# Determination and characterization of the causal agent of *Pinus radiata* needle blight in Chile

**by**

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# DECLARATION

I, the undersigned, hereby declare that the thesis submitted herewith for the degree *Phylosophiae Doctor* to the University of Pretoria, contains my own independent work and has hitherto not been submitted for any degree at any other University.

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# PREFACE

In 2004, 70 ha of *Pinus radiata* plantations experienced a serious and devastating defoliation on the Arauco coast of Chile. Despite extensive effort in the few years following the onset of the problem, the cause was not found. The problem spread rapidly until 60000 ha had been damaged in 2006. The affected area is principally close to the coast and the level of damage varies across the region. As the cause of the problem was not known, the damage was locally referred to "Daño Foliar del Pino" (DFP) which translated into English means "Foliar damage on pine" reflecting the symptoms of the disease.

In 2007, a study was undertaken that included an exhaustive series of isolations using a wide range of methods. The outcome of this investigation was that a *Phytophthora* sp. was consistently associated with the disease symptoms. This result was most surprising as no *Phytophthora* sp. had ever been previously implicated in a defoliation disease of pines. However, the consistent association of the organism with the symptoms and the fact *Phytophthora* spp. are primary plant pathogens, provided good evidence that this organism was the cause of DFP. The aim of this thesis was firstly to determine the identity of the *Phytophthora* isolated. In addition, it was important to provide concrete experimental evidence that this *Phytophthora* sp. was the causal agent of DFP. Further priorities in the study of DFP were to determine the population structure of the new *Phytophthora* species should it prove to be pathogenic, and to investigate possible control strategies against the disease.

In the first chapter of this thesis, a general review of *Phytophthora* app. associated with forest ecosystems is presented. Several species of *Phytophthora* are associated with serious damage in native forests worldwide, and a major motivation of this chapter was to summarize the information related to the impact of some of the key *Phytophthora* pathogens, and management strategies used against them. Information related to *Phytophthora* spp. that affect forest plantations was also summarized and compared to the situation in natural forests. A complete compilation of species associated with common plantation tree species, namely *Pinus*, *Eucalyptus* and other forest plantation species, is also presented.

In Chapter Two, the damage caused by DFP is characterized and the identity of the causal agent determined. Samples collected in several localities in Chile were cultured in growth media specific for *Phytophthora* spp. The isolates were identified using morphological characterization and comparisons of mtDNA and rDNA sequences. The pathogen was found to represent a species of *Phytophthora* and designated *P. pinifolia*. Pathogenicity trials were conducted to evaluate its ability to cause disease on *P. radiata* and thus satisfy Koch's postulates.

A molecular based method for the rapid identification of *P. pinifolia* as developed and this work is presented in Chapter Three. This technique will be essential for the fast and cost effective identification of the pathogen that will be needed for epidemiological studies necessary to determine effective management strategies. In addition, accurate identification of *P. pinifolia* is needed for quarantine activities, where large volumes of samples must be screened. Species-specific primers based on DNA sequences for two regions (ITS and Ypt1) were developed. A PCR-RFLP protocol developed previously to identify several *Phytophthora* spp., was also evaluated for its capacity to discriminate *P. pinifolia* from other *Phytophthora* spp.

Population genetic diversity can provide clues pertaining to the origin of pathogens. Understanding this diversity is important in assessing the value of strategies such selecting for disease tolerant material in disease management. The population diversity of *P. pinifolia* was determined using AFLPs (Chapter 4). A collection of isolates representing a wide distribution of *P. pinifolia* cultures from diseased pines in Chile were used to determine the genetic diversity of this pathogen. In addition several techniques were also used in attempts to induce the sexual state of *P. pinifolia*, in the hope of determining the mating types of the isolates should the pathogen be determined to be heterothallic.

In Chapter Five of this thesis, a fungicide trial was executed to test several common fungicides used in the management of other *Phytophthora* spp. Additional techniques were also evaluated, such as *in vitro* hyphal growth inhibition by mixing fungicides into growth medium. Protective and curative fungicide effects of the same fungicides tested *in vitro*, were also evaluated at the same doses in inoculations on *P. radiata* seedlings.

The chapters in this thesis represent separate, yet linked studies on *P. pinifolia* and are presented in the format of stand-alone manuscripts. Due to the nature of this style, however, there is some unavoidable repetition in the text, especially within the introduction sections of each chapter. Chapters two and three, of this thesis have already appeared in the international literature. Chapters one, four and five have either been submitted or are in preparation for publication.

# Chapter 1

## Literature review:

## *Phytophthora* spp. in forest ecosystems

## ABSTRACT

Approximately 4 billion ha of forests cover about 30 % of the globe. These forests include native and planted trees, and provide source of wood, pulp, fuelwood, fibre and non-wood forest products. Pests and diseases are an important component of the ecology of native forests, but they can also be detrimental to the quality, productivity and sustainability of planted and natural forests. *Phytophthora* spp. are amongst the many important biotic agents that damage principally in native forests. The most ubiquitous species is *P. cinnamomi,* which has for example destroyed large areas of native *Eucalyptus* forest in Australia. Other species include *P. ramorum* that has caused dramatic damage to the native coastal forests of California and Oregon (USA), *Phytophthora alni* and *P. lateralis* that have virtually wiped out *Alnus* spp. and *Chamaecyparis lawsoliana* forests, respectively, in several areas where these trees are native. In the case of the plantation forestry industry, *P. cinnamomi* affects important forest species both in the nursery and in established plantations, however, in the case of established trees, the damage is usually associated with flooded areas or stressed trees. The recent appearance of the very destructive *P. pinifolia* in *Pinus radiata* plantations in Chile, is of considerable concern as it threatens one of the most important *Pinus* spp, utilized in plantations in many parts of the world. Information presented in this review, suggests that, as a consequence of climate change, conversion of natural vegetation for agriculture and forestry, and the increasing movement of goods will lead to increased damage to forests and plantations due to *Phytophthora* spp. In this regard, it is likely that new pathogen host encounters, host shifts and the emergence of hybrid species will be important. Ultimately, this seems likely to result in serious disease problems and possibly also the extinction of trees in some natural situations.

## 1.0 Introduction

Approximately 4 billion ha of forests cover about 30 % of the globe (FAO, 2007). These forests ecosystems are important repositories of terrestrial biodiversity and they play a key role in safeguarding the natural, climatic and cultural processes on which life depends (Herrmann, 2006). A forest ecosystem is defined as "a dynamic complex of plant, animal and micro–organism communities and their abiotic environment, interacting as a functional unit, where trees are a key component of the system" (FAO, 2005). Such forests also provide a variety of valuable products such as timber, fuelwood, fibre and non–wood forest products (FAO, 2005). In this regard, they play an important role in contributing to the livelihoods of rural communities (FAO, 2009).

A **native** forest is defined as a "stand composed predominantly of native tree species established naturally. This can include assisted natural regeneration, excluding stands that are visibly offspring/descendants of planted trees" (FAO, 2005). "A native forest where there are no clearly visible indications of human activity and where ecological processes are not significantly disturbed are defined as **primary** forests "(FAO, 2005). These native forests represent approximately 36 % of all forests globally, with the largest areas found in Latin America and the Caribbean, followed by North America (FAO, 2007). Forest plantations established using native species have been mainly established with the purpose of restoration or to extract products other than wood (Huang *et al*., 1994; Santos & Luz, 2007). Such trees are also used for the restoration of forests damaged as a consequence of biotic (Anagnostakis, 1987) or abiotic factors (Jung & Blaschke, 2004) that have substantially diminished forested areas.

A forest **plantation** is considered as a "stand in which trees have been established by planting or/and deliberate seedling or coppicing (where the coppicing is of previously planted trees) with either native species or non–native species that have even–aged and regular spacing" (FAO, 2005). Forest plantations are primarily established for the production of timber or fuel wood and these provided about 50 % of the total wood production worldwide (FAO, 2007). Some plantations have been established to reduce erosion, fix carbon, or other environmental, economic, or social benefits (Brockerhoff *et al*., 2008; FAO, 2005). Conifers dominate the productive planted forest category, accounting for 54 % of the plantation area reported in 2005 (FAO, 2007). These

plantations contributed to about half the industrial round wood production of approximately 1.6 billion cubic meters in 2004 and a total trade value in wood products reaching approximately US$327 billion (FAO, 2007). This represents 3.7 % of global trade value in all commodity products.

Pests and diseases represent one of the greatest threats to both planted and natural forests. In natural forests, they play a key role in the dynamics of the ecosystem, form important components of the nutrient cycle, contribute to acceleration of plant succession and influence the rate and direction of nutrient transfer between vegetation and plant litter (Burgess & Wingfield, 2002; Gilbert, 2002; Lovett *et al*., 2004; Morehouse *et al*., 2008; Schowalter, 1981; Swank *et al*., 1981). However, they can also have a negative influence on the development and sustainability of forests by "diminishing health, vigor, survival, yield and quality of wood and non–wood products, and influence wildlife habitat, recreation, aesthetics and cultural value" (FAO, 2009). In plantations, pests and diseases result in the limitation of plantation programs and they have led to the abandonment of tree species or varieties (Hunter *et al*., 2004; Wingfield, 2003; Wingfield *et al*., 2006). They can lead to the necessity to clear cut large areas dominated by infested trees (FAO, 2009) and in some cases, the use of large quantities of pesticides (Friedman, 2006; Pimentel *et al*., 1993; Soares & Porto, 2009). These can result in very significant negative economic impacts (Holmes *et al*., 2009; Pimentel *et al*., 2000; 2005).

Of the many pathogens and pests that are detrimental to forest ecosystems, diseases caused by species in the oomycete genus *Phytophthora* provide examples of those resulting in some of the most serious epidemics. In this regard, the damage caused to forest ecosystems by *Phytophthora* spp. has been principally associated with natural forests (Brasier *et al*., 2004; Hansen *et al*., 2000; Rizzo *et al*., 2002; Weste & Taylor, 1971). In some cases, damage has been such that it has led to the devastation and even extinction of certain tree species (Hansen *et al*., 2000; Weste & Marks, 1987), thus substantially changing the structure and composition of the forest (Ellison *et al*., 2005; Peters & Weste, 1997; Rizzo *et al*., 2005).

In the case of plantation forestry, *Phytophthora* spp. are well known as the causal agents of especially pre– and post–emergence damping off in forest nurseries as well as in the

first years of plantation development (Heather *et al*., 1977; Linde *et al*., 1994; Newhook & Podger, 1972; Reglinski *et al.*, 2009). However, few examples exist where *Phytophthora* spp. have caused serious disease problems on mature trees in plantations (Ali *et al*., 1999; Chavarriaga *et al*., 2007; Durán *et al*., 2008; Newhook & Podger, 1972; Wingfield & Knox–Davies, 1980).

*Phytophthora* spp. are well adapted to survive and proliferate in forest ecosystems and they can persist unnoticed in an environment for long periods of time. They spread in forests by means of their reproductive structures, and they are well adapted to survive in the soil. Those species with the capacity to spread aerially, utilize wind and rain to move the sporangia in the canopy, thus reinfecting the same tree or moving to new trees (Davidson *et al*., 2003a; 2005; 2008; Hansen *et al*., 2008). For those species that disperse via the soil, the sporangia release zoospores that are able to swim in soil associated water to the host (Drenth & Guest, 2004; Hardman, 2007; Staples, 2002; van Walker & van West, 2007; West *et al*., 2002). Asexual chlamydospores and the sexual oogonia are the most important resting structures for *Phytophthora* spp. and they can survive for many years in the soil until water, animals or soil movement bring them into contact with a host (Drenth & Guest, 2004; Erwin & Ribeiro, 1996; Hemmes & Lerma, 1985; McCarren *et al*., 2005). Rivers and stream channels also represent an important means of spread for both aerial and soil–associated infection strategies. Sporangia moved in rivers and streams can germinate directly by forming hyphae that penetrate the host surface, or indirectly where motile zoospores initiate new infections on the host (Erwin & Ribeiro, 1996; Hardham, 2007; Judelson & Blanco, 2005).

The objective of this review is to summarize current knowledge regarding *Phytophthora* spp. associated with forest ecosystems. Various *Phytophthora* spp. are found in natural forests (Table 1). However, for the purposes of this review only a suite of important species such as *P. cinnamomi, P. lateralis*, *P. ramorum* and *P. alni*, are used as examples to highlight the economic and biological impact of *Phytophthora* spp. on natural forest ecosystems. This review also treats the *Phytophthora* spp. that are important to plantation forestry. This includes those in plantations of *Pinus* spp., *Eucalyptus* spp. and other tree species and includes both in the nursery and the field environment. This review also considers control strategies used in the management of *Phytophthora* spp. in forest plantation and natural forests.

## 2.0 Impact of *Phytophthora* spp. on forest ecosystems

The impact of *P. cinnamomi* on Jarrah (*Eucalyptus marginata*) forests of Australia (Podger *et al*., 1965; Weste, 1974; Weste & Marks, 1987) is historically the most important example of a *Phytophthora* sp. associated with natural forest ecosystems (Erwin & Ribeiro, 1996). Numerous other examples exist of *Phytophthora* spp. associated with damage to natural forest ecosystems (Table 1). Important examples include *Phytophthora quercina* causing root rot of European oaks (Jung *et al*., 1999), *P. alni* killing riparian alders in Europe (Brasier *et al*., 2004a) and *P. ramorum* (Werres *et al*., 2001) that is a devastating invasive alien pathogen in native forests in the Western United States (Prospero *et al*., 2007; Rizzo *et al*., 2005).

In terms of the commercial forestry industry, *Phytophthora* spp. have typically been associated with damage in nurseries, where several species result in pre– and/or post–emergence damping off (Belisario, 1990; Butcher *et al*., 1984; Davison *et al*., 1994; Erwin & Ribeiro, 1996; Heather *et al*., 1977; Newhook & Podger, 1972; Reglinski *et al*., 2009; Sánchez *et al*., 2002; Viljoen *et al*., 1992; Von Broembsen, 1984a). In established forest plantations, *Phytophthora* damage is most commonly associated with poorly drained or waterlogged areas manifested by root and collar rot (Ali *et al*., 1999; Bumbievis, 1976; Heather & Pratt, 1975; Linde *et al*., 1994; Newhook & Podger, 1972). Of concern, however, is the emergence of a recent *Phytophthora* disease in Chile of aerial parts of young and adult trees in the field (Durán *et al*., 2008).

More than 40 species of *Phytophthora* have been reported in forest ecosystems. Some of these species have caused substantial damage, while others have not been associated with any disease symptoms (Table 1). Interestingly, a substantial number of these have been described in the last ten years and were associated with tree decline and mortality in both natural and planted forests (Balci *et al*., 2008b; Belbahri *et al*., 2006; Brasier *et al*., 2003; Brasier *et al*., 2004; Brasier *et al*., 2005; Dick *et al*., 2006; Hansen *et al*., 2003; Greslebin *et al*., 2007; Jung *et al*., 1999; Jung *et al*., 2002; Jung *et al*., 2003; Jung & Nechwatal, 2008; Maseko *et al*., 2007; Reeser *et al*., 2007). This illustrates a lack of knowledge related to *Phytophthora* spp. in native forest ecosystems and particularly of the role that these organisms play in the ecology of important natural ecosystems.

The recognition of many new *Phytophthora* diseases in recent years does not only reflect the emergence of new diseases. Certainly, one of the key elements of this trend is the improved selective media (Jefferes & Martin, 1986; Erwin & Ribeiro, 1996; Solel & Pinkas, 1984) that have made it possible to more readily isolate *Phytophthora* pathogens previously masked by other fungi. Furthermore, the emergence of new techniques and especially DNA sequence–based comparisons to recognize previously cryptic species (Burgess *et al*., 2009; Cooke *et al*., 1999; Schena *et al*., 2008) has also contributed to the recognition of new *Phytophthora* spp. Studies in previously unexplored habitats (eg. riparian ecosystems, rivers, forest soil), and surveys carried out in natural forests to detect the presence of pathogens of quarantine importance, have also substantially influenced the discovery of new *Phytophthora* diseases or at least the presence of *Phytophthora* spp. in new environments (Balci & Halmschlager, 2003a; Brasier, 2009; Cooke *et al*., 2007; Hansen, 2008; Hansen & Delatour, 1999; Hansen *et al*., 2003; Jung *et al*., 1996).

Despite the great species diversity for *Phytophthora* that has been exposed in recent years, the role of *Phytophthora* spp. as plant pathogens in a particular ecosystem has only been resolved for a small number of these species (Brasier *et al*., 2004a; Brasier *et al*., 2005; Jung, 2009; Rizzo *et al*., 2002). Studying the examples of species for which a large volume of data are available, will facilitate our understanding of other, recently emerging *Phytophthora* disease epidemics in both native and planted forests. While the disease dynamics may differ between natural forests and plantations, comparisons between these two systems could yield new information and strategies for the management of *Phytophthora* spp.

## 2.1 *Phytophthora* spp. associated with native forests

### 2.1.1 *Phytophthora cinnamomi*

Of all the *Phytophthora* spp., *P. cinnamomi* is the most ubiquitous species, causing diseases in many parts of the world and on over 3000 hosts (Hardman, 2005; Weste, 2003; Weste *et al*, 2002; Zentmyer, 1980). The pathogen is found on many tree crops, plantation and forest trees (Erwin & Ribeiro, 1996). Several destructive diseases are associated with *P. cinnamomi* including root rot of avocado, blight and canker of

macadamia, heart and root rots in pineapple, crown rots and dieback of a variety of shrubs and trees in nurseries and natural ecosystems (Drenth & Guest, 2004; Erwin & Ribeiro, 1996; Newhook & Podger, 1972; Shearer *et al*., 2007; Weste & Marks, 1987; Zentmyer, 1980).

The damage caused by *P. cinnamomi* in the Australian native forests during the course of the last 30 to 40 years has been the most dramatic and extensive example of the effect of *Phytophthora* spp. in natural forest ecosystems (Erwin & Ribeiro, 1996; Weste, 1974; Weste, 2003; Weste *et al*., 2002). There, it has affected principally the jarrah (*Eucalyptus marginata*) forests (Davison & Tay, 1987; Erwin & Ribeiro, 1996, Podger *et al*., 1965; Weste, 1974). An important complication of the presence of *P. cinnamomi* on jarrah is that it also kills most other plants within the community, resulting in the death of 50 % of the species within a few weeks and producing chlorosis and dieback in many others (Weste & Taylor, 1971).

The surface affected by *P. cinnamomi* in Australia increased dramatically from the first report of the pathogen, with 282000 ha affected in 1975 and a subsequent increase in affected areas of 20000 ha per year. By 1982, 14 % of the Australian forest was affected by *P. cinnamomi* (Weste & Marks, 1987). In the state of Victoria, close to 12000 ha are affected (Weste & Marks, 1987), while in Western Australia, 600000 ha were infested by 2008 (Dunstan *et al*., 2008). In 2009, the Australian Government reported that *P. cinnamomi* infests thousands of hectares of native vegetation in Western Australia, Victoria, Tasmania, and South Australia (Commonwealth of Australia, 2009).

The effect of the devastation caused by *P. cinnamomi* on some of the most important native forests in Australia has been poorly documented both in the ecological and economic sense. The south–western part of Western Australia is an internationally recognized biodiversity hotspot. However, ecologically there is a lack of knowledge regarding the direct (vegetation mortality) and indirect (destruction of the habitat) effect that *P. cinnamomi* has on the ecosystem (Shearer *et al.*, 2007).

The economic impact of *P. cinnamomi* via tree mortality is virtually impossible to quantify because the damage is in native forests (Guest, 2002). However, it can be estimated to amount to billions of dollars (Tommerup *et al*., 1999). Additionally, the

cost associated with management or mitigation of damage might be used as indicators of economic impact. For instance, in Western Australia, the cost of control was at least US$3 million in 1989 (Shearer *et al*., 2004). When a highly valuable plant is threatened with extinction due to *P. cinnamomi* infection, it must be moved to a healthy site and this could cost at least US$85000 (Shearer *et al*., 2004). The destruction of forests has also put the water supply to the city of Perth under risk (Weste & Marks, 1987) due to an increase in salt concentrations (Ecos, 1978). Furthermore, as a consequence of mineral exploration and mining activities, the removed soil has spread the inoculum of *P. cinnamomi* to new areas and into the jarrah forests. To address these problems, management activities incorporated by mining companies (Colquhoun & Kerp, 2007; Howard *et al.*, 1998; O'Gara *et al.*, 1997) to rehabilitate the soils after mining bauxite were estimated to be more than US$100000 until 1999 (Old & Dudzinski, 1999).

Other than in Australia, *P. cinnamomi* has been associated with decline of native forests globally (Table 1). In Europe, *P. cinnamomi* is associated with oak decline in Portugal, Spain, France and Italy (Table 1) and it is possibly present in all areas of Mediterranean Europe (Brasier, 1996; Robin *et al*., 2001). It has been implicated as a possible factor contributing to several forest diseases in North America, for instance it has been reported causing root disease in *Pinus* spp., *Castanea dentata* and *Abies* spp., and bleeding canker in *Quercus* spp. (Barnard *et al*., 1993; Benson & Grand, 2000; Crandall *et al.*, 1945; Garbelotto *et al*., 2006; Mirchetich *et al*., 1977; Tainter, 1997; Tainter & Baker, 1996: Wood & Tainter, 2002; Zentmyer, 1980). It has also been isolated from soil in several states in the USA (Balci *et al*., 2008b). In Mexico, it has been implicated in the decline of several *Quercus* spp. (Tainter *et al.*, 2000; Williams *et al.*, 2008). In South Africa, *P. cinnammomi* has invaded natural woody ecosystems particularly in the Western Cape Province where it has caused devastating losses particularly to native Proteaceae (Von Broembsen & Kruger, 1985).

Details regarding the origin and worldwide movement of *P. cinnamomi* are relatively scarce. Von Broembsen & Kruger (1985), proposed a South African origin for *P. cinnamomi*, based on the presence of the pathogen in undisturbed areas and absence of symptoms or damage in the vegetation associated. However, subsequent studies, based on molecular analyses, indicated that the *P. cinnamomi* populations in South Africa have a low diversity, characteristic of an introduced organism (Linde *et al.*, 1997;

1999). Other authors, have hypothesized that *P. cinnamomi* is native to Papua New Guinea based on high genetic diversity of A1 mating type strains there (Arentz & Simpson, 1986; Linde *et al*., 1999; Old *et al*., 1984). From there it could have spread through the rest of the world via the movement of plants with soil (Shepherd, 1975; Williams *et al*., 2009). In Europe, *P. cinnamomi* was probably introduced at the beginning of the Nineteenth Century, causing a massive epidemic on European chestnut (*Castanea sativa*) in southern Europe (Brasier, 1996). The first introduction to North America could have been in the Sixteenth Century with the first settlers (Zentmyer, 1977). In Australia and New Zealand, the first report of *P. cinnamomi* was in 1887 causing wilt and top rot of pineapple in Queensland (Newhook & Podger, 1972). It was subsequently isolated from Proteaceae, Leguminosae or conifer species in almost all the states of Australia and in most of New Zealand by the 1960`s (Newhook & Podger, 1972). In 1921, a few patches of dead jarrah trees were noticed east of Perth (Ecos, 1978), but the cause was not identified to be *P. cinnamomi* until mid 1960`s (Ecos, 1978; Podger *et al*., 1965; Shepherd, 1975; Weste & Taylor, 1971).

### 2.1.2 *Phytophthora lateralis*

*Chamaecyparis lawsoniana* (Port–Orford–cedar) is the largest member of the Cypress family (Cupressaceae). It is native to a small area of coastal northern California and southern Oregon (Zobel & Hawk, 1980; Zobel *et al*., 1985) and used in products such as boat hulls, venetian blinds, battery separators, and arrow shafts because of its straight grain and decay resistance (Zobel *et al*., 1985). All ages of Port–Orford–cedar are severely affected by *P. lateralis* (Hansen *et al*., 2000; Sniezko *et al*., 2003; Woodhall & Sansford, 2006; Zobel *et al*., 1985), with mortality occurring within 2–3 weeks in the case of seedlings and 1–4 years for older trees (Hansen *et al*., 2000; Jules *et al*., 2002; Oh *et al*., 2006; Zobel *et al*., 1985).

The first report of disease symptoms associated with *P. lateralis* was in 1923 in a nursery in Seattle (Tucker & Milbrath, 1942; Zobel *et al*., 1985). In the 1940`s, the disease was epidemic in ornamental *C. lawsoniana* production stands (Zobel *et al.* 1985). In 1952, *P. lateralis* was first reported in native *C. lawsoniana* forest (Sinkiewicz & Jules, 2003; Zobel *et al*., 1985), and then spread rapidly throughout the natural range of *C. lawsoniana* (Jules *et al*., 2002). Currently it is present along the West Coast of the United States and Canada (Hansen *et al*., 2000; Oh *et al*., 2006). The rapid spread of the

disease was substantially influenced by road building and timber harvesting in the 1960`s and 1970`s (Hansen *et al.*, 2000).

More than thirteen hosts other than *C. lawsoniana* have been reported to be susceptible to *P. lateralis*. These hosts belong to the Actinidaceae, Apocynaceae, Ericaceae, Rosaceae, and Taxaceae (Woodhall & Sansford, 2006) and interestingly, are thus different to the Cupressaceae to which *C. lawsoniana* belongs. Of these reports, *Taxus brevifolia* (DeNitto & Kliejunas, 1991) was infected within the area where infestation of *C. lawsoniana* has occurred (Hansen *et al.*, 1999). Nevertheless, Woodhall & Sansford, (2006) suggested that hosts other than cedars (*C. lawsoniana* or *Chamaecyparis* spp.) and *T. brevifolia* probably represent incorrect reports arising from misidentification of the pathogen.

*Phytophthora lateralis* has substantially reduced the wood market and the availability of *C. lawsoniana* nursery plants. This tree species is the only native conifer that can be exported as raw logs from federal forests in the United States (Zobel *et al.*, 1985). At the height of the export market, harvest volumes exceeded 40 million board feet annually, and *C. lawsoniana* sold for up to US$ 6000 per thousand board feet, on the stump (Hansen *et al.*, 2000). This disease has been so severe in some localities that nurseries were forced to abandon the production of *C. lawsoniana* seedlings (Erwin & Ribeiro, 1996). Monetary losses due to disease caused by *P. lateralis* have amounted to millions of dollars (Hansen *et al.*, 2000). Ecosystem function in riparian areas was probably also altered due to loss of the cedar overstory (Murray & Hansen, 1997). Mortality in old–growth forests was estimated to have peaked in the early 1970's at just under 10 million board feet annually and since then to have gradually declined to about 5 million board feet (Zobel *et al.*, 1985). This reduction has been primarily due to depletion of the resource, but also to slower spread of the pathogen (Zobel *et al.*, 1985).

The origin of *P. lateralis* is unknown. Nevertheless, due to its high impact it is thought to be an introduced pathogen in the natural range of *C. lawsoniana* (Hansen *et al.*, 2000; Sinkiewicz & Jules, 2003). A proposed origin is Asia e. g. Taiwan, China or Japan (Brasier, 2008b) based on the high resistance of the Asiatic species of *Chamaecyparis* to root rot (Zobel *et al.*, 1985). However, Murray & Hansen (1997) support the hypothesis of Zobel *et al.* (1985) that *P. lateralis* might be native to northwestern North

America, which represent the northern present range of *C. lawsoniana*, based on the tolerance to the infection detected in *T. brevifolia.* The European (French) origin of *P. lateralis* proposed by Erwin & Ribeiro (1996) based on available records of the disease in France, was discarded by Hansen *et al.*, (2000). It is now widely accepted that the disease occurrences in France represented an introduction event that was eradicated (Brasier, 2008b).

### 2.1.3 *Phytophthora ramorum*

Starting in 1995, tan oak (*Lithocarpus densiflorus*), coast live oak (*Quercus agrifolia*), and California black oak (*Quercus kelloggii*) were reported to be dying suddenly in Central California (Garbelotto *et al*., 2001; Rizzo & Garbelotto, 2003; Rizzo *et al*., 2002). Rizzo *et al*. (2002) identified *P. ramorum*, a species previously described in Europe on *Rhododendron* spp. and *Viburnum* spp. (Werres *et al*., 2001), as the causal agent of sudden oak death. Since then, it has been detected in southwestern Oregon forests (Goheen *et al*., 2002; Ivors *et al*., 2004; Rizzo *et al*., 2002; Rizzo *et al*., 2005), streams in the eastern of USA as well in Washington State (Johnson, 2009), nurseries on the west coast of North America (Hansen *et al*., 2003b), and nurseries and landscape plantings in Europe (Brasier *et al*., 2004b; Hansen *et al*., 2008; Werres *et al.,* 2001).

More than 100 species of both wild and ornamental plants are recorded as naturally infected hosts of *P. ramorum* (Denman *et al*., 2005; Mascheretti *et al*., 2008). These are principally in the Ericaceae (e.g. species of *Rhododendron* and *Vaccinium*) that is widely used as ornamental plant in gardens worldwide (Werres *et al.*, 2001), and Fagaceae (e.g. species of *Quercus* and tanoak) (Hansen *et al.,* 2005). These species are dominant in many forests and serve as ecological keystone species that provide food and habitat for animals, serve as fire breaks, and host mycorrhizal networks (Davison *et al*., 2005). The impact of *P. ramorum* on ecological systems is thus great and there is great concern regarding its potential spread to susceptible species in western forests, as to susceptible forests around the world (Hansen *et al*., 2008).

*Phytophthora ramorum* infects aerial parts of its host plants (Hansen *et al*., 2005). This characteristic is different to the majority of *Phytophthora* spp. that are considered soil pathogens (Erwin & Ribeiro, 1996; Hansen *et al*., 2008), but similar to a small number of recently described *Phytophthora* spp. such as *P. ipomoeae* (Flier *et al*., 2002), *P.*

*nemorosa* (Hansen *et al*., 2003a), *P. kernoviae* (Brasier *et al*., 2005), *P. captiosa*, *P. fallax* (Dick *et al*., 2006), *P. foliorum* (Donahoo *et al*., 2006) and *P. pinifolia* (Durán *et al*., 2008) that affect aerial plant parts, causing leaf blight (Brasier *et al*., 2005; Donahoo *et al*., 2006; Flier *et al*., 2002; Hansen *et al*., 2003a), crown die–back (Dick *et al*., 2006) or stem lesions (Brasier *et al*., 2005; Hansen *et al*., 2003a) on forest trees (Table 1).

In USA forest ecosystems, three different diseases have been described associated with *P. ramorum* infection. This is because the pathogen manifests itself with different symptoms when infecting different parts of the plant or infecting different hosts. ***Phytophthora* canker** (sudden oak death) affects stem of several members of native Fagaceae, **ramorum leaf blight** occurs on several species including *U. californica,* and **ramorum dieback** is a symptom occurring on branches of several species including *Q. ilex*, on species as *Lithocarpus densiflorus* all the three symptoms are observed (Bienapfl *et al*., 2005; Davidson *et al*., 2003b; Hansen *et al*., 2002; Hansen *et al*., 2005; Prospero *et al*., 2007). Interestingly, *U. californica* leaves are the most important source of inoculum in the forest and this tree has been associated with a major and rapid spread of disease in Californian forests (Davidson *et al*., 2005; Fichtner *et al*., 2007). In Europe, the pathogen mainly affects ornamental plants in nurseries where it causes leaf blight. However, it has also been found causing cankers and leaf blights on a small number of tree species and in some established plantings of shrubs, especially *Rhododendron* spp. and, in a limited number of countries (Defra, 2006).

The ecological impact of *P. ramorum* in forests has been estimated using numbers of lost trees and wood lost. In the Big Sur region of coastal California, *P. ramorum* has killed more than 200000 *Quercus* spp. and *L. densiflorus* trees with a total loss of at least 12,650 $m^2$ of tree basal area, which represent 20 % of available host trees in the impacted forest stands (Meentemeyer *et al*., 2008). In greater California, the potential impact of the pathogen was estimated by Barret *et al*. (2006), where they determined that three of the most susceptible tree species (*Q. kelloggi, Q. agrifolia,* and *L. densiflorus*) predominate in 608000 ha over 12 counties that are currently under quarantine control. If a large percentage of these trees are affected, the management and activities to control *P. ramorum* would most likely amount to many millions of dollars.

In general, the economic impact of losses in nurseries due to *P. ramorum* in USA is estimated to be in the tens of millions of dollars (Grünwald *et al*., 2008). Economic losses due to *P. ramorum* in nurseries are associated with management, prevention practices and loss of plants. Nursery plants must be certified as free of disease before they can be moved outside the nursery (Grünwald *et al*., 2008; USDA–APHIS, 2002; 2004; 2006). This certification process for nurseries has been implemented with a total cost on the West Coast of the U.S.A. of more than US$ 6 million up to 2005 (Reinherd, 2006). Losses due to affected plants that must be destroyed, amounted to over 1 million camellias, worth US$ 9 million, in one nursery in Southern California alone (Alexander, 2006), US$ 2 million in an eradication program in nurseries in SW Oregon, and US$ 727,100 in direct losses to nurseries in Oregon since 2005 (Hall *et al.*, 2008). During 2004–2005, plants destroyed for quarantine purposes were estimated at US$ 423,043 in Washington State nurseries (Dart & Chastagner, 2007). Even when plants are not killed, infection renders ornamental plants unmarketable and the potential exists that the disease can spread to harm the nursery, landscape, and floral plant industries of the USA that are worth $16 billion (McGinnis, 2008). If *P. ramorum* were introduced into Canada, the management costs have been estimated to be US$ 5 million annually (Elliot *et al*., 2008).

The origin of *P. ramorum* is unknown (Goss *et al*., 2009a; Mascheretti *et al*., 2008; Rizzo *et al.*, 2005). However, the predominant hypothesis is that the pathogen originated in Asia (Brasier, 2008b; Brasier *et al*., 2004; Goheen *et al*., 2006; Martin & Tooley, 2003). From there, it could have been introduced into North America and Europe on ornamental rhododendrons (Brasier, 2008b). Molecular population analyses, show a limited genetic diversity of the North American and European population, but show that both populations are distint from each other (Goss *et al*., 2009a; Ivors *et al*., 2004; 2006). Brasier (2008b), proposed that there has been a single introduction into Europe, from where it subsequently spread rapidly via the nursery trade to many countries in Europe (Woodhall & Sansford, 2006.). In North America, current data suggest that the pathogen was introduced in the west coast of USA, maybe as a single event, and from there it migrated, most likely with ornamental plants, to the east where it has been recorded in nurseries over 20 states, with no reports in forests (Goss *et al.*, 2009b; Grünwald *et al*., 2008; Stokstad, 2004).

### 2.1.4 *Phytophthora alni*

Since 1993, a *Phytophthora* sp. was recognized as being associated with an aggressive root and collar rot of native riparian, nursery and shelterbelt alder (*Alnus* spp.) trees in Europe (Brasier & Kirk, 2001; Brasier *et al*., 1995; 1999). The trees affected by the alder *Phytophthora*, displayed symptoms typical to those of *Phytophthora* root and collar rot, namely abnormally small, sparse and often yellowish foliage, die–back of the crowns, early and often excessive fruit production with unusually small cones (Jung & Blaschke, 2004). Tongue–shaped lesions were also found in the inner bark and the cambium and extended up to 3 m from the stem base finally killing the tree (Jung & Blaschke, 2004; Jung *et al*., 2007).

*Alnus* spp. are important trees in riparian ecosystems because they are well adapted to flooding (Hughes *et al*., 1997) and stabilize river banks with their substantial and deep root systems. These trees also play a role in water purification, absorbing phosphorus and some heavy metals such as zinc and copper (Claessens, 2003; Jonsson *et al*., 2004; Pulford & Watson, 2003; Radoux *et al*., 1997; Salinas *et al*., 2000). A change in the composition of the riparian ecosystems, resulting from the death of many Alder trees, will affect the ability of these ecosystems to cycle and mitigate nutrient fluxes originating upstream and upslope, and the associated vegetation and fauna (Pinay *et al*., 2002; Richardson *et al*., 2007).

Alder *Phytophthora* causes substantial mortality of *Alnus* spp. in several countries of Europe. A survey of *A. glunitinosa* undertaken in 1994, revealed that 3.9 % of trees exhibited symptoms of *Phytophthora* disease and an additional 1.2 % of the trees were dead in southern England and eastern Wales (Gibbs *et al*., 1999). Surveys after 1996 showed that for a native *A. glutinosa* population of 585000 trees from rivers in England and Wales, 32,800 (5.6 %) were symptomatic and 13,500 (2.3 %) were dead (Gibbs *et al*., 1999). Elsewhere in Europe, the alder *Phytophthora* was identified causing decline of *A. glutinosa* and *A. incana* in 60–year–old stands in the Czech Republic (Cerny *et al*., 2008), and a survey conducted along the Moselle River in northeastern France in 2000 showed that approximately 20 % of the alders were diseased or dead (Streito *et al*., 2002).

Studies showed that alder *Phytophthora* represent a species complex. The complex includes *Phytophthora alni* subsp. *alni* (Paa), *P. alni* subsp. *uniformis* (Pau), and *P. alni* subsp. *multiformes* (Pam) (Bakonyi *et al*., 2006; Brasier *et al*., 2004a). These species are characterized by clear morphologic differences, but they were described as sub–species because phylogenetic studies showed that they group in the same clade based on isozyme alleles and rDNA ITS sequences (Brasier *et al.*, 2004a). A further complication has been the proposal that these sub–species originated from natural hybridizations, probably between *P. cambivora* and *P. fragariae* (Brasier *et al.*, 1999; 2004a). Based on gene genealogies (Ioos *et al.*, 2006) and microsatellite patterns (Ioos *et al*., 2007a), it has been proposed that only *Paa* is a genuine hybrid taxon, originating from a hybridization between *Pau* and *Pam* (Ioos *et al*., 2007b). However, the taxonomic and hybrid status of *Pam* remains unclear, while the genetic features of *Pau* do not fit with a hybrid origin (Ioos *et al*, 2006; 2007a; 2007b).

No conclusive information is available related at the origin of three sub–species of *P. alni.* The proposed parents of the hybrids, namely *P. cambivora* and *P. fragariae*, are believed to be exotic to Europe and it is thus unclear where the first hybrid was formed (Érsek & Nagy, 2008). However, all three sub–species of *P. alni* occur only in Europe. The current distribution for *Paa* is the UK, Ireland, France, Belgium, The Netherlands, Sweden, Germany, Austria, Hungary (Brasier *et al*., 2004a) and the Czech Republic (Cerny *et al*., 2008). *Pau* occurs in Sweden, Latvia, Germany, Austria, Italy and Hungary (Brasier *et al*., 2004a) and was recently found in Alaska in soil beneath *Alnus incaica* (Adams *et al*., 2008). *Pam* has been found in The Netherlands, UK and Germany (Brasier *et al*., 2004a). The distributions of *Pau, Pau* and *Pam* can thus overlap with the other sub–species and all three sub–species are probably spreading to other parts of Europe as well as other continents such as America (Adams *et al*., 2008; Brasier *et al*., 2004a). The only hosts of these organisms are *Alnus* spp. and they are found in the soil surrounding the trees (Adams *et al*., 2008; Ioos *et al*., 2007b). Thus far, Alaska represents the only area where one of these sub–species (*Pau*) occurs outside Europe and it is unknown whether it has been introduced or whether it is native to the area (Adams *et al*., 2008).

### 2.2 *Phytophthora* spp. damaging forest plantations

Two of the most important tree genera in commercial forest plantations are *Pinus* and *Eucalyptus* spp. In 2005, these two genera represented 40 % of the area of productive planted forests in the world (FAO, 2007). These trees provide the foundation for the production of paper pulp, round wood, and other commodities in many countries in the world (FAO, 2007). Several other tree species are also planted for the production of timber and pulp (FAO, 2007; 2008) and with multipurpose objectives (King, 1987). The importance of these species should not be underestimated as they represent 60 % of plantations worldwide (FAO, 2007).

*Phytophthora* spp. cause diseases in at least one component of the production cycle in most countries that are involved in plantation forestry (Bloomberg, 1985; Gibson, 1972; Hepting & Cowling, 1977; Nienhaus & Castello, 1989; Shipton, 1977; Wallace, 1978). This is usually represent damage due to root disease in nurseries or in the first year after planting (Table 1). The following section provides a summary and discussion relating to the association between *Phytophthora* spp. and some of the most important forest plantation species.

### 2.2.1 *Phytophthora* diseases in *Pinus* nurseries and plantations

In nurseries, seedlings of *Pinus* are highly susceptible to infection by *Phytophthora* spp., usually originating in infested soil (Newhook & Podger, 1972). The most common of these is *P. cinnamomi* (Table 1) that infects seedlings of several *Pinus* spp. in several countries (Butcher *et al*., 1984; Davison *et al*., 1994). Other *Phytophthora* spp. causing damage in nurseries include *P. drechsleri*, *P. cryptogea* and *P. citrophthora* (Table 1). These species mostly cause root rot and pre– or post– emergence damping off (Erwin & Ribeiro, 1996; Heather *et al*., 1977; Sánchez *et al*., 2002; Viljoen *et al*., 1992).

Young *Pinus* plantations are highly susceptible to infection by *Phytophthora* spp. until the third year when the youngest trees are most susceptible, making the damage strongly related to age of the plantation (Newhook & Podger, 1972). Many poor quality and wetter areas of the jarrah forest in Australia that were cleared for *P. radiata* plantations in the mid– and late– 1970`s, became infected with *P. cinnamomi* and many pine deaths occurred in especially young plantations (Davison *et al*., 1994). In these sites, mortality was as high as 170 per hectare per annum in the first year after planting, decreasing to about two trees per hectare per year at 5–years–old (Davison *et al*., 1994).

Other *Phytophthora* spp. affecting young pine plantations are *P. cryptogea, P. cinnamomi, P. cambivora, P. megasperma* and *P. drechsleri* (Table 1). Of these, *P. drechsleri* was associated with the death of 3–year–old *P. radiata* under wet conditions in New South Wales, Australia (Heather & Pratt, 1975). All of these *Phytophthora* spp. are found in the soil and associated with root and lower trunk damage.

Adult pine trees are usually resistant to infection by *Phytophthora* spp., but stress conditions can enhance susceptibility of trees for instance water logging is usually associated with *Phytophthora* infections (Bumbievis, 1976). That was considered the key factor associated with the death and decline of *P. radiata* on wet sites and where trees were infected by *P. cinnamomi* and *P. cryptogea* in South Australia (Bumbievis, 1976; Newhook & Podger, 1972). A similar situation was found with the decline of *P. radiata* and *P. pinaster* in New Zealand caused by *P. cinnamomi* (Ali *et al*., 1999; Newhook & Podger, 1972). Other stress factors predisposing adult plantations to *Phytophthora* spp. are low quality sites and inappropriate silvicultural practices. Likewise, root rot and death of *P. radiata* (40–50 year–old trees) has been associated with infections caused by *P. cinnamomi* in South Africa (Wingfield & Knox–Davies, 1980). Stress arising from culling and pruning during silvicultural practices, induced infections by *P. cinnamomi* in a 6–yr–old stand of *P. radiata*, causing mortality of 18 trees per hectare per year in a plot in Australia (Davison *et al*., 1994).

A *Phytophthora* species, assigned with the name *P. pinifolia*, has recently emerged as the causal agent of a serious above–ground disease in *P. radiata* plantations in Chile (Chapter 2). The disease increased rapidly between 2004 and 2006 when the largest area, close to 60000 ha, was affected. During 2007 and 2008, the affected area decreased significantly and in most cases has been confined to plantations close to the coast (Chapter 2; 3). The disease is typified by needle infections, exudation of resin at the bases of needle brachyblasts and, in younger trees, necrotic lesions in the cambium, which eventually girdle the branches. The disease results in the death of seedlings, while young trees and mature trees can also succumb after a few years of successive infection. This is probably hastened by opportunistic fungi such as *Diplodia pinea.* A characteristic of the pathogen is distinct host–specificity, because *P. pinaster* and *Pseudotsuga menziessii*, growing in close proximity to severely infected trees, show no symptoms (Chapter 2).

*Phytophthora pinifolia* is the first *Phytophthora* sp. to be discovered causing extensive damage to needles and branches, without obvious soil related symptoms. It also spreads extremely rapidly due to infection of the aerial parts of trees. Infections occur without an obvious association with stress, which is a common feature of other *Phytophthora* spp. attacking *Pinus* spp. There is, furthermore, a clear pattern to the infections with trees on east- facing slopes more seriously affected. Thus, *P. pinifolia* infections are clearly related to humidity and temperature conditions, which is very similar to *P. ramorum* (Rizzo *et al*., 2005). *Phytophthora pinifolia* is known only from Chile and its origin remains unknown. The severity of the disease that it has caused in Chile and on one of the most widely planted *Pinus* spp. in the world is of great concern globally.

### 2.2.2 *Phytophthora* diseases in *Eucalyptus* nurseries and plantations

*Phytophthora* spp. are responsible for root rot and cankers, or leaf blight on the aerial parts of plants in *Eucalyptus* nurseries. For example, *Phytophthora nicotianae* var. *nicotianae* has been found on *E. globulus, E. delegatensis,* and *E. regnans* attacking leaves, petioles and shoots in Italy (Belisario, 1990; Belisario, 1993). In South Africa, irrigation from *P. cinnamomi*–infected river water (Von Broembsen, 1984b) could have introduced the pathogen into the nurseries (Viljoen *et al*., 1992). There have thus been various reports of *P. cinnamomi* associated root rot of *E. citriodora, E. fastigata, E. grandis* (Von Broembsen, 1984a) and *E. lehmanii* (van der Merwe & van Wyk, 1973) from nurseries in South Africa. However, a survey carried out in plants with root rot grown in hydroponics cultivation systems, which is an environmentally suitable for *Phytophthora* spp., due to high humidity and optimal temperature, showed the presence of *P. cinnamomi* and *P. nicotianae* to be unexpectedly low with incidences of 0.1 and 0.3 %, respectively. This was compared to the higher incidence of the most predominant pathogen, a *Cylindrocladium* sp. (Lombard, 2004).

In *Eucalyptus* plantations, *Phytophthora* spp. have been associated principally with root and collar rot. In South Africa, symptoms in one–year–old plantations include chlorosis of the leaves, gum exudation from collar cankers and finally the death of the plant (Maseko *et al*., 2001). In these cases, *Phytophthora* spp. known to be associated with collar and root rot include *P. boehmeriae, P. cinnamomi* and *P. nicotianae* (Linde *et al.*, 1994; Maseko *et al*., 2001; Von Broembsen, 1984a; Wingfield & Knox–Davies, 1980).

Tree mortality in plantations, are presented in small areas prone to water–logging during the rainy seasons (Maseko *et al*., 2007). The species *E. fastigata* and *E. fraxinoides* have been severely damaged by *P. cinnamomi* in South Africa (Wingfield & Knox–Davies, 1980). Despite their very desireable pulping characteristics, they are no longer planted due to their high level of susceptibility (Linde *et al*., 1994). In the province of KwaZulu–Natal, *P. nicotinae* was recovered from 45 % of soil samples and 60 % of diseased plants, and *P. cinnamomi* was recovered from 5 % of soil samples and 12 % of diseased plant material in several recently established plantations of *E. smithii* (Maseko *et al*., 2001). This is one of the few cases where *P. cinnamomi*, despite its presence, was not the principal cause of mortality of its host.

New species of *Phytophthora* causing disease in *Eucalyptus* plantations have been described recently. In South Africa, two new species where identified causing damage to *Eucalyptus* spp. These include *P. frigida,* causing root and collar rot disease of *E. dunnii and E. smithii*, and *P. alticola,* causing stem canker of *E. macarthurii* and disease in *E. dunnii* and *E. bajensis* (Maseko *et al*., 2007). Two new *Phytophthora* spp. have also been identified causing disease of leaves and petioles, seed capsules, peduncles and twigs in plantations of non–native *Eucalyptus* spp. in New Zealand. The pathogens in this case were described as *P. captiosa*, on *E. botryoides* and *E. saligna*; and *P. fallax* on *E. delegatensis, E. fastigata, E. nitens*, and *E. regnans* (Dick *et al*., 2006). In Australia, *P. multivora* has been isolated from soil surrounding declining or dead *Eucalyptus gomphocephala*, *E. marginata* and *Agonis flexuosa* trees and another 13 plant species, and from the fine roots of *E. marginata* and from collar lesions on *Banksia attenuate* in Western Australia. The organism has been shown as athogenic to bark and cambium of *E. gomphocephala* and *E. marginata* (Scott *et al*., 2009). The origin of these newly described *Phytophthora* spp. is unknown but they very likely represent new host encounters for native pathogens.

### 2.2.3 *Phytophthora* diseases in plantations of other tree species

Various tree species other than *Pinus* spp. and *Eucalyptus* spp. have bee used to establish plantations. In these plantations several *Phytophthora* spp. have been reported although there is very little information for them.

#### 2.2.3.1 *Alnus* plantations

Species of *Alnus* have been widely used in Europe for afforestation since the 1980's. The principal reason for planting these trees is to ameliorate degraded soil. This is because *Alnus* spp. fix nitrogen and display rapid growth and can also be used as a source of biomass for energy production (Hall & Burgess 1990; Jørgensen *et al*., 2005). In Bavaria (Germany), some of these plantations are subsidized by the EU governments to recover former agricultural land, and for to stabilize degraded soil and banks of white–water rivers in the Alps. Other plantations of *Alnus* spp. have been established to replace trees killed by a devastating storm in January 1990 (Jung & Blaschke, 2004).

Mortality of planted alders, caused by a previously unknown lethal root and collar rot, has been reported in these stands since 1993 (Jung & Blaschke, 2004; Jung *et al*., 2007). The causal agents are species in the *P. alni* complex, as previously discussed (Brasier *et al.,* 2004a; Jung & Blaschke, 2004; Jung *et al*., 2000; Jung *et al*., 2007). The *P. alni* complex has been frequently detected in natural and planted stands and was presumably spread by the movement of contaminated plant material from nurseries to the field (Jung & Blaschke, 2004; Jung *et al*., 2007). Using the current data where the disease is present, a geographic information system (GIS) and coarse–resolution auxiliary data were used to develop a model of the disease spread in Bavaria (Jung *et al.*, 2007). This model concluded that approximately 1.4 million ha (73.5 %) from the 1.9 million ha of planted forests in Bavaria have a high potential for root and collar rot caused by members of the *P. alni* complex, while 564000 ha (28.56 %) have a high potential to remain healthy.

#### 2.2.3.2 *Castanea dentata* (American chestnut)

The American Chestnut was almost wiped out by the fungus *Cryphonectria parasitica* in several parts of eastern North America during the 20th century (Anagnostakis, 1987). Historically, *P. cinnamomi* caused the second most severe mortalities of *C. dentata* after the damage caused for *C. parasitica* (Schlarbaum *et al*., 2004). The pathogen was introduced into Georgia in the 1800`s, and as a consequence, *C. dentata* was almost eliminated from wet, poorly–drained soils and soils having a heavy clay content by the turn of the 20th century (Crandall *et al*., 1945).

An extensive breeding program has been established against chestnut blight (Hebard, 2006). Through this program the first of these blight–resistant chestnut seedling should

be ready for out planting in the relatively near future and in the hope of restoring American chestnut that were devastated by chestnut blight (Hebard, 2006; Rhoades *et al*., 2003; 2009). However, some of the resistant trees planted in the preliminary trials became infected by *P. cinnamomi* (Rhoades *et al*., 2003; Jacobs, 2007) and there is concern that resistance to *C. parasitica* will not be sufficient to allow the recovering of the American chestnut.

Symptoms caused by *P. cinnamomi* on *C. dentata* included root and collar rot, dieback of branches and whole seedlings, and defoliation of seedling and young trees. This resulted in 60 % seedling mortality within 4 months of planted chestnut seedlings in some sites in the Kentucky's Knobs and Cumberland Plateau regions, Pennsylvania, Tennessee and North Carolina (Rhoades *et al.*, 2003). These infections limited the establishment of new patches or the conservation of old groves in some cases (Vettraino *et al*., 2001). There is thus a serious concern that chestnut trees resistant to *C. parasitica* are susceptible to *P. cinnamomi* (Rhoades *et al*., 2003) and resistance to *P. cinnamomi* should also be incorporated in breeding programs (Jacobs, 2007).

#### 2.2.3.3 *Castanea sativa* (Sweet chestnut)

*Castanea sativa* is the only species of the genus *Castanea* occurring in Europe (Conedera *et al*., 2004a; 2004b; Fineschi *et al.*, 2000). Currently, *C. sativa* is planted for timber and chestnut production, with more than 2.5 million ha mainly concentrated in France and Italy (Conedera *et al*., 2004b). Pests and diseases complicate the management of *C. sativa* plantations and orchards (Bissegger *et al.,* 1997; Pridnya *et al.*, 1996; Vannini & Vettraino, 2001).

Ink disease associated with *Phytophthora* infections is one of the most destructive disease affecting *C. sativa* and has been recorded in several countries of Europe (Vannini & Vettraino, 2001). The disease is characterized by the presence of root and collar rot of seedlings in nurseries and adult trees in the field (Gomes–Laranjo *et al*., 2004; Vettraino *et al.*, 2005). The most characteristic, and where the name is originated, is a necroses of the main roots, which can spread to the collar and the trunk with black exudates (Vettraino *et al*., 2005). Principally *P. cinnamomi*, and less commonly *P. cambivora* are considered as the causal agents of ink disease of *C. sativa* in Europe (Gomes–Laranjo *et al*., 2004; Maurel *et al*, 2001; Vettraino *et al.*, 2005). Recently, two

other *Phytophthora* species, namely *P. citricola* and *P. cactorum*, were also found to be present in declining chestnut stands suffering from ink disease in a restricted area of Italy (Vettraino *et al*., 2001; 2005). However, their roles as causal agents of ink disease have not yet been proven even though they produced necrotic lesions in pathogenicity tests (Vettraino *et al*., 2001).

The economic and ecological impact of Ink Disease have been remarkable when it spreads into orchards, forests, coppices or natural lands dominated by chestnut (Vettraino *et al.*, 2005). The diseases is one of the most important factors associated with the reduction of *C. sativa* in Portugal reducing the resource from the 70000 ha in the 1950`s to the current 30000 ha (Abreu *et al.*, 1999). A study carried out in the Padrela Region of northern of Portugal has shown that from 1995 to 2004, the disease incidence increased by 18 %, in the area under study and affect most of the new plantations established in those years (Martins *et al*., 2007).

#### 2.2.3.4 *Acacia mearnsii* (Black wattle)

*Acacia mearnsii* is native to Australia and is planted extensively in Brazil and South Africa. This species is considered as a multipurpose crop established for the production of tannin, paper, cellulose, plywood, charcoal and firewood (Huang *et al*., 1994; Santos & Luz, 2007). It is estimated that more than 500000 ha of *A. mearnsii* are planted worldwide (Santos & Luz, 2007).

Gummosis and a disease known as black butt is one of the most important constraints to the propagation of *A. mearnsii* in plantations (Roux & Wingfield, 1997; Santos & Luz, 2006; 2007). The disease reduces bark quality and tannin yield, hinders stripping of the bark and could kill trees (Abdanur *et al*., 2003; Roux *et al*., 1995; Santos & Luz, 2006, 2007). In seven–year–old plantations of *A. mearnsii* in Rio Grande do Sul (Brazil) the incidence of Gummosis range from 23 % to 48 % (Santos & Luz, 2006).

Several fungal species and *Phytophthora* spp. are involved in gummosis, with *P. nicotianae* and *P. boehmeriae* as the major causal agents (Roux & Wingfield, 1997; Roux *et al.*, 1995; Santos & Luz, 2006; Santos *et al.*, 2005; Wingfield & Kemp 1993; Zeilemaker, 1971). Other species isolated from the lesions are *P. meadii*, *Fusarium* spp., *Rhizoctonia* spp. and *Pestalotia* spp., although their roles in the disease are not

clear (Roux *et al.*, 1995). In South Africa, Roux & Wingfield (1997) proposed that, although *Phytophthora* spp. are mainly involved in the gummosis of *A. mearnsii*, they are not the single cause of the disease, and they speculated that the infection by *P. nicotinae* provides entry sites for other opportunistic pathogens such as *Botryosphaeria* spp. and *Diplodia* spp.

### 3.0 Control of *Phytophthora* spp. diseases in forest nurseries and plantations

The impact of *Phytophthora* spp. on forest ecosystems may be localized or spread over large areas. *Phytophthora* spp. can also spread easily and the anthropogenic influences on this movement have commonly been illustrated. Control strategies for *Phytophthora* spp. and the diseases they cause are mostly focused on exclusion of the pathogen. Where this fails, chemicals targeted at the various stages in the lifecycle of the particular *Phytophthora* spp. involved are used. In the case of small foci of infection and where early detection has been made, *Phytophthora* spp. can be eradicated and monitored. This is especially true in nurseries. However, many of the examples of diseases discussed in this review have shown that control and management requires large scale operations and monitoring, which may not be feasible nor make a significant difference, although spread of the pathogen may be impeded.

The control of *Phytophthora* spp. must be understood in terms of integrated management, where all the options need be considered to develop an efficient and low cost program (Erwin & Ribeiro, 1996). The term 'integrated implies incorporation of natural enemy/antagonist levels into decision–making, detection, monitoring and prediction and the use of compatible and non–disruptive tactics that preserve these agents" (Ehler, 2006). Often, management of these pathogens also entails large scale operations involving scientists representing numerous disciplines, industry representatives and also the public. This has been clearly illustrated in efforts to restrict the movement of *P. ramorum* in nurseries and forests, and where serious quarantine embargos have been needed for forests, between different parts of a country and between countries.

Many of the *Phytophthora* spp. causing epidemics in forests also occur on other hosts used in agriculture or on ornamental plants. This facilitates their spread and impedes

monitoring and management. Examples are found in the case of *P. cinnamomi* causing death of *Eucalyptus* spp., but that was first important in the pineapple industry. Likewise *P. ramorum* that is a pathogen of rhododendron plants important in the nursery trade, gained the opportunity to encounter native and highly susceptible hosts in Californian forests. Because they have been well–studied in forest ecosystems, *P. cinnamomi*, *P. ramorum*, *P. lateralis* and *P. alni* provide outstanding model species to illustrate management strategies applied for *Phytophthora* spp. in general.

## 3.1 Exclusion of the pathogen

### 3.1.1 Quarantine

The most important approach to disease control lies in quarantine (Cotton & Van Riel, 1993; Salama & Abd–Elgawad, 2003). At a large scale level, such as in the case of continents or countries, quarantine measures are based on the regulations determined by plant protection organizations or plant health services (Schrader & Unger, 2003). The regulations must address international agreements, and these are under the supervision of the FAO (Food and Agriculture Organization of the United Nations). These regulations prohibit in the most of the cases, the movement of untreated plants, vegetable material and soil into countries (FAO, 2006). The main limitation of quarantine procedures is the fact that the restrictions are based on organisms that are contained in lists and as a consequence, unlisted organisms are not regulated (Brasier, 2008a; 2008b). To deal with this situation, several phytosanitary measures have horizontal effects that are effective not only against the introduction of listed quarantine pests, but also against many other species that may “hitchhike” on the imported plants or plant products (Schrader & Unger, 2003). Example of success of these mesures are Australia and New Zealand, where the strict restriction of the importantion of possibles sources of inoculum, and those potential host, have lead that this countries are free of several diseases, incluidng several *Phytophhora* spp. (Hüberli *et al*., 2008).

At a local level, quarantine measures are focused on avoiding the movement of infested materials into disease–free areas. Restrictions in the movement of soil and machinery have been implemented because it is known that these are sources of spread for *Phytophthora* spp. (Hansen *et al*., 2000; Weste & Marks, 1987). For instance, road closure in *P. lateralis* infested areas with hikers and cars, or in areas infested with *P.*

*cinnamomi* residue management in mines have been put in operation (Hansen *et al*., 2000; Hardy *et al*., 2001; Shearer *et al.,* 2006; Sinkiewicz & Jules, 2003; Weste & Marks, 1987). For *P. ramorum* and *P. alni* destruction of infected plant material, has been a successful strategy to limit the spread of disease caused by these pathogens (Jung & Blaschke, 2004; Osterbauer *et al*., 2004; Rizzo *et al*., 2005).

Brasier (2009) has estimated that 10 to 20 % of all *Phytophthora* spp. have been described. This implies that 80 to 90 % of *Phytophthora* spp. still move freely and can be regulated only when they are described after disease symptoms are noticed (Brasier, 2008b). Thus, current *Phytophthora* spp. causing most damage in forest ecosystems, such as *P. ramorum* and *P. cinnamomi*, have been introduced into new environments while their origins still remain unknown. Their presence in new environments can consequently be considered a failure of current quarantine procedures, including those with horizontal effect (Brasier, 2008a). Thus, despite the quarantine regulation imposed in USA to limit the movement of *P. ramorum*, plants were moved from an infested nursery in California to 783 garden centres in 39 states in 2003 and more than 11 nurseries where reported as positive for the presence of the pathogen (Goss *et al*., 2009b; Stokstad, 2004).

### 3.1.2 Eradication

Once a pathogenic *Phytophthora* spp. has been introduced and established in a new ecosystem or plantation, the next step in the control chain is an attempt at eradication or at least containment (Heesterbeek & Zadoks, 1987). This is more successful if the disease problem is acted upon early. However, eradication programs for forests imply huge costs and large scale environmental impacts (Davidson *et al.*, 2005; Waring & O'Hara, 2005). An example here has been the cut and burn approach used in Humboldt county and Oregon State in SA against *P. ramorum* or the aerial spraying of fungicides used in Australia against *P. cinnamomi* (Rizzo *et al*., 2005; Shearer & Fairman, 2007b). One trial in Australia has illustrated the potential for eradication of *P. cinnamomi* from the field through elimination of infested plant material, fungicide and fumigant treatments together with containment barriers to protect threatened vegetation (Dunstan *et al*., 2008). Nevertheless, major eradication efforts against *P. ramorum* and *P. cinnamomi* (Colquhoun & Hardy, 2000; Ribeiro & Linderman, 1991; Rizzo *et al*., 2005) have not succeeded in eliminating these pathogens (Colquhoun & Hardy, 2000;

Prospero *et al.*, 2007; Rizzo *et al.*, 2005; Slawson *et al.*, 2006) although they have reduced the spread and expansion of infested areas (Davidson *et al.*, 2008; Goheen *et al.*, 2008; Kanaskie *et al.*, 2008).

The complex biology and life cycles of *Phytophthora* spp. makes them difficult to eradicate once established in the field. The resting structures that *Phytophthora* spp. produce limit of measure by which the pathogens can be eliminated. These structures can survive in extreme conditions for a long period of time (Erwin & Ribeiro, 1996; Hansen & Hamm, 1996; Hwang & Ko, 1978). Since many, *Phytophthora* spp. have a soil phase (Drenth & Guest, 2004), complete elimination from the soil is unlikely due to the fact that fire or fungicides have a limited capacity to penetrate into the soil (Munnecke & Van Gundy, 1979). For example, *P. cinnamomi* has been detected in roots 2 m deep and in water 5 m deep (Hill *et al.*, 1995). Likewise, *P. ramorum* has been isolated from soil 20 cm deep (Aveskamp *et al.*, 2005). Also, the presence and spread of zoospores in water tanks or stream water (Davidson *et al.*, 2005; Jung & Blaschke, 2004) makes the use of fungicides or other suppression measures impractical due to the high impact it will have on the surrounding ecosystems. Furthermore, once a *Phytophthora* spp. have become established in a forestry situation, it is virtually impossible to eradicate them. This is well illustrated in the case of *P. lateralis* in the native range of *C. lawsoniana* (Hansen *et al*. 2000).

Eradication has been most successful in nurseries. Successful examples include the eradication of *P. ramorum* in Oregon nurseries (Grünwald *et al.*, 2008; Osterbauer *et al.*, 2004; Prospero *et al.*, 2007), *P. alni* complex in Europe (Jung & Blaschke, 2004), *P. lateralis* in USA (Hansen *et al.*, 2000; Woodhall & Sansford, 2006) and Europe (Brasier, 2008a). Eradication from nurseries involves the elimination of the infected material and the use of healthy plants as source of parental material (Jung & Blaschke, 2004; Woodhall & Sansford, 2006).

### 3.2 Elimination or reduction of pathogen inoculum

#### 3.2.1 Chemical control

Fungicides represent an important component of management strategies against *Phytophthora* spp. Several fungicides have been tested in forests, and these have been

applied as soil drenches, stem injections, topical bark applications, foliar treatments and aerial sprays in ultra low volumes (Chastagner *et al.*, 2008; Garbelotto *et al.*, 2009; Garbelotto *et al*., 2007; Linderman & Davis, 2008; Orlikowski, 2004; Shearer & Fairman, 2007a; 2007b; Shearer *et al*., 2006). They have thus been effective in reducing the disease spread, protecting the trees against infection or reducing the damage to infected trees.

Several types of fungicides have proved to be useful against *Phytophthora* spp. The most effective fungicides for the control of *P. cinnamomi* in nurseries and in the field are phenylamides (e.g. metalaxyl) and the phosphonates (e.g. fosteyl–Al) (Fenn & Coffey, 1984; Hardman, 2005). Chloropicrin, mancozeb, nabam and zineb have been reported to be effective as soil drenches in nurseries to protect *C. lawsoniana* seedlings against *P. lateralis* (Woodhall & Sansford, 2006). For *P. ramorum*, Cyazofamid, dimethomorph, mefenoxam, pyraclostrobin and fenamidone applied on foliage have been evaluated in nurseries with different levels of control (Tjosvold *et al*., 2006).

The most extensive program to manage *Phytophthora* spp. with fungicides in forests has been in Australia where *P. cinnamomi* were managed with phosphite (Hardy *et al*., 2001; Shearer *et al*., 2006; Tynan *et al*., 2001; Wilkinson *et al*., 2001b). This systemic fungicide (Guest & Grant, 1991; Fairbanks *et al.*, 2002) is currently being applied as foliar sprays, aerial mists or trunk injections (Barret *et al.*, 2003; Fairbanks *et al*., 2002; Shearer & Fairman, 2007b; Shearer *et al*., 2006;) and can protect trees for extended periods of time, even years (Shearer & Fairman, 2007a; Tynan *et al.*, 2001). Based on the impressive results obtained in Australia, phosphite trials were established with promising results to manage *P. cinnamomi* causing Oak decline in Spain (Fernandez–Escobar *et al*., 1999) and *P. ramorum* in California (Garbelotto *et al*., 2007).

Fungicide management strategies have not yet been developed for the *P. alni* complex or for *P. lateralis*. This is probably due to a reticence to apply fungicides in the native environment. Nevertheless, chemical control has been is suggested as part of the strategy in the establishment of resistant varieties of *C. lawsoniana* during the first year, when the plants are most susceptible (Oh *et al.*, 2006).

The use of fungicides in *Phytophthora* control programs have resulted in the emergence of resistant pathogen strains (Coffey *et al*., 1984; Davidse *et al.*, 1981; Dobrowolski *et al.*, 2008; Gisi & Cohen, 1996; Wilkinson *et al.*, 2001a). Resistance to metalaxyl in several species of *Phytophthora* is the most documented case where resistance arises after an intensive use of fungicides (Gisi & Cohen, 1996). It is worrying that Dobrowolski *et al.* (2008) detected resistance in some lineages of *P. cinnamomi* against phosphite in avocado plantations in Australia. If these lineages were to move into the forest, they could seriously impede current management strategies against *P. cinnamomi* in jarrah forests.

The negative environmental impact of chemical fungicides will always be a concern. In this regard, phosphite has commonly been accepted as non–hazardous. Yet some adverse effects such as a reduction in root and shoot development, growth abnormalities and reduction in pollen production have been detected in some tree species after several years of use (Aberton *et al.* 1999; Barret *et al*., 2002; Fairbanks *et al*., 2002; Hardman, 2005).

### 3.2.2 Biological control

Biological control is defined as "the use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be" (Eilenberg *et al*., 2001). This approach has been proposed as an alternative to the use of fungicides for the control of *Phytophthora* spp. in some situations (Reglinski & Dick, 2005). Strategies where biological control is included have demonstrated as having potential for the management of diseases in nurseries (Reglinski & Dick, 2005).

In agriculture, several organisms including *Bacillus* spp, *Pseudomonas* spp. and species of *Trichoderma* spp, have been tested for the biological control of *Phytophthora* spp. with different level of success (Ezziyyani et al 2007; Lee *et al*., 2008; Sang *et al*., 2008). Control with endophytes has also been proposed as a possible biological approach for the control of *Phytophthora* spp. (Hanada *et al*., 2008). Nevertheless, the development and extensive use of biological control for *Phytophthora* spp. has been disappointing. This is most likely due to the difficulties involved in producing inoculum, complications

involved in rapid inoculation of the host plants and the inability of the biological control agents to survive and compete in the soil environment (Erwin & Ribeiro, 1996).

*Phytophthora cinnamomi* has been most studied in terms of biological control in forests. Several biological process have been reported, such as colonization and lysis by bacteria (Nesbitt *et al*, 1981), mycoprasitism and colonization of oospores by the fungus *Catenaria anguillulae* (Daft & Tsao, 1984), antagonism by a basidiomycete fungus (Pratt, 1971) growth inhibition by mycorrhiza (Malajczuk, 1988), the use of animal manures that stimulate the activity of endospore–forming bacteria (Aryantha *et al*., 2000), and antagonism from *Acacia* spp. root exudates (D`Souza *et al.,* 2004; 2005; Whitfield *et al.,* 1981). However, these results generally emerged from *in vitro* or glasshouse studies and there were insufficient subsequent field evaluations. The biological control of *P. cinnamomi* is thus currently not applied in native *Eucalyptus* forests (D`Souza *et al*., 2004). However, *Trichoderma* spp. have been shown to have potential as control agents and are applied to nursery stock as part of a management strategy (Reglinski & Dick, 2005).

Preliminary studies with biological agents were promising for the management of *P. ramorum.* The bacteria *Bacillus subtilis* and *Streptomyces lydicus* were effective in commercial formulations to control growth in plates and the level of infection in detached leaves inoculation in the laboratory (Elliott & Shamoun, 2008). Nevertheless, the study of Linderman & Davis, (2006) showed that these bacteria were ineffective in controlling infections on detached leaves.

Once large areas of land are affected, control using biological agents is difficult or not viable. Podger & Keane (2000) indicated that it is unlikely that native organisms present in an infested area are able to reduce *P. cinnamomi* populations in forests. This same conclusion might thus be extrapolated for *P. ramorum*, *P. alni* and *P. lateralis* that are affecting large areas of native forest. When native organisms are not successful as biological control agent, release of imported antagonist microorganisms could be an option, nevertheless the release of alien microorganism in a native environment could also be extremely dangerous because potential untargeted effect (Podger & Keane, 2000).

### 3.3 Silvicultural treatment

#### 3.3.1 Elimination of susceptible trees

Using this approach, the primary intention is to reduce inoculum. For example, the main source of inoculum of *P. ramorum* in the Redwood and Mixed–Evergreen Forest in California, are the leaves of California bay laurel (*U. californica*) (Davidson *et al.*, 2005; 2008). Thus, Valachovic *et al.* (2008) proposed the elimination of bay laurel, including the use of fire to eliminate seeds and further regeneration, to reduce the inoculum source and protect the remnant forest. This measure, would have a small cost with a high level of benefit, although it will affect the forest structure. Nevertheless, the results of these trials are not complete and it is not possible to recommend this treatment at the present time.

#### 3.3.2 Reduce stand density

Humidity is a key factor in the *Phytophthora* infection process. This fact can be specifically exploited to reduce infection by aerial *Phytophthora* spp. More specifically, where it is possible to reduce the amount and duration of water present on leaves, it has been shown to be a key factor to reduce that infection due to *P. ramorum* (Davidson *et al*., 2006). Reduction in tree density in the forest allows solar radiation to reach the remaining canopy and aids wind movement within the forest (Burdon & Chilvers, 1982; Bulman *et al.*, 2004). These factors may thus explain why low levels of new infections have been found on natural regeneration or on trees surviving within areas where the tree density was drastically reduced by *P. ramorum* (Goheen *et al.*, 2008).

#### 3.3.3 Use of fire

Prescribed burning is a routine strategy used to manage forests in the Appalachian Mountains. Studies carried out after fires have shown that propagules of *P. cinnamomi* were not eliminated or even significantly reduced from the soil, and consequently is not recommended as a strategy for *Phytophthora* disease management (McLaughlin *et al*. 2009). Nevertheless, the wildfire that affected Oregon and northwest California in 2002, reduced the presence of *P. lateralis* in soil (Betlejewski, 2009). This is presumably due to the soil heat pulse from the fire, and the darkening of the soil after the fire that increase the soil temperature and thus reduces *Phytophthora* propagules (Betlejewski, 2009). The latter author has also proposed that fire could be used as strategy to manage

*P. lateralis*. A study by Moritz & Odion (2005) suggested that areas burned from 1950, are less susceptible to the disease caused by *P. ramorum*. The causes are not clear, but changes in soil chemistry that promote the intake of minerals such as calcium, magnesium, phosphorous for the tree and a change in the soil pH have been suggested as important factors. Further studies should be conducted to evaluate this strategy as a management practice for *Phytophthora* spp. in natural forest ecosystems, particulary for aerial-borne *Phytophthora* spp. .

### 3.4 Genetic resistance

Several strategies have been developed to produce trees that are genetically resistant to infection by pathogens. For example, crossing a susceptible and a resistant species, to generate hybrids with the phenotypic characteristic of the susceptible species, but that are resistant to disease, has been used with some success to develop *C. dentata* tres resistant to infection by *C. parasitica* (Hebard, 2006). In the case of *Phytophthora* spp. in forests, individual trees grow in infested in areas provide a potential source of resistant genotypes. A danger here is that few genotypes are resistant and this implies a narrow genetic diversity available to re–establish the natural forest. Thus, the genetic homogeneity of the host can result in dramatic losses when the pathogen overcomes the resistance.

In areas affected by *P. cinnamomi* in Australia some individual *E. marginata* trees that are resistant to the pathogen, have been selected as parents for progeny tests. The seedlings of those trees where tested for resistance and resistant seedlings have either been planted or used to produce clonal jarrah seed orchards (Hüberli *et al*., 2002). Some trees from these selections have been used to evaluate the heritable resistance in glasshouse inoculation tests using zoospore suspensions. A positive correlation was found with those observed in the field when these trees were established in naturally infected areas (Bennet *et al*., 1993; Cahill *et al*., 1993; Stukely & Crane 1994; Stukely *et al*., 2007a). In trials reported by Stukely *et al*. (2007a), the clones still showed resistance to *P. cinnamomi* in the field after 13 years.

One of the most successful programs involving breeding for resistance has been for *C. lawsoniana* against *P. lateralis.* This program began in 1989 based on some trees that survived natural exposure to the pathogen (Hansen *et al.*, 1989; Oh & Hansen, 2007; Oh *et al.*, 2006; Sinkiewicz & Jules, 2003) and more than 1000 trees were selected for resistance (Oh *et al.*, 2006). The high hereditability of the resistance (Hansen *et al.*, 2000) provided a framework for the restoration of *C. lawsoniana* in its original habitat (Hansen *et al*., 2000). Field trials are underway to evaluate the resistance of the selected families to infection by *P. lateralis* in the field (Sniezko *et al*., 2009).

The first attempts to detect genetic resistance in trees for infection against *P. ramorum* have begun. The available data, indicate that infection by *P. ramorum* results in irregular mortality of *Q. agrifolia* and that differences in susceptibility occur in *U. californica* to infection by *P. ramorum* (Anacker *et al*., 2008; Dodd *et al*., 2005). In addition, and despite tha high succeptibility of *L. densiflorus* to *P. ramorum,* has been reported that lone trees remain healthy in the middle of highly infested stand, indicanting a natural resitence to the disease (Hayden & Garbeloto, 2005). Because only a small number of trees have resistance to the disease in a severely affected forestry, it is possible that resistance is present in very few individuals, and thus is not a common characteristic of the population where it belongs (Anacker *et al*., 2008; Dodd *et al*., 2005). Clearly, there is justification for additional studies to search for resistance in tree species most seriously threatened by *P. ramorum*.

## 4.0 Conclusions

*Phytophthora* spp. represent some of the most important plant pathogens in the world. Their success appears to rest strongly on their ability to spread both locally and globally. They are very effectively spread by wind, rain, streams, irrigation water, soil, and contaminated plant material. Sporangia represent the propagules most important for the spread of aerial *Phytophthora* spp. In the case of *Phytophthora* spp. that occur in the soil, all the reproductive structures are able to move into new areas with soil, water or plant material, to produce new disease foci. Interestingly aerial *Phytophthora* spp. can be moved in infested soil but soil dwelling *Phytophthora* spp. do not typically have an aerial form of dispersal.

*Phytophthora* spp. include those with broad host ranges, such as *P. cinnamomi*, and others that are highly host specific, such as *P. alni*. Those with broad host ranges associated with forests also infect agricultural or ornamental plants, making their management and containment an important challenge. As is demonstrated by *P. cinnamomi* and *P. ramorum*, the ability to infect numerous hosts is an important advantage to establish in new areas. The opposite situation is represented by those species with a narrow host range, such as *P. lateralis* and *P. alni* that are present only in the areas where their hosts are found. Nevertheless, for both of the wide and narrow host range situations, once the species is in contact with the host it can produce a devastating effect.

Tree diseases caused by *Phytophthora* spp. occur in natural ecosystems, orcharads and in forest plantations. Those in natural ecosystems have caused devastating losses with little prospect for recovery. In plantations and orchards, most of the diseases are associated with seedlings and young trees, which are relatively easier to manage. An important advantage of plantations and orhards lies in the possibility to use management and silvicultural strategies, such as fungicides or species rotation, which is situation not possible in natural ecosystems. Nevertheless, the use of phosphite in natural jarrah forests in Australia highlights the fact that a balance can be reached between the environment, choice of the correct strategy and the necessity to protect the forest environment, possibly even from extinction.

New *Phytophthora* diseases of trees appear to be increasing in number dramatically. These diseases include those caused by native *Phytophthora* spp. that have undergone host shifts to infect trees planted for ornamental and commercial forestry. It is more worrying that new *Phytophthora* diseases are appearing that are clearly caused by introduced species. In most cases, the origins of these pathogens are not known.

All evidence suggests that there will be increasing numbers of *Phytophthora* diseases in the future. As a consequence of climate change, exploration of new areas and a change in the natural vegetation to agriculture or forestry, novel encounters between *Phytophthora* spp. and hosts will be promoted. Also, *Phytophthora* spp. will be moved to new niches in both native and planted forests due to the increasing movement goods

and as consequence of the worldwide trade, possibly limiting or causing the extinction of plants and trees.

The chapters of this thesis treat a new and serious disease of *Pinus radiata* that first emerged in Chilean plantations in 2004. An initial step was to determine the cause of the disease. This was subsequently shown to be a new species of *Phytophthora*. The remaining chapters include the first studies to be undertaken on this serious pathogen that will certainly emerge as one of the most important constraints in the future to global forestry.

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**Table 1.** *Phytophthora* spp. associated with forest ecosystems

| Species | Presence (1) | Association (2) | Hosts / associated | Geographic distribution | Selected references |
|---|---|---|---|---|---|
| *P. alni* subsp. *alni* | F-N | S, CR, RR | *Alnus glutinosa, A. incana* and *A. cordata.* | Germany, UK, Ireland, France, Belgium, The Netherlands, Sweden, Germany, Austria, and Hungary. | Brasier *et al.*, 2004a; Jung & Blaschke, 2004 |
| *P. alni* subsp. *multiformis* | F-N | CR, RR | *Alnus glutinosa* (Dutch, German and UK variant), *A. incana* (Dutch and German variant) and *A. cordata* (Dutch variant) | The Netherlands and Germany (Dutch varian), Germany (German variety), UK (UK variety) | Brasier *et al*., 2004a |
| *P. alni* subsp. *uniformis* | F-N | S, CR, RR | *Alnus glutinosa*, *A. incana* and *A. cordata.* | Sweden, Latvia, Germany, Austria, Italy, Hungary. | Brasier *et al*., 2004a; Jung & Blaschke, 2004 |
| *P. alticola* | F | CR, RR | *Eucalyptus dunnii, E. macarthurii, E. badjensis* | South Africa | Maseko *et al*., 2007 |
| *P. austrocedrae* | F | SC, RR | *Austrocedrus chilensis* | Argentina | Greslebin *et al*., 2007 |
| *P. boehmeriae* | F | CR, RR, G, S | *Pinus* sp., *Eucalyptus smithii* | Australia, South Africa | D`Souza *et al*., 1997; Erwin & Ribeiro, 1996; Maseko *et al.*, 2007; Roux & Wingfield, 1997; Roux *et al*., 1995; Santos & Luz, 2006; Santos *et al*., 2005; Wingfield & Kemp, 1993; Zeilemaker, 1971 |
| *P. cactorum* | F-N | SR, CR, RR, F, S, D | 154 genera in 54 families (e.g *Eucalyptus* spp., *Pinus* spp., *Fagus* spp., *Quercus* spp.) | Cosmopolitan | Jung & Blaschke, 2004; Jung, 2009; Jung *et al*., 1996; Jönsson *et al*., 2003; Lakatos & Szabo, 2007; Vettraino *et al*., 2001; 2005; Vettraino *et al*., 2002 |
| *P. cambivora* | F-N | CR, RR, SC, S | 30 genera in 19 families *(Eucalyptus* spp., *Fagus* spp, *Alnus* spp, *Picea* spp., *Quercus* spp., Pinus spp.) | Cosmopolitan | Erwin & Ribeiro, 1997; Balci *et al.*, 2007; Jönsson *et al*., 2003; Jung, 2009; Jung & Blaschke, 2004; Jung *et al*., 1996; Jung *et al.*, 2002; Reeser *et al.*, 2009; Vettraino *et al.*, 2002; Balci *et al*., 2008a |
| *P. capcici* | F | S | *Pinus* sp. | China | Zeng *et al*., 2009 |

| | | | | | |
|---|---|---|---|---|---|
| *P. captiosa* | F | F, S | *Eucalyptus saligna, E. botryoides* | New Zealand | Dick *et al*., 2006 |
| *P. cinnamomi* | F-N | S, CR, RR, SC | 266 genera in 90 families (e.g. *Eucalyptus* spp., *Pinus* spp) | Cosmopolitan | Balci & Halmschlanger 2003b; Balci *et al.*, 2007; Barnard *et al*., 1993; Benson & Grand 2000; Brasier *et al*., 1993; Crandall *et al*., 1945; Erwin & Ribeiro, 1996; Gallego *et al*., 1999; Sánchez *et al.*, 2002; Vettraino *et al*., 2002; Zentmyer, 1980 |
| *P. citricola* | F-N | RR, SC | 75 genera in 38 families (e.g. *Eucalyptus* spp., *Pinus* spp., *Fagus* spp., *Quercus* spp., *Alnus* spp.) | Cosmopolitan | Balci & Halmschlanger 2003a; 2003b; Balci *et al.*, 2007; Balci *et al.*, 2008a; Erwin & Ribeiro, 1997; Hansen & Delatour, 1999; Jung, 2009; Jung & Blaschke, 2004; Jung *et al*., 1996; Lakatos & Szabo, 2009; Vettraino *et al*., 2002; |
| *P. citrophthora* | F-N | SC, SR, RR | *Eucalyptus* spp., *Taxus* spp. | USA; USA; Argentina ; China | Erwin & Ribeiro, 1996; Schwingle *et al.*, 2007; Zeng *et al*., 2009 |
| *P. cryptogea* | F-N | RR, D, S | *Eucalyptus* spp; *Pinus* spp; *Quercus* spp. | Australia, Italy, Turkey | Balci & Halmschlanger 2003b; Erwin & Ribeiro, 1997; Vettraino *et al*., 2002 |
| *P. drechsleri* | N-F | RR, SA | 113 genera in 40 families (e.g. *Eucalyptus* spp., *Pinus* spp.) | Cosmopolitan | Erwin & Ribeiro, 1997; Heather & Pratt, 1975 |
| *P. europaea* | F | S, RR | *Quercus macrocapra, Q. palustris, Q. alba, Q. montana, Q. velutina, Q. rubra* | Germany , France, Austria, USA | Balci & Halmschlanger 2003a; Balci *et al.*, 2007; Balci *et al*., 2008; Jung *et al.*, 2002; |
| *P. fallax* | F | F | *Eucalyptus regnans, E. delegatensis, E. fastigata, E. nitens* | New Zealand | Dick *et al.*, 2006 |
| *P. frigida* | F | RR, CR | *Eucalyptus smithii, Acacia decurrens* | South Africa | Maseko *et al.*, 2007 |
| *P. gallica* | F | S | *Quercus robur, Phragmites australis* | France, Germany | Jung & Nechwatal, 2008 |

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|---|---|---|---|---|---|
| *P. gonapodyides* | F-N | S, W, SC, RR | 13 genera in 11 families (e.g. *Abies spp., Pseudotsuga menziesii, Tsuga mertensiana)* | Cosmopolitan | Balci & Halmschlanger 2003a; 2003b; Hansen & Delatour, 1999; Jung, 2009; Jung & Blaschke, 2004; Jung *et al*., 1996; Lakatos & Szabo, 2009; Reeser *et al*., 2009; Vettraino *et al*., 2002; |
| *P. hevae* | F | RR, S, W | *Pinus* sp. | Australia, China | Erwin & Ribeiro, 1996; Zeng *et al*., 2009 |
| *P. inflata* | F | SC | *Ulmus* spp. | England, Canada, USA | Jung *et al*., 2005 |
| *P. insolita* | F | S, W | Forest | China | Zeng *et al*., 2009 |
| *P. inundata* | F-N | W, S, RR | *Alnus* spp., *Salix matsudana*, *Xanthorrhoea preissii* | Denmark, France, Spain, UK, USA, Hungary, Denmark, Australia | Brasier *et al*., 2003; Hüberli *et al*., 2009; Lakatos & Szabo, 2009; Stukely *et al*., 2007b; |
| *P. katsurae* | F | S, SC | *Castanea crenata* | Ivory Coast, Japan, Korea, Taiwan, Jamaica, USA, Papua New Guinea; China | Ko & Chang, 1979; Oh *et al*., 2008; Zeng et al., 2008 |
| *P. kernoviae* | F | SC | Several host including *Fagus sylvatica, Quercus robur* | UK; New Zealand | Brasier, 2008b; Brasier *et al*., 2005 |
| *P. lateralis* | F-N | RR | *Chamaecyparis lawsoniana, Taxus brevifolia* | Canada, USA | DeNitto & Kliejunas 1991; Hansen *et al*., 2000; Murray & Hansen, 1997; Woodhall & Sansford, 2006; Zobel *et al* 1985 |
| *P. megasperma* | F-N | RR, W, S | *Picea* spp., *Abies* spp., *Quercus* spp., *Alnus* spp. *Pinus* spp. | Cosmopolitan | Erwin & Ribeiro, 1997; Hansen & Delatour, 1999; Jung & Blaschke, 2004; Jung *et al*., 2002; Lakatos & Szabo, 2009; Vettraino *et al*., 2002 |
| *P. multivora* | F | RR, S, CR | *Eucalyptus gomphocephala, E. marginata, Agonis flexuosa,* | Australia | Scott *et al*., 2009 |
| *P. nemorosa* | F | SC, F | *Umbellularia californica, Sequoia sempervirens, Lithocarpus densiflorus,* | North America | Hansen *et al*., 2003a; Reeser *et al*., 2009; Wickland *et al*., 2008 |

| | | | | | |
|---|---|---|---|---|---|
| | | | *Quercus agrifolia* | | |
| *P. nicotianae* | F-N | G, RR, CR SA | *Acacia* spp, *Pinus* spp., *Eucalyptus* spp. | Cosmopolitan | Erwin & Ribeiro, 1997; Linde *et al.*, 1994; Maseko *et al.*, 2001; Roux & Wingfield, 1997; Roux *et al.*, 1995; Santos & Luz, 2006; Santos *et al.*, 2005; Von Broembsen, 1984a; Wingfield & Knox–Davies, 1980; Wingfield & Kemp, 1993; Zeilemaker, 1971; Zeng *et al.*, 2009 |
| *P. plurivora* | F-N | CR, RR, SC | More than 40 species (e.g. *Abies* spp., *Pinus* spp., *Quercus* spp.) | Cosmopolitan | Jung & Burgess 2009 |
| *P. polonica* | F | S | *Alnus glutinosa* | Poland | Belbahri *et al.*, 2006 |
| *P. pseudosyringae* | F | CR, RR, SC, S | *Alnus glutinosa, Fagus sylvatica, Quercus spp. , U. californica, Lithocarpus densiflora, Sequoia sempervirens* | Germany, Spain, USA, France, Italy | Jung, 2009; Jung *et al.*, 2003; Martin & Tooley, 2003; Reeser *et al.*, 2009; Wickland *et al.*, 2008 |
| *P. pseudotsugae* | F | RR | *Pseudotsuga menziesii* | USA | Hamm & Hansen, 1983; Hamm & Hansen, 1987 |
| *P. pseudotsugae* | F | S | *Quercus* spp. | France,Germany, UK | Jung *et al.*, 2002; Jung *et al.*, 2003 |
| *P. quercina* | F-N | RR, S | *Alnus glutinosa, Quercus* spp | Turkey, Germany, Hungary, Italy, USA | Balci & Halmschlanger 2003a; 2003b; Hansen & Delatour, 1999; Jung & Blaschke, 2004; Jung *et al.*, 1999; Jung *et al.*, 2002; Jönsson *et al.*, 2003; Vettraino *et al.*, 2002 |
| *P. quercetorum* | F-N | RR, S | *Quercus macrocapra, Q. palustris, Q. montana, Q. rubra, Q. phellos* | USA | Balci *et al.*, 2008a |
| *P. ramorum* | F-N | F, SC, S, SA | 26 genera in 17 families (e.g. *Quercus spp, Umbellularia californica,* | Europe, North America | Davidson *et al.*, 2005; 2008; Mascheretti *et al.*, 2008; Rizzo *et al.*, 2005 |

| | | | | | |
|---|---|---|---|---|---|
| | | | *Lithocarpus densiflora*) | | |
| *P. siskiyouensis* | F | S, W, SC | *Alnus spp, Umbellularia californica, Lithocarpus densiflora* | USA, Australia | Reeser *et al*., 2007; |
| *P. syringae* | F | S | 29 genera in 14 families (e.g. *Q. robur, Q. petraea, Q. pubescens, Q. cerris*) | Austria, Italy, Morocco, South Africa, Korea, Canada, USA, Argentina, Brazil, Peru | Balci & Halmschlanger, 2003a; Vettraino *et al*., 2002 |
| *P. uliginosa* | F | CR, S | *Quercus robur*, *Q. petrae, Fagus sylvatica* | Poland, Germany | Jung, 2009, Jung *et al*., 2002 |

(1) F: Forest; N: Forest nursery

(2) CR: Collar rot; D: Decline; F: Aerial part of the three; G: Stem gumossis; RR: Root rot; SA: Aerial damage in seedling; SR: Seedling root rot; SC: Stem canker; S: soil; W: stream bank.

# Chapter 2

## *Phytophthora pinifolia* sp. nov. associated with a serious needle disease of *Pinus radiata* in Chile

## ABSTRACT

During the course of the past three years, a new disease of *Pinus radiata*, referred to as 'Daño Foliar del Pino' (DFP) has appeared in the Arauco province of Chile and subsequently spread to other areas. The disease is typified by needle infections, exudation of resin at the bases of the needle brachyblasts and, in younger trees, necrotic lesions in the cambium, which eventually girdle the branches. The disease causes the death of young seedlings and mature trees can also succumb after a few years of successive infection, probably hastened by opportunistic fungi such as *Diplodia pinea*. Isolations on selective medium for *Phytophthora* spp. led to the consistent isolation of a *Phytophthora* sp. from needle tissue. DNA sequence comparisons for the ITS rDNA and *cox* II gene regions, and morphological observation showed that this oomycete represents a previously undescribed species for which the name *Phytophthora pinifolia* sp. nov. is provided. This new species is characterized by unbranched sporangiophores, and non-papillate, sub-globose to ovoid sporangia that are occasionally free from the sporangiophore with medium length pedicels. Despite using a number of oospore inducing techniques, oogonia/antheridia were not observed in isolates of *P. pinifolia*. Pathogenicity trials with *P. pinifolia* showed that it is pathogenic to *P. radiata* and causes rapid death of the succulent apical parts of young plants. *Phytophthora pinifolia* is the first *Phytophthora* known to be associated with needles and shoots of a *Pinus* sp. and its aerial habit is well matched with the occurrence and symptoms of DFP in Chile.

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## 1.0 Introduction

Forestry plantations based on non-native trees, particularly in the tropics and Southern Hemisphere, represent substantial business enterprises of great international relevance (Boyle *et al*., 1999). These forestry companies primarily plant species of *Pinus*, *Eucalyptus* and *Acacia*, and they produce solid–wood products and pulp for paper and rayon production. Growth of the trees, used to sustain wood and fiber farms, is in many cases exceptional and typically far beyond that associated with the trees in their natural environment (Richardson *et al*., 2007). This is widely attributed to the separation of the trees from their natural enemies (Brockerhoff *et al*., 2006; Wingfield, 2003). Thus, one of the greatest threats to plantation forestry based on non-native trees clearly lies in dangers surrounding the accidental introduction of pests and pathogens (Brockerhoff *et al*., 2006; Wingfield *et al*., 2001, 2006), or native pests and diseases that can affect these exotic hosts (Carroll, 2007; Slippers *et al* ., 2005).

*Pinus radiata* (Monterey pine), one of the most widely planted forest trees in the Southern Hemisphere, has its origin on the California coast (USA) and nearby islands (Richardson *et al*., 2007; Rogers *et al*., 2006). Within its native range, it is ecologically restricted to coastal Mediterranean climates, growing in only five locations; three along central coastal California and two on Mexican islands of Baja California (Rogers, 2004; Rogers *et al* ., 2006). Consistent with its native range, *P. radiata* has performed best in other areas of the world that have a Mediterranean climate, including parts of South Africa, Australia, Chile, New Zealand and Spain (Rogers, 2004). Chile has a substantial investment in plantations of *P. radiata*, which was introduced for ornamental purposes in 1885, and has grown exceptionally well over a relatively long period (Toro & Gessel, 1999). In surface area, Chilean *P. radiata* plantations represent more than one third of the total area planted to this species in the world, with approximately 1·5 million ha

established and forming the basis of a major industry important to the economy of the country (Guerrero & Bustamante, 2007).

Several important pests and diseases occur on *P. radiata* plantations in Chile. Needle blight caused by *Dothistroma septosporum* (Barnes *et al*., 2004) and the pine shoot moth *Rhyacionia buoliana* (Lanfranco, 2000) have resulted in the most serious sanitary problems on *P. radiata* in Chilean plantations (Ahumada, 2003). *Fusarium circinatum*, the causal agent of pitch canker in *P. radiata* plantations in Spain and South Africa (Coutinho *et al*., 2007; Landeras *et al*., 2005), occurs in forestry nurseries in Chile, but has not been found in the field (Jacobs *et al*., 2007)

In February 2004, an unusual tree mortality appeared in a 6-year-old *P. radiata* stand of about 70 ha on the Arauco coast of Chile. In October 2004 and in the same area, a serious needle blight disease was observed and associated with this mortality. The damage increased dramatically expanding to approximately 60 000 ha by the end of 2006. The *P. radiata* needle blight disease in Chile has been referred to as 'Daño Foliar del Pino' (DFP) and it is generally accepted to be the most serious problem yet to have affected pine forestry in the country.

The overall pattern of disease development, the species of tree affected and the symptoms provided a robust indication that DFP is caused by a biotic agent. Previous isolations from disease symptoms on routine growing media, such as streptomycin–amended 2% malt extract agar (MEA), yielded many fungi including *Strasseria geniculata* , *Allantophomopsis lycopodina* , *Phoma herbarum* , *Microsphaeropsis olivaceae* , *Diplodia pinea* and *Pestalotiopsis* spp. *Strasseria geniculata* and *A. lycopodina* were also commonly found sporulating at the bases of needles in the early stages of infection. However, inoculation of these fungi onto the needles and stems of young and old *P. radiata* plants with spore suspensions or with mycelial plugs, did not result in any symptoms (unpublished data). Thus none of these fungi appear to be responsible for the disease and they probably represent opportunistic endophytes or secondary inhabitants.

Isolations from diseased needles in July 2007 on *Phytophthora*– selective media consistently yielded a *Phytophthora* species (http://www.fabinet.up.ac.za/tpcp/

pinifolia). There are more than 80 species in the genus *Phytophthora* (www.phytophthoradb.org) and most are destructive plant pathogens (Goodwin, 1997). Thus, the close association of a *Phytophthora* sp. with the needle and shoot disease in Chile suggested that this could be the causal agent. The aim of this study was to describe DFP and to characterize the *Phytophthora* species isolated from diseased tissue based on morphology and DNA sequence comparisons. In addition, the ability of the oomycete to cause disease was considered in pathogenicity tests using young *P. radiata* plants.

## 2.0 Materials and Methods

### Disease occurrence and symptoms

Symptoms of DFP were first observed on the Arauco coast at Paillacahue (37°17′40′′S; 73°36′44′′W) on 18-year-old *P. radiata* trees in October 2004 and they have subsequently been monitored regularly. During this period, the disease expanded from an area of 70 ha to approximately 60 000 ha at the end of 2006 with varying levels of damage depending on specific sites (Fig. 1a–b). Symptoms were thus studied over a period of four years and their description reflects observations over a relatively long period of time.

### Isolations from infected tissue

Isolations were made from the bases of newly infected needles, the resinous bands on needles, as well from infected phloem tissue below infected needles on the branches and stems. For isolations, a sharp scalpel was used to cut small (1 mm) pieces of infected tissue that were transferred to plates of modified selective CMA–NARP (17 g L –1 corn meal agar [Difco], amended with 1 mg $L^{-1}$ nystatin, 100 mg $L^{-1}$ ampicillin, 100 mg $L^{-1}$ pentachloronitrobenzene (PCNB) and 10 mg $L^{-1}$ rifampicin) (Shearer & Dillon, 1995). Plates were incubated at 20 °C for 10 days. Resultant colonies were transferred to carrot agar (CA) and maintained at 20 °C (Erwin & Ribeiro, 1996). All cultures used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria and representative isolates have been deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, and the World Phytophthora Genetic Resource Collection (WPC), University of California, Riverside, CA, USA.

**DNA sequence comparisons**

Five isolates of the *Phytophthora* sp. (CMW 26667–26671), tentatively identified based on mycelial characteristics and the presence of sporangia, were selected for DNA extractions. DNA was extracted from mycelium scraped from the surface of agar plates using the PrepMan Ultra sample preparation reagent (Applied Biosystems) following the manufacturer's instructions. The ITS region of the rDNA was amplified using the primers ITS6 and ITS4 (White *et al*., 1990). Primer ITS6 is similar to ITS5 (White *et al*., 1990), but was modified according to the *P. megasperma* 18S rDNA sequence to allow more efficient amplification in *Phytophthora* spp. (Cooke & Duncan, 1997; Cooke *et al*., 2000). The *cox* II region was amplified using the primers FM35 and FM58 (Martin, 2000). Amplifications were performed in an iCycler thermocycler (Bio–Rad). The thermocycle sequence for ITS and *cox* II regions was as follows: an initial denaturation step of 2 min at 95 °C was followed by 30 cycles of 20 s denaturation at 95 °C, 25 s of annealing at 55 °C and 50 s extension at 72 °C, and a final extension step of 72 °C for 10 min (Cooke *et al*., 2000). The PCR reaction mixture (25 µL) consisted of 10–20 ng of template DNA, 200 µm dNTPs, 1U *Taq* DNA polymerase, 10X buffer (Roche Molecular Biochemicals), 1.5mm $MgCl_2$, and 25 ng of each primer. Successful amplification was confirmed by gel electrophoresis (1% agarose gel stained with ethidium bromide and visualized under UV light). PCR products were purified on Sephadex columns (Multiscreen HV, Millipore) 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 TM Big Dye terminator sequencing reaction kit following the manufacturer's instructions (Perkin–Elmer, Applied BioSystems). Sequencing was done using an ABI 3100TM automated DNA sequencer. The forward and reverse sequences were assembled using the software Vector NTI AdvanceTM v.10.3 (Invitrogen Corporation), and aligned using the software MEGA v. 3.0 (Kumar *et al*., 2004) followed by manual improvement of the alignment. The sequences were subjected to an NCBI BLAST search (http:// www.ncbi.nlm.nih.gov/BLAST/) prior to phylogenetic analyses to identify the closest related sequences.

The obtained sequences for the *Phytophthora* spp. were compiled into DNA sequence data matrixes. The ITS sequences were incorporated in a DNA sequence dataset that represents the diversity of the genus *Phytophthora*, produced by Cooke *et al*. (2000) and obtained from TreeBASE (www.treebase.org; Matrix #M751). Additional DNA sequences for *Phytophthora ramorum* (Martin & Tooley, 2003), *P. inundata* (Brasier *et al*., 2003a), and *P. austrocedrae* (Greslebin *et al*., 2007) were added. Similarly, the *cox* II sequences were incorporated in a DNA sequence dataset representative of the genus *Phytophthora* published by Martin & Tooley (2003) (Treebase matrix # M1228), and DNA sequences of *P. ramorum* (Ivors *et al*., 2004) were added. *Cox* II sequences corresponding to the region used here were not available for *P. inundata*, *P. humicola* and *P. austrocedrae*. Maximum parsimonious trees were obtained with PAUP (Phylogenetic Analysis Using Parsimony) version 4·0b10 (Swofford, 2002), with heuristic searches of only informative characters and tree bisection and reconstruction (TBR) as branch–swapping algorithm (random stepwise addition). Gaps were treated as missing, and all characters were unordered and of equal weight. Maxtrees were unlimited, branches of zero length were collapsed, and all multiple, equally parsimonious trees were saved. Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices and g1–value) were determined (Hillis & Huelsenbeck, 1992). Branch supports were determined using 1000 bootstrap replicates (Felsenstein, 1985). Phylogenetic species hypotheses were also tested using distance analyses with the neighbour joining algorithm (implemented in MEGA v. 3·0 [Kumar *et al*., 2004]), and compared to maximum parsimony analysis. Individual sequences generated in this study have been deposited in GenBank.

**Morphology**

To induce the production of fruiting structures, isolates were grown on either CA or V8 agar (354 mL V8 juice, buffered with 5 g of CaCO3, 15 g of agar, 1 litre with distilled water) (Erwin & Ribeiro, 1996). For sporangia, blocks (5 × 5 mm) were cut from the edges of actively growing 7-day-old CA and V8 cultures, and immersed directly into non-sterile soil water (Erwin & Ribeiro, 1996), with modifications (60 g soil in 1 litre of water, allowing the soil to settle for 8 h and filtering the supernatant through four layers of cheese cloth). Blocks of agar bearing mycelium in the soil-water mixture were incubated at room temperature (20–22 °C) for 12 hrs and then transferred to a 4 °C incubator for 4 hrs. Cultures were then returned to room temperature and inspected

every 30 min for the presence of fruiting structures. In an attempt to produce oogonia, pure cultures growing in CA and V8 were incubated in the dark at 20 °C and 25 °C and checked at 14 and 30 d. Isolates were also paired with each other in all combinations on the same media and under the same incubation conditions, and observed at 14 and 30 days.

The morphology of the *Phytophthora* sp. was studied using conventional microscopy techniques. Structures were mounted in 85% lactic acid. Twenty measurements of all structures were made, but fifty measurements were taken for the isolate representing the ex-type. Measurements and photography were done with a HRc Axiocam digital camera system with Axiovision 4·6 software (Carl Zeiss Ltd.).

To test for the optimum growth temperature, five isolates (CMW 26667–26671) were transferred to two types of growing media (V8 and CA) with five replicates for each isolate. Circular agar blocks (3 mm diam) were placed at the centres of 90 mm diameter Petri dishes and these were incubated at temperatures between 10 and 35°C at five degree intervals, in the dark. Colony growth was assessed after 10 days by taking two diameter measurements at right angles to each other for each replicate plate. The averages were computed and the data statistically compared with a one–way anova test using Statistica v. 6 (StatSoft Inc., 2004).

**Pathogenicity tests**

A pathogenicity test was conducted using the same *Phytophthora* isolates (CMW 26667–26671) used in the DNA sequence comparisons. The isolates were grown on CA at 20°C for two weeks prior to inoculation. Inoculations were conducted in Chile where *P. radiata* seedlings, approximately 12-month-old, were used. A total of 70 plants, approximately 400 mm high and 6 mm collar diameter, were acclimatized into the screening facilities with artificial day light at approximately 20–25°C for two weeks prior to inoculation. Small (3 mm diam) discs of bark were removed from the apical parts of the stems where the tissue was green and plugs of mycelium of similar size, taken from the edges of actively growing cultures, were placed into the wounds. Discs of clean CA were used as negative controls. Ten plants were inoculated for each of the cultures. Inoculation wounds were covered with Parafilm (Pechiney Plastic) to reduce desiccation and contamination.

Two weeks after inoculation, the outer bark was removed from the inoculation points with a sterile scalpel, and the inner bark lesion lengths (mm) were measured. Small pieces of wood from the leading edges of the lesions were plated onto NARP medium to ensure that the inoculated organism was associated with the lesions. The means of the measured lesions were compared with a oneway anova test using Statistica v. 6 (StatSoft Inc., 2004).

## 3.0 Results

### Disease occurrence and symptoms

The symptoms of DFP on mature trees, up to 18-yearsold, begin with a reddening of the past year's needles in early winter (Fig. 2a–b). The first needles to display symptoms are those on the lower sides of the branches (Fig. 2d). Needles die and assume a distinctly grey colour and begin to fall. Initially, dead and dying needles are retained on the branches giving the trees the appearance of having been severely scorched. Needles then fall from the trees, which can be almost entirely defoliated. New needle growth is not affected and the trees appear to recover until infection re-occurs in the following season. After two or three years of defoliation, trees occasionally die and this appears to be hastened due to infection by *D. pinea*, which is a well-known opportunistic pathogen (Swart & Wingfield, 1991).

One of the earliest symptoms on affected needles is the emergence of dark resinous bands on the green needles, which appear transparent when viewed with backlighting (Fig. 2c). These bands are found at various positions, either close to the bases or higher up on the needles. Drops of resin are often found at the base of the needles (Fig. 2f) and the tissue within the papery brachyblasts is commonly collapsed and has a light grey colour. Needles often collapse from their bases just above the branches and hang at right angles from the branches. When the bark is removed, a distinct brown or reddish brown discolouration can be seen in the phloem and cambium, particularly where it is associated with dying needles (Fig. 2g–i)

Symptoms on young trees between one and 4-year-old appear different to those on mature trees. Damage to young trees is most common and most severe where stands occur alongside larger trees affected by DFP. One of the first and most obvious symptoms to appear on young trees is where young growing terminal shoots wilt and die rapidly (Fig. 2e–j). Lesions are typically found on these shoots or on the needles associated with them, depending on the time of the year. The damage resulting in the wilted growing shoots is typically found on parts of branches or shoots a short distance below the obviously affected tissue. Close inspection reveals needles with symptoms similar to those on older trees with infected bases leading to girdling of the stems. Dark resinous bands can be found on the needles in the early stages of symptom development and infection appears to be concentrated within the papery brachyblasts at the base of the needle fascicles.

On younger trees, necrosis of the cambium is more pronounced than that on mature trees and the impact of infection is commonly more severe. Resin can be found exuding profusely from the brachyblasts or the bases of the needles (Fig. 2h). Branches can have large numbers of needles hanging at right angles, apparently from their bases and infections begin on the lower sides of branches. Removal of the outer bark reveals distinct lesions below the needle bases and in many cases, due to multiple infections, they coalesce to form cankers in the phloem and outer cambium. These cankers result in girdling of the stems or branches and wilting of the needles and shoots proximal to them. Naturally regenerated plants and newly planted seedlings are equally affected by DFP, and appear to wilt and die rapidly due to their small size.

The pine needle blight in Chile has a distinctly seasonal pattern of occurrence. Trees begin to show symptoms in early winter from about July onwards when the temperatures begin to drop to between 6 and 12°C. This is also the start of the rainy season and there appears to be a very close association of the disease with rainfall. A spatial analysis based on data from 2006 (data not shown), has shown that the southern slopes (higher humidity, most free water and low sun radiation) are most severely affected (Fig. 1c).

**DNA sequence analyses**

GenBank BLAST searches using ITS and *cox* II sequences for the *Phytophthora* isolates from *P. radiata* in Chile all indicated that the organism is unequivocally a species of *Phytophthora*, with the highest similarity to sequences obtained for isolates of *P. megasperma* and *P. gonapodyides*. These searches included sequences of undescribed lineages in Clade 6 defined by Brasier *et al*. (2003b). The ITS dataset contained a total of 935 characters after alignment, 492 of which were constant, 187 variable and parsimony uninformative, and 256 were variable and parsimony informative. Heuristic searches using the TBR algorithm found 10 most parsimonious trees (g1 = –0·406866, tree length = 996 steps; consistency index (CI) = 0·603; retention index (RI) = 0·806; rescaled consistency index (RC) = 0·486). These trees differed only in re-arrangements within the major clades and not between them, and consequently one was chosen for presentation (Fig. 3). The clades identified using these analyses were identical to those revealed by neighbour-joining analysis (data not shown).

The *cox* II dataset had a total of 568 characters after alignment, of which 403 were constant, 46 were variable and parsimony uninformative, and 119 were variable and parsimony informative. Parsimony analysis resulted in 4 most parsimonious trees (Fig. 4) with 494 steps (g1 = –1·149137; CI = 0·496, RI = 0·677, RC = 0·336). The clades identified by parsimony analysis were identical to those revealed by neighbour-joining analysis (data not shown).

Both the analyses of the ITS and *cox* II datasets revealed that the isolates from *P. radiata* in Chile resided in a well resolved clade (100% bootstrap support) distinct from all other *Phytophthora* spp. (Figs 3–4). In both trees, the Chilean isolates grouped in Clade 6 of the *Phytophthora* phylogeny described by Cooke *et al*. (2000), with *P. megasperma* and *P. gonapodyides* its closest relatives. In the ITS tree, the isolates from *P. radiata* in Chile grouped in a sub-clade with *P. megasperma* and *P. gonapodyides* (93% bootstrap support) separate from *P. humicola* and *P. inundata* (100% bootstrap support). In the *cox* II tree, the isolates from *P. radiata* in Chile form a distinct clade (99% bootstrap support) with *P. megasperma* and *P. gonapodyides*, separate from all other *Phytophthora* spp. currently available in GenBank.

**Morphology**

Cultures on CMA-NARP (Fig. 5a–b) were submerged and had coenocytic hyphae typical of *Phytophthora* spp. with a coralloid morphology (Fig. 6a–b), while CA and V8 cultures were fluffy with a regular margin to rosaceous or petallate margin (Fig. 5c–f). Sporangia formed abundantly in soil water, but more rarely in sporangium inducing solutions, such as Petri Solution, and they were generally absent in pure culture. Sporangia were borne on predominantly unbranched sporangiophores and were non-papillate, sub-globose to ovoid (Fig. 6c–d), releasing the zoospores (Fig. 6i–j) or occasionally germinating directly with apical elongations (Fig. 6e–f). Free sporangia with pedicels were occasionally observed in the medium immersed in soil water and were usually released after the liquid medium was stirred (Fig. 6g–h). Despite regular observation of plates, including those where isolates of the *Phytophthora* sp. were paired with each other in all possible combinations, sexual structures of the oomycete where not observed.

**Taxonomy**

The *Phytophthora* sp. isolated from the bases of newly infected needles, the resinous bands on needles and infected phloem tissue below infected needles of *P. radiata* in Chile, resides in Group 6 of the phylogeny-based classification of Cooke *et al*. (2000). Other species in this group include *P. gonapodyides*, *P. megasperma, P. humicola* and *P. inundata.* These species are all ecologically and morphologically different to the *Phytophthora* sp. from Chile (Table 1) despite their phylogenetic affinity. For example, they are all soil fungi whereas the Chilean *Phytophthora* sp. has an aerial habit, with occasionally caducous sporangia that do not proliferate internally or externally unlike those found in *P. gonapodyides*, *P. megasperma, P. inundata* and *P. humicola*. DNA sequence comparisons, the ecology and the morphology of the *Phytophthora* sp. from diseased *P. radiata* needles in Chile thus provide convincing evidence that it represents a new taxon. The following description is provided for it.

*Phytophthora pinifolia* A. Durán, Gryzenh. & M.J. Wingf., sp. nov., MB 511870, Figs 5–6. Etymology: ‘pinifolia’ refers to the occurrence of the organism on the needles of *Pinus radiata*.

*Coloniae* in CA et V8 albae mycelio laxo, margine regulari, rosoide vel petalloide, appressae vel in medio immersae; crescunt optime in 25 ºC (minime 10 ºC, maxime 30

ºC), diametro maxime 45–55 mm post 4 hebdomades; in CMA–NARP mycelio immerso pariete valde irregulari. *Hyphae* coralloideae tumoribus paucis singulis 4–8 µm diametro, interdum cup hyphis radiatis. *Sporangiophorae* 28–44 µm, mediocriter 36 µm longae, simplices. *Sporangia* terminalia, semiglobosa vel ovoidea, non papillata, 39–61 × 27–45 µm, mediocriter 48 × 35 µm, aliquando proxime elongatione apicali poro exitus 15 µm lato germinantia, aliquando liber pedicellis 14–32 µm, mediocriter 23 µm longis.

*Colonies* on CA and V8 white with fluffy aerial mycelium, with a regular border or a rosaceous to petallate pattern, appressed or submerged in the medium, optimal temperature for growth 25 ºC (min 10 ºC; max 30 ºC), plates never completely covered but reaching maximum diam (45–55 mm) on CA in 4 weeks. On CMA-NARP, mycelium submerged with a very irregular border. *Hyphae* coralloid with unusual single spherical swellings, sometimes with radiating hyphae, swellings 4–8 µm diam. *Sporangiophores* 28–44 µm long (avg. 36 µm), simple. *Sporangia* produced abundantly in soil extract water and rare in culture; in soil extract water mostly occurring within agar plugs; terminal, semi-globose to ovoid, nonpapillate, 39–61 × 27–45 µm (avg. 48 × 35 µm), occasionally germinating directly with apical elongation or releasing zoospores directly, exit pore 15 µm wide, occasionally free with pedicels 14–32 µm (avg. 23 µm) long.

*Specimens examined*: CHILE. Arauco province, Arauco, Llico plantation, *Pinus radiata*, July 2007, M.J. Wingfield (**holotype** PREM 59887, ex–holotype isolate CMW 26668/CBS 122924/WPC P16100; paratypes PREM 59888, PREM 58889; living cultures CMW 26667/CBS 122923/WPC P16101, CMW 26669/CBS 122922/WPC P16102).

*Distribution* – Arauco province, Chile.

**Pathogenicity**

Distinct lesions developed on *P. radiata* shoots 15 days after inoculation with *P. pinifolia* isolates. In contrast, no lesions developed on shoots inoculated as negative controls. The average lengths of the lesions for the treated shoots ranged from 15 mm

(CMW 26669) to 35 mm (CMW 26671) (Fig. 7) with no significant difference between the isolates. Inoculated shoots wilted and the needles turned brown, in a manner very similar to symptoms on infected shoots observed in nature (Fig. 8a–b). *Phytophthora pinifolia* was consistently re-isolated from the lesions at various positions across the necrotic area, and the identity of the isolates was confirmed using DNA sequence comparisons.

**4.0 Discussion**

This study considered a serious new needle blight disease of *P. radiata* in Chile and led to the discovery of a *Phytophthora* sp. consistently isolated from affected needles, needle bases and stems of young seedlings. Furthermore, DNA sequence comparisons and morphological observations provided convincing evidence to show that the oomycete represents an undescribed species of *Phytophthora*, which was provided with the name *P. pinifolia*. Pathogenicity tests with a suite of isolates also showed that *P. pinifolia* is able to rapidly kill inoculated shoots, leading to symptoms similar to those seen on naturally infected trees in Chile.

The habitat of *P. pinifolia* is unusual in that it is the only species of this genus known to infect green shoots and needles of a *Pinus* sp. Results of this study, based on a consistent association with infected needles, as well as pathogenicity tests, have provided convincing evidence that it is the primary cause of the needle disease and ultimately death of young trees. All evidence, including consistent isolation from the needles with symptoms, suggests that *P. pinifolia* is also the cause of the needle cast on older *P. radiata* trees. It has, however, not been possible to induce disease symptoms without wounding and a technique to test pathogenicity under natural conditions must still be developed.

While various *Phytophthora* spp. have been isolated from *Pinus* spp. (Chavarriaga *et al*., 2007; Erwin & Ribeiro, 1996; Sánchez *et al*., 2002), only *P. cinammomi* (Ali *et al*., 1999), *P. citricola* (Sandlin *et al*., 1992), *P. cryptogea* (Bumbieris, 1976) and *P. drechsleri* (Heather & Pratt, 1975) have been reported to be associated with *P. radiata*. All are soil–borne pathogens and they do not infect pine shoots and needles. The disease

of *P. radiata* in Chile is clearly a new pine disease and it should not easily be confused with other *Phytophthora* diseases or indeed, any other pine needle disease.

The discovery of an aerial *Phytophthora* sp. causing a serious disease of *P. radiata* in Chile, adds to a number of new and serious tree-infecting aerial *Phytophthora* spp. that have recently been discovered. The most dramatic of these has been *P. ramorum* which was described from Germany and the Netherlands in 2001 (Werres *et al*., 2001). This pathogen was discovered in the USA in 2001 (Rizzo *et al*., 2002) where it has caused death of woody plants and particularity of tanoak (*Lithocarpus densiflorus*) and coast live oak (*Quercus agrifolia*) (Rizzo *et al*., 2005). Another example is the more recently discovered *P. kernoviae*, which infects *Fagus sylvatica* and *Rhododendron ponticum* in England (Brasier *et al*., 2005; Brown & Brasier, 2007). These newly discovered aerial *Phytophthora* spp. are apparently emerging as seriously threatening tree pathogens in various parts of the world (Denman *et al*., 2006) and the discovery of *P. pinifolia* contributes to this view.

The closest relatives of *P. pinifolia* based on DNA sequence data are *P. megasperma, P. gonapodyides*, *P. humicola* and *P. inundata*. These species all reside in Clade 6 in the phylogenetic classification of *Phytophthora* spp. (Cooke *et al*., 2000). Members of this clade mostly occur in forest or riparian ecosystems and cultures are mostly sexually sterile or inbreeding (Brasier *et al*., 2003b; Kroon *et al*., 2004). These species are unlikely to be confused with *P. pinifolia* because they are all soil–borne pathogens (Brasier *et al*., 2003b; Brasier *et al*., 1993; Ko & Ann, 1985; Hansen & Maxwell, 1991) and all have nested or extended sporangium proliferation and are noncaducus, in this regard very different to *P. pinifolia* (Table 1). It is consequently easy to discriminate between *P. pinifolia* and other species of *Phytophthora* even in the absence of oogonia, simply on the basis of habit and morphology of sporangia.

The discovery of a *Phythophthora* species consistently associated with symptoms of DFP in Chile, fits the aetiology of the disease. For example, DFP appears in close association with low temperatures and wet conditions. A particularly obvious symptom is that needles on the lower sides of branches die first and this is an area where free water typically accumulates. The disease is also more obvious on the southern hill slopes than on northern slopes, and thus in areas that are cool and moist. These

conditions are all conducive to the formation of sporangia and the spread and development of the disease (Erwin & Ribeiro, 1996).

While the symptoms of DFP in Chile are consistent with the biology of an aerial *Phytophthora* sp., there are many questions regarding the biology of the pathogen that remain to be answered. While it is assumed that the sporangia are the infective propagules, this has yet to be shown experimentally. These structures were not abundant in culture and more natural conditions under which to produce them will need to be developed. Likewise, infection studies with zoospores and the infection biology and life cycle of the pathogen remain to be understood.

The association of a new species of *Phytophthora* with a serious pine needle blight disease in Chile raises the important question as to where it might have originated. One hypothesis regarding the origin of *P. pinifolia* would be that it is native and reproducing sexually on other hosts in Chile. In this regard, it would have undergone a 'host-jump' to *P. radiata*, a phenomenon quite common in tree pathogens (Slippers *et al*., 2005). The absence of sexual structures in cultures of *P. pinifolia* could then be explained by the absence of conditions necessary for sexual reproduction in the laboratory. An alternative hypothesis is that the oomycete is represented by a single mating type in Chile, and it is thus not able to undergo sexual recombination. This could imply that *P. pinifolia* has been introduced into Chile, as was the case with *P. infestans* that was accidentally introduced into Europe as a single mating type (Goodwin, 1997).

The origin of *P. pinifolia* on a native plant in Chile seems unlikely because there are no native pines in Chile and a host jump to *P. radiata* would imply a pathogen with a broad host range. The fact that only *P. radiata* is infected and that *P. pinaster* and *Pseudotsuga menziessii*, growing in close proximity, show no symptoms suggests that it has a limited host range and is, restricted to one or only a small number of pine species. Studies are continuing to consider the sexuality in *P. pinifolia*, to investigate its population biology and diversity, and to search for potential native hosts and origin of *P. pinifolia* in Chile.

The pine needle and shoot blight in Chile known as DFP has emerged and spread rapidly to cover an area of approximately 60 000 ha in only three years. It is currently

the most important problem affecting *P. radiata* plantations in Chile and it seriously threatens the local forestry industry and consequently the economy of the country. Opportunities exist to select other *Pinus* spp. for plantation development and screening of species suited to the area is currently underway. There is early evidence to suggest that different genotypes of *P. radiata* differ in susceptibility to infection and this offers opportunities to reduce the impact for DFP. Alternatively, there are also possibilities for chemical control of the disease, for example through the application of fungicides.

## 5.0 References

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**Table 1** Comparison of ecological and morphological characteristics of *Phytophthora pinifolia* and other *Phytophthora* species in Clade 6.

| Characteristics | *Phytophthora pinifolia* | *Phytophthora gonapodyides*[a] | *Phytophthora humicola*[a] | *Phytophthora inundata*[a] | *Phytophthora megasperma*[a] |
|---|---|---|---|---|---|
| **Habitat** | Aerial parts of *Pinus radiata* | Soil, root | Soil | Soil, root, river water or from pond debris | Soil, root |
| **Occurring on *Pinus*** | Yes | No | No | No | No |
| **Caducous** | Yes | No | No | No | No |
| **Sporangial proliferation** | None | Internal or internal nested or external proliferation | Mostly internal proliferation | Internal proliferation | Internal proliferation |
| **Sporangium** | Non–papillate | Non–papillate | Non–papillate | Non–papillate | Non–papillate |
| **Hyphal swellings** | Yes | No | Yes | No | Yes |
| **Hyphal swelling morphology** | Spherical, radiating hyphae | n/a | Spherical, radiating hyphae | n/a | Rounded or angular, in chains or clusters |
| **Sexuality** | unknown | Heterothallic | Homothallic | Partially heterothallic | Mostly homothallic |

[a] From Erwin & Ribeiro (1996), Brasier *et al*. (2003a).

**Figure 1**. Aerial views of *Pinus radiata* plantations affected by 'Daño Foliar del Pino' (DFP) on the Arauco coast of Chile. (a) Extensive damage due to needle blight. (b) Unaffected native vegetation (white arrow) alongside affected pine stands. (c) Severe infection on the eastern slopes (black arrow) of a plantation with little damage on western the slopes (white arrow).

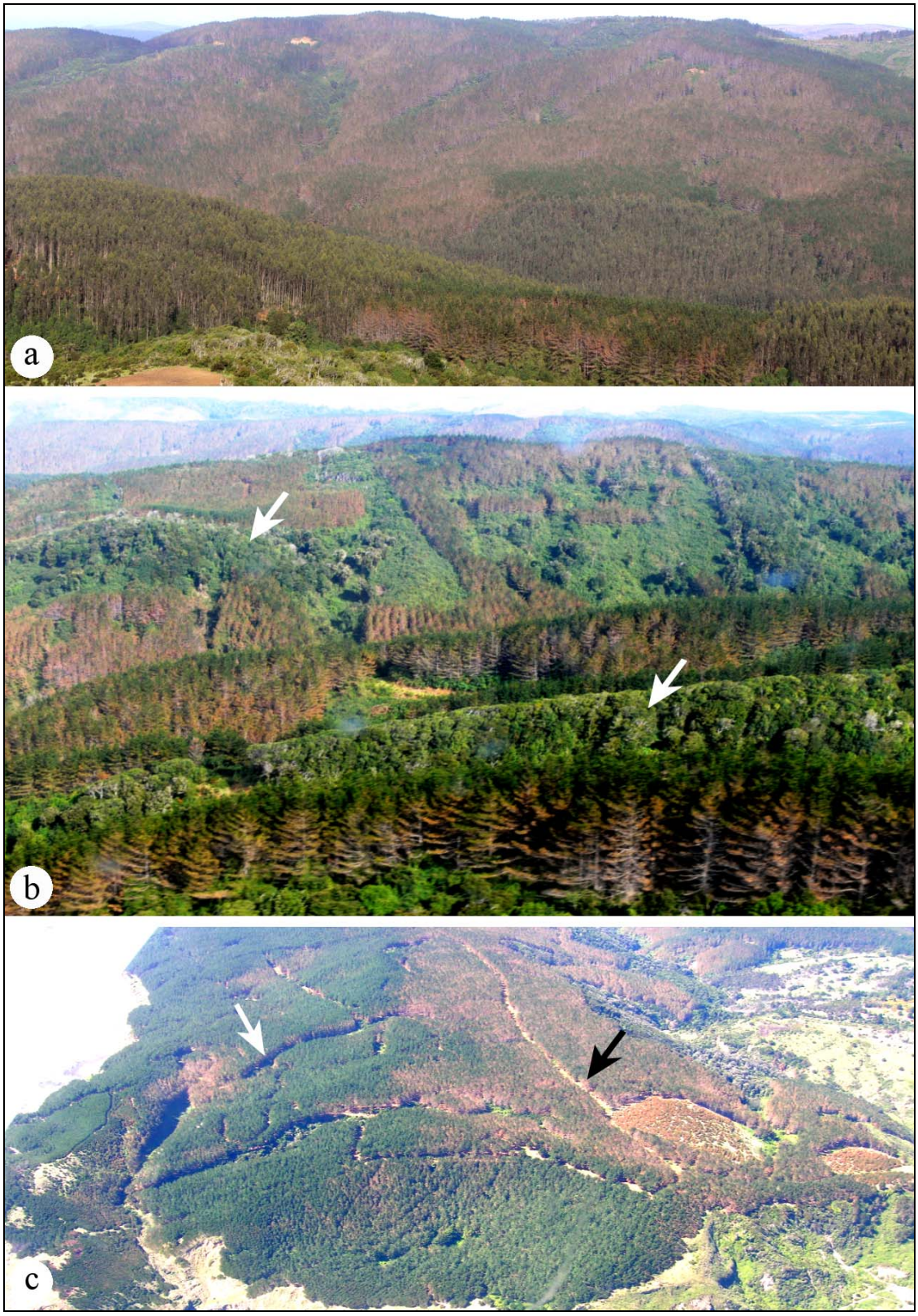


**Figure 2**. Symptoms on *Pinus radiata* associated with infection by *Phytophthora pinifolia*. (a) Dying trees with scorched appearance. (b) New growth at branch tips (arrow) after heavy infection of the previous season's needles. (c) Black band on a needle representing one of earliest symptoms of infection. (d) Dead needles on the lower and less affected needles on the upper side of a branch. (e) Wilted shoots on a young tree, resulting from cankers lower down the stem and associated with infection of needles. (f) Resin flowing from the bases of infected needles. (g) Lesions in cambium at the bases of infected needles. (h) Copious resin exudation on a stem associated with infected needles. (i) Cankers developing from the coalescence of lesions at the needle bases. (j) Naturally regenerated trees showing needle blight and tip death.

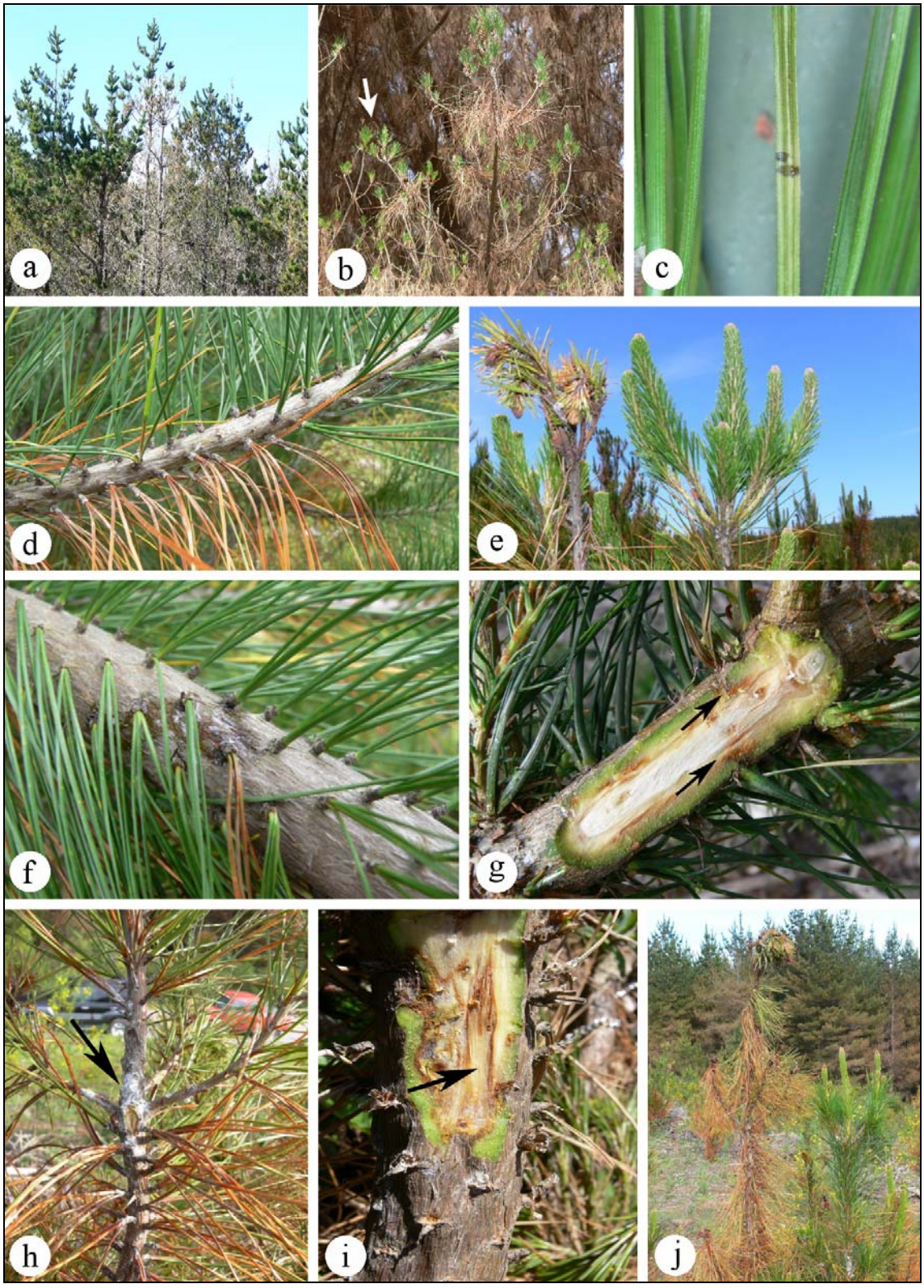


**Figure 3**. One of 10 most parsimonious trees having the same overall topology obtained through analysis of ITS rDNA sequence data, reflecting hypotheses of phylogenetic relationships for *Phytophthora pinifolia* and other *Phytophthora* species. The tree is rooted to *Pythium aphanidermatum*. Bootstrap support values (1000 replicates) are given at the branch nodes.

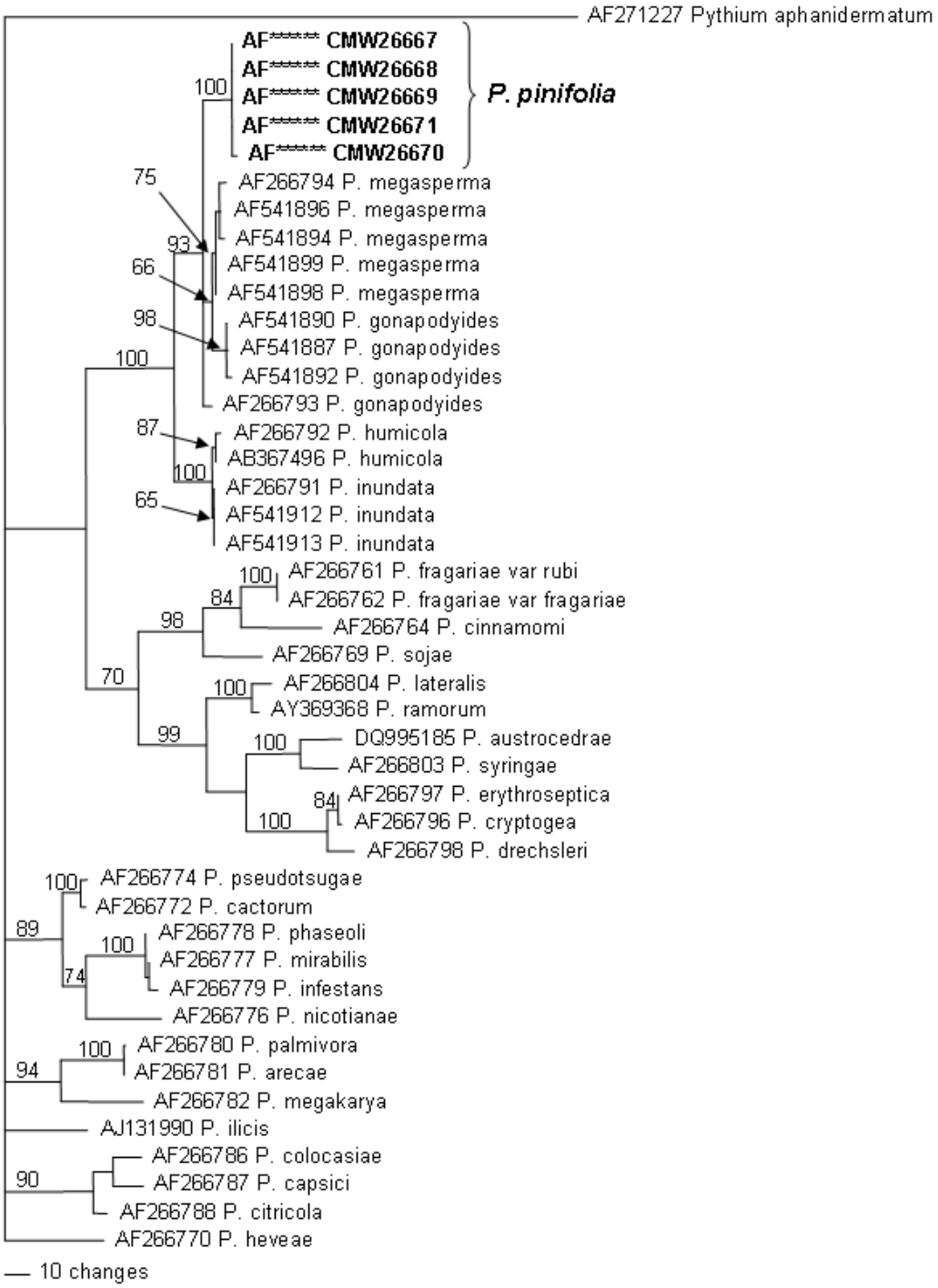


**Figure 4**. The most parsimonious tree obtained through analysis of *cox* II mtDNA sequence data. The tree is rooted to *Pythium ultimum* and shows inferred phylogenetic relationships among *Phytophthora* species and *Phytophthora pinifolia*. Bootstrap support values (1000 replicates) are given at the branch nodes.

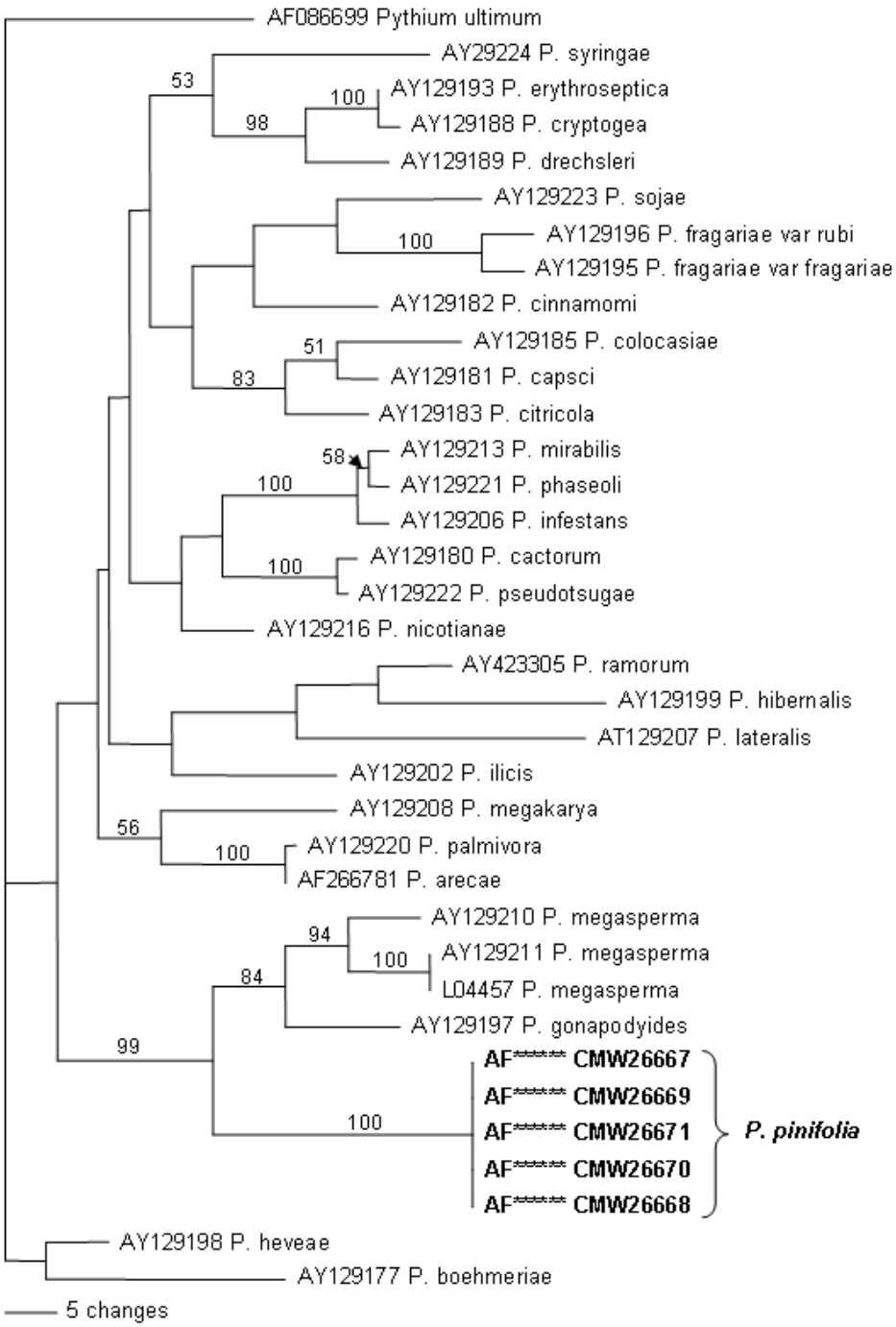


**Figure 5**. Colony morphology of *Phytophthora pinifolia* at 20°C on CMA-NARP (a–b) carrot agar (c–d) and V8 Juice agar (e–f) after six (left column) and three (right column) weeks of growth.

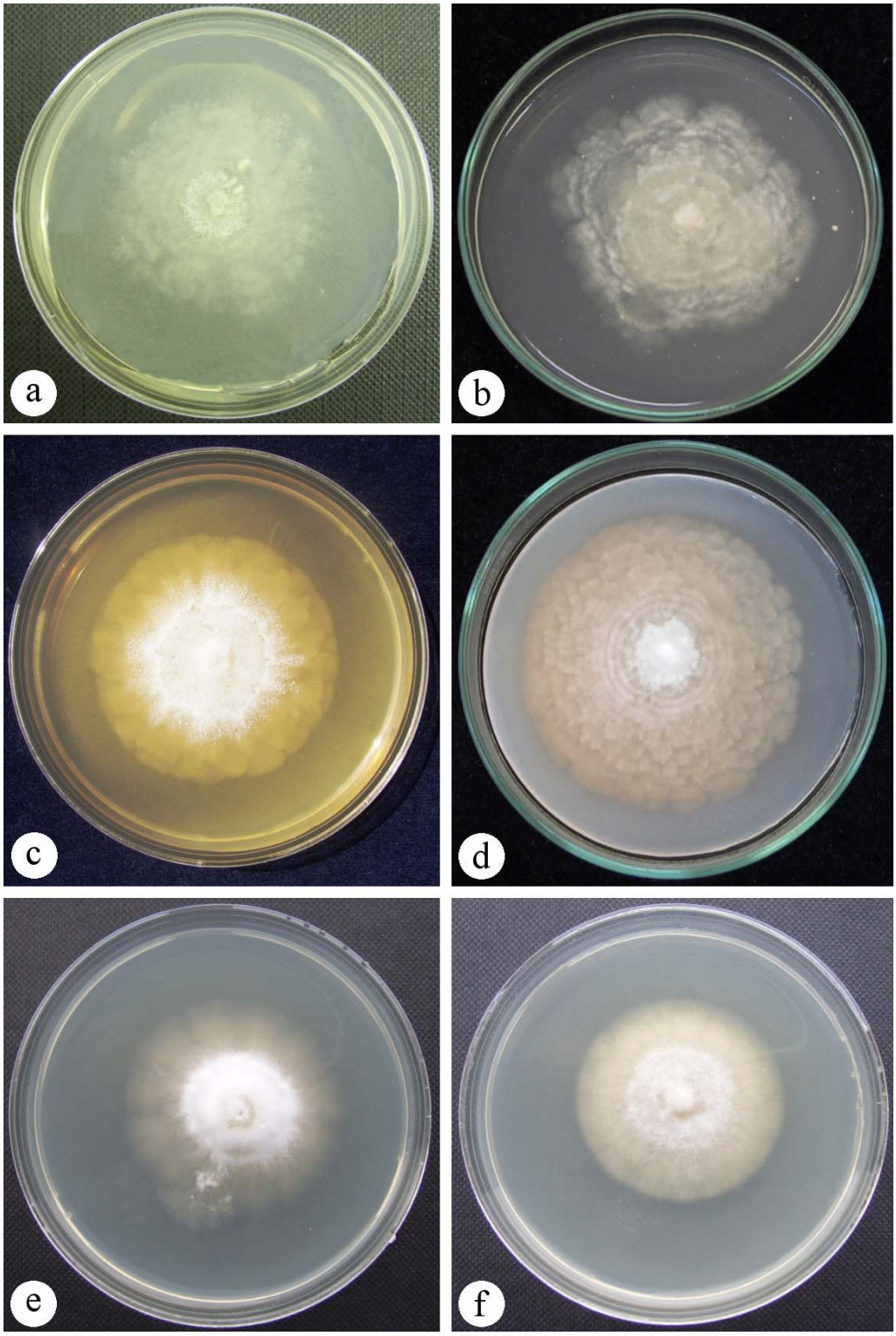


**Figure 6**. Morphology of *Phytophthora pinifolia*. (a) Coenocytic hyphae. (b) Spherical hyphal swelling with radiating hyphae. (c–h) Different shapes of non-papillate sporangia, (c–d) unbranched sporangiophores, sporangia with direct germination (e–f) and non-papillate free sporangia with pedicles (g–h). (i–j) Sporangia releasing zoospores. Scale bars = 20 µm.

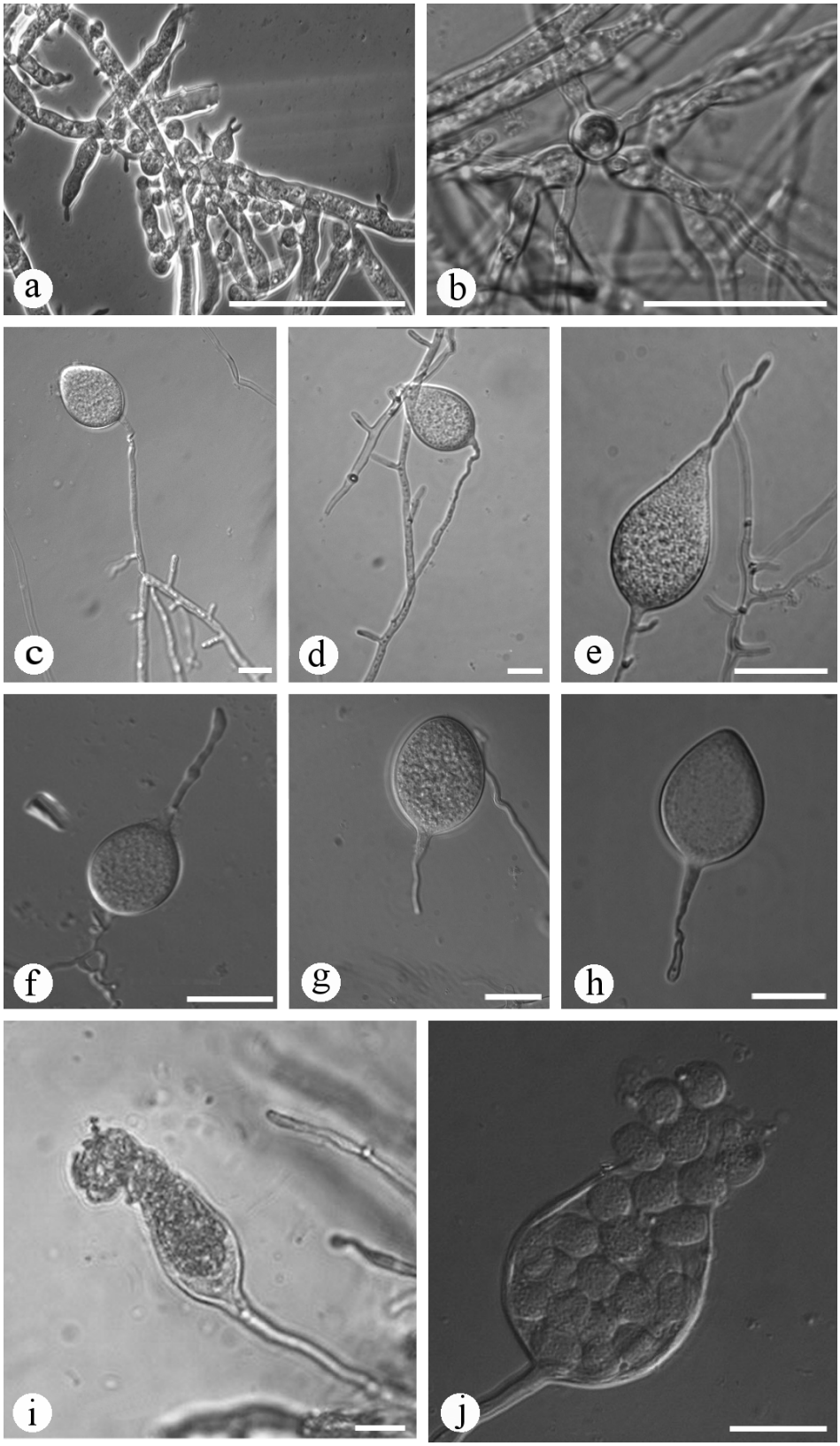


**Figure 7**. Histogram showing average lesion lengths for *Pinus radiata* plants 15 days after inoculation with *Phytophthora pinifolia* isolates (CMW 26667-26671) or a sterile agar control.

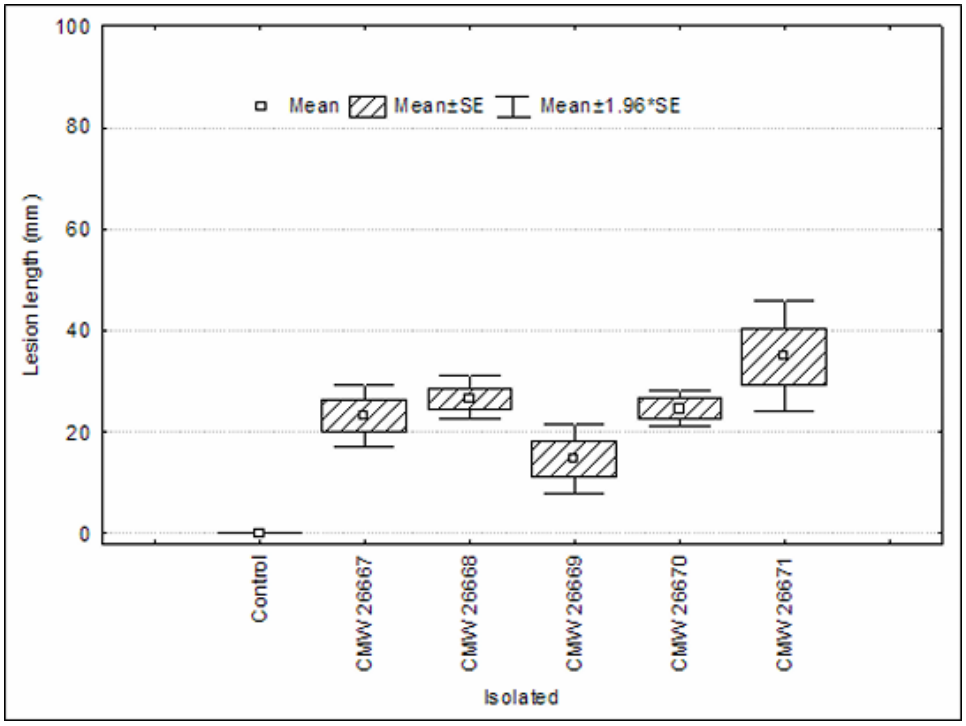
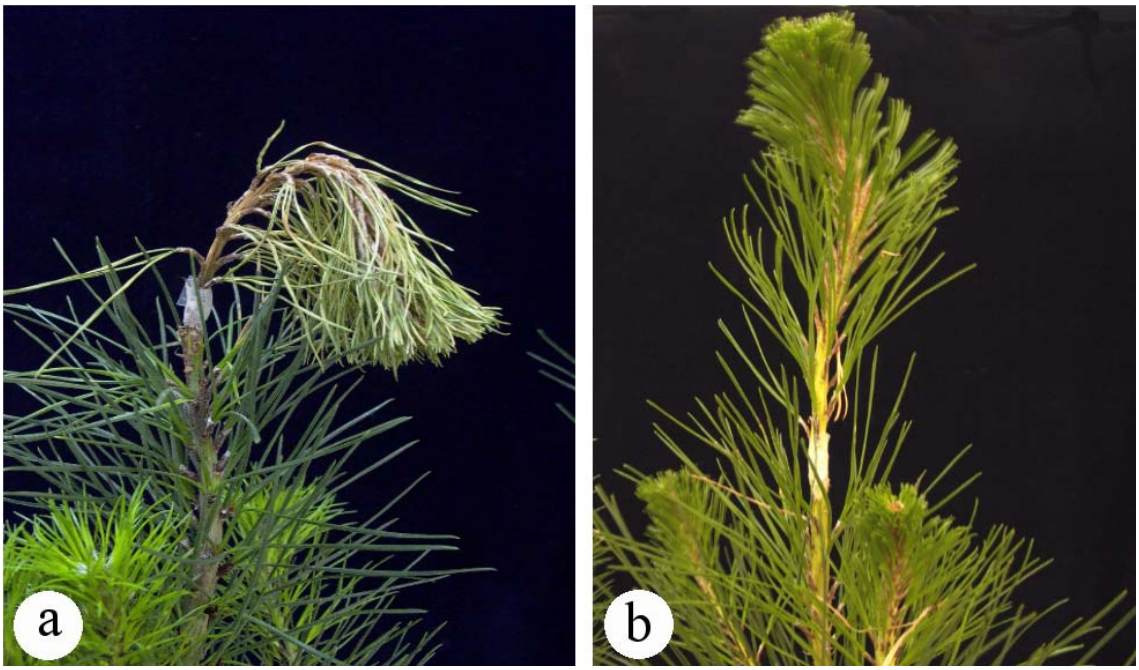


**Figure 8**. Symptoms on *Pinus radiata* plants 15 days after inoculation (a) with *Phytophthora pinifolia* or control (b).



# Chapter 3

## DNA–based method for rapid identification of the pine pathogen, *Phytophthora pinifolia*

## ABSTRACT

*Phytophthora pinifolia* causes a needle and shoot disease of *Pinus radiata*, referred to as "Daño foliar del Pino" (DFP). This newly discovered disease requires intensive research efforts that necessitate the processing of large numbers of samples for which accurate identification, oftentimes by people not experienced in *Phytophthora* taxonomy, is required. The aim of this study was, therefore, to develop species–specific primers for *P. pinifolia* that amplify the ITS region of the ribosomal operon and the nuclear *Ypt*1 gene, respectively. The primers were tested over several *Phytophthora* spp., as well as fungi isolated from *P. radiata*. In all cases, only *P. pinifolia* was amplified. In addition to the species–specific primers, a PCR–RFLP protocol using available *Phytophthora* genus–specific primers was also used to generate a species–specific profile for *P. pinifolia*. This provided a characteristic profile that allows the identification of *P. pinifolia*, and it could also discriminate between 27 different species of *Phytophthora*. Both techniques reported in this study make it possible to identify large numbers of *P. pinifolia* cultures accurately and efficiently, and will be important for both quarantine work and biological research on this important new pathogen.

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## 1.0 Introduction

*Phytophthora pinifolia* Durán, Gryzenh. & M.J.Wingf is the causal agent of a recently discovered needle and shoot disease on *Pinus radiata* (Durán *et al*., 2008). The disease is known as "Daño Foliar del Pino" (DFP) and subsequent to its first detection in 2004, extensive research was conducted to determine its cause. The disease increased rapidly between 2004 to 2006 when the largest area was affected. The affected area has decreased significantly during 2007 and 2008, and were confined to plantations close to the coast in most cases.

In adult trees, symptoms start in the lower crown and gradually spread from the central to distal part of the foliage. The infection may result in almost complete defoliation of the trees during the winter and spring season. In seedlings, damage is characterized by the rapid death of the growing terminal shoots due to girdling cankers that develop on the young stems, which can ultimately lead to death of the entire plant. A characteristic symptom in all the age groups is black bands on the needles that represent one of the earliest symptoms of infection (Durán *et al*., 2008).

*Phytophthora pinifolia* resides in Clade 6 of the most contemporary phylogeny for *Phytophthora* spp. (Cooke *et al*., 2000a; Kroon *et al*., 2004). This placement is unusual, because it is the only species of the Clade 6 without a known soil–borne phase and it also lacks nested or extended sporangium proliferation. Furthermore, it is the first *Phytophthora* sp. to be described to cause a pine needle and shoot disease.

The discovery of a new pine needle and shoot disease of *P. radiata* requires urgent and intensive research in order to understand the biology of the pathogen and to develop effective management procedures. Such studies will generate very large numbers of samples where the presence or absence of *P. pinifolia* will need to be accurately

determined. In this regard, *Phytophthora* spp. are difficult to identify with certainty and accurate diagnoses typically require experienced taxonomists (Cooke *et al*., 2000b; Duncan & Cooke, 2002; Martin *et al*., 2000). In order to facilitate the accurate identification of *P. pinifolia* in large numbers of samples, an urgent need has arisen to have robust procedures available for rapid and accurate diagnoses, including those by non–specialists.

Contemporary identification of *Phytophthora* spp. generally includes molecular methods and particularly DNA sequence comparisons (Schena & Cooke, 2006). These methods have also led to the discovery of many new *Phytophthora* spp. that would not have been easily recognised using classical morphology–based techniques (Cooke *et al*., 1999; Schubert *et al*., 1999). DNA–based methods have, furthermore, given rise to protocols for rapid identification of some of the most important *Phytophthora* spp. (Drenth *et al*., 2006; Martin & Tooley, 2004; Schena *et al*., 2006). These protocols have made it possible to identify species from large numbers of samples generated from surveys, which are focussed on epidemiology and quarantine (Cooke *et al*., 2007; Hayden *et al*., 2004; Williams *et al*., 2009).

The aim of this study was to develop species–specific primers for PCR identification of *P. pinifolia.* Furthermore, a PCR–RFLP protocol developed by Drenth *et al.* (2006), that can detect and differentiate between 27 *Phytophthora* spp., was applied to *P. pinifolia* to determine whether this test would be useful to distinguish this species in addition to other important *Phytophthora* spp. which may be isolated during field surveys.

## 2.0 Materials and Methods

### Isolates

Cultures of fifty *Phytophthora* spp. related to *P. pinifolia*, as well as various fungi commonly isolated from *P. radiata* needles, were used in this study (Table 1). The *Phytophthora* spp. were grown on carrot agar (CA) (Erwin & Ribeiro, 1996), amended with β–sitosterol (0,02 gr/L) at 25ºC for 10 days. Isolates of fungi were grown on potato dextrose agar (PDA) (20 g $L^{-1}$Biolab, Merck, Midrand, South Africa) at 20ºC for 7–10

days. All the isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

**Primer development**

*DNA extraction*

For all the samples used in this study, DNA was extracted from mycelium scraped from the surface of agar plates using PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. The concentration of isolated DNA for each culture was determined with a Nanodrop ND 1000 spectrophotometer and NanoDrop 3.2.1 software (NanoDrop Technologies Inc. Rockland, DE) and adjusted to 50 ng/µL with sterile water.

*DNA sequencing*

Twenty isolates of *P. pinifolia*, including the ex–type culture (Table 1), collected in February 2007 were used for DNA analysis.

The ITS region of the rDNA was amplified using the primers ITS1 and ITS4 (White *et al*., 1990). The ras-related protein gene *Ypt*1 of the nuclear DNA was amplified using the primers Yph1F and Yph2R (Schena *et al*., 2006). The PCR reaction mixtures and reaction conditions were the same as those described by Durán *et al.* (2008). Successful amplification was confirmed by gel electrophoresis (1 % agarose gel stained with ethidium bromide and visualized under UV light). PCR products were purified through Sephadex G–50 (Sigma–Aldrich, St. Louis, MO) in Centri–sep Spin Columns (Princeton Separations, Adelphia, NJ) following the manufacturer's instructions to remove excess primers and nucleotides.

PCR products were sequenced with the forward and reverse primers used in the amplification reactions. Reactions were performed using an ABI PRISM$^{TM}$ Big Dye terminator sequencing reaction kit following the manufacturer's instructions (Perkin–Elmer, Applied BioSystems). Sequencing was done using an ABI 3100$^{TM}$ automated DNA sequencer and the sequences obtained were verified using the software programme, MEGA 3.0 (Kumar *et al.*, 2004). Individual sequences generated in this study were deposited in GenBank (Table 2).

*Primer design*

For both the ITS region and *Ypt*1 gene, a data matrix was compiled using sequences obtained in this study for *P. pinifolia* and those retrieved from GenBank and published for other *Phytophthora* spp. (Table 2). For the ITS region the sequences generated in this study were added to the sequence data from Durán *et al*. (2008) and for the *Ypt*1 gene, to the sequence data from Schena *et al*. (2006) .

Sequences were aligned using the software MEGA 3.0 (Kumar *et al*., 2004) and the alignment of all sequences was also checked visually. The *P. pinifolia*–specific PCR primers were developed using the software Primer3–web v. 0.3.0 (Rozen & Skaletsky, 2000). The primer sequences were checked for possible sequence homology with other DNA sequences using a BLAST search in GenBank (NCBI, Bethesda, MD).

*Primer Testing*

The primers were tested using PCR reactions with *P. pinifolia* DNA extracted from the same twenty isolates from which the rDNA sequences were used for primer design, as well as for 30 additional *P. pinifolia* isolates that had been identified separately using rDNA sequence data. Species that are phylogenetically closest related to *P. pinifolia*, namely *P. humicola, P. gonapodyides, P. megasperma* and *P. inundata*, and other *Phytophthora* spp. available in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (Table 1), were also tested. Fungal species isolated from *P. radiata* needles as well as other fungal species isolated from conifers and present in CMW were also included (Table 1).

To assess the sensitivity of the PCR reaction to detect *P. pinifolia*, DNA extracted from pure cultures was serially diluted with sterile water to yield final concentrations ranging from 10 ng/µL to 1 fg/µL of DNA and amplified with both sets of primers. Sterile water replaced template DNA to provide a negative control. The PCR product was visualised using gel electrophoresis.

**PCR–RFLP**

Twenty isolates of *P. pinifolia* (Table 1) were analysed using the RFLP identification protocol described by Drenth *et al*. (2006). In addition, 15 *Phytophthora* spp. including the species that are phylogenetically most closely related to *P. pinifolia* (Table 1) were

tested using this procedure. The PCR reactions and the digestions with the enzymes *Msp*I, *Rsa*I and *Taq*I were performed in an iCycler thermocycler (Bio–Rad). The digested DNA was size fractionated using 3 % agarose gels containing ethidium bromide at 120V for 90 minutes and visualized under UV light.

## 3.0 Results

### Primer development

Amplification of the ITS regions of the *P. pinifolia* isolates generated fragments of approximately 811 bp and amplification of the *Ypt*1 gene yielded fragments of 568 bp. The sequences for both the ITS region and *Ypt*1 were identical in all the isolates tested. Based on the sequences obtained, the following specie–specific primers for *P. pinifolia* were identified: Pfoli1F (5′–GCTCTATCGCGAGCGTTT–3′) and Pfoli1R (5′–CGCAAATGACTGAAAAAGCA–3′) for the ITS region, and Yfoli1F (5′–CAGGCTGGACTCTGCTCTTC–3′) and Yfoli1R (5′–CCCACTACACAAGAGAGTTAGTTTT–3′) for the *Ypt*1 gene. BLAST searches with these primer sequences against the GenBank database showed that no organisms had sequence motifs identical to those of the primers designed for *P. pinifolia.*

PCR amplification from DNA of *P. pinifolia* isolates was successful using both sets of primers (Pfoli1F─Pfoli1R) and (Yfoli1F─Yfoli1R). Primer set Pfoli1F─Pfoli1R produced a fragment of 557 bp, and primer set Yfoli1F─Yfoli1R produced a fragment of 216 bp. Sequencing of these fragments confirmed that the region amplified was that of the targeted locus. No amplification was observed in the negative control, with DNA of other *Phytophthora* spp. (Fig. 1) or the fungi tested (data not shown). The sensitivity test showed that the Pfoli1F and Pfoli1R primer set produced a visually identifiable amplicon from 2.5 pg/µL of DNA, while the Yfoli1F and Yfoli1R primer set produced a visually identifiable amplicon from 100 pg/µL of DNA.

### PCR–RFLP

Amplification of *P. pinifolia* with the primers A2F and I2R, produced a fragment of 813 bp, with no fragment visible in the negative control. In the RFLP profile (Fig. 2), fragments for the *P. pinifolia* isolates digested with the enzymes *Msp*I, *Rsa*I and *Taq*I were produce the same RFLP profile such is distinct from those of the other

*Phytophthora* spp. described by Drenth *et al.* (2006) and those that are phylogenetically most closely related to *P. pinifolia* (Table 3).

**4.0 Discussion**

In this study, two sets of primers were developed that will provide an accurate and rapid identification of *P. pinifolia* from cultures. Both the ITS rDNA region and *Ypt*1 gene were targeted to produce these species–specific primers, while the combined assays increased the reliability of the identification. Both sets of primers were able to discriminate *P. pinifolia* from the *Phytophthora* spp. known to be phylogenetically most closely related to it and residing in phylogenetic Clade 6 of Brasier *et al*. (2003). They also distinguished *P. pinifolia* from a number of other *Phytophthora* spp. and fungus isolated from forest environments.

The primer set Pfoli1F and Pfoli1R, designed from the ITS region, was able to detect the *P. pinifolia* DNA from very small samples (2.5 pg/µL). This level of detection sensitivity compares favourably with that previously reported for *P. citricola* (Schubert *et al.*, 1999) and *P. cinammomi* (Drenth *et al.*, 2006). The amount of DNA corresponded to the DNA content of approximately two nuclei based on the approximate size of the *Phytophthora* genome (Drenth *et al.*, 2006).

The choice of the ITS region to develop species–specific PCR primers for *P. pinifolia* was based on the fact that this region has previously been used to develop specific detection methods for *P. quercina*, *P. citricola*, *P. cambivora*, (Schubert *et al.*, 1999), *P. ramorum* (Hayden *et al.*, 2004), *P. nicotinae* (Grote *et al*., 2002) and *P. megasperma* (Nigro *et al*., 2005). A short–coming of the ITS locus is that in some circumstances, it fails to discriminate among closely related taxa (Bowman *et al*., 2007; Schena & Cooke, 2006) although there was no evidence of this problem in the case of *P. pinifolia*.

Using the primer set Yfoli1F and Yfoli1R designed for the ras–related protein gene *Ypt*1, DNA could be amplified from *P. pinifolia* samples as small as 100 pg/µL. This level of sensitivity is forty times lower than that needed for detection using the Pfoli1F and Pfoli1R primers based on the ITS region. This lower level of sensitivity may either be due to the fact that the *Ypt*1 gene is present only as a single copy in the genome

(Chen & Roxby, 1996), compared with the multiple copies of the rDNA locus and/or the design of the oligo nucleotide primers for the assay. The detection sensitivity for the primers Yfoli1F and Yfoli1R was lower than that described for *P. inundata* and *P. megasperma* using the species–specific primers developed for these species (Schena *et al*., 2008), but similar to the detection limit for species–specific primers designed by Schena *et al*. (2008) for *P. cambivora, P. cinnamomi, P. lateralis, P. nemorosa* and *P. psychrosphila*.

The PCR–RFLP profile for *P. pinifolia*, obtained using primers A2F and I2R developed by Drenth *et al*. (2006), was unique compared to those described for the *Phytophthora* spp. used in their development. These primers can thus be used together with those developed in this study to provide an added base for identification of *P. pinifolia* isolates. The fact that we were able to produce profiles for *P. gonapodyides, P. megasperma*, *P. drechsleri*, *P. palmivora* and *P. cinnamomi* identical to those produced by Drenth *et al*. (2006) also emphasises the reliability of this method.

The diagnostic procedures described in this study, allow for the screening and reliable identification of large numbers of *P. pinifolia* of isolates in a short period of time. The level of specificity observed with our assays for *P. pinifolia* makes additional sequencing steps unnecessary for reliable species identifications. These techniques should be valuable in augmenting research on the biology and epidemiology of this important new pathogen for which very little knowledge is currently available as well for quarantine procedures.

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**Table 1.** Cultures of *Phytophthora* spp. and various fungal species associated with pines that were used to test the specificity of the species-specific primer sets Pfoli1F and Pfoli1R, and Yfoli1F and Yfoli1R

| Species | Isolate numbers |
|---|---|
| *P. alticola* | CMW 19423 |
| *P. arecae* | CMW 19436, CMW 19437, CMW 19438 |
| *P. boehmeriae* | CMW 19440, CMW 19441 |
| *P. cactorum* | CMW 21922 |
| *P. cinnamomi* | CMW 29596/UQ 3648, CMW 29606/UQ 789, CMW 29608/UQ 828, CMW 29607/UQ 795 |
| *P. colocasiae* | CMW 20201 |
| *P. cryptogea* | CMW 19410 |
| *P. drechsleri* | CMW 28870/CBS 292.35, CMW 28869/CBS 359.52, CMW 29592/P1899, CMW 29594/P3808 |
| *P. frigida* | CMW 19428 |
| *P. gonapodyides* | CMW 28872/CBS 117379, CMW 28871/CBS 113346 |
| *P. humicola* | CMW 28867/CBS 114082, CMW 28866/CBS 200.81 |
| *P. inundata* | CMW 28868/CBS 217.85, CMW 29595/P8478 |
| *P. megasperma* | CMW 28865/CBS 686.79, CMW 28864/CBS 687.79, CMW 29593/P3724 |
| *P. nicotinae* | CMW 6991 |
| *P. pinifolia* | CMW 26667/CBS 122923, CMW 26668/CBS 122924, CMW 26669/CBS 122922, CMW 26670, CMW 26671, CMW 30228, CMW 30229, CMW 30230, CMW 30231, CMW 30232, CMW 30233, CMW 30234, CMW 30235, CMW 30236, CMW 30237, CMW 30238, CMW 30239, CMW 30240, CMW 30241, CMW 30242 |
| *P. palmivora* | CMW 29601/UQ 3694, CMW 29602/UQ 3716, CMW 29603/UQ 3717, CMW 29604/UQ 3718 |
| *Phoma herbarum* | CMW 24391 |
| *Trichoderma koningii* | CMW 24389 |
| *Podospora* sp. | CMW 24385 |
| *Allantophomopsis cytisporea* | CMW 25545/CBS 262.85, CMW 25546/CBS 109.22 |
| *Allantophomopsis lycopodina* | CMW 25547/CBS 361.68 |
| *Phacidium coniferarum* | CMW 25548/CBS 841.91, CMW 25549/CBS 437.71, CMW 25550/CBS 562.63, CMW 25551/CBS 322.53, CMW 25552/CBS 321.53 |
| *Phacidium fennicum* | CMW 25556/CBS 457.83 |
| *Phacidium infestans* | CMW 25557/CBS 265.31, CMW 25558/CBS 264.31, CMW 25559/CBS 263.31, CMW 26234/CBS 396.48 |
| *Phacidium lacerum* | CMW 25560/CBS 400.81, CMW 25561/CBS 761.73, CMW 25562/CBS 557.70, CMW 25563/CBS 540.70, CMW 25564/CBS 371.62 |
| *Phacidium vaccinii* | CMW 25567/CBS 444.71 |
| *Strasseria geniculata* | CMW 25568/CBS 442.82, CMW 25569/CBS 441.82 |
| *Sphaeropsis sapinea* | CMW 14971, CMW 14972, CMW 14973, CMW 14974, CMW 14975 |
| *Botryosphaeria* sp. | CMW 10530, CMW 10531, CMW 10532, CMW 10533, CMW 10534 |
| *Dothistroma septosporum* | CMW 8611, CMW 9304, CMW 10247 |

- Culture collection: CMW (FABI, South Africa); CBS (Centraalbureau voor Schimmelcultures, The Netherlands); P (World *Phytophthora* culture collection, USA); UQ (*Phytophthora* culture collection, University of Queensland).

**Table 2**. GenBank accession numbers of *Phyphthora* spp. sequences used to design specific primers

| Species | *Ypt*1 | ITS |
|---|---|---|
| *P. alni* subsp. *alni* | DQ270297[d] | |
| *P. alni* subsp. *multiformis* | DQ270307[d] | |
| *P. alni* subsp. *uniformis* | DQ270301[d] | |
| *P. alni* subsp. *uniformis* | DQ270300[d] | |
| *P. arecae*[a] | | AF266781[a] |
| *P. austrocedrae* | | DQ995185[e] |
| *P. boehmeriae* | DQ270324[d] | |
| *P. cactorum* | DQ162960[d], DQ270309[d] | AF266772[a] |
| *P. cambivora* | DQ162954[d], DQ162955[d] | |
| *P. capsici* | DQ162972[d] | AF266787[a] |
| *P. cinnamomi* | DQ162959[d] | AF266764[a] |
| *P. citricola* | DQ162968[d], DQ162969[d] | AF266788[a] |
| *P. citrophthora* | DQ162973[d] | |
| *P. colocasiae* | | AF266786[a] |
| *P. cryptogea* | DQ162987[d] | AF266796[a] |
| *P. drechsleri* | DQ162989[d] | AF266798[b] |
| *P. erythroseptica* | | AF266797[b] |
| *P. europaea* | DQ162952[d] | |
| *P. fragariae* var. *fragariae* | DQ162950[d], DQ270306[d] | AF266762[a] |
| *P. fragariae* var. *rubi* | DQ270305[d], DQ162951[d] | AF266761[a] |
| *P. gonapodyides* | | AF541892[b], AF541888[b], AF266793[a], AF541891[b], AF541890[b], AF541889[b] |
| *P. heveae* | | AF266770[a] |
| *P. idaei* | DQ270312[d], DQ270313[d] | |
| *P. ilicis* | DQ162962[d], DQ162963[d] | AJ131990[a] |

| Species | *Ypt*1 | ITS |
|---|---|---|
| *P. infestans* | DQ162961[d] | AF266779[a] |
| *P. insolita* | DQ162974[d] | |
| *P. inundata* | DQ162982[d], DQ162983[d] | |
| *P. katsurae* | DQ162980[d] | |
| *P. kernoviae* | DQ162975[d], DQ270322[d] | AY940661[c] |
| *P. lateralis* | DQ162991[d] | AF266804[a] |
| *P. medicaginis* | DQ162990[d] | |
| *P. megakarya* | | AF266782[a] |
| *P. megasperma* | DQ162986[d] | AF266794[a], AF541893[b], AF541894[b], AF541896[b], AF541895[b] |
| *P. mirabilis* | | AF266777[a] |
| *P. nemorosa* | DQ162965[d] | |
| *P. nicotianae* | DQ162981[d] | AF266776[a] |
| *P. palmivora* | | AF266780[a] |
| *P. phaseoli* | | AF266778[a] |
| *P. pinifolia* | **FJ754188, FJ754189, FJ754190, FJ754191, FJ754192** | EU725805[f], EU725806[f], EU725807[f], EU725808[f], EU725809[f] |
| *P. pistaciae* | DQ162957[d] | |
| *P. pseudosyringae* | DQ162966[d], DQ162967[d] | AF266774[a] |
| *P. psychrophila* | DQ162964[d] | |
| *P. quercina* | DQ162976[d], DQ162977[d] | |
| *P. ramorum* | DQ162992[d], DQ270319[d] | AY369368[a] |
| *P. sojae* | DQ162958[d] | AF266769[a] |
| *P. syringae* | | AF266803[a] |

Sequence reference: [a]Cooke *et al*., 2000a; [b]Brasier *et al*., 2003; [c]Brasier *et al*., 2005; [d]Schena *et al*., 2006; [e]Greslebin *et al*., 2007; [f]Durán *et al*., 2008.

**Table 3.** Amplicon sizes after amplification with genus specific primer A2F and I2R and fragment sizes (bp) of different *Phytophthora* species after restriction digests with three different restriction enzymes

| *Phytophthora* species | Isolate number | PCR amplicon | *Msp*I band sizes | *Rsa*I band sizes | *Taq*I band sizes |
|---|---|---|---|---|---|
| *P. pinifolia* | CMW 26668/CBS 122924 | 811 | 331, 244, 125, 111 | 369, 154, 171, 107, 10 | 150, 143, 127, 118, 89, 64, 59, 53, 8 |
| *P. gonapodyides* | CMW 28871/CBS 113346 | 819 | 334, 147, 125, 115, 98 | 372, 172, 158, 107, 10 | 150, 143, 143, 130, 118, 68, 59, 8 |
| *P. humicola* | CMW 28866/CBS 200.81 | 817 | 334, 260, 125, 98 | 372, 156, 151, 107, 21, 10 | 157, 143, 130, 118, 90, 67, 59, 53 |
| *P. inundata* | CMW 29595/P 8478 | 817 | 334, 260, 125, 98 | 372, 172, 156, 107, 10 | 157, 143, 130, 118, 90, 67, 59, 53 |
| *P. megasperma* | CMW 28864/CBS 687.79 | 814 | 334, 143, 125, 114, 98 | 372, 325, 107, 10 | 149, 143, 140, 130, 118, 67, 59, 8 |

**Figure 1**. Agarose gel stained with ethidium bromide and visualised under UV light, showing the PCR product of *Phytophthora pinifolia* (CMW 26667-CMW 26670) and several other *Phytophthora* spp. with the species specific primer sets Pfoli1F and Pfoli1R (A) and Yfoli1F and Yfoli1R (B).

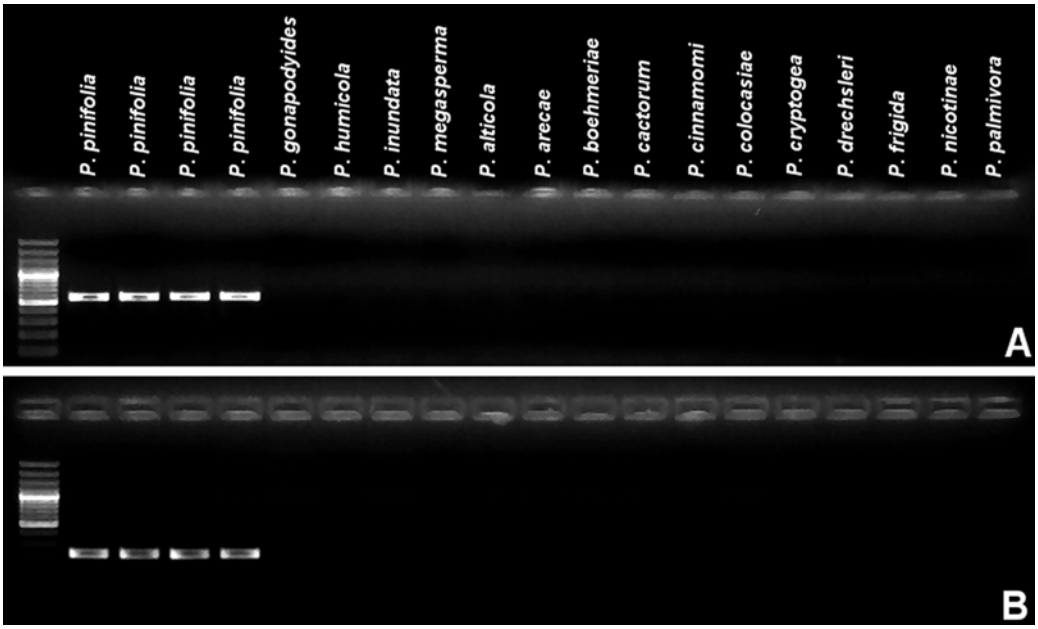
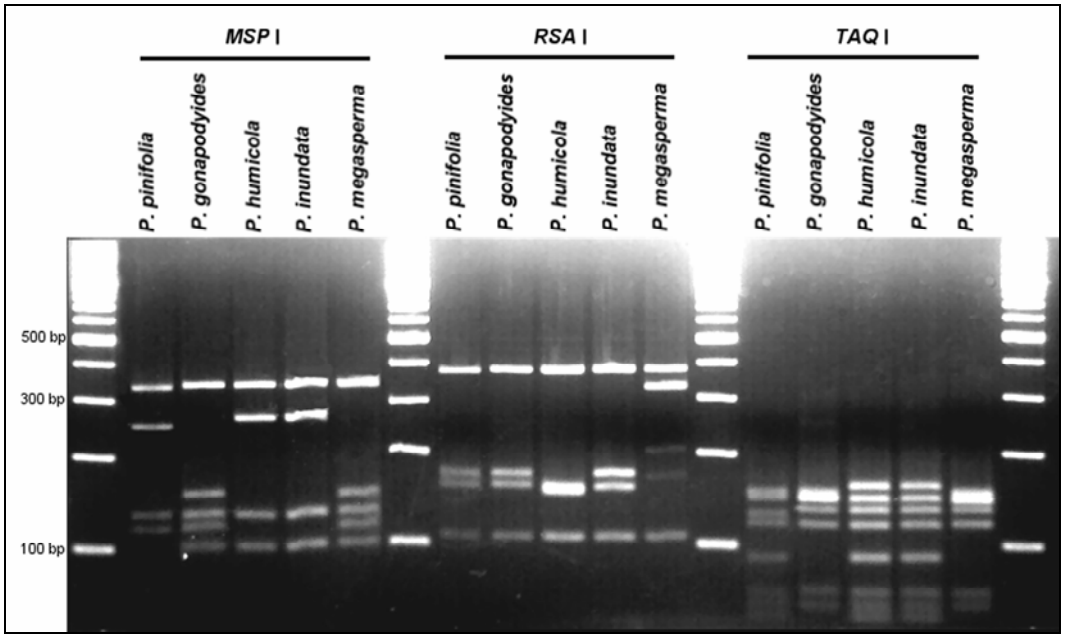


**Figure 2**. Agarose gel showing ITS amplicons of *Phytophthora* spp. belonging to Clade 6 amplified using *Phytophthora* genus–specific primer A2F and I2R (Drenth *et al*., 2006) and digested with restriction enzymes *Msp*I, *Rsa*I and *Taq*I, after PCR. Lane 1, 7, 13 and 19: 100 bp ladder.



# Chapter 4

## AFLP analysis reveals a clonal population of *Phytophthora pinifolia* in Chile

## ABSTRACT

*Phytophthora pinifolia* is the causal agent of the recently discovered needle disease of *Pinus radiata* in Chile, referred to as "Daño Foliar del Pino" (DFP). The genetic structure of the pathogen population is unknown which hinders our understanding of its sudden appearance and rapid spread in Chile since 2004. In this study, a population of eighty eight cultures of *P. pinifolia* isolated from *P. radiata* at several localities in Chile was evaluated for genotypic diversity using amplified fragment length polymorphisms (AFLP). Results of the AFLP analyses showed that the *P. pinifolia* population in Chile consists of a single genotype with no genetic differentiation based on geography, year of isolation or the part of the tree from which the isolates were obtained. Mating experiments did not lead to the production of gametangia suggesting that the organism is most likely sterile. The fact that a single clonal genotype dominates the population of *P. pinifolia* in Chile supports the hypothesis that *P. pinifolia* was recently introduced into this country and that its impact is due to a new and susceptible host encounter.

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## 1.0 Introduction

*Phytophthora pinifolia* causes a needle disease of *Pinus radiata* in plantations in Chile. The disease, locally known as Daño Foliar del Pino (DFP) affects *P. radiata* (Monterrey pine) of all ages. In young and adult trees, infection occurs in current year needles towards the end of the growing season, resulting in severe needle loss. In seedlings the disease is characterized by the rapid death of young terminal shoots and death of the entire plant (Durán *et al.*, 2008).

DFP was first observed in Raqui, on the Arauco coast of Chile in 2004 and the pathogen rapidly spread from the initial outbreak, confined to an area of 70 ha, to 60,000 ha in 2006 (Durán *et al.,* 2009). Between 2007 and 2008 the affected area has reduced, and is now confined to road borders and specific zones in the plantation, which are in most of the cases closest to the coast (Durán *et al.,* 2009). The *Phytophthora* sp. causing DFP was new to science at the time of the initial outbreak of the disease, but is now known to reside in Clade 6 in the phylogeny of *Phytophthora* spp. presented by Cooke *et al*. (2000). The behavior of *P. pinifolia* is unusual because it is the only species residing in Clade 6 without a known soil-borne phase and it also lacks nested or extended sporangium proliferation. Furthermore, it is the only *Phytophthora* sp. known to cause a foliar disease of *Pinus* spp. in plantations (Durán *et al.,* 2008; 2009).

Observations in the field suggest that *P. pinifolia* is specific to *P. radiata.* Other conifer species planted in the affected areas remain asymptomatic. For example, *P. pinaster* and *Pseudotsuga menziessii*, do not show any signs of disease in Chile in areas where *P. radiata* is heavily infected by *P. pinifolia* (Durán *et al*., 2008).

At present, nothing is known regarding the population genetic structure of the *P. pinifolia* population in Chile. Management strategies such as plant breeding and selection programs, urgently needed to reduce the impact of DFP rely on information concerning the genetic make up of the pathogen population. Studies considering the genetic structure of populations of other *Phytophthora* spp. have provided evidence in support of an introduction hypothesis for species such as *P. cinnamom*i in Australia and South Africa (Linde *et al*. 1997, 1999) and *P. ramorum* in USA and UK (Ivors *et al.* 2006; Prospero *et al*. 2007). Knowledge of the genetic diversity of *P. pinifolia* might also provide clues to the possible origin of the pathogen.

The production of gametangia in culture represents the standard technique to determine the sexual status of *Phytophthora* spp. (Erwin & Ribeiro 1996). The production of oospores in single cultures is representative of a homothallic species, while the requirement of a culture of the opposite mating type (A1 or A2) is indicative of heterothallic species (Erwin & Ribeiro 1996; Judelson & Blanco 2005). Where no gametangia are produced under a wide range of conditions, *Phytophthora* spp. are considered sterile or silent (Brasier *et al*., 1993; 1999). Previous experiments on *P. pinifolia* failed to produce oospores despite repeated attempts to do so (Durán *et al.*, 2008). Hence, additional studies are required to more reliably determine the nature of the sexuality in *P. pinifolia*.

The fact that *P. radiata* is not native to Chile, and that plantations in this country have been free of this diseases for more than a century suggests that the *P. pinifolia* pathogen population may have recently been introduced into the country. An introduced pathogen such as *P. pinifolia* in Chile, would be expected to show a low level of genetic diversity due to the founder effect which makes the population go through a significant bottleneck (Parker & Gilbert 2004). A good example of such a situation is *P. infestans*, where a single A1 mating type isolate escaped from its ancestral home in central Mexico in the 1840's giving rise to the global distribution of only a single clone (Fry *et al*., 1993). Studies on populations from the centre of origin of *P. infestans* revealed very high levels of gene and genotypic diversity (Grünwald *et al*., 2005). A second global migration of this pathogen took place in the 1980's, including both mating types which gave rise to a sexually reproducing, and thus far more variable pathogen populations (Drenth *et al*., 1994; Fry *et al*. 1992; 1993). The presence of high levels of gene and

genotypic diversity in populations of *P. pinifolia* would also be indicative of sexual reproduction involving both mating types, and/or a very high level of gene flow due to continuous introduction of the pathogen over time (McDonald & Linde 2002).

In order to gain insight into the structure, reproductive biology and origin of the *P. pinifolia* population in *P. radiata* plantations in Chile our first aim was to determine the genetic diversity of the population using AFLP analysis. The resulting data would allow us to test the hypothesis if the pathogen population was introduced. Our second aim was to test a wider range of experimental conditions than those used in Durán *et al.* (2008) under which gametangial production may occur, to more rigorously determine the possibility and likelihood of sexual reproduction in this population. Determining the population genetic structure of the pathogen population will assist in the development of disease management strategies and the identification and deployment of potential resistance to this important plant pathogen.

## 2.0 Materials and Methods

### Isolates

Eighty eight isolates of *P. pinifolia* were obtained from several locations in Chile (Fig. 1) that represent a broad geographic range of the occurrence of DFP, with more than 600 km between the two most distant locations. Isolations were made from symptomatic *P. radiata* needles (Table 1) as described by Durán *et al*., (2008). Hyphal tip cultures were made from each isolate on V8 agar (Erwin & Ribeiro, 1996). All the isolates are maintained in $H_20$ and 10% glycerol at room temperature in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

### DNA extraction

Three 5 mm agar blocks were transferred from the edges of active growing cultures to 65 mm plates with 20 ml of V8 broth and these were incubated for two weeks at 25 ºC. The mycelium was harvested from these cultures, excluding the agar blocks, under sterile conditions, air dried for 5 minutes in the plate lid and transferred to 2 ml Eppendorf tubes. The harvested mycelium was washed by filling the tubes with 1.5 ml double autoclaved distilled $H_20$ and vortexed for 30 sec. The tubes were then

centrifuged for 5 min at 5000 rpm and the water removed with a pipette, taking care to eliminate the solid pieces generated from the V8 broth at the bases of the tubes. This washing step was repeated twice. Cleaned mycelium was freeze dried (24 h) and ground to a fine powder using the Retsch GmbH MM301 mixer mill (Haan, Germany) for 5 min (1/30 mhz).

DNA was extracted using the protocol described by Goodwin *et al.* (1992) and resuspended in 50 µL of nuclease free water. Successful DNA extraction was confirmed by gel electrophoresis (1% agarose gel stained with ethidium bromide and visualized under UV light). The DNA concentration was determined using a Nanodrop ND 1000 spectrophotometer and NanoDrop 3.2.1 software (NanoDrop Technologies Inc. Rockland, DE) and adjusted to 30 ng/µL with nuclease-free water. To confirm the suitability of the DNA to perform PCR and to confirm the identity of each isolate, the extracted DNA was used as template in amplification with the species specific primers Pfoli1F and Pfoli1R, developed by Durán *et al.,* (2009), and following the same protocols described therein.

**Amplified Fragment Length Polymorphism (AFLP) analysis**

Eighty eight isolates were selected for the analysis, with eight isolates duplicated as internal controls to ensure reproducibility of the banding patterns (Table 1). AFLP profiles were generated from the extracted DNA using the method described by Vos *et al*. (1995) with minor modifications. These included the initial DNA concentration, the restriction and ligation incubation times. From the diluted genomic DNA, 5 µL (150 ng) was digested with 2 units of the restriction endonuclease *Eco*RI (Fermentas, Vilnius, Lithuania) and *Mse*I (New England Biolabs, Beverly, Massachusetts) for 3 h at 37 °C. The digested fragments were ligated to the adapters: *Eco*RI: (5' CTC GTA GAC TGC GTA CC/CAT CTG ACG CAT GGT TAA 5') and *Mse*I: (5' GAC GAT GAG TCC TGA G/TAC TCA GGA CTC AT 5') (Vos *et al*., 1995) using T4 DNA ligase (Fermentas, Vilnius, Lithuania) for 3 h at 22 °C.

The DNA fragments were subjected to an initial pre-amplification step with the reaction mixture including 0.3 µM *Eco*RI + A (5' GAC TGC GTA CCA ATT CA 3') primer, 0.3 µM *Mse*I + C (5' GAT GAG TCC TGA GTA AC 3') primer (Vos *et al*. 1995), 0.2 mM dNTPs, PCR buffer containing 1.5 mM $MgCl_2$, 0.6 U *Taq* Polymerase (Roche

Diagnostics, Mannheim, Germany) and 5 µL of the restriction-ligation mix. The pre-amplification PCR was carried out using the following conditions: 30 sec at 72 ºC followed by 25 cycles of 30 sec at 94 ºC; 30 sec at 56 ºC; one min, plus one second per cycle, at 72ºC, and a final cycle of two min at 72ºC. Successful restriction, ligation and amplification were confirmed by gel electrophoresis (1% agarose gel stained with ethidium bromide and visualized under UV light).

The product of the pre-amplification step was diluted 1/10 with nuclease free water, and used as template for the subsequent final amplification step. The reaction mixture included 5 µL of the diluted pre-amplification product, PCR buffer, 0.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.04 µM Infrared dye (IRD) labeled (Li-COR, Lincoln, NE, USA) *Eco*RI + 2 primer (E), 0.25 µM *Mse*I + 2 primer (M) and 0.6 U *Taq* Polymerase (Roche Diagnostics). Reaction conditions for the final amplification step were 13 cycles for 10 sec at 94ºC; 30 sec at 65ºC with temperature decreasing by 0.7 ºC per cycle during subsequent cycles; one min at 72 ºC; followed by 23 cycles of 10 sec at 94 ºC; 30 sec at 56 ºC; one min, plus one second per cycle, at 72 ºC and a final cycle of one min at 72 ºC. The enzyme restriction, ligation, pre-amplification and final amplification was carried out in 200 µl Eppendorf tubes in an Eppendorf Mastercycler ep (Eppendorf, Hamburg, Germany).

AFLP fragment analysis was performed on a model 4200 LI-COR automated DNA sequencer with the electrophoresis parameters similar to those described by Wingfield *et al.* (2009). Before the final amplification with all the samples, a screening test was done using 4 isolates (CMW 26667, CMW 26668, CMW 26669, CMW 26670) and 16 primer combinations (M+AA, M+AG, M+AC, M+CC in combination of each E+AA, E+AC, E+TC, E+CC). Based on the clarity of the resulting fingerprinting profiles (allowing unambiguous scoring) and reproducibility, four primer combinations (M+AA─E+TC, M+AG─E+AA, M+AG─E+AC, M+AA─E+AA) were selected and used to evaluate the larger group of isolates. From the digital image, bands were visually evaluated with JelMarker Software V1.3 Demo (Softgenetics, State College, PA).

**Gametangial induction**

The ability of *P. pinifolia* to produce selfed gametangia was evaluated on various growth media. Three isolates of *P. pinifolia*, including the ex-type isolate (CMW 26668) and two paratypes (CMW 26667, CMW 26669), where grown for two weeks in V8A (Erwin & Ribeiro 1996) at 25 ºC in the dark. A single block (5 × 5 mm) of agar bearing mycelium was transferred to the centers of a 65 mm Petri dishes containing either CA, CMA, OMA (Erwin & Ribeiro, 1996), V8A, WA (15 g agar $L^{-1}$), growth medium including *P. radiata* needles (PRN) and *P. radiata* needle extract (PRNE) with 10 replicates. To prepare the PRN, 5 pieces of 5 mm long autoclaved *P. radiata* needle, where scattered randomly on the surface of V8A, CMA and CA growing media, before the agar solidified. The PRNE was made by mixing V8A and CA, respectively, with *P. radiata* needle broth that was prepared by taking fifty grams of fresh, cut *P. radiata* needles, autoclaving this with 800 ml of distilled water and then filtering the broth through a double layer of cheese cloth. For the V8A-PRNE, 150 ml of centrifuged V8 and 15 g of agar were added to 800 ml of PRN extract, and made up to one liter before autoclaving. The CA-PRNE was made by adding 300 ml of cheese cloth filtered carrot juice (200 g carrot, crushed in a blender and boiled for 20 min) and 15 g of agar. Cultures were maintained at 25 ºC in the dark and inspected every three weeks for the presence of gametangia.

*Phytophthora pinifolia* isolates were tested for their ability to produce gametangia in crosses with tester strains of other *Phytophthora* spp. of opposite mating types (Erwin & Ribeiro, 1996). Three *P. pinifolia* isolates (CMW 26667, CMW 26668, CMW 26669) were paired with known A1 and A2 tester isolates of *P. cambivora*, *P. cinnamoni*, *P. crytogea, P. drechsleri, P. palmivora* (Table 2). Agar plates (CA, CMA, OMA, V8A or WA) were inoculated with a block (5 × 5 mm) of *P. pinifolia* culture placed 15 mm from the edge of a 65 mm Petri dish and maintained at 25 ºC in the dark for one week prior to the start of these tests. The tester isolates were transferred to the opposite sides of the plate, 15 mm from the edge, and incubated in the dark at 25 ºC and inspected for the presence of gametangia every three weeks. Ten replicates where made in each treatment. Also as control the tester isolates where crossed in the same way as described.

**3.0 Results**

**AFLP analysis**

A total of 200 clearly resolved bands were visually evaluated from the digital images emerging from the four different primer combinations. The profiles for the isolates with the primers M+AA─E+TC, M+AG─E+AA, M+AG─E+AC, M+AA─E+AA, which were generated during the primers screening phase, were the same as those generated for the same isolates when they were evaluated together with the rest of the population. The 8 isolates duplicated as internal controls, had the same profiles in both samples for the four primer combinations evaluated.

Other than two isolates, all the 86 isolates included in this study had identical profiles for all of the primer combinations used. For isolates CMW 30000 and CMW 30001, one band was not detected for the primer combination (M+AA─E+TC). This difference was considered to be a polymorphic site. Other than this inconsistency, these isolates were identical to all other isolates in this study for the other three primer combinations used; M+AG─E+AA, M+AG─E+AC, M+AA─E+AA.

**Gametangial induction**

After 12 weeks of growth on a wide variety of media, including those amended with pine needle tissue, no sign of gametangia was found in *P. pinifolia* cultures. Likewise, where *P. pinifolia* isolates were crossed with A1 and A2 mating type testers for *P. cambivora*, *P. cinnamoni*, *P. crytogea, P. drechsleri,* or *P. palmivora*, there were no signs of gametangia in the *P. pinifolia* cultures or in the tester strains. All tester strains produced oospores in all cases when mated with testers of the opposite mating type of the same species.

**4.0 Discussion**

This is the first study to consider the genetic structure of the population of the new and important pine pathogen *P. pinifolia*. Results of this study based on a broadly collected population of *P. pinifolia* isolates from its currently known geographic distribution in Chile, showed that the pathogen population is represented by a single genotype. Isolates included in this study were broadly sampled from across the area in which DFP occurs and they were also collected at different times of the year as well as in different years. The clonal nature of the pathogen population provides compelling evidence that the

pathogen has spread rapidly from a single entry point, with the first detection in Raqui in 2004 (Durán *et al*., 2008). The population structure of *P. pinifolia* observed in this paper is indicative of an introduced pathogen population (Goodwin *et al*., 1994; Ivors *et al.*, 2004; Loo, 2009).

Two isolates (CMW 33987 and CMW 34012) included in this study had a single missing band for primer (M+AA─E+TC). This difference is equivalent to 0.5% of the scored bands and is a much lower level of diversity than that found in other *Phytophthora* spp. such as *P. infestans* (Goodwin *et al*., 1994), *P. cinnamomi* (Linde *et al*., 1997; 1999) and *P. ramorum* (Ivors *et al*., 2004; 2006) that have been defined as clonal. As the majority of mutations are deleterious (Goodwin, 1997), the missing band in isolates CMW 33000 and CMW 33001 could be consequence of recent mutations in the *P. pinifolia* population.

*Phytophthora pinifolia* in Chile represents a single genotype that has spread rapidly and has had a major impact on *P. radiata* production in Chile. It is typical for introduced pathogens to have very limited genetic diversity. For example, *P. infestans* spread around the world as one genotype for more than one hundred years (Goodwin *et al*., 1994; Goodwin & Drenth, 1997), *P. ramorum* in USA and Europe and *P. cinnamomi* in Australia and South Africa are also introduced pathogens showing very low levels of genetic diversity (Ivors *et al*., 2006; Linde *et al*., 1997; Prospero *et al*., 2007). Genetic bottlenecks giving rise to reduced levels of genetic diversity may have some long term evolutionary disadvantages as is often hypothesized by ecologists (Kolar & Lodge 2001). The fact remains that most epidemics in the plant and animal world caused by viruses, bacteria and fungi are almost all caused by pathogen populations showing low levels of genetic diversity in the initial expansionary phase of the epidemic. Once a specific genotype of a potential invasive pathogen finds a suitable agricultural or forestry environment in which to become established, it can spread rapidly and cause significant damage, because the host and environment are often uniform as well (Drenth, 2004; Parker & Gilbert 2004).

Efforts to induce sexual structures in *P. pinifolia* on different media in this study were not successful. Likewise crossing of isolates with defined mating tester strains of several species of *Phytophthora* spp., including *P. drechsleri* and *P. cambivora* that

have been shown to induce sexual structures in other *Phytophthora* species (Brasier *et al*., 2003a; 2003b; Brasier & Kirk, 2004) did not induce the production of gametangia. *Phytophthora pinifolia* resides in Clade 6 of the *Phytophthora* phylogeny (Cooke *et al*., 2000; Durán *et al*., 2008) and there was a possibility that sexual structures might have been induced, as has been the case for *P. inundata* and *P. gonapodyides* (Brasier *et al*., 2003a) that also reside in this clade. Furthermore, no sexual structures have been observed in *P. pinifolia* cultures isolated routinely in Chile. This absence of sexual structures might suggest that *P. pinifolia* is heterothallic and that only a single genotype of one mating type is currently present in the country. Based on the results of this study, an alternative explanation for the absence of gametangia, is that the organism is sterile, similar to the recently described species *P. gallica* (Jung & Nechwatal 2008). This characteristic is found in approximately 4% of *Phytophthora* spp. (Cooke *et al.,* 2000). In Clade 6 where *P. pinifolia* resides, 50% of the taxa examined by Brasier *et al*. (2003a) are fully sterile, or sterile, but act as silent A1 mating type strain. Sterility in *Phytophthora* spp. is not fully understood and it could be an adaptation for adverse environments (Brasier *et al*., 2003b) although whether this is the case for *P. pinifolia* remains to be determined. A third hypothesis is that the mating type system in the clade 6 group show some slight differences and as such does not respond to components produced by the opposite mating types of other *Phytophthora* species. This can easily be tested if strains of the opposite mating type from the center or origin are paired with the ones currently under investigation.

The absence of any sign of the pine disease caused by *P. pinifolia* prior to 2004, and the rapid development of the disease subsequent to its first appearance in Chile, is typical of an introduced pathogen that is exposed to a highly susceptible host grown over vast areas in monoculture (Drenth, 2004). The presence of a single dominant clone in the *P. pinifolia* population in Chile further supports the hypothesis that this is a newly introduced pathogen. An alternative hypothesis is that *P. pinifolia* is native to Chile and that it has undergone a host shift (Slippers *et al*., 2005) from a native plant. Such host shifts might also result in a clonal population of a pathogen on a new host. However, a review of literature on *Phytophthora* epidemics (Brasier *et al*., 2005; Denman *et al*., 2006; Hansen *et al*., 2000; Ivors *et al.,* 2004, 2006; Prospero *et al*., 2007), as well as emerging plant diseases in general (Anderson *et al*., 2004), indicates that introduction is by far the most common explanation for such patterns. Hence, our data supports the

hypothesis that *P. pinifolia* is an introduced pathogen in Chile. The origin of *P. pinifolia* is unknown, but we hypothesize that it has most likely originated in an area where *Pinus* spp. are native, such as Mexico, and where cool moist conditions might favor infection.

*Phytophthora pinifolia* is an important pathogen of *P. radiata* and this study provides compelling evidence that it has been introduced into Chile. *Pinus radiata* appears to be uniformly susceptible to infection which occurs on seedlings, young as well as mature trees (Durán *et al*., 2008). The pathogen represents a very serious threat to *P. radiata* and probably related species. *Pinus radiata* is one of the most widely planted *Pinus* spp. in plantations in the southern hemisphere and the fact that the pathogen might have been accidentally introduced into Chile suggests that it has the capacity to be introduced into other areas of the world where *P. radiata* is grown in plantations.

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**Table 1.** Origin and isolation date of eighty eight *Phytophthora pinifolia* cultures used in this study.

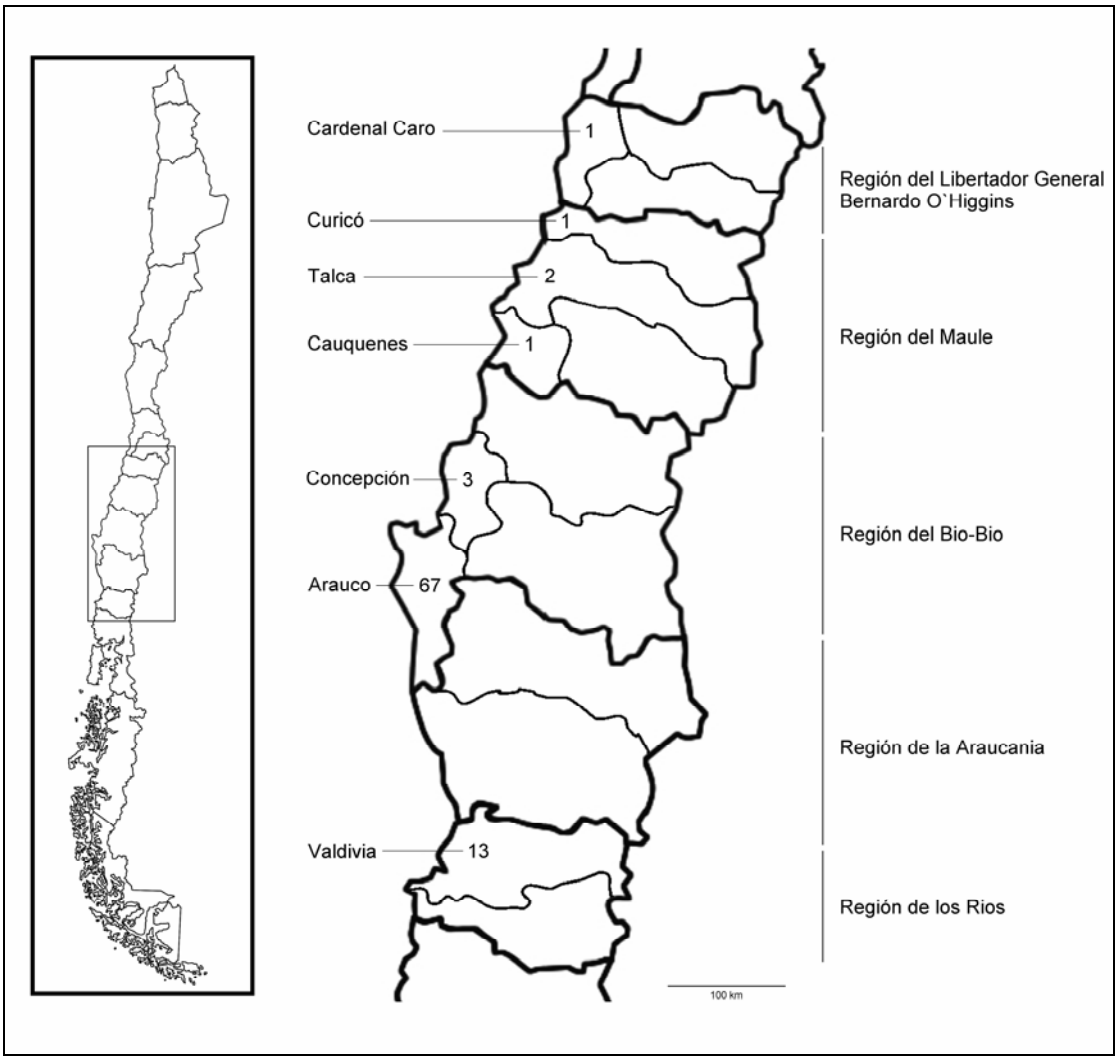
| Province | Compartment | 2007 | | | 2008 | | | | | | 2009 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | June | August | October | May | June | July | August | September | October | April | May |
| Cardenal Caro | Sta Consuelo | | | | | | | 1 | | | | |
| Curicó | El Alamo | | | | | | | 1 | | | | |
| Talca | Quivolgo | | | | | | 1 | | | | | |
| | Risco Afuera | | | | | | | 1 | | | | |
| Cauquenes | Bellavista | | | | | | | | 1 | | | |
| Concepción | La Posada | | | | | | | 1 | | | | |
| | Las Palmas | | | | | | | | 1 | | | |
| | Malal Aparte | | | | | | | 1 | | | | |
| Arauco | Camaron | 1 | | 17 | | 5 | 1 | | | | | |
| | Cordillera | | | | | | | 1 | | | | |
| | La Colcha | | | | | | | 1 | | | | |
| | Llico | | 5 | | | | | | | | | |
| | Llico Pira 03 | | | 14 | | | | 1 | | 1 | | |
| | Llico Pira 94 | | | | | 5 | | 1 | | | | 1 |
| | Molino del Sol | | | | | | | 1 | | | | |
| | Nalcahue | | | | | | 1 | | | | | |
| | Paillacahue | | | | 1 | | | 1 | | | 1 | |
| | Quidico | | | | | | 1 | | | | 1 | |
| | Quilachanquín | | | | | | | 1 | | | | |
| | Rumena | | | | | | 1 | | | | | |
| | Trana 3 | | | | | | 1 | | | 1 | | |
| | Trehuaco | | | | | | 1 | | | | | |
| | Tropen Efa | | | | | | | | | | | 1 |
| Valdivia | El Duero | | | | | | | | 1 | | | |
| | Naguilan | | | | | | | | 1 | | | |
| | Rochela 4 | | | | | | | | 1 | | | |
| | San Guillermo | | | | | | | | 1 | | | |
| | Sta Clara | | | | | | | | 1 | | | |
| | Tres Chiflones | | | 8 | | | | | | | | |

**Table 2.** Isolates of different *Phytophthora* species used for the mating tests.

| *Phytophthora* spp. | Isolate number [1] | | Mating Type | Host or source | Location |
|---|---|---|---|---|---|
| *P. crytogea* | P29 | CMW 33002 | A2 | *Begonia* sp. | Germany |
| *P. crytogea* | P30 | CMW 33003 | A1 | *Lewisia* sp. | Germany |
| *P. cambivora* | P31 | CMW 33004 | A2 | Soil (*Quercus robur*) | Germany |
| *P. cambivora* | P32 | CMW 33005 | A1 | *Chameacyparis lawsoliana* | Germany |
| *P. cinnamoni* | P33 | CMW 33006 | A1 | *Camelian* sp. | Germany |
| *P. cinnamoni* | P34 | CMW 33007 | A2 | *Rhododendron simisii* | Germany |
| *P. drechsleri* | P35 | CMW 33008 | A2 | *Beta vulgaris* | Iran |
| *P. drechsleri* | P36 | CMW 33009 | A1 | *Hedera* sp. | Germany |
| *P. palmivora* | UQ 3689 | CMW 29599 | A1 | *Howea forsteriana* | Australia |
| *P. palmivora* | UQ 3694 | CMW 29601 | A1 | *Arecastrum romanzoffianum* | Australia |
| *P. palmivora* | UQ 3716 | CMW 29602 | A1 | *Palm* spp. | Australia |
| *P. palmivora* | UQ 3717 | CMW 29603 | A2 | Soil | Australia |
| *P. palmivora* | UQ 3718 | CMW 29604 | A2 | Soil | Australia |
| *P. cinnamomi* | UQ 3652 | CMW 29598 | A2 | *Ananas comosus* | Australia |
| *P. cinnamomi* | UQ 3651 | CMW 29597 | A2 | *Ananas comosus* | Australia |
| *P. cinnamomi* | UQ 3648 | CMW 29596 | A2 | *Ananas comosus* | Australia |
| *P. cinnamomi* | UQ 789 | CMW 29606 | A1 | *Eucalyptus gummifera* | Australia |
| *P. cinnamomi* | UQ 795 | CMW 29607 | A1 | Soil | Australia |
| *P. drechsleri* | WPC1899 | CMW 29592 | A1 | *Beta vulgaris* | USA |
| *P. drechsleri* | WPC3808 | CMW 29594 | A1 | *Vigna unguiculata* | USA |

(1) Isolates with P numbers were provided by Prof. Everett M. Hansen, Oregon State University, Corvallis, OR, USA; those with UQ notations are from the: *Phytophthora* culture collection, University of Queensland, Brisbane, Queensland, Australia, those with WPC numbers from the: World *Phytophthora* culture collection, University of California, Riverside, CA, USA and the CMW numbers represent the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

**Figure 1.** Map of Chile and total number of *Phytophthora pinifolia* isolates obtained in each province.



# Chapter 5

## Evaluation of fungicides for the control of *Phytophthora pinifolia* under *in vitro* and *in vivo* conditions

## ABSTRACT

*Phytophthora pinifolia* causes the needle disease in Chile known as DFP. The disease affects *Pinus radiata* of all ages, with seedlings being most susceptible. In this study the products, mefanoxem, phosphorous acid and fosetyl-Al, where evaluated in four doses to control *P. pinifolia*. A first step was to evaluate the doses recommended by the manufacturers *in vitro* at full strength and various dilutions. The same doses were then evaluated in two separate trials where the products, were tested on plants pre-inoculated with the pathogen to assess the curative effect and then by applying the fungicides one week after the inoculation. Results of the *in vitro* trial showed that all the fungicides completely inhibited the growth of *P. pinifolia* in all the doses evaluated. The study in planta showed that mefenoxam was the most effective fungicides both as a preventative and curative. The results of this study show that fungicides can be used as part of the management strategy against *P. pinifolia.*

## 1.0 Introduction

*Pinus radiata* is one of the most widely planted non-native tree species in the world (Richardson *et al*., 2007). Plantations of this tree have been established for the production of solid wood products and pulp (Sutton, 1999), mostly in regions with a Mediterranean climate such as Australia, Chile, New Zealand, South Africa and Spain (Rogers, 2004). Of these, one third of the global *P. radiata* plantation area is in Chile (Guerrero & Bustamante, 2007) where trees are characterized by rapid growth and high levels of productivity. This superb performance is commonly attributed to the separation of the trees from their most important natural enemies (Richardson, 1998; Wingfield *et al*., 2001).

Recently, a new disease referred to as "Daño Foliar del Pino" (DFP) appeared in coastal plantations located in the central part of Chile (Durán *et al.,* 2008; Chapter 2). A new species of *Phytophthora* described as *P. pinifolia* A. Durán, Gryzenh. & M.J. Wingf. was shown to be the causal agent of this important disease and this is the only *Phytophthora* specie known to infect the foliage of *Pinus* spp. (Durán *et al.,* 2008).

DFP affects trees of all ages, with the most important damage in young plants causing rapid mortality (Durán *et al.,* 2008; 2009/ Chapter 3). Recent studies have shown that *P. pinifolia* has a clonal population structure in Chile, providing support for the view that the pathogen has been introduced into the country (Chapter 4). This would facilitate disease management options such as breeding for resistance where selected disease tolerant planting stock would most likely have strong durability (McDonald & Linde 2002). Furthermore, efficacy of fungicides could be greater due to the genetic uniformity of the pathogen (Georgopoulos & Skylakakis 1986).

Fungicide treatments represent the most widely employed strategy to manage diseases caused by *Phytophthora* in the forestry environment. Of the fungicides applied, phosphites such as fosteyl-Al and phenylamides (e.g. metalaxyl) (Hardman, 2005), are commonly used in nurseries or in field situations where they are applied as soil drenches, stem injections, topical bark applications, foliar treatments or aerial sprays (Shearer *et al*., 2006; Garbelotto *et al*., 2007; Shearer & Fairman, 2007; Linderman & Davis, 2008). The most extensive program to manage a *Phytophthora* sp. in forests has

been in Australia where phosphite has been applied to curb the devastation of the native vegetation caused by *Phytophthora cinnamomi* Rands (Hardy *et al*., 2001; Wilkinson *et al*., 2001; Shearer *et al*., 2006). The fungicide is applied as foliar sprays, aerial mists or trunk injections (Shearer *et al*., 2006; Shearer & Fairman, 2007) and long term protection has been achieved (Shearer & Fairman, 2007).

Phosphate, has a double action including a direct phytotoxic effect and inducing resistance in treated plants (Smillie *et al*, 1989; Linderman & Davis, 2008). Phosphite contains a P-H bond and is found in phosphonic acid (in the form of mono and di-basic sodium, potassium, and ammonium salts of phosphorous acid) or fosetyl-Al [(aluminium tris (o-ethyl phosphonate)] (Hardy *et al*., 2001). Within treated plants phosphite stimulates the production of phenolic compounds that are linked to resistance against infection by *Phytophthora* (Smillie *et al*, 1989). In contrast to phosphate, phenylamide fungicides such as metalaxyl and mefenoxam (metalaxyl-M) inhibit ribosomal RNA synthesis in members of the Peronosporales (Gisi & Sierotzki, 2008).

*Pinus radiata* is the most important forest plantation tree in Chile and it contributes substantially to the national economy of the country. The damage to this crop by *P. pinifolia* requires urgent action and the development of management strategies to reduce the impact of the pathogen. In this regard, fungicides must be considered as one of the tools to achieve effective management. The aim of this study was thus to evaluate mefanoxem, phosphorous acid and fosetil-Al *in vitro* and *in planta* under laboratory conditions for the control of *P. pinifolia*.

## 2.0 Material and Method

Isolates

Three isolates (CMW 32001, 32003, 32004) of *P. pinifolia* collected from *P. radiata* needles from the provinces of Talca, Valdivia and Arauco in July and September 2008 and May 2009 respectively, were used in this study. The identities of the isolates were confirmed with species-specific primers developed by Durán *et al*. (2009). Isolates were grown on 10% V8 agar (100 ml $L^{-1}$ of clarified V8 juice [Campbell Co, USA] and 15 $g.L^{-1}$ agar [Biolab, South Africa]) (Erwin & Ribeiro, 1996) in 90 mm Petri dishes for two weeks at 25 ºC and in the dark. All the trials described in this study, were carried

out under quarantine conditions at the Bioforest S.A. research facilities, Concepción (Chile).

*In vitro* screening

Mefanoxem, phosphorous acid and fosetyl-Al, were evaluated for the reduction of hyphal growth in plates, in four doses each (Table 1). The doses were determined as those recommended by the manufacturer, and half, a quarter and an eighth of the recommended doses. The fungicides were dissolved in sterile water, filtered using syringe filters (0.45 µm), and added to autoclaved 10% V8 agar when the temperature had dropped to between 40 – 50 ºC. The medium was then dispensed into 90 mm Petri dishes with approximately 15 ml per dish. In order to establish negative controls for the experiments, identical plates were made but without fungicides. Plates were inoculated with 5 mm diam mycelial plugs taken from the actively growing edges of the three two-week-old isolates, placed at the center of each test plate and incubated at 25 ºC in the dark. Five replicates were made for each isolate and treatment. Colony growth was measured after 20 days by taking two diameter measurements at right angles to each other for each plate. Colony diameter was analyzed with a two-way ANOVA to test for the effect of treatment and isolate.

*In planta* trial

The protective or curative ability of the same fungicides at the same doses used in the *in vitro* trial, were evaluated *in planta* in the Bioforestr S.A. disease screening facilities in Chile. *Pinus radiata* seedlings (*ca* 40 – 50 cm high, *ca* 8 – 14 mm collar diameter) were acclimatized for two weeks prior to inoculation in the laboratory, which were subjected to artificial day light of approximately 12 hours duration, maintained at approximately 18 – 22 ºC and daily irrigated by root immersion. Inoculations were made by removing a disc of bark (4 mm diam) in the upper half of the plant and placing a plug of mycelium of similar size taken from the edge of an actively growing colony, into the wounds. Inoculation wounds were covered with Parafilm (Pechiney Plastic Packaging, Inc., IL, USA) to reduce desiccation and contamination. Ten plants where inoculated for each isolate in each treatment.

The preventive effect of the fungicides was evaluated by spraying the foliage of the plants to run-off with the fungicides one week before the *P. pinifolia* inoculations. On a

different set of plants, the curative effect was evaluated by inoculating the plants one week before the application of the same fungicide spray treatments. Evaluation in both cases was made by measuring the lesion lengths at 30 days after the inoculation. For the protective and curative treatments, a positive control was established by inoculating each *P. pinifolia* isolate onto 10 plants not treated with fungicides.

The lesion lengths associated with inoculation of *P. pinifolia* in the case of both the curative and the preventive treatment experiments were log transformed. Each trial separately was analyzed with a two-way ANOVA to test for the effect of treatment and isolate. Further pairwise comparisons testing for effect of treatment were conducted using Fisher's least significant difference procedure ($P = 0.05$). Analyses were performed using STATISTICA V6 (StatSoft, Inc. 2004).

## 3.0 Results

*In vitro* screening

Treatments with all three fungicides and at four different doses resulted in the complete inhibition of growth of *P. pinifolia* isolates. In contrast, radial growth of the pathogen on the control plates ranged from 53 – 55 mm (mean 54 mm) at the 20 days. There were no significant difference in the colony growth between the three different isolates evaluated ($P < 0.05$).

*In planta* trial

The three *P. pinifolia* isolates evaluated all resulted in lesions of varying size on the *P. radiata* seedlings tested. In the control treatments where plants were not sprayed with fungicides, the *P. pinifolia* isolates resulted in lesions an average of 91.3 mm long. It was possible to re-isolate *P. pinifolia* from the lesions associated with the inoculations showing that the organism was responsible for the symptoms observed in the control as well as in in the fungicide treatment evaluated.

In the preventive trial where plants were treated with fungicide before inoculation, the mefenoxam doses of 0.03, 0.06, 0.13 and 0.25 g a.i./L gave complete protection with no lesions developing after inoculation with *P. pinifolia* isolates (Fig 1). These results were not included in the statistical analyses. The phosphorous acid treatment at doses of 0.56,

1.12 and 2,25 g a.i./L reduced the lesion size compared with the control, but at the highest dose evaluated (4.49 g a.i./L) no difference were found in comparison to the control ($P$<0.05). Fosetyl-Al treated lesions did not differ from the control at doses of 0.63 and 2.5 g a.i./L, but longer lesions than those recorded for the control at doses of 0.31 and 1.25 g a.i./L (Fig. 1).

In the trial to test the curative effect of the various fungicides, smaller lesions developed where all of the doses of mefenoxam were applied. The lesion length in the treatment with phosphorous acid at doses of 0.2, 0.4, 0.8 and 1.6 g a.i/L, did not differ significantly from the control ($P$<0.05). In the case of the fosetyl-Al treatment, smaller lesions than the control were producded at doses of 0.3 and 2.5 g a.i./L, while lesion sizes did not differ at 0,63 g a.i./L and longer lesions were produced with a dose of 1.25 g a.i./L (Fig. 1).

**4.0 Discussion**

The results of this study show that fungicides could be used to reduce or fully protect *P. radiata* seedlings against damage caused by *P. pinifolia*. Mefenoxam was the most effective fungicide in both the preventive and curative trials at all the doses tested. Moreover, the treatment of mefanoxam as a preventative treatement also gave positive results. These results are similar to those of Linderman & Davis (2008) where 13 fungicides where tested against 5 species of *Phytophthora*, and the most significant reduction in lesion size was achieved with mefanoxam, and limited or no size reduction was found with fosetyl-Al and phosphorus acid. These fungicides could thus be used as part of a management strategy in recently established plantations, especially in area with high levels of infection, where the damage caused by *P. pinifolia* is important and often lethal (Durán *et al.,* 2008).

*Phytophthora pinifolia* is a recently described species, and there are no recommendations available relating to the fungicides that could be used to protect seedlings against infection. Thus, before applying the fungicides to plants, a first step was to evaluate the best doses for application in a laboratory trial. The results showed that in the case of mefanoxem, phosphorus acid and fosetyl-Al, a dosage equal to one

eighth of the dilution recommended by the producer gave total inhibition in the hyphal growth.

*In planta* evaluation with mefenoxam provided the most consistent reduction in lesion length in both the preventive and curative trials. This chemical might thus be effective in reducing the colonization of plant tissue by *P. pinifolia* in a manner similar to that for *P. ramorum* (Garbelotto *et al.* 2008). Mefenoxam used at the dosage suggested by the manufacturer could be used as either a protectant or a curative agent where stands are at risk to infection by *P. pinifolia*. The residual effect of treatment with mefenoxam on *P. radiata* seedlings was not tested in this study. However, results on Rhododendron treated for infection by *P. ramorum* have shown that the chemical can remain active until 8 weeks after treatment (Linderman & Davis, 2008). It would thus be possible to treat *P. radiata* seedlings in the nursery before they are dispatched to the field to achieve protection against infection during establishment.

Application of phosphorus acid in both the preventive and curative treatments did not reduce lesion development for *P. pinifolia* compared with the control inoculations. This compound has been widely used and with good results in the management of *P. cinnamomi* in Australia (Hardy *et al* 2001; Shearer *et al*., 2006), including aerial applications in infected *E. marginata* forests (Shearer & Fairman, 2007). Also in Australia, the application of potassium phosphonate was effective in reducing root disease in *P. radiata* seedlings inoculated with *P. cinnamomi* and against natural infections in nurseries when applied as a soil drench (Ali *et al*., 1999; Reglinski *et al*., 2009).

Phosphorus acid is thought to stimulate the production of resistance compounds in plants (Smillie *et al*, 1989). In this study, the inoculations with mycelial plugs into the stems of plants represent an aggressive manner to introduce the pathogen into the plant. It is possible that resistance compounds stimulated by phosphorus acid were less effective against the established lesions tested here than would have been the case in naturally infected young tissue. Also, concentrations of phosphorus acid are thought to be higher in regions of the plant undergoing rapid growth, such as the roots and shoots (Hardy *et al*., 2001). This might explain why the fungicide is effective in the case of *P. cinnamomi* that infects roots (Ali *et al*., 1999) but not in the case of the stem-infecting pathogens *P. ramorum* (Garbelotto *et al*., 2007) and *P. pinifolia*.

The trails using Fosetyl-Al produced better results as a curative than as a protective against infection by *P. pinifolia*. In previous studies, this fungicide has been effective both in *in vitro* and *in planta* trials for some species of *Phytophthora* (Fenn & Coffey, 1984), but not in others such as *P. infestans* (Mont.) de Bary (Fenn and Coffey 1984). Results of the present study suggest that at the dose recommended by the manufacturer, fosetyl-Al could slow lesion development but that it is apparently not useful as a protectant. These results are similar to those of Belisario *et al.* (2009) showing that fosetyl-Al is effective against *P. cinnamomi* but only after it has become established.

The different results for the *in vitro* and *in planta* trials in this study could be related to the physiology of *P. radiata*. This would be consistent with the study of Cooke & Little (2001) who showed that fosetyl-Al was effective for controlling late blight caused by *P. infestans* in tomato but not in potato, possibly due to differences in the uptake translocation and/or metabolism of fosetyl-Al in these two plants. Clearly *P. pinifolia* is sensitive to fosetyl-Al and phosphite as indicated in the *in vitro* tests but *in planta*, its sensitivity is apparently influenced by the host.

A disadvantage of treating plants with a fungicide such as mefenoxam is the possibility of resistance emerging in the pathogen population. Several studies have thus shown that extensive use of mefenoxam in the field can lead to the emergence of resistant lineages of *Phytophthora* spp. (Hwang & Benson 2005; Lamour *et al*., 2003). Nonetheless, the same study suggested that mefenoxam applications, combined with other fungicides, can be an important strategy for disease management. In the case of *P. pinifolia*, the pathogen is clonal in Chile and lacks sexual re-combination (Chapter Four). This would reduce the possibility that fungicide resistant pathogen strains will emerge as the result of genetic segregation in a short time frame. Some clonal variation and genetic mutations can however, occur in clonal populations and with sufficient selective pressure resistant strains could arise over time.

The results of this study will need to be validated in the field and thus under natural conditions and with aerial spray. Damage by *P. pinifolia* in the field appears to increase at the end of the winter and at the start of Spring. In Chile, plantations are established in winter when rain provides moisture for growth. In some areas, plants do not become

infected immediately after establishment, but infection only occurs the following spring. In these cases, it might be possible to apply the results of this study by using the fungicides in protective, treatments. In this way, plants would be able to become established at the very sensitive young age in the absence of serious damage by *P. pinifolia*.

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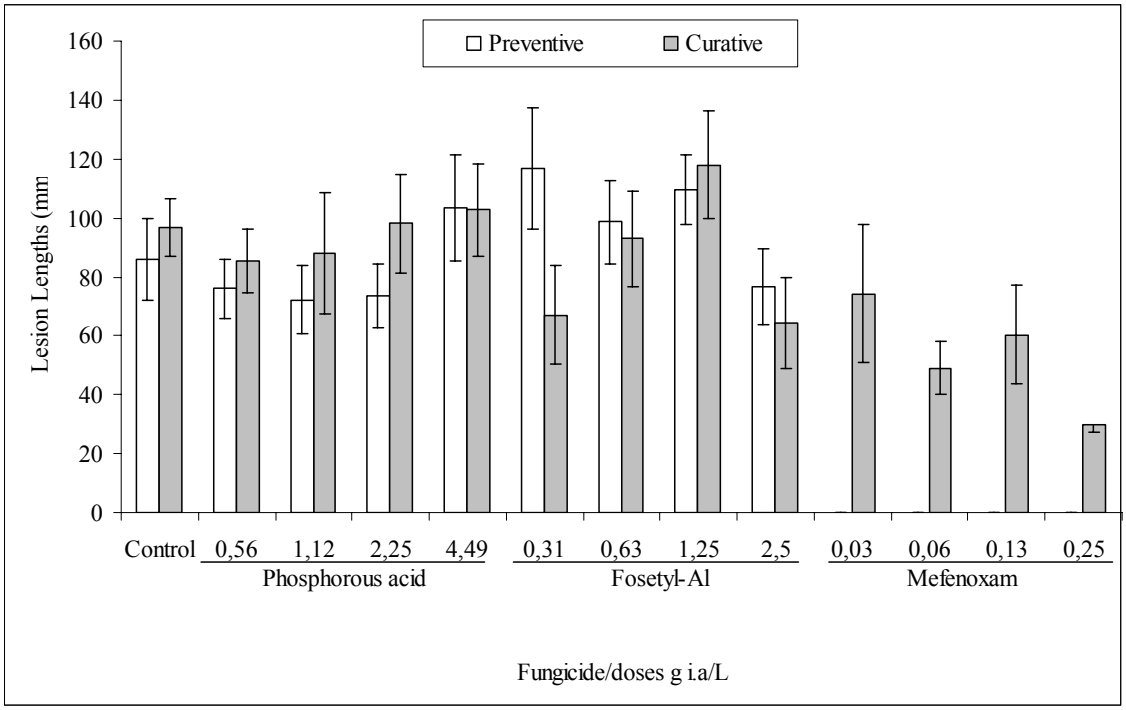
**Table1**. Fungicides and doses used in this study.

| Active Ingredient (a.i) | Comercial designatin | Dosage | g a.i./L |
|---|---|---|---|
| Phosphorous acid | Phostrol | 1 | 0,200 |
| | “ | 2 | 0,400 |
| | “ | 3 | 0,800 |
| | “ | 4* | 1,600 |
| Fosetyl-Al | Aliette 80 WP | 1 | 0,250 |
| | “ | 2 | 0,500 |
| | “ | 3 | 1,000 |
| | “ | 4* | 2,000 |
| Mefenoxam | Ridomil Gold 480 SL | 1 | 0,015 |
| | “ | 2 | 0,030 |
| | “ | 3 | 0,060 |
| | “ | 4* | 0,120 |

* Dose 4 is the manufacture`s recommended rate

**Figure 1**. Lesion length average (with bars showing standard deviation) caused by the three isolate of *Phytophthora pinifolia* on 30 *Pinus radiata* seedlings in a greenhouse after preventive (1 week prior to inoculation) and curative (1 week after inoculation) application of three fungicides at four doses.



## SUMMARY

The leaf and shoot blight disease known locally as Daño Foliar del Pino (DFP) is the most important problem affecting Chilean *Pinus radiata* plantations. The disease is typified by needle infections, exudation of resin at the bases of needle brachyblasts and necrotic lesions in the cambium of younger trees, which eventually girdle branches. The disease causes death of seedlings young trees and mature trees can also succumb after a few years of successive infection. DFP emerged very recently and its cause was unknown. Studies that make up this thesis led to the identification of the causal agent of DFP, which was described as the new species *Phytophthora pinifolia* A. Durán, Gryzenh. & M.J. Wingf. This is the first discovery of a *Phytophthora* sp. infecting needles and shoots of a *Pinus* sp. anywhere in the world. Two diagnostic procedures were developed to screen large numbers of samples rapidly and cost effectively for the pathogen. Population genetic studies showed that the *P. pinifolia* population in Chile is a single clonal lineage that exists in the absence of sexual recombination. This suggests strongly that *P. pinifolia* is an introduced pathogen in Chile although its origin and natural host-range is uknown. In order to promote effective management of *P. pinifolia*, three fungicides were screened *in vitro* and *in planta*. Mefenoxam was shown to be the most effective compound, also providing the most consistent results. DFP is recognized as one of the most important disease problems to strike non-native pine plantation forestry in recent times. Studies in this thesis represent the first ever on the disease and its causal agent and they provide a foundation for the many studies that must follow to ensure that *P. pinifolia* does not lead to disease disasters elsewhere in the world.