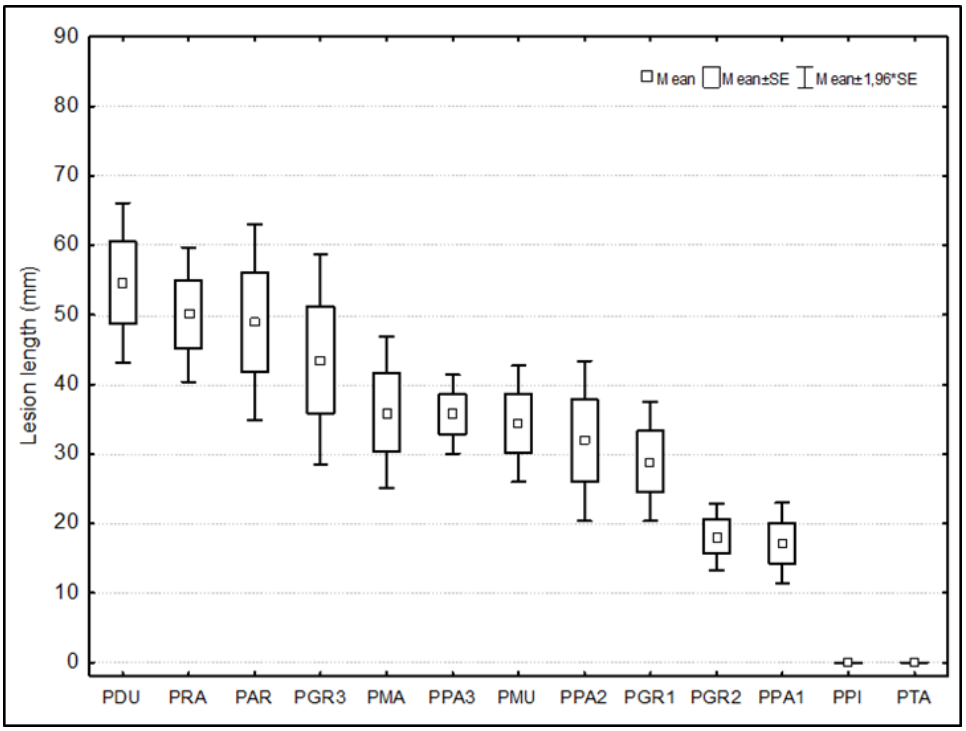


Figure 1**.** Average lesion lengths on 13 *Pinus* spp. (or varieties) inoculated with *P. pinifolia* isolate (CMW33986) into the succulent stem tissue with a plug of agar covered with mycelium or sterile agar in the case of the controls. PDU = *Pinus durangensis*; PRA = *P. radiata*; PAR = *P. arizonica*; PGR3 = *P. greggii* var *greggii*; PMA = *P. maximinoi*; PPA3 = *P. patula* var *patula*; PMU = *P. muricata*; PPA2 = *P. patula* var *longipedunculata*; PGR1 = *P. greggii* mix of families; PGR2 = *P. greggi* var *australis*; PPA1 = *P. patula* mix of families; PPI = *P. pinaster* and PTA = *P. taeda.*

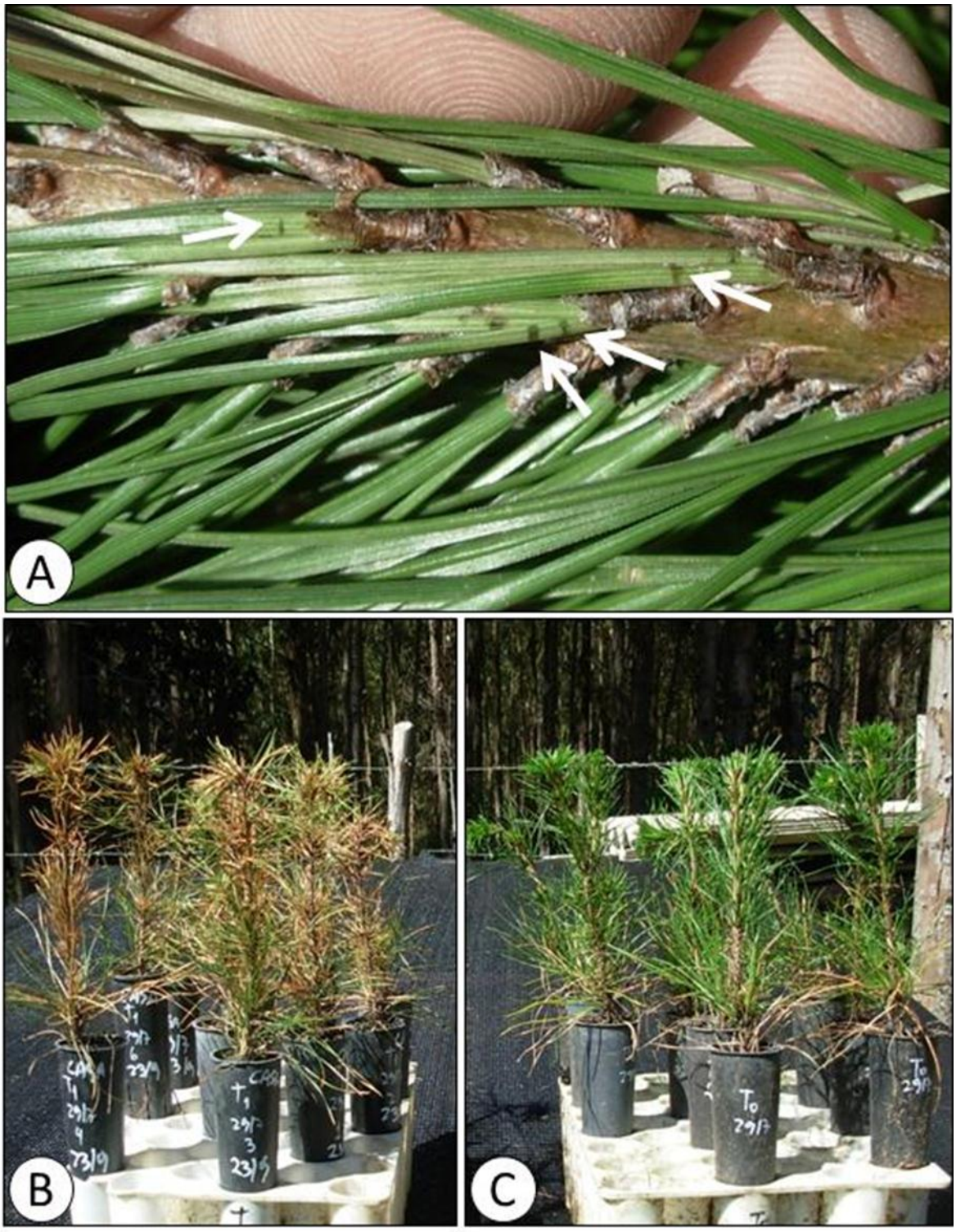


Figure 2. Seedlings used in the inoculation trials. A) Seedlings with resinous band (black bands) highlighted with the white arrows, after the inoculation. B) Seedlings inoculated with *P. pinifolia* zoospore suspensions with dying or brown foliage, and C) seedlings used as controls and inoculated with sterile water with green non-infected foliage.

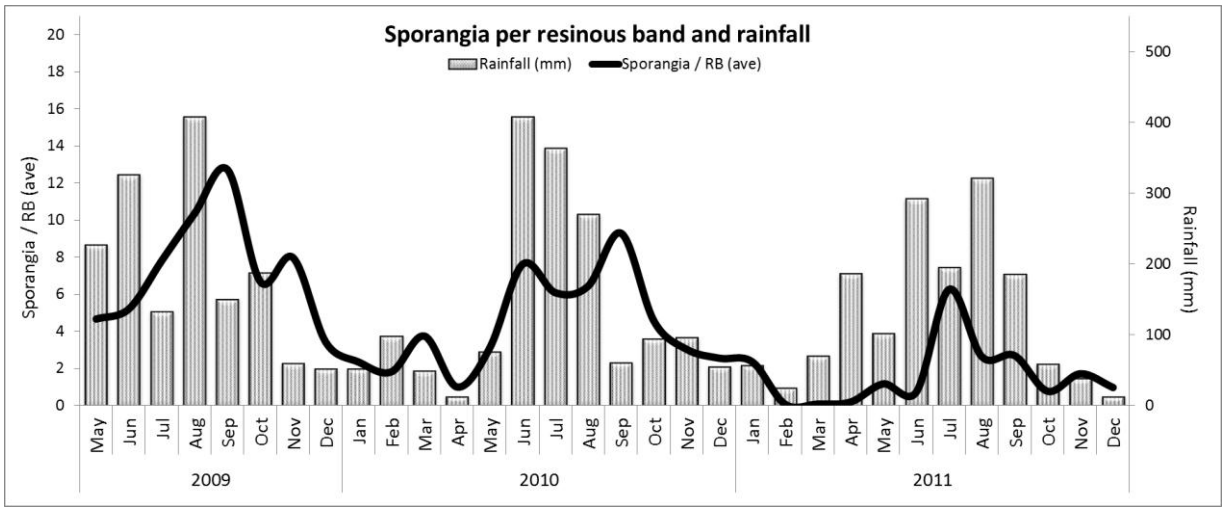


Figure 3. Mean monthly rainfall in 2009, 2010 and 2011 (bars) and the average number of sporangia on resinous bands (RB), (solid line).

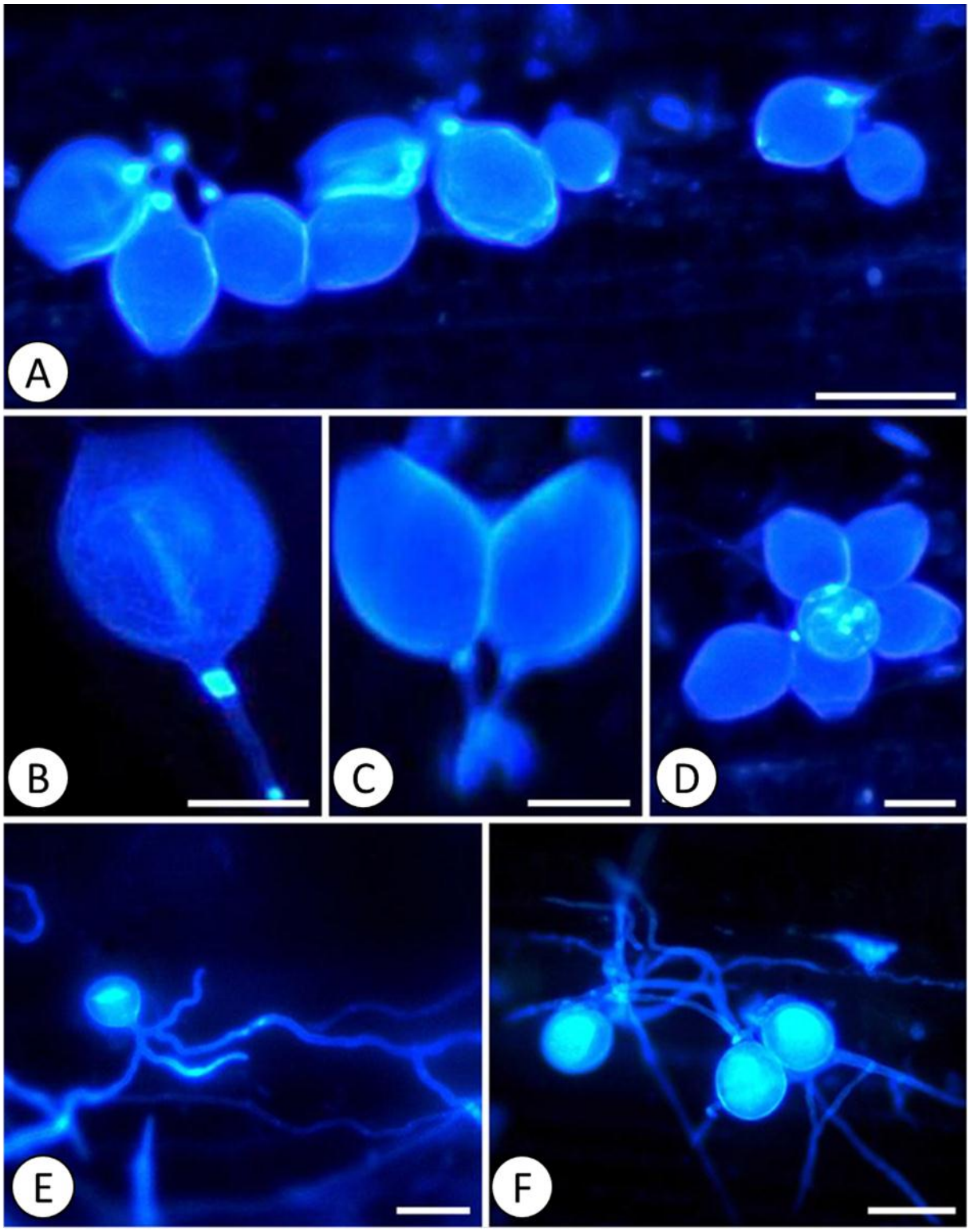


Figure 4. Sporangia A-D) and germinating zoospore E-F) seen on the surface of *P. radiata* needles having symptoms of infection by *P. pinifolia* (resinous bands) viewed with fluorescence microscopy). Scale bars are: A = 50 µm, B-D = 25 µm and E-F = 10 µm.

# Chapter 3

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

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


## 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.


## 1. 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 & 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’ S, 72° 31’ W) and in some young plantations in the Maule region (Constitución locality: 35° 25’ S, 71° 39’ 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 & 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 (*Chamaecyparys 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 & Hansen 1987; Hansen et al. 1989; Greslebin & 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 & Hansen 1987; Rizzo et al. 2002; Davidson et al. 2003; Garbelotto et al.

2003; Brasier et al. 2005; Brown & Brasier 2007; Parke et al. 2007; Moralejo et al. 2009; Greslebin & 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 & 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.

## 2. Materials and Methods

### 2.1 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 & 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 hematocytometer. The final zoospore suspension was prepared by adding autoclaved distilled water to adjust the concentration to approximately 5 x $10^4$ 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 & Ribeiro 1996). The number of colony-forming units was counted and the zoospore viability recorded.

## 2.2 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 x $10^4$ 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 1). 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 $mm^2$) 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' 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 placed on the surface of plastic trays, 10 cm above the ground, to prevent flooding due to rainfall (Figure 1). 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 $mm^2$ 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).

## 2.3 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 1).

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 1), 75 pieces of bark approximately 10 $cm^2$, each including resinous cankers or evidence of a potential infection (Figure 1) 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 (Table 2). 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.

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 $mm^2$) were selected randomly from the lesions. The wood discs were analyzed by taking five wood pieces (2-3 $mm^2$) 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 $mm^2$) 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.

## 3. Results

### 3.1 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 1) 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.

### 3.2 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 of 70 % (462 from 660) of the samples. *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.

## 4. 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 & 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 & 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 &

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.

## 5. 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.

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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%) |

Table 2. Origin of samples analyzed and the presence of *P. pinifolia*.

| No of trees examined | Type of material | Samples (No) | Cultures (No) | *P. pinifolia* (+) (No) | (%) |
|---|---|---|---|---|---|
| 45 | Foliage | 165 | 660 | 462 | 70,0 |
| | Bark | 75 | 375 | 6 | 1,6 |
| | Wood bolts | 36 | 130 | 0 | 0,0 |
| | Lumber | 40 | 200 | 0 | 0,0 |

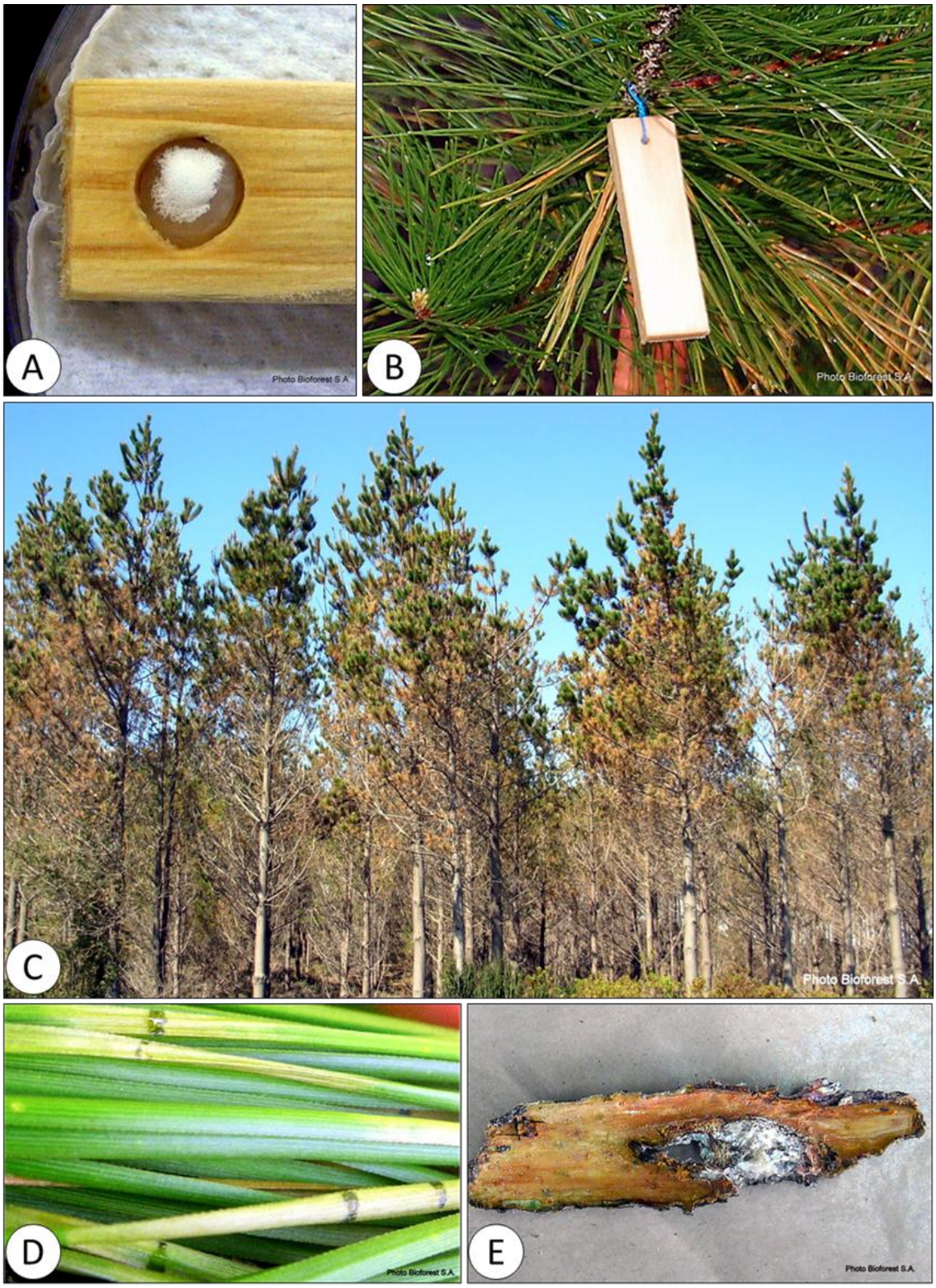


Figure 1. Symptoms of *P. pinifolia* infection and material used in isolations. A) Punched sample in the wood with a disc of colonized agar. B) Wood block tied to infected apical part of tree optimizing exposure to natural inoculum. C) Infected *P. radiata* plantation selected for sampling timber. D) Branch with infected needles showing black bands typical of infection E) Inner part of a bark sample showing a typical lesion in the cambium.

# Chapter 4

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# *Phytophthora pinifolia*: the cause of Daño Foliar del Pino on *Pinus radiata* in Chile

## 1. Introduction

*Phytophthora pinifolia* was first observed in *Pinus radiata* plantations in 2004 in the coastal areas of the Arauco province of Chile. *P. pinifolia* causes a disease known locally as "Daño Foliar del Pino (DFP)" which translates as Pine Foliar Damage. *P. pinifolia* is unusual because it is the only known *Phytophthora* infecting the needles and succulent tissue of any *Pinus* spp. (Durán et al. 2008). *P. pinifolia* was formally described in 2008 and the origin is unknown (www.fabinet.up.ac.za/tpcp/pinifolia; Durán et al. 2008).

Symptoms of DFP were first observed in July of 2004 and by October of 2004 there was mortality of one and two-year-old plantations (Durán et al. 2008; Ahumada et al. 2013). Currently, DFP infected trees are present in plantations in the coastal Biobío and Los Ríos regions and in some young plantations in the Maule region. Annual monitoring indicates affected areas increased from 3 300 ha in 2004 to 54 000 ha in 2006 and by 2007 had decreased to about 2 000 ha and have remained at this level through 2011 (R. Ahumada, unpublished). The overall reduction is likely due to multiple factors including non-conducive environmental conditions, removal of infected trees over large areas, and implementation of research-based strategies to reduce the impact of DFP. Research-based strategies include the use models to define risk levels at different sites, methods to select tolerant planting stock, and appropriately timed chemical treatments.

## 2. Phylogeny

*Phytophthora pinifolia* has un-branched sporangiophores and non-papillate, sub-globose to ovoid sporangia. Sequence analysis of the ITS region places it into Group 6 of the phylogeny-based classification of Cooke et al. (2000) and its closest relatives are *P. megasperma*, *P. gonapodyides*, *P. thermophile* and *P. litoralis*. Other Group 6 species include *P. humicola, P. inundata*, *P. gibbosa*, *P. gregata*, *P. rosacearum* and several un-described species (Cooke et al. 2000; Jung et al. 2011). Group 6 species are either sterile

or inbreeding and occur primarily in forest or riparian ecosystems (Brasier et al. 2003; Kroon et al. 2004). *P. pinifolia* is ecologically and morphologically distinct because other Group 6 species are soilborne and *P. pinifolia* is found above ground. In addition, *P. pinifolia* occasionally produces caducous sporangia that do not proliferate internally or externally; a trait distinct from *P*. *gonapodyides*, *P*. *megasperma, P. inundata* and *P. humicola*.

## 3. Disease characteristics and spatial distribution

*Phytophthora pinifolia* attacks the needles and the cambium of succulent tissues. Infected needles die relatively quickly (Figure 1) and may lead to defoliation (Figure 1) (Durán et al. 2008). Chile has a Mediterranean climate and symptoms are generally observed from autumn (May) to late spring (November) during the rainy season. Infected needles often have black bands (Figure 2) which are resinous translucent areas (Figure 2). The tops of infected trees have a grayish appearance and turn brown at the end of spring. Infected trees in one and two year-old plantations typically die while trees in three to six-year-old plantations suffer needle damage and defoliation and survive. In adult plantations (older than six years), DFP is limited to the needles and the succulent tissue of young branches and the trees do not die (Durán et al. 2008). *P. pinifolia* does not survive in the felled green timber of infected trees and wood from infected trees is not a risk for subsequent infections. Since 2008, ongoing investigations by Bioforest (a research company of the Arauco Group) indicate that *P. pinifolia* is not transported with timber from Chile (Ahumada et al. 2012).

In Chile, *P. pinifolia* is found primarily in coastal areas from Constitución to Valdivia with the highest incidence in the Arauco province. Areas with high levels of infection have high humidity for most of the year due to their proximity to the Pacific coast. Environmental conditions substantially influence disease severity and disease is most severe in plantations with a southern exposure where the trees remain cooler and moister for longer periods (Figure 1). The spread of DFP in trees older than 2 years has a clear pattern where disease starts in the lower canopy which is cool and wet due to less sunlight and

then progresses upward. The highest levels of infection are observed from June to September, followed by defoliation from July to November.

## 4. Isolations and identification

*Phytophthora pinifolia* can be isolated on CARP media (corn meal agar specific medium with 0.01 g benomyl, 0.01 g pimaricin, 0.2 g ampicillin, 0.01 g rifampicin) and recovery from freshly infected needles, particularly those with resinous bands, is relatively simple and mycelium is visible in 5 to 7 days. In addition, sporangia and zoospores can be visualized by placing infected needles on a glass slide in calcofluor fluorescent brightener (0.001 %) for 30 seconds and examining them microscopically under a fluorescent light (Figure 2). Isolates grown on V8 and CA media are characterized by fluffy mycelium with regular to rosaceous or petallate margins and growth is optimal at 25 °C. The sporangia are occasionally detached from the medium length pedicels (Durán et al. 2008). Oogonia and antheridia have not been observed and *P. pinifolia* is thought to be sterile (Durán et al. 2008).

Molecular identification of *P. pinifolia* can be accomplished using species–specific PCR primers or a PCR–RFLP assay (Durán et al. 2009). There are two *P. pinifolia*-specific primer sets. One is based on the internal transcribed spacer (ITS) region of the rRNA gene and the other is based on the ras–related protein gene *Ypt*1 (Durán et al. 2009). The PCR–RFLP assay was developed according to Drenth et al. (2006) and results in a unique profile compared to other *Phytophthora* spp. In Chile, *P. pinifolia* is routinely identified using genomic DNA from infected plants using either species–specific primers or the PCR–RFLP assay.

A large number of isolates of *P. pinifolia* have been collected in Chile and analyzed using AFLP markers. Results indicate a single clonal lineage is dominant and the overall population structure is clonal (Durán et al. 2010). *P. pinifolia* appeared suddenly in Chile in 2004 across a very limited area and it is likely an introduced pathogen. Interestingly, other *Pinus* species and conifers (eg. *P. pinaster* and *Pseudotsuga menziesii*) found in areas with DFP are not infected and it appears that *P. pinifolia* is host specific. While the

origin of *P. pinifolia* is currently unknown, it may originate from areas where *P. radiata* or related species are native. Future studies are needed to determine the origin of *P. pinifolia* as this might prevent its accidental movement to new environments.

## 5. Epidemiology

*Phytophthora pinifolia* is spread via sporangia. Sporangia are produced on green needles and on dead needles in the trees or that fall to the forest floor. The majority of sporangia are produced during the winter months and needle wetness and temperature are key factors for infection and the development of DFP symptoms. Along the coast of the Arauco province, the frequent mist and rain and conducive temperatures favor the development of DFP symptoms (Del Pozo & Del Canto 1999).

The incidence of new infections has been monitored using healthy trap plants and new infections occur throughout the year with the highest incidence during the wet winter and spring months. The area affected by DFP on the Arauco coast province has varied considerably between 2006 and 2011 from a high of 54 000 ha to the current low of 2 000 ha. The number of days favorable for *Phytophthora* development has been estimated using the Hyre model (Hyre 1954) and there were 141 favorable days in 2006 and between 51 and 60 favorable days between 2007 and 2011. Clearly, wet weather favors disease development.

## 6. Management of the disease

The management of DFP in *P. radiata* stands includes selection of clones tolerant to the infection, selection of sites that do not have the conditions for disease development and use of specific fungicides. Tolerant clones are identified by placing young clonal plants under the canopies of trees with high levels of infection. Clones exhibit a range of tolerance and are ranked to select tolerant clones for planting in high risk areas. The selection of tolerant clones is a long term programme, but it is already providing promising results. In addition, rapid laboratory and greenhouse screening techniques are being developed to improve the efficiency of selecting tolerant clones.

Low risk sites are identified based on knowledge gained from monitoring areas with severe disease incidence (roughly 120 000 ha from 2005 – 2009). The monitoring includes annual aerial photography of the same areas when symptoms are the most severe (October). Monthly climatic data is used to estimate the dew point and relative humidity and a model has been developed to estimate the amount of time each month where the temperature is above 7 °C, the minimum temperature where *P. pinifolia* can grow. Another model estimates the length of time with > 90 % relative humidity (assumed to be a condition favourable for *P. pinifolia* infection). The models confirm that the coastal area of the Arauco province (also called Arauco Gulf) is the most favourable for infection by *P. pinifolia* and aerial surveys in 2006 confirmed a direct relationship between the number of days favourable for infection and the amount of damage observed.

Several fungicides have been tested for DFP control. Systemic products such as metalaxyl and mefenoxam (phenylamides), propamocarb HCL (carbamate), dimetomorph and mandipropamid (carboxylic acid amides) and other fungicides including fosetyl-aluminium (ethyl phosphonate) and salts of phosphorous acid (phosphite) have been considered for their efficacy to reduce infection by *P. pinifolia*. The use of chemicals in young plantations consistently reduced the damage of DFP. Several trials carried out between 2008 and 2011 indicate spraying with phenylamides and mefenoxam two to four times per year reduced symptoms of DFP by up to 90 % and plant mortality to less than 5 %. Other formulations of mefenoxam with chlorotalonil and mancozeb, sprayed four times during the year, reduced DFP by 77 % and 96 % and mortality to 2 % and 14 % respectively.

Phosphites sprayed alone or alternated with phenylamides showed promising results for the management of *P. pinifolia* in the field. Phosphites sprayed four times per year alone gave a control of between 73 % to 99 % with 700 cc/hL and 1 400 cc/hL, respectively. The variation in results could be a consequence of the different chemicals formulations. The use of phosphites in rotation with other fungicides could reduce the emergence of resistant strains of *P. pinifolia* to phenylamides. An important advantage of phosphites is the translocation between both phloem and xylem (Guest & Grant 1991). Fosetyl-Al and phosphite are less effective than metalaxyl-Mz (metalaxyl) and metalaxyl-M

(mefenoxam). In addition, phosphites are treated as fertilizers and have a low environmental impact.

To achieve the best control, application of fungicides should occur before the resinous bands on the needles are first observed. On the Arauco coast, applications are made from April to July in plantations older than one year. For establishment of plantations, plants are treated in the nursery before they are planted. For areas with high risk of infection, plants are treated every three months with phosphites to prevent the development of DFP. Adult plantations are only treated with phosphite if located in the highest risk areas.

## 7. Conclusions and future research

*Phytophthora pinifolia* has been studied intensely since it was first recognized. Damage was severe in commercial plantations and much of the work has occurred in the private sector. A better understanding of the climatic conditions favouring disease has made it possible to develop strategies for management, which, thus far, appear to be working. The clonal nature of the pathogen in Chile and its apparent specificity to *Pinus* spp. suggests that it was accidentally introduced into the country. Studies to determine its origin may be helpful to identify pathways of introduction and reduce the risk of *P. pinifolia* being introduced into other areas of the world.

*Phytophthora pinifolia* is one of the most important pathogens to affect plantation-grown pines in the last decade and it may be a threat to *P. radiata* in other parts of the world. This includes areas where *P. radiata* is planted as a non-native and where the species is native. Very little is known regarding the susceptibility of other *Pinus* spp., but preliminary studies suggest that relatives of *P. radiata* are susceptible to infection (Ahumada, unpublished data). Clearly, continued research is needed to prevent the spread of *P. pinifolia* to new areas.

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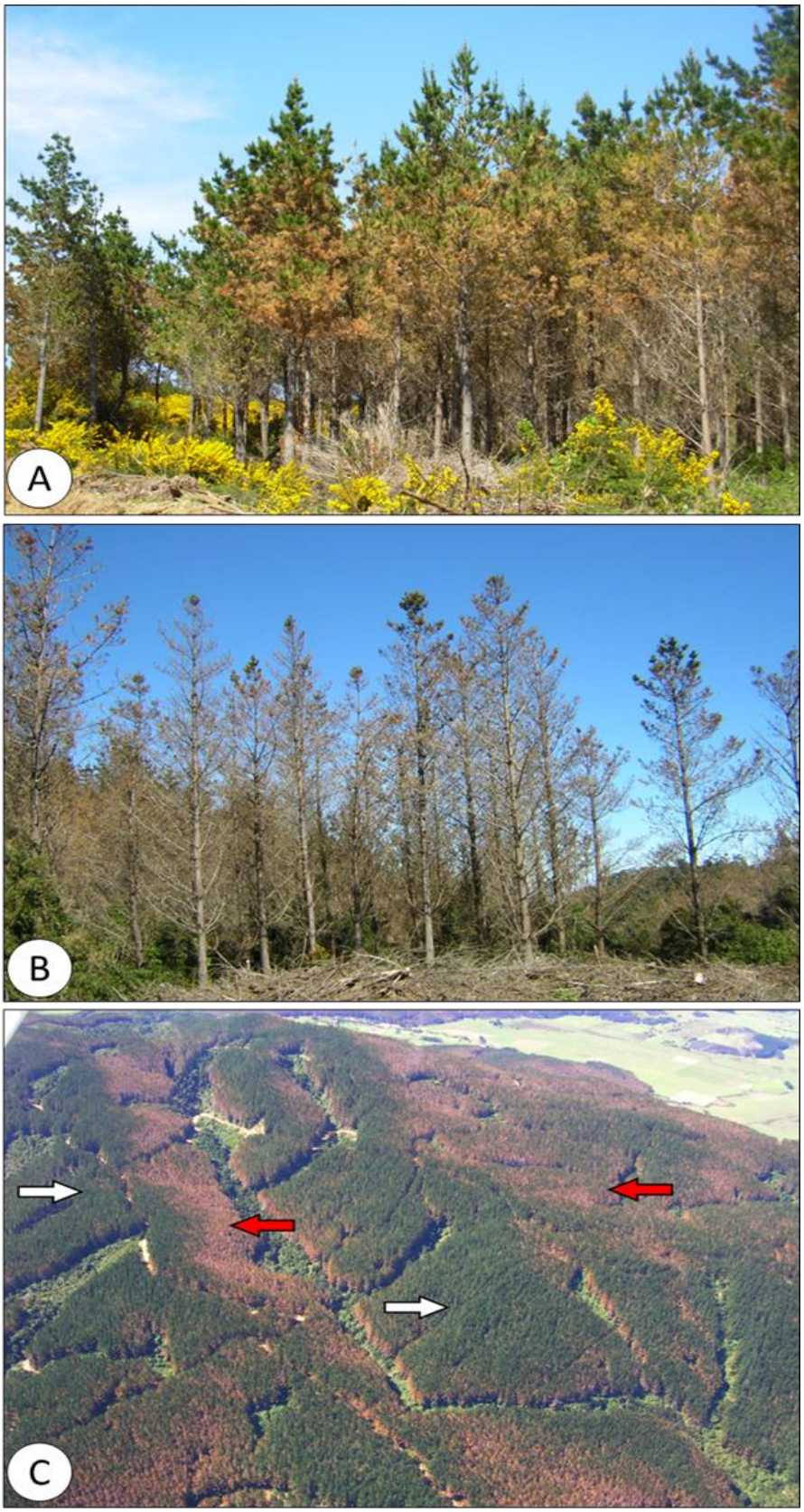


Figure 1. Symptoms of *P. pinifolia* on *Pinus radiata*. A) Infected adult trees, B) defoliated trees, and C) aerial photo of infected trees with a southern exposure (red arrows) and adjacent healthy trees with a northern exposure (white arrows).

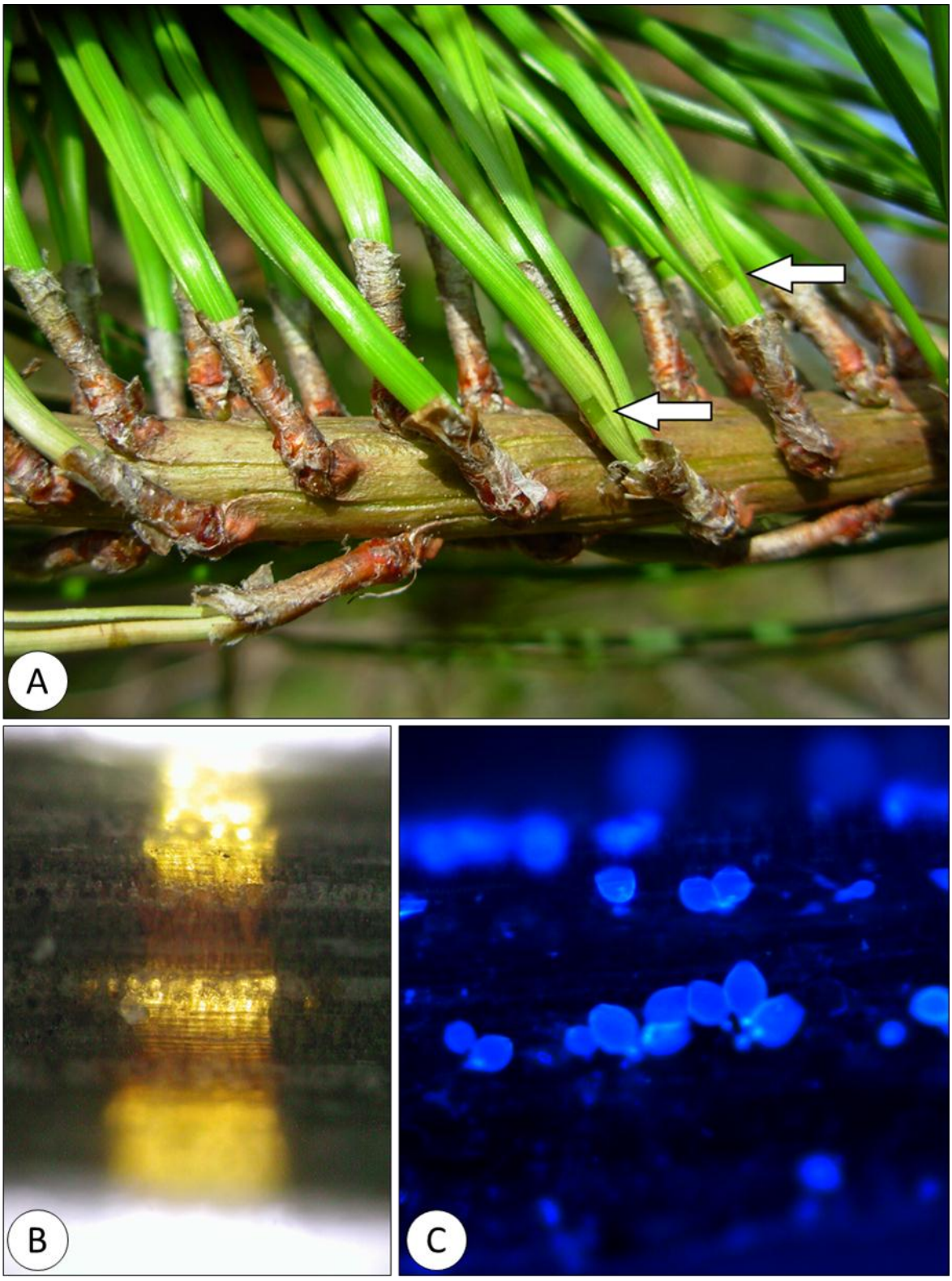


Figure 2. *Phytophthora pinifolia* on needles of *P. radiata*. A) infected needle with black bands (arrows), B) close-up of the resinous translucent band, and C) sporangia on the needle surface observed by fluorescent light microscopy.

# Chapter 5

## Genetic diversity and population biology of *Fusarium circinatum* in Chile


### Abstract

*Fusarium circinatum* causes the disease known as pitch canker on *Pinus* species. During the course of the last decade, this pathogen has appeared for the first time in various parts of the world and has become an important concern to both pine nursery production and plantation forestry. In order to understand the pathways facilitating the movement of *F. circinatum*, studies on its population diversity, structure, epidemiology and ecology have been conducted in the USA, South Africa and Spain. However, very little is known regarding these important issues in countries of South America. Here we characterized the genetic diversity of 71 isolates of *F. circinatum* from three nurseries in Chile using microsatellite and mating type markers. The data revealed a relatively low level of gene and genotypic diversity in these populations, and all isolates were of the *MAT-2* mating type. There was also no structure observed in the distribution of the diversity amongst the nurseries. Interestingly, all three nursery populations contained two lineages of the fungus, potentially from different source populations. Overall, the results suggest a limited introduction of the pathogen into Chile, which is at present apparently reproducing clonally. A representative selection of 19 isolates showed a range in aggressiveness on *P. radiata* seedlings. These results underscore the importance of continued quarantine to avoid further introductions.


### 1. Introduction

*Fusarium circinatum* (Nirenberg & O'Donnell 1998) (sexual state = *Gibberella circinata*), the causal agent of pitch canker, is one of the most important pathogens in *Pinus radiata* plantations worldwide (Gordon et al. 2006a; Wingfield et al. 2008). Pitch canker is known on more than 60 pine species (Hodge & Dvorak 2000) and also on *Pseudotsuga menziesii*, the only known non-pine host (Storer et al. 1997; Gordon et al. 2006b). The disease derives this name from the characteristic exudation of resin at the site of infection and was first reported on *Pinus virginiana* in North Carolina, USA (Hepting & Roth 1946), after which it spread to the south-eastern USA and eventually to California (McCain et al.

1987). *Fusarium circinatum* is now found on five continents and has been reported from Haiti (Hepting & Roth 1953), Japan (Kobayashi & Muramoto 1989), South Africa (Viljoen et al. 1994), Mexico (Guerra-Santos 1999), South Korea (Lee et al. 2000), Chile (Wingfield et al. 2002), Spain (Landeras et al. 2005), France (EPPO 2006), Italy (Carlucci et al. 2007), Uruguay (Alonso & Bettucci 2009), Portugal (Bragança et al. 2009) and Colombia (Steenkamp et al. 2012).

Symptoms of pitch canker include wilting and discolouration of needles, branch and tip die-back (Storer et al. 1995; Gordon et al. 2001) and resinous cankers on the stems and branches of trees (Barnard & Blakeslee 1980; Barrows-Broaddus & Dwinell 1985). Mature cones and seeds, flowers, shoots, bases of hedge plants or seedlings in nurseries can also be affected (Barrows-Broaddus & Dwinell 1985; Correll et al. 1991; Carey & Kelly 1994; Viljoen et al. 1994). Most of the symptoms in nursery plants appear when the plants are under stress, which is common due to transplanting or intensive hedge production. The economic losses in nurseries and commercial plantations have not been quantified, but is estimated to be many millions of US$ annually, due to costs of monitoring, silvicultural activities, reduction of growth, the timber quality, seed production and mortality (Barrows-Broaddus & Dwinell 1985; Pérez-Sierra et al. 2007).

*Fusarium circinatum* was first discovered in Chile on *P. radiata* hedge plants in nurseries located in the Biobío region in 2001 (Wingfield et al. 2002). Later the fungus was also isolated from containerised and open root *P. radiata* plants in nurseries, distributed from Constitución (35° 20’ S, 72° 25’ W) in El Maule region to Valdivia (39° 48’ S, 73° 14’ W) in Los Ríos region (SAG 2003; González 2007). The pathogen is now subject to official control regulations, enforced by the Agriculture and Livestock Service (Servicio Agrícola y Ganadero, SAG). The direct economic impact calculated based on the 4.3 million (0.65 % of the total production) plants destroyed between 2006 and 2012, as part of the quarantine regulations, had an estimated cost of US$ 540,000 (R. Ahumada, unpublished information).

Control of *F. circinatum* in nurseries is achieved mainly through hygiene and chemical control to prevent new disease outbreaks (González 2007; Wingfield et al. 2008; Van Wyk

et al. 2012). In plantations, pruning or thinning of infected trees and other actions to avoid stress by careful site selection and appropriate planting density can be useful (Wingfield et al. 2008). An important longer term strategy, however, lies in selecting *Pinus* breeding material tolerant to infection following several previous studies showing variation in tolerance to *F. circinatum* infection amongst *Pinus* spp., provenances, families, clones and hybrids (Storer et al. 1999; Roux et al. 2007; Dvorak et al. 2009; Mitchell et al. 2012). For example, *P. radiata* has been shown to vary in susceptibility levels to *F. circinatum* at the clonal and family level (Storer et al. 1999; Roux et al. 2007). Furthermore, certain lineages of *P. oocarpa* and *P. tecunumanii* (referred to as low elevation) were highly tolerant to *F. circinatum* infection (Dvorak et al. 2009), as was *P. elliottii* x *P. caribea*, *P. patula* x *P. oocarpa* and *P. patula* x *P. tecunumanii* in South Africa (Roux et al. 2007; Mitchell et al. 2012).

Knowledge of the mating strategy and population genetics of *F. circinatum* can have implications for disease control and tree breeding (Wingfield et al. 2008). *Fusarium circinatum* is known to be heterothallic with two mating types (Kerényi et al. 1999; Britz et al. 1999). Molecular protocols were developed to distinguish the mating types (Steenkamp et al. 2000; Wallace & Covert 2000; Schweigkofler et al. 2004) and have been used to characterise the mating strategies for populations of *F. circinatum* isolates from Chile, Mexico, Spain, South Africa and the USA (Britz et al. 1998; Covert el al. 1999; Jacobs et al. 2007; Pérez-Sierra et al. 2007; Iturritxa et al. 2011; Berbagal et al. 2013). In most of these studies both mating types were reported, which was also true for Chile although only six isolates were considered and only one was of the *MAT-1* type (Jacobs et al. 2007). This suggests that the potential for sexual reproduction exists in all these environments, increasing the evolutionary potential of these populations and their ability to overcome resistance and to adapt to variable host and environmental factors.

Two sets of polymorphic microsatellite markers (17 loci in total) have been developed for population genetic studies of *F. circinatum* (Wikler & Gordon 2000; Britz et al. 2002; Santana et al. 2009). These markers have been used to analyse a number of *F. circinatum* populations from around the world to characterize patterns of introduction and population biology (Wikler & Gordon 2000; Iturritxa et al. 2011; Berbegal et al. 2013).

These studies suggest that Mexico, which has the highest diversity, is potentially the origin of this pathogen (Wingfield et al. 2008). The data from these studies suggest that once the pathogen moved out of Mexico, the invasive populations served as the main source of introductions and that there have been multiple introductions into some regions, such as Spain (2) and South Africa (> 1) (Britz et al. 2005; Berbegal et al. 2013). Furthermore, these data supported the possibility of sexual reproduction (indicated by mating type markers) in the USA, South Africa and Mexico, while they revealed likely clonal reproduction in Northern Spain (Wikler & Gordon 2000; Britz et al. 2005; Berbegal et al. 2013).

Very little is known regarding the population structure of *F. circinatum* in South America where the fungus has been reported in Chile, Uruguay and Colombia. In this study we determined the genetic diversity and population structure of a *F. circinatum* population from Chile by assessing 71 isolates from three geographically isolated populations in the country and using 10 different primer pairs of microsatellite markers. We also considered the reproductive biology of *F. circinatum* by characterizing the diversity and distribution of mating type loci in the isolates. Furthermore, the variation in aggressiveness of *F. circinatum* isolates representing the Chilean population was tested on *P. radiata*.

## 2. Material and methods

### 2.1 Sampling and fungal isolates

A total of 71 isolates of *F. circinatum* were collected from *P. radiata* hedge plants, as well as cuttings and seedlings displaying symptoms of infection. The sources of isolates included plant material with typical foliage wilting and resin accumulation at the bases of plants, with depression or abnormal growth in the resin soaked areas. When the bark was removed, diseased tissue could normally be identified as brown or darkly stained (Figure 1). Isolates were obtained from three different nurseries belonging to the Arauco forestry company, namely Quivolgo (31 isolates), Las Cruces (30 isolates) and Los Castaños (10 isolates). The three nurseries were selected because they produce *P. radiata* plants, they were known to be infected by *F. circinatum* and they represented the major geographic

regions where this pine species is propagated. One nursery was located in Constitución (35° 19’ S, 72° 24’ W), which represents the northern distribution of the *F. circinatum*; another in Coronel (36° 55' S, 73° 9' W) and at the centre of the distribution of the pathogen and the third nursery was near Valdivia (39° 43' S, 73° 6' W) in the southern part of the range of distribution of the pathogen in Chile (Figure 2). All the isolates used in this study have been maintained in the culture collection of Bioforest and were selected to represent isolations made between 2002 and 2012. A duplicate set of isolates has been deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

Primary isolations from the symptomatic tissue were made by placing a small piece of pitch-soaked pine tissue on *Fusarium* selective medium (Nash & Snyder 1964). Cultures were incubated at 18–22 °C under normal day light period. The plates were inspected regularly for fungal growth and all the colonies with typical *Fusarium* morphology were transferred to half-strength potato dextrose agar (PDA) (Merck, Germany). Single conidial cultures were made and stored at –20 °C on sterile filter paper.

## 2.2 DNA extraction, species identification and mating type assignment

The DNA samples were prepared by transferring each of the 71 isolates to PDA and incubating the fungi at 18–22 °C for 7 days. About 20 mg of the mycelium was harvested for each isolate and homogenized in 100 µl PrepMan$^{TM}$ Ultra preparation reagent (Applied Biosystems, Foster City, CA, USA). The reaction mixtures were then shaken using a Vortex mixer for 30 seconds, heated at 100 °C for 10 minutes and centrifuged. Supernatants containing the extracted DNAs were transferred to new tubes and stored at –20 °C until required.

All the isolates were originally identified using the diagnostic PCRs with *F. circinatum*-specific oligonucleotide CIRC primers (Schweigkofler et al. 2004). The species identity of all isolates was then confirmed using restriction fragment length polymorphism (RFLP) and analysis of the histone H3 PCR-amplified (Steenkamp et al. 1999). For the histone H3 PCR-RFLP analysis, PCR reactions were performed using the primers H3-1a (5'-

ACTAAGCAGACCGCCCGCAGG-3') and H3-1b (5'-GCGGGCGAGC-TGGATGTCCTT-3'). The PCR reaction mixture of 25 µl included 1 x PCR buffer with $MgCl_2$, 200 µM of each dNTP, 5 µM of each primer, 25 ng template DNA and 1 U *Taq* polymerase (Roche Pharmaceuticals, Germany). The PCR reaction conditions were an initial denaturation at 93 °C for 3 min, followed by 35 cycles of denaturation at 93 °C for 1 min, annealing at 65 °C for 1 min and elongation at 72 °C for 1 min. A final extension was performed at 72 °C for 5 min. Histone H3 PCR products obtained for the 71 isolates were digested with restriction enzymes *Cfo*I and *Dde*I (Roche Pharmaceuticals, Germany). Consecutive enzymatic digestion was performed by addition of 5U *Cfo*I to 15 µl of the unpurified PCR product followed by incubation at 37 °C for 3 h. Subsequently, the sodium chloride concentration was adjusted to 100 mM and 5 U of *Dde*I was added to the reaction mixture. This was followed by further incubation at 37 °C for 5 h. The digested fragments were separated by electrophoresis in 2 % agarose gels at 100 Volt for 45 minutes, and visualized under UV light.

The mating types of the isolates were determined using the PCR-based method described by Steenkamp et al. (2000). This method involves the use of two primer sets that specifically target the conserved alpha and HMG (high mobility group) domains in *MAT*-1 and *MAT*-2 individuals (Martin et al. 2011). The *MAT* idiomorphs were amplified with GFmat1a (5'-GTTCATCAAAGGGCAAGCG-3'), GFmat1b (5'-TAAGCGCCTCTTAACGCCTTC-3'), GFmat2c (5'-AGCGTCATTATT-CGATCAAG-3') and GFmat2d (5'-CTACGTTGAGAGCTGTACAG-3') developed by Steenkamp et al. (2000). Each 25 µl of the PCR mixture contained 1 x PCR buffer with $MgCl_2$, 200 µM of each dNTP, 0.1 µM of each primer, 25 ng template DNA and 0.5 U Roche *Taq* polymerase (Roche Pharmaceuticals, Germany). The PCR reaction conditions were an initial denaturation at 92 °C for 1 min, followed by 35 cycles of denaturation at 92 °C for 30 s, annealing at 63 °C for 30 s and elongation at 72 °C for 30 s. A final elongation step was done at 72 °C for 5 min. Gel electrophoresis was performed as described above.

## 2.3 Microsatellite PCRs, genescan and allele scoring

DNA was extracted from all isolates using PrepMan™ as described above and the microsatellite loci were amplified using ten pairs of fluorescently labelled primers (Santana et al. 2009). PCR reactions and conditions were as described in Santana et al. (2009). PCR amplicons were separated by 2 % agarose gel electrophoresis, stained with GelRed™ (Biotium, CA, USA) and visualised under UV light.

Microsatellite PCR products were size-separated on an ABI Prism™ 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) including the internal size standard GeneScan™ 500 LIZ™ (Applied Biosystems, Warrington, UK). Alleles for each microsatellite locus were determined using the GeneMapper® v4.0 software package (Applied Biosystems). Allele sizes were manually interrogated at each locus within the GeneMapper program. The analysis was done using the results of the allelic diversity of the SSR loci by assigning different letters to individual alleles for each of the loci.

## 2.4 Population genetic analyses

Allele frequency at each locus and allele diversity was calculated using the program POPGENE (Yeh et al. 1999). Genotypic diversity (*G*) was estimated using the equation $G = (1 \times (\sum Pi^2)^{-1}$ where *Pi* is the observed frequency of the $i^{th}$ genotype (Stoddart & Taylor 1988). Maximum percentage genotype diversity $G^* = (G/N) \times 100$, where N = number of isolate, was determined. A Chi-square ($x^2$) test was used to determine the differences in the frequencies of alleles, which were calculated for each locus across all populations. The $x^2$ values obtained were compared using the contingency table at $P = 0.001$ to determine the significance of the allele frequency at each locus.

To infer and assign population structure to the 71 isolates analysed, the software programme STRUCTURE 2.3 (Pritchard et al. 2000) was used. The Monte Carlo Markov Chain (MCMC) program was run with 100 000 replicates with initial burn-in of 20 000 for *K* ranging from 1 to 6 at 20 iterations. Clusters were assigned with cluster identity > 75 %.

The program Multilocus (Agapow & Burt 2000) was used to calculate population differentiation theta ($\Theta$) between the populations, with an estimate of Wright's Fst as $\Theta$ = (Q–1) / (1–q) where "Q" is the probability that two alleles from the same population are the same and "q" is the probability that two alleles from different populations are the same (Weir 1997). When $\Theta$ = 0, allele frequencies are equal between two populations, meaning that two populations are identical. On the other hand, when $\Theta$ = 1, the two populations are completely isolated and there are no alleles shared between them. The statistical significance of $\Theta$ was determined by comparing the observed value with 1 000 randomized datasets using a probability value of $P < 0.05$. The null-hypothesis that there is no difference between two populations was rejected when significant difference between populations ($P < 0.05$) was observed.

To measure the extent of linkage disequilibrium within different populations by quantifying the amount of recombination among a set of loci and detecting association between alleles at the different loci, the Index Association ($I_A$) and rBarD ($\bar{r}_D$) were calculated using the program Multilocus (Agapow & Burt 2000). The observed values $I_A$ and $\bar{r}_D$ were compared to 1 000 randomized datasets with a significance level of $P < 0.05$. The null-hypothesis was that the alleles are randomly associated, which is typical of a population that is expected to randomly recombine. When the observed $I_A$ and $\bar{r}_D$ were significantly different from those obtained from the randomized datasets, the null-hypothesis was rejected. In contrast, when the observed values of $I_A$ and $\bar{r}_D$ were not significantly different or fell within the distribution range of randomized values, the null-hypothesis of random association of alleles was accepted.

## 2.5 Pathogenicity tests

To determine the relative aggressiveness (the quantitative component of pathogenicity) of different *F. circinatum* isolates, 19 isolates were selected to represent a broad sampling of the genetic diversity of isolates from amongst the three geographical regions sampled (Table 1). The SSR data were used to ensure that the selection was representative of the population genetic diversity by selecting from the different Multilocus haplotypes. Fourteen-month-old plants representing an equal mixture of 20 operational *P. radiata*

clones with an average height of 34 cm and average diam. at soil level of 6 mm were chosen for inoculation. One plant of each of the 20 clones was used for inoculation by each of the 19 isolates, and the control (400 plants in total). The inoculation trial was conducted at the Bioforest screening facility in Concepción, which was set to provide a photoperiod of 12 h of artificial light, 75 % relative humidity, temperatures between 18–22 °C and irrigation to the containers daily for 1 h (Figure 5).

The inoculum was prepared on the same day that the plants were inoculated, using the 19 selected isolates grown on half strength PDA medium at 18–22 °C for 10 days prior to inoculation. After the cultures had been flooded with 5 mL of sterile water, the mycelium was gently removed with a glass "hockey stick" and rinsed in a glass beaker to reach a homogeneous suspension. The spore concentration was determined using a hematocytometer and adjusted to approximately 5 x $10^4$ spores $ml^{-1}$.

The inoculation was carried out using a technique similar to that used to select *Pinus* spp. for tolerance to infection by *F. circinatum* (Mitchell et al. 2012) by excising the top 2 cm of the apical shoot from each plant with sharp scissors disinfected with 70 % ethanol after every ten plants to avoid cross contamination (Figure 5). Ten microliters of the suspension containing approximately 500 spores was applied to the wounded surface of each plant using a 20 µl micropipette (Figure 5). The same procedure was used for the control inoculations except that sterile water was applied to the cut plant surfaces. The plants were examined weekly for the development of symptoms and the lesions were measured from point of inoculations to the advancing edge of the lesion after 6 weeks.

Re–isolation was attempted from 30 % of the inoculated plants. Small pieces (< 5 $mm^2$) of succulent infected tissue from the leading edges of the lesions were plated onto PDA and developing cultures were purified. The identity of 10 % of the resulting cultures that were characteristic of *F. circinatum* based on morphology was confirmed using the PCR-CIRC specific primers (Schweigkofler et al. 2004).

Data for each of the isolates was analyzed to evaluate their relative aggressiveness using the linear model of analysis of variance (ANOVA) and means were separated based on

LSD (Least Significant Difference) using the software Statistica V10 for Windows (StatSoft Inc. 2004).

## 3. Results

### 3.1 DNA extraction, species identification and mating type assignment

All 71 isolates included in this study were confirmed as being those of *F. circinatum* using PCR-RFLP digestion of the histone H3 gene. The PCR amplicon digested with the restriction enzymes *CfoI* and *DdeI* produced the two expected fragments of approximately 232 bp and 250 bp in the all isolates analysed (Table 1). The PCR identification of the mating types consistently showed that all 71 isolates were of the *MAT-2* type and *MAT-1* type was absent from all isolates used in this study (Table 1).

### 3.2 Population genetic analyses

Four loci (FCM-16, FCM-20, FCM-24 and FCM-25) were monomorphic amongst the 71 isolates of *F. circinatum* from Chile and were excluded from further analyses. For the six polymorphic loci analysed, the number of alleles ranged from 2 to 9 (Table 2). Eleven of the 22 (50 %) alleles were shared between all the populations. The Quivolgo and Las Cruces populations had the same number of unique alleles (n = 4), while that from Los Castaños did not have any unique alleles (Table 2).

For the three populations of *F. circinatum* analysed, the *G* values were 0.48 in Quivolgo, 0.73 in Las Cruces and 0.78 in Los Castaños. The genotypic diversity (*G*) for the combined populations was 0.33 and the maximum genotypic diversity (*G**) was 0.47 %. The highest maximum genotypic diversity (*G**) was 7.8 % in the Los Castaños population, followed by the Las Cruces population with 2.5 % and the Quivolgo population with 1.6 % (Table 2). Seventeen multilocus haplotypes (*MLH*) were observed for the three populations amongst the 71 isolates, with 10 from Quivolgo (31 isolates), 11 from Las Cruces (30 isolates) and 5 from Los Castaños (10 isolates) (Table 2). The mean gene diversity (*H*) for all six loci across all populations of *F. circinatum* ranged from 0.22 to 0.316 for the clone-

corrected populations, with 0.316 for Quivolgo, 0.308 for Las Cruces and 0.22 for Los Castaños (Table 3).

Analysis of population differentiation ($\Theta$) showed no significant difference between the three populations at $P < 0.05$. Low level of genetic distance was determined between Quivolgo and Las Cruces ($\Theta = 0.021$) and also between Quivolgo and Los Castaños ($\Theta = 0.071$), while the highest value of the genetic distance was between Las Cruces and Los Castaños ($\Theta = 0.213$) (Table 4). The greatest geographical distance was between Quivolgo and Los Castaños (500 km) and Las Cruces is approximately 200 km from Quivolgo and 300 km from Los Castaños (Figure 2). All the isolates used in this study resided into two groups based on the Structure analysis. Both groups were present in all three nursery populations (Figure 3).

The observed $I_A$ and $\bar{r}D$ values for the clone corrected data of the combined population were 1.03 and 0.25, respectively, and both values were significantly different from the range of 1 000 randomly recombined datasets (Figure 4). Similar results were obtained for the $I_A$ and $\bar{r}D$ values for each of the three populations analysed separately (Figure 4). The null-hypothesis that the alleles are randomly associated and that the population is expected to undergo out-crossing was thus rejected.

### 3.3 Pathogenicity tests

All the isolates tested for aggressiveness in the inoculation trial produced lesions on the treated plants while no symptoms were observed in the controls (Figure 5). Three of the plants used in the inoculation experiment died from the base and were removed from the analysis because the cause of the death was not associated with the inoculation. All the re-isolations yielded *F. circinatum* based on culture identification and confirmed using PCR- specific primers.

Lesions on the individual inoculated plants ranged between 6.5 mm and 58.5 mm. Significant differences ($p < 0.001$) were found between plants inoculated with *F. circinatum* isolates and the controls (Figure 6). The isolates showed a range in

aggressiveness, producing average lesion lengths from 22 to 38.4 mm. The results showed differences in the lesion length produced with a range of approximately 16.4 mm on average, with a minimum average value of 22 mm and a maximum average value of 38.4 mm between the 19 isolates assessed (Figure 6).

## 4. Discussion

Results of this study showed that *F. circinatum* isolates collected across a wide geographical and temporal range in Chile had a relatively low level of gene and genotypic diversity. Furthermore, all of these isolates represented a single mating type. These results suggest that the fungus was introduced into the country with limited diversity and has subsequently been reproducing asexually. The introduction was most likely initially into the Biobío region where it was first detected (Wingfield et al. 2002). The spread of the pathogen from the Biobío region to the North and the South has most likely been through the movement of planting material.

The gene (*H*) and genotypic diversity (*G*) for *F. circinatum* in Chile was fairly low (*H* = 0.22 to 0.31 and *G* = 0.33). These values are close to those reported in a recent study using some of the same microsatellite markers, but that used only ten isolates from of unknown origin in Chile (Berbegal et al. 2013). This is in contrast to results obtained using the same markers for the pathogen from Mexico, Portugal, South Africa and USA where populations displayed high genotypic diversity (*G*) values (0.9 to 1.0) and populations from Spain, France and Japan ranged between 0.5 and 0.7 (Berbegal et al. 2013). Similarly, earlier studies on *F. circinatum,* where different polymorphic markers were applied to samples from USA (Florida), Mexico, South Africa and Japan, showed the highest genotypic diversity value for South African (0.92) and Mexican (0.82) populations. This was in contrast to collections from USA (California and Florida) that had lower values (0.4 and 0.61) than those found in the present study (Wikler & Gordon 2000). These previous studies suggested fairly large or multiple introductions of *F. circinatum* that reduced the effects of genetic bottlenecks. The opposite appears to be true in Chile, where the lower diversity (*G* = 0.33) appears to support a smaller number of introductions of the pathogen or a genetic bottleneck with a greater impact. This pattern

has also most likely been influenced by the strict monitoring and quarantine regulations applied to all imported *Pinus* material into Chile.

The data from this study suggest that *F. circinatum* is reproducing clonally at least in the nurseries of Chile sampled in this study. Firstly, all 71 isolates were of the *MAT-2* mating type. This result differed from that of a previous study where only six samples from a nursery located near the Las Cruces nursery were considered and where a single isolate was of the *MAT*-1 mating type (Jacobs et al. 2007). The specific isolate thought to be of the *MAT-1* type was, however, shown to be *MAT*-2 in the present study. Furthermore, the association between alleles (significant $I_A$ values compared to a randomized datasets), the fact that less than 8 % of the potential genotypes are present given the gene diversity, and the fact that many genotypes are repeated over time and space (e.g. haplotype "AAAABAAEAA" was isolated 22 times and from all three nurseries) support the lack of sexual reproduction in the Chilean *F. circinatum* population. These results are in contrast to those from various other parts of the word where both mating types occur and where levels of genotype diversity are relatively high (Wallace & Covert 2000; Schweigkofler et al. 2004; Berbegal et al. 2013).

Structure analysis suggested that the *F. circinatum* isolates from Chile belonged to two distinct evolutionary lineages. Interestingly, isolates representing both lineages were found in all three nurseries, but despite their co-existence at these locations, there was no evidence of introgression between these lineages. This is clear from structure analyses, but also manual inspection of the data showing fixed alleles in both groups without evidence of re-combination. These results add further credence to the view that this population is reproducing predominantly clonally. Whether these two lineages were introduced together or independently from independent origins is not clear at this stage, but it represents an intriguing and important question for further consideration.

Comparison of the data generated in this study, with those from Berbegal et al. (2013) has implications for the hypothesized routes of introduction of *F. circinatum* into Chile. Berbegal et al. (2013) found two distinct lineages for *F. circinatum* in Spain, and concluded that this indicates two introductions and subsequent clonal reproduction.

These authors found only one of the lineages from Chile. Other countries where this same lineage has been found include Mexico, South Africa, USA, Spain and Portugal. The fact that Berbegal et al. (2013) did not find the second lineage in Chile is likely due to the fact that they considered a very small number of isolates (n = 10). They did, however, find the second lineage from samples collected in Uruguay, the USA, South Africa, Spain and Japan. Overall, the results of the present and other studies suggest that these two lineages are common in invasive populations around the world and points to the spread between these invasive populations. In Chile, it is expected that the introduction of these lineages was more or less simultaneous, as both lineages are found amongst samples from early collections in 2003 and 2004, respectively.

The results of this study show the value of quarantine to limit the genetic (and resulting phenotypic) diversity and evolutionary potential of introduced pathogens such as *F. circinatum*. Movement of planting stock between countries can contribute to the introduction of new genotypes and, more crucially a diversity of mating types, which could have major impacts on control efforts such as resistance breeding programs (Wingfield et al. 2008). Chile allows for the importation of *Pinus* spp. seed, but follows a strict protocol of one-year quarantine post entry (SAG 2008). Various other countries such as New Zealand have imposed even stricter controls, for example by prohibiting the entry of any material, including seeds, given the uncertainty of the effectiveness of treatments against *F. circinatum* (Storer et al. 1998; Wingfield et al. 2008).

There was considerable variation in the aggressiveness in the *F. circinatum* population in Chile, as displayed by relative lesion length in the inoculation experiment. This variability in aggressiveness illustrates the importance of careful selection of isolates to use when testing potential breeding material for resistance. This effect might be even more pronounced in countries where variation in genetic diversity is substantially higher.

Population genetic data made it possible to select a suite of isolates that represent the diversity of the population of *F. circinatum* in Chile and to utilize these to test for resistance in *P. radiata* breeding material. Similar studies conducted in the USA and South Africa using *P. radiata* families and clones also showed that despite its high level of

susceptibility to *F. circinatum*, there are clear differences in the susceptibility of the genetic material (Storer et al. 1999; Roux et al. 2007).

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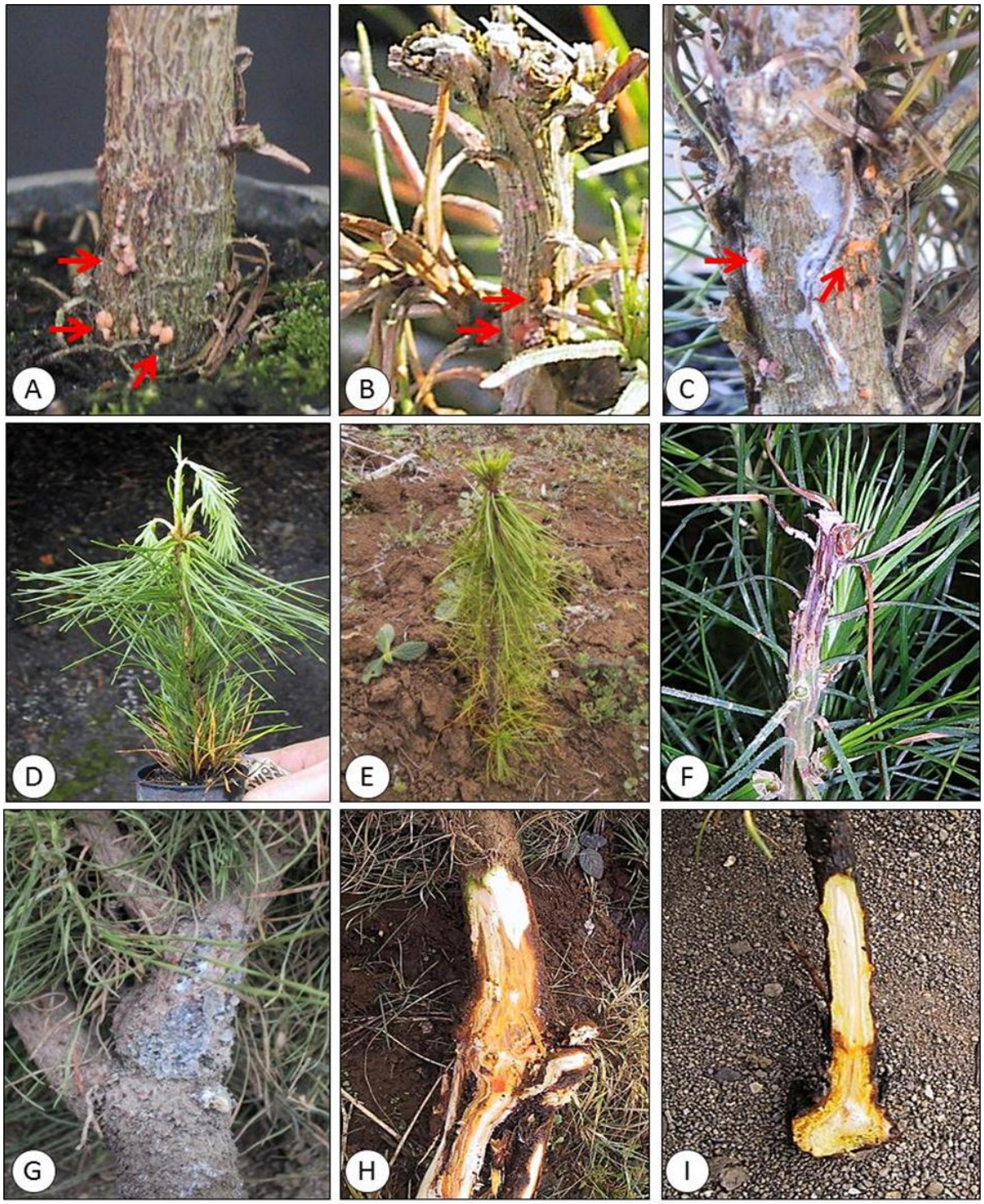


Figure 1. *Pinus radiata* plants with characteristic signs and symptoms of *F. circinatum* infection. A – C) Cuttings plants with presence of typical fruiting bodies (red arrow shows sporodoquia). D – F) Containarised and open root cuttings with symptoms of *F. circinatum* infection either in the collar or aerial (F). G) Resin in the collar and base of a cutting infected and H – I) Discoloration of the tissue infected by *F. circinatum*.

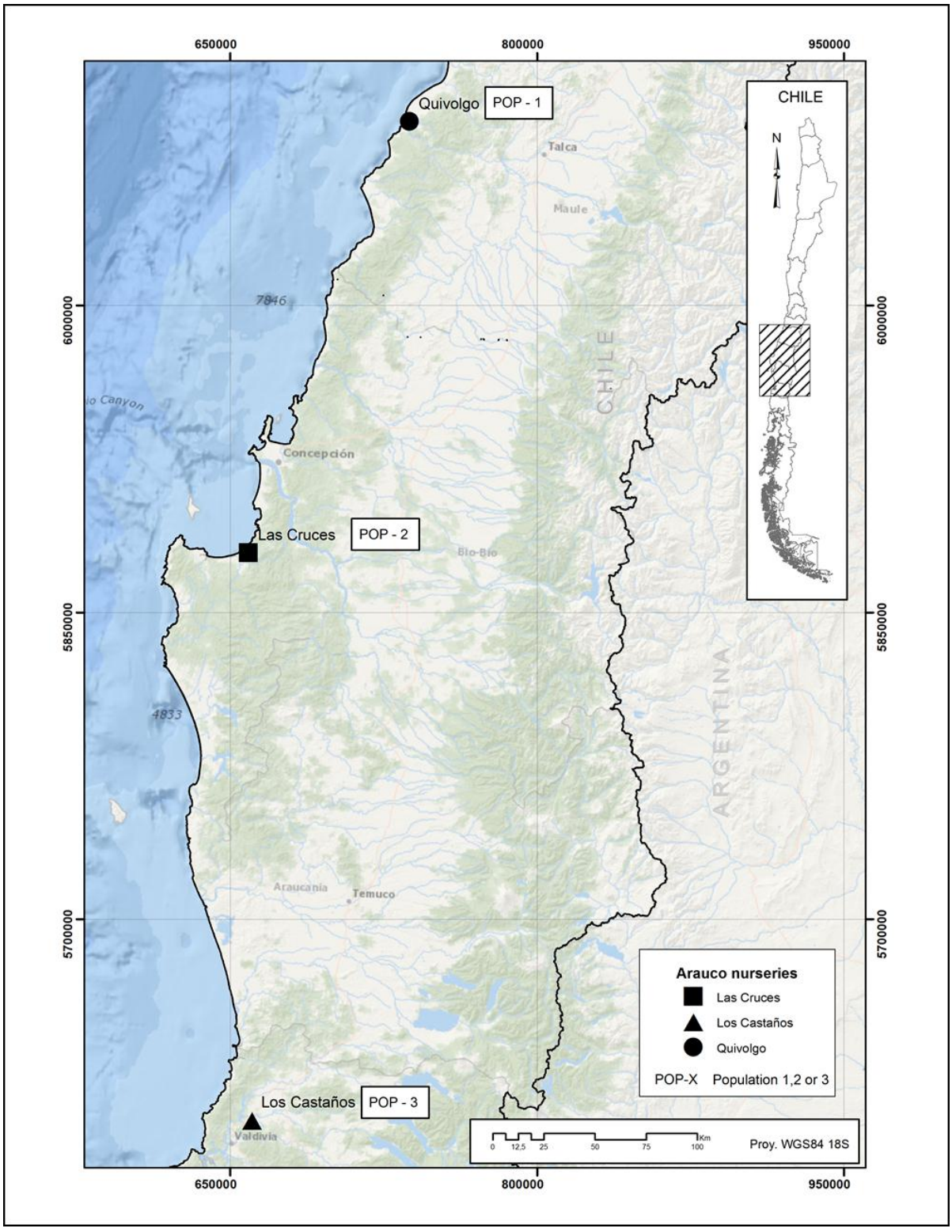


Figure 2. Geographical distribution of the Arauco nurseries sampled for the population studies of *F. circinatum*.

Table 1. Description of the isolates used in the analyses.

| Nursery of origin | Year of isolation | Assigned population | *F. circinatum* identity | | Mating types | | Isolates for pathogenicity test | |
|---|---|---|---|---|---|---|---|---|
| | | | RFLP-H3 | PCR-CIRC | MAT-1 | MAT-2 | No | CMW |
| Quivolgo | 2004 - 2012 | Pop-1 | + | + | 0 | 31 | 9 | 40017, 40018, 40019, 40020, 40021, 40022, 40023, 40024, 40031 |
| Las Cruces | 2003<br>2005 - 2010<br>2012 | Pop-2 | + | + | 0 | 30 | 6 | 40025, 40026, 40027, 40032, 40034, 40035 |
| Los Castaños | 2002<br>2005<br>2008 - 2011 | Pop-3 | + | + | 0 | 10 | 4 | 40028, 40029, 40030, 40033 |

Table 2. Allele frequencies and genotypic diversity in the *F. circinatum* populations from different nurseries.

| Locus | Allele | Quivolgo | Las Cruces | Los Castaños |
|---|---|---|---|---|
| FCM-2 | 1 | 0.967 | 0.966 | 1.000 |
| | 2 | 0.032 | | |
| | 3 | | 0.033 | |
| FCM-3 | 1 | 0.516 | 0.666 | 0.200 |
| | 2 | 0.483 | 0.333 | 0.800 |
| FCM-4 | 1 | 0.516 | 0.666 | 0.200 |
| | 2 | 0.354 | 0.100 | 0.600 |
| | 3 | 0.129 | 0.200 | 0.200 |
| | 4 | | 0.033 | |
| FCM-6 | 1 | 0.967 | 1.000 | 1.000 |
| | 2 | 0.032 | | |
| FCM-7 | 1 | | 0.033 | |
| | 2 | 1.000 | 0.966 | 1.000 |
| FCM-23 | 1 | 0.419 | 0.233 | 0.700 |
| | 2 | 0.064 | 0.066 | |
| | 3 | | 0.033 | 0.100 |
| | 4 | 0.032 | | |
| | 5 | 0.354 | 0.333 | 0.100 |
| | 6 | 0.032 | 0.200 | |
| | 7 | 0.064 | 0.100 | 0.100 |
| | 8 | 0.032 | | |
| | 9 | | 0.033 | |
| G | | 0.483 | 0.738 | 0.781 |
| G* | | 1.558 | 2.459 | 7.813 |
| MLH | | 10 | 11 | 5 |
| N | | 31 | 30 | 10 |

G = Genotypic diversity (Stoddart & Taylor 1988), G* = Percentage maximum diversity, MLH = Moltilocus haplotype, N = Number of isolates per population.

Table 3. Gene diversity (H) and chi-square tests for differences in allele frequencies for 10 SSR loci across clone corrected populations of *F. circinatum*.

| Locus | Gene diversity (H) | | | Chi ($x^2$) | *df* |
|---|---|---|---|---|---|
| | Quivolgo | Las Cruces | Los Castaños | | |
| FCM-2 | 0.062 | 0.064 | 0.000 | 2.66 | 4 |
| FCM-3 | 0.499 | 0.444 | 0.320 | 6.64 | 2 |
| FCM-4 | 0.591 | 0.504 | 0.560 | 12.67 | 6 |
| FCM-6 | 0.062 | 0.000 | 0.000 | 1.30 | 2 |
| FCM-7 | 0.000 | 0.064 | 0.000 | 1.38 | 2 |
| FCM-23 | 0.686 | 0.777 | 0.490 | 19.28 | 16 |
| Mean | 0.316 | 0.308 | 0.220 | | |

*Significant difference at $P = 0.001$, *df* = degree of freedom

Table 4. Population differentiations ($\Theta$) among *F. circinatum* populations from three different locations in Chile.

| Nurseries | Quivolgo | Las Cruces | Los Castaños |
|---|---|---|---|
| Quivolgo | ….. | 0.021 | 0.071 |
| Las Cruces | ….. | ….. | 0.213 |
| Los Castaños | ….. | ….. | ….. |

*Significant difference at $P < 0.05$

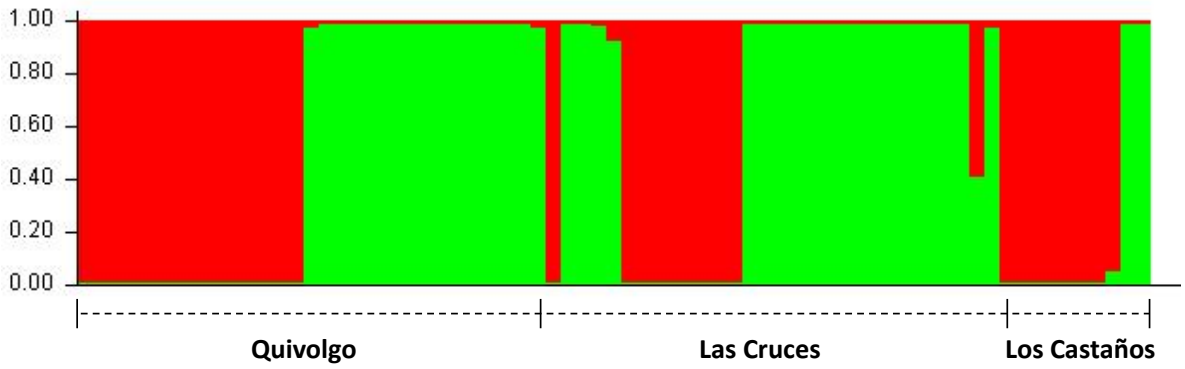


Figure 3. Affiliation of individual genotypes of *F. circinatum* assessed by Structure 2.3 and separated into two groups represented by two colours which suggest two populations. The origin of the isolates used is the nurseries described under the bar graph.

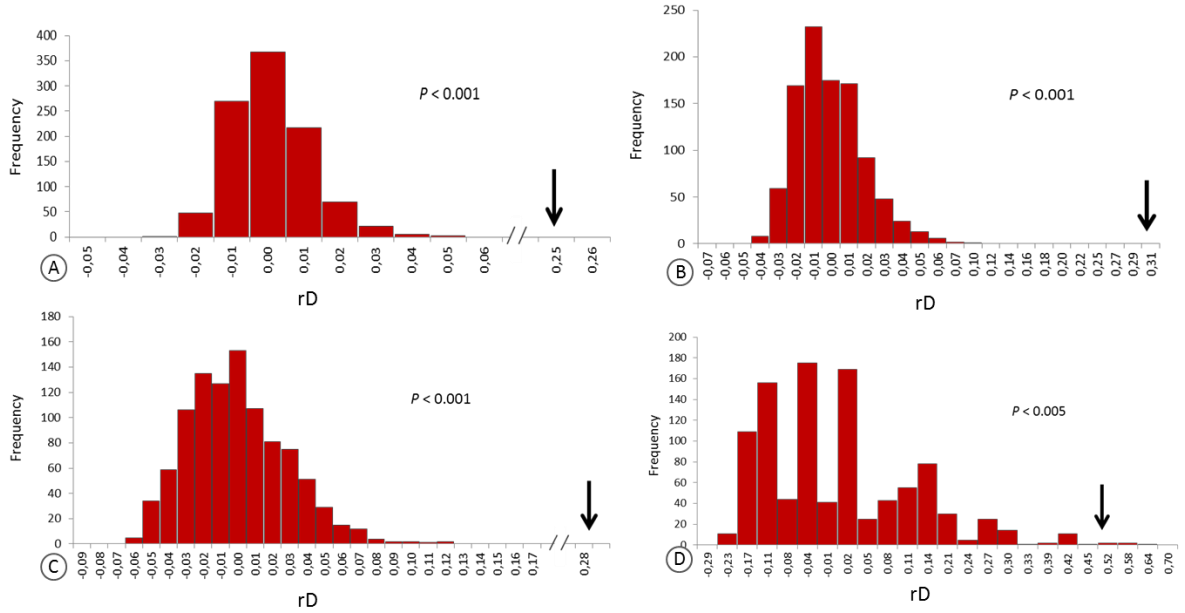


Figure 4. Histogram made with the $\bar{r}D$ frequencies obtained after 1000 time randomized datasets from *F. circinatum* population from Chile. A) The whole population, B) Population from Quivolgo, C) Las Cruces and D) Los Castaños. Arrow indicated the observed $\bar{r}D$ values. *P* is the probability to accept the differences ($P < 0.05$, the $H_o$ is rejected).

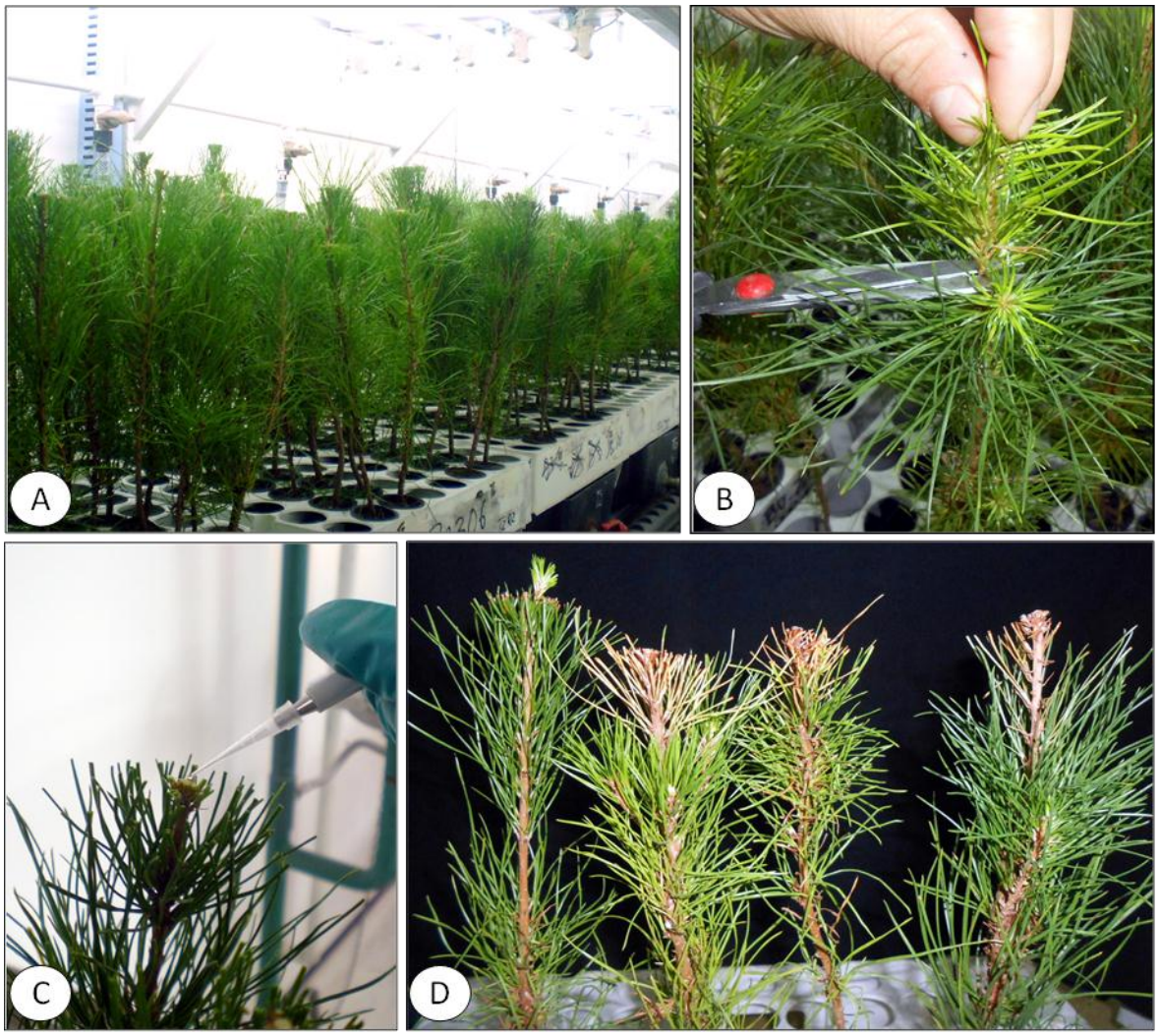


Figure 5. Inoculation trial with *F. circinatum* isolates on *P. radiata* plants. A) Plant material used for the inoculation into the quarantine facilities at Bioforest laboratory. B) The apical bud is cut to create a homogenize inoculation wound on all plants. C) Inoculation of *F. circinatum* suspension using a micropipette. D) Symptoms of *F. circinatum* infection on *P. radiata* plants six weeks after inoculation (one control (left) and three inoculated plants).

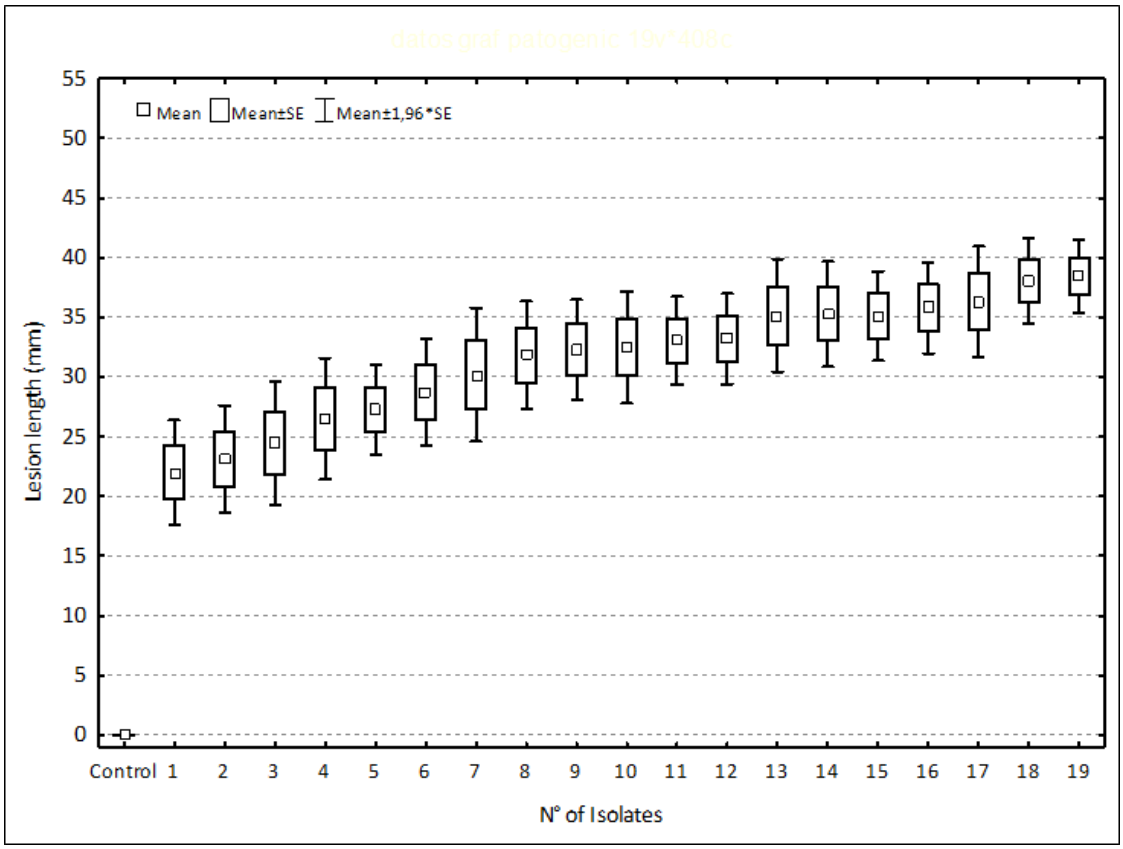


Figure 6. Average lesion lengths (mm) and standard error, after the inoculation of 19 *F. circinatum* isolates on *P. radiata* clones. Each isolate was inoculated into one plant of each of the same 20 clones. Lesion lengths (mm) grouped by isolates and the control.

# Chapter 6

# Molecular identification, incidence and pathogenicity of flute canker on *Pinus radiata* in Chile


## Abstract

Neonectria flute canker is a well-known disease of *Pinus radiata* in New Zealand caused by the fungus *Neonectria fuckeliana*. The same disease symptoms have been reported recently from Chile, but the identity of the fungus has been made based only on morphological features. There is also very little known regarding the epidemiology of the pathogen to develop potential control strategies. In this study, a collection of 300 isolates from symptoms of flute canker were obtained from across the distribution of the pathogen in the south of Chile. Thirteen of the isolates were identified using ITS rDNA data and compared with sequences of isolates from New Zealand and Europe. Isolates were also characterized based on cultural morphology, and this grouping was used to select isolates for an inoculation trial. The results confirmed that the fungus in Chile is the same as that found elsewhere in the world, namely *N. fuckeliana*. Sequences from the Chile population were identical to those for isolates from New Zealand and Europe. Four different morphotypes were identified and there was significant variation in aggressiveness amongst the isolates selected. The fungus was also present across the areas that were assessed and the disease was shown to have increased in incidence between 2009 and 2010. Since 2010, a strategy has been employed to reduce the damage by avoiding pruning during the winter.


## 1. Introduction

*Neonectria fuckeliana* (C. Booth) Castl. & Rossman (*Nectria fuckeliana* C. Booth) is an ascomycete fungus that causes flute canker on conifers (Dick & Crane 2009). The species is believed to be native to the Northern Hemisphere (Europe and North America) on conifers such as *Picea abies* in Europe (Huse 1981) and *Abies* spp. and *Picea* spp. in North America (Smerlis 1969; Schultz & Parmeter 1990). The fungus is frequently associated with die-back on *Abies concolor* (white fir) in Europe and western North America (Roll-Hansen & Roll-Hansen 1979; Callan 1997).

*Neonectria fuckeliana* is characterized by the production of masses of red perithecia on the surface of cankers. The ascospores produced from these fruiting structures are the most important dispersal propagules (Vasiliauskas & Stenlid 1997). The fungus also has two asexual forms; one *Acremonium*-like and the other known as a *Cylindrocarpon cylindroides* var *tenue* (Vasiliauskas & Stenlid 1997; Castlebury et al. 2006).

Neonectria flute canker of *Pinus radiata* D. Don is an important disease in New Zealand where this pine species is widely propagated (NZFOA 2009, INFOR 2011). The disease was first reported in the south Island of New Zealand in 2003 (Gadgil et al. 2003), but the disease symptoms had been observed several years earlier (Dick & Crane 2009). A disease with similar symptoms was first found on *P. radiata* in Chile in the late 1990's, but only officially reported in 2009 from plantations between 7 and 15-years-old (Morales 2009). Neonectria flute canker disease is characterized by cracks in the bark, depressed stem cankers, or a shallow to deep flutes (narrow grooves) that can extend for several meters along the trunks of infected trees (Dick & Crane 2009; Hopkins et al. 2009). Although *N. fuckeliana* does not typically kill trees, stem breakage can occur at infected whorls and the quality of the wood is affected (Gadgil et al. 2003; Hopkins et al. 2008). Pruning wounds and branch stubs are the main infection points for *N. fuckeliana* (Bulman 2009). In this regard, it is important to recognise that a large proportion of the *P. radiata* grown in both New Zealand and Chile is managed to produce clear wood free of knots and that this is achieved by intensively pruning trees. The fluting damage is most serious when branch stub sizes are larger than 60 mm (Bulman 2009). Winter pruning also results in more infection than in summer, due to the lower temperatures and the wet conditions in winter that favour infection (Bulman 2009). After the trees are pruned and infection occurs, the disease develops slowly and the fruiting bodies appear after four to nine months, depending on the season when the trees were pruned (Power & Ramsfield 2007; Bulman 2009). In Chile, the disease has also been associated with animal damage to trees, as well as with wind breakage of branches that produce wounds that allow infection to occur.

The original identification of *N. fuckeliana* in Chile was based on morphological characteristics and the associated symptoms. The aim of this study was firstly to

characterize the fungus using DNA sequence analysis and to compare the data with those for isolates from other regions in the world. Secondly, we considered the pathogenicity of the fungus to *P. radiata* planting stock used in Chile. The distribution of the disease in *P. radiata* plantations in this country was also characterized.

## 2. Materials and methods

### 2.1 Disease symptoms

Neonectria flute canker symptoms were detected in 2008 in two different *P. radiata* plantations distributed between the Los Ríos (39° 48’ 30” S, 73° 14’ 30” W) and Los Lagos (41° 28’ 18” S, 72° 56’ 12” W) regions in the southern distribution of *P. radiata* in Chile (Morales 2009). During an operational health survey in this region in 2008, symptoms typical of neonectria flute canker were detected in pruned *P. radiata* stands between 7 and 9-years-old. A survey was conducted to characterize the symptoms of the disease in this region.

### 2.2 Monitoring and sample collection

A systematic assessment was done of the incidence of flute canker in 2009 and 2010. Areas of approximately 4 000 ha (2009) and 5 000 ha (2010) of *P. radiata* plantations between five to 10-years-old were monitored. The plots assessed over these two years covered a north to south distance of approximately 220 km, from Temuco to Osorno cities, approximately. The assessment was carried out using a series of plots distributed every 25 ha across all pruned plantations in this region belonging to the Arauco Company. In each plot, a total of 30 trees were assessed and these were distributed in three parallel lines of 10 trees each, separated by three rows. Thus, a total of 159 and 205 plots were assessed in 2009 and 2010, respectively.

The incidence of Neonectria flute canker was recorded as ‘0’ = no symptoms and ‘1’ = flute canker present. For severity, symptoms were characterised as (1) low = cankers less than 20 cm long, (2) medium = cankers between 20 to 40 cm long, and (3) high = cankers

more than 40 cm long (Figure 1). Lesions were scored between whorls up to approximately 5.5 m, which corresponded to the pruning height. The longest lesions were scored per stem if more than one canker was present. Analyses of the data were done by calculating the annual average for the plots. The age of the plantations was also considered as a variable.

During the monitoring undertaken in October and November 2009, small sections (3 x 3 cm) of bark with symptoms of flute canker disease and bearing fruiting bodies of the pathogen were collected from ten trees in 10 of the assessed plots, taking three samples per tree. Samples were maintained at 4 °C in brown paper envelopes in plastic containers and transported to the laboratory for isolations and further analyses.

### 2.3 Fungal isolations, DNA extraction and sequencing

Primary isolations from the symptomatic material were made by cutting a small piece of bark (1 x 1 cm) bearing abundant fruiting bodies for further examination. The typical red perithecia were dissected using a sterile scalpel to expose the ascospore masses, which were transferred to 2 % water agar (WA) in Petri dishes using a sterile needle and the plates were incubated at 25 °C during 12 h in dark (Crane et al. 2009). Single germinating ascospores on the agar surface were identified using a dissection microscope and these were individually transferred onto 2 % malt extract agar (MEA) and incubated at 22-25 °C and exposed to 12 h cycles of cool-white fluorescent illumination and darkness, for twenty-one days. Culture morphology was recorded and used to group isolates. At least two isolates, with a maximum of four isolates per cultural morphotype were randomly selected for molecular characterization. The main morphological characteristics of cultural morphotypes were recorded, including colony colour, which was assessed using the colour charts of Rayner (1970) (Table 1).

Mycelium of actively growing cultures was scraped from the surface of the agar plates using a sterile scalpel and ground to a fine powder using liquid nitrogen. DNA was extracted from the ground mycelium using PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The

Internal Transcribed Spacer (ITS) region of the rRNA operon was amplified using primers ITS1 (5' CTT GGT CAT TTA GAG GAA GTA A 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') (White et al. 1990).

The PCR reaction mixture contained 1 x PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 1 mM of each dNTP, 0.4 μM of each primer (Integrated DNA Technologies (IDT), Iowa, USA), 1 U Taq DNA polymerase (Promega corporation, Wisconsin, USA), 30 ng of template DNA. Sterile deionized water was used to make up the total volume of the reaction to 25 μl.

Amplifications were performed in an iCycler thermocycler (Bio-Rad, Hercules, CA, USA). The thermocycle sequence started with an initial denaturation at 95 °C for 5 min, followed by 30 cycles of template denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s, followed by extension at 72 °C for 30 s. A final elongation of 5 min at 72 °C completed the program. PCR products were visualised on 1 % agarose gels in a TAE buffer electrophoresis system, stained with ethidium bromide and visualized with UV light.

PCR products were purified on Sephadex columns (Multiscreen HV, Millipore, Bedford, USA) following the manufacturer's recommendations, to remove excess primers and nucleotides. These PCR products were sequenced with the forward and reverse primers used in the amplification reactions. Reactions were performed using an ABI PRISM™ Big Dye terminator sequencing reaction kit following the manufacturer's instructions (Perkin-Elmer, Applied BioSystems, Foster City, USA). Sequencing was done using an ABI 3100™ automated DNA sequencer.

## 2.4 Phylogenetic analyses

Sequences of the isolates were edited using Vector NTI 11 (Lu & Moriyama 2004). DNA sequences for species previously published were retrieved from GenBank (http://www.ncbi.nlm.gov). Sequences for New Zealand collections were supplied by Dr. R. Ganley (Scion). The data matrices were aligned online using MAFFT (http://align.bmr.kyushuu.ac.jp/mafft/online/server/) version 6 (Katoh et al. 2005) and

checked manually for alignment errors. *Bionectria ochroleuca* was used as the outgroup taxon.

Phylogenetic analyses of the sequence data were made using Maximum Parsimony (MP) and Maximum Likelihood (ML) in PAUP* v.4.0b10 (Swofford 2002). A maximum parsimony (MP) genealogy was constructed with the heuristic search option (100 random taxa additions, tree bisection and reconstruction (TBR)) in PAUP. The uninformative aligned regions within the data set were removed from the analyses, gaps were treated as a fifth character and all characters were unordered and of equal weight. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the tree obtained was evaluated by 1 000 bootstrap replications. Other measures such as tree length (TL), rescaled consistency index (RC), and the retention index (RI) (Hillis & Huelsenbeck 1992) were also recorded.

For ML analyses, the best nucleotide substitution models were selected with Model test 3.7 (Posada & Buckley 2004). The model for GTR + G (G = 0.4180, I = 0.0) was chosen for the datasets. The ML analyses were performed in PAUP v.4.0b10 and confidence levels were determined with 1 000 bootstrap replications. The consensus trees were constructed in MEGA v.3.0 (Kumar et al. 2004) and posterior probabilities were assigned to branches using a 60 % majority rule.

## 2.5 Pathogenicity tests

Pathogenicity tests were conducted using four isolates of *N. fuckeliana*, including one representative of each of the cultural morphotypes that had been identified. All isolates used in the inoculations trials were grown on MEA at 22 °C for two weeks prior to inoculation. Preservation and maintenance of the cultures was the same as that used for the identification of isolates described above.

Inoculations were conducted on 1-year-old cuttings representing an equal a mix of three *P. radiata* families with unknown levels of susceptibility to neonectria flute canker. Each *P. radiata* family was replicated ten times per treatment; therefore, each treatment

included a total of 30 plants. In total, one hundred and twenty plants were inoculated for each of the four selected isolates (one from each morphotype) to test for relative aggressiveness, and thirty plants were inoculated as controls. The plants were acclimatized in a room at approximately 18-22 °C for two weeks prior the inoculation. Stems of plants were surface disinfected in the area to be inoculated using 70 % EtOH, prior to inoculation. Small (3 mm diam.) discs of bark were removed from the middle parts of the stems, where the tissue was somewhat lignified, using a cork borer. Plugs of mycelium of similar size, taken from the actively growing edges of cultures, were placed into the wounds. Wounds were covered with Parafilm (Pechiney plastic packing, Chicago, USA) to reduce desiccation and to avoid contamination.

Six weeks after inoculation, symptom characteristics and lesion lengths (mm) were recorded. Small pieces of wood from lesions on 30 % of the plants were plated onto MEA to re-isolate the inoculated fungus. A linear model of analysis of variance (ANOVA) and means were separated based on LSD (Least Significant Difference) using the software STATISTICA V10 for Windows (StatSoft Inc., 2004).

## 3. Results

### 3.1 Disease symptoms

The symptoms typically included depressions in the stems that originated from the pruned whorls, and masses of red perithecia that resembled those of *N. fuckeliana* were common on diseased tissue (Figure 2). The disease symptoms showed a broad range of severities (Figure 2). The majority of the symptoms (> 95 %) were observed on pruned trees. Fruiting bodies were observed either along the flute in the trunk or more commonly on the pruned whorl. No symptoms were observed in the foliage of any trees. The first visible symptoms of infection were a slight depression of the pruned area, which gradually lengthened and deepened as the cambium growth was affected in the infected area. Between four to six months after infection, a wide range of damage was visible, ranging from slight damage to deep grooves on the stems of trees.

## 3.2 Monitoring and sample collection

Neonectria flute canker was found in an average of 10.1 % of the total plots assessed in 2009 and 13.1 % in 2010. The range of disease incidence was between 3.3 % and 66.7 % for 2009 and between 3.3 % and 73.3 % for 2010, respectively. neonectria flute canker was distributed across the whole of the surveyed region (Figure 3). The incidence of the disease was the lowest in the youngest age class (five-year-old plantations; 3 % in 2009 and 2.7 % in 2010) and the highest in the older age classes (10-year-old plantations; 16.3 % in 2009, and 20.4 % in the nine-years-old plantation in 2010) (Figure 5). The severity increased between 2009 and 2010. The increase of the average severity categories was reflected in the lower (1) and medium (2) categories, while the highest damage class (3) showed a slight reduction from 1.5 % to 1.4 % between 2009 and 2010 (Figure 6).

## 3.3 Fungal isolations, DNA extraction and sequencing

A total of 300 isolates were obtained from ten different plantations monitored. All isolates originated from perithecia either on the cankers or on the pruning stubs. Four distinct cultural morphotypes could be distinguished amongst the cultures (Table 1; Figure 4).

A total of 13 isolates were selected for sequencing to validate the identity of the causal agent, representing two to four representatives of each cultural morphotype. Six of these isolates have been deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

## 3.4 Phylogenetic analysis

The ITS sequence dataset contained 445 characters. A single most parsimonious tree was generated with a retention index (RI) of 0.913, rescaled consistency index (RC) of 0.813, tree length (TL) of 165.7 and homoplasy index (HI = 0.109) (TreeBase Accession No. S12358). The sequences from isolates from Chile grouped in the same clade as sequences from New Zealand isolates representing *N. fuckeliana* (Figure 7). This clade also included

two sequences of *N. fuckeliana* deposited in GenBank, which were isolated from *Picea* spp. in Europe. Most of the isolates in this clade were identical, or nearly identical, and could not be distinguished based on geographic origin or cultural morphotype.

### 3.5 Pathogenicity tests

All isolates tested for pathogenicity produced lesions on the outer bark, but more clearly in the xylem of the inoculated plants (Figure 8). The most common symptoms observed were the exudation of resin and discoloration around the inoculation points. No symptoms developed on plants inoculated as controls.

The lesions observed in the xylem were very variable in length. The mean inner lesion lengths on the plants inoculated could be grouped into two distinct groups. The first group (morphotypes C and D) gave rise to lesions ranging for 10.6 to 10.9 mm on average, with no significant difference between them. The second group (morphotypes A and B) had lesions ranging on average between 18.4 to 23.6 mm, also without a significant difference between them, but the two groups differed significantly from each other. The pathogen was recovered from 40 % (N = 18) of the plants sampled.

## 4. Discussion

Results of this study provided evidence based on ITS rDNA sequence data that flute canker on *P. radiata* in Chile is caused by *N. fuckeliana*. Furthermore, the data showed that isolates of the fungus from Chile were identical to those from New Zealand and Europe. The disease in plantations was shown to be present in about 10 % of the assessed plots, but the severity was relatively low. It was also obvious that neonectria flute canker is directly associated with pruning or natural wounds, as has been found in New Zealand (Dick & Crane 2009) and in areas of the Northern Hemisphere on *Picea* and *Abies* species (Huse 1981).

Neonectria flute canker was first reported from Toltén in the Araucanía Region (Morales 2009). Similar symptoms were, however, observed in 1998 between Los Ríos and Los

Lagos regions, more than 60 km south of the area where the disease was first reported (A. Rotella pers. comm.). It is thus possible that the disease has been present in Chile since the late 1990's, but it evidently did not cause significant damage and was thus not noticed. It is possible that the symptoms were not recognised by foresters when the disease first appeared and before damage became more widespread and evident.

During the course of this study, neonectria flute canker was recorded across a 220 km area from the north of the area where the disease was first found (near Temuco city; 38° 46' S) to the southernmost point of its apparent distribution (near Osorno city; 40° 39' S). *Neonectria fuckeliana* occurs further south in New Zealand (from 41° 53' S to 46° 28' S), where it was first reported from the south Island (Dick & Crane 2009) and from where it has gradually spread northwards (Bulman 2007). In New Zealand the fungus has been detected in *P. radiata* plantations in regions with average annual rainfall above 500 mm (Crane et al. 2009), whereas in Chile the current distribution is restricted to regions where the average rainfall is above 1 000 mm per year (INE 2010). This suggests that the fungus could be expected to also spread further northwards in Chile where the largest areas of *P. radiata* are concentrated. Given this possibility, it will be important to further improve procedures to ensure early detection, as well as to gain knowledge of the epidemiology and biology of this pathogen in Chile. Subsequent to the completion of this study and during the preparation of this manuscript, symptoms of *N. fuckeliana* were identified in the Biobío region, which would then be the most northerly distribution of the disease in plantation forestry in Chile (R. Gómez pers. comm.).

The incidence of neonectria flute canker increased between 2009 and 2010. This was probably due to the continued pruning during the winter in some areas. Research from New Zealand (Bulman 2009) and Chile (R. Ahumada, unpublished data) strongly suggest that all pruning should be withheld during the winter when the main infection occurs. In New Zealand, neonectria flute canker disease is almost exclusively associated with pruned branches. The results of a six-year-long investigation showed that pruning in winter results in substantially more damage than when this procedure is carried out in summer (Bulman 2009). This matches the fact that double the number of spores is produced in late autumn and winter than in spring and summer (Crane et al. 2009).

The inoculation trial carried out in this study showed that isolates of *N. fuckeliana* varied significantly in their aggressiveness. This variation might contribute to the variation in the level of symptom severity observed in the field. The other source of variation could also be differences in susceptibility or response of the host trees. Although this was not tested here, inoculation trials conducted in a greenhouse and in the field in New Zealand have also shown marked differences between the responses of individual trees to infection (Dick & Crane 2009).

It is important to recognise that we were able to re-isolate *N. fuckeliana* from only 40 % of the inoculated plants and a similar low level of isolation (7-24 %) has been recorded in New Zealand (Dick et al. 2011). These results reflect a problem in reproducing disease symptoms in small plants, and raises questions relating to how these results might be interpreted (Dick et al. 2011). Given the importance of resistance breeding to reduce the impact of this disease, and because pruning wounds cannot entirely be avoided, it will be important to pursue efforts to develop more effective inoculation protocols to screen *P. radiata* clones or hybrids for resistance to neonectria flute canker.

The low incidence of neonectria flute canker reported in this study suggests that it is not a particularly important disease in Chile. It does, however, cause significant damage in some situations. This justifies further investigations to better understand the factors that influence distribution of the disease, modes of infection and its potential impact. It will also be important to continue to monitor the incidence of the disease and to evaluate changes in incidence and severity across different plantation areas. The selection of resistant genetic material, especially through the deployment of hybrids, is one option to reduce the impact of neonectria flute canker and it should also be possible to select planting sites for the production of high value wood where pruning is required and where the disease is less likely to be a problem.

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Table 1. Characteristics of the *Neonectria fuckeliana* morphotypes based on culture morphology, that were used to select isolates for use pathogenicity tests.

| Isolates | Morphotypes | N° of isolates per type | Characteristics of the colony [1] |
|---|---|---|---|
| CMW37274 | A | 10 | Circular with fimbriate edges, cottony, dense up to the half of the colony and becoming sparse towards the edge, above luteous (17b), sterile, no effect on medium. |
| CMW37275 | B | 4 | Had even edges with mycelium densely, fluffy, white up to the half of the colony and becoming orange (13i) and pale luteous (17d), sterile and resulting in no discoloration of the medium. |
| CMW37276 | C | 4 | Cottony, dense mycelium and becoming sparse towards the edges, above salmon (11'd), sterile and no colour changes were seen in the media. |
| CMW37278 | D | 5 | Flat and cottony mycelium which was dense in the central 2/3 and becoming sparse towards the edges, salmon (11'd) coloured with an amber (19'i) tint, sterile and having no effect on colour of the medium. |

[1] Codes for colony colour refer to Rayner (1970).

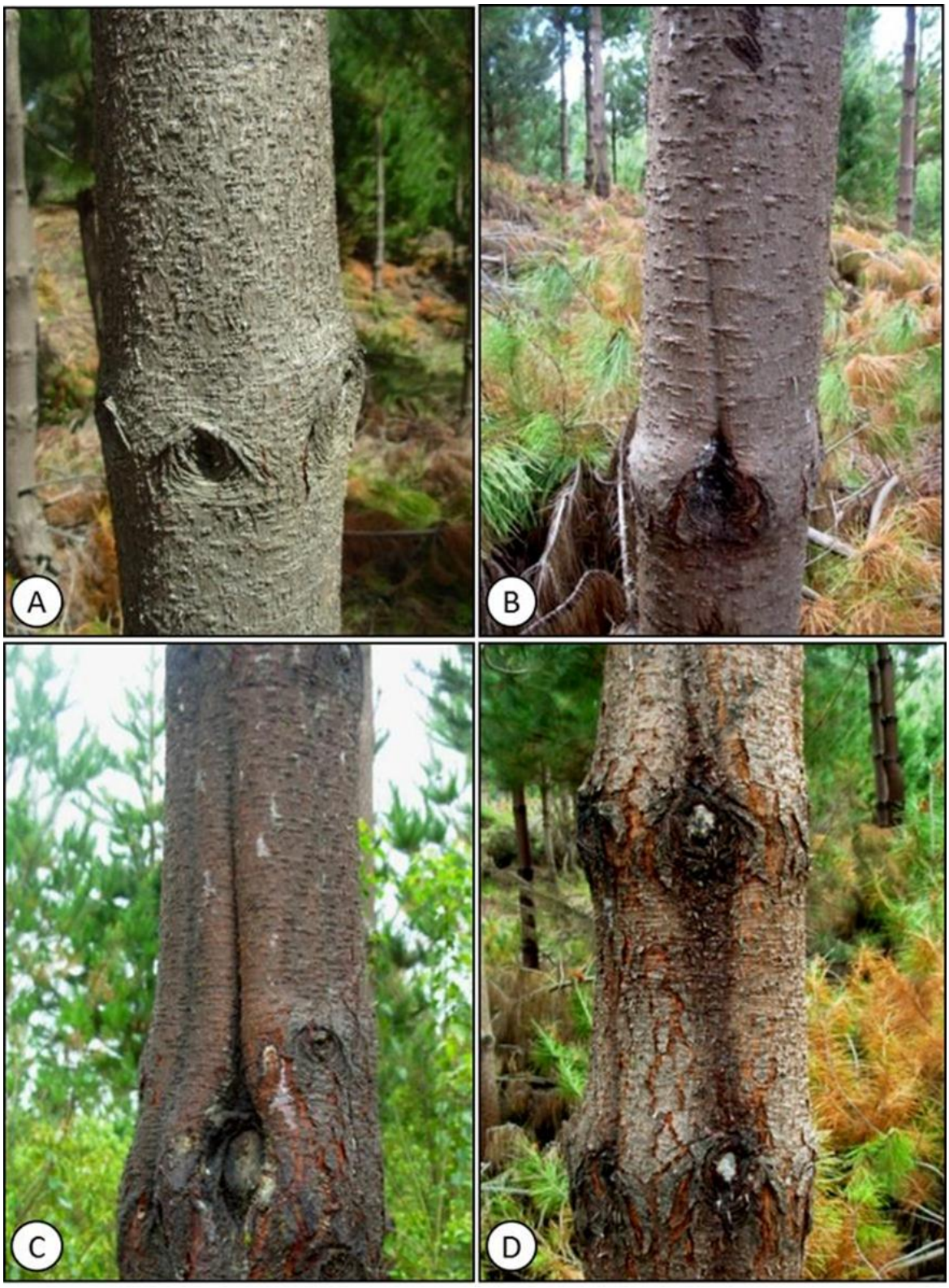


Figure 1. Classification of *Neonectria fuckeliana* damage in *Pinus radiata* plantations used in the operational monitoring. A) Level **0** = No damage (Healthy trunk). B) Level **1** = Low damage (Minor groove < 20 cm). C) Level **2** = Medium damage (Defined groove between 20 to 40 cm) and D) Level **3** = Severe damage (Deep groove and deformation > 40 cm).


Figure 2. Symptoms caused by *Neonectria fuckeliana* infection of *Pinus radiata* trees. A – E) Varying levels of damage. F-G) Red fruiting bodies (perithecia) normally found either in the pruned whorl (F) or along the flute canker (G).

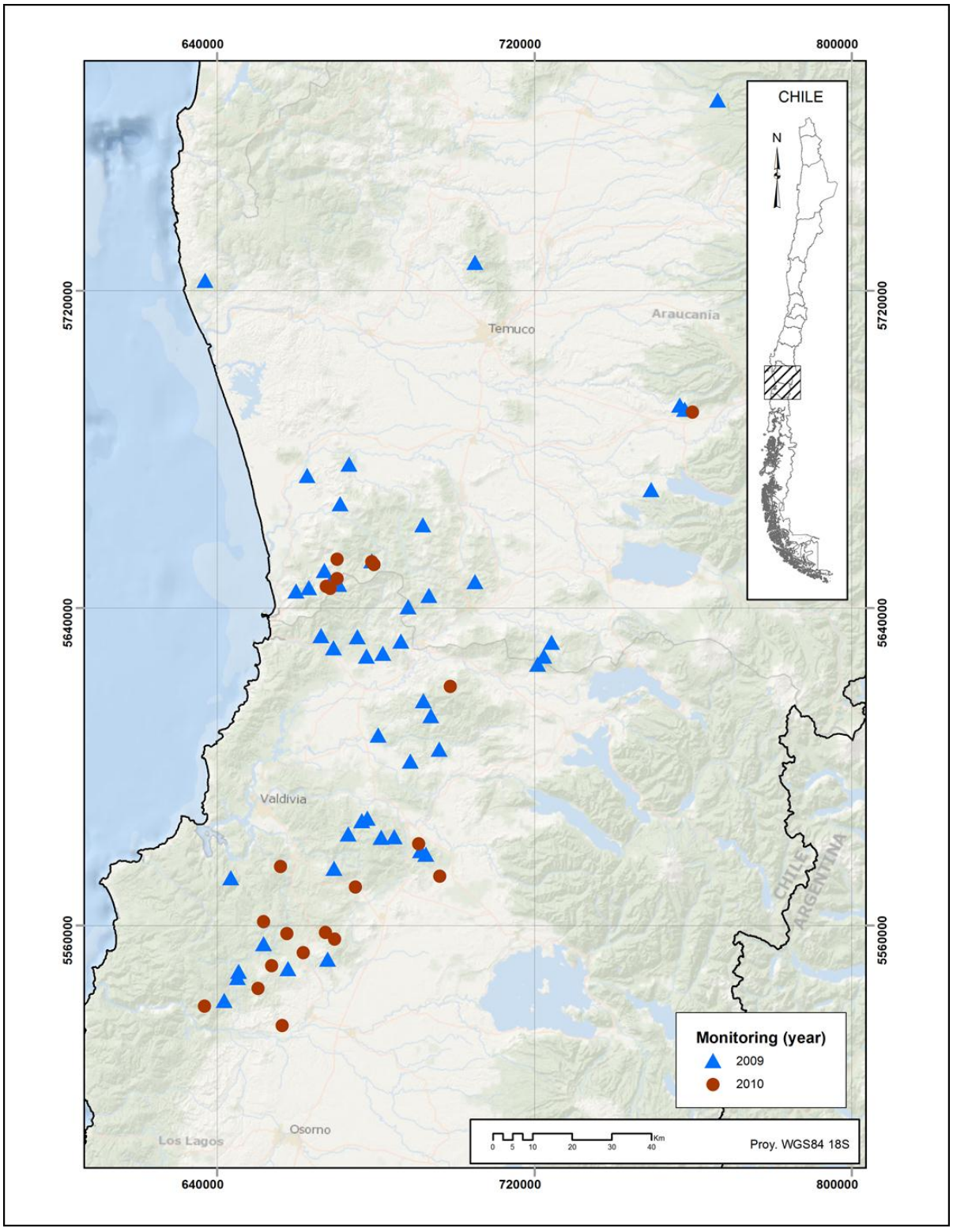


Figure 3. Distribution of the neonectria flute canker in *P. radiata* plantations in Chile, based on monitoring conducted in 2009 and 2010.

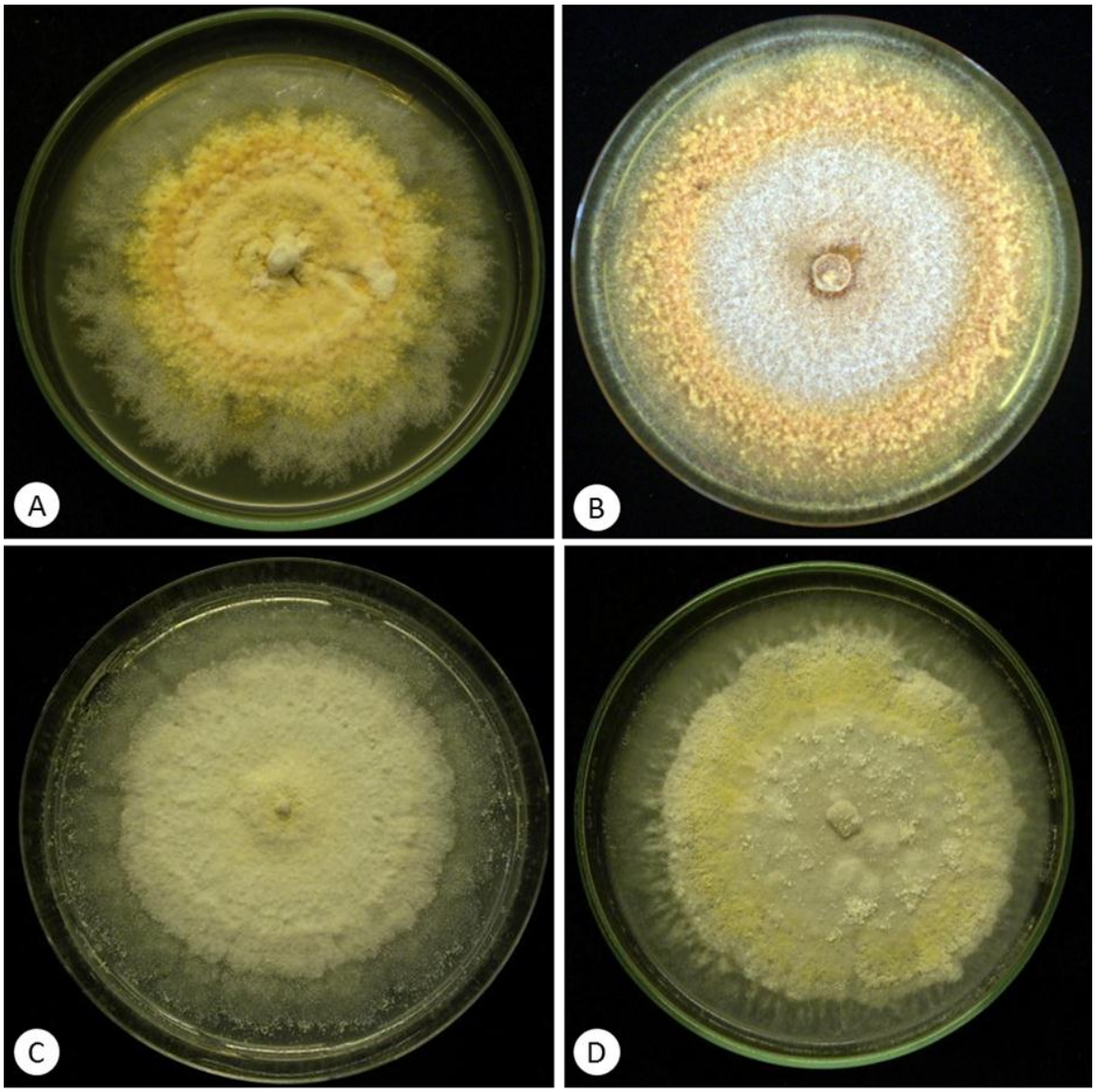


Figure 4. Four morphotypes of *N. fuckeliana* found in this study and used to group isolates for subsequent DNA sequence analyses and pathogenicity tests.

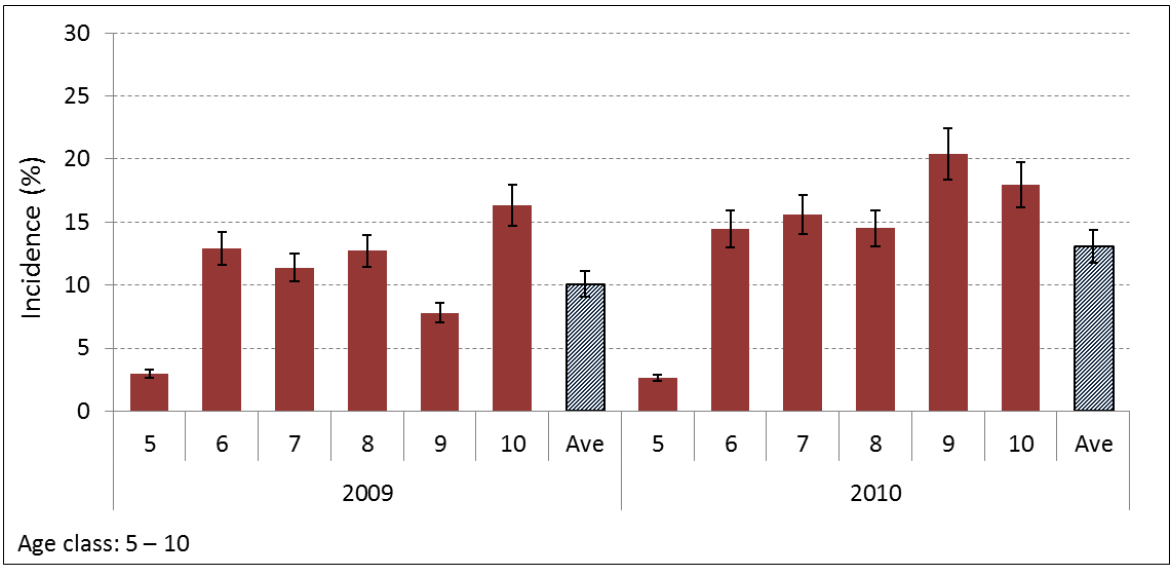


Figure 5. Average percentage incidence (with bars showing standard error) of neonectria flute canker on *Pinus radiata* plantations during 2009 and 2010. The incidence was calculated for the all plots (average per annum = Ave) and grouped by the range of age (5 to 10 years old) classes assessed of Forestal Valdivia plantations in Chile.

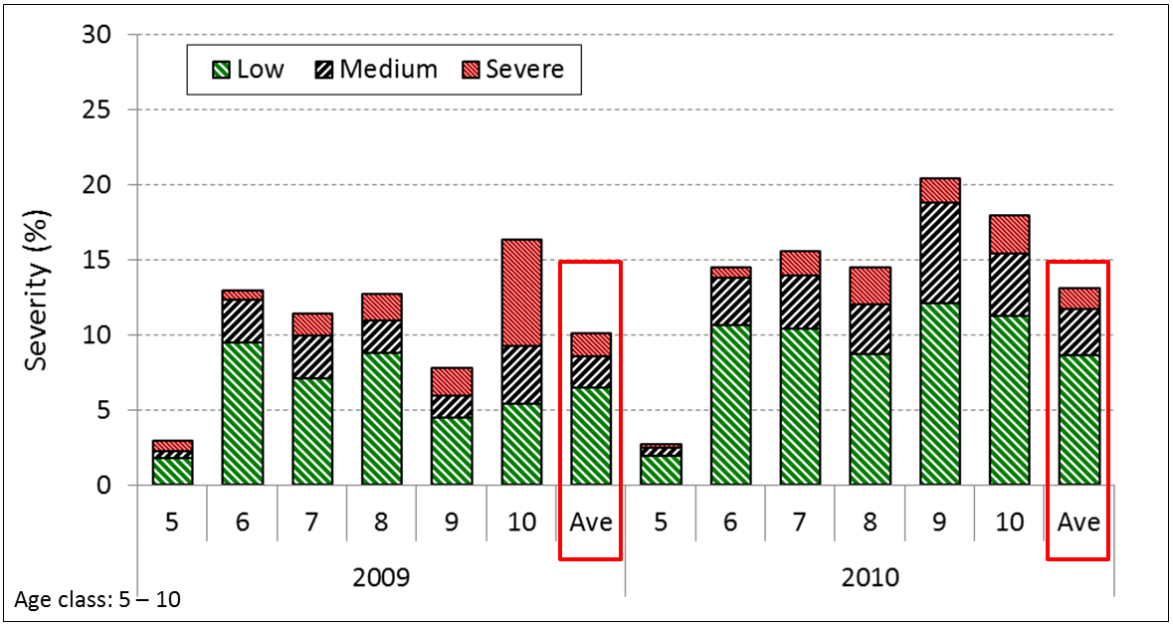


Figure 6. Average severity of neonectria flute canker of *P. radiata* plantations assessed. The severity was calculated for the all plots and grouped by the range of age (5 to 10 years old) assessed of Forestal Valdivia plantations in Chile. Red rectangles indicate the average per annum and per category of severity.

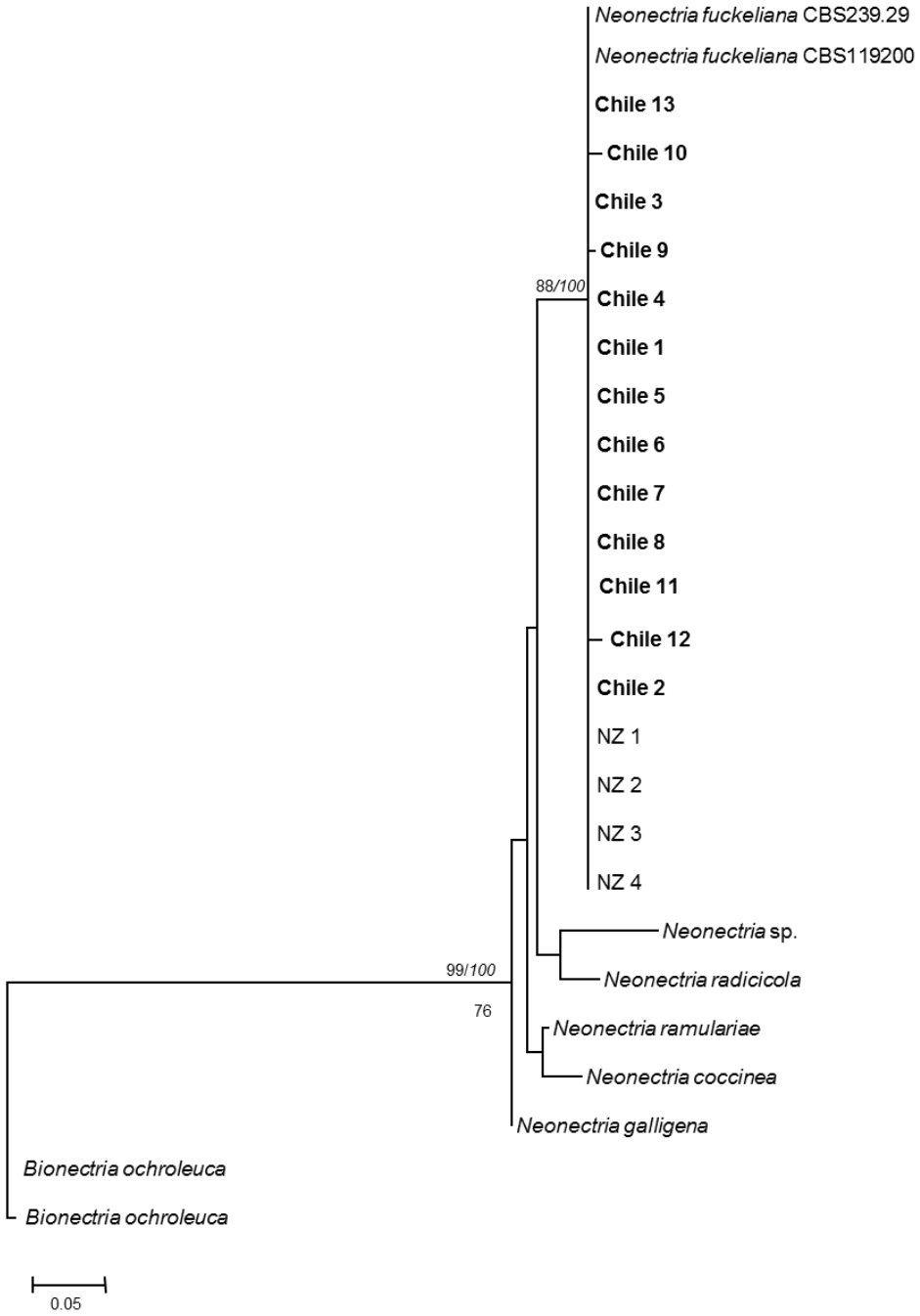


Figure 7. Phylogenetic tree drawn using Maximum Likelihood (ML) analysis of the ITS ribosomal DNA data set. Bootstrap values for ML (roman) and Maximum Parsimony (italics) above 76 % are given at the nodes. The tree was rooted to *Bionectria ochroleuca* (S9A6 and ATT093). Isolates sequenced in this study are indicated in bold. Sequences from New Zealand were provided by Dra Rebecca Ganley from Scion.

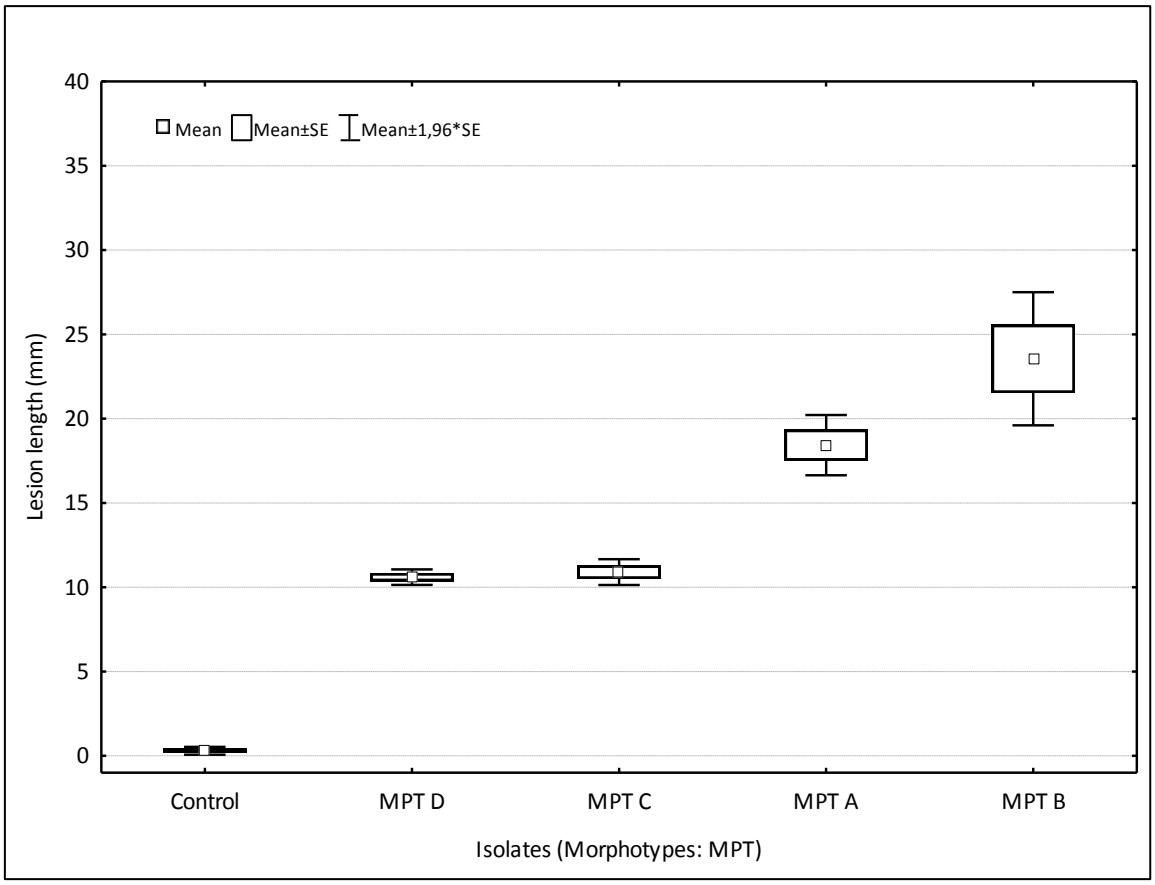


Figure 8. Average lesion lengths (mm) after inoculation of four isolates of *N. fuckeliana* onto 30 *P. radiata* plants representing a mixture of 3 families. The four isolates represent different morphotypes of the pathogen with morphotypes C and D being substantially less aggressive than those of morphotypes A and B.

# Summary

The timber and pulp industry is an important part of the economy of Chile, and it relies on *Pinus radiata* plantations as its most important crop. For this reason it is crucially important to develop capacity and knowledge to deal with the ever increasing number of diseases of *P. radiata*, which represent one of the greatest threats to the sustainability of this industry. This thesis included investigations on the three main pathogens currently affecting *P. radiata* in nurseries (*Fusarium circinatum* and *Phytophthora pinifolia*) and in plantations (*P. pinifolia* and *Neonectria fuckeliana*) in Chile. Studies focused on issues such as correct identification, population diversity and epidemiology as important basic knowledge that will contribute towards the understanding of the pathogens and improved management decisions. Importantly, tools were developed to prove Koch's postulates for *P. pinifolia* as the causal agent of DFP. Sporangia of this pathogen were shown to remain on the needles for extended periods of time and to mainly disperse during the rainy season. Important for the exportation of timber, it was shown that green sawn lumber taken from trees infected by *P. pinifolia* or from infected pine plantations displayed no evidence of the pathogen surviving in this material. Finally, the potential was demonstrated for combining data on site selection for planting, the use of disease-tolerant clones and the application of effective chemical products for the management of DFP as part of an integrated strategy. For *F. circinatum*, a population genetic study revealed a relatively low level of gene and genotypic diversity, including only one mating type, in Chilean populations. These data, together with the predominantly asexual reproduction of the pathogen, and demonstrated variation in resistance of the trees, illustrates the potential of resistance breeding strategies. It also illustrates the importance of quarantine to prevent the introduction of more diversity of the pathogen. *Neonectria fuckeliana* was confirmed to be the causal agent of flute canker across all the areas assessed. The knowledge generated through this thesis on key pathogens of *P. radiata* in Chile will form an important part of the knowledge foundation on which management decisions will be taken in future.