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Pathogens in commercial *Eucalyptus* plantations in Chile, with special reference to *Mycosphaerella* and *Botryosphaeria* species

Submitted by

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DECLARATION

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

Rodrigo Ahumada

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PREFACE

Commercial plantations in Chile are comprised of exotic *Pinus* and *Eucalyptus* species, which make up approximately 2.1 million ha. *Eucalyptus* spp. represent about 17 % of the total, and the most widely planted species are *E. globulus* and *E. nitens*. Both species have been considered as relatively disease free in Chile, despite their susceptibility to several insects and diseases, elsewhere in the world. In Chile, leaf spot diseases are considered the most important sanitary problem associated with commercial *Eucalyptus* plantations and *Mycosphaerella* spp. appear to be the fungi causing the most significant damage. *Botryosphaeria* spp. have also been considered as a potential threat to *Eucalyptus* plantations, especially based on experience in other countries, where these fungi severely damage stressed trees. Very little research has been conducted on *Eucalyptus* diseases in Chile and the aim of this thesis was to expand our knowledge of this field. The more specific aim was to provide robust identifications for the species of *Mycosphaerella* and *Botryosphaeria* associated with disease symptoms in commercial *Eucalyptus* plantations in Chile.

In the first chapter of this thesis, a general review of forestry activities in Chile is presented. Special emphasis is placed on matters pertaining to tree health. Information is summarized separately for *Pinus* and *Eucalyptus* species and is organized in such a way that insect pests and disease problems are treated separately. A review of the available literature on tree pest and disease problems in Chile is provided to serve as a foundation for research undertaken in this thesis.

Prior to undertaking studies in this thesis, very little research had been conducted on forest pathogens. In the second chapter of the thesis, I have undertaken a study to identify the most common *Mycosphaerella* spp. associated with leaf spot diseases in Chile. The taxonomy of these fungi is known to be very complicated and it was previously based exclusively on morphological characteristics. However, recent studies have applied DNA sequencing techniques to this process. My aim has thus been to use DNA sequence data, together with morphological characters to identify *Mycosphaerella* spp. associated with leaf diseases of *Eucalyptus* in Chile. Ultimately, the intention has also been to gain some perspective on the relative importance of the various species of these fungi in Chile and to reduce losses caused by them. To achieve this objective, diseased adult and juvenile *E.*

globulus and *E. nitens* leaves were collected from plantations in Northern-central and Southern-central Chile. Species of *Mycosphaerella* were identified based on ascospore germination patterns, cultural characteristics and sequence data from the Internal Transcribed Spacer (ITS) region of the rRNA operon.

Botryosphaeria spp. have thus far not been considered as serious pathogens in *Eucalyptus* plantations in Chile. This is despite the fact that they can be highly damaging elsewhere in the world. However, very little is known regarding their presence or potential to cause disease. The rapid growth of commercial *Eucalyptus* plantations has resulted in an increase in new pests and diseases. Of these, Botryosphaeria dieback disease has begun to appear in young and adult trees more frequently in Chile. Therefore, the aim of studies presented in Chapter III of this thesis, was to identify the *Botryosphaeria* spp. associated with dieback disease of commercial *Eucalyptus* plantations. Symptomatic branches were thus collected from *E. globulus* and *E. nitens* plantations in Northern-central and Southern-central Chile. Species of *Botryosphaeria* were subsequently identified based on morphological characteristics and sequence data from the Internal Transcribe Spacer (ITS) and β-tubulin regions of the ribosomal RNA operon. A greenhouse pathogenicity trial was also conducted using the various *Botryosphaeria* spp. identified, to evaluate their relative importance in Chile.

The primary aim of this thesis is to provide a better understanding of *Mycosphaerella* and *Botryosphaeria* species that are currently damaging *Eucalyptus* spp. in commercial plantations in Chile. The study is the first of its kind and it will hopefully also form a foundation for further studies of *Eucalyptus* diseases in the country. Knowledge of contemporary techniques to identify pathogens has also been learned and this will also be useful to forestry researchers in Chile in the future.

Chapter 1

PLANTATION FORESTRY IN CHILE WITH PARTICULAR REFERENCE TO DISEASE AND INSECT PROBLEMS

1.0 INTRODUCTION

Chile, one of the largest forestry exporters in the world, has a rapidly growing forestry industry, based on exotic commercial plantations of *Pinus radiata* D. Don and various *Eucalyptus* spp. (Lowy 1995). Approximately 11 % of the Country's land is forested and about 2.1 million ha are being used for commercial plantations (WFI 1999). Almost all commercial plantations are linked to industry and these are all managed by private companies. The most important forestry products are pulp, paper, wood chips, log and sawn lumber, with an estimated value of US$ 2 205 million that was exported in 2001 (INFOR 2002). The major export markets are in countries of Asia, Europe and South America (Lowy 1995, INFOR 2001, WFI 1999).

Pinus radiata was introduced into Chile from California (USA) at the end of the 1800s and is the most widely planted species. This is because the species grows rapidly in a variety of environmental conditions and on a wide diversity of sites (Cerda & Nuñez 1998). The second most commonly planted trees are species of *Eucalyptus*, which were introduced into Chile in the 1800's from Australia. Propagation of both *Pinus* and *Eucalyptus* species in plantations has been subsidised by the Chilean government. Currently, there are approximately, 1.5 million ha of *P. radiata* and 360 000 ha of primarily *Eucalyptus globulus* Labill. and *Eucalyptus nitens* (Deane & Maid.) Maid. planted in Chile (INFOR 2000, Kliejunas *et al.* 2001).

Thus far, needle blight caused by *Dothistroma septospora* Hulbary and the pine shoot moth *Rhyacionia buoliana* Dennis et Schiff. have resulted in the most serious sanitary problems on *Pinus radiata* in Chilean plantations. The pitch canker fungus *Fusarium circinatum* Nirenberg and O'Donell (= *F. subglutinans* (Wollenw. and Reinking) Nelson *et al.* f.sp. *pini* Correll *et al.*), was detected late in 2001 and although it is still confined to nurseries, its presence in the country is of considerable concern (Wingfield *et al.* 2002). *Dothistroma septospora* and *R. buoliana* are well known, and it is important to understand the biology and management options for these and other pests and diseases present in Chile. However, Chile is also threatened by a large number of pests and diseases that do not yet occur in the country. It is, therefore, crucial to have a clear understanding of these potential threats.

Eucalyptus plantations have been damaged by several pathogens and insects in the past, but the most severe damage has been caused by species of *Mycosphaerella* Johanson. Two of the most widely planted species in Chile, *E. globulus* and *E. nitens* are both susceptible to Mycosphaerella leaf disease (MLD), which results in leaf necrosis and defoliation (Carnegie 2000, Dungey *et al.* 1997). *Mycosphaerella* spp. are well known as important pathogens of *Eucalyptus* spp. MLD is one of the most important constraints to *Eucalyptus* propagation in various parts of the world, where it can be responsible for severe reduction in tree growth (Crous 1998). Studies in *E. nitens* plantations in South Africa, have shown that defoliation caused by *Mycosphaerella* leaf spot caused a 38% drop in tree height and a loss of 25% in diameter (Lundquist & Purnell 1987). In countries such as New Zealand, the disease has been more serious on seedlings and saplings than on adult trees (Ganapathi 1979).

With the exception of Dothistroma needle blight and the pine shoot moth (*R. buoliana*), very little research has been conducted on pests and diseases in commercial forestry plantations in Chile. In the future, it will be important to build a sustainable internal capacity to deal with pests and diseases present in the country. In addition, attention will also need to be given to those pests and diseases that are likely to appear in the future. The aim of this review is to summarize the available literature pertaining to diseases and insect pests that have been associated with damage to forest plantations in Chile. In addition, I hope to provide a background to the studies on two diseases of *Eucalyptus* that are treated in subsequent chapters of this thesis.

2.0 FOREST PLANTATIONS IN CHILE

Continental Chile extends approximately 4 300 km in length and is 427 km wide at its widest point. The country is divided into 12 administrative regions from North to South (17° 30' S - 56° S). Chile is 75.5 million ha in size and approximately 11 % of this area is afforested (Lowy 1995). This forested area is comprised of approximately 4.75 million ha of unprotected native forest, 1.4 million ha of protected native forest and approximately 7.5 million ha of commercially productive native forest (Brownie 2001). The Southern-central part of Chile (35° S - 42° S) is considered to be the most important area for commercial forestry. This is because it provides conditions most suitable for the growth of forest tree species such as *P. radiata* and *Eucalyptus* spp. (Jayawickrama, Schlatter & Escobar 1993).

Extensive establishment of commercial plantations began in the 1970's with the implementation of government incentives. These substantially promoted the expansion of the forest products industry and subsidised the majority of costs for reforestation and plantation establishment (Brownie 2001, WFI 1999). The rate of tree planting has been high, with 65 413 ha of *P. radiata* and 11 953 ha of *Eucalyptus* spp. planted in 1978, in contrast to 59 411 ha of *P. radiata* and 36 781 ha of *Eucalyptus* spp. planted in 2000. The highest level of establishment of *P. radiata* was in 1981 when 88 529 ha were planted. Likewise, in 1993 approximately 53 293 ha of *Eucalyptus* spp. were planted. Currently commercial plantations make up approximately 2.1 million ha (INFOR 2002). Most of these plantations are concentrated between Northern-central to Southern-central regions of (approximately from V to X administrative regions) Chile (Figure 1).

Almost all commercial plantations in Chile are industrial and all are privately owned. Larger timber companies have purchased smaller companies. For example, in 1977, 14 companies owned 88 % of the forest land and by the end of the 1980's, four groups controlled over 90 % of the timber industry (Lowy 1995). Currently, two large forestry groups, Forestal Arauco and CMPC, own approximately 50 % of *P. radiata* forests and 75 % of all pulp production in the country (Brownie 2001). Chile is the largest exporter of wood chips into Latin America and is the world's third-largest chip exporter, after Australia and USA. Japan is the largest importer of Chilean wood chips, followed by Finland (Lowy 1995).

Forest products represent the second most important Chilean export product after copper. The forestry industry has focused mainly on pulp, paper, wood chips, log exports, sawn lumber, boards, furnishing and packaging (Lowy 1995, WFI 1999). The total volume of forest products exported in 2001 was estimated at US$ 2 205 million. The major market for the Chilean forestry industry was Asia with approximately 50 % of the total trade, Europe (20 %) and South America (17 %). Pulp and paper are the principal export products with 54 % of the total, including wood chips (9.8 %), sawn lumber (9.4 %) and logs (6.1 %) (WFI 1999, INFOR 2001).

2.1 *Pinus radiata*

Pinus radiata (radiata pine or Monterrey pine), was introduced into Chile from California towards the end of 1800's. It is now the most widely planted species for forestry and comprises approximately 1.5 million ha (74.1 %) of the commercial plantations (INFOR 2000). This species grows well in areas with poor soil and low rainfall and is planted on various land types, other than deserts, swamps, and areas subjected to snow and low temperatures (Cerda & Nuñez 1998, WFI 1999). Most forestry companies consider the optimum rotation age for a well-managed plantation of *P. radiata* to be between 18 to 25 years for pulpwood and 25 to 35 years for solid timber (Balocchi, Ahumada & Ramirez 1999, INFOR 2000). *Pinus radiata* can produce up to 25 m^3/ha/yr of wood and the average yield per hectare at harvest is 470 m^3 (Williams 1998). During 2000, the consumption of *P. radiata* was approximately 18.8 million m^3. The future stock of wood during the next rotation has been calculated at approximately 64.3 million m^3 (INFOR 2001).

2.2 *Eucalyptus spp.*

Eucalyptus spp. were introduced into Chile in 1823 and an extensive forest base was rapidly developed in the early 1900's (FAO 1979, Lopez *et al.* 2001). Commercial planting of *Eucalytus* spp. began in the 1930's to complement coal mining (Jayawickrama *et al.* 1993). As the global demand for wood increased in the 1980's, government loans subsidised the planting of additional *Eucalyptus* trees. Currently almost 360 000 ha of *Eucalyptus* spp. have been established in Chile and more than two-thirds of the plantations are under 5-years-old (INFOR 2001, Kliejunas *et al.* 2001).

The most widely planted *Eucalyptus* species in Chile are *E. globulus*, which is distributed, from central to Southern Chile and *E. nitens*, planted in colder areas. *E. globulus*, is adaptable to a wide range of climatic and soil conditions, flourishing along the coast and inland areas that are not subjected to severe frost. They grow best in areas with 800 mm or more of precipitation (Jayawickrama *et al.* 1993). *E. nitens* has also been widely planted due to its rapid growth, desirable pulping properties, and frost tolerance. It is estimated that both *E. globulus* and *E. nitens* can grow at 40 m^3/ha/yr on sites such as the coastal area of the Southern-central region of Chile (Kliejunas *et al.* 2001). Rotations between 8 to 10 years are envisaged for pulpwood production and up to 25 years for solid wood production

(Jayawickrama *et al.* 1993). Other species of *Eucalyptus* such as *E. camaldulensis* Dehnhardt, *E. cladocolyx* F. Müeller and *E. siderexylon* A. Cunningham: Woolls are planted in the drier, northern and central areas of Chile. *Eucalyptus regnans* F. Muell., *E. fastigata* Deane & Maiden, *E. delegatensis* R.T. Barker and *E. viminalis* Labill. have also shown promise in the South of Chile (Jayawickrama *et al.* 1993, INFOR 1997).

In addition to the most important products obtained from *Eucalyptus* spp., other less important uses include fuel, veneers, mouldings, fence posts, firewood, and structural elements (Jayawickrama *et al.* 1993). The consumption of *Eucalyptus* products during 2000 in Chile was approximately 4 million m^3 (INFOR 2001). Harvest of plantation-grown *Eucalyptus* spp. is predicted to increase from 2 million m^3 in 1995 to more than 7 million m^3 by 2004 (Brownie 1996). The future stock of wood for the next rotation (10 years) is calculated at approximately 27.4 million m^3 (INFOR 2002).

3.0 INSECT AND DISEASE PROBLEMS IN COMMERCIAL PLANTATIONS

Pinus and *Eucalyptus* species in Chilean forestry have not been without pest and pathogen problems. During the last 15 years several insects and diseases have been detected in the country. Forestry companies have been conducting surveys, plantation assessments and strategies including chemical and biological control against these pest and disease problems in order to reduce their impact. Commercial plantations were considered virtually free from insect pests and diseases for several decades (Alfaro & Singh 1997). Viewed in the context of the number of diseases and insects that could damage *P. radiata* and *Eucalyptus* spp. in Chile, the country has been relatively fortunate in having avoided the impact of many of these pests and diseases (Wingfield 2001, unpublished report).

Almost every year since 1997, a new pest or disease has been detected in *Pinus* or *Eucalyptus* plantations in Chile (Table 2). This unpredictable increase of various problems, can be attributed to the increase in the extent of commercial plantations, an increase in world trade in unmanufactured wood articles, as well as, an increase of tourist activity (Gonzalez & Parra 1994, Tkacz 2002). The main pest and pathogen problems in both *Pinus* and *Eucalyptus* plantations are described in the following section.

3.1 *Pinus* Plantations

Pinus radiata has been considered as a healthy species in Chile, despite its high susceptibility to several insect pests and diseases, such as the pine shoot moth (*R. buoliana*), Dothistroma needle blight (*D. septospora*) and Pitch canker (*F. circinatum*). More exotic than native insects have been considered important in terms of damage they may cause to commercial plantations of *P. radiata*. Lanfranco (2000) classified these species according to their taxonomic group, origin, type of damage, and risk or importance to commercial plantations (Table 1).

3.1.1 Insects

Chile is considered relatively fortunate in terms of the presence of insect pests in commercial *Pinus* plantations (Alfaro & Singh 1997). Since 1981, when *Hylurgus ligniperda* Fabr. and *Hylastes ater* Payk. were detected on commercially grown trees, only two introduced pest species have been reported. Of these, *R. buoliana* is by far the most important insect pest affecting Chilean plantations. In addition *Sirex noctilio* Fabr. is a species that has recently reached Chile and is one of the species with a high potential to cause damage (Iede, Penteado & Schaitza 1998, Rawlings & Wilson 1988). Native insects have also been reported in *P. radiata* plantations and some of them have resulted in significant but infrequent damage (Lanfranco 2000).

Bark Beetles

Many forest entomologists consider bark beetles to be one of the most economically important groups of forest insects. They infest logs or pine cargo crates containing strips of bark and in this way, they have been spread to various countries (Sato 1975, Ciesla 1993). In 1981, the first exotic insect pests were detected in Chilean *P. radiata* plantations. These were two bark beetle (Coleoptera: Scolytidae) species (*H. ligniperda* and *H. ater*). Later, *Orthotomicus erosus* Wollaston was also introduced into Chile (1983), apparently in containers from Europe (Ciesla 1993, Lanfranco 2000).

Bark beetles are known to vector pathogenic fungi. These include the genera *Ophiostoma* H. & P. Sydow, *Ceratocystis* Ellis & Halsted and *Leptographium* Lagerberg & Melin

(Upadhyay 1981, Wingfield, Seifert & Webber 1993). Some of these fungi can lead to serious tree diseases, and the majority cause blue stain of timber (Jacobs & Wingfield 2001). In Chile, emerging *H. ater* adults feed on the roots of one-to-two year old seedlings and on the root collars, causing tree mortality (Ciesla 1993). However, these insects have not resulted in severe damage in commercial plantations in Chile. They are, however, considered as potentially damaging in the USA (Ide *et al.* 2000, Tkacz 2002). These bark beetles could potentially be effective vectors for black stain root disease (*Leptographium wageneri* (Kendrick) M.J. Wingf.), which is one of the most serious pathogens of conifers in the western USA (Jacobs & Wingfield 2001, Tkacz 2002).

Recently, two ophiostomatoid fungi, *Ophiostoma ips* (Rumbold) Nannfeldt and *Ceratocystiopsis minuta* (Siem.) Upadhyay & Kendrick have been associated with *H. ligniperda* in Chile (Zhou *et al.* 2002). *Hylastes ater* has also been associated with other ophiostomatoid fungi such as *Ophiostoma geleiformis* (Bakshi) Mathiesen-Käärik, *O. huntii* (Rob.-Jeffr.) de Hoog & R. J. Scheff. and a *Pesotum* sp. (Zhou *et al.* 2002). Studies are currently underway to determine the full spectrum of fungal associates of *H. ater* and *H. ligniperda*, as well as to determine the potential for these insects to vector the pitch canker pathogen, *F. circinatum*.

Pine shoot moth

The European Pine Shoot Moth (EPSM) *R. buoliana* was the second exotic pine-infesting insect to be detected in Chile. This insect was first found in the country in 1985 (Espinoza, Lobos & Gomez 1986). Thus far, the pest has resulted in the most severe impact to commercial Chilean forestry (Cogollor & Ahumada 1990, Lanfranco 2000). The characteristic symptoms associated with EPSM is the presence of resin in the shoots, chlorosis of the shoot tip and needles and tip dieback, caused by larval feeding and mining, resulting in shoot destruction (Figure 2). When the attack is in the main shoot or stem, some trees can recover but the majority will be deformed (Figure 2), (Ahumada & Smith 1993, Alzamora *et al.* 1996, Ide & Lanfranco 1996).

Reduction in growth of trees due to EPSM has been calculated to be approximately 20 - 40%, thus causing a substantial overall loss of production (Alvarez de Araya, Ramirez & Parra 1991). The distribution of *R. buoliana* has expanded at a rate of approximately 50

km/year. This relatively local movement is attributed to adult insect dispersal, which is facilitated by wind currents and, cloud mass movement. In addition, insects have been recorded to be moved on vegetative material (branches and small trees) to be transported between different areas in Chile (CONAF 1998, Bioforest unpublished report).

A substantial effort has been instituted by the various forestry companies, collectively with the Chilean Forest Service as the lead agency, to control *R. buoliana*. This includes chemical control and, silvicultural management to reduce pest populations. Actions also include biological control measures using both a native (*Coccygomimus fuscipes* Brullé; Hymenoptera: Ichneumonidae) and an introduced parasite (*Orgilus obscurator* Ness.;Hymenoptera, Braconidae) (Lanfranco & Cerda 1986, Lanfranco 1996, Bravo & Ahumada 1998a). Good success has been achieved in Chile with these biological control agents (Ahumada, Alfaro & Ojeda 1999). Ten years after the first introduction of the exotic parasite from Europe, parasitism levels of 80 % have been achieved (Ahumada & Brieva 2000).

Sirex wood wasp

Sirex noctilio was detected at the beginning of 2001 in the central part of Chile. Its high potential to damage pine plantations, is due to its capacity to attack and spread more than 100 km/yr. (Tribe 1997). However, in Chile the insect infestation is still concentrated around the area where it was originally detected (SAG 2001, unpublished report) and has not caused any damage in commercial plantations. Both forest service and forestry companies have already prepared a strategy for its control, based on biological control with the nematode *Deladenus siricidicola* Bedding and improved plantation management. These strategies have proven affective in management of this pest in Australia, Brazil and New Zealand (Rawlings & Wilson 1988, Haugen & Underdown 1990, Iede, Penteado & Schaitza 1998).

Native insects

Some native insects have developed the capacity to damage pine plantations in Chile. These insects result in damage, particularly associated with long periods of drought, poor site quality, stress due to inadequate silvicultural practices or opportunistic behaviour of

the insects (Lanfranco 2000). Very little research has been undertaken in order to understand the impact of these native pest outbreaks. The most important damage has been caused by species of *Ormiscodes*, *Rhyephenes*, *Antandrus*, *Coniungoptera* (Lewis 1996, Lanfranco 2000). Bioforest, a research company of the Arauco Forestry group, has been studying the life cycle of two native insects, *Antandrus viridis* Blanchard (*Acrididae*) and *Coniungoptera nothofagi* Rentz & Gurney (*Tettigonidae*), as well their impact in commercial plantations (Ahumada & Brieva 2001).

3.1.2 Diseases

Disease caused by introduced pathogens have not been less important than insect pests in terms of impact on commercial plantations. Some of the more important pathogens are *Sphaeropsis sapinea* (Fr.) Dyko & Sutton, *D. septospora* and recently *F. circinatum* (Wingfield 2001, 2002 unpublished reports). Other less important pathogens in plantations include *Cyclaneusma minus* (Butin) DiCosmo, Peredo & Minter, *Ceratocystis picea* (Munch) Bakshi (=*Ophiostoma piceae* (Münch) H. & P. Sydow), *C. pilifera* (Fr.) C. Moreau (=*Ophiostoma pilifera* (Fries) H. & P. Sydow) and *Armillaria mellea* (Vahl ex Fr.) Kumm. *sensu lato*. In nurseries, fungi such as *Macrophomina phaseolina* (Tassi) Goid., *Colletotrichum acutatum* Simmds. f. *sp. pinea* Dingley & Gilmour, *F. oxysporum* Snyder & Hansen, have been recorded. None of these fungi are considered to be associated with serious problems at this time (Mujica & Vergara 1980, Butin & Peredo 1986).

Recently, Zhou *et al.* (2002) isolated five Ophiostomatoid fungi *C. minuta* and *O. ips* from *H. ligniperda* and *O. geleiformis*, *O. huntii* and *O. quercus* (Georgévitch) Nannfeldt from galleries and *H. ater*. These are the first records of the fungi from Chile. Further studies on these fungi are planned and these will especially concentrate on their importance.

***Sphaeropsis sapinea* shoot blight**

The first pathogen reported in Chilean *P. radiata* plantations was *S. sapinea*, which was detected in 1942 (Gonzalez & Jorquera 1997, Lanfranco 2000). Since its detection, it has only been a problem occasionally, specifically after high stress situations, where trees have displayed dieback in several commercial plantations (Butin & Peredo 1986). One of the concerns with this disease is that, the pathogen has the capacity to enter through prunning

wounds and if this should occur, these trees might be more susceptible to attack by the Sirex woodwasp.

Dothistroma needle blight

Dothistroma septospora (teleomorph: *Mycosphaerella pini* Rostr.) has been the most important disease affecting Chilean commercial plantations of *P. radiata*. This tree species is considered as one of the most susceptible species to Dothistroma needle blight (Evans 1984, Butin & Peredo 1986). The disease was first reported from Chile in 1965 (Dubin 1965) and the pathogen is present in most of the commercial plantation areas (35° S - 42° S), especially in the Southern (below 41° S) area of Chile (Lanco, Valdivia, Los Lagos, La Union).

Because Dothistroma needle blight is the most important disease affecting commercial pine plantations, much research has been conducted since the disease was first reported in Chile. This research has been focused on biology, life cycle, mechanism of infection, foliage loss, management and economic impact (Bustamante 2000). However, much remains to be known regarding the disease in Chile, especially issues pertaining to impact and control.

Dothistroma needle blight occurs when the conidia of *D. septospora* are liberated from the fungal fruiting bodies into a film of water, from where they are dispersed by water-droplets (Gibson, Christensen & Munga 1964). Conidia, which land on needles of susceptible host species germinate, and penetrate through the stomata (Gadgil 1967). In Kaingaroa, New Zealand, spray trials on 13-year-old *P. radiata* trees has indicated that trees with 25 % or more infection, sprayed on three occasions, have 30 to 40 m^3/ha more volume at the end of a rotation, compared to unsprayed trees (Kershaw *et al.* 1988). Other studies have shown that the height of the trees is affected if the defoliation is greater than 30 % to 40 % (Van der Pas 1981). Similarly, the increment of the tree is decreased by 50%, if the infection level is more than 50 % of the active foliage (Van der Pas 1981, Whyte 1969). In Chile, several studies have shown that with infection levels lower than 25 %, effects on growth are unimportant. However, if the infection levels are greater than 50 %, the annual increment is strongly affected (Perez 1973, Contreras 1988, Hauer 2000, Bioforest 2000 unpublished reports).

Studies carried out in Chile have shown that Dothistroma needle-blight levels start to increase in winter and reach a peak in early summer. Thereafter, they drop in late summer, and remain stable in autumn, before starting to increase again in winter (Bulman, Gadgil & Ahumada 2000, unpublished report). Research conducted in the USA has shown that *Pinus nigra* Arnold. and *P. ponderosa* (Watson) Weber, are two of the most susceptible pines in central and eastern USA, while *P. radiata* and *P. contorta* (Engelm.) Critchfield are severely affected in California and the Pacific Northwest (Christensen 1998, Peterson 1965, Peterson 1982, Peterson & Wysong 1986, Sinclair, Llyon & Johnson 1987). In both cases those species suffer severe infection during wet periods from May to October (Christensen 1998).

The control strategy for Dothistroma needle blight used in the USA, New Zealand and Chile has been similar. Aerial application of chemicals based on copper fungicides has been implemented as one of the most important control measures (Bulman & Gadgil, pers. comm.). In all three countries, two spray applications have been used when the infection has been greater than 30 % of the tree crowns. In USA, the first application is made in later spring (May), and the second in midsummer (July) (Peterson 1982). In New Zealand, the first application is also between spring and summer (Bulman & Gadgil, pers. comm.). The situation in Chile does not differ much from that in the USA and New Zealand and, chemical control is applied in the late spring and summer. New Zealand and Chile have implemented an intensive silvicultural management (pruning and thinning) in most of the commercial plantations and the positive impact of these silvicultural strategies is known (Bioforest 2000, unpublished report). In the past ten-years, thousands of hectares (between 3 to 10-year-old) were sprayed once or twice per year, depending on infection levels. Particularly in young plantations, the amount of inoculum was drastically reduced (Bioforest 2000, unpublished report) (Figure 3).

Experience in the past with selection of trees resistant to Dothistroma needle blight, has been inconsistent. However, evidence from New Zealand, Australia and USA suggests that different families of *P. radiata* differ in their susceptibility to *D. septospora* (Contreras 1988, Elmudesi 1992), (Figure 4). This aspect of the disease deserves significantly more attention in the future (Peterson 1982, Wingfield 2001, unpublished report).

Pitch canker

The most recent pathogen to be detected on pine trees in Chile is the pitch canker fungus, *F. circinatum*, which was first reported in 2001 (Wingfield *et al.* 2002). Pitch canker is one of the most serious fungal diseases affecting *Pinus* spp. in the world (Viljoen *et al.* 1997a, Britz *et al.* 2001, Wingfield *et al.* 2001, 2002). At present, very little is known about the pathogen in Chile and damage has only been to *P. radiata* plants in nurseries (Figure 5). The disease has, as yet, not been found on older trees in plantations (Wingfield *et al.* 2002). Nonetheless, the presence of the disease in Chile is of substantial concern, not only to this country but also to other countries growing *P. radiata* (Wingfield 2001, 2002, unpublished reports).

Fusarium circinatum has caused significant losses on a wide variety of pine species (Dwinell, Barrows-Broaddus & Kuhlman 1985, Viljoen *et al.* 1995, 1997b, Gordon, Storer & Wood 2001). The pathogen has been isolated from different parts of the trees, including stems, branches, cones and root collars of seedlings (Viljoen 1994, Britz *et al.* 2001). At present, it is difficult to predict how pitch canker will affect the commercial plantations in Chile. One concern is that the pathogen might be able to enter wounds made by insects such as *R. buoliana* and *H. ater*. These aspects of the disease in Chile will need to receive careful study in the future (Wingfield 2001, 2002, unpublished reports).

Armillaria root rot

Armillaria spp. are pathogens with a worldwide distribution, that usually exist as saprophytes on stems and roots, but can also infect living tissue (Butin & Peredo 1986, Pronos & Patton 1977). The disease usually referred to as Armillaria root rot, causes reduction in growth, yellowing and browning of foliage until death. Damage is especially severe on stressed trees. In *P. radiata* plantations in Chile, however, this pathogen has not been important (Osorio, pers. comm.)

Native pathogens

Thus far, the Chilean literature has registered very little information regarding important disease outbreaks caused by native pathogens. Little research has been conducted in order to identify and understand native fungi, adapted or present in these productive plantations.

3.2 *Eucalyptus* Plantations

In contrast to pine plantations, very little attention has been given to pests and diseases in plantations of *Eucalyptus* spp. Since the early 1980's preliminary assessments have been conducted in order to determine the general disease condition within Chilean *Eucalyptus* plantations. One of the first national assessments of *Eucalyptus* plantations was made in 1993 by Government agencies and private forestry companies. From these evaluations, it was found that Eucalyptus leaf spot was the most important problem associated with commercial *Eucalytus* plantations. In contrast there was no evidence of any significant insect problems (CONAF 1994, CONAF 2002).

3.2.1 Insects

Eucalyptus Longhorn borer

One of the first insects reported affecting *Eucalyptus* spp. in Chile, was *Phoracantha semipunctata* Fabricius (Coleoptera: Cerambycidae). This insect was discovered in 1973 on *E. globulus* in the central region of Chile, where it began to cause problems in plantations subjected to water stress, due to low precipitation or to soils with poor water retention (Cogollor 1986, 1991). This insect has been well studied and its life cycle, symptoms, damage and control strategies are well understood (Cogollor 1986). In 1998, the parasite *Avetianella longoi* Siscaro (Hymenoptera: Encyrtidae), was introduced into Chile from South Africa and biological control has already proven useful (SAG, unpublished report).

In 1997, *Phoracantha recurva* Newman (Coleoptera: Cerambycidae), was detected in Chile for the first time (Perez & Pinar 1999). This species has not been a problem and has been confined to the area where it was first detected (Beeche, pers. comm.).

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Australian weevil

Gonipterus scutellatus Gyllenhal (Coleoptera: Curculionidae) was detected for the first time in Chile in 1998, and it was apparently introduced from Argentina (Bravo & Ahumada 1998b, SAG 1999). Like *P. semipunctata*, *G. scutellatus* is native to Australia and both adult and larval stages feed on the foliage of a wide range of *Eucalyptus* spp. It particularly favours *E. globulus* and *E. viminalis*. There have been few serious outbreaks of this insect and it is not regarded as a major pest of *Eucalyptus* in Australia. However, it has become a major pest in many countries where *Eucalyptus* spp. are grown as an exotic plantation species (Ojeda 1994). After the initial detection of *G. scutellatus*, some small areas planted with *E. globulus* showed damage produced by larvae and adults of *Gonipterus* (Figure 6). The first action undertaken was to spray the affected area with insecticides (SAG 1999). Subsequently the biocontrol agent, *Anaphes nitens* Girault (Hymenoptera: Mymaridae), was also introduced into Chile from South Africa. Thus far, *G. scutellatus* has been confined in the area where it was originally detected and, the population has remained at a low level (Beeche, pers. comm.).

Blue Gum Psyllid

The insect pest most recently detected on *Eucalyptus* in Chile, is the blue gum psyllid, *Ctenarytaina eucalypti* Maskell, (Homoptera: Psyllidae), which was reported in 1999. Since its original detection, the psyllid has expanded its distribution in a short time and now occurs throughout all commercial plantations of Chile (Lanfranco & Dungey 2001). Clusters of yellow eggs are deposited at the bases of the terminal leaves and axial buds. The nymphs settle and feed on the leaves or stems, where the eggs are deposited. The nymphs secrete wax-coated honeydew spheres as well as copious amounts of waxy filaments, which can conceal them from view. Feeding by psyllids causes acute damage, such as inhibition of new shoot formation and distortion in the shape of new leaves (Dahlsten *et al.* 1993, Hodkinson 1999). A variety of control measures have been taken in order to reduce the damage caused by the *C. eucalypti* insects in commercial plantations. These include chemical application using pyrethroids and biological control through releasing *Psyllaephagus pilosus* Noyes (Hym. Encyrtidae). In addition, the impact of the pest on *Eucalyptus* plantations is uncertain and is currently being evaluated (Bioforest, unpublished reports).

3.2.2 Diseases

Very little is known regarding *Eucalyptus* diseases in Chile. Indeed, this provides the key motivation for undertaking the studies presented in this thesis. The following section provides a brief review of the pathogens known to occur. Some of the studies that have been undertaken, have not been published and there is generally a paucity of knowledge of this topic.

One of the first sanitary assessments in *Eucalyptus* plantations in Chile was made by Mujica & Vergara (1980). In this study, the authors found the fungi *Fairmaniella leprosa* (Fairm.) Petrak. & Syd. and *Botrytis cinerea* Pers. damaging *E. globulus* foliage. Ten years later, Peredo (1990) reported the presence of *Hainesia lythri* (Desm.) Hohn. and subsequently, Gonzalez & Parra (1994), identified *Mycosphaerella cryptica* (Cooke) Hansf., *M. molleriana* (Thüm.) Lindau, *Harknessia globosa* B. Sutton, *Sonderhenia eucalypticola* (A.R. Davis) H.Swart & Walker, *Microthyrium* spp., *Pestalotiopsis* spp. and *Phoma* on *Eucalyptus globulus*.

Wingfield *et al.* (1995) provided a detailed description of several leaf pathogens of *Eucalyptus* spp. in Chile. They detected species such as *Aulographina eucalypti* (Cook & Mass) Arx & Muller, which is a well-known pathogen of older *Eucalyptus* foliage. In Chile it has, however, not been a problem but has been the cause of serious leaf spot in Australia, Brazil, New Zealand, United Kingdom and South Africa (Wall & Keane 1984, Ferreiras 1989, Dick 1982, Spooner 1981, Crous 1998). The second potentially important pathogen that Wingfield *et al.* (1995) found was a *Ceuthospora* sp. from *Eucalyptus* leaves and leaf litter. Little is known, however, of its pathogenicity. *Fairmaniella leprosa* was also found on *E. globulus* leaves, causing round to irregular, dark brown leaf spots, and appeared to be a common but unimportant leaf inhabitant (Wingfield *et al.* 1995). *Harknessia globosa* was isolated by Gonzalez & Parra (1994) and although this species appeared to be pathogenic, it was associated with only small lesions on *Eucalyptus* foliage.

Two species of *Mycosphaerella*, *M. cryptica* and *M. walkeri*, were isolated and identified through the observation of pseudothecia and acervuli of their anamorph (*Colletogloeopsis nubilosum* (Ganap. & Corbin) Crous & M.J.Wingf. and *Sonderhenia eucalypticola*, respectively). *Mycosphaerella cryptica* occurred on both juvenile and adult leaves of *E.*

globulus and *E. nitens* (Wingfield *et al.* 1995). This species causes lesions on leaves that vary from regular, circular spots to larger blotches (Park & Keane 1982) and is considered as one of the most destructive species on mature *Eucalyptus* foliage (Carnegie 2000). *Mycosphaerella walkeri* and its anamorph *S. eucalypticola* were also collected from *E. globulus* and *E. nitens* and often occurred in association with *M. cryptica*. Another species that was identified by Wingfield *et al.* (1995) was *Thyriopsis sphaerospora* Marasas, which according these authors, appears not to be a serious pathogen.

An epidemiological study of *Eucalyptus* foliar diseases was carried out by Gonzalez (1997) in different areas in the southern part of Chile, during 1996 and 1997. In this study, the health of species such as, *E. globulus*, *E. nitens*, *E. camaldulensis*, *E. viminales* and *E. delegatensis* was assessed. Gonzalez (1997) found *S. eucalypticola* on *E. globulus* and *E. viminalis*. Also, like Gonzales and Parra (1994) and Wingfield *et al.* (1995), he found *H. globosa* (*E. globulus* and *E. viminalis*) and *F. leprosa* (*E. globulus* and *E. viminalis*). Additionally, he was able to identify species of *Harknessia* (*E. globulus* and *E. viminalis*), *Coleophoma* sp. (*E. globulus* and *E. viminalis*), *Coniella minima* (Sutton & Thaung) Sutton (*E. viminalis*), *Pestalotiopsis* sp. (*E. delegatensis*), *Septoria* sp. (*E. globulus*), *Oidium* sp. (*E. globulus* and *E. nitens*) and *Hainesia lythri* (*E. globulus*). Gonzalez (1997) also found species belonging to the *Mycosphaerella* complex, such as *M. cryptica* on juvenile and adult leaves and from *E. globulus, E. nitens, E. viminalis* and *E. delegatensis*. *Mycosphaerella molleriana* was only found on juvenile leaves from *E. globulus* and *E. nitens* and *M. walkeri* on juvenile leaves from *E. globulus* and *E. viminalis*. *Mycosphaerella parva* was found only on *E. globulus*. In contrast to Wingfield *et al.* (1995), Gonzalez (1997) did not detect *A. eucalypti*.

Muñoz (1999) identified 29 pathogens (11 *Acomycetes* and 18 *Deuteromycetes*) from leaves of six *Eucalyptus* species. This study was based on morphological features, ascospore germination patterns and the presence of anamorph stages. Fifteen fungi were identified to species level; 11 to genus level and three were only assigned to class level. Of the 29 species identified, eight represented new records for Chile, including: *Botryosphaeria dothidea* (Mougeot ex Fries) Cesati & de Notaris, *Coleophoma empetri* (Rostr.) Petrak., *C. minima, H. eucalypti*, *Microsphaeropsis callista* (H. Syd) Sutton, *Sonderhenia eucalyptorum* (Hansf.) Swart and Walker, *Thyrinula eucalypti* (Cooke and Mass.) Swart and *Zoellneria eucalypti* (= *Torrendiella eucalypti auctt.*) Berk. and Dennis.

Another species found on both juvenile and adult leaves on *E. nitens* was a *Sphaerulina* sp., that according to the author was associated with *M. cryptica*. Muñoz (1999), also found *M. cryptica* on different ages of *E. globulus* leaves, including adult and intermediate leaves on *E. camaldulensis* and juvenile and adult leaves of *E. nitens*, *E. viminalis* and *E. delegatensis*.

Gonzalez (2000, unpublished report) examined different plantations of *E. globulus* with severe defoliation of both juvenile and adult leaves. *Mycosphaerella cryptica*, *M. molleriana* (as a synonym of *M. nubilosa*), *M. walkeri*, *S. eucalypticola* and *H. globosa* were identified in this study. Mohammed (2001, unpublished report), as well as Gonzalez (2000, unpublished report) surveyed similar areas of planted *E. globulus* and *E. nites* and identified *M. cryptica*, *M. walkeri*, *Harknesia* sp., *H. globosa*, *S. eucalypticola*, *Ceuthospora* and *Cladosporium*. However, all of these identifications, as well as others previously, were based only on morphological characteristics and ascospore germination patterns of *Mycosphaerella* ascospores.

Wingfield (2001, unpublished report) confirmed that the serious defoliation problem observed on juvenile leaves of *E. globulus* during the year 2000 was associated with two species of *Mycosphaerella*. One of these was tentatively identified as *M. marksii*. The other included smaller *Sonderhenia* type spots caused by *M. walkeri*, that has an asexual state known as *Sonderhenia eucalypticola*. However, he suggested that neither *Mycosphaerella* species nor any other biotic agent had caused the defoliation of adult *E. globulus* leaves. Wingfield (2001, unpublished report) also observed that some of the worst affected stands were on the best sites. *Eucalyptus nitens* stands, however, always showed lower levels of MLD than *E. globulus* stands. One site in Southern-central Chile (Valdivia area), distinct leaf spots that are rather different to those typical of *Mycosphaerella* infections was observed. According to the initial observations of Wingfield (2001), the fungus most closely associated with these spots is a species of *Sphaerulina*, which is characterized by 3-celled ascospores. Wingfield (2001, unpublished report), also noted a relatively serious dieback problem on *E. globulus* hedge plants apparently caused by the opportunistic basidiomycete, *Chondrostereum purpureum* (Pers:Fr) Pouzar, but did not find any evidence of *Mycosphaerella cryptica* or *M. nubilosa* on *Eucalyptus* spp. in Chile, as had been suggested previously.

4.0 CONCLUSIONS

As compared with the extent of damage that might potentially occur, Chile has been relatively fortunate in not having been seriously affected by diseases or pests in its extensive plantations of *P. radiata*. However, Dothistroma needle blight and Pine shoot moth have caused substantial damage and their impact might be viewed as an indication of the importance of pests and diseases in the country.

Eucalyptus plantations are relatively new in Chile and are much less extensive than those of *Pinus* spp. Thus far, the most important problem that has been recorded on these trees is Mycosphaerella leaf disease, which appears to be most important on juvenile leaves in *E. globulus* plantations, particularly in the Northern-central area of Chile (Arauco area).

All indications are that new pests and diseases are appearing in Chile with regular or even increased frequency. This is a trend that is likely to continue. The recent discovery of the devastating pitch canker pathogen in Chile is an example of the level of problems that can appear, relatively rapidly. Special attention will need to be given to protecting the forestry resource. Pests and diseases will be one of the most important constraints to the sustainability of exotic plantation forestry in the future and every effort must be made to reduce this threat.

Very little research has been conducted on *Eucalyptus* diseases in Chile. The studies that make up this thesis were, therefore, intended to improve this situation. They focus on *Mycosphaerella* spp. associated with leaf blight and *Botryosphaeria* spp. associated with tip dieback, both of which have been important in recent years. It is thus hoped that these studies will significantly improve our knowledge of the diseases associated with these complexes of pathogens.

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Table 1. Insects that cause damage on *Pinus radiata* plantations in Chile.

Order	Family	Specie	Origin	Damage	Risk Level
Coleoptera	*Anobiidae*	*Ernobius mollis*	Exotic	Bark	Low
	Buprestidae	*Buprestis novenmanculata*	Exotic	Bark	Low
	Cerambycidae	*Callideriphus laetus*	Native	Bark	Low
		Colobura alboplagiata	Native	Bark	Low
	Curculionidae	*Rhyephenes maillei*	Native	Bark	Low
	Scolytidae	*Hylastes ater*	Exotic	Bark	Low
		Hylurgus ligniperda	Exotic	Bark	Medium
		Orthotomicus erosus	Exotic	Bark	Low
		Xyleborinus saxeseni	Exotic	Bark	Low
Homoptera	*Adelgidae*	*Pineus borneri*	Exotic	Tissue	Low
Hymenoptera	*Siricidae*	*Urocerus gigas*	Exotic	Wood	Low
		Sirex noctilio	Exotic	Wood	High
Lepidoptera	*Psychidae*	*Thanatopsyche chilensis*	Native	Crown	Low
	Saturniidae	*Ormiscodes cinnamomea*	Native	Crown	Low
	Tortricidae	*Rhyacionia buoliana*	Exotic	Shoot	High
Orthoptera	*Acrididae*	*Antandrus viridis*	Native	Defoliation	Low
	Tettigoniidae	*Coniungoptera nothofagi*	Native	Defoliation	Low
Phasmatodea	*Pseudophasmatidae*	*Bacunculus phyllopus*	Native	Defoliation	Low

Table 2. Chronological order of detection of pests and pathogens in Chile. Graphic representation from the data.

Insect or Pathogen	Year of detection	Host
Pineus borneri	1900	*P. radiata*
Buprestis novenmaculata	1940	*P. radiata*
Sphaeropsis sapinea	1942	*P. radiata*
Xyleborinus saxeseni	1953	*P. radiata*
Ernobius mollis	1963	*P. radiata*
Dothistroma septospora	1965	*P. radiata*
Phoracantha semipunctata	1973	*Eucalyptus spp.*
Hylurgus ligniperda	1981	*P. radiata*
Hylastes ater	1981	*P. radiata*
Orthotomicus erosus	1983	*P. radiata*
Rhyacionia buoliana	1995	*P. radiata*
Phoracantha recurva	1997	*Eucalyptus spp.*
Gonipterus scutellatus	1998	*Eucalyptus spp.*
Cteranytaina eucalypti	1999	*Eucalyptus spp.*
Sirex noctilio	2001	*P. radiata*
Fusarium circinatum	2001	*P. radiata*

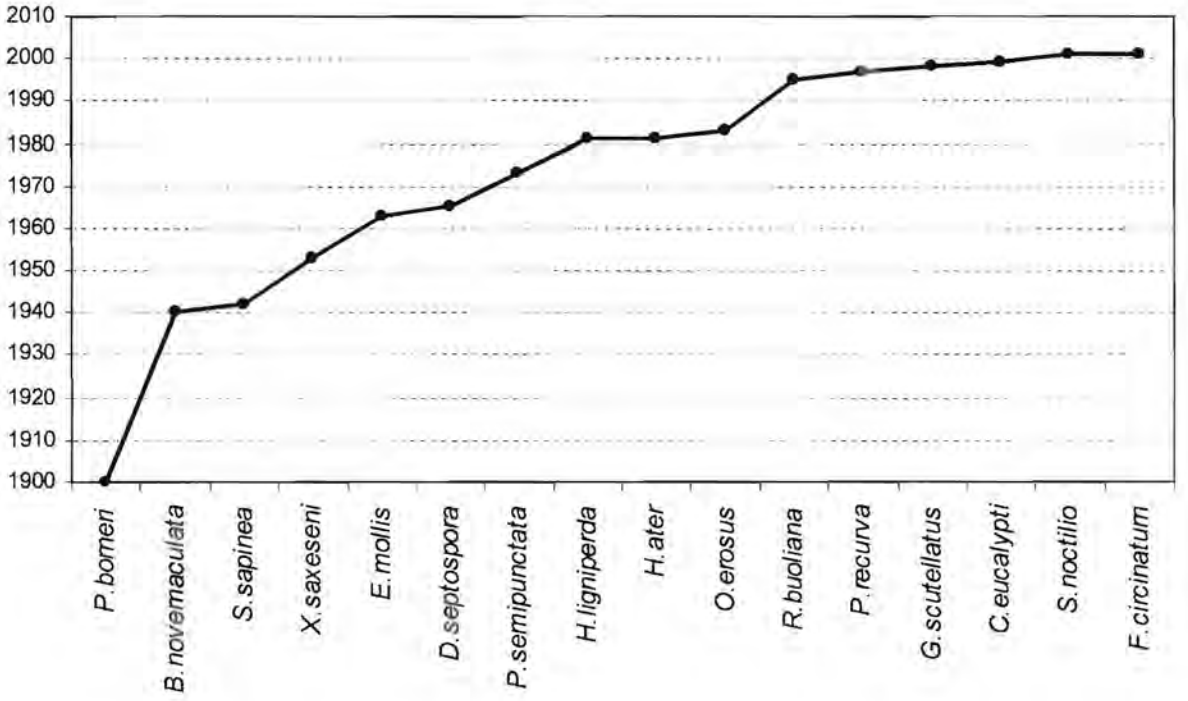

Figure 1. Administrative regions (from I to XII), geographic distribution and area of the commercial plantations in Chile (1000 x ha).

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Plantations (1000 x ha) **Administrative Regions of Chile**

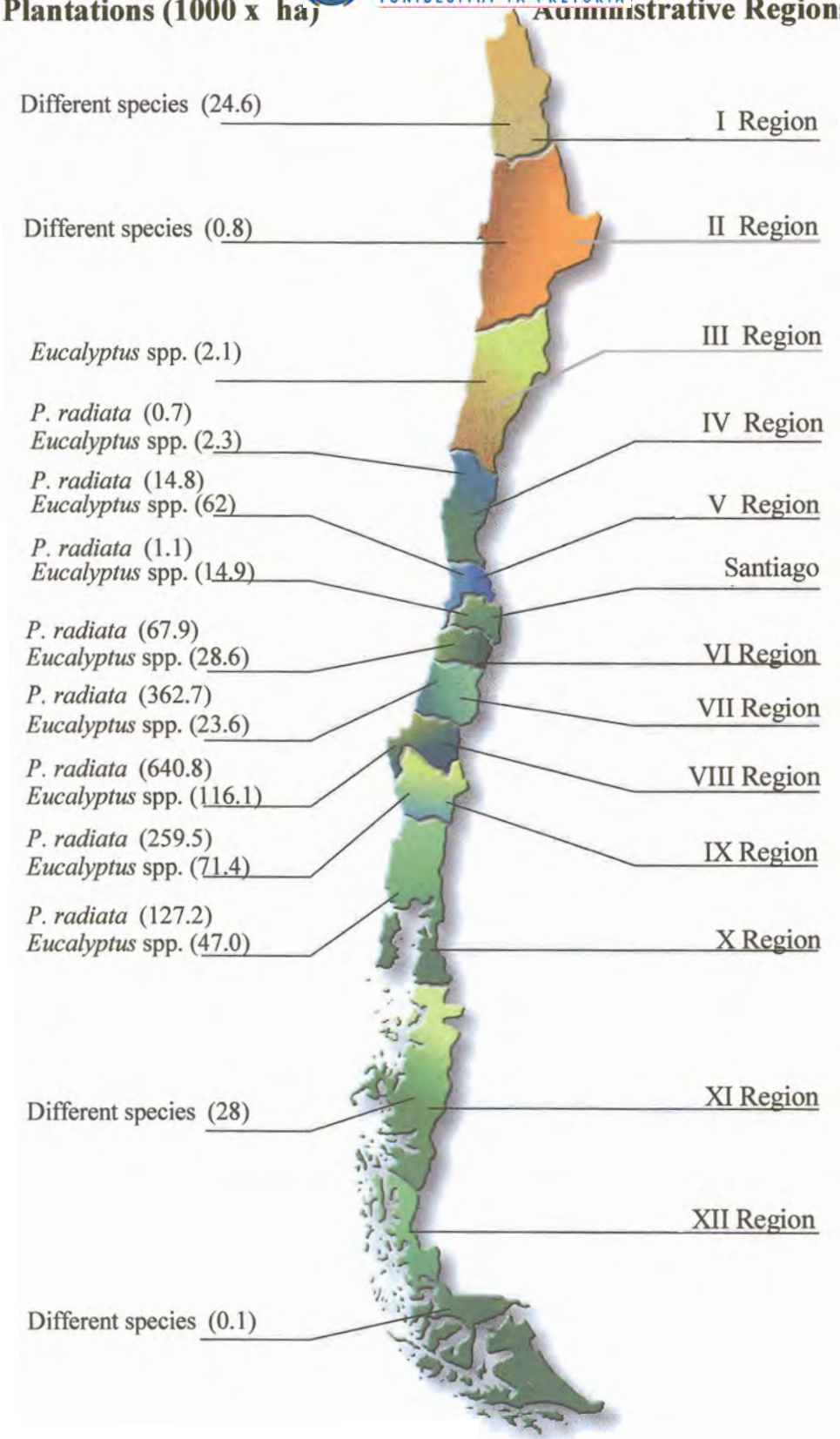

Figure 2. *Rhyacionia buoliana* on *Pinus radiata*. (**A**) Larvae feeding on a pine shoot (**B**) Initial deformation and shoot damaged (**C**) Chlorotic shoot due to a high infestation level (**D**) Apical deformation (**E**) Stem deformation.

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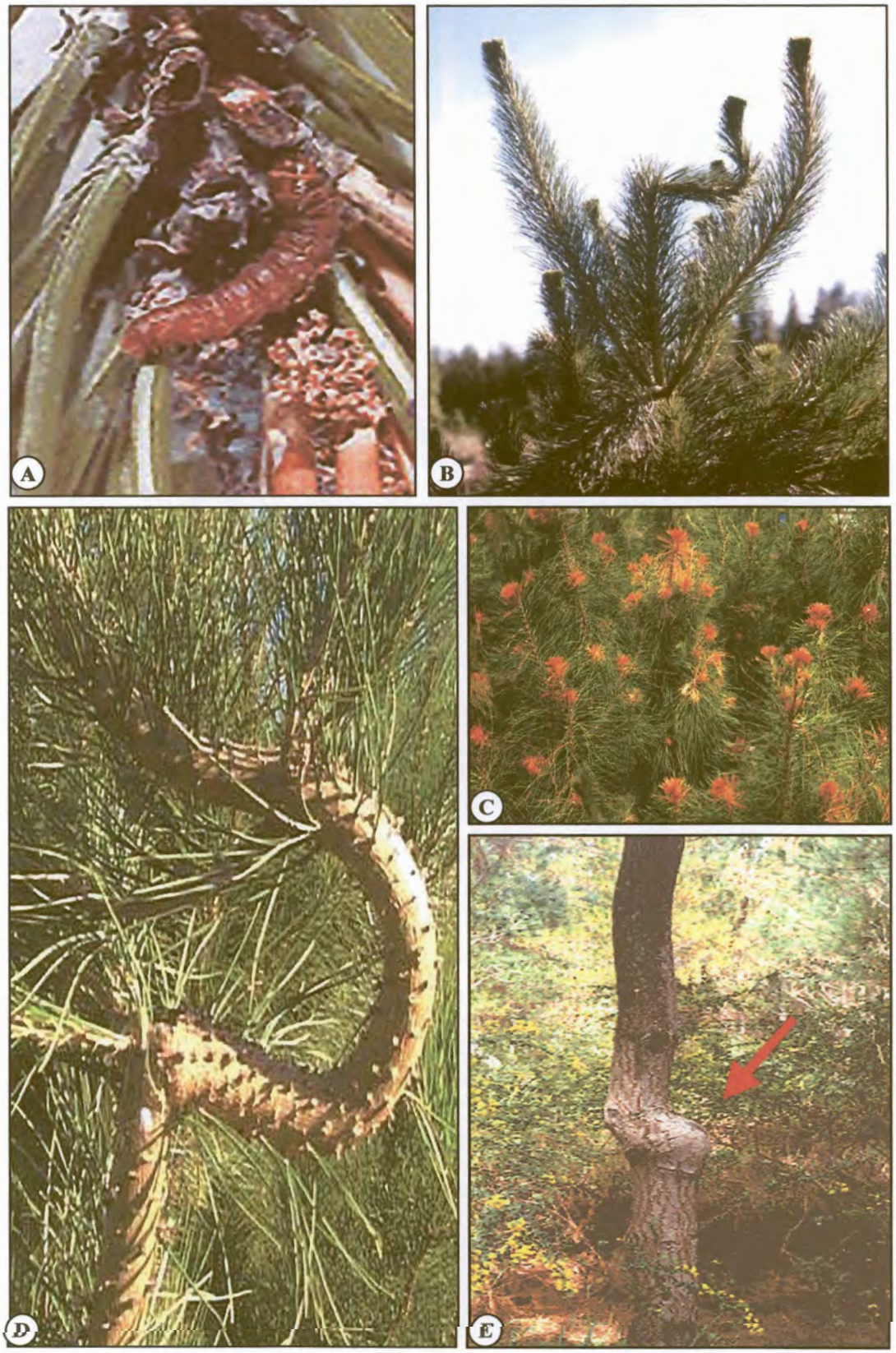

Figure 3. Damage caused by *Dothistroma septospora* (**A**) Plantation with silvicultural management (thinning and pruning) and chemical control (**B**) Plantation without management and chemical control.

A

B

Figure 4. Susceptibility to *Dothistroma* (**A**) Tree with low susceptibility (**B**, **C**) Trees with high susceptibility to *Dothistroma* attack.

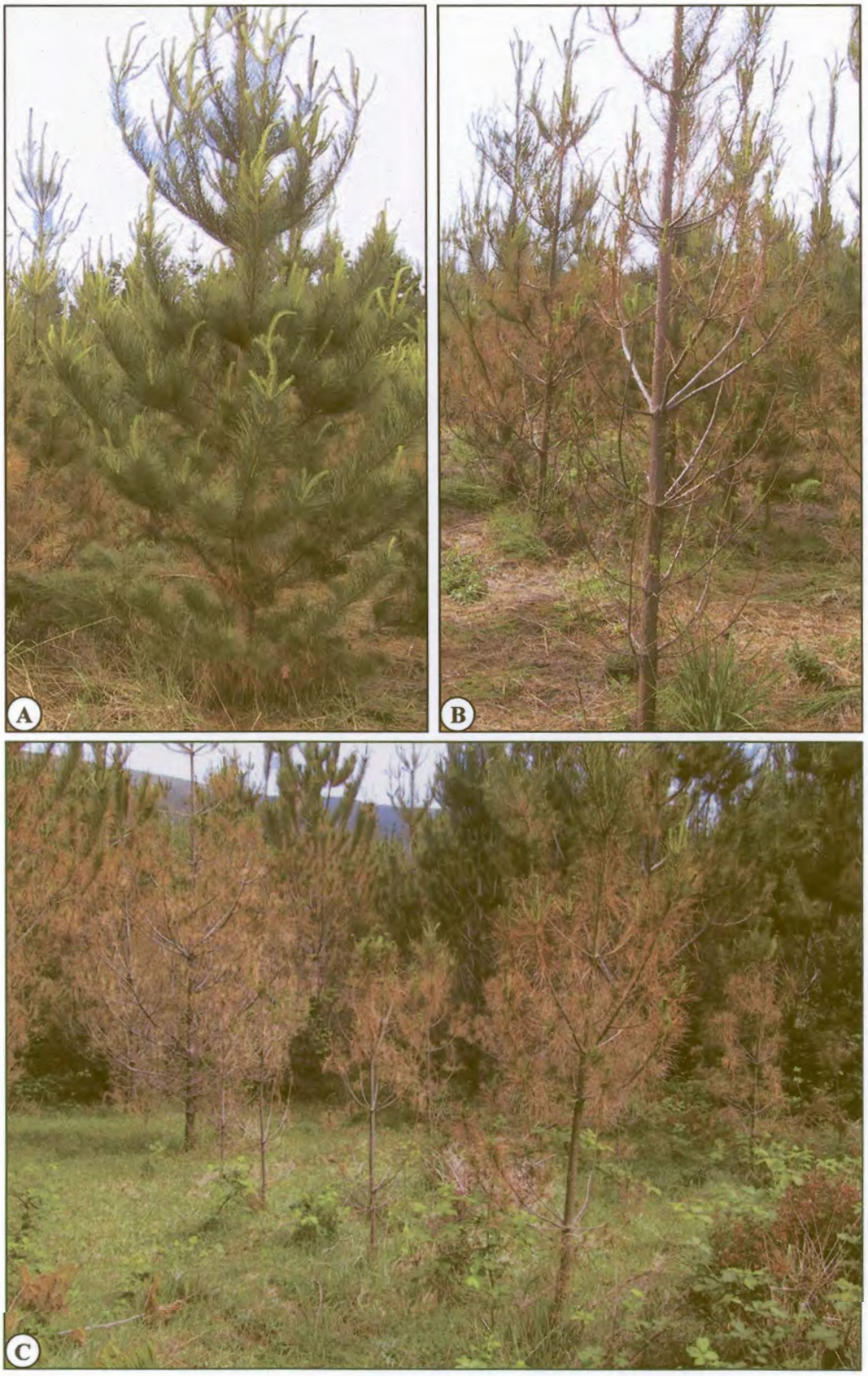
A
B
C

Figure 5.Pitch canker present in Chilean nurseries (**A**) Plants dead by *Fusarium circinatum* attack (**B**) Pitch canker symptoms in dying plants in a nursery from Chile.

A

B

Figure 6. *Gonipterus scuttelatus* on *Eucalyptus globulus* plantations in Chile (**A**) Adult insect (**B**) Larvae (**C**) Characteristic damage in leaves due to adult feeding (**D**) Damage on the plantation where the insect was detected in Chile.

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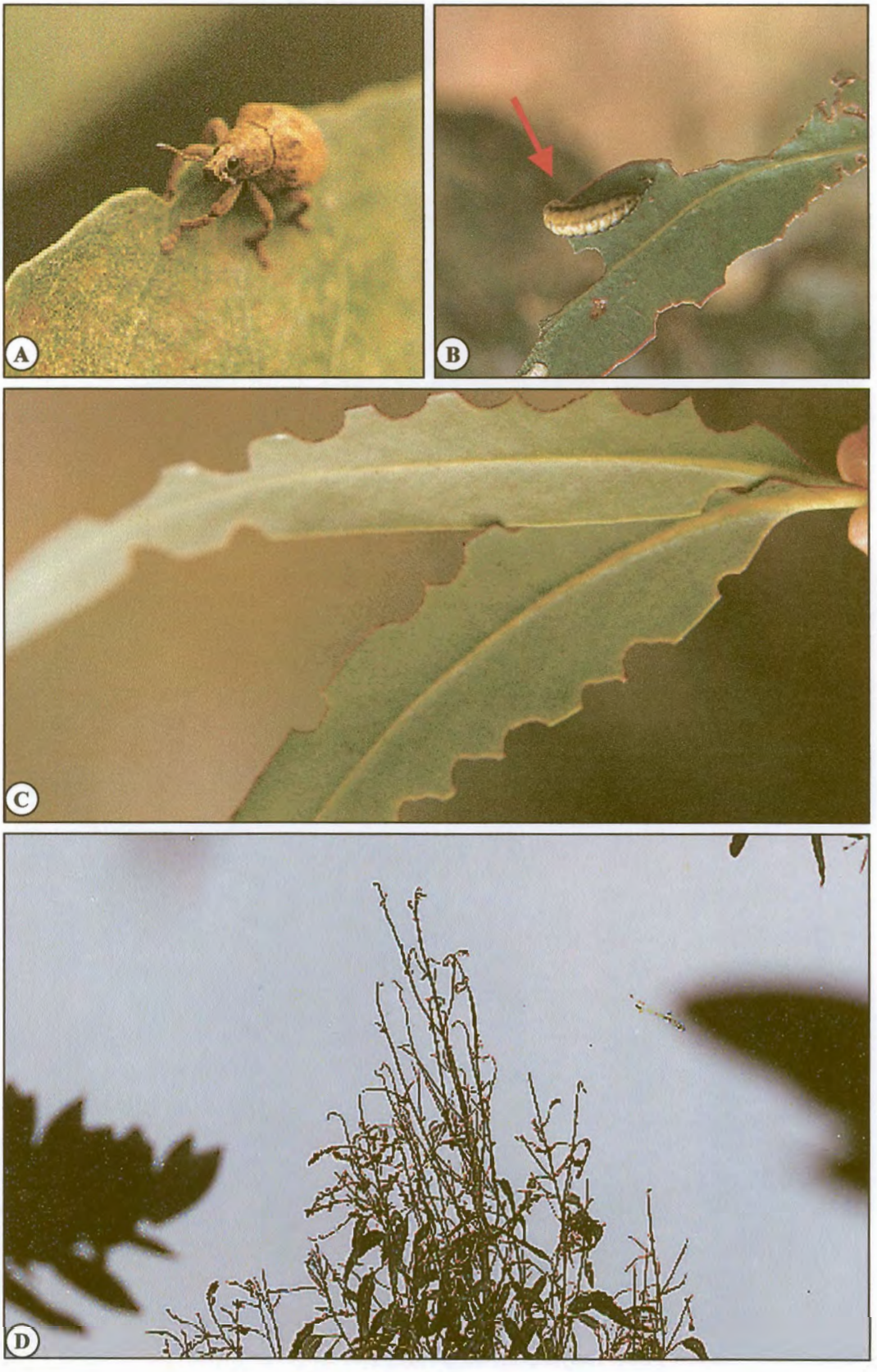

Chapter 2

MYCOSPHAERELLA SPECIES CAUSING LEAF BLOTCH DISEASE ON *EUCALYPTUS* SPP. IN CHILE

ABSTRACT

Chile is one of the largest exporters of wood products produced in exotic plantations of *Pinus radiata* and *Eucalyptus* spp. *Eucalyptus globulus* and *E. nitens* are primarily propagated and these represent the second largest commercial plantation resource, after *P. Radiata*. With the expansion of *Eucalyptus* plantations, new disease problems have emerged in Chile. Of these, *Mycosphaerella* leaf disease (MLD) has recently been recognized as one of the most important threats. Little research has been conducted on *Mycosphaerella* spp. on *Eucalyptus* in Chile and their identity is poorly understood. The aim of this study was, to collect and identity these fungi. Diseased adult and juvenile *E. globulus* and *E. nitens* leaves were collected from plantations in Northern-central and Southern-central Chile. Species of *Mycosphaerella* were identified based on ascospore germination patterns, cultural characteristics and sequence data from the Internal Transcribed Spacer (ITS) region of the ribosomal RNA (rRNA) operon. Results showed clearly that six *Mycosphaerella* spp. are associated with *Eucalyptus* leaf disease in the plantations surveyed. These species include *M. africana, M. grandis, M. lateralis, M. molleriana, M. parkii* and *M. walkeri*. Five of these species, *M. africana, M. grandis, M. lateralis, M. molleriana,* and *M. parkii* represent new records for Chile. Previous suggestions that the important pathogens, *M. cryptica* and *M. nubilosa* are present in Chile have not been confirmed in this study. We are currently considering the importance of the *Mycosphaerella* spp. identified in this study, and this must be investigated in order to develop effective management strategies to reduce their impact.

INTRODUCTION

Chile is one of the largest producers of wood products in the world and this industry depends on rapidly growing exotic *Pinus radiata* D. Don and *Eucalyptus* spp. A total of 11% of the country's land is forested and about 2.1 million ha is utilised for commercial plantations (Lowy 1995, WFI 1999). *Eucalyptus* spp. are the second most commonly planted forest tree species. *Eucalyptus* trees were introduced into Chile in 1823 but commercial planting only started in the 1930's. Currently, there are almost 360 000 ha of *Eucalyptus* spp. established in Chile (Jayawickrama, Schlatter & Escobar 1993, INFOR 2002).

Eucalyptus spp. in Chile were considered relatively healthy for several decades until the outbreaks of leaf blotch and leaf spot disease caused by *Mycosphaerella* spp. This newly recorded problems suggest that pressure due to disease and pest problems is increasing markedly in Chile. Forest protection will clearly need to be augmented to ensure the long-term sustainability of forestry in the country.

Eucalyptus globulus and *E. nitens* are two of the most widely planted species in Chile. *E. globulus* has been damaged by several pathogens and insects in the past, but the most severe damage has been caused by species of *Mycosphaerella* Johanson. These fungi are well known as important pathogens of *Eucalyptus* spp. elsewhere and in some countries, they are considered amongst the most important constraints to *Eucalyptus* propagation (Carnegie 2000, Crous 1998, Dungey *et al.* 1997).

The genus *Mycosphaerella* is one of the largest genera of Ascomycetes. Approximately 2000 species have been described and have generaly been identified based on the hosts that they infect (Corlett 1991, 1995). The taxonomy and identification of *Mycosphaerella* spp. is complicated. Numerous characters, such as host, lesion characteristics, ascospore germination patterns, cultural characteristics and anamorph-teleomorph connections have been used to identify these fungi (Crous 1998, 1999, Crous *et al.* 1999, 2001). However, the wide variation within species and the overlap between them has led to confusion in the identification process (Carnegie 2000, Hunter 2002). Recently, molecular techniques and particularly analysis of DNA sequences have contributed considerably to the identification

of *Mycosphaerella* spp. (Crous *et al.* 1999, Stewart *et al.* 1999, Crous *et al.* 2000, Carnegie *et al.* 2001, Hunter 2002).

The aim of this study was to identify the *Mycosphaerella* species causing damage on *Eucalyptus* plantations in Chile. To achieve this objective, surveys were conducted in *E. globulus* and *E. nitens* plantations located in the Northern-central and Southern-central area of Chile. Samples were then examined and *Mycosphaerella* spp. identified using traditional morphological techniques as well as DNA sequence comparisons.

MATERIALS AND METHODS

Collection of Samples

Between April 2001 and April 2002, leaves showing symptoms of MLD were collected from *Eucalyptus* trees in Chile. These samples were collected from *E. globulus* and *E. nitens* trees located in 24 different plantations. The plantations were located in different regions of the Northern and Southern parts of the forestry region (Figure 1). Fifteen infected leaves were collected from each sampled tree and these included both juvenile and adult leaves. Leaves were stored at 4 °C in brown paper envelopes. The samples were then transported to the laboratory for further identification.

Isolations and preparation of cultures

Five leaves from each sample were chosen for the isolation of *Mycosphaerella* spp. Lesions were excised and placed in Petri dishes with water for two hours, after which they were attached, using double-sided adhesive tape, to the lids of 60 mm Petri dishes over 2% malt extract agar (MEA) and incubated in the dark at room temperature, for 24 hours. After incubation, single germinating ascospores were observed on the agar surface and these were individually transferred onto 2% MEA and incubated at 25 °C under cool white light. Cultures were generally slow growing and required about two months before culture morphology could be described. Isolates were grouped based on colour and mycelial characteristics of cultures on MEA. To produce asexual states, cultures were grown on Carnation Leaf Agar (CLA) [1% water agar with sterilized carnation leaves placed onto

medium] and incubated at 25 °C under ultra-violet light (nuv, 250 nm) as suggested by Crous (1998).

DNA extraction and PCR amplification

Mycelium from pure cultures was scraped from the surface of cultures using a sterile scalpel and freeze dried. The dried mycelium was ground to a fine powder using liquid nitrogen. The method used to isolate DNA was as described by Raeder and Broda (1985), with minor modifications. Extracted nucleic acids were precipitated from the aqueous layer using two volumes of absolute ethanol. The nucleic acids were pelleted using centrifugation and washed with 70% ethanol. The resultant nucleic acid pellet was then dissolved in water and 4 µl RNaseA (10 µg/µl) (Sigma, USA) was added to digest the RNA. The resulting DNA was quantified by UV light visualization after electrophoresis on a 1% agarose gel stained with ethidium bromide. A Beckman DU Series 60 Spectrophotometer (Beckman, Germany) was used to calculate the DNA concentration of all DNA samples.

The Internal Transcribed Spacer (ITS) region of the rRNA operon was targeted for amplification using primers ITS1 (5' – TCC GTA GGT GAA CCT GCG G – 3'), and LR1 (5' – GGT TGG TTT CTT TTC CT – 3'), (White, Bruns & Taylor 1990, Vilgalys and Hester 1990). The PCR reaction mixture consisted of 50 µl total volume reaction and contained PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3) supplied by Roche Diagnostic, South Africa, 2.5 mM of each dNTP (Roche Diagnostic, SA), 0.2 µM of primers ITS1 and LR1 (MWG Biotech, Germany), 2.5 U Taq DNA polymerase (Roche Diagnostic, SA). Sterile deionised water was used to make the reaction volume up to 50 µl.

The PCR reaction consisted of an initial denaturation step at 96 °C for 2 min, followed by 40 cycles of template denaturation at 94 °C for 30 s, primer annealing at 56 °C for 30 s and extension at 72 °C for 1 min. A final elongation of 10 min at 72 °C completed the program. Following the PCR reaction, the amplicons were visualised by electrophoresis on 2 % agarose gels in TAE buffer, stained with ethidium bromide and visualized with UV light. A 100 bp (base pair) molecular marker (Roche Diagnostic, SA) was used to determine the sizes of PCR products. PCR products were purified using the High Pure PCR product Purification Kit (Roche Diagnostics, Germany).

DNA sequencing and phylogenetic analysis

Purified PCR products were sequenced using the ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems), following the protocols recommended by the manufacturer. One forward primer ITS1 and two reverse primers ITS2 and LR1, were used for the reactions (White *et al.* 1990, Vilgalys & Hester 1990). Sequence reactions were run using Polyacrylamide Gel Electrophoresis (PAGE) on an ABI PRISM™ 377 and capillary electrophoresis on an ABI PRISM™ 3100 Autosequencer (Perkin-Elmer Applied Biosystems).

Sequences were analysed using Sequence Navigator version 1.0.1 (Perkin-Elmer Applied Biosystems, USA). Sequence alignments were conducted manually and gaps were inserted wherever necessary. The phylogenetic analysis of the aligned sequences was determined using Phylogenetic Analysis Using Parsimony (PAUP version 4.0b1) (Swofford 1998). All characters in the analysis were given equal weight and the MULPAR option was effective. The heuristic search function based on parsimony with tree bisection reconnection (TBR) was used to generate a phylogram. Branch supports were calculated by performing a Bootstrap analysis of a 1000 replications on the aligned sequences. All resulting trees were rooted to *Ramulispora anguoides* (Nirenberg) Crous, which has been shown to be an effective outgroup taxon for *Mycosphaerella* (Crous *et al.* 2001, Hunter 2002).

Morphological characteristics

All isolates were characterized based on lesion type, ascospore germination patterns, cultural morphology and anamorph production, following the methodology described by Crous (1998). The characteristics of the lesions were noted and grouped based on their shape, colour, and position of pseudothecia (abaxial or adaxial) on the leaf. Several pseudothecia were taken from each lesion and mounted in lactophenol on glass microscope slides. These were then used for comparisons of characteristics of teleomorph structures.

Ascospore germination patterns were also examined at the time of making cultures. Here, single germinating ascospores were isolated from MEA and then mounted in lactophenol on microscope slides and examined based on the characteristics described by Crous (1998). All of the cultures obtained from isolations were grouped based on morphology and thus in

terms of colour, size after growth at 25 °C on MEA for 60 days and, texture of the cultures. Between two to ten representative cultures were then chosen for further identification using DNA sequencing.

RESULTS

Isolations and preparation of cultures

All leaves used in the identification process were collected from 24 different commercial plantations, including those of *E. globulus* and *E. nitens*. Symptoms were variable depending on host and whether the samples were taken from juvenile or adult leaves (Figure 2). Approximately 360 juvenile and adult leaves with obvious symptoms of MLD were ultimately examined. Most of the *Mycosphaerella* infections were obtained from the Northern-central distribution of the surveyed areas, and from juvenile leaves (Table 1).

A total of 276 cultures were obtained representing 92 isolations from diseased *Eucalyptus* leaves. Of these, 80 sets of leaves were from *E. globulus* and 12 were from *E. nitens*. All isolates included in this study have been deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative isolates have also been deposited in the Cetraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands.

Phylogenetic analysis

Amplification of the ITS region of the rRNA operon resulted in amplification products of approximately 600 bp for all isolates. A total of 19 taxa were used in the phylogenetic analysis the sequence alignment resulted in 655 characters. Of these, 396 were constant, 82 were parsimony-uninformative and 181 parsimony-informative. The analysis produced 4 most parsimonious trees with a length of 438 steps. For all trees the consistency index (CI = 0.822), retention index (RI = 0.874) and the homoplasy index (HI = 0.178) was calculated using the heuristic search option. After 1000 Bootstrap replications a consensus tree was obtained that showed the same topology as the most parsimonious trees generated from the heuristic search (Figure 3).

Phylogenetic analysis showed that isolates from *Eucalyptus* leaves in Chile, represent six species of *Mycosphaerella*. These included *M. africana*, *M. grandis*, *M. lateralis*, *M. molleriana*, *M. parkii*, and *M. walkeri*. The six *Mycosphaerella* species identified in this study were grouped into two clades. The first group includes *M. africana*, *M. grandis*, *M. molleriana*, *M. parkii*, and *M. walkeri* within the larger *Mycosphaerella* clade. These species were isolated from *E. globulus* and *E. nitens*. The second group included only *M. lateralis*, which, lies outside the larger *Mycosphaerella* clade and was isolated only from *E. nitens*.

Morphological characteristics

Symptomatic leaves were prevalent on juvenile foliage of *E. globulus* and exhibited a wide range of colours, often with raised brown to dark brown borders (Figure 4). Black pseudothecia were observed on both abaxial and adaxial leaf surfaces, however, most of them were present on the adaxial surfaces of leaves. Most of the cultures obtained from single germinating ascospores were characterized by slow growth. All cultures were grouped according their morphological characteristics (culture morphology, ascospore germination pattern, identity of anamorph and symptoms on leaves) into 10 different morphotypes. Based on morphological characteristics and phylogenetic analysis, the following species were identified.

Mycosphaerella africana Crous & M.J. Wingf.

Mycosphaerella africana was isolated from two plantations (Raqui Reserva 4 and La Colcha) both located in the Arauco area in the Northern central distribution of *Eucalyptus* plantations. In total, 5 isolates were obtained from juvenile leaves of *E. globulus* trees planted in 1999. Leaf spots were distributed through the lamina and were visible as round to irregular spots, light brown, also surrounded by light brown to brown edges (Figure 5) but not dark borders as described by Crous (1998). Black sub epidermal pseudothecia were observed on both sides of the lamina (Figure 5). Ascopore germination was observed after 24 h on MEA and was characterized according to Crous (1998) as Type G (Figure 5). Culture growth was approximately 30 – 40 mm diam. on MEA after 2 months and white-pink fluffy aerial mycelium was observed (Figure 5). No anamorph fruiting structures were observed in the cultures.

Mycosphaerella grandis Carnegie & Keane

Mycosphaerella grandis was isolated from juvenile and adult leaves on both *E. globulus* and *E. nitens* in plantations at many different locations in Northern and Southern areas of Chile. This species was the predominant species, with 71 isolates that represented 68 % of the total isolations in this study. Leaf spots were amphigenous, distributed irregularly through the lamina (Figure 6). Lesions were light brown, to brown with raised purple to brown borders and irregular in size and shape (Figure 6). Ascospores were similar in shape with Type F germination patterns (Crous 1998) (Figure 6). Cultures had colonies green to dark green (Figure 6) and no anamorph was identified in the cultures.

Mycosphaerella lateralis Crous & M.J. Wingf.

Mycosphaerella lateralis was identified only from juvenile leaves of *E. nitens* in plantations, located at Santa Isabel in Southern-central Chile near Valdivia. Eight isolates in this study represented this species. Leaf spots were amphigenous (Figure 7) and lesions were sub-circular to circular in shape and grey to brown in colour, surrounded by dark-brown borders (Figure 7). Ascospores of this species germinated from both poles, with long germ tubes and were typical Type I patterns as described by Crous (1998), (Figure 7). Colonies were grey to light brown and turned brown with increased incubation. Colonies had a smooth appearance and showed some folding (Figure 7). The anamorph *Uwebraunia lateralis* Crous & M.J. Wingf. was rarely found sporulating on Carnation Leaf Agar after two weeks of incubation at 25 °C under near ultra-violet light.

Mycosphaerella molleriana (Thüm.) Lindau in Engler & Prantl.

Mycosphaerella molleriana was identified from juvenile leaves of *E. globulus* in plantations at Raqui Reserva 4 and San Ricardo, both located in Northern-central area (Arauco). Ten isolates of this species were collected. The species was characterised by amphigenous leaf spots, which were irregular in shape and confluent at the middle and borders of the leaf laminas (Figure 8). Lesions were light brown with raised brown borders, bearing black subepidermal pseudothecia (Figure 8). Ascospore germination was observed after 24 h on MEA and fitted the Type C pattern (Figure 8) of Crous (1998). Aerial hyphae were gray to light green, with fluffy white mycelium towards the margins (Figure 8). The

anamorph *Colletogloeopsis molleriana* Crous & M.J. Wingf. was found sporulating on MEA after two weeks incubation at 25 °C under ultra-violet light.

Mycosphaerella parkii Crous & M.J. Wingf., F.A. Ferreira & Alfenas

Mycosphaerella parkii was identified from juvenile leaves on *E. globulus* only at the Raqui Reserva 4 plantation in the Arauco area. Seven isolates were obtained of this species. Leaf spots were amphigenous and they were irregular in shape (Figure 9). The lesions were light brown at the center and turned light brown towards the raised borders (Figure 9). In most lesions, black aggregated and immersed pseudothecia were seen. Ascospores showed the Type D germination pattern (Crous 1998), (Figure 9). The cultures were grey-green to dark green, with radial cracks and white spots around the border of mycelium (Figure 9). The anamorph *Stenella parkii* Crous & Alfenas was not found in Carnation leaf agar culture.

Mycosphaerella walkeri R.F. Park & Keane

Mycosphaerella walkeri was identified from juvenile leaves of *E. globulus* in the plantations San Ricardo, La Colcha, Lomas de la Madera and Raqui, in the Northern-central forestry area. This *Mycosphaerella* species was also isolated from both juvenile and adult *E. globulus* leaves in the Southern-central area. Leaf spots were amphigenous, round to confluent and irregular, however, most of the lesions observed were circular with purple margins (Figure 10). Ascospore germination was of the Type C described by Crous (1998) and Carnegie (2000), (Figure 10). Cultures had white aerial hyphae, surrounded by dark-green submerged mycelium (Figure 10). The anamorph *Sonderhenia eucalypticola* (A.R. Davis) H. Swart & J. Walker. was frequently isolated from the diseased leaves.

DISCUSSION

Prior to this study, virtually nothing was known regarding the identity of *Mycosphaerella* species associated with leaf disease of *Eucalyptus* spp. in Chile. Those identifications previously reported, were based on symptoms and morphological structures on lesions. Thus, results of this study have provided the first attempt at detailed identification of these important pathogens in Chile.

Results of this study, supported by DNA sequence comparisons have given rise to the identification of six species. These included *M. africana*, *M. grandis*, *M. lateralis*, *M. molleriana*, *M. parkii*, and *M. walkeri*. *M. africana*, *M. grandis*, *M. lateralis*, *M. molleriana*, and *M. parkii* are reported for the first time from Chile.

Mycosphaerella leaf disease caused high levels of defoliation on *Eucalyptus* spp. during the 2000 growth season, in both the Northern-central and Southern-central area of Chile. In the Arauco area (Northern-central) the problem was confined to juvenile leaves, while in the Valdivia area (Southern-central) the problem was recognized from both juvenile and adult leaves of *Eucalyptus* spp. Although the results of the current study have shown that *M. grandis* and *M. walkeri* are present on adult leaves, we now believe that the severe damage observed in the 2000 season was not caused only by MLD. At that time it was suggested that the aggressive *M. cryptica* (Cooke) Hansf. was responsible for the disease (Gonzalez 2000, unpublished report, Mohammed 2001, unpublished report). We have shown clearly that this is not so, and there is no evidence to suggest that *Mycosphaerella* spp. contributed to the defoliation problems. Rather, current views are that the adult leaf loss was mainly due to physiological disturbance, probably linked to frost damage or other environmental factors.

In the Northern-central (Arauco) area of Chile, we were able to identify five species of *Mycosphaerella*. It is impossible to know which of these is more or less important in the defoliation of juvenile *E. globulus* leaves in the area. *Mycosphaerella grandis* was clearly the most predominant species in all areas (68 %). We thus believe that this species is an important component of the *Eucalyptus* leaf disease. *Mycosphaerella grandis* is known only from Tasmania, where it causes leaf disease on *E. grandis*, *E. globulus* and *E. nitens* (Carnegie & Keane 1998). This species showed very similar germination patterns and culture characteristics to *M. juvenis* that is one of the more important species occurring in South Africa (Crous & Wingfield 1996, Crous 1998, Hunter 2002). This identification constitutes the first report of *M. grandis* in Chile as well as South America. Further studies are thus required to evaluate its relative importance.

Mycosphaerella africana, *M. molleriana* and *M. parkii*, were identified only from juvenile leaves on *E. globulus* in the Arauco area. Their presence was infrequent and concentrated in Raqui Reserva 4, La Colcha and San Ricardo. These species were frequently found

together with *M. grandis* and were not easy to distinguish based on the symptoms or type of lesions that they produced on leaves. *Mycosphaerella africana* is known from Colombia (Crous 1998) and its appearance in Chile, which is geographically close to Colombia, is perhaps not surprising. The fungus has also been reported in Portugal, South Africa and Zambia (Crous 1998). There is, however, no evidence that this species is an important pathogen.

Mycosphaerella molleriana has been reported in the USA (California), Portugal and this name has been used by previous authors (Gonzalez & Parra 1994, Muñoz 1999) as present in Chile. However, in the past, this fungus was thought to be a synonym of *M. nubilosa* (Crous, Wingfield & Park 1990), that later was rejected by Crous and Wingfield (1996). Hunter (2002), using molecular techniques, has also shown that this species represents a distinct taxon. *Mycosphaerella nubilosa* is recognised as a major pathogen of *E. globulus* and *E. nitens* (Dick & Gadgil 1983, Park 1988, Milgate *et al.* 2001) but the confusion in the taxonomy of *M. molleriana* and *M. nubilosa* makes it difficult to assess the importance of *M. molleriana*. Thus, *M. molleriana* was reported before, but as a synonym of *M. nubilosa*, so this is the first clear report, which is supported by both morphological and DNA sequence data.

Mycosphaerella parkii has previously been recorded from Brazil, Colombia and Indonesia (Crous 1998). Together with *M. africana* and *M. molleriana*, it appeared infrequently in this study and is unlikely to have been important in defoliation. This species is also reported from Chile for the first time.

Mycosphaerella lateralis, was isolated only from *E. nitens* plantations in the Valdivia area. It was present on juvenile leaves of *E. nitens* but there were no signs of severe defoliation. We, therefore, do not believe that it is particularly important. This species has previously been reported from South Africa and Zambia (Crous 1998) and also from Australia (Maxwell *et al.* 2000). This is the first record of the fungus in South America including Chile.

Mycosphaerella walkeri was common in Chile although it is usually found in the asexual (*Sonderhenia*) state and was not commonly isolated using the technique used to collect *Mycosphaerella* spp. in this study. The fungus causes small discrete spots on the juvenile

and less commonly adult leaves of *E. globulus* and *E. nitens*. It has been reported from Australia, New Zealand, Portugal, Equator, and Colombia, and has also previously been reported from Chile (Crous 1998, Wingfield *et. al* 1995).

Effective management of MLD on *Eucalyptus* in Chile will be important and necessary for the forestry activity in the future. Thus far, losses due to defoliation caused by MLD have not been quantified and this deficiency must be addressed. In order to achieve effective management strategies of this disease, it will also be important to have greater knowledge of the pathogen and rapid screening techniques for the different species. Selection for resistant clones or hybrids should be initiated in the future, however, it is also important to achieve a better understanding related to MLB and its effect on *Eucalyptus* plantations.

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Table 1. *Mycosphaerella* spp. identified from *Eucalyptus* plantations in Chile.

Collected from	Host	Foliage	*Mycosphaerella spp.* identified	Anamorph	Distribution
Arauco Concepcion (Northern-Central area)	*E. globulus*	Juvenile	*M. africana*	Unknown	Colombia, Portugal, South Africa, Zambia
			M. grandis	Unknown	Australia
			M. molleriana	*Colletogloeopsis molleriana*	USA (California), Portugal
			M. parkii	*Stenella parkii*	Brazil, Colombia, Indonesia
			M. walkeri	*Sonderhenia eucalypticola*	Australia, Chile, New Zealand
Valdivia Los Lagos (Southern-Central area)	*E. globulus*	Juvenile	*M. grandis*	Unknown	Australia
			M. lateralis	*Uwebraunia lateralis*	South Africa, Zambia
			M. walkeri	*Sonderhenia eucalypticola*	Australia, Chile, New Zealand
		Adult	*M. grandis*	Unknown	Australia
	E. nitens	Juvenile	*M. lateralis*	*Uwebraunia lateralis*	South Africa, Zambia

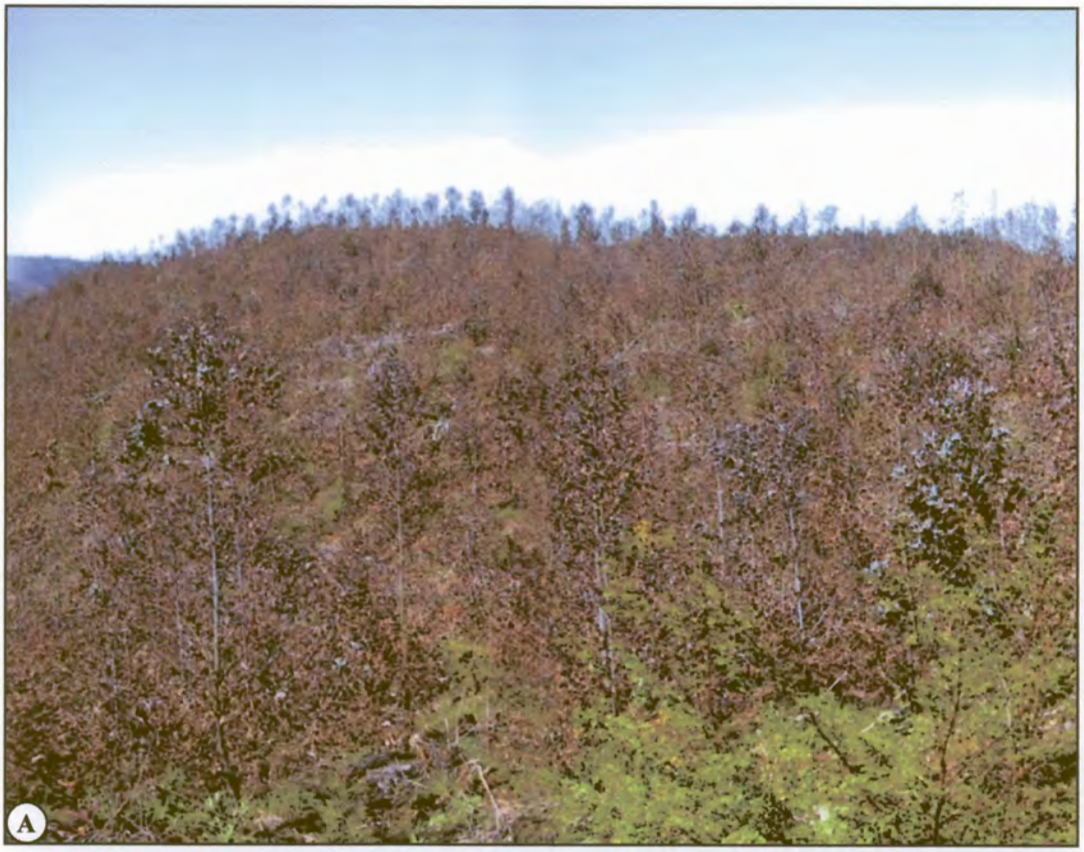
Crous (1998) & Carnegie (2000).

Figure 1. Geographic distribution of *Eucalyptus* commercial plantations in Chile where samples were collected.

1: Concepcion and Arauco areas (northern-central)

2: Valdivia area (southern-central)

Figure 2. Commercial plantations of *Eucalyptus globulus* affected by MLD. (**A**) Juvenile foliage (**B**) Adult foliage.


A

B

Figure 3. Phylogram based on aligned ITS sequence data from the rRNA operon of *Mycosphaerella* spp. isolated from *E. globulus* and *E. nitens* from Chile (CI = 0.822, RI = 0.874, HI = 0,178) inferred using heuristic and branch swapping options of PAUP version 4.0b1. Bootstrap support of 1000 replications is listed above the branches.

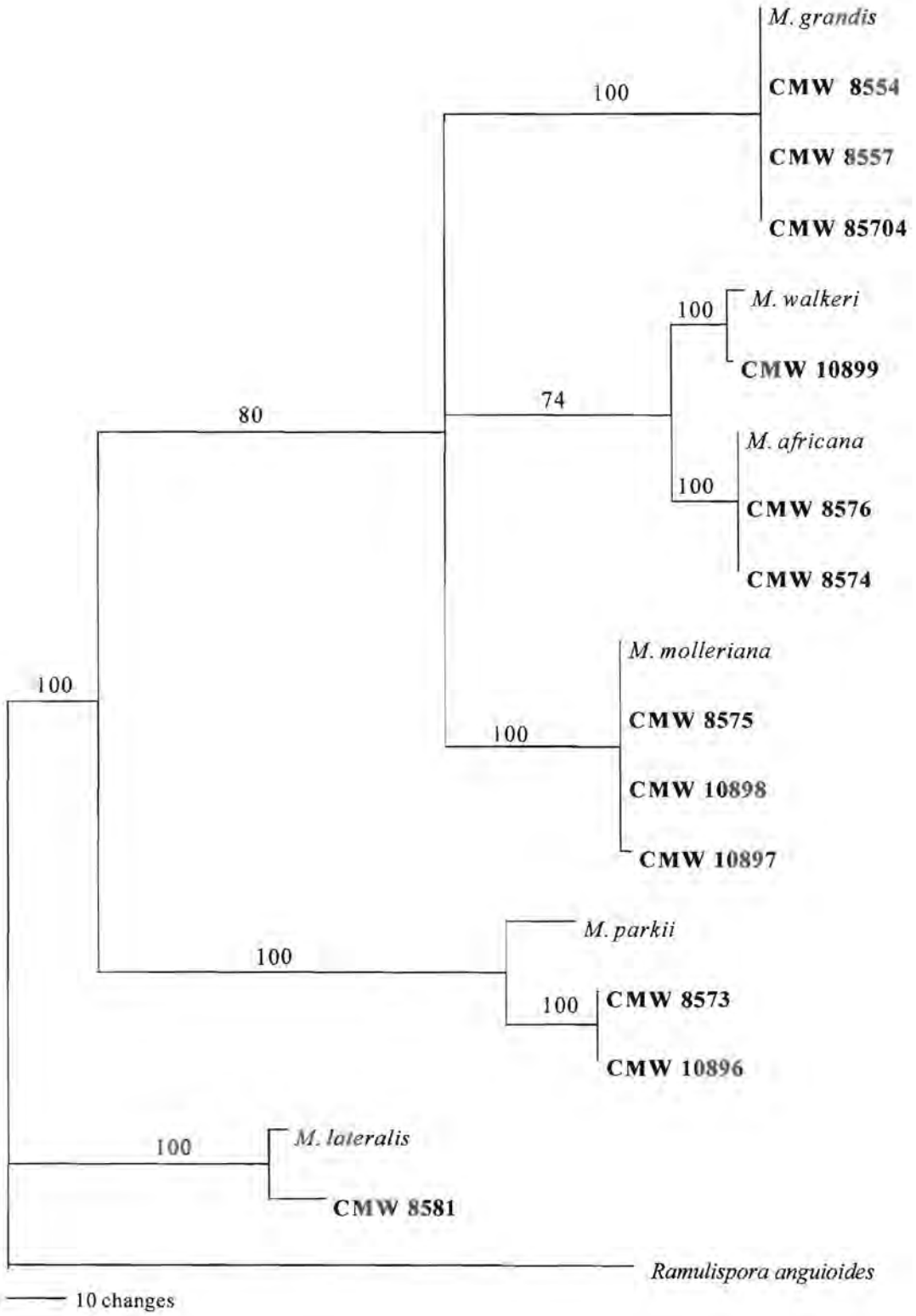

M. grandis
CMW 8554
CMW 8557
CMW 85704
M. walkeri
CMW 10899
M. africana
CMW 8576
CMW 8574
M. molleriana
CMW 8575
CMW 10898
CMW 10897
M. parkii
CMW 8573
CMW 10896
M. lateralis
CMW 8581
Ramulispora anguioides
100
100
74
80
100
100
100
100
100
10 changes

Figure 4. Leaf symptoms associated with infections by *Mycosphaerella* species isolated from Chile. (**A**) Juvenile leaves of *E. globulus* from the Northern-central area (Arauco). (**B**) Adult leaves of *E. globulus* from the Southern-central area (Valdivia).

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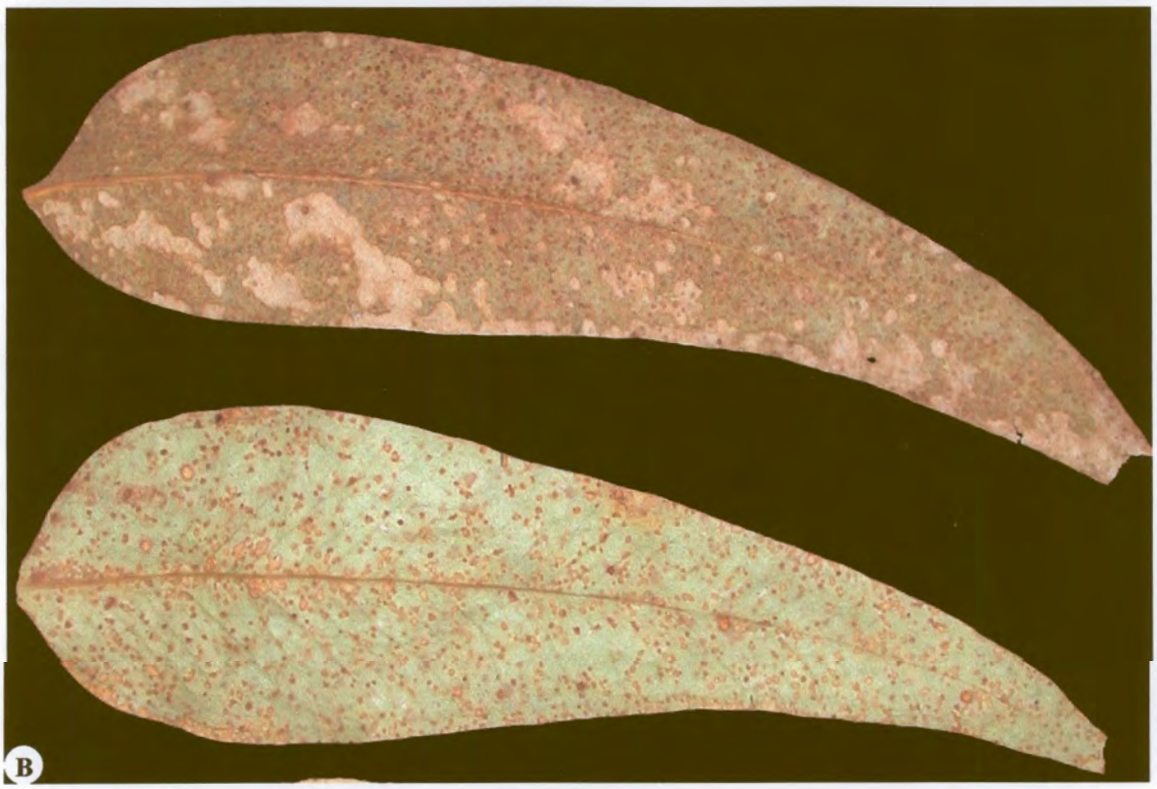

Figure 5. *Mycosphaerella africana*. (**A**) *E. globulus* leaf showing symptoms. (**B**) Lesion of *M. africana*. (C) Ascospore germination pattern. (**D**) Culture of *M. africana*.

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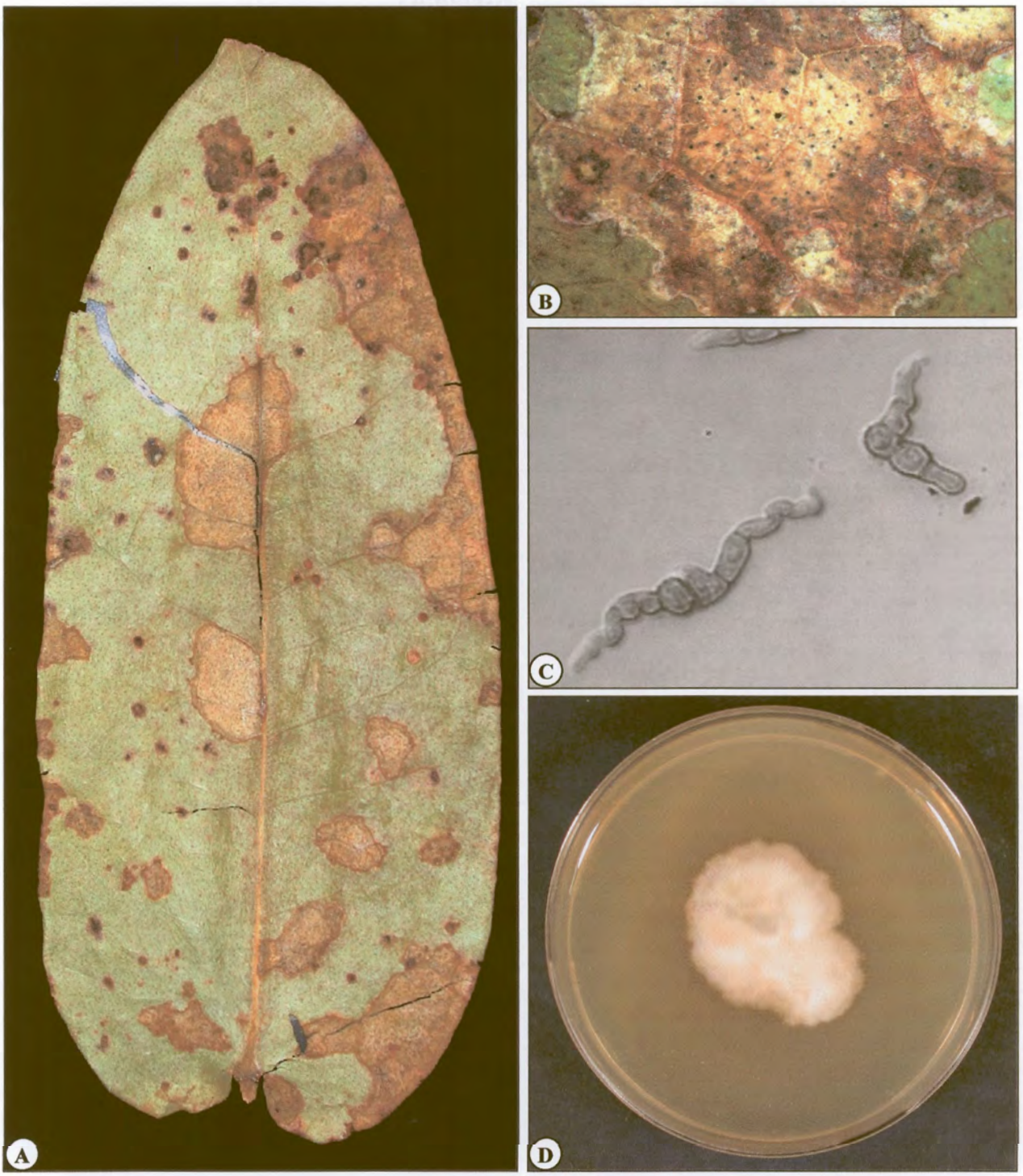

Figure 6. *Mycosphaerella grandis*. (**A**) *E. globulus* leaf with symptoms. (**B**) Lesion. (**C**) Ascospore germination pattern. (**D**) Culture characteristics.

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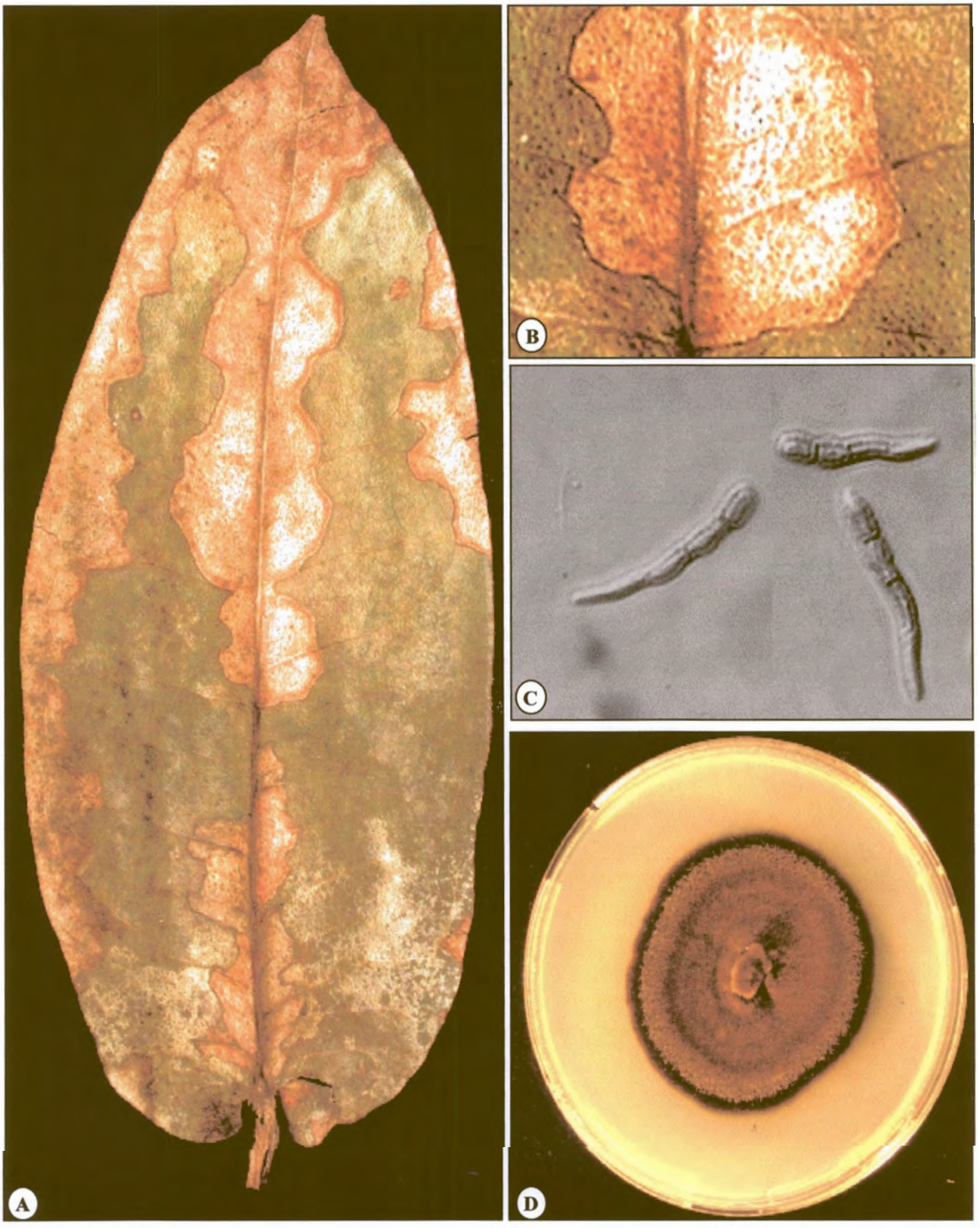

Figure 7. *Mycosphaerella lateralis* **(A)** *E. globulus* leaf with symptoms. **(B)** Lesion. **(C)** Ascospore germination pattern. **(D)** Culture characteristics.

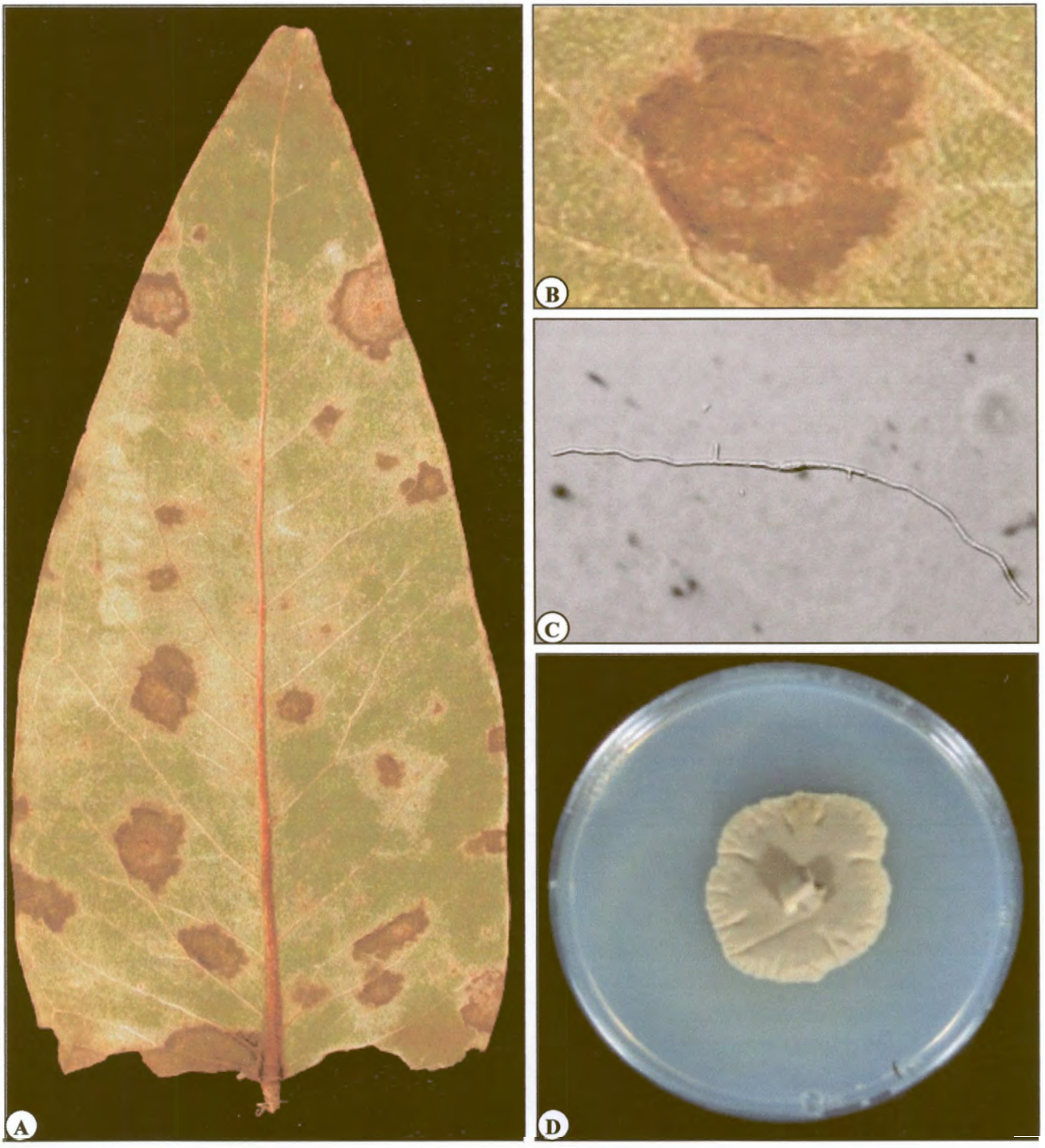
A
B
C
D

Figure 8. *Mycosphaerella molleriana* (**A**) *E. globulus* leaf with symptoms. (**B**) Lesion. (**C**) Ascospore germination pattern. (**D**) Culture characteristics.

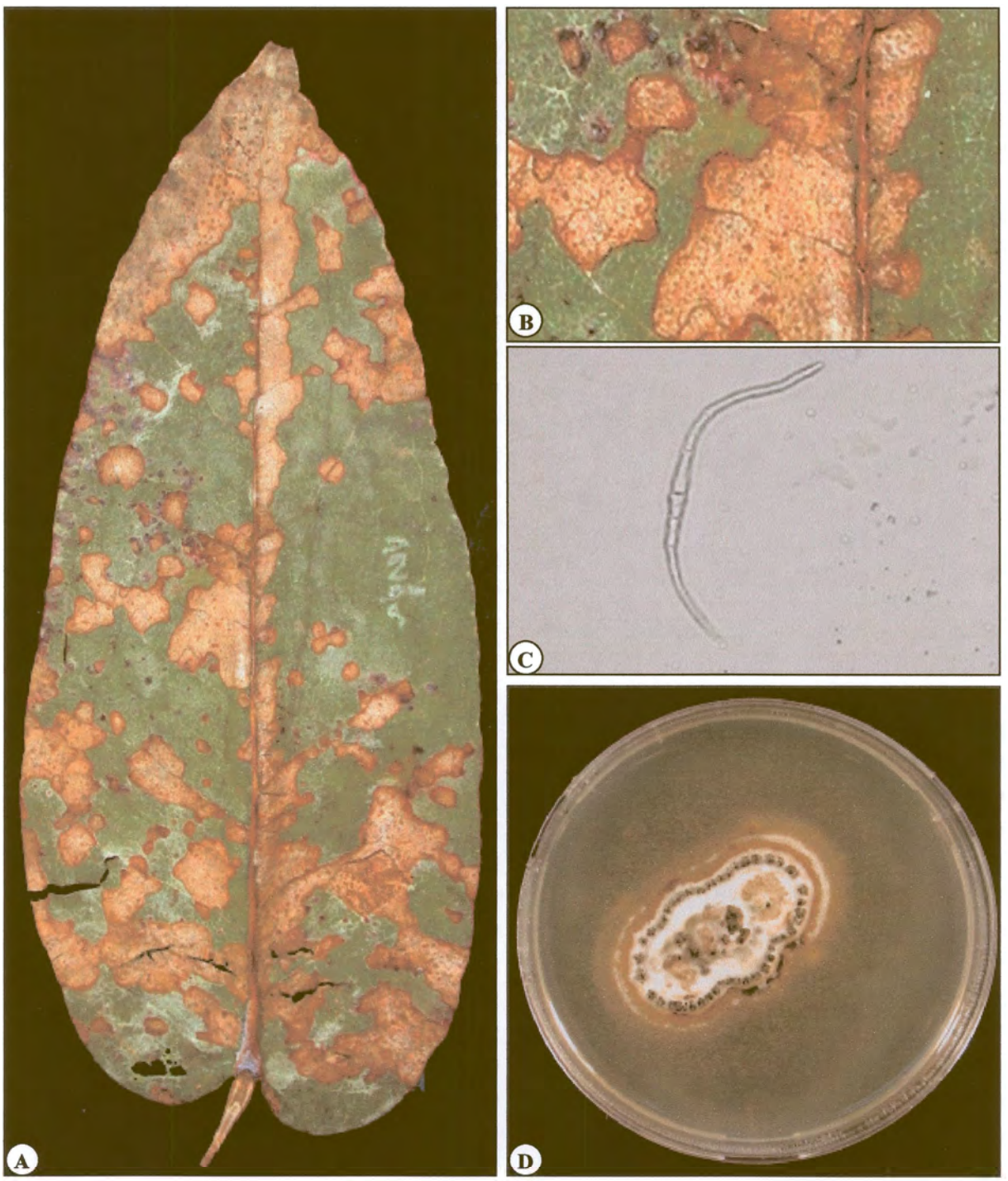
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Figure 9. *Mycosphaerella parkii* **(A)** *E. globulus* leaf with symptoms. **(B)** Lesion. **(C)** Ascospore germination pattern. **(D)** Culture characteristics.

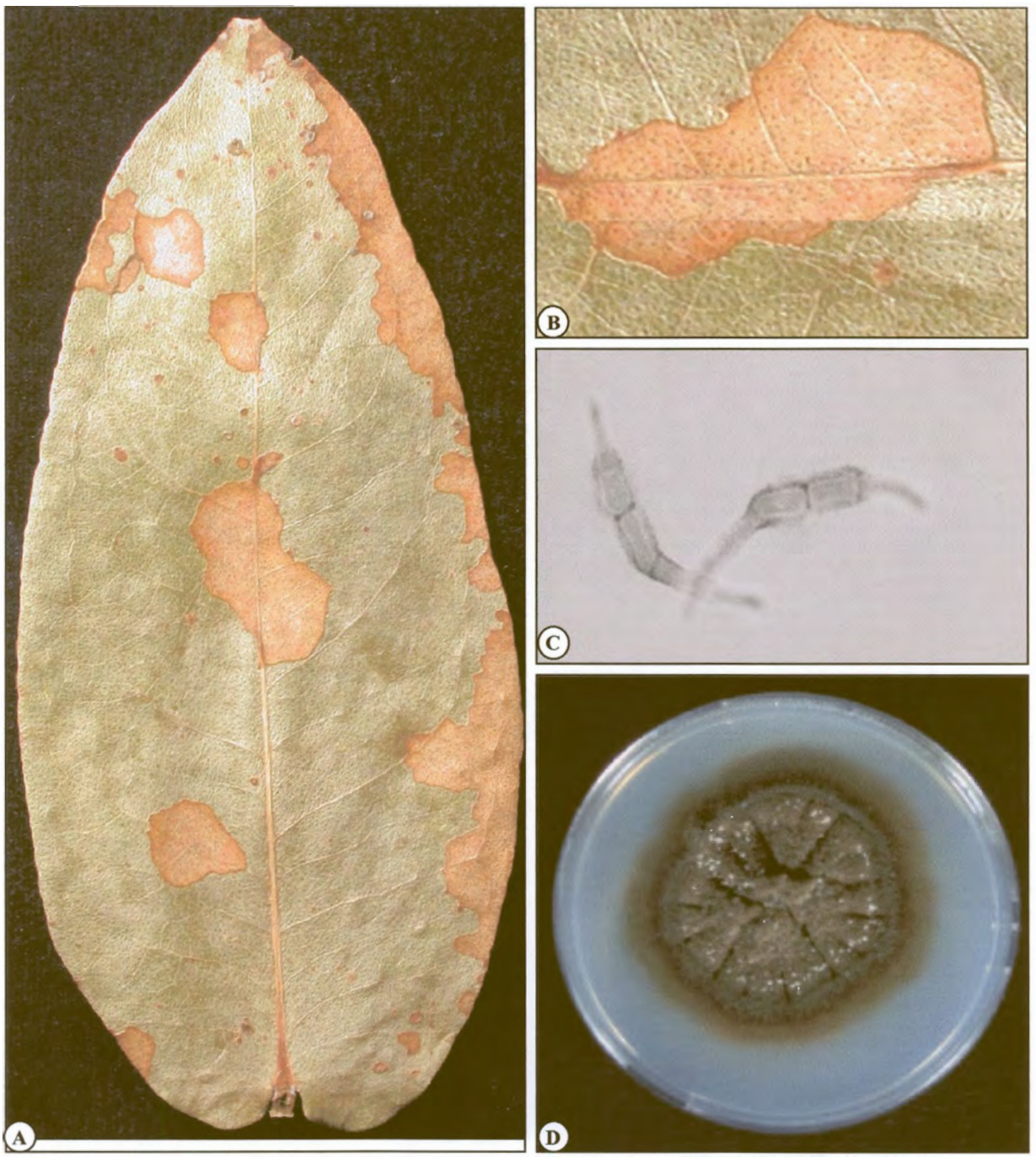
A
B
C
D

Figure 10. *Mycosphaerella walkeri* (**A**) *E. globulus* leaf with symptoms. (**B**) Lesion. (**C**) Ascospore germination pattern. (**D**) Culture characteristics.

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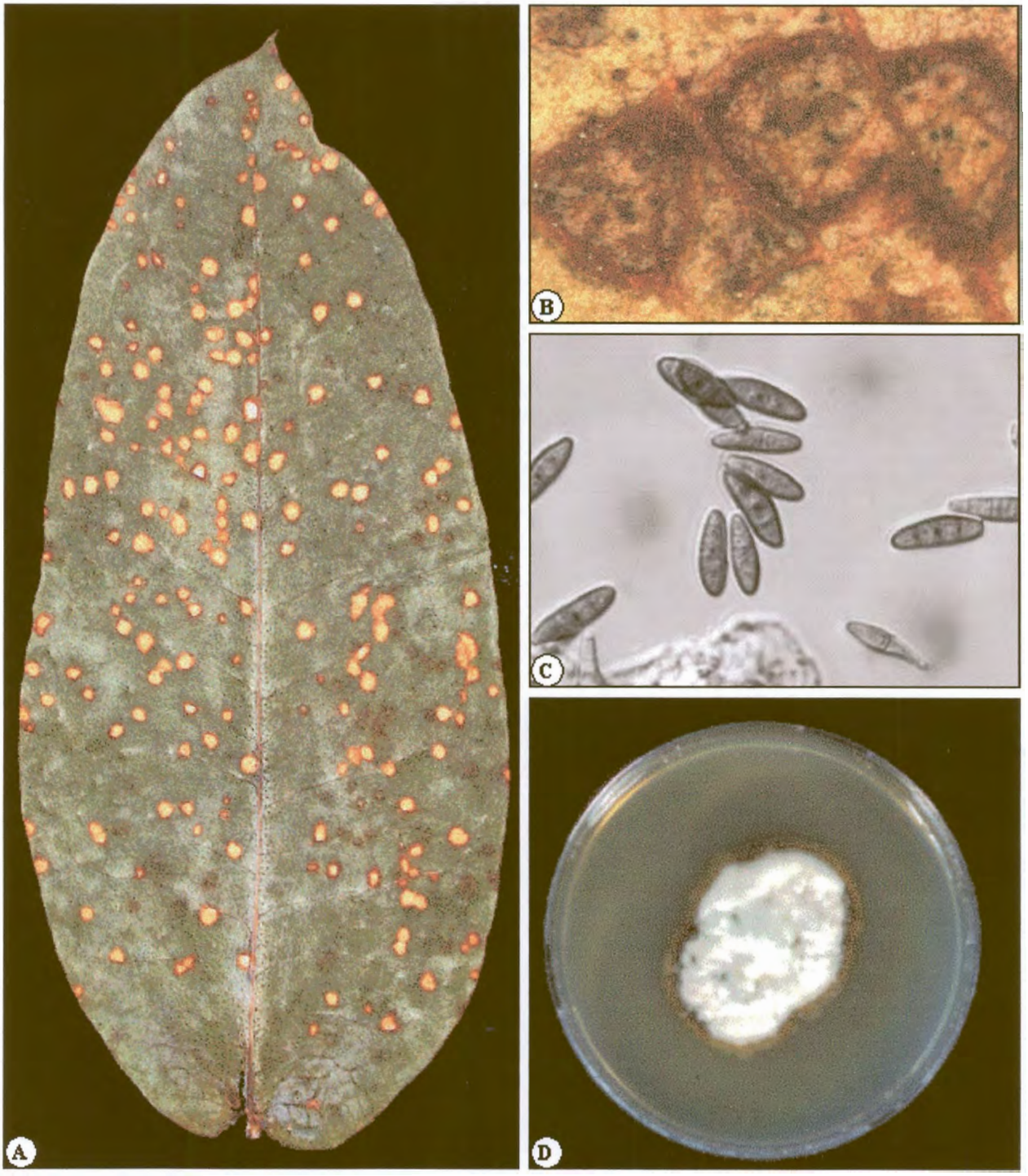

Figure 11: ITS DNA sequences of *Mycosphaerella* species generated using primers ITS1, ITS2 and LR1. Sequence data set contains sequences of *Mycosphaerella* spp. isolated from *Eucalyptus* spp. from Chile.

```
                                10        20        30        40
M.grandis                       TCCGTAGGTG AACCTGCGGA GGGATCATTA CC-GAG---T
CMW 8554                        TCCGTAGGTG AACCTGCGGA GGGATCATTA CC-GAG---T
CMW 8557                        TCCGTAGGTG AACCTGCGGA GGGATCATTA CC-GAG---T
CMW 8570                        TCCGTAGGTG AACCTGCGGA GGGATCATTA CC-GAG---T
M.walkeri STU2768               TCGGTAGGTG AACCTGCGGA GGGATCATTA CC-GAG---T
CMW 10899                       TCCGTAGGTG AACCTGCGGA GGGATCATTA CC-GAG---T
M.africana CMW4945              TCCGTAGGTG AACCTGCGGA GGGATCATTA CT-GAG---T
CMW 8576                        TCCGTAGGTG AACCTGCGGA GGGATCATTA CT-GAG---T
CMW 8574                        TCCGTAGGTG AACCTGCGGA GGGATCATT- CT-GA----T
M.molleriana CMW2734            TCCGTAGGTG AACCTGCGGA GGGATCATTA CT-GAG---T
CMW 8575                        TCCGTAGGTG AACCTGCGGA GGGATCATTA CT-GAG---T
CMW 10898                       TCCGTAGGTG AACCTGCGGA GGGATCATTA CT-GAG---T
CMW 10897                       TCCGTAGGTG AACCTGCGGA GGGATCATTA CT-GAG---T
M.parkii CMW3358                TCCGTAGGTG AACCTGCGGA GGGATCATTA CT-GAG---T
CMW 8573                        TCCGTAGGTG AACCTGCGGA GGGATCATTA CC-GAG---T
CMW 10896                       TCCGTAGGT- AACCTGCGGA GGGATCATTA CC-GAG---T
M.lateralis CMW4935             TCCGTAGGTG AACCTGCGGA GGGATCATTA CCAGA-----
CMW 8581                        TCGAAGGGTG AACCTGCGGA GGGATCATTA CCAGA-----
Ramulispora anguioides          TCCGTAGGTG AACCTGCGGA AGGATCATTA ATAGAGCAAT

                                50        60        70        80
M.grandis                       GAGGGCCT-- -CCGGGCT-C G------ACC TC----CAAC
CMW 8554                        GAGGGCCT-- -CCGGGCT-C G------ACC TC----CAAC
CMW 8557                        GAGGGCCT-- -CCGGGCT-C G------ACC TC----CAAC
CMW 8570                        GAGGGCCT-- -CCGGGCT-C G------ACC TC----CAAC
M.walkeri STU2768               GAGGGCC--- -CCGGCC--C G------ACC TC----CAAC
CMW 10899                       GAGGGCC--- -CCGGCC--C G------ACC TC----CAAC
M.africana CMW4945              GAGGGC-T-C AC-G-CC--C G------ACC TC----CAAC
CMW 8576                        GAGGGC-T-C AC-G-CC--C G------ACC TC----CAAC
CMW 8574                        GAGGGC-T-C AC-G-CC--C G------ACC TC----CAAC
M.molleriana CMW2734            GAGGGC--GC A-AG-CC--C G------ACC TC----CAAC
CMW 8575                        GAGGGC--GC A-AG-CC--C G------ACC TC----CAAC
CMW 10898                       GAGGGC--GC A-AG-CC--C G------ACC TC----CAAC
CMW 10897                       GAGGGC--GC A-AG-CC--C G------ACC TC----CAAC
M.parkii CMW3358                GAGGGTTT-C ACCG--C--C G------ACC TC----CAAC
CMW 8573                        GAGGGCCC-- -CCGGCC--C G------ACC TC----CAAC
CMW 10896                       GAGGGCCC-- -CCGGCC--C G------ACC TC----CAAC
M.lateralis CMW4935             -AG------- ACGCCTCGGC GGAAACGCCG GGG---CCTT
CMW 8581                        -AG------- ACGCTCTGGC GGAAACGCCG GGG---CCTT
Ramulispora anguioides          GAGCGTCAG- -CGCCCCGGG AGCAATCCTG GGGGCCACCC
```

```
                                  90         100        110        120

M.grandis                 CCC------- ---ATT---- GT-----AT- TCC--GAC--

CMW 8554                  CCC------- ---ATT---- GT-----AT- TCC--GAC--
CMW 8557                  CCC------- ---ATT---- GT-----AT- TCC--GAC--
CMW 8570                  CCC------- ---ATT---- GT-----AT- TCC--GAC--
M.walkeri STU2768         CCT------- ----TT---- GT-------- -----GAA--
CMW 10899                 CCT------- ----TT---- GT-------- -----GGA--
M.africana CMW4945        CCT------- ----TT---- GT-------- -----GAA--
CMW 8576                  CCT------- ----TT---- GT-------- -----GAA--
CMW 8574                  CCT------- ----TT---- GT-------- -----GAA--
M.molleriana CMW2734      CCC------- ---AT----- GT------T- TC--------
CMW 8575                  CCC------- ---AT----- GT------T- TC--------
CMW 10898                 CCC------- ---AT----- GT------T- TC--------
CMW 10897                 CCC------- ---AT----- GT------T- TC--------
M.parkii CMW3358          CCT------- ----TT---- GT-------- -----GAA--
CMW 8573                  CCT------- ----TT---- GT-------- -----GAA--
CMW 10896                 CCT------- ----TT---- GT-------- -----GAA--
M.lateralis CMW4935       CGTCCAACCC TTTG-TGAAC GT-----ATC TC--------
CMW 8581                  CGTCCAACCC TTTG-TGAAC GT-----ATC TC--------
Ramulispora anguioides    TCCTCGGAGG GTTTAGAGAC GTCGAGCCTC TCGGAGAAGC

                                  130        140        150        160

M.grandis                 ---------- ---------- -----CTC-- -----TTGTT
CMW 8554                  ---------- ---------- -----CTC-- -----TTGTT
CMW 8557                  ---------- ---------- -----CTC-- -----TTGTT
CMW 8570                  ---------- ---------- -----CTC-- -----TTGTT
M.walkeri STU2768         ---------- --CCCAA--- -----CT--- -----T-GTT
CMW 10899                 ---------- --CCCAA--- -----CT--- -----T-GTT
M.africana CMW4945        ---------- ---CCAA--- -----CTC-- -----T-GTT
CMW 8576                  ---------- ---CCAA--- -----CTC-- -----T-GTT
CMW 8574                  ---------- ---CCAA--- -----CTC-- -----T-GTT
M.molleriana CMW2734      ---------- ----CAAA-C CAC------- -------GTT
CMW 8575                  ---------- ----CAAA-C CAC------- -------GTT
CMW 10898                 ---------- ----CAAA-C CAC------- -------GTT
CMW 10897                 ---------- ----CAAA-C CAC------- -------GTT
M.parkii CMW3358          ---------- ---CCACAA- --CTT----- -------GTT
CMW 8573                  ---------- ----CAC--- ---TTCTC-- -------GTT
CMW 10896                 ---------- ----CAC--- ---TTCTC-- -------GTT
M.lateralis CMW4935       ---------- ---------- ---------- ------TATT
CMW 8581                  ---------- ---------- ---------- ------TATT
Ramulispora anguioides    TCGGTTCAGA CCTCCACCCT TGAATAAAAA ACCTTTGTTG
```

```
                                170        180        190        200

M.grandis                       GCCTCGG--G GGCGACCCG- GCC------T TCGGGCG--T
CMW 8554                        GCCTCGG--G GGCGACCCG- GCC------T TCGGGCG--T
CMW 8557                        GCCTCGG--G GGCGACCCG- GCC------T TCGGGCG--T
CMW 8570                        GCCTCGG--G GGCGACCCG- GCC------T TCGGGCG--T
M.walkeri STU2768               GCTTCGG--G GGCGACCCT- GCCGTC---- TCGGCGGC-G
CMW 10899                       GCTTCGG--G GGCGACCCT- GCCGTC---- TCGGCGGC-G
M.africana CMW4945              GCTTCGG--G GGCGACCCC- GCCGT----T TCGGCGAC--
CMW 8576                        GCTTCGG--G GGCGACCCC- GCCGT----T TCGGCGAC--
CMW 8574                        GCTTCGG--G GGCGACCCC- GCCGT----T TCGGCGAC--
M.molleriana CMW2734            GCCTCGG--G GGCGACCCG- GCCG------ CCGCGCCG--
CMW 8575                        GCCTCGG--G GGCGACCCG- GCCG------ CCGCGCCG--
CMW 10898                       GCCTCGG--G GGCGACCCG- GCCG------ CCGCGCCG--
CMW 10897                       GCCTCGG--G GGCGACCCG- GCCG------ CCGCGCCG--
M.parkii CMW3358                GCTTCGG--G GGCGACCCT- GCCG-----T TCGCGGCATC
CMW 8573                        GCTTCGG--G GGCGACCCT- GCCG------ TCCCGGCGCC
CMW 10896                       GCTTCGG--G GGCGACCCT- GCCG------ TCCCGGCGCC
M.lateralis CMW4935             GCCCCGG--G GG-AACCC-C GCCTGTCAT- ---GGGCGTG
CMW 8581                        GCCCCGG--G GG-AACCC-C GCCTGTCAT- ---GGGCGTG
Ramulispora anguioides          CTTTGGCAGG ACGCCTCGCG CCAGCGGCTT CGGCTGTTGA

                                210        220        230        240

M.grandis                       CGGG-GCCCC CGGTGGACCA -TC---AAAC TCTGCATCT-
CMW 8554                        CGGG-GCCCC CGGTGGACCA -TC---AAAC TCTGCATCT-
CMW 8557                        CGGG-GCCCC CGGTGGACCA -TC---AAAC TCTGCATCT-
CMW 8570                        CGGG-GCCCC CGGTGGACCA -TC---AAAC TCTGCATCT-
M.walkeri STU2768               CGGC-GCCCC CGGAGG-CC- CTC---AAAC ACTGCATCC-
CMW 10899                       CGGC-GCCCC CGGAGG-CC- CTC---AAAC ACTGCATCC-
M.africana CMW4945              -GGCGGCCCC CGGAGGT-CA -TC---AAAC ACTGCATC--
CMW 8576                        -GGC-GCCCC CGGAGGT-CA -TC---AAAC ACTGCATC--
CMW 8574                        -GGC-GCCCC CGGAGGT-CA -TC---AAAC ACTGCATC--
M.molleriana CMW2734            -GG--GCCCC CGGTGGACCC -TC----AAC TCTGCATC--
CMW 8575                        -GG--GCCCC CGGTGGACCC CTC----AAC TCTGCATC--
CMW 10898                       -GG--GCCCC CGGTGGACCC CTC----AAC TCTGCATC--
CMW 10897                       -GG--GCCCC CGGTGGACCC CTC----AAC TCTGCATC--
M.parkii CMW3358                -G-C-GCCCC CGGAGGA--- -T-ACTTAAC CCTGCATCAT
CMW 8573                        GG-C-GCCCC CGGAGGAC-- ---ACCCAAC ACTGCATCCA
CMW 10896                       GG-C-GCCCC CGGAGGAC-- ---ACCCAAC ACTGCATCCA
M.lateralis CMW4935             GG-C----CC CCGGTGGCCA --ACTCAAAC TCTGTTTTTA
CMW 8581                        GG-C----CC CCGGCGGCCA --CTTCAAAC TCTGTTTTTA
Ramulispora anguioides          GTGCCTGCCA GAGG--ACCA -----CAACT CTTGTTTTTA
```

```
                                250        260        270        280

M.grandis                       --TTGACGTC TGA------- -------GTA AAT-A---TT
CMW 8554                        --TTGACGTC TGA------- -------GTA AAT-A---TT
CMW 8557                        --TTGACGTC TGA------- -------GTA AAT-A---TT
CMW 8570                        --TTGACGTC TGA------- -------GTA AAT-A---TT
M.walkeri STU2768               --TCGCGTCG GAGTCTCA-- -------GTA AATGAAA---
CMW 10899                       --TCGCGTCG GAGTCTCA-- -------GTA AATGAAA---
M.africana CMW4945              T-TTGCGTCG GAGTCTTA-- -----AAGTA AATT-AAA--
CMW 8576                        T-TTGCGTCG GAGTCTTA-- -----AAGTA AATTTAAA--
CMW 8574                        T-TTGCGTCG GAGTCTTA-- -----AAGTA AATTTAAA--
M.molleriana CMW2734            T-CTGCGTCT GAGTCACAAA ATC--AA-TC AAT-------
CMW 8575                        T-CTGCGTCT GAGTCACAAA ATC--AA-TC AAT-------
CMW 10898                       T-CTGCGTCT GAGTCACAAA ATC--AA-TC AAT-------
CMW 10897                       T-CTGCGTCT GAGTCACAAA ATC--AA-TC AAT-------
M.parkii CMW3358                TGCGT--CG- --GAGTAATT TT---ATT-- AAT-ACA-T-
CMW 8573                        T--G---CGT CGGAGTGATT TT-GTA---- AATCAAAA--
CMW 10896                       T--G---CGT CGGAGTGATT TT-GTA---- AATCAAAA--
M.lateralis CMW4935             T-TGCCGTCT --GAGTAACA AAC--A---- AATCAAAA--
CMW 8581                        T-TGCCGTCT --GAGTAACA AAC--A---- AATTAAAA--
Ramulispora anguioides          GTG-ATGTCT GAGTACTAT- -----AT--- AAT---AGTT

                                290        300        310        320

M.grandis                       GAATCAATCA AAACTTTTAA CAACGGATCT CTTGGTTCTG
CMW 8554                        GAATCAATCA AAACTTTTAA CAACGGATCT CTTGGTTCTG
CMW 8557                        GAATCAATCA AAACTTTTAA CAACGGATCT CTTGGTTCTG
CMW 8570                        GAATCAATCA AAACTTTTAA CAACGGATCT CTTGGTTCTG
M.walkeri STU2768               --------CA AAACTTTCAA CAACGAATCT CTTGGTTCTG
CMW 10899                       --------CA AAACTTTCAA CAACGGATCT CTTGGTTCTG
M.africana CMW4945              --------CA AAACTTTCAA CAACGGATCT CTTGGTTCTG
CMW 8576                        --------CA AAACTTTCAA CAACGGATCT CTTGGTTCTG
CMW 8574                        --------CA AAACTTTCAA CAACGGATCT CTTGGTTCTG
M.molleriana CMW2734            --------CA AAACTTTCAA CAACGGATCT CTTGGTTCTG
CMW 8575                        --------CA AAACTTTCAA CAACGGATCT CTTGGTTCTG
CMW 10898                       --------CA AAACTTTCAA CAACGGATCT CTTGGTTCTG
CMW 10897                       --------CA AAACTTTCAA CAACGGATCT CTTGGTTCTG
M.parkii CMW3358                ---------A AAACTTTCAA CAACGGATCT CTTGGTTCTG
CMW 8573                        --------CA AAACTTTCAA CAACGGATCT CTTGGTTCTG
CMW 10896                       --------CA AAACTTTCAA CAACGGATCT CTTGGTTCTG
M.lateralis CMW4935             --------CA AAACTTTCAA CAACGGATCT CTTGGTTCTG
CMW 8581                        --------CA AAACTTTCAA CAACGGATCT CTTGGTTCTG
Ramulispora anguioides          ---------A AAACTTTCAA CAACGGATCT CTTGGTTCTG
```

```
                                330        340        350        360

M.grandis                       GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG
CMW 8554                        GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG
CMW 8557                        GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG
CMW 8570                        GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG
M.walkeri STU2768               GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG
CMW 10899                       GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG
M.africana CMW4945              GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG
CMW 8576                        GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG
CMW 8574                        GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG
M.molleriana CMW2734            GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG
CMW 8575                        GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG
CMW 10898                       GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG
CMW 10897                       GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG
M.parkii CMW3358                GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG
CMW 8573                        GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG
CMW 10896                       GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG
M.lateralis CMW4935             GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG
CMW 8581                        GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG
Ramulispora anguioides          GCATCGATGA AGAACGCAGC GAAATGCGAT AAGTAATGTG

                                370        380        390        400

M.grandis                       AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC
CMW 8554                        AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC
CMW 8557                        AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC
CMW 8570                        AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC
M.walkeri STU2768               AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC
CMW 10899                       AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC
M.africana CMW4945              AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC
CMW 8576                        AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC
CMW 8574                        AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC
M.molleriana CMW2734            AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC
CMW 8575                        AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC
CMW 10898                       AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC
CMW 10897                       AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC
M.parkii CMW3358                AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC
CMW 8573                        AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC
CMW 10896                       AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC
M.lateralis CMW4935             AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC
CMW 8581                        AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC
Ramulispora anguioides          AATTGCAGAA TTCAGTGAAT CATCGAATCT TTGAACGCAC
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                                410        420        430        440

M.grandis                       ATTGCGCCCC TCGGTATTCC GAGGGGCATG CCTGTTCGAG
CMW 8554                        ATTGCGCCCC TCGGTATTCC GAGGGGCATG CCTGTTCGAG
CMW 8557                        ATTGCGCCCC TCGGTATTCC GAGGGGCATG CCTGTTCGAG
CMW 8570                        ATTGCGCCCC TCGGTATTCC GAGGGGCATG CCTGTTCGAG
M.walkeri STU2768               ATTGCGCCCT CTGGTATTCC GGGGGGCATG CCTGTTCGAG
CMW 10899                       ATTGCGCCCT CTGGTATTCC GGGGGGCATG CCTGTTCGAG
M.africana CMW4945              ATTGCGCCCC GTGGTATTCC GCGGGGCATG CCTGTTCGAG
CMW 8576                        ATTGCGCCCC GTGGTATTCC GCGGGGCATG CCTGTTCGAG
CMW 8574                        ATTGCGCCCC GTGGTATTCC GCGGGGCATG CCTGTTCGAG
M.molleriana CMW2734            ATTGCGCCCT CTGGTATTCC GGAGGGCATG CCTGTTCGAG
CMW 8575                        ATTGCGCCCT CTGGTATTCC GGAGGGCATG CCTGTTCGAG
CMW 10898                       ATTGCGCCCT CTGGTATTCC GGAGGGCATG CCTGTTCGAG
CMW 10897                       ATTGCGCCCT CTGGTATTCC GGAGGGCATG CCTGTTCGAG
M.parkii CMW3358                ATTGCGCCCC GTGGTATTCC GCGGGGCATG CCTGTTCGAG
CMW 8573                        ATTGCGCCCC GTGGTATTCC GCGGGGCATG CCTGTTCGAG
CMW 10896                       ATTGCGCCCC GTGGTATTCC GCGGGGCATG CCTGTTCGAG
M.lateralis CMW4935             ATTGCGCCCC CTGGTATTCC GGGGGGCATG CCTGTTCGAG
CMW 8581                        ATTGCGCCCC CTGGTATTCC GGGGGGCATG CCTGTTCGAG
Ramulispora anguioides          ATTGCGCCCT CTGGTATTCC GGGGGGCATG CCTGTTCGAG

                                450        460        470        480

M.grandis                       CGTCATTTCA CCAC-TCAAG C-CTG-GCTT GGTATTGGGC
CMW 8554                        CGTCATTTCA CCAC-TCAAG C-CTG-GCTT GGTATTGGGC
CMW 8557                        CGTCATTTCA CCAC-TCAAG C-CTG-GCTT GGTATTGGGC
CMW 8570                        CGTCATTTCA CCAC-TCAAG C-CTG-GCTT GGTATTGGGC
M.walkeri STU2768               CGTCATTTCA CCAC-TCAAG C-CTG-GCTT GGTATTGGGC
CMW 10899                       CGTCATTTCA CCAC-TCAAG C-CTG-GCTT GGTATTGGGC
M.africana CMW4945              CGTCATTTCA CCAC-TCAAG C-CTA-GCTT GGTATTGGGC
CMW 8576                        CGTCATTTCA CCAC-TCAAG C-CTA-GCTT GGTATTGGGC
CMW 8574                        CGTCATTTCA CCAC-TCAAG C-CTA-GCTT GGTATTGGGC
M.molleriana CMW2734            CGTCATTACA CCAC-TCCGG C-CTC-GCTG GGTATTGGGC
CMW 8575                        CGTCATTACA CCAC-TCCGG C-CTC-GCTG GGTATTGGGC
CMW 10898                       CGTCATTACA CCAC-TCCGG C-CTC-GCTG GGTATTGGGC
CMW 10897                       CGTCATTACA CCAC-TCCGG C-CTC-GCTG GGTATTGGGC
M.parkii CMW3358                CGTCATTTCA CCAC-TCGAG T-CTGA-CTC GGTATTGGGC
CMW 8573                        CGTCATTTCA CCAC-TCGAG T-CTGA-CTC GGTATTGGGC
CMW 10896                       CGTCATTTCA CCAC-TCGAG T-CTGA-CTC GGTATTGGGC
M.lateralis CMW4935             CGTCATTACA ACCAATCCAG C-CCC-GCTG GGTATTGGGC
CMW 8581                        CGTCATTGCA ACCAATCCAG C-CCC-GCTG GGTATTGGGC
Ramulispora anguioides          CGTCATTATA ACCACTCAAG CTCTC-GCTT GGTATTGGGG
```

```
                                490         500         510         520

M.grandis                       GCCGCGG-TT T---GCCGCG CGCCTCAAAG TCT---CCGG
CMW 8554                        GCCGCGG-TT T---GCCGCG CGCCTCAAAG TCT---CCGG
CMW 8557                        GCCGCGG-TT T---GCCGCG CGCCTCAAAG TCT---CCGG
CMW 8570                        GCCGCGG-TT T---GCCGCG CGCCTCAAAG TCT---CCGG
M.walkeri STU2768               GTCGCGG-T- ----GCCGCG CGCCTCAAAG TCTT--CCGG
CMW 10899                       GTCGCGG-T- ----GCCGCG CGCCTCAAAG TCTT--CCGG
M.africana CMW4945              GTCGCGG-TT -----CCGCG CGCCTTAAAG TCT---CCGG
CMW 8576                        GTCGCGG-TT -----CCGCG CGCCTTAAAG TCT---CCGG
CMW 8574                        GTCGCGG-TT -----CCGCG CGCCTTAAAG TCT---CCGG
M.molleriana CMW2734            GCCGCGGC-- --CT-CCGCG CGCCTCGAAG TCT---CCGG
CMW 8575                        GCCGCGGC-- --CT-CCGCG CGCCTCGAAG TCT---CCGG
CMW 10898                       GCCGCGGC-- --CT-CCGCG CGCCTCGAAG TCT---CCGG
CMW 10897                       GCCGCGGC-- --CT-CCGCG CGCCTCGAAG TCT---CCGG
M.parkii CMW3358                GTCGCGGCTT -CC-GCCGCG CGCCTCAAAG TCT---CCGG
CMW 8573                        GCCGCGCA-- -CC-GCCGCG CGCCTCAAAG TC---CCCGG
CMW 10896                       GCCGCGCA-- -CC-GCCGCG CGCCTCAAAG TC---CCCGG
M.lateralis CMW4935             GTCGCGGC-- --CTGCCGCG CGCCTCAAAG TCTT---CGG
CMW 8581                        GTCGCGGC-- --CTGCCGCG CGCCTCAAAG TCTA---CGG
Ramulispora anguioides          TTCGCGG-TT T-C-GC-G-G --CCTCTAAA -CTCA---GT

                                530         540         550         560

M.grandis                       CTG-AGCCAA CT---GTCTC TAAGCGTTGT GGTTTAA---
CMW 8554                        CTG-AGCCAA CT---GTCTC TAAGCGTTGT GGTTTAA---
CMW 8557                        CTG-AGCCAA CT---GTCTC TAAGCGTTGT GGTTTAA---
CMW 8570                        CTG-AGCCAA CT---GTCTC TAAGCGTTGT GGTTTAA---
M.walkeri STU2768               GTG-AGCTG- --CCCGTCTC CAAGCGTTGT GGCGACT---
CMW 10899                       CTG-AGCTG- --CCCGTCTC CAAGCGTTGT GGCGACT---
M.africana CMW4945              CTG-AGCAGT T---CGTCTC TAAGCGTTGT GGCATAT---
CMW 8576                        CTG-AGCAGT T---CGTCTC TAAGCGTTGT GGCATAT---
CMW 8574                        CTG-AGCAGT T---CGTCTC TAAGCGTTGT GGCATAT---
M.molleriana CMW2734            CCG-AGCCGA C---CGTCTC CAAGCGTTGT GGCACAACTG
CMW 8575                        CCG-AGCCGA C---CGTCTC CAAGCGTTGT GGCACAACTG
CMW 10898                       CCG-AGCCGA C---CGTCTC CAAGCGTTGT GGCACAACTG
CMW 10897                       CCG-AGCCGA C---CGTCTC CAAGCGTTGT GGCACAACTG
M.parkii CMW3358                CTG-GGCAG- C--CCGTCTC CGAGCGTTGT GGCATCACAG
CMW 8573                        CTG-GGCGGG C--CCGTCCC CGAGCGTTGT GATCTCACAG
CMW 10896                       CTG-GGCGGG C--CCGTCCC CGAGCGTTGT GATCTCACAG
M.lateralis CMW4935             CGGAAGCCG- --CCCGTTCC TCTGCGTGAT GACACATCG-
CMW 8581                        CGGAAGCCG- --CCCGTTCC TCTGCGTGAT GACACATCG-
Ramulispora anguioides          GGCGG---TG --CCTGTCGG CTCTACGCGT AGTAATAC--
```

```
                                570        580        590        600

M.grandis                       -TCATC-C-G C-TT-GTGAG AT-CGAA--G GCGA--CGGC
CMW 8554                        -TCATC-C-G C-TT-GTGAG AT-CGAA--G GCGA--CGGC
CMW 8557                        -TCATC-C-G C-TT-GTGAG AT-CGAA--G GCGA--CGGC
CMW 8570                        -TCATC-C-G C-TT-GTGAG AT-CGAA--G GCGA--CGGC
M.walkeri STU2768               -A-TTCGCTT CG----GGG- -CGCGGG-CG GC--CGCGGC
CMW 10899                       -A-TTCGCTT CG----GGG- -CGCGGG-CG GC--CGCGGC
M.africana CMW4945              -ATTTCGCT- -G--AAAGAG TT-CGGGACG GCT-------
CMW 8576                        -ATTTCGCT- -G--AA-GAG TT-CGG-ACG GCT-------
CMW 8574                        -ATTTCGCT- -G--AA-GAG TT-CGG-ACG GCT-------
M.molleriana CMW2734            TT--TCGCTT TC----GGG- AC-CGGTCTG GCGGCGCGCC
CMW 8575                        TT--TCGCTT TC----GGG- AC-CGGTCTG GCGGCGCGCC
CMW 10898                       TT--TCGCTT TC----GGG- AC-CGGTCTG GCGGCGCGCC
CMW 10897                       TT--TCGCTT TC----GGG- AC-CGGTCTG GCGGCGCGCC
M.parkii CMW3358                TTC-TCGCTA ------GGG- AGTCGCGGAC GGCGTCGGCC
CMW 8573                        T-C-TCGCTA ------GGG- AGTCGCGTCC GCCGGCGGCC
CMW 10896                       T-C-TCGCTA ------GGG- AGTCGCGTCC GCCGGCGGCC
M.lateralis CMW4935             ----TCGCTT ------GGGA -CACGGGGGT GAGCGCCCGG
CMW 8581                        ----TCGCTT ------GGGA -CACGGGGGT GAGCGCCCGG
Ramulispora anguioides          TCC-TCGCGA T-----TGAG TCCGGTAGGT TTACTTGCCA

                                610        620        630        640

M.grandis                       C---------G TTAAA-CTTA TTCA---AAG GTTGACCTCG
CMW 8554                        C---------G TTAAA-CTTA TTCA---AAG GTTGACCTCG
CMW 8557                        C---------G TTAAA-CTTA TTCA---AAG GTTGACCTCG
CMW 8570                        C---------G TTAAA-CTTA TTCA---AAG GTTGACCTCG
M.walkeri STU2768               C---------G TTAAATCTTT CAC----AAG GTTGACCTCG
CMW 10899                       C---------G TTAAATCTTT CAC----AAG GTTGACCTCG
M.africana CMW4945              -TTTGGCC-G TTAAATCTTT CTT---AAAG GTTGACCTCG
CMW 8576                        -TTTGGCC-G TTAAATCTTT CTT----AAG GTTGACCTCG
CMW 8574                        -TTTGGCC-G TTAAATCTTT CTT----AAG GTTGACCTCG
M.molleriana CMW2734            ----------G TTAAACCCTT -TCAC-AAAG GTTGACCTCG
CMW 8575                        ----------G TTAAACCCTT -TCACCAAAG GTTGACCTCG
CMW 10898                       ----------G TTAAACCCTT -T-ACCAAAG GTTGACCTCG
CMW 10897                       ----------G TTAAACCCGA -TCACCAAAG GTTGACCTCG
M.parkii CMW3358                ----------G TTAAATACCC --CATCAAAG GTTGACCTCG
CMW 8573                        ----------G TTAAACACCC -CCATCACAG GTTGACCTCG
CMW 10896                       ----------G TTAAACACCC -CCATCACAG GTTGACCTCG
M.lateralis CMW4935             A-AAACATCG GCGGAGACGT CGATTTCAAG GTTGACCTCG
CMW 8581                        A-AAACATCG GCGGAGACGT CGATTTCAAG GTTGACCTCG
Ramulispora anguioides          ACAACC--C- CC--A-A-TT --TTTTACAG GTTGACCTCG
```

650 655

M.grandis	GATCAGGTAG GGATA
CMW 8554	GATCAGGTAG GGATA
CMW 8557	GATCAGGTAG GGATA
CMW 8570	GATCAGGTAG GGATA
M.walkeri STU2768	GATCAGGTAG GGATA
CMW 10899	GATCAGGTAG GGATA
M.africana CMW4945	GATCAGGTAG GGATA
CMW 8576	GATCAGGTAG GGATA
CMW 8574	GATCAGGTAG GGATA
M.molleriana CMW2734	GATCAGGTAG GGATA
CMW 8575	GATCAGGTAG GGATA
CMW 10898	GATCAGGTAG GGATA
CMW 10897	GATCAGGTAG GGATA
M.parkii CMW3358	GATCAGGTAG GGATA
CMW 8573	GATCAGGTAG GGATA
CMW 10896	GATCAGGTAG GGATA
M.lateralis CMW4935	GATCAGGTAG GGATA
CMW 8581	GATCAGGTAG GGATA
Ramulispora anguioides	GATCAGGTAG GGATA

Chapter 3

BOTRYOSPHAERIA SPECIES CAUSING DIEBACK ON *EUCALYPTUS* SPP. IN CHILE

ABSTRACT

Eucalytus spp. represent the second largest forest plantation species in Chile with almost 360 000 ha already planted to these trees. The forestry industry has growing rapidly in this country, basing its *Eucalyptus* production mainly on *Eucalyptus globulus* and *E. nitens*. The increase in commercial plantations has resulted in a natural increase of new pests and diseases. Of these, Eucalyptus dieback associated with *Botryosphaeria* spp. in young and adult trees has been recognized as one of the diseases threatening *Eucalyptus* plantations in Chile. Very little research has been conducted on *Botryosphaeria* spp. on *Eucalyptus* and little is known regarding their identity or biology on this host. The aim of this chapter was, therefore, to identify *Botryosphaeria* spp. associated with dieback on commercially planted *Eucalyptus* trees in Chile. Symptomatic branches were collected from *E. globulus* and *E. nitens* plantations in Southern-central Chile, where dieback was severe. Species of *Botryosphaeria* were identified based on morphological characteristics and sequence data from the Internal Transcribed Spacer (ITS) region of the ribosomal RNA operon and the β-tubulin gene. Results showed that three *Botryosphaeria* species are present on *Eucalyptus* in the surveyed area. These species include *B. parva*, *B. eucalyptorum* and an undescribed species of *Botryosphaeria*. All three species represent new records for Chile. Pathogenicity trials conducted in greenhouses showed a significant difference in virulence between the three species with *B. parva* as the most virulent.

INTRODUCTION

Eucalyptus L' Heritier is one of the largest genera in the Myrtaceae containing approximately 700 species (Potts & Pederick 2000). These species are native to Australia, Papua New Guinea, Indonesia and surrounding islands (Poynton 1979). *Eucalyptus* spp. are well recognized for the excellent quality and versatility of their wood, and the variety of other purposes for which they can be utilised. They have thus become one of the world's most widely planted forestry crops (Poynton 1979, Turnbull 1991). Approximately 10 million ha of *Eucalyptus* spp. had been planted by the year 2000, for commercial timber production, with a yield of more than 30 million tons per year (Turnbull 1991).

Eucalyptus spp. were introduced into Chile in the early 1800's, and since the 1980's when the Chilean government implemented a subsidy on tree planting, they have become the second most commonly planted trees, after *Pinus radiata* D. Don. (Jayawickrama, Schlatter & Escobar 1993; Kliejunas *et al.* 2001). Approximately 360 000 ha of commercially planted *Eucalyptus* trees have been established in Chile (INFOR 2001). The most widely planted species are *E. globulus* Labill. and *E. nitens* (Deane & Maid.) Maid, however, species such as *E. camaldulensis* Dehnhardt, *E. cladocolyx* F. Mueller and *E. siderexylon* A. Cunningham, *E. regnans* F. Muell., *E. fastigata* Deane & Maiden, *E. delegatensis* R.T. Barker and *E. viminalis* Labill. have also been planted in some areas (Jayawickrama *et al.* 1993, INFOR 1997).

Disease problems have not been serious on *Eucalyptus* in Chile in the past, but they are beginning to appear quite rapidly. The most important disease on *Eucalyptus* in the country is Mycosphaerella leaf disease (MLD), caused by a variety of *Mycosphaerella* spp. This disease is particularly important on juvenile leaves of *E. globulus* (see Chapter II). Various less important diseases have been reported in *Eucalyptus* plantations in Chile, but very little research has been conducted to determine the identity of the pathogens or to assess the damage that they cause (Wingfield 2001, unpublished report). In a recent survey conducted by the Bioforest S.A.- Arauco companies, symptoms of dieback on *E. globulus* and *E. nitens* plantations were noted as a common problem. Preliminary studies showed that the symptoms were consistently associated with *Botryosphaeria* spp.

The genus *Botryosphaeria* Ces. & De Not. (Pleosporales, Loculoascomycetes, *Botryosphaeriaceae*) includes 143 species with anamorph states such as *Fusicoccum* Corda in Sturm., *Diplodia* Fr. in Mont. and *Lasiodiplodia* Ellis & Everh. (Von Arx 1987, Jacobs & Rehner 1998, Denman *et al.* 2000). These species are mainly latent pathogens that colonise plant tissue after stress or senescence. Occasionally, they cause extensive disease in different woody hosts and this is usually associated with high levels of susceptibility and stress (Schoeneweiss 1979, Zhonghua & Michailides 2001). *Botryosphaeria* spp. are well known for their cosmopolitan distribution and wide host range, producing stem cankers, tip dieback, kino exudation and various other symptoms on *Eucalyptus* spp. (Von Arx & Müller 1954, Von Arx 1987, Denman *et al.* 2000).

Disease associated with *Botryosphaeria* spp. is spread from one plantation to another by conidia or ascospores, which are dispersed via rain splash and wind (Cresswell & Milholland 1988, Pusey 1989). *Botryosphaeria* spp. penetrate trees through wounds or natural openings, even though the infection is usually latent (Caroll 1988). Wounds, serve as primary infection sites, therefore, insects, animals, hail, windstorms and cultural practices can increase the opportunity for the infection to develop in trees (Brown & Hendrix 1981, Swart, Wingfield & Knox-Davis 1987, Cresswell & Milholland 1988). Symptoms of the disease are normally expressed when the host is stressed. Some authors, however, have been able to isolate *Botryosphaeria* spp. from asymptomatic plant tissue, confirming that the pathogen is commonly present in healthy host tissue (Luttrell 1950, Fisher, Petrini & Sutton 1993, Roux 1998). The early view that these fungi are typical wound infecting opportunities appears not to be sound and that infection of healthy tissue and disease manifestation after stress seems more common.

Botryosphaeria spp. have been recognised as important pathogens of *Eucalyptus* spp. in various parts of the world (Barnard *et al.* 1987, Shearer, Tippett & Bartle 1987, Smith, Wingfield & Petrini 1996, Roux *et al.* 2000, Roux *et al.* 2001). Of these *B. dothidea* (Moug.: Fr.) Ces. & De Not. causes stem canker (Barnard *et al.* 1987, Smith, Kemp & Wingfield 1994) and the more recently described *B. eucalyptorum* Crous, H. Smith et M. J. Wingf. causes dieback (Smith *et al.* 2001). To the best of our knowledge, the only *Botryosphaeria* spp. recorded on *Eucalyptus* in Chile is *B. dothidea* (Gonzalez 1997, Munoz 1999), which was found on leaves of *E. camaldulensis* Dehn. The only case of severe dieback has been observed on *E. nitens* trees in the Southern-central area (Valdivia

– Lanco – Los Lagos areas) of Chile during May of 2000, where approximately 30 % of the trees showed tip death. However, most trees recovered their foliage during the following growing season (Brito, pers. comm).

The taxonomy of *Botryosphaeria* spp. is confusing and teleomorph structures are rarely observed in nature or produced in culture. Therefore, identification of these fungi has generally been based on morphological characteristics of the anamorph states, although this approach is also complicated by the similarities between conidia in various species and changes in conidial morphology with age of these spores (Jacobs & Rehner 1998, Smith & Stanosz 2001, Zhou & Stanosz 2001). Recent research has combined morphological characteristics and various molecular techniques such as RAPD (Smith & Stanosz 2001) and ISSR markers (Powell *et al.* 1996, Zhou & Stanosz 2001), RFLP's (Powell *et al.* 1996) and analysis of sequence data from the ITS regions of the ribosomal RNA operon (Jacobs & Rehner 1998, Denman *et al.* 2000), the β-tubulin genes and translation elongation factor 1-α (EF1-α) gene (Smith *et al.* 2001, Slippers *et al.* 2002). These contemporary techniques and particularly DNA sequence data have given rise to an entirely new perspective on the taxonomy of *Botryosphaeria* spp.

The aim of this study was to identify the *Botryosphaeria* species associated with dieback of *Eucalyptus* in Chilean plantations. This was achieved by collecting symptomatic tissue from trees in plantations in various parts of Chile, and subsequently studying specimens and cultures linked to these collections. Identifications were made based on morphological characteristics of the fungi as well as analysis of DNA sequence data.

MATERIALS AND METHODS

Collection of Samples

Surveys were conducted in *Eucalyptus* plantations in Chile during April and October 2001. Small branches and stem pieces displaying symptoms of dieback were collected from trees in Southern-central Chile. Samples were collected from symptomatic tissue on *E. globulus* and *E. nitens* trees, located in eight commercial plantations (Figure 1). Ten samples were collected from each tree and five trees were sampled from each plantation. Samples were

maintained at 4 °C in brown paper envelopes. They were then transported to the laboratory for further study.

Isolations and preparation of cultures

Five branches from each tree were selected for the isolation of *Botryosphaeria* spp. Using a sterile scalpel, the surface of the bark was removed to expose the spore masses of pseudothecia or pycnidia. Spore masses were then lifted from the structures using a sterile needle and transferred to 60 mm Petri dishes containing 2 % malt extract agar (MEA) and incubated at 25 °C, under cool white light in an incubator for seven to ten days.

For the production of anamorph structures, mycelium was transferred to 2 % water agar (WA) with sterilised pine needles placed on the surface of the medium and incubated at 25 °C with 12 hour cycles of near ultra-violet (nuv, 250 nm) light and dark (Sutton 1980, Johnson 1992, Crous & Palm 1999).

Morphological characteristics

Pycnidia developed on pine needles within approximately ten days of incubation. Conidia were taken from these structures and used for morphological characterization as described by Johnson (1992) and, Crous & Palm (1999). Conidia were spread on the surface of WA medium and after 12 to 24 hours of incubation, single germinating conidia were transferred to malt extract agar (MEA). Pycnidia that formed on pine needles were broken with a scalpel to liberate the conidia, which were mounted in lactophenol on glass microscope slides. Conidia were examined under a Zeiss light microscope. The average length of 20 conidia was measured for each isolate studied. Between five and ten cultures representing three groups based on conidial morphology were then chosen for further identification using DNA sequencing (Table 1).

DNA extraction and PCR amplification

Mycelium from actively growing MEA cultures was scraped from the surface of the agar plates using a sterile scalpel and lyophilized for 24 hours. The dried mycelium was ground to a fine powder, using liquid nitrogen. DNA was extracted using the method described by Raeder and Broda (1985), with minor modifications. Thus 800 µl of extraction buffer

(200mM Tris-HCl pH 8.3, 150mM NaCl, 25mM EDTA pH8.0, SDS 0.5%) was added to the ground mycelium, and mixed. Phenol and chloroform (v/v 5:3) was added to all samples, vortexed and centrifuged three times. Extracted nucleic acids were precipitated from the aqueous layer using two volumes of absolute ethanol. Nucleic acids were pelleted by centrifugation and washed with 70% ethanol. The dry nucleic acid pellets were then re-suspended in water and 4 µl RNaseA (10 µg/µl) (Sigma, USA) was added to digest any residual RNA. The resulting DNA was quantified by UV light visualization after electrophoresis in a 1% agarose gel stained with ethidium bromide. A Beckman DU Series 60 Spectrophotometer (Beckman, Germany) was used to calculate the DNA concentration of all DNA samples.

The Internal Transcribed Spacer (ITS) region of the rRNA operon, were amplified using primers ITS1 (5' TCC GTA GGT GAA CCT GCG G 3'), and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') (White *et al.* 1990). Part of the ß-tubulin 2-gene region was amplified for selected isolates using primers Bt 2a (5' GGT AAC CAA ATC GGT GCT GCT TTC 3') and Bt 2b (5' ACC CTC AGT GTA GTG ACC CTT GGC 3') (Glass & Donaldson 1995).

The same amplification and sequencing protocol was used to amplify both the ITS and ß-tubulin regions. The PCR reaction mixture contained 1 X PCR buffer and $MgCl_2$ (10 mM Tris-HCl, pH 8.0, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3) (Roche Diagnostic, South Africa), 0.2 mM of each dNTP (Roche Diagnostics, SA), 0.15 µM of primers (MWG Biotech, Germany), 0.5U Expand™ High Fidelity Taq DNA polymerase (Roche Molecular Biochemicals, Almeda, CA). Sterile deionized water was used to make up the total volume of the reaction to 50 µl.

The PCR reaction consisted of an initial denaturation step at 96 °C for 1 min, followed by 35 cycles of template denaturation at 94 °C for 30 s, primer annealing at 56 °C for 30 s, for ITS and ß-tubulin, followed by extension at 72 °C for 90 sec. A step up of 5 sec. elongation was added with each cycle after the first 25 cycles. A final elongation of 10 min at 72 °C completed the program. PCR products were purified using the High Pure PCR product purification Kit (Roche Diagnostics, Germany). PCR products were visualised on 2% agarose gels in a TAE buffer electrophoresis system, stained with ethidium bromide and

visualized with UV light. A 100 bp (base pair) molecular marker (Roche Diagnostic, SA) was used to determine the sizes of PCR products.

DNA sequencing and phylogenetic analysis

PCR products were sequenced in forward and reverse directions using the primers ITS1, ITS4, Bt 2a and Bt 2b. Sequencing reactions were performed using the ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems), following the protocols of the manufacturer. One forward primer (ITS1) and one reverse primer (ITS4) was used for the ITS region and a forward primer (Bt 2a) and one reverse primer (Bt 2b), was used for the ß-tubulin gene region (White *et al.* 1990). Sequence reactions were run using Polyacrylamide Gel Electrophoresis (PAGE) on an ABI PRISM™ 377 and capillary electrophoresis on an ABI PRISM™ 3100 Autosequencer (Perkin-Elmer Applied Biosystems).

Sequences were analysed using Sequence Navigator version 1.0.1 (Perkin-Elmer Applied Biosystems, USA). Sequences were aligned manually and gaps were inserted wherever necessary. A partition homogeneity test was used to test the congruence and combinability of the two data sets (Huelsenbeck, Bull & Cunningham 1996). The data sets were congruent and thus, ITS and ß-tubulin sequence data sets were combined. Phylogenetic analysis of the aligned sequences were determined using Phylogenetic Analysis Using Parsimony (PAUP version 4.0b1) (Swofford 1998). All characters in the analysis were given equal weight and the MULPAR option was effective. The heuristic search function based on parsimony with tree bisection reconnection (TBR) was used to generate a phylogram. Branch supports were calculated by performing a Bootstrap analysis on 1000 replications of the aligned sequences.

To determine the phylogenetic relationships and the identities of the *Botryosphaeria* spp., sequences of known *Botryosphaeria* spp. were obtained from GenBank as well as from the study of Slippers *et al.* (2002), and included in the alignment for comparative purposes. All resulting trees were rooted to *Botryosphaeria rhodina* (Cooke) Arx, which has been shown (Slippers, pers. comm.) to be a more effective outgroup taxon for this group than *Guignardia philoprina* (Ellis) Viala & Ravaz used in various previous studies (Jacobs 2002, Slippers *et al.* 2002).

Greenhouse Pathogenicity test

A pathogenicity test was conducted using two isolates for each of the three *Botryosphaeria* species identified, based on morphology and sequence data. The isolates representing these three species were randomly selected from the larger group of isolates (Table 2). All six isolates that were used in the inoculations were grown on MEA at 25 °C for one week prior to inoculation. Preservation and maintenance of the cultures was the same as that used for the identification studies.

Inoculations were conducted on a *E. grandis* Hill ex Maid. clone (ZG14) known to be susceptible to Botryosphaeria canker. A total of 70 trees were acclimatised in a phytotron at approximately 22 - 25 °C for three weeks prior to inoculation.

Ten trees were inoculated with each of the six isolates selected for testing and ten trees were inoculated as controls. Stems of trees to be inoculated were surface disinfested with 70% EtOH prior to inoculation. Bark disks were removed between two nodes, using a cork borer (4 mm diam.). Mycelium plugs from the edges of actively growing cultures or sterile MEA plates (controls) were then placed into the wounds made with the cork borer. Wounds were covered with Parafilm (Pechiney plastic packing, Chicago, USA) to reduce desiccation and to avoid contamination.

Seven weeks after inoculation, lesion lengths (mm) were recorded. Small pieces of wood from the lesion were plated onto MEA to re-isolate the inoculated fungus. Analysis of data was conducted for all isolates separately and also by grouping the isolates by species, using the linear model of analysis of variance (ANOVA) and means were separated based on LSD (Least Significant Difference) using the STATISTICA software for Windows (StatSoft, Inc., 1995).

RESULTS

Isolations and preparation of cultures

All samples used in this study originated from eight plantations, and included six *E. globulus* and two *E. nitens* trees. Symptoms were very similar, showing a death of young

trees or dead tips in adult trees (Figure 2). Newly developing symptoms were not common. In most cases, the material was collected from trees that were damaged one or two years before the surveys were conducted.

A total of 60 cultures were obtained from the various isolations made and 20 of these isolates were selected to identify the *Botryosphaeria* spp. present in the sampled area. All isolates sequenced in this study have been deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1).

Morphological characteristics

All primary isolations were made from bark pieces bearing teleomorph structures of *Botryosphaeria* spp. (Figure 3). Purified cultures were obtained from these primary isolations and they appeared to display very little difference in cultural morphology on MEA (Figure 3). All 20 isolates produced pycnidia on sterile pine needle (Figure 3). Single conidial cultures produced from these structures were used for subsequent sequencing and pathogenicity tests.

All conidia observed under the microscope were hyaline and characteristic of *Fusicoccum* spp. The average length of these conidia ranged from 19.3 to 26.0 µm, while the widths range from 6.7 to 7.8 µm (Figure 4). Three groups could be defined based on conidial size.

DNA sequencing and phylogenetic analysis

A total of 19 taxa were used for the sequencing analysis. A fragment of approximately 520 bp was amplified for the ITS region for all isolates. The amplicon produced for the ß-tubulin amplification was 440 bp. A partition homogeneity test to determine whether it is possible to combine both datasets showed that they are combinable ($p = 0.46$). The combined data set comprised 985 characters after alignment. A heuristic search was done on the combined data set. Of the 985 characters, 770 were constant, 30 of the variable characters were parsimony-uninformative and 185 were parsimony-informative. A single most parsimonious tree was generated with a Consistency index (CI = 0.888), retention

index (RI = 0.935) and homoplasy index (HI = 0.112). A bootstrap analysis of 1000 replications was done.

From the sequence analysis, seven clades emerged. Clade I includes isolates of *B. ribis* Grossenb. & Dugg. Two Chilean isolates (CMW 10532 & CMW 10549) were grouped in clade II, representing *B. parva* Pennycook & Samuels. Clade III contained *B. lutea*, while clade IV contained two Chilean isolates (CMW 10541 & CMW 10542) representing *B. eucalyptorum*. Clade V included two Chilean isolates (CMW 10530 & CMW 10531), as well as two isolates from Australia representing an undescribed *Botryosphaeria* sp. Clade VI and VII contained *B. dothidea* and *B. obtusa* (Schwein.) Shoem., respectively (Figure 5).

Greenhouse Pathogenicity test

All isolates tested for pathogenicity produced lesions on the outer bark, but more clearly in the xylem of the inoculated trees. No symptoms developed on trees inoculated as controls (Figure 6). The lesions observed in the xylem of the inoculated trees varied greatly in size and were not homogeneous. Thus, the variability between them was very high in some cases.

Mean inner lesion lengths on the trees inoculated with *Botryosphaeria* spp. showed a range of 21.5 mm for isolate CMW 10542 to 42.2 mm for isolate CMW 10532 (Table 2). Significant differences ($p < 0.001$) were found between control inoculations and trees inoculated (Table 2, Figure 7). The analysis showed three distinct groups with different lesion lengths. The first group of isolates (CMW 10547, CMW 10533, CMW 10532 and CMW 10539) gave rise to lesions ranging for 33.2 to 42.2 mm in length. The second group of isolates (CMW 10542 & CMW 10537) had lesions ranging in average length between 21.5 to 24.8 mm and the control gave rise to lesions and average of 9.1 mm including the size of the wound made by the cork borer (Table 2, Figure 7).

DISCUSSION

In this study, we have considered, for the first time, *Botryosphaeria* spp. associated with dieback on *Eucalyptus* in Chile. The results have clearly shown that three species of *Botryosphaeria* are associated with these symptoms. They included *B. eucalyptorum*, *B. parva* and an undescribed *Botryosphaeria* sp. We have also been able to show that these fungi differ in their pathogenicity and probably contribute, in varying degrees, to the symptoms observed in the field.

Botryosphaeria eucalyptorum and *B. parva* are well known worldwide from *Eucalyptus* spp.(Smith *et al.* 2001, Jacobs 2002), but they have not previously been recorded in Chile. The third species identified in this study represents an undescribed species that has significant sequence identity with other isolates from Australia and South Africa (Slippers *et al.* 2002). This fungus now requires formal description. Prior reports of *B. dothidea* from *Eucalyptus* in Chile (Gonzalez 1997, Munoz 1999) could not be confirmed and we doubt that this species is present.

Botryosphaeria eucalyptorum (anamorph *Fusicoccum eucalyptorum* Smith and M.J. Wingf.) has recently been reported causing cankers on the main stem in *E. grandis* and *E. nitens* plantations in South Africa (Smith *et al.* 2001). Smith *et al.* (2001), were also able to demonstrate that the pathogenicity of *B. eucalyptorum* appears to be much lower than that of other species such as *B. dothidea*. The results reported in the study conducted by Smith *et al.* (2001) are in accordance with those obtained in this thesis where *B. eucalyptorum* produced smaller lesion sizes that the other species tested.

Botryosphaeria parva was first described in 1985 from Kiwifruit in New Zealand (Pennycook & Samuels 1985). It is now well known worldwide as a pathogen of woody plants and fruit trees such as Mango trees in South Africa (Von Arx 1987, Jacobs 2002). Controversy with respect to the identity of this species has been clarified by Slippers *et al.* (2002) using combined data from the ITS region with ß-tubulin and/or Elongation factor sequence data. From this thesis, it is clear that *B. parva* is completely different to *B. ribis* and this is supported by the combined analysis of the ITS region and ß-tubulin gene sequences. *Botryosphaeria parva* was the species with the highest average lesion lengths in the inoculation trials. These results were also similar with those recently obtained by

Nakabonge (pers. comm.) on Ugandan *Eucalyptus* trees where *B. parva* was most pathogenic.

From morphological studies we have observed few cultural differences between *B. eucalyptorum*, *B. parva* and the unidentified *Botryosphaeria* sp. Morphological characteristics of conidia based on size and shape, gave us an orientation with respect to the identity of the species mentioned. Slippers *et al.* (2002) observed that not all conidia share similar characteristics and that it is generally necessary to combine both morphological characteristics and sequence data to obtain reliable identifications. Our results support this view.

This study has shown that Botryosphaeria dieback is probably not a particularly important disease in Chile. It does, however, occur in isolated situations and it will be important to monitor its presence in plantations. The fact that species such as *B. eucalyptorum* that cause serious disease in countries such as South Africa, is of concern. Certainly, if conditions become conducive for disease development, this fungus is likely to cause serious damage (Shearer *et al.* 1987, Fisher *et al.* 1993, Smith *et al.* 1996, Smith *et al.* 2001, Roux *et al.* 2001). Although this study has shown that the three species of *Botryosphaeria* are pathogenic to a single *E. grandis* clone, it will be important to assess pathogenicity on *E. nitens* and *E. globulus* grown in Chile.

Botryosphaeria spp. are well known opportunistic pathogens of *Eucalyptus* spp., especially when the trees are under stress (Smith *et al.* 1996, Smith *et al.* 2001). Strategies to reduce the stress and selection of resistant clones and use of hybrids would provide opportunities to avoid disease development in the future. In Chile, it will be necessary to select planting sites not conducive to disease, especially when less resistant material is planted. Future activities should also include more extensive surveys to achieve a better understanding of the presence and distribution of *Botryosphaeria* spp. and their potential to negatively impact on Chilean forest production.

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Table 1. *Botryosphaeria* isolates from Chile used in this study for identification porposes.

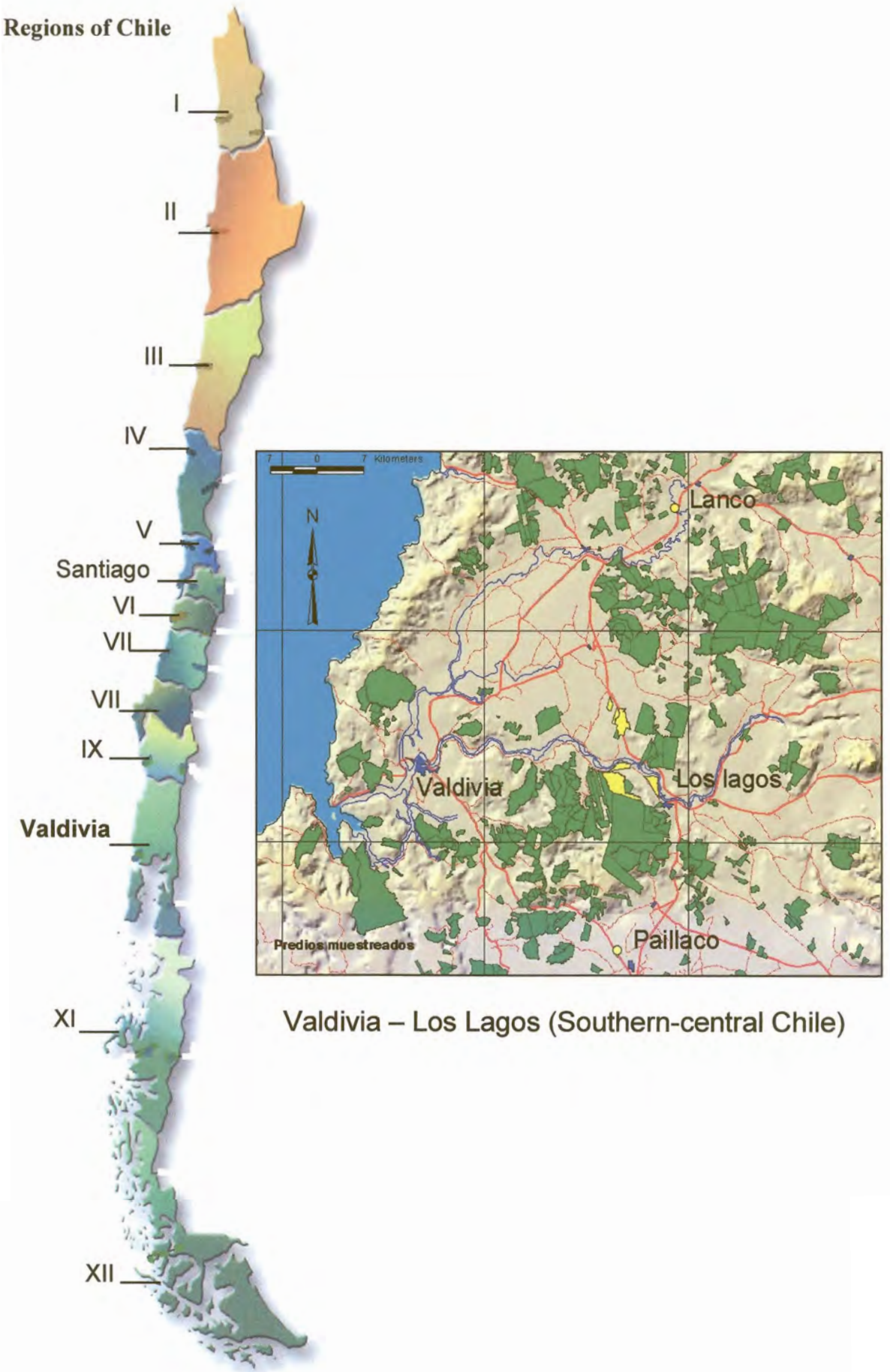
Culture No	Identity	Host	Collection Area	Collector	Date of Isolation
CMW 10530	*Botryosphaeria* sp.	*E. globulus*	Valdivia	R. Ahumada	July 2002
CMW 10531	*B.* sp.	*E. globulus*	Valdivia	R. Ahumada	July 2002
CMW 10532	*B. parva*	*E. globulus*	Valdivia	R. Ahumada	July 2002
CMW 10533	*B.* sp.	*E. globulus*	Valdivia	M.J. Wingfield	July 2002
CMW 10534	*B. eucalyptorum*	*E. globulus*	Valdivia	R. Ahumada	July 2002
CMW 10535	*B.* sp.	*E. globulus*	Valdivia	R. Ahumada	July 2002
CMW 10536	*B.* sp.	*E. globulus*	Valdivia	R. Ahumada	July 2002
CMW 10537	*B.* sp.	*E. globulus*	Valdivia	R. Ahumada	July 2002
CMW 10538	*B. parva*	*E. globulus*	Concepción	M.J. Wingfield	July 2002
CMW 10539	*B. parva*	*E. globulus*	Concepción	M.J. Wingfield	July 2002
CMW 10540	*B. eucalyptorum*	*E. globulus*	Los Lagos	R. Ahumada	October 2002
CMW 10541	*B. eucalyptorum*	*E. globulus*	Los Lagos	M.J. Wingfield	October 2002
CMW 10542	*B. eucalyptorum*	*E. globulus*	Los Lagos	M.J. Wingfield	October 2002
CMW 10543	Not identified	*E. globulus*	Los Lagos	R. Ahumada	October 2002
CMW 10544	Not identified	*E. globulus*	Los Lagos	R. Ahumada	October 2002
CMW 10545	*B.* sp.	*E. globulus*	Valdivia	M.J. Wingfield	October 2002
CMW 10546	*B. eucalyptorum*	*E. globulus*	Valdivia	M.J. Wingfield	October 2002
CMW 10547	*B. eucalyptorum*	*E. nitens*	Valdivia	R. Ahumada	October 2002
CMW 10548	*B.* sp.	*E. nitens*	Valdivia	R. Ahumada	October 2002
CMW 10549	*B. parva*	*E. nitens*	Valdivia	M.J. Wingfield	October 2002

Table 2. Lesion lengths on trees of a *E. grandis* clone (ZG14), resulting from inoculation with three *Botryosphaeria* spp. from Chile.

Isolates	Identity	Lesion Lengths (mm) Min	Max	Means[1]	
Control	Pure MEA	8	11	9.1	a
CMW 10547	*B. eucalyptorum*	22	52	36.1	cd
CMW 10542	*B. eucalyptorum*	12	46	21.5	b
CMW 10537	*B.* sp.	13	49	24.8	b
CMW 10533	*B.* sp.	21	65	38.7	cd
CMW 10532	*B. parva*	11	66	42.2	d
CMW 10539	*B. parva*	8	66	33.2	c

[1] Lesion lengths represent the mean of ten replications per isolates. Means with different letters are significantly different (LSD, $p < 0.005$).

Figure 1. Geographic distribution of commercial *Eucalyptus* plantations in Chile, showing sampling areas.



Valdivia – Los Lagos (Southern-central Chile)

Figure 2. Commercial plantation of *Eucalyptus* spp. affected by *Botryosphaeria* spp. in Southern-central area of Chile (Valdivia, Los Lagos).

Figure 3. Morphology of *Botryosphaeria* spp. found in Chile. **(A)** Perithecia on *Eucalyptus* bark from which isolations were made. **(B)** Asci and ascospores. **(C)** Pycnidia on sterile pine needles. **(D)** Examples of differences in culture morphology from the single conidia.

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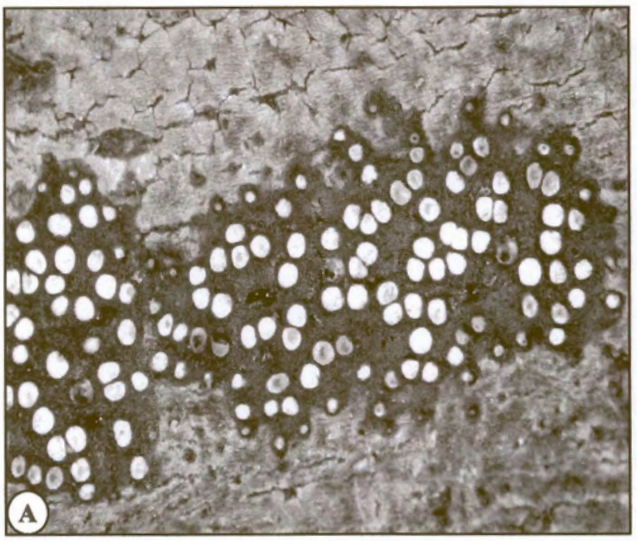

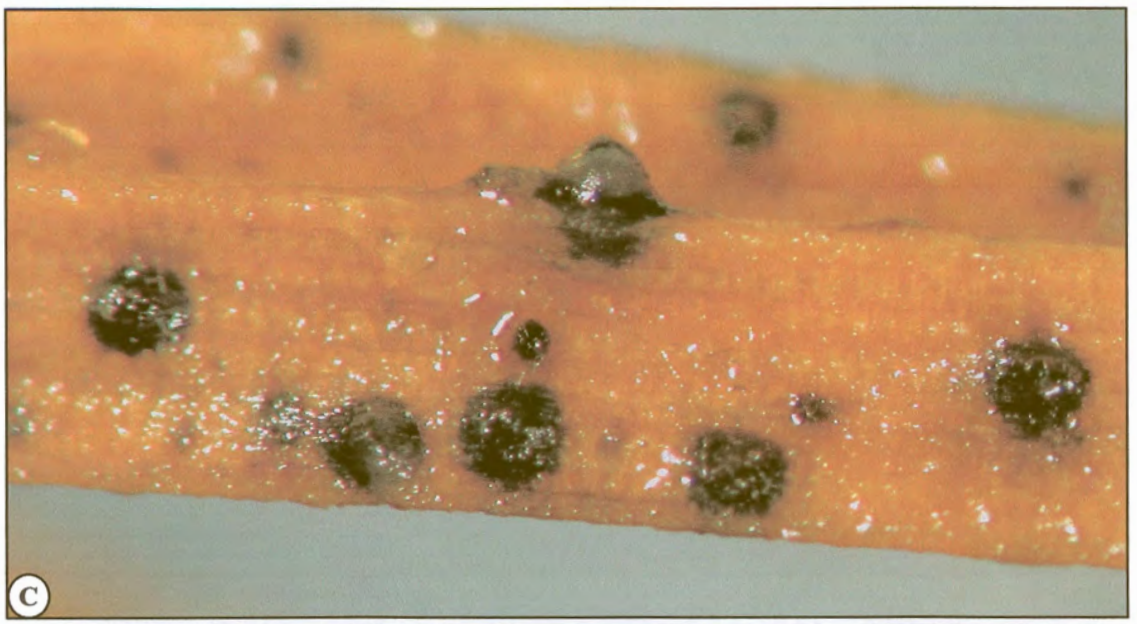

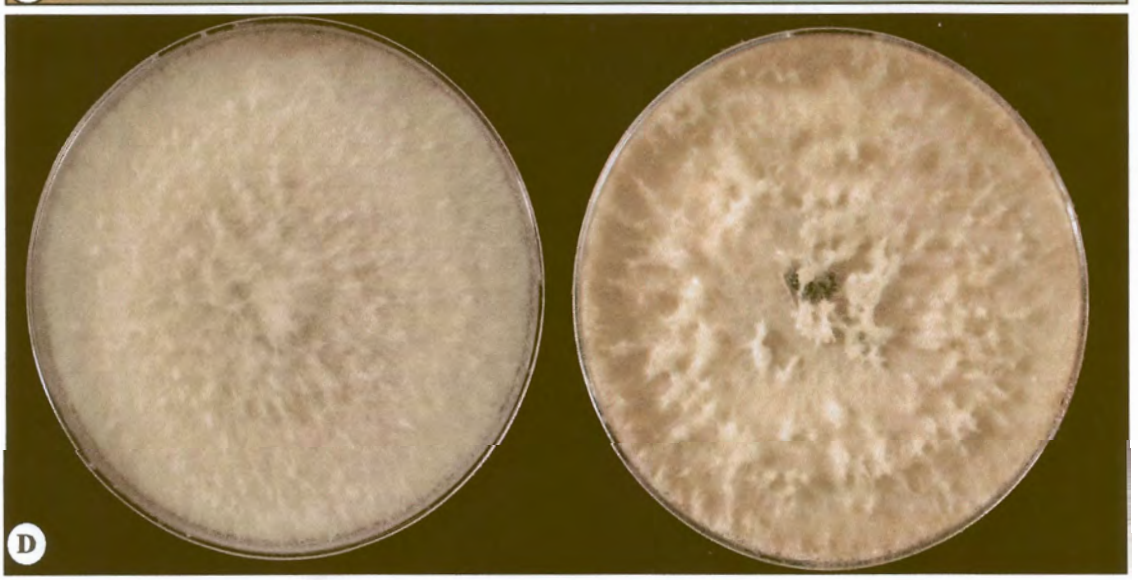

Figure 4. Conidia of the three species identified from *Eucalyptus* plantations in Chile, namely **(A)** *B. parva*. **(B)** *B. eucalyptorum* and **(C)** *Botryophaeria* sp. (Bars = 10 µm).

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Figure 5. Phylogram generated from combined ITS and β-tubulin sequences from the rRNA operon of seven *Botryosphaeria* spp. (p = 0.46, CI = 0.888, RI = 0.935, HI = 0.112) inferred using heuristic search and branch swapping options of PAUP version 4.0b1. Bootstrap support from 1000 replications is given above the branches and branches length below the branches. The tree is rooted to the outgroup, *B. rhodina*.

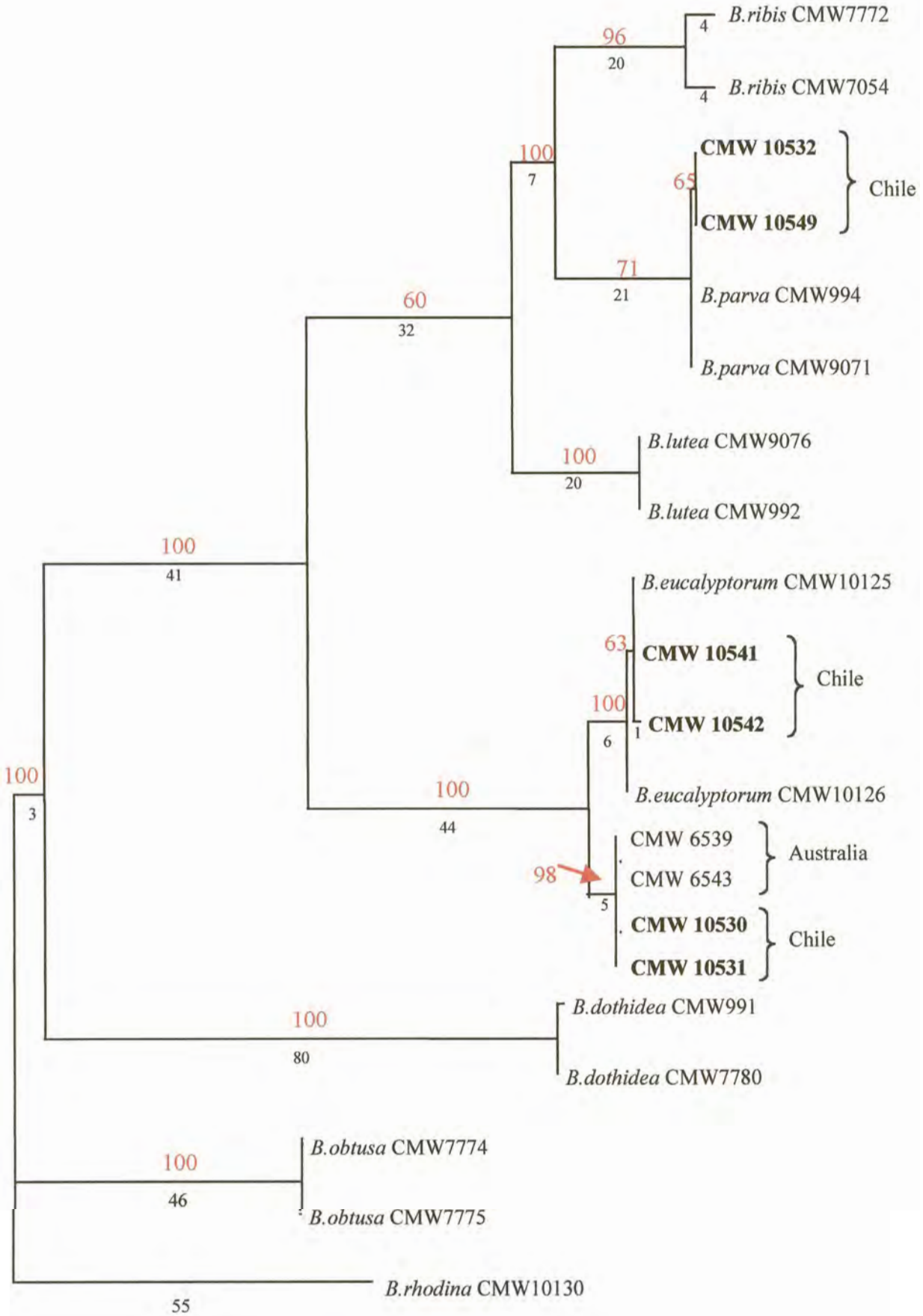
B.ribis CMW7772
B.ribis CMW7054
CMW 10532
CMW 10549
Chile
B.parva CMW994
B.parva CMW9071
B.lutea CMW9076
B.lutea CMW992
B.eucalyptorum CMW10125
CMW 10541
CMW 10542
Chile
B.eucalyptorum CMW10126
CMW 6539
CMW 6543
Australia
CMW 10530
CMW 10531
Chile
B.dothidea CMW991
B.dothidea CMW7780
B.obtusa CMW7774
B.obtusa CMW7775
B.rhodina CMW10130
96
20
4
4
100
7
65
71
21
60
32
100
20
100
41
63
100
6
1
100
3
100
44
98
5
100
80
100
46
55

Figure 6. Lesions observed in the xylem of an *E. grandis* clone, seven weeks after inoculation with three *Botryosphaeria* spp. and a control.

Control CMW 10547 CMW 10542 CMW 10537 CMW 10533 CMW 10532 CMW 10539

Figure 7. Average lesion lengths (mm) after inoculation of six isolates, representing the three *Botryosphaeria* spp. identified from Chile, and a control on an *E. grandis* clone.

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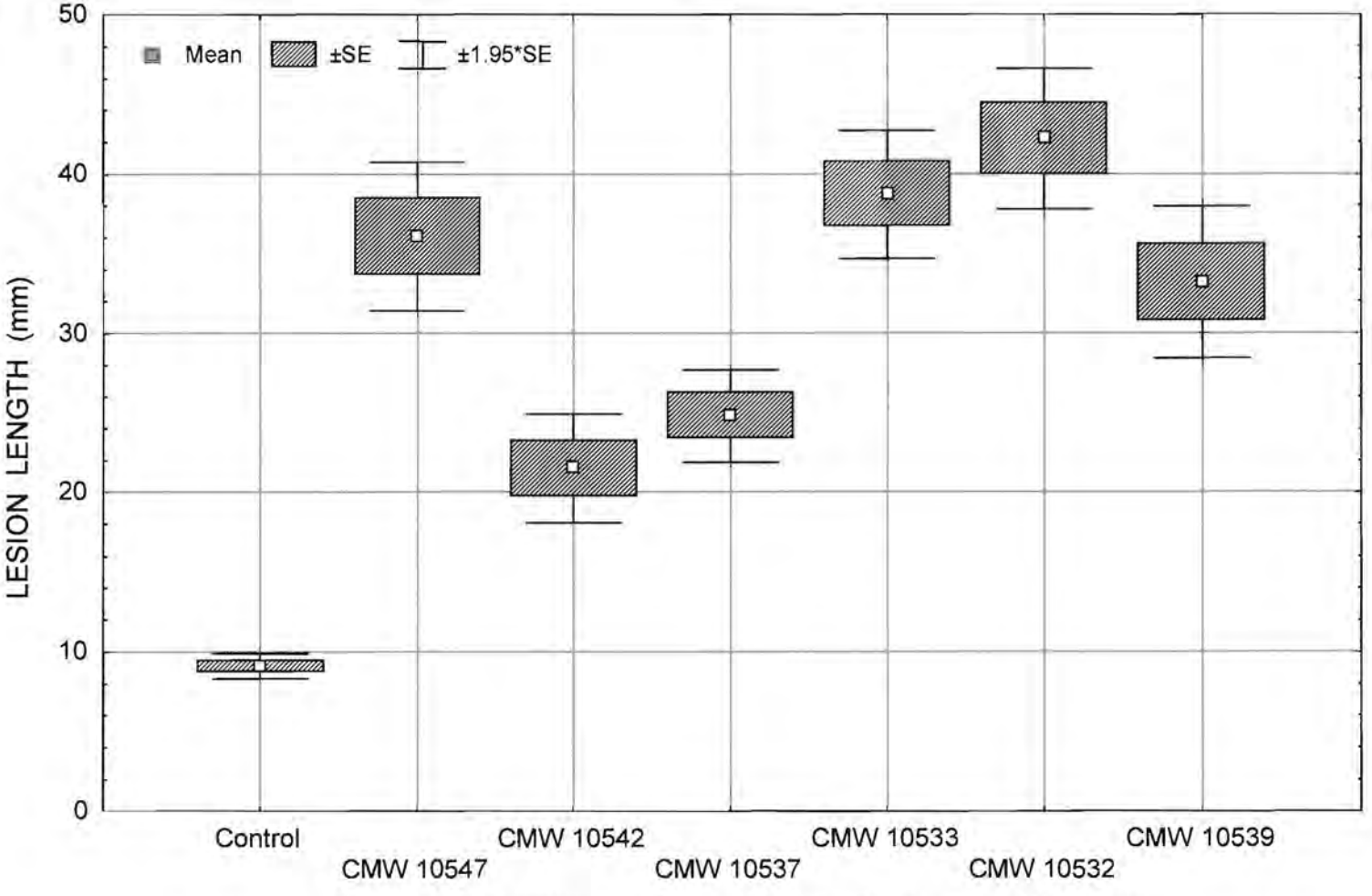

Figure 8. Combined ITS and β-tubulin sequences of *Botryosphaeria* species, consisted of 985 characteres of equal weight, with 771 constant characters of which 185 were parsimony-informative. Seuqence data set contains sequences of *Botryosphaeria* spp. isolated from *Eucalyptus* spp. from Chile.

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                                  10         20         30         40
B.ribis CMW7772                   GGAAGGATCA TTACCGAGTT GATTCGAGCT CCGGCTCGAC
B.ribis CMW7054                   GGAAGGATCA TTACCGAGTT GATTCGAGCT CCGGCTCGAC
CMW 10532                         GGAAGGATCA TTACCGAGTT GATTCGAGCT CCGGCTCGAC
CMW 10549                         GGAAGGATCA TTACCGAGTT GATTCGAGCT CCGGCTCGAC
B.parva CMW994                    GGAAGGATCA TTACCGAGTT GATTCGAGCT CCGGCTCGAC
B.parva CMW9071                   GGAAGGATCA TTACCGAGTT GATTCGAGCT CCGGCTCGAC
B.eucalyptorum CMW10125           GGAAGGATCA TTACCGAGTT GACTCGAGCT CCGGCTCGAC
B.eucalyptorum CMW10126           GGAAGGATCA TTACCGAGTT GACTCGAGCT CCGGCTCGAC
CMW 10541                         GGAAGGATCA TTACCGAGTT GACTCGAGCT CCGGCTCGAC
CMW 10542                         GGAAGGATCA TTACCGAGTT GACTCGAGCT CCGGCTCGAC
CMW 10530                         GGAAGGATCA TTACCGAGTT GACTCGAGCT CCGGCTCGAC
CMW 10531                         GGAAGGATCA TTACCGAGTT GACTCGAGCT CCGGCTCGAC
B.lutea CMW9076                   GGAAGGATCA TTACCGAGTT GATTCGAGCT CCGGCTCGAC
B.lutea CMW992                    GGAAGGATCA TTACCGAGTT GATTCGAGCT CCGGCTCGAC
B.dothidea CMW991                 GGAAGGATCA TTACCGAGTT GATTCGGGCT CCGGCCCGA-
B.dothidea CMW7780                GGAAGGATCA TTACCGAGTT GATTCGGGCT CCGGCCCGA-
B.obtusa CMW7774                  GGAAGNATCA TTACCGAGTT -CT-CGGGCT TCGGCTCGAA
B.obtusa CMW7775                  GGAAGGATCA TTACCGAGTT -CT-CGGGCT TCGGCTCGAA
B.rhodina CMW10130                GGAAGGATCA TTACCGAGTT --TTCGGGCT TCGGCTCGAC

                                  50         60         70         80
B.ribis CMW7772                   TC-TCCCACC CAATGTGTAC CTACCTCTGT TGCTTTGGCG
B.ribis CMW7054                   TC-TCCCACC CAATGTGTAC CTACCTCTGT TGCTTTGGCG
CMW 10532                         TC-TCCCACC CAATGTGTAC CTACCTCTGT TGCTTTGGCG
CMW 10549                         TC-TCCCACC CAATGTGTAC CTACCTCTGT TGCTTTGGCG
B.parva CMW994                    TC-TCCCACC CAATGTGTAC CTACCTCTGT TGCTTTGGCG
B.parva CMW9071                   TC-TCCCACC CAATGTGTAC CTACCTCTGT TGCTTTGGCG
B.eucalyptorum CMW10125           TC-TCCCACC CTATGTGTAC CTACCTCTGT TGCTTTGGCG
B.eucalyptorum CMW10126           TC-TCCCACC CTATGTGTAC CTACCTCTGT TGCTTTGGCG
CMW 10541                         TC-TCCCACC CTATGTGTAC CTACCTCTGT TGCTTTGGCG
CMW 10542                         TC-TCCCACC CTATGTGTAC CTACCTCTGT TGCTTTGGCG
CMW 10530                         TC-TCCCACC CTATGTGTAC CTACCTCTGT TGCTTTGGCG
CMW 10531                         TC-TCCCACC CTATGTGTAC CTACCTCTGT TGCTTTGGCG
B.lutea CMW9076                   TC-TCCCACC CCATGTGTAC CTACCTCTGT TGCTTTGGCG
B.lutea CMW992                    TC-TCCCACC CCATGTGTAC CTACCTCTGT TGCTTTGGCG
B.dothidea CMW991                 TCCTCCCACC CTTTGTGTAC CTACCTCTGT TGCTTTGGCG
B.dothidea CMW7780                TCCTCCCACC CTTTGTGTAC CTACCTCTGT TGCTTTGGCG
B.obtusa CMW7774                  TC-TCCCACC CTTTGTGAAC ATACCTCTGT TGCTTTGGCG
B.obtusa CMW7775                  TC-TCCCACC CTTTGTGAAC ATACCTCTGT TGCTTTGGCG
B.rhodina CMW10130                TC-TNCCACC CTTTGTGAAC GTACCTCTGT TGCTTTGGCG
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                                  90         100        110        120
B.ribis CMW7772                   GGCCGCGGTC CT--CCGC-A CCGG-CGCCC -TT--CG-GG
B.ribis CMW7054                   GGCCGCGGTC CT--CCGC-A CCGG-CGCCC -TT--CG-GG
CMW 10532                         GGCCGCGGTC CT--CCGC-A CCGG-CGCCC -TT--CG-GG
CMW 10549                         GGCCGCGGTC CT--CCGC-A CCGG-CGCCC -TT--CG-GG
B.parva CMW994                    GGCCGCGGTC CT--CCGC-A CCGG-CGCCC -TT--CG-GG
B.parva CMW9071                   GGCCGCGGTC CT--CCGC-A CCGG-CGCCC -TT--CG-GG
B.eucalyptorum CMW10125           GGCCGCGGTC CT--CCGC-A CCGG-CTCCC TTT---G-GG
B.eucalyptorum CMW10126           GGCCGCGGTC CT--CCGC-A CCGG-CTCCC TTT---G-GG
CMW 10541                         GGCCGCGGTC CT--CCGC-A CCGG-CTCCC TTT---G-GG
CMW 10542                         GGCCGCGGTC CT--CCGC-A CCGG-CTCCC TTT---G-GG
CMW 10530                         GGCCGCGGTC CT--CCGC-A CCGG-CCCCC TTT---G-GG
CMW 10531                         GGCCGCGGTC CT--CCGC-A CCGG-CCCCC TTT---G-GG
B.lutea CMW9076                   GGCCGCGGTC CT--CCGC-A CCGACCCCCG -TT--CG-GG
B.lutea CMW992                    GGCCGCGGTC CT--CCGC-A CCGACCCCCG -TT--CG-GG
B.dothidea CMW991                 GGCCGCGGTC CT--CCGCGG CCGGCCCCCC -TCCCCG-GG
B.dothidea CMW7780                GGCCGCGGTC CT--CCGCGG CCGGCCCCCC -TCCCCG-GG
B.obtusa CMW7774                  G-C-----TC TTTGCCGCGA --GGAGGCCC -T---CGCGG
B.obtusa CMW7775                  G-C-----TC TTTGCCGCGA --GGAGGCCC -T---CGCGG
B.rhodina CMW10130                G-C-----TC -------CG- ---------- ----------

                                  130        140        150        160
B.ribis CMW7772                   GGGGCTGGCC A--GCGC--- CCGCCAGAGG ACCAT-AAAA
B.ribis CMW7054                   GGGGCTGGCC A--GCGC--- CCGCCAGAGG ACCAT-AAAA
CMW 10532                         GGG-CTGGCC A--GCGC--- CCGCCAGAGG ACCAT-AAAA
CMW 10549                         GGG-CTGGCC A--GCGC--- CCGCCAGAGG ACCAT-AAAA
B.parva CMW994                    GGG-CTGGCC A--GCGC--- CCGCCAGAGG ACCAT-AAAA
B.parva CMW9071                   GGG-CTGGCC A--GCGC--- CCGCCAGAGG ACCAT-AAAA
B.eucalyptorum CMW10125           GG--CTGGCC A--GCGT--- CCGCCAGAGG ACCA-CAAAA
B.eucalyptorum CMW10126           GG--CTGGCC A--GCGT--- CCGCCAGAGG ACCA-CAAAA
CMW 10541                         GG--CTGGCC A--GCGT--- CCGCCAGAGG ACCA-CAAAA
CMW 10542                         GG--CTGGCC A--GCGT--- CCGCCAGAGG ACCA-CAAAA
CMW 10530                         GG--CTGGCC A--GCGC--- CCGCCAGAGG ACCA-CAAAA
CMW 10531                         GG--CTGGCC A--GCGC--- CCGCCAGAGG ACCA-CAAAA
B.lutea CMW9076                   GGG-CCGGCC A--GCGC--- CCGCCAGAGG ACCA-CAAAA
B.lutea CMW992                    GGG-CCGGCC A--GCGC--- CCGCCAGAGG ACCA-CAAAA
B.dothidea CMW991                 GGG--TGGCC A--GCGC--- CCGCCAGAGG ACCATCAAA-
B.dothidea CMW7780                GGG--TGGCC A--GCGC--- CCGCCAGAGG ACCATCAAA-
B.obtusa CMW7774                  GCC--CCCCC -GCGCGCTTT CCGCCAGAGG ACCTTCAAA-
B.obtusa CMW7775                  GCC--CCCCC -GCGCGCTTT CCGCCAGAGG ACCTTCAAA-
B.rhodina CMW10130                ---------- ---------- CCGCCAAAGG ACCTTCAAA-
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                                170        180        190        200
B.ribis CMW7772                 CTCCAGTCAG TGAAC-TTCG CAGTCTGAAA AACAAGTTAA
B.ribis CMW7054                 CTCCAGTCAG TGAAC-TTCG CAGTCTGAAA AACAAGTTAA
CMW 10532                       CTCCAGTCAG TGAAC-TTCG CAGTCTGAAA AACAAGTTAA
CMW 10549                       CTCCAGTCAG TGAAC-TTCG CAGTCTGAAA AACAAGTTAA
B.parva CMW994                  CTCCAGTCAG TGAAC-TTCG CAGTCTGAAA AACAAGTTAA
B.parva CMW9071                 CTCCAGTCAG TGAAC-TTCG CAGTCTGAAA AACAAGTTAA
B.eucalyptorum CMW10125         CTCCAGTCAG TAAACGTT-G CAGTCTGAAA AACAAGTTAA
B.eucalyptorum CMW10126         CTCCAGTCAG TAAACGTT-G CAGTCTGAAA AACAAGTTAA
CMW 10541                       CTCCAGTCAG TAAACGTT-G CAGTCTGAAA AACAAGTTAA
CMW 10542                       CTCCAGTCAG TAAACGTT-G CAGTCTGAAA AACAAGTTAA
CMW 10530                       CTCCAGTCAG TAAACGTT-G CAGTCTGAAA ACCAAGTTAA
CMW 10531                       CTCCAGTCAG TAAACGTT-G CAGTCTGAAA ACCAAGTTAA
B.lutea CMW9076                 CTCCAGTCAG TAAACG-TCG CAGTCTGAGA AACAAGTTAA
B.lutea CMW992                  CTCCACTCAC TAAACG-TCC CAGTCTCAGA AACAAGTTAA
B.dothidea CMW991               CTCCAGTCAG TAAACGAT-G CAGTCTGAAA AACAT-TTAA
B.dothidea CMW7780              CTCCAGTCAG TAAACGAT-G CAGTCTGAAA AACAT-TTAA
B.obtusa CMW7774                CTCCAGTCAG TAAACG-TCG ACGTCTGATA AACAAGTTAA
B.obtusa CMW7775                CTCCAGTCAG TAAACG-TCG ACGTCTGATA AACAAGTTAA
B.rhodina CMW10130              CTCCAGTCAG TAAACGCA-G ACGTCTGATA AACAAGTTAA

                                210        220        230        240
B.ribis CMW7772                 TAAACTAAAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA
B.ribis CMW7054                 TAAACTAAAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA
CMW 10532                       TAAACTAAAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA
CMW 10549                       TAAACTAAAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA
B.parva CMW994                  TAAACTAAAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA
B.parva CMW9071                 TAAACTAAAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA
B.eucalyptorum CMW10125         TAAACTAAAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA
B.eucalyptorum CMW10126         TAAACTAAAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA
CMW 10541                       TAAACTAAAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA
CMW 10542                       TAAACTAAAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA
CMW 10530                       TAAACTAAAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA
CMW 10531                       TAAACTAAAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA
B.lutea CMW9076                 TAAACTAAAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA
B.lutea CMW992                  TAAACTAAAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA
B.dothidea CMW991               TAAACTAAAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA
B.dothidea CMW7780              TAAACTAAAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA
B.obtusa CMW7774                TAAACTAAAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA
B.obtusa CMW7775                TAAACTAAAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA
B.rhodina CMW10130              TAAACTAAAA CTTTCAACAA CGGATCTCTT GGTTCTGGCA
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                                  250         260         270         280
B.ribis CMW7772                   TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT
B.ribis CMW7054                   TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT
CMW 10532                         TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT
CMW 10549                         TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT
B.parva CMW994                    TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT
B.parva CMW9071                   TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT
B.eucalyptorum CMW10125           TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT
B.eucalyptorum CMW10126           TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT
CMW 10541                         TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT
CMW 10542                         TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT
CMW 10530                         TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT
CMW 10531                         TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT
B.lutea CMW9076                   TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT
B.lutea CMW992                    TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT
B.dothidea CMW991                 TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT
B.dothidea CMW7780                TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT
B.obtusa CMW7774                  TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT
B.obtusa CMW7775                  TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT
B.rhodina CMW10130                TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT

                                  290         300         310         320
B.ribis CMW7772                   TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT
B.ribis CMW7054                   TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT
CMW 10532                         TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT
CMW 10549                         TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT
B.parva CMW994                    TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT
B.parva CMW9071                   TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT
B.eucalyptorum CMW10125           TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT
B.eucalyptorum CMW10126           TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT
CMW 10541                         TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT
CMW 10542                         TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT
CMW 10530                         TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT
CMW 10531                         TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT
B.lutea CMW9076                   TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT
B.lutea CMW992                    TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT
B.dothidea CMW991                 TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT
B.dothidea CMW7780                TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT
B.obtusa CMW7774                  TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT
B.obtusa CMW7775                  TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT
B.rhodina CMW10130                TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT
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                                330        340        350        360

B.ribis CMW7772                 GCGCCCCTTG GTATTCCGAG GGGCATGCCT GTTCGAGCGT
B.ribis CMW7054                 GCGCCCCTTG GTATTCCGAG GGGCATGCCT GTTCGAGCGT
CMW 10532                       GCGCCCCTTG GTATTCCGAG GGGCATGCCT GTTCGAGCGT
CMW 10549                       GCGCCCCTTG GTATTCCGAG GGGCATGCCT GTTCGAGCGT
B.parva CMW994                  GCGCCCCTTG GTATTCCGAG GGGCATGCCT GTTCGAGCGT
B.parva CMW9071                 GCGCCCCTTG GTATTCCGAG GGGCATGCCT GTTCGAGCGT
B.eucalyptorum CMW10125         GCGCCCCTTG GTATTCCGAG GGGCATGCCT GTTCGAGCGT
B.eucalyptorum CMW10126         GCGCCCCTTG GTATTCCGAG GGGCATGCCT GTTCGAGCGT
CMW 10541                       GCGCCCCTTG GTATTCCGAG GGGCATGCCT GTTCGAGCGT
CMW 10542                       GCGCCCCTTG GTATTCCGAG GGGCATGCCT GTTCGAGCGT
CMW 10530                       GCGCCCCTTG GTATTCCGAG GGGCATGCCT GTTCGAGCGT
CMW 10531                       GCGCCCCTTG GTATTCCGAG GGGCATGCCT GTTCGAGCGT
B.lutea CMW9076                 GCGCCCCTTG GTATTCCGAG GGGCATGCCT GTTCGAGCGT
B.lutea CMW992                  GCGCCCCTTG GTATTCCGAG GGGCATGCCT GTTCGAGCGT
B.dothidea CMW991               GCGCCCTTTG GTATTCCGAA GGGCATGCCT GTTCGAGCGT
B.dothidea CMW7780              GCGCCCTTTG GTATTCCGAA GGGCATGCCT GTTCGAGCGT
B.obtusa CMW7774                GCGCCCCCTG GCATTCCGGG GGGCATGCCT GTTCGAGCGT
B.obtusa CMW7775                GCGCCCCCTG GCATTCCGGG GGGCATGCCT GTTCGAGCGT
B.rhodina CMW10130              GCGCCCCTTG GTATTCCGGG GGGCATGCCT GTTCGAGCGT

                                370        380        390        400

B.ribis CMW7772                 CATTTCAACC CTCAAGCT-C TGCTTGGTAT TGGGCTCCGT
B.ribis CMW7054                 CATTTCAACC CTCAAGCT-C TGCTTGGTAT TGGGCTCCGT
CMW 10532                       CATTTCAACC CTCAAGCT-C TGCTTGGTAT TGGGCCTCGT
CMW 10549                       CATTTCAACC CTCAAGCT-C TGCTTGGTAT TGGGCCTCGT
B.parva CMW994                  CATTTCAACC CTCAAGCT-C TGCTTGGTAT TGGGCCCCGT
B.parva CMW9071                 CATTTCAACC CTCAAGCT-C TGCTTGGTAT TGGGCCCCGT
B.eucalyptorum CMW10125         CATTTCAACC CTCAAGCTT- TGCTTGGTAT TGGGCCCCGT
B.eucalyptorum CMW10126         CATTTCAACC CTCAAGCTT- TGCTTGGTAT TGGGCCCCGT
CMW 10541                       CATTTCAACC CTCAAGCTT- TGCTTGGTAT TGGGCCCCGT
CMW 10542                       CATTTCAACC CTCAAGCTT- TGCTTGGTAT TGGGCCCCGT
CMW 10530                       CATTTCAACC CTCAAGCT-C TGCTTGGTAT TGGGCTCCGT
CMW 10531                       CATTTCAACC CTCAAGCT-C TGCTTGGTAT TGGGCTCCGT
B.lutea CMW9076                 CATTTCAACC CTCAAGCT-C TGCTTGGTAT TGGGCTCCGT
B.lutea CMW992                  CATTTCAACC CTCAAGCT-C TGCTTGGTAT TGGGCTCCGT
B.dothidea CMW991               CATTACAACC CTCAAGCT-C TGCTTGGTAT TGGGCACCGT
B.dothidea CMW7780              CATTACAACC CTCAAGCT-C TGCTTGGTAT TGGGCACCGT
B.obtusa CMW7774                CATTACAACC CTCAAGCT-C TGCTTGGTAT TGGGCGCCGT
B.obtusa CMW7775                CATTACAACC CTCAAGCT-C TGCTTGGTAT TGGGCGCCGT
B.rhodina CMW10130              CATTACAACC CTCAAGCT-C TGCTTGGAAT TGGGCACCGT
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                                410        420        430        440
B.ribis CMW7772                 CCTCCA---C GGACGCGCCT TAAAGACCTC GGCGGTGGC-
B.ribis CMW7054                 CCTCCA---C GGACGCGCCT TAAAGACCTC GGCGGTGGC-
CMW 10532                       CCTCCA---C GGACGCGCCT TAAAGACCTC GGCGGTGGC-
CMW 10549                       CCTCCA---C GGACGCGCCT TAAAGACCTC GGCGGTGGC-
B.parva CMW994                  CCTCCA---C GGACGCGCCT TAAAGACCTC GGCGGTGGC-
B.parva CMW9071                 CCTCCA---C GGACGCGCCT TAAAGACCTC GGCGGTGGC-
B.eucalyptorum CMW10125         CCT-CT--GT GGACGCGCCT CAAAGACCTC GGCGGTGGC-
B.eucalyptorum CMW10126         CCT-CT--GT GGACGCGCCT CAAAGACCTC GGCGGTGGC-
CMW 10541                       CCT-CT--GT GGACGCGCCT CAAAGACCTC GGCGGTGGC-
CMW 10542                       CCT-CT--GT GGACGCGCCT CAAA-ACCTC GGCGGTGGC-
CMW 10530                       CCT-CT--GT GGACGCGCCT CAAAGACCTC GGCGGTGGC-
CMW 10531                       CCT-CT--GT GGACGCGCCT CAAAGACCTC GGCGGTGGC-
B.lutea CMW9076                 CCT-CT--GT GGACGCGCCT CGAAGACCTC GGCGGTGGC-
B.lutea CMW992                  CCT-CT--GT GGACGCGCCT CGAAGACCTC GGCGGTGGC-
B.dothidea CMW991               CCT--T-TGC GGGCGCGCCT CAAAGACCTC GGCGGTGGC-
B.dothidea CMW7780              CCT--T-TGC GGGCGCGCCT CAAAGACCTC GGCGGTGGC-
B.obtusa CMW7774                CCT-CTCTGC GGACGCGCCT TAAAGACCTC GGCGGTGGCT
B.obtusa CMW7775                CCT-CTCTGC GGACGCGCCT TAAAGACCTC GGCGGTGGCT
B.rhodina CMW10130              CCT-CACTGC GGACGCGCCT CAAAGACCTC GGCGGTGGCT

                                450        460        470        480
B.ribis CMW7772                 GTCTTGCC-T CAAGCGTAGT AGAAAA--CA CCTCGCTTTG
B.ribis CMW7054                 GTCTTGCC-T CAAGCGTAGT AGAAAA--CA CCTCGCTTTG
CMW 10532                       GTCTTGCC-T CAAGCGTAGT AGAAAA--CA CCTCGCTTTG
CMW 10549                       GTCTTGCC-T CAAGCGTAGT AGAAAA--CA CCTCGCTTTG
B.parva CMW994                  GTCTTGCC-T CAAGCGTAGT AGAAAA--CA CCTCGCTTTG
B.parva CMW9071                 GTCTTGCC-T CAAGCGTAGT AGAAAA--CA CCTCGCTTTG
B.eucalyptorum CMW10125         GTCTTGCC-T CAAGCGTAGT AGAA-AT-CA CCTCGCTTTG
B.eucalyptorum CMW10126         GTCTTGCC-T CAAGCGTAGT AGAA-AT-CA CCTCGCTTTG
CMW 10541                       GTCTTGCC-T CAAGCGTAGT AGAA-AT-CA CCTCGCTTTG
CMW 10542                       GTCTTGCC-T CAAGCGTAGT AGAA-AT-CA CCTCGCTTTG
CMW 10530                       GTCTTGCC-T CAAGCGTAGT AGAA-AT-CA CCTCGCTTTG
CMW 10531                       GTCTTGCC-T CAAGCGTAGT AGAA-AT-CA CCTCGCTTTG
B.lutea CMW9076                 GTCTTGCC-T CAAGCGTAGT AGAAAA--CA CCTCGCTTTG
B.lutea CMW992                  GTCTTGCC-T CAAGCGTAGT AGAAAA--CA CCTCGCTTTG
B.dothidea CMW991               GTCTTGCC-T CAAGCGTAGT AGAACATACA TCTCGCTTCG
B.dothidea CMW7780              GTCTTGCC-T CAAGCGTAGT AGAACATACA TCTCGCTTCG
B.obtusa CMW7774                GTTCAGCCCT CAAGCGTAGT AGAA--TACA CCTCGCTTTG
B.obtusa CMW7775                GTTCAGCCCT CAAGCGTAGT AGAA--TACA CCTCGCTTTG
B.rhodina CMW10130              GTTCAGCCCT CAAGCGTAGT AGAA--TACA CCTCGCTTTG
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                                570        580        590        600

B.ribis CMW7772                 TCTGGTTTGT TGCCAAAACA CTCCCGCTCC CGCGCCCCC-
B.ribis CMW7054                 TCTGGTTTGT TGCCAAAACA CTCCCGCTCC CGCGCCCCC-
CMW 10532                       TCTGGTTTGT TGCCAAAACA CTCCCGCTCC CGCGCCCCC-
CMW 10549                       TCTGGTTTGT TGCCAAAACA CTCCCGCTCC CGCGCCCCC-
B.parva CMW994                  TCTGGTTTGT TGCCAAAACA CTCCCGCTCC CGCGCCCCC-
B.parva CMW9071                 TCTGGTTTGT TGCCAAAACA CTCCCGCTCC CGCGCCCCC-
B.eucalyptorum CMW10125         TCTGGTTTGT TGCCAAAACA CTCTCGCTCC TGCGCCCCC-
B.eucalyptorum CMW10126         TCTGGTTTGT TGCCAAAACA CTCTCGCTCC TGCGCCCCC-
CMW 10541                       TCTGGTTTGT TGCCAAAACA CTCTCGCTCC TGCGCCCCC-
CMW 10542                       TCTGGTTTGT TGCCAAAACA CTCTCGCTCC TGCGCCCCC-
CMW 10530                       TCTGGTTTGT TGCCAAAACA CTCTCGCTCC TGCGCCCCC-
CMW 10531                       TCTGGTTTGT TGCCAAAACA CTCTCGCTCC TGCGCCCCC-
B.lutea CMW9076                 TCTGGTTTGT TGCCAAAACA CTGCCGCTCC CGCGCCCCC-
B.lutea CMW992                  TCTGGTTTGT TGCCAAAACA CTGCCGCTCC CGCGCCCCC-
B.dothidea CMW991               TCTGGTTTGT TGCCAAAACA C-CC-GCTCC CGCGCCCCC-
B.dothidea CMW7780              TCTGGTTTGT TGCCAAAACA C-CC-GCTCC CGCGCCCCC-
B.obtusa CMW7774                TCTGGTTTGT TGCCAAAACA CTCCCGCTGC CGCGCCCCCC
B.obtusa CMW7775                TCTGGTTTGT TGCCAAAACA CTCCCGCTGC CGCGCCCCCC
B.rhodina CMW10130              TCTGGTTTGT TGCCAAAACA CTCCTGCTCC TGCGCCCCCC

                                610        620        630        640

B.ribis CMW7772                 GCTGACGCGA ATCGACACCA CAGGCAGACC ATTTCCGGCG
B.ribis CMW7054                 GCTGACGCGA ATCGACACCA CAGGCAGACC ATTTCCGGCG
CMW 10532                       GCTGACGCGA ATCGACACCA CAGGCAGACC ATTTCTGGCG
CMW 10549                       GCTGACGCGA ATCGACACCA CAGGCAGACC ATTTCTGGCG
B.parva CMW994                  GCTGACGCGA ATCGACACCA CAGGCAGACC ATTTCTGGCG
B.parva CMW9071                 GCTGACGCGA ATCGACACCA CAGGCAGACC ATTTCTGGCG
B.eucalyptorum CMW10125         GCTGACGCGA ATCGACACCA TAGGCAGACC ATTTCTGGTG
B.eucalyptorum CMW10126         GCTGACGCGA ATCGACACCA CAGGCAGACC ATTTCTGGTG
CMW 10541                       GCTGACGCGA ATCGACACCA TAGGCAGACC ATTTCTGGTG
CMW 10542                       GCTGACGCGA ATCGACACCA TAGGCAGACC ATTTCTGGTG
CMW 10530                       GCTGACGCGA ATCGACACCA CAGGCAGACC ATTTCTGGCG
CMW 10531                       GCTGACGCGA ATCGACACCA CAGGCAGACC ATTTCTGGCG
B.lutea CMW9076                 GCTGACGCGA ATCGACACCG CAGGCAGACC ATTTCTGGCG
B.lutea CMW992                  GCTGACGCGA ATCGACACCG CAGGCAGACC ATTTCTGGCG
B.dothidea CMW991               GCTAACGCGA ATCGACACCA CAGGCAGACC ATCTCCGGCG
B.dothidea CMW7780              GCTAACGCGA ATCGACACCA CAGGCAGACC ATCTCCGGCG
B.obtusa CMW7774                GCTGACGCCA ATCGACACCA CAGGCAGACT ATCTCTGGCG
B.obtusa CMW7775                GCTGACGCCA ATCGACACCA CAGGCAGACT ATCTCTGGCG
B.rhodina CMW10130              GCTGACGG-A AGCGACACCA TAGGCAGACC ATCTCCGGCG
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                                650        660        670        680
B.ribis CMW7772                 AGCACGGCCT GGACGGCTCT GGCGTGTGAG TCTGCGCCGT
B.ribis CMW7054                 AGCACGGCCT GGACGGCTCT GGCGTGTGAG TCTGCGCCGT
CMW 10532                       AGCACGGCCT GGACGGCTCT GGCGTGTAAG TCTGCGCCGT
CMW 10549                       AGCACGGCCT GGACGGCTCT GGCGTGTAAG TCTGCGCCGT
B.parva CMW994                  AGCACGGCCT GGACGGCTCT GGCGTGTAAG TCTGCGCCGT
B.parva CMW9071                 AGCACGGCCT GGACGGCTCT GGCGTGTAAG TCTGCGCCGT
B.eucalyptorum CMW10125         AACACGGCCT GGACGGCTCT GGCGTGTAAG TCTGCGCCGT
B.eucalyptorum CMW10126         AACACGGCCT GGACGGCTCT GGCGTGTAAG TCTGCGCCGT
CMW 10541                       AACACGGCCT GGACGGCTCT GGCGTGTAAG TCTGCGCCGT
CMW 10542                       AACACGGCCT GGACGGCTCT GGCGTGTAAG TCTGCGCCGT
CMW 10530                       AACACGGCCT GGACGGCTCT GGCGTGTAAG TCTGCGCCGT
CMW 10531                       AACACGGCCT GGACGGCTCT GGCGTGTAAG TCTGCGCCGT
B.lutea CMW9076                 AGCACGGCCT GGACGGCTCT GGCGTGTAAG TCTGCGCCGT
B.lutea CMW992                  AGCACGGCCT GGACGGCTCT GGCGTGTAAG TCTGCGCCGT
B.dothidea CMW991               AGCACGGCCT GGACGGCTCT GGCGTGTAAG TCTGCATC-A
B.dothidea CMW7780              AGCACGGCCT GGACGGCTCT GGCGTGTAAG TCTGCATC-A
B.obtusa CMW7774                AGCACGGCCT GGACGGCTCC GGCGTGTAAG TTTGCG-C-T
B.obtusa CMW7775                AGCACGGCCT GGACGGCTCC GGCGTGTAAG TTTGCG-C-T
B.rhodina CMW10130              AGCACGGCCT GGATGGCTCC GGTGTGTAAG TGTGCG-CC-

                                690        700        710        720
B.ribis CMW7772                 TTC----CCG CGC-----GA A--TGGCAAT GGCTGACCC-
B.ribis CMW7054                 TTC----CCG CGC-----GA A--TGGCAAT GGCTGACCC-
CMW 10532                       TTC----CCG CGC-----GA A--TGGCAAT GGCTGACCC-
CMW 10549                       TTC----CCG CGC-----GA A--TGGCAAT GGCTGACCC-
B.parva CMW994                  TTC----CCG CGC-----GA A--TGGCAAT GGCTGACCC-
B.parva CMW9071                 TTC----CCG CGC-----GA A--TGGCAAT GGCTGACCC-
B.eucalyptorum CMW10125         TTC----CCG CTC-----GA A--TGGCAAT GGCTGACCC-
B.eucalyptorum CMW10126         TTC----CCG CTC-----GA A--TGGCAAT GGCTGACCC-
CMW 10541                       TTC----CCG CTC-----GA A--TGGCAAT GGCTGACCC-
CMW 10542                       TTC----CCG CTC-----GA A--TGGCAAT GGCTGACCC-
CMW 10530                       TTC----CCG CTC-----GA A--TGGCAAT GGCTGACCC-
CMW 10531                       TTC----CCG CTC-----GA A--TGGCAAT GGCTGACCC-
B.lutea CMW9076                 TTCTT----G CGC-----GA A--TGGCAAT GGCTGACCC-
B.lutea CMW992                  TTCTT----G CGC-----GA A--TGGCAAT GGCTGACCC-
B.dothidea CMW991               TTCTCA---G CG-TGGGAGA ACAT--CAAT GACT-AAACT
B.dothidea CMW7780              TTCTCA---G CG-TGGGAGA ACAT--CAAT GACT-AAACT
B.obtusa CMW7774                GTCTTTGCCG CGCTC----- ---TG-CAAT CGCTGACCCT
B.obtusa CMW7775                GTCTTTGCCG CGCTC----- ---TG-CAAT CGCTGACCCT
B.rhodina CMW10130              TTCTCCGCCG CG-------- -CATGGCAAT CGCTGAC-CT
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                                730        740        750        760

B.ribis CMW7772                 GTAGCAGCTA CAATGGCACC TCCGACCTGC AGCTCGAGCG
B.ribis CMW7054                 GTAGCAGCTA CAATGGCACC TCCGACCTGC AGCTCGAGCG
CMW 10532                       GCAGCAGCTA CAATGGCACC TCCGACCTGC AGCTCGAGCG
CMW 10549                       GCAGCAGCTA CAATGGCACC TCCGACCTGC AGCTCGAGCG
B.parva CMW994                  GCAGCAGCTA CAATGGCACC TCCGACCTGC AGCTCGAGCG
B.parva CMW9071                 GCAGCAGCTA CAATGGCACC TCCGACCTGC AGCTCGAGCG
B.eucalyptorum CMW10125         GCAACAGCTA CAATGGCACC TCCGACCTCC AGCTCGAGCG
B.eucalyptorum CMW10126         GCAACAGCTA CAATGGCACC TCCGACCTCC AGCTCGAGCG
CMW 10541                       GCAACAGCTA CAATGGCACC TCCGACCTCC AGCTCGAGCG
CMW 10542                       GCAACAGCTA CAATGGCACC TCCGACCTCC AGCTCGAGCG
CMW 10530                       GCAACAGCTA CAATGGCACC TCCGACCTCC AGCTCGAGCG
CMW 10531                       GCAACAGCTA CAATGGCACC TCCGACCTCC AGCTCGAGCG
B.lutea CMW9076                 GCAGCAGCTA CAATGGCACC TCCGACCTCC AGCTCGAGCG
B.lutea CMW992                  GCAGCAGCTA CAATGGCACC TCCGACCTCC AGCTCGAGCG
B.dothidea CMW991               GTAGCAGCTA CAATGGCACC TCGGACCTTC AGCTCGAGCG
B.dothidea CMW7780              GTAGCAGCTA CAATGGCACC TCGGACCTTC AGCTCGAGCG
B.obtusa CMW7774                TG-GCAGCTA CAATGGCACC TCCGACCTCC AGCTGGAGCG
B.obtusa CMW7775                TG-GCAGCTA CAATGGCACC TCCGACCTCC AGCTGGAGCG
B.rhodina CMW10130              GTAGCAGCTA CAATGGCACT TCGGACCTCC AACTGGAGCG

                                770        780        790        800

B.ribis CMW7772                 CATGAACGTC TACTTCAACG AGGTACTCTC TCACTAATTG
B.ribis CMW7054                 CATGAACGTC TACTTCAACG AGGTACTCTC TCACTAATTG
CMW 10532                       CATGAACGTC TACTTCAACG AGGTACTCTC TCACTAATTG
CMW 10549                       CATGAACGTC TACTTCAACG AGGTACTCTC TCACTAATTG
B.parva CMW994                  CATGAACGTC TACTTCAACG AGGTACTCTC TCACTAATTG
B.parva CMW9071                 CATGAACGTC TACTTCAACG AGGTACTCTC TCACTAATTG
B.eucalyptorum CMW10125         CATGAACGTC TACTTCAACG AGGTACTCTC TCACTAATCA
B.eucalyptorum CMW10126         CATGAACGTC TACTTCAACG AGGTACTCTC TCACTAATCA
CMW 10541                       CATGAACGTC TACTTCAACG AGGTACTCTC TCACTAATCA
CMW 10542                       CATGAACGTC TACTTCAACG AGGTACTCTC TCACTAATCA
CMW 10530                       CATGAACGTC TACTTCAACG AGGTACTCTC TCACTAATCA
CMW 10531                       CATGAACGTC TACTTCAACG AGGTACTCTC TCACTAATCA
B.lutea CMW9076                 CATGAACGTC TACTTCAACG AGGTACTCTC TCACTAATCG
B.lutea CMW992                  CATGAACGTC TACTTCAACG AGGTACTCTC TCACTAATCG
B.dothidea CMW991               CATGAACGTC TATTTCAACG AGGTACTCTC TCACTAATTA
B.dothidea CMW7780              CATGAACGTC TATTTCAACG AGGTACTCTC TCACTAATTA
B.obtusa CMW7774                CATGAACGTC TACTTCAACG AGGTACTCTC T-ACTAGTTA
B.obtusa CMW7775                CATGAACGTC TACTTCAACG AGGTACTCTC T-ACTAGTTA
B.rhodina CMW10130              CATGAACGTC TACTTCAACG AGGTACTCTC TCCATAATTA
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                                  810        820        830        840
B.ribis CMW7772                   CACAAACACG TAAAGTATGG CAATCTTCTG AACGCGCAGC
B.ribis CMW7054                   CACAAACACG TAAAGTATGG CAATCTTCTG AACGCGCAGC
CMW 10532                         CACAAACACG TAAAGTATGG CAATCTTCTG AACGCGCAGC
CMW 10549                         CACAAACACG TAAAGTATGG CAATCTTCTG AACGCGCAGC
B.parva CMW994                    CACAAACACG TAAAGTATGG CAATCTTCTG AACGCGCAGC
B.parva CMW9071                   CACAAACACG TAAAGTATGG CAATCTTCTG AACGCGCAGC
B.eucalyptorum CMW10125           CACAAACACA TAAAGTATGG CAATCTTCTG AACGCGCAGC
B.eucalyptorum CMW10126           CACAAACACA TAAAGTATGG CAATCTTCTG AACGCGCAGC
CMW 10541                         CACAAACACA TAAAGTATGG CAATCTTCTG AACGCGCAGC
CMW 10542                         CACAAACACA TAAAGTATGG CAATCTTCTG AACGCGCAGC
CMW 10530                         CACAAACACG TAAAGTATGG CAATCTTCTG AACGCGCAGC
CMW 10531                         CACAAACACG TAAAGTATGG CAATCTTCTG AACGCGCAGC
B.lutea CMW9076                   CACGAACACG TAAAGTATGG CAATCTTCTG AACGCGCAGC
B.lutea CMW992                    CACGAACACG TAAAGTATGG CAATCTTCTG AACGCGCAGC
B.dothidea CMW991                 GACAAACACG TAAAGTATGG CAATCTTCTG AACGCGCAGC
B.dothidea CMW7780                GACAAACACG TAAAGTATGG CAATCTTCTG AACGCGCAGC
B.obtusa CMW7774                  GACAAACACG TAAAGTATGG CAATCTTCTG AACGCGCAGC
B.obtusa CMW7775                  GACAAACACG TAAAGTATGG CAATCTTCTG AACGCGCAGC
B.rhodina CMW10130                GACAAACACG TAAAGTATGG CAATCTTCTG AACGCGCAGC

                                  850        860        870        880
B.ribis CMW7772                   AGGCGTCCAA CAACAAGTAC GTTCCTCGTG CCGTCCTCGT
B.ribis CMW7054                   AGGCGTCCAA CAACAAGTAC GTTCCTCGTG CCGTCCTCGT
CMW 10532                         AGGCGTCCAA CAACAAGTAC GTTCCTCGTG CCGTCCTCGT
CMW 10549                         AGGCGTCCAA CAACAAGTAC GTTCCTCGTG CCGTCCTCGT
B.parva CMW994                    AGGCGTCCAA CAACAAGTAC GTTCCTCGTG CCGTCCTCGT
B.parva CMW9071                   AGGCGTCCAA CAACAAGTAC GTTCCTCGTG CCGTCCTCGT
B.eucalyptorum CMW10125           AGGCGTCCAA CAACAAGTAC GTTCCTCGTG CCGTCCTCGT
B.eucalyptorum CMW10126           AGGCGTCCAA CAACAAGTAC GTTCCTCGTG CCGTCCTCGT
CMW 10541                         AGGCGTCCAA CAACAAGTAC GTTCCTCGTG CCGTCCTCGT
CMW 10542                         AGGCGTCCAA CAACAAGTAC GTTCCTCGTG CCGTCCTCGT
CMW 10530                         AGGCGTCCAA CAACAAATAC GTTCCTCGTG CCGTCCTCGT
CMW 10531                         AGGCGTCCAA CAACAAATAC GTTCCTCGTG CCGTCCTCGT
B.lutea CMW9076                   AGGCGTCGAA CAACAAGTAC GTTCCTCGTG CCGTCCTCGT
B.lutea CMW992                    AGGCGTCGAA CAACAAGTAC GTTCCTCGTG CCGTCCTCGT
B.dothidea CMW991                 AGGCGTCCAA CAACAAGTAC GTTCCTCGTG CCGTCCTCGT
B.dothidea CMW7780                AGGCGTCCAA CAACAAGTAC GTTCCTCGTG CCGTCCTCGT
B.obtusa CMW7774                  AGGCATCCAA CAATAAGTAC GTTCCTCGTG CTGTCCTCGT
B.obtusa CMW7775                  AGGCATCCAA CAATAAGTAC GTTCCTCGTG CTGTCCTCGT
B.rhodina CMW10130                AGGCGTCCAA CAACAAGTAC GTTCCTCGTG CTGTCCTCGT
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                                890        900        910        920

B.ribis CMW7772                 CGACCTCGAG CCCGGCACCA TGGATGCCGT CCGCGCCGGC
B.ribis CMW7054                 CGACCTCGAG CCCGGCACCA TGGATGCCGT CCGCGCCGGC
CMW 10532                       CGACCTCGAG CCCGGCACCA TGGATGCCGT CCGCGCCGGC
CMW 10549                       CGACCTCGAG CCCGGCACCA TGGATGCCGT CCGCGCCGGC
B.parva CMW994                  CGACCTCGAG CCCGGCACCA TGGATGCCGT CCGCGCCGGC
B.parva CMW9071                 CGACCTCGAG CCCGGCACCA TGGATGCCGT CCGCGCCGGC
B.eucalyptorum CMW10125         TGACCTCGAG CCCGGCACCA TGGATGCCGT CCGCGCCGGC
B.eucalyptorum CMW10126         TGACCTCGAG CCCGGCACCA TGGATGCCGT CCGCGCCGGC
CMW 10541                       TGACCTCGAG CCCGGCACCA TGGATGCCGT CCGCGCCGGC
CMW 10542                       TGACCTCGAG CCCGGCACCA TGGATGCCGT CCGCGCCGGC
CMW 10530                       TGACCTCGAG CCTGGCACCA TGGATGCCGT CCGCGCCGGC
CMW 10531                       TGACCTCGAG CCTGGCACCA TGGATGCCGT CCGCGCCGGC
B.lutea CMW9076                 CGACCTCGAG CCCGGCACCA TGGATGCCGT CCGCGCCGGC
B.lutea CMW992                  CGACCTCGAG CCCGGCACCA TGGATGCCGT CCGCGCCGGC
B.dothidea CMW991               CGACCTCGAG CCCGGCACGA TGGATGCTGT CCGCGCCGGC
B.dothidea CMW7780              CGACCTCGAG CCCGGCACGA TGGATGCCGT CCGCGCCGGC
B.obtusa CMW7774                TGACCTCGAG CCCGGCACCA TGGATGCCGT CCGCGCCGGC
B.obtusa CMW7775                TGACCTCGAG CCCGGCACCA TGGATGCCGT CCGCGCCGGC
B.rhodina CMW10130              CGACCTCGAG CCCGGCACCA TGGATGCCGT CCGCGCCGGC

                                930        940        950        960

B.ribis CMW7772                 CCCTTCGGCC AGCTCTTCCG CCCTGACAAC TTCGTCTTCG
B.ribis CMW7054                 CCCTTCGGCC AGCTCTTCCG CCCTGACAAC TTCGTCTTCG
CMW 10532                       CCCTTCGGCC AGCTCTTCCG CCCTGACAAC TTCGTCTTCG
CMW 10549                       CCCTTCGGCC AGCTCTTCCG CCCTGACAAC TTCGTCTTCG
B.parva CMW994                  CCCTTCGGCC AGCTCTTCCG CCCTGACAAC TTCGTCTTCG
B.parva CMW9071                 CCCTTCGGCC AGCTCTTCCG CCCTGACAAC TTCGTCTTCG
B.eucalyptorum CMW10125         CCCTTCGGCC AGCTCTTCCG CCCCGACAAC TTTGTCTTCG
B.eucalyptorum CMW10126         CCCTTCGGCC AGCTCTTCCG CCCCGACAAC TTTGTCTTCG
CMW 10541                       CCCTTCGGCC AGCTCTTCCG CCCCGACAAC TTTGTCTTCG
CMW 10542                       CCCTTCGGCC AGCTCTTCCG CCCCGACAAC TTTGTCTTCG
CMW 10530                       CCCTTCGGCC AGCTCTTCCG CCCCGACAAC TTTGTCTTCG
CMW 10531                       CCCTTCGGCC AGCTCTTCCG CCCCGACAAC TTTGTCTTCG
B.lutea CMW9076                 CCCTTCGGCC AGCTCTTCCG CCCCGACAAC TTTGTCTTCG
B.lutea CMW992                  CCCTTCGGCC AGCTCTTCCG CCCCGACAAC TTTGTCTTCG
B.dothidea CMW991               CCCTTCGGCC AGCTTTTCCG CCCCGACAAC TTCGTCTTCG
B.dothidea CMW7780              CCCTTCGGCC AGCTTTTCCG CCCCGACAAC TTCGTCTTCG
B.obtusa CMW7774                CCCTTCGGCC AGCTCTTCCG TCCCGACAAC TTCGTTTTCG
B.obtusa CMW7775                CCCTTCGGCC AGCTCTTCCG TCCCGACAAC TTCGTTTTCG
B.rhodina CMW10130              CCCTTCGGCC AGCTCTTCCG CCCCGACAAC TTCGTCTTCG
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                                970       980    985
B.ribis CMW7772                 GTCAGTCTGG CGCCGGTAAC AACTG
B.ribis CMW7054                 GTCAGTCTGG CGCCGGTAAC AACTG
CMW 10532                       GTCAGTCTGG CGCCGGTAAC AACTG
CMW 10549                       GTCAGNNNNN NNNNNNNNNN NNNNN
B.parva CMW994                  GTCAGTCTGG CGCCGGTAAC AACTG
B.parva CMW9071                 GTCAGNNNNN NNNNNNNNNN NNNNN
B.eucalyptorum CMW10125         GCCAGTCTGG TGCCGGTAAC AATTG
B.eucalyptorum CMW10126         GCCAGTCTGG TGCCGGTAAC AATTG
CMW 10541                       GCCAGTCTGG TGCCGGTAAC AATTG
CMW 10542                       GCCAGTCTGG TGCCGGTAAC AATTG
CMW 10530                       GCCAGTCTGG TGCCGGTAAC AATTG
CMW 10531                       GCCAGTCTGG TGCCGGTAAC AATTG
B.lutea CMW9076                 GCCAGTCTGG TGCCGGTAAC AACTG
B.lutea CMW992                  GCCAGTCTGG TGCCGGTAAC AACTG
B.dothidea CMW991               GTCAGTCCGG TGCCGGTANN NNNNN
B.dothidea CMW7780              GTCAGTCCGG TGCCGGTAAC AACTG
B.obtusa CMW7774                GCCAGTCTGG TGCCGGTAAC AACTG
B.obtusa CMW7775                GCCAGTCTGG TGCCGGTAAC AACTG
B.rhodina CMW10130              GCCAGTCTGG TGCCGGTAAC AACTG
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SUMMARY

Eucalyptus spp. represent the second largest commercial plantation species in Chile. Despite this fact, very little is known regarding diseases of these trees in the country. The research presented in this thesis is focused on two groups of pathogens. These include, *Mycosphaerella* spp. that are the cause of severe damage to leaves and *Botryosphaeria* spp. which infect shoots and stems of *Eucalyptus globulus* and *E. nitens* in Chile. The aim of the study has also been to expand the base of knowledge pertaining to *Eucalyptus* diseases and to utilise contemporary techniques to identify pathogens.

The first chapter of this thesis presents a general overview of *Pinus* and *Eucalyptus* plantation forestry in Chile, with special attention being given to the influence of pests and diseases. This review has shown that most of the existing research has been conducted on *P. radiata* plantations, where a relatively solid base of information exists. In contrast, it is clear that there is a lack of information regarding *Eucalyptus* diseases. Most of the taxonomy used to identify pathogens in Chile has been based on morphological characters. Phylogenetic analyses have generally not been utilised, prior to the studies presented in this thesis. Outcomes of this thesis also emphasise the need, not only to understand the importance of tree pathogens, but also the need to publish data pertaining to pests and diseases affecting commercial plantations in Chile. In this way, forestry research in the country will be more extensively exposed to the research groups elsewhere in the world and opportunities for collaboration will be increased.

In the second chapter of this thesis, it was possible to provide important information pertaining to the identification of *Mycosphaerella* spp. associated with leaf disease of *E. globulus* and *E. nitens* in Chile. Thus, six *Mycosphaerella* spp. were shown to be associated with *Eucalyptus* leaf disease in the plantations that were surveyed. These species include *M. africana, M. grandis, M. lateralis, M. molleriana, M. parkii* and *M. walkeri*, of which only *M. walkeri* has been reported in Chile previously. *Mycosphaerella grandis* appeared to be the most predominant species, which was present in northern and southern-central Chile, on *E. globulus* and *E. nitens* and also in juvenile and adult leaves. This species has also been reported as a cause of serious defoliation in Australia. *Mycosphaerella africana, M. lateralis, M. molleriana, M. parkii* and *M. walkeri* are

apparently not associated with the severe defoliation that has been observed during the last two years in Chile. However, to confirm this, it will be important to conduct further investigations and disease surveys. Previous suggestions that the important pathogens, *M. cryptica* and *M. nubilosa* are present in Chile have not been confirmed. This is encouranging, considering that these are two of the most pathogenic *Mycosphaerella* species on *Eucalyptus*. Knowledge resulting from this study will now be used to develop effective management strategies to reduce the impact caused by *Mycosphaerella* on *Eucalyptus* forestry in Chile.

The third chapter of this thesis deals with disease known as Botryosphaeria dieback. This disease appears to be increasing in importance in Chile and the species of *Botryosphaeria* associated with the disease have, as yet, not been identified. Results of this study using conventional morphological techniques and analysis of DNA sequences have shown that three *Botryosphaeria* species are present in the surveyed, which included *E. globulus* and *E. nitens* plantations in northern and southern-central Chile. These species include *B. parva*, *B. eucalyptorum* and an undescribed species (*B. sp.*). Previous reports based on morphology, identifying *B. dothidea* were probably incorrect. The three species identified in this study all represent new records for Chile. Pathogenicity trials conducted in a greenhouse showed that all of the species are pathogenic, but *B. parva* appeared to be the most virulent and more so than *B. eucalyptorum*. It will be important, in the future to re-evaluate the pathogenicity of those species on mature *E. globulus* and *E. nitens* in Chile. Even though, *Botryosphaeria* spp. are considered as opportunistic pathogens, it is important to carefully select plantation sites to reduce potential stress that may be placed on the trees. It will also be important in the future to select resistant planting stock that is able to withstand infections by *Botryospaheria* spp.

The knowledge generated from studies that make up this thesis will certainly be an important step in the process of developing a forest pathology program in Chile. New techniques for the identification of tree pathogens have been acquired and it is hoped that these can be further applied in the Chilean forestry situation in the future. Little attention was given to control of the pathogens studied in this thesis and this will be an important step in the future. In conclusion, it is hoped that the studies in this thesis will stimulate further research on *Eucalyptus* diseases in Chile and promote the field of forest pathology in the country.