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Die-back of cold tolerant eucalypts associated with *Phytophthora* spp. in South Africa

By

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TABLE OF CONTENTS **Page**

TABLE OF CONTENTS CONT **Page**

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Declaration

I, Bruce O'clive Zwelibanzi Maseko, declare that this thesis, which I hereby submit for the degree Philosophiae Doctor at the University of Pretoria, is my own work and has not been submitted by me for a degree at this or any other tertiary institution

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Preface

Chapter 1: is a comprehensive review of the literature, which covers the taxonomy of the genus *Phytophthora*, methods used to control and identify *Phytophthora* spp. and an overview of root and collar rot of cold tolerant eucalypts in South Africa.

Chapter 2: deals with extensive surveys conducted in several *Eucalyptus* plantations in the Mpumalanga and KwaZulu-Natal Provinces of South Africa. *P. nicotianae* rather than *P. cinnamomi* was consistently and more frequently isolated from the rhizosphere soil and infected root collars of cold-tolerant *Eucalyptus* spp. However, pathogenicity tests showed that *P. nicotianae* isolates were less pathogenic than isolates of *P. cinnamomi.*

Chapter 3: deals with the role of *Phytophthora* root rot disease in the poor establishment and growth of *E. smithii*. Results obtained in this study indicated that three *Phytophthora* spp., namely *P. nicotianae*, *P. boehmeriae,* and the recently described *P. frigida*, are involved in the poor establishment of *E. smithii* in re-established study sites investigated. None of the above *Phytophthora* spp. were recovered from the virgin grassland site. Nevertheless, 20% seedling mortality was recorded at this *Phytophthora*-free site and seedling mortality did not differ significantly from the other three re-established sites investigated. Seedling mortality and absence of *Phytophthora* spp. on the virgin grassland site suggested that other factors play a role the poor establishment of *E. smithii* seedlings on the study sites investigated.

Chapter 4: deals with the development of reliable and robust screening methods of a large number *E. smithii* seedlings for tolerance to *P. nicotianae* and *P. cinnamomi*. Preliminary data indicated that total phenolics could be use as a potential robust tool for screening large number of seedlings. Three highly tolerant and susceptible *E. smithii* half-sib families were identified using the stem inoculation technique.

Chapter 5: is a taxonomic description of two previously unknown species of *Phytophthora* namely; *P. frigida* and *P. alticola*, associated with root and collar rot disease of cold tolerant eucalypts.

Chapter One

Die-back of cold-tolerant eucalypts associated with *Phytophthora* spp. in South Africa: A literature review

1 Introduction

The genus *Eucalyptus* (commonly known as 'eucalypts' or 'gum trees'), belongs to the large plant family *Myrtaceae* which consists of at least 700 species (Brooker 2000). The majority of the species are endemic to Australia with an exception of four species which are found in neighbouring Australasian islands (Ladiges *et al*. 2003). New species continue to be discovered and more are likely to be described in the future as robust molecular techniques become available to discriminate between similar sorts (Hill & Johnson 2000, Hill *et al.* 2001). Despite extensive review and taxonomic revision the taxonomy of *Eucalyptus* remains incomplete (Griffin *et al*. 1988), *Angophora* and *Corymbia* were included as subgenera (Brooker 2000). However, this was critised by Ladiges & Udovicic (2000). A view, consistent with both morphological (Hill & Johnson 1995) and molecular studies (Ladiges *et al*. 1995, Sale *et al*. 1993, Udovicic *et al*. 1995, Steane *et al.* 1999), suggests that in broad terms the eucalypts consist of three major lineages namely, the *Angophora* and *Corymbia* (bloodwoods, ghost gums) lineage and the *Eucalyptus sensu stricto* lineage. The latter comprises three major subgroups, namely, the *Symphyomyrtus*, *Eudesmia,* and *Monocalyptus* (Hill & Johnson 1995, Ladiges *et al*. 1995).

Eucalypts are versatile trees that grow in a variety of climatic conditions and in a diverse range of habitats (Turnbull 2000). They were initially introduced into the tropical and subtropical regions of Africa and South America for fuel wood, windbreaks, and land reclamation purposes since the 1900s (Poynton 1979, Evans 1982, Potts & Pederick 2000). Over the years, eucalypts have become a major source material for diverse commercial forest products such as pulpwood, fibreboard, sawn timber, poles, mine timber props, charcoal, honey and essential oils (Sedjo 1999, Turnbull 1999). A combination of biological factors such as wood properties, high productivity and versatile ecological adaptation have made eucalypts the most widely planted hardwood trees in many parts of the world (Turnbull 2000). As such, eucalypts are grown as a plantation species in several countries in Africa, Asia, South America and New Zealand, as well as throughout its extensive natural range in Australia (Eldridge *et al.* 1994). Eucalypt plantations are rapidly expanding and the total area under plantation is estimated to be at least 18 million hectares in 90 countries (Carle *et al*. 2002, FAO 2000).

High-yielding and genetically improved *E. camaldulensis*, *E. grandis, E. globules*, *E. urophylla*, and their inter-specific hybrids are leading sources of hardwood throughout the world (Campinhos 1999).

South Africa has a long history of planting eucalypts, since it does not have its own natural timber resources (Zwolinski & Bayley 2001). It is amongst the leading *Eucalyptus* growing countries in the world (Schönau *et al*. 1994, Owen & van der Zel 2000). These plantations are distributed along the eastern coastline of South Africa, and comprise an assortment of species and hybrids that are planted in various habitats with diverse climatic conditions (Forestry South Africa 2008). Eucalypts in South African plantations are harvested at short rotation periods of 7-8 years for production of pulpwood and other timber products (Poynton 1981). Because of its suitability for such purposes, improved *E. grandis* and its various clones, crosses and hybrids are the most commonly planted genotypes (Wingfield *et al*, 2002). These constitute more than 75% of commercial eucalypt plantings in South Africa, and the total area planted to *E. grandis* is at least 290, 155 ha (Forestry South Africa 2008). *E. grandis* is a preferred forest tree species for commercial forestry in subtropical areas because it grows fast, has good form and versatile wood and pulp properties (Turnbull & Pryor 1978). It is amongst the best *Eucalyptus* spp. for short rotation forestry and is also planted extensively in South America (Eldridge *et al.* 1994). *E. grandis* performs very well and produces good yield per hectare when planted at correct sites (Eldridge *et al.* 1994). However, it grows slowly or even fails to establish if planted at cold or frost prone sites (Swain & Gardner 2003). Consequently, for plantations in such areas foresters are forced to make use of species or hybrids with great tolerance of low temperatures (Swain & Gardner 2003).

Cold-tolerant eucalypts were first introduced into South Africa at the turn of the 20th century for the production of mine roof support columns (Poynton 1979). Afforestation using cold-tolerant gums has increased steadily over the past decade due to increased demand for pulp and paper, promoting the rapid expansion of forestry into marginal or high altitude areas that are unsuitable for *E. grandis* (Edwards, 2000, Swain *et al*. 2000, Swain & Gardner 2003). Today cold-tolerant eucalypts cover a total area of more than 201,779 hectares in South Africa (Forestry South Africa 2008). Cold-tolerant *Eucalyptus* spp. commonly planted include *E. dunnii*, *E. nitens* and *E. macarthurii* (Darrow 1984). Less commonly planted eucalypts include *E. cloeziana, E. elata*, *E. fastigata, E. saligna* and *E. smithii.* While these species enjoy an advantage over *E. grandis* in terms of cold tolerance, their commercial value is limited by inferior pulp and fibre quality (Clarke *et al*. 1999). Hence, there is a need to find alternative cold-tolerant eucalypts suitable for high altitude areas in South Africa (Little & Gardner, 2003). *E. fraxinoides*, *E. smithii* and *E. oreades* are cold-tolerant with a potential for commercial propagation in such areas (Clarke *et al*. 1999). They grow vigorously and produce high quality pulp and paper compared to other *Eucalyptus* spp., including *E. grandis* (Clarke 1995, Clarke *et al*. 1999). However, they have an important shortcoming. This is that they are all susceptible to root and collar rot (Linde *et al*. 1994a, Wingfield & Roux 2000). A number of soilborne pathogens

belonging to the genus *Pythium* and *Phytophthora* have been reported to cause this disease. The following section presents an overview of the genus *Phytophthora* and its taxonomic history. This is followed by a description of *Phytophthora* die-back among eucalypts in plantations and strategies employed to manage this disease. The development of effective disease management strategies depends on a comprehensive understanding of the biology and epidemiology of the pathogen, which in turn requires insight into the genetic variation between and within *Phytophthora* spp. This review concludes, therefore, with a discussion of morphological and non-morphological methods used to distinguish between species, sub-species, and varieties within the genus.

2 Overview of the genus *Phytophthora*

The genus *Phytophthora* belongs to a diverse and primitive group of 'fungus-like' mycelial organisms, commonly referred to as "water moulds" (Agrios 1997). In 1996 there were approximately 54 described species (Erwin & Riberio 1996). The number of described species and designated taxon has increased rapidly in the past 10 years due to molecular identification of cryptic species and large scale environmental surveys in natural ecosystems and there are now over 105 recognised species and taxa (Brasier, 2008). Its name (Greek: *Phyton*, plant + *phtheiro*, destroyer) derives from the fact that it includes some of the most destructive plant pathogens (Judelson & Blanco 2005). The type species, *P. infestans*, destroyed Ireland's potato crop leading to a famine during the 19th century (Gregory 1983, Bourke 1991). Even today late blight is active and widespread and is responsible for high losses in potato production in many parts of the world (Duncan 1999).

Phytophthora spp. have long been classified as prokaryotic fungi under the Order Pythiales and Phylum Oomycota (Waterhouse 1973) because they are physiologically and morphologically similar to true fungi (Erwin & Riberio 1996). However, they represent a distant evolutionally line (Dick 1990a, 1997, Barr 1992, Cavalier-Smith 1998) and unique biological features distinct from true fungi (Erwin *et al.* 1983). For example, the genus *Phytophthora* differs from true fungi by having a diploid life cycle, non-septate mycelia, different cell wall and membrane structure, a requirement for external source of sterols for sporulation and thiamine for growth and different pathways for synthesizing lysine (Hendrix 1970, Bartnicki-Garcia & Wang 1983, Elliott 1983). Oomycetes including members of the genus *Phytophthora* have been reclassified under a recently described kingdom Stramenopila (or Chromista) (Cavalier-Smith 1998, Yoon 2002) based on their close evolutionary relationship with biflagellate, heterokont brown algae (Chromophyte algae), which possess tinsel-like flagellae (Gunderson *et al.* 1987, Förster *et al.* 1990a, Brasier & Hansen 1992, Paquin *et al.* 1995). However, according to Barr (1992) the similarity between oomycetes and algae was first postulated in 1858 by Pringsheim and this hypothesis is supported by sequence data of the small subunit of the RNA (Ariztia *et al*. 1991, Wainright *et al.* 1993). Recent analysis of the nuclear large subunit ribosomal DNA sequence data indicate that the genus *Phytophthora* is more closely related to the genera

Peronophythora, *Bremia* and *Plasmopara* and not *Pythium* as previously thought (Riethmüller *et al*. 2002). Although oomycetes, including *Pythium* and *Phytophthora,* are unrelated to fungi, Dick (1997) and Money (1998) recommends that the Oomycetes should be, for practical purposes, still be treated as fungi. Therefore, for the purpose of this review, the convention term(s) "fungi or fungal-like" are used to refer to *Phytophthora*.

2.1 Disease cycle of *Phytophthora*

Phytophthora spp. produce motile biflagellate zoospores that differentiate within the sporangium. When abundant water is available, zoospore move upward (negative geotaxis) towards the surface of the soils and are chemically attracted to roots of the host plant and readily initiate infection (Zentmyer 1961, Hinch & Weste 1979, Carlile 1983). The zoospores encyst and germinate to produce germ tubes, which infect and penetrate fine roots and progress to larger roots (Shearer *et al*. 1981, Zentmyer 1980).

Phytophthora spp. can either be homothallic or heterothallic. The heterothallic species produce thick walled oospores (sexual spores) when fertilisation of two opposite compatible mating types (A1 and A2) occurs. The homothallic species have the ability to form oospores independently without crossing. Oospores form one or more germ tubes, which initiate mycelial growth, or sporangia that produce either zoopores or chlamydospores. Oospores and chlamydospores can survive in the soil for many years (Duncan 1980, Duncan & Cowan 1980). Limited information is available about the dormancy of oospores and chlamydospores and the factors that activate their germination.

2.2 Taxonomic history of the genus *Phytophthora*

The history of the taxonomy of the genus *Phytophthora* began with the description of *P. infestans* by Anton de Bary in 1876 (Erwin & Riberio 1996). According to historical accounts by Erwin & Riberio (1996), in 1931 Tucker produced the first monograph, based on 21 species, and proposed a classification system based on morphology, physiology and pathology. In 1963, Waterhouse produced a taxonomic key in which 41 species were divided into six groups based on the shape and size of sporangia and attachment of antheridia to the oogonium. However, the taxonomic relevance of some morphological characteristics such as the position of antheridia has been challenged recently by Hüberli *et al*. (1997) and Gao *et al*. (1998). The revised tubular keys of Newhook *et al*. (1976), Stamps *et al*. 1990 and Erwin & Riberio (1996) were used for many years as traditional references for the

taxonomy and systematics of more than 50 *Phytophthora* species. Other taxonomic keys specific to countries such Taiwan and South Africa were published (Wager 1931, 1941, Ho *et al*. 1995).

Presently, the number of described *Phytophthora* spp. has increased rapidly and recently the taxonomy of the genus *Phytophthora* underwent major revision (Cooke *et al.* 2000, Kroon *et al*. 2004). This is mainly due to improved molecular techniques (Lévesque *et al*. 1998, Drenth *et al*. 2006, Bailey *et al.* 2002). However, other factors such as nursery trade of plant material and their associated pathogens across countries have been reported to play a role (Wingfield *et al*. 2001, Brasier *et al*. 2004, Brasier 2008). In 1996 less than 20% of *Phytophthora* species were known from forests and natural ecosystems (Brasier 2008, Erwin & Riberio 1996). Since 2000, over 50 new species have been described or are under description. The majority of these new species are from forest ecosystems (Brasier 2008).

3 Impact of *Phytophthora* diseases

The range of plants attacked by members of the genus *Phytophthora* spp. is enormous. Species such as *P. cinnamomi*, *P. nicotianae* and *P. cactorum* have a very broad host range, although others such as *P. infestans* and *P. sojae* are restricted to few host plants (Zentmyer 1983). Much damage is caused by these pathogens to native host species in natural environments for example in Western Australia, large areas of the native jarrah (*Eucalyptus marginata*) forest have been destroyed by *P. cinnamomi* (Podger *et al*.1965, Newhook & Podger 1972, Podger 1972, Weste & Marks 1987, Shearer & Tippett 1989, Shearer *et al.* 2004) as well as more recent outbreaks in other parts of the world (Tainter *et al.* 2000, Gallego *et al.* 1999). In addition, this pathogen also attacks a number of rare, susceptible Australian endemic species such as *Banksia* spp. (Shearer & Dillon 1995, 1996, Peters & Weste, 1997, Scott *et al*. 2009, Burgess *et al*. 2009). According to published reports, more than 2000 plant species are susceptible to *P. cinnamomi* in South-Western Australia alone (Wills 1993, McDougall *et al*. 2001, 2005).

There is an increasing concern regarding the impact of *Phytophthora* on food and wood fibre production worldwide. The recently described *P. ramorum* previously known only from two countries in Europe on *Rhododendron* and *Viburnum* killed large numbers of native *Quercus* spp. in California and Oregon and has jumped to other hosts such as redwoods in the United States (Rizzo *et al.* 2002, Tooley & Kyde 2007, Grünwald *et al*. 2008). It is evident from the above examples that plant disease epidemics caused by invasive pathogens such as *P. cinnamomi* can cause widespread death and decline of several susceptible native plant and tree hosts.

Hybridization between different *Phytophthora* spp. is possibly resulting in new host specific species (Brasier *et al*. 1990, 1995, Ioos 2006). Several new and often aggressive *Phytophthora* spp. continue to be discovered in Europe (Jung *et al.* 1999, 2002, 2003, Brasier *et al.* 2004, 2005) and other parts of the world (Greslebin *et al*. 2007, Maseko *et al* 2007, Durán *et al.* 2008, Scott *et al*. 2009), resulting in expanded lists of susceptible hosts (Mirabolfathy *et al.* 2001, Fier *et al.* 2002, Polashock *et. al*. 2005). These recent discoveries reflect an increasing threat posed by these pathogens in native and plantation forests. As knowledge is increased and molecular diagnostic tools are refined, new species will be discovered.

In South Africa, *Phytophthora* related diseases are widespread in several agricultural crops, ornamentals and horticulture, forest tree species. More than 15 *Phytophthora* spp. have been reported from 74 different hosts in Southern Africa (Crous *et al*. 2000, 2006). Susceptible agricultural and ornamental crops include *Persea americana* (Darvas *et al*. 1987), *Musa acuminata* (Thompson 1981), *Gypsophila paniculata* (Thompson & Naudé 1992), *Brassica oleracea* (Thompson & Phillips 1988), citrus (Thompson *et al*. 1995), *Vitis vinifer*a (Marais 1979, 1980 Halleen *et al*. 2003), *Medicago falcata* (Botha 1993), *Nicotiana tabacum* (van Jaarsveld 2001), *Lycopersicom esculantum* (Ferreira *et al*. 1991) and *Allium cepa* (von Maltitz & von Broembsen 1984). Susceptible exotic forest tree species include black wattle (*Acacia mearnsii*) (Zeijlemaker 1971), *Pinus* and *Eucalyptus* spp. (Donald & von Broembsen 1977, Darvas *et al*. 1978, Wingfield & Knox-Davies 1980). Indigenous tree hosts susceptible to *Phytophthora* root rot include *Leucadendron argenteum* (van Wyk 1973), *Ocotea bullata* (von Broembsen *et al*. 1986, Lubbe & Geldenhuys 1990, Lubbe & Mostert 1991) and the Clanwilliam cedar (*Widdringtonia cedarbergensis)* (Wingfield *et al.* 1988). *Phytophthora cinnamomi*, in particular is a serious treat to the several exotic hosts and indigenous flora (fynbos) endemic to the South Western Cape (von Broembsen 1984). However, very little is known about the interaction between *Phytophthora* spp., and native forest species in South Africa. Whereas, the interaction of *P. cinnamoni* and native forests has been extensively studied in Australia (Weste & Marks 1987, Shearer *et al.* 2000, Shearer & Tippett 1989). The Western Cape Province of South Africa and Western Australia share the mediterranean climate and closely related vegetation type including, *Proteaceae* family (*Protea* and *Banksia*), which are reported to be highly susceptible to *Phytophthora* root rot (Burrows 1985, von Broembsen & Brits 1985). Thus, the potential threat of *P. cinnamoni* to the indigenous fynbos vegetation of the south Western Cape still requires extensive research.

Diseases of exotic forest species associated with *Phytophthora* in South Africa have been the subject of several recent reviews (Linde *et al.* 1994a, Roux *et al*. 1995). *P.nicotianae* is mainly associated with black butt of black wattle (Roux *et al*. 1995). Other known pathogens associated with this disease include include *P. boehmeriae* and *P. meadii* (Roux *et al*. 1997). *P. cinnamoni* is the main causal agent of root and collar rot of cold-tolerant eucalypts in South Africa (Linde *et al*. 1994b). However, *P. boehmeriae* and *P. nicotianae* have also been recorded as causal agents of eucalypt collar and root rot

include (Linde *et al*. 1994b, Maseko *et al.* 2001). In the past, *Phytophthora* spp. were mostly identified by examining morphological features but more recently molecular techniques have been employed.

3.1 *Phytophthora* die-back of eucalypts

A number of *Phytophthora* spp., have been reported as serious soil-borne pathogens associated with native eucalypts (Newhook & Podger 1972, Pratt & Heather 1973, Fagg 1987, Shearer *et al.* 1987a) and in eucalypt plantations in many countries (Shearer & Smith 2000). Some infect young eucalypt seedlings in nurseries leading to pre-emergence damping-off and post-emergence premature death of seedlings leading to serious losses of planting stock (Marks & Kassaby 1976, von Broembsen 1984, Bayley & Snell 1997, Brown & Ferreira 2000). In addition, *Phytophthora* spp. also cause root and collar rot disease of older seedlings in plantations leading to poor growth and in some cases early death (Shearer & Smith 2000). In Australia and South Africa, collar rot of eucalypts associated with *Phytophthora* spp. in mining rehabilitation and plantation sites is confined to areas, which are often subject to occasional waterlogging (Jarvel 1998, Colquhoun & Elliot 2000, Colquhoun & Hardy 2000).

Although, a number of *Phytophthora* spp. have been isolated from eucalypt forests, *P. cinnamoni* is considered the single most destructive pathogen associated with eucalypts and their associated understorey species in Western Australia (Shearer & Smith 2000). In contrast, *P. cinnamoni* seldom causes diseases of eucalypt in nurseries and plantations in Australia and in several eucalypt growing countries because resistant eucalypts are usually planted (Brown & Ferreira 2000). In South Africa, for example, the commonly grown *E. grandis* is less prone to *Phytophthora* root rot in comparison to the susceptible cold-tolerant eucalypts (Wingfield & Knox-Davies 1980). The role of other *Phytophthora* spp., in native and plantation forests in less known, however, their potential role as serious pathogens is becoming more significant as they are associated with new disease outbreaks in *Eucalyptus* nurseries (Belisario 1993) and in plantations (Maseko *et al.* 2001).

3.1.1 *Phytophthora* related diseases in *Eucalyptus* nurseries

Nursery propagation of eucalypt seedlings is the key phase of any eucalypt-planting programme (Eldridge *et al*. 1994). Losses of plating stock due to *Phytophthora* related diseases can thus severely affect a planting programme (Brown 2000). The nursery conditions in general are conducive to the development of disease epidemics caused by *Phytophthora* spp. Over watering, poor drainage and high seedling density are some of the main factors contributing to the rapid spread of waterborne pathogens in nurseries (Old *et al*. 2003). Eucalypt clones grown in hydroponic nurseries in particular,

are even more vulnerable to *Phytophthora* related disease outbreaks (Lombard 2004), especially if untreated water is used.

Damping-off is one of the most important nursery diseases caused by a wide range of soil borne pathogens including *Phytophthora* spp. (Brown & Ferreira 2000). They are involved in pre-emergence and post-emergence damping-off disease affecting eucalypts and also cause root and collar rot diseases of older seedlings (Gibson 1975, Brown & Ferreira 2000). *P. cinnamomi* and *P. nicotianae* are common nursery pathogens found in forest nurseries in South Africa (Lombard 2004). There are, however, few reports of nursery diseases attributed to *Phytophthora* spp. in South Africa. Damping-off was once considered to be the most important disease of exotic forest trees including *Eucalyptus* seedlings in nurseries (Lückhoff 1964, Heather *et al*. 1977, Darvas *et al.* 1978, von Broembsen 1984). However, most outbreaks were consistently traced to poor nursery practices (Brown 2000). Damping-off has also been a problem in the propagation of eucalypt seedings in nurseries in Australia in the past and *Phytophthora* and *Pythium* spp. were implicated in plant death (Brown & Wylie 1991).

3.1.2 *Phytophthora* related disease symptoms in *Eucalyptus* plantations

Eucalypt plantations differ from natural forests in a number of respects. The latter consist of a variety of species and thus are more complex than man-made forests (Gadgil *et al.* 2000). *Eucalyptus* plantations usually consist of a single species planted on an industrial scale. *Eucalyptus* plantations represent monocultures, especially where the trees are clonally propagated and they are consequently more vulnerable than native forests to invasion by pests and pathogens (Wingfield *et al*. 1991, Gadgil & Bain 1999). The rapid turnover cycle also allows a build up of pathogens because the soil ecology is in a constant state of disturbance.

In South Africa, *P. cinnamomi*, a pathogen believed to be introduced (Linde *et al.* 1997) into South Africa, is the most destructive and aggressive pathogen associated with die-back of exotic eucalypts (Wingfield & Roux 2000). However, recently *P. nicotianae* rather than *P. cinnamomi* was consistently isolated from dying cold-tolerant eucalypts (Maseko *et al.* 2001). *P. nicotianae* also causes the disease known as black butt on the non-native *Acacia mearnsii* de Wild in South Africa (Zeijlemaker 1971, Roux & Wingfield 1997).

The initial symptoms of *Phytophthora* die-back occur below ground and thus often go unnoticed because *Phytophthora* spp. do not form visible fruiting structures in infected tree host. The expression of advanced disease symptoms such as wilting and die-back in eucalypts are observed long after the initial infection has taken place (Shearer & Tippett 1989). Infected root collars and roots usually have

water-soaked discoloured lesions, although asymptomatic infection of *P. cinnamoni* in *E. marginata* has been demonstrated (O'Gara *et al.* 1996, 1997, Hüberli *et al*. 2000). The most obvious above ground disease symptoms associated with eucalypts as the result of *Phytophthora* infection is a gradual wilting of the leaves resulting from girdling of the root collar leading to canopy thinning, die-back and ultimately death of the tree. If the bark is removed, brown lesions, extending from the roots are often observed. Other disease symptoms include root rot, gum exudation from diseased tissue and the formation of epicormic shoots. Death and poor growth of eucalypts in plantations is localised and is characterized by the presence of patches of dead trees. Reports suggest that eucalypt die-back is complex in nature and is possibly caused by a number of pathogens including *Phytophthora* and *Pythium* spp. (Linde *et al.* 1994b, Wingfield *et al.* 2001).

3.1.2 Physiological and anatomical response to bark injury

Infection of eucalypts by *P. cinnamomi* induces a series of physiological responses in the host. The initial physiological responses are stimulation of defence mechanisms aimed at preventing the spread of the pathogen (Dixon *et al*. 1994). Tolerance to *Phytophthora* spp. in eucalypts is associated with the ability of the host to limit colonization and necrosis of the infected tissue (Byrt & Holland 1978, Cahill *et al.* 1989). Resistance of eucalypts is often expressed by the inhibition of *Phytophthora* spp. in secondary root tissue (Tippett *et al*. 1985). Accumulation of secondary plant metabolites such as lignin and phenolic compounds are usually associated with this type of reaction in eucalypts (Tippett & Malajczuk 1979, Cahill *et al*. 1993). The formation of the wound induced periderm tissue in the secondary tissue of eucalypts also plays an important role in resistance (Tippett *et al.* 1983, Tippett & Hill 1984).

A second suite of physiological responses by the host occurs when *Phytophthora* spp. invade roots and prevent the uptake and absorption of water and minerals (Cahill *et al*. 1986, Tippett *et al*. 1987, Weste & Marks 1987). Responses include production of tyloses (Dawson & Weste 1984), increased respiration and cell wall permeability, and leakage of electrolytes (Cahill & Weste 1983, Cahill *et al*. 1985b). Increased production of tyloses has also been associated with waterlogging in *E. marginata* seedlings (Davison & Tay 1987). The increase in nutrient exudates and production of tyloses following infection by *Phytophthora* spp. is greater on susceptible than on tolerant eucalypts (Weste & Marks 1987).

Secondary metabolites such as glucans are secreted by some *Phytophthora* spp. and have been reported to induce wilting in eucalypts (Halsall 1978). However, glucan is not associated with the pathogenicity of *Phytophthora* spp. (Cahill *et al*. 1985a). *Phytophthora*-mediated hormonal imbalance

is also suspected to play a major role in the development of symptoms in susceptible eucalypts (Cahill *et al*. 1985a).

3.1.3 Variation of disease susceptibility amongst *Eucalyptus* spp.

Several studies have shown that eucalypts differ significantly in their response to infection by *Phytophthora* spp., especially *P. cinnamomi* (Weste & Taylor 1971, Marks *et al.* 1972, Marks *et al*. 1973, Pratt *et al.* 1973, Tippett *et al.* 1985). *Eucalyptus* spp. belonging to the subgenus *Monocalyptus* are more susceptible to *P. cinnamomi* than species which belong to the subgenus *Symphyomyrtus* or the genus *Corymbia* (Weste & Marks 1974, Noble 1989). In South Africa, *E. fastigata (Monocalyptus)* has been reported to be susceptible to *P. cinnamomi* while *E. grandis (Symphyomyrtus)* is tolerant (Wingfield & Knox-Davies 1980). During the 1980s, *Phytophthora* disease outbreaks led to the termination of the commercial propagation and planting of *E. fastigata* in South Africa (Eldridge *et al.* 1994). *E. fastigata* has since been gradually replaced by species more tolerant to infection by *Phytophthora* spp. (Linde *et al.* 1994a).

Significant variation in tolerance to *P. cinnamomi* appears to exist amongst susceptible *Monocalyptus* subgenera. Evidence from greenhouse inoculation studies have shown that certain individual trees within the susceptible *E. marginata* half-sib families exhibit some degree of tolerance to *P. cinnamomi* (Stukely & Crane 1994). Variation in tolerance to *P. cinnamomi* has also been demonstrated on *E. regnans* F. Muell (Marks *et al.* 1981, Harris *et al*. 1985) and on *E. marginata* clones (Cahill *et al*. 1992, Bennett *et al*. 1993, Hüberli *et al*. 2002a, 2002b).

3.1.5 Variation in pathogenicity among *Phytophthora* isolates

Pathogenicity refers to the ability of a pathogen to cause a disease in a specific host (Bos & Parlevliet 1995), while virulence refers to the degree of pathogenicity (Agrios 1997). Shaner *et al.* (1992) employs a more precise definition of virulence, using the term to denote the genetic ability of a pathogen or race to overcome genetically determined host resistance. In this review, however, the term "virulence" will not be used in this sense because virulence genes have not been clearly defined for *Eucalyptus.*

Selection and breeding for tolerance to *Phytophthora* spp. in susceptible *Eucalyptus* spp. must take into account the pathogenicity of the *Phytophthora* spp. involved (Shearer *et al.* 2000). In view of this fact, comprehensive studies on the variation of *P. cinnamomi* isolates on eucalypts have been conducted in Australia (Dudzinski *et al.* 1993, Hüberli 1995, Hüberli *et al.* 2001b) and in South Africa

(Linde *et al.* 1999b, Linde *et al.* 2001, Robin *et al*. 1998). There are several reports on the pathogenicity of *P. cinnamomi* and other *Phytophthora* spp. on eucalypts (Marks & Kassaby 1976, Shearer *et al*. 1988) and other forest tree hosts (Weste 1975, Hamm & Hansen 1982, Hansen *et al*. 1999, Jung *et al.* 1999). However, very little is known about the interaction of the combined effect between *P. cinnamomi* and other *Phytophthora* spp. in forest ecosystems where they coexist.

3.1.6 Assessment host tolerance and pathogenicity of *Phytophthora* spp.

Pathogenicity tests have been conducted on young eucalypts in tissue culture (Cahill *et al.* 1992), in pot trials under greenhouse conditions (Butcher *et al*. 1984, Marks *et al.* 1972, Maseko 1999) and in the field on young trees (Marks *et al.* 1981, Wolfaardt *et al.* 1997, Linde *et al.* 1999b, Linde *et al.* 2001). These tests are used to distinguish between eucalypts susceptible or tolerant to *Phytophthora* spp. Excised stems from eucalypts and other susceptible hosts have also been used in determining the pathogenicity of *P. cinnamomi* isolates (Dixon *et al*. 1984, Lundquist & Baxter 1985, Shearer *et al.* 1987a, Gabor & Coffey 1991, Tynan *et al*. 1998, Hüberli *at al*. 2002b). From these studies it is evident that there is great variation in pathogenicity among *P. cinnamomi* isolates.

In wound inoculation studies, variation in resistance to *Phytophthora* is assessed by the degree of lesion extension (Marks *et al.* 1981). Although this method has been widely used in the past, it has shortcomings as it bypasses the bark that is a natural barrier to pathogen invasion. Studies by O'Gara *et al*. (1996, 1997) and Lucas *et al.* (2002) have shown that *P. cinnamomi* does not require wounds to infect *E. marginata.* The extent to which the bark of trees is able to withstand infection, therefore, presents a source of variation in their resistance to *Phytophthora* that is not taken into account by wound inoculation studies.

4 Role of environmental factors in the development of *Phytophthora* die-back on eucalypts

Several stress-inducing factors influence the growth and development of eucalypts in indigenous forest and plantations. These factors include biotic and abiotic agents, and can predispose trees to infection by *Phytophthora* spp. Biotic factors include other pathogens and pests. Abiotic factors, on the other hand, include the health and nutritional status of eucalypts as well as extremes in temperature and soil moisture, which are discussed in detail below.

4.1 Soil Moisture

The life-cycle of soil borne *Phytophthora* spp. is largely dependent on moisture, which influences development in soil as well as in host tissue (Smith & Marks 1982, Tippett *et al.* 1987, Bunny *et al*. 1995). Different moisture conditions affect the pathogen and host in different ways, and their impact

on the likelihood of *Phytophthora* infection is sometimes complex. It is useful to emphasize two moisture conditions: surface ponding and waterlogging. In the former, the oxygen content of water is typically high. In the latter, soil is saturated with water creating hypoxic and potentially anoxic conditions, especially in poorly drained soils.

Surface ponding provides an ideal opportunity for *Phytophthora* collar infection because it wets the bark of the tree collars while high oxygen concentrations remain. Waterlogging, by contrast, has been reported to inhibit the activity of *P. cinnamomi* as this species cannot sporulate under low oxygen conditions (Burgess *et al.* 1998). However, waterlogging also induces stressful conditions that harm eucalypts and predispose them to invasion by *Phytophthora* spp. (Newhook & Podger 1972, Duniway 1979). Waterlogging has an inhibitory effect on the growth and vigour of eucalypts, especially on poorly drained sites (Burgess *et al.* 1999), and the effects of waterlogging alone can be sufficient to cause die-back of *E. marginata* (Davison & Tay 1987).

The harmful effects of waterlogging on eucalypts in indigenous and plantations forests are well documented (Newhook & Podger 1972, Duniway 1983, Bell 1999, Niknam & McComb 2000). Eucalypts have been found to differ in their ability to tolerate waterlogged conditions. Species residing in the *Monocalyptus* for example, are more sensitive to waterlogging than species of *Symphyomyrtus* (Niknam & McComb 2000). The physiological and genetic mechanisms involved in waterlogging tolerance in eucalypts are not clearly understood.

4.2 Temperature

Temperature is one of the most important abiotic factors infuencing the development of *Phytophthora*-related diseases in plants. Seasonal variation in particular, determines the time of *Phytophthora* disease outbreaks, which are usually associated with wet soil conditions and moderate temperatures. For example, in Western Australia *Phytophthora* die-back in the jarrah forests is severe during winter and spring when both the temperature and soil moisture are highly favourable to *P. cinamomi* infection (Weste & Marks 1987).

All major components of the *Phytophthora* life cycle are influenced by seasonal variables such as temperature and moisture (Duniway 1983). Temperature directly influences the sporangia formation and germination, inoculum production and survival in the soil (Shearer & Tippett 1989). Thus temperature plays an important role in the epidemiology of the *Phytophthora*-related diseases since it influences the pathogen directly in the soil and within the host tissue (Shearer & Tippett 1989). The temperature-growth relationships of *P. cinnamomi* in culture and in secondary phloem tissue of *E. marginata* have also been studied in detail (Shearer *et al*. 1987b). In a study conducted by Grant and Byrt (1984), the extent to which host tissue is colonized was reported to be dependent on temperature

in both tolerant and susceptible eucalypts. Hüberli *et al* (2002a) shows how temperature affects the growth of *P. cinnamomi* within one host (cloned jarrah); as temperature got into the optimal levels for the pathogen, resistant clones were as susceptible as susceptible clones of jarrah.

4.3 Soil nutrition

Nutrition plays an important role in the growth and survival of eucalypts. The nutrition of eucalypts in their native environment and in plantations has been subjected to comprehensive reviews (Herbert 1992, Attiwill & Adams 1996). Nutrient deficiencies of eucalypts lead to reduced growth and increased susceptibility to diseases (Showdon 2000). Eucalypts growing on sites with poor nutritional status have been found to be more susceptible to *P. cinnamomi* (Tippett *et al*. 1989). Calcium, however, has been reported to enhance resistance of eucalypts towards *P. cinnamomi* in the field and in glasshouse conditions (Boughton *et al* 1978, Halsall 1980, Cahill *et al.* 1986).

5 Management of *Phytophthora* die-back in eucalypt plantations

Effective management of *Phytophthora* die-back is essential, as it is one of the destructive diseases in eucalypt-growing countries (Wingfield 1990, Gadgil *et al.* 2000). Prevention is essentially the basis of all management strategies used in *Eucalyptus* plantations because chemical control is not financially viable. Integrated disease management strategies involving quarantine, silvicultural practices and planting of disease resistant trees are currently used in eucalypt plantations.

5.1 Quarantine and sanitation

Quarantine and sanitation measures are effective disease control strategies used in many eucalypt nurseries and forest operations. They are aimed at preventing accidental introduction of a pathogens and pests into disease-free areas. A combination of three quarantine and sanitation methods is used in forest nurseries. These are pathogen eradication, reduction of pathogen propagules, and prevention of pathogen spread (Old *et al*. 2003).

Pathogen eradication measures include all practices aimed at eliminating pathogens from all nursery operations. Examples of such practices include decontamination of nursery equipment, pre-treatment of seedlings and seeds (Donald & Lundquist 1988) and the use of steam-sterilized trays (Donald *et al.* 1994, Myburgh 2000). Pathogen reduction methods are aimed at reducing the inoculum levels of the *Phytophthora* spp. in forest nurseries, and include the use of composted bark media (Hoitink *et al*. 1976, 1977, Hoitink 1980, Hoitink & Fahy 1986, Hardy & Sivasithamparam 1991). Measures aimed at preventing the spread of pathogens in forest nurseries include chemical and biological control.

Systemic fungicides such as metalaxyl and fosetly-Al are used to control *Phytophthora* root and collar rot in nurseries (Ali Smith & Guest 1999, Hardy *et al*. 2001, Wilkinson *et al.* 2001).

5.2 Silvicultural practices

Intensive management of eucalypts plantations through silvicultural strategies enhances tree growth and vigour and thus reduces the risk of diseases (Denison & Kietzka 1993). Silvicultural practices such as appropriate site preparation, timely fertilization, and weed control enhance seedling survival and vigour during establishment (Donald 1987, Gadgil *et al.* 2000). Eradication of pathogens through careful use of controlled burning; hygienic thinning and pruning are effective disease control strategies.

Correctly matching species to sites is a commonly used disease avoidance strategy. As was mentioned earlier, different eucalypts have different site requirements (Herbert 2000). When a *Eucalyptus* sp. is planted on unsuitable sites, it is prone to stress and damage by pathogens. Hence, the use of appropriate site and species selection can prevent or limit disease losses (Herbert 1994, Louw 1999).

5.3 Breeding and selection for disease resistance

Breeding for disease resistance is one of the most important aspects of any *Eucalyptus* improvement programme worldwide. Many such breeding programmes were started several decades ago with initial emphasis on improving phenotypic traits such as growth rate, stem form and wood density. *E. grandis* breeding programmes in Brazil and in South Africa serve as classic examples (van Wyk 1973, Eldridge *et al.* 1994). Originally, eucalypt breeding programmes in South Africa did not pay much attention to disease resistance. Due to the increased impact of fungal pathogens, however, this strategy is now an integral part of all eucalypt breeding programmes (Gadgil & Bain 1999, Gadgil *et al.* 2000). A measure of the success of such programmes is the fact that many eucalypt plantations remain disease free for several years until new pathogens are accidentally introduced from other countries or jump from native hosts (Gadgil *et al.* 2000).

Disease resistance in forest trees has been subjected to many reviews (Bingham *et al*. 1971, Carson & Carson 1989, Namkoong 1991). Significant advances have been made in controlling fungal disease through selection and breeding programmes of forest trees (Bazzigher 1981, Nomkoong *et al*. 1988, Smalley & Guries 1993). Successful screening techniques for tolerance to *P. cinnamomi* have been reported on pine (Butcher *et al.* 1984) and on eucalypts (Stukely & Crane 1994) and a good correlation between greenhouse and field trials have been achieved. Although complete resistance towards *P. cinnamomi* on susceptible eucalypts has not been achieved, planting of tolerant trees has proven successful enough to justify intensive selection efforts (Stukely *et al*. 2007).

5.4 Importance of maintaining genetic diversity

The importance of genetic diversity in forest tree species has been reviewed in the past (Namkoong *et al* 1998, Namkoong 1991). As was pointed out earlier, plantations are usually made up of a single species and are thus less diverse than mixed indigenous forests. This makes them more vulnerable to diseases (Chou 1981, Wingfield *et al.* 1991, Simpson Simpson & Podger 2000). Maintaining genetic diversity in planting stock minimizes the risk of serious losses due to diseases (Namkoong 1991, Wingfield *et al.* 2001).

5.5 Knowledge of the disease epidemiology

Sound knowledge of the biology of the pathogen is a prerequisite to any disease control strategy (Wingfield *et al.* 2001). The biology of *P. cinnamomi* in native Australian forests has been extensively studied (Shearer & Tippett 1989, Weste & Marks 1987), although little is known about the biology of other *Phytophthora* spp. in native forests. Population diversity studies are also helpful in determining the origins and reproduction strategies of pathogens (Linde *et al.* 1997, Dobrowolski *et al.* 2002, 2003). The epidemiology of pathogens plays a particularly important role in disease management because it has a direct impact on disease control strategies (Ristaino & Gumpertz 2000). Modern molecular techniques offer a powerful array of tools for studying these aspects of *Phytophthora* spp., and are discussed in the following section.

6 Identification of *Phytophthora* spp.

As has been mentioned earlier, taxonomic keys currently used for the identification of *Phytophthora* spp. are based on morphological groups, where each group encompasses species with closely related morphological characteristics. However, the identification and detection of *Phytophthora* spp. based on morphological characteristics is laborious, time consuming and expensive, and often requires specialized skill (Duncan & Cooke 2002). *Phytophthora* spp. usually require specific growth media and baiting techniques (Erwin & Riberio 1996), making it difficult to isolate *Phytophthora* spp. from soil or old infected plant material. Morphological classification relies on the production of pure cultures and induction of various diagnostic structures such as sporangia, chlamydospores, oogonia and antheridia. These key diagnostic structures can be variable within and between species (Erwin 1983) under different environmental conditions (Hendrix 1967, Brasier & Griffin 1979, Alizadeh & Tsao 1985).

Other limitations of using morphology to identify *Phytophthora* spp. include the difficulty of applying the type species concept as proposed by the International Code of Botanical Nomenclature (Brasier 1991). Preserving dried *Phytophthora* type cultures on infected host tissue and retaining the original

state of the culture remain a challege. There are also various unresolved taxonomic problems with the genus *Phytophthora*. Examples include the debates on the conspecificity of *P. nicotianae*, var. *nicotianae* and var. *parasitica* (Tucker 1931, Waterhouse 1963 and *P. crytogea* and *P. drechsleri* (Bumbieris 1974, Ho & Jong 1986). Species such as *P. megasperma* (Hansen 1991b, Hansen & Maxwell 1991a, Hansen *et al*. 2009) and *P. palmivora* have been reported as being species complexes.

6.1 Non-morphological classification methods

Given the shortcomings associated with the classification of *Phytophthora* spp. based on morphological characteristics alone, non-morphological methods are receiving increasing attention. Such methods include physiological and molecular techniques. Physiological characteristics that are useful for distinguishing subspecies and varieties within the genus include temperature-growth relationships, host specificity and chemical sensitivity (Shepherd 1976, Jung *et al.* 1999). Physiological characteristics are incorporated in taxonomic keys to support morphological variation between *Phytophthora* spp (Stamps *et al*. 1990). Each group within the genus *Phytophthora* consists of species with closely related physiological characteristics (Erwin & Riberio 1996). Response to different temperatures is one of the criteria used in taxonomic keys to distinguish between varieties within species (Newhook *et al*. 1978, Stamps *et al*. 1990, Erwin & Riberio 1996). *Phytophthora* spp. such as *P. infestans*, *P. mirabilis* and *P. phaseoli* are morphologically similar but can be distinguished on the basis of their host specificity (Brasier & Hansen. 1992). Hymexazol and malachite green have been successfully used to differentiate species and subspecies within Group I (Kennedy & Duncan 1995, Jung *et al.* 1999).

While the incorporation of physiological characteristics has helped to overcome some of the difficulties inherent in classification on purely morphological grounds, some taxonomic questions remain unresolved. Relatively, few *Phytophthora* spp. accurately match defined morphological groups (I–VI). The heterothallic species *P. capsici* and the homothallic *P. megasperma*, for example, exhibit complexity and appear to consist of clusters of biological species and discrete population units (Hansen *et al.* 1986). The term "biological species" refers to actual or potential interbreeding populations that are impossible to differentiate morphologically (Hawksworth *et al.* 1995). Brasier (1991) proposed a biological species concept in order to accommodate the changing status of species within species complexes. According to Brasier (1991) hybridization is another complicating factor that can hamper the morphological and physiological identification process. Part of the solution to these problems involves use of population analysis and complementary molecular methods.

Modern molecular techniques allow interpretation of useful DNA sequence information that is essential for the diagnosis and control of plant pathogens (Schena *et al*. 2004, Crous, 2005) Molecular tools, especially the Polymerase Chain Reaction (PCR), automated DNA sequencing and

bioinformatics have revolutionized the identification of many fungal pathogens, including *Phytophthora* (Cooke *et al.* 2000, Duncan & Cooke 2002, Kamoun *et al.* 2002). Universal primers published by White *et al.* (1990) fuelled the growth of fungal databases housing sequence data. The Internal Transcribed Spacer (ITS) regions of ribosomal RNA the ribosomal DNA has been used extensively for the identification of *Phytophthora* and other oomycete genera (Cooke *et al* 2000, de Cock & Lévesque 2004, Lévesque & de Cock 2004). It is now recognised that there are 10 ITS clades within *Phytophthora* which are not specifically correlated with particular unique morphological features Cooke *et al.* 2000). Accurate and rapid techniques to detect and identify *Phytophthora* spp. have been extensively investigated in recent years because of their importance as pathogens. Molecular techniques have been useful in confirming the validity of species within the genus *Phytophthora* and also provide a better resolution between morphology and genetic relationships amongst species (Martin *et al*. 2000). In the following sections, various *Phytophthora* detection and identification methods are discussed with special reference to *Phytophthora* disease management.

6.2 Methods for isolation, detection, and identification of Phytophthora

6.2.1 Serological methods

Phytophthora zoospores are attracted to a wide range of chemicals released from the roots of host plants (Deacon & Donaldson 1993). Serological methods can be used to monitor or detect *Phytophthora* spp. Antibodies derived from antisera against the whole organism are called polyclonal, and those derived antisera from single cloned cells are called monoclonal antibodies and thus are more specific than polyclonal antibodies (Hardham *et al*. 1986, 1991). Various serological methods have become valuable tools for research and commercial purposes. The advantages of serological methods over traditional baiting methods include rapid identification, low detection levels, detection before the disease symptoms are expressed by the host plant and accurate detection of *Phytophthora* regardless of the presence of other microorganisms (Erwin & Riberio 1996). However, the disadvantages of using serological methods are that they do not distinguish between dead and living propagules (Timmer *et al*. 1993) and may produce false positive results by detecting common antigens from related microorganisms. Thus they should always be used in conjunction with direct isolation methods (Macdonald *et al*. 1990). Robold & Hardham (1998) reported species specific monoclonal antibodies that react only with surface components on zoospores and cysts of *P. nicotianae*. Similarly, Hardham *et al*. (1986) and Ferraris *et al*. (2004) were also able to produce monoclonal antibodies binding only to cysts or zoospores of *P. cinnamomi* or and not to those of *P. parasitica* or *P. vignae*. Surface components of zoospores and cysts of *Phytophthora* and *Pythium* spp., have a range of specificities. Thus, they may have an important value in the taxonomy and phylogeny of species, as well as use in disease diagnosis (Hardham *et al*. 1994).

6.2.2 DNA probe detection methods

Cloned DNA fragments can be used as species-specific probes to detect *Phytophthora* spp. Goodwin *et al*. (1989, 1990b) used DNA probes to detect and identify *P. parasitica* and *P. citrophthora* among a range of different species. They also used DNA hybridization probes to detect *P. parasitica* directly from plant tissue (Goodwin *et al.* 1990a). Judelson & Messenger-Routh (1996) used a cloned DNA fragment of *P. cinnamomi* to quantitatively detect the pathogen. A number of DNA sequences have been generated through phylogenetic studies of *Phytophthora* and related genera sequence data have become available making it possible to design specific primers (Briard *et al.* 1990, Cooke *et al.* 1996, Crawford *et al.*1996, Lee & Taylor 1992). Examples of primer hybridization probes for *Phytophthora* spp. have been reported by Lee & Taylor (1992) and Lee *et al*. 1993).

6.2.3 Multigene phylogeny of *Phytophthora* species

Molecular studies based on single gene regions, especially the ITS region has been used extensively to infer phylogenetic relationships between species within the genus *Phytophthora* (Cooke *et al*. 2000, Martin & Tooley 2003, Blair *et al*. 2008). The ITS region, in particular is a widely method used routinely for identification of *Phytophthora* spp. (Drenth 2006). Modification of the universal ITS1 or ITS2 primers has been used in designing species-specific probes for detecting *P. citricola* in inoculated plants (Schubert *et. al*. 1999) or used in combination with the ITS for designing species-specific probes (Lee *et al.*1993, Coelho *et al.* 1997, Shen *et al.* 2005). Many species however, remain incompletely described and require more thorough molecular analysis (Brasier & Hansen 1992).

A multiple gene phylogeny based on combined nuclear DNA (translation elongation factor 1α; β-tubulin) and mitochondrial DNA (cytochrome c oxidase subunit 1; NADH dehydrogenase subunit 1) sequence data has been reported for the genus *Phytophthora* (Kroon *et al*. 2004). Recently, Schena & Cooke (2006) also reported multigene phylogeny of *Phytophthora* spp. based on intergenic spacer (IGS) regions of rDNA (rDNA-IGS), ras-related protein (Ypt1) gene and four regions of mitochondrial DNA (mt-IGS). Blair *at al*. (2008) produced a phylogeny based on 7 loci derived from the complete genome sequence of *P. ramorum, P. sojae*, and *P. infestans.* The resultant phylogeny is remarkably similar to that obtained from ITS sequence data alone. A number of species-specific molecular markers have been developed for *Phytophthora* spp.

6.2.4 Protein electrophoresis

Protein electrophoresis differentiates species on the basis of the protein profiles generated when mycelial proteins are dispersed into a medium and subjected to an electric current. Early application of

this technique in *Phytophthora* systematics was pioneered by Clare & Zentmyer (1966). Since then, protein electrophoresis has proven useful in distinguishing *Phytophthora* spp. (Boccas & Zentmyer 1976, Boccas 1981, Erselius & de Vallavieille 1984, Erselius & Shaw 1982, Bielenin *et al.* 1988, Chowdappa & Chandramohanan 1995) and resolving conspecific species (Bielenin *et al.* 1988, Masago *et al.* 1989) and morphospecies within *P. megasperma* species complex (Irwin & Dale 1982, Hansen *et al.* 1986). Protein electrophoresis is generally used to confirm species identification and thus provide support in cases where morphological data is not clear (Erwin & Riberio 1996). However, this technique generates a large number of bands that are often difficult to score and biosynthesis of some proteins is not constant (Förster & Coffey 1991).

6.2.5 Isozymes

Isozymes are alternative forms of an enzyme that have the same catalytic function (Spielman 1991). Isozymes can also be used to differentiate species (Latorre *et al.* 1995, Mills *et al*. 1991, Oudemans & Coffey 1991a, 1991b, Oudemans *et al*. 1994), biotypes (Nygaard *et al.* 1989) or prove conspecificity (Oudemans & Coffey 1991c, Mchau & Coffey 1994b) within the genus *Phytophthora.* However, they are generally used to study the genetic diversity within population of various *Phytophthora* spp. (Micales *et al*. 1986, Old *et al*. 1984, 1988, Goodwin 1997, Linde *et al.* 1997). Isozymes are less complex than protein profiles and are easy to differentiate and interpret. Isozymes represent the end-products of specific gene functions.

6.2.6 Restriction Fragment Length Polymorphisms (RFLPs)

Mitochondrial DNA (mtDNA) or chromosomal DNA (chrDNA) isolated from the species of interest is digested with restriction endonuclease enzymes, which cut the DNA at specific sites resulting in fragments of varying lengths. The size of the resulting fragments is visually estimated using molecular markers. *Phytophthora* spp. produce different fragment profiles that can be used to distinguish them. RFLPs are routinely used to study the genetic relationship between biotypes and species in the genus *Phytophthora* (Förster *et al.* 1987, 1988 1989, Förster & Coffey 1991). RFLPs are also useful in differentiating between morphologically distinct *Phytophthora* spp. (Panabiéres *et al.* 1989, Förster & Coffey 1991, Mills *et al.* 1991, de Cock *et al.* 1996) or similar species (Förster, *et al.* 1990b, Mirabolfathy *et al.* 2001). Other applications of RFLPs include population studies of pathogen races (Drenth *et al.* 1996, Lebreton & Andrivon 1998, Ordonez *et al.* 2000, Purvis *et al.* 2001) and pathogen populations from different host and locations (Linde *et al.* 1999a) and represent products of fuctional genes.

6.2.7 Random Amplified Polymorphic DNA (RAPDs)

RAPD markers represent a technique that involves random amplification of DNA of a chosen organism using short oligonucleotide primers. The resulting products are separated by electrophoresis and visually inspected and analysed (Karp *et al*. 1996). RAPDs have been used extensively in *Phytophthora* taxonomy to study the genetic variation within and between species. Examples include studies conducted by Linde *et al*. (1999a), Nyasse *et a*l. (1999) and Purwantara *et al*. (2001) who respectively compared the genetic diversity of *P. cinnamomi*, *P megakarya* and *P. clandestina* isolates from different geographic origins. RAPDs have been successfully used to differentiate between pathogen races (Chang *et al*. 1996, Förster, Tyler & Coffey 1994, Meng *et al*. 1999, Punja *et al*. 1998), morphological groups (Cooke *et al*. 1996, Jung *et al*. 1999, Elena & Paplomatas 1999, Goodwin *et al*. 1999) and to compare isolates of the same species from different hosts (Lilja *et al*. 1998) or geographic origins (Cooke *et al*. 1999).

6.2.8 Amplified Fragment Length Polymorphisms (AFLPs)

AFLP is a fingerprinting method involving restriction digests of genomic DNA, ligation of oligonucleotide adapters and selective amplification products of restriction fragments (Vos *et al.* 1995). AFLP can be a useful tool in discriminating *Phytophthora* spp., especially when used to compliment other molecular techniques (Mirabolfathy *et al.* 2001, Werres *et al.* 2001). AFLPs have been used successfully in detecting genetic variation in fungi (Majer *et al.* 1996) and in characterizing hybrid isolates of *P. nicotianae* and *P. cactorum* (Bonants *et al.* 2000). However, they have not been extensively used in *Phytophthora* taxonomy.

7 Conclusion

South Africa has inadequate natural timber resources to supply industrial demand and thus depends on plantations of non-native species such as eucalypts. The history of eucalypt plantations in South Africa dates back more than one hundred years. Improved *E. grandis* and it's various hybrids and clones are the most commonly planted species. During the past decades, forestry has been expanding towards marginal areas which are unsuitable for *E. grandis,* consequently there is an increased demand for altenative species.

Small areas of cold-tolerant eucalypts are planted at high altitude in South Africa. However, the afforestation potential of the majority of these species is limited by the root rot complex involving *P.*

cinnamomi. The role of *P. cinnamomi* in causing disease of many forest species has been studied extensively. However, very little is known about the role of other *Phytophthora* spp. associated with the die-back of cold-tolerant eucalypts in South Africa.

The taxonomy of the genus *Phytophthora* has undergone extensive revision. This revision commenced with the reclassification of Oomycetes and closely related genera under a recently described kingdom Chromista. For years scientists have relied on traditional taxonomic keys to identify *Phytophthora* spp. However, inherent problems associated with variation of morphological features within and between species have resulted in some areas of confused taxonomy. The advent of various molecular tools, especially comparisons based on sequences of the ITS regions of the rDNA operon has advanced the taxonomy of the genus *Phytophthora*. Many species thought to be different or similar have since been found to be either co specific or belonging to species complexes respectively. In addition, a number of hybrid *Phytophthora* spp. have emerged in many parts of the world, resulting in wider host range and further taxonomic confusion. Understanding the genetic diversity of *Phytophthora* spp. remains an area deserving considerably more study.

The expression of *Phytophthora* related disease symptoms differs significantly between native eucalypt forests and man-made plantations. Plantations are established in monoculture and are genetically uniform and more prone to invasion by aggressive pathogens such *P. cinnamomi*. The development of *Phytophthora*-related disease symptoms in eucalypt plantations depends on a number of constantly changing abiotic and biotic factors. These factors are strongly influenced by the species involved and its pathogenicity, level on infestation and prevailing environmental conditions. *Eucalyptus* spp. also differ significantly in their tolerance to *Phytophthora* spp. Species belonging to the sub-genus *Monocalyptus* are generally more susceptible to *Phytophthora* root rot than those belonging to the *Symphyomyrtus*.

Disease management strategies used in reducing the impact of *Phytophthora* root rot in *Eucalyptus* plantations are primarily preventative in nature because chemical control is not economical. Disease control strategies mainly involve resistance breeding, use of various silvicultural practices and quarantine. Accurate identification of the pathogen (s) involved as well as sound understanding of the population structure and the biology of the pathogen are fundamental to successful breeding programme. With little being known about *Phytophthora* spp. associated with eucalypts in South Africa the following work addressed the taxonomy of *Phytophthora* spp. and interactions with eucalypt hosts.

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Chapter Two

Die-back of cold-tolerant *Eucalyptus* associated with *P. nicotianae* and *P. cinnamomi* in South Africa

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ABSTRACT

Phytophthora root and collar rot is a widespread disease associated with cold-tolerant *Eucalyptus* spp. in South Africa. Various soil borne pathogens, including *P. cinnamomi* have been reported as the causal agents of this disease. In recent years, *P. nicotianae* rather than *P. cinnamomi* has consistently been isolated from the rhizosphere and from infected root collars of cold-tolerant *Eucalyptus* spp. During 2000–2003, sampling was undertaken in several *Eucalyptus* plantations in the Mpumalanga and KwaZulu-Natal Provinces of South Africa to determine the primary cause of root disease on these trees. The aim of this study was to identify the species responsible for the disease outbreaks and assess the potential threat posed by *P. nicotianae* to cold-tolerant *Eucalyptus* spp. A large number of *P. nicotianae* and *P. cinnamomi* isolates, from diseased trees as well as from the soil surrounding their roots were obtained and identified based on morphology, mating type and sequence data for the ITS region of the ribosomal DNA operon. The pathogenicity of isolates of these species was determined by inoculating one-year old *Eucalyptus smithii* in the field. Results obtained in this study showed that *P. nicotianae* and *P. cinnamomi* are commonly associated with symptoms on dying trees in the sampled areas. The former species was more frequently isolated from soil and diseased trees than *P. cinnamomi*. Pathogenicity tests showed that *P. nicotianae* isolates were less aggressive than those of *P. cinnamomi.* However, overall results suggest that *P. nicotianae* was the more common causal agent of the die-back outbreaks that occurred during the period under investigation.

Keywords: *Phytophthora* die-back, disease tolerance, stems inoculation

1. Introduction

Cold-tolerant *Eucalyptus* spp. were first introduced into South Africa more than a century ago for the production of mine timber props (Poynton 1979). Planting the *Eucalyptus* spp. has increased steadily over the years, mainly due to the expansion of the forestry industry and to an increased demand for raw material for pulpwood (Darrow 1984a, 1984b). Cold-tolerant *Eucalyptus* spp. are planted in high altitude (above 1000 m), areas in Mpumalanga and KwaZulu-Natal, which are prone to frost and thus unsuitable for the commonly grown *E. grandis* (Schönau *et al*. 1994).

One of the factors that impacts negatively on the propagation of cold-tolerant *Eucalyptus* in South Africa is root and collar rot (Wingfield *et al*. 2001). A primary disease symptom is death of the root cambium which results in a lesion that commonly extends up into the collar region (Fig 1). Advanced disease symptoms on affected trees include chlorosis and wilting of leaves, gum exudation from cankers at the tree collar and eventually death. In the past, this disease problem resulted in large-scale losses in forest nurseries and plantations in South Africa (Lundquist & Baxter 1985, Wingfield & Roux 2000). During the 1980s, breeding programmes that used highly susceptible species such as *E. fastigata* and *E. elata* were abandoned due to root rot related disease problems (Herbert 1994). Other species reported to be susceptible to collar and root rot include *E. fraxinoides* and *E. smithii* (Linde *et al.* 1994).

In South Africa, root and collar rot of *Eucalyptus* has been associated with species of *Phytophthora* and *Pythium* (Wingfield *et al*. 2001). Amongst the *Phytophthora* species reported to affect this host, *P. cinnamomi* is the most destructive causing die-back of cold-tolerant *Eucalyptus* (Linde *et al*. 1999a). In a previous study, Linde *et al* (1994) obtained a small number of isolates of *P. nicotianae* from *Eucalyptus* forest soil. These isolates were amongst the most aggressive when inoculated on *E. grandis* and *E. fastigata.*

Other than a brief preliminary report (Maseko *et al.* 2001),very little is known about the effect of *P. nicotianae* on cold-tolerant *Eucalyptus* spp. in South Africa. The aim of this study was to investigate the distribution of *Phytophthora* spp. in *Eucalyptus* plantations in Mpumalanga and KwaZulu-Natal Provinces of South Africa. *Eucalyptus* plantations were tested for the presence of *Phytophthora* spp. which were then identified using molecular technology. Pathogenicity tests using selected *P. nicotianae* and *P. cinnamomi* isolates were conducted on *E. smithii* to assess their pathogenicity and the potential threat they pose to cold-tolerant *Eucalyptus* spp. in plantations

2. Materials and Methods

2.1 Sampling

During 2000 and 2003, 66 commercial *Eucalyptus* stands located in 31 sites in the Mpumalanga and KwaZulu-Natal Provinces of South Africa were selected for investigation (Fig 2). Stands in commercial plantations as well as small private holdings were chosen. Sampling was carried out from August to April when the soil temperature and moisture are reported conducive to the isolation of *Phytophthora* spp. (Weste & Vithanage 1979).

Four of the most common and important cold-tolerant *Eucalyptus* spp. were considered in this study. These included *E. nitens*, *E. macarthurii*, *E. dunnii* and *E. smithii.* However, a greater number of *E. smithii* stands were sampled because of the economic importance of this host and its high susceptibility to *Phytophthora* root rot. During the course of this study, diseased plant material was also received by the diagnostic clinic of the Tree Protection Cooperative Programme, University of Pretoria, and isolates of *Phytophthora* spp. from these samples were included in this work.

Trees displaying symptoms of root and collar rot were tested for the presence of *Phytophthora* species. In total, 564 diseased trees and 320 soil samples were collected and tested for the presence of *Phytophthora* spp. Isolations were made from trees showing symptoms of root and collar rot and foliage chlorosis, whenever possible. *Phytophthora*-affected areas were characterised by patches of dead or dying plants. Soil and fine root samples were taken from the root zone of dying tress. In stands with healthy trees, soil samples with fine roots were taken from the rhizosphere of randomly selected visually healthy trees. At each sampling site, four randomly selected soil samples from a depth of about 10 cm were collected, mixed and pooled. Symptomatic plants were uprooted and placed in plastic bags for later diagnosis. Soil and plant samples were assayed within 48 hours of collection. The remaining samples were stored in a cool room at 5 °C for baiting, especially in cases where plants showed die-back symptoms but no *Phytophthora* spp. were retrieved.

Soil in each sample was divided into two equal portions (100 g) and placed into labelled 350 ml plastic containers. Dry soil samples were pre-moistened for two days to induce the growth of dormant spores. Soil which contained fine root was flooded with deionised water and then baited with citrus leaf pieces and *E. sieberi* cotyledons as described by Grimm & Alexander (1973) and Marks & Kassaby (1974) and incubated at room temperature in the dark for 3–4 days. Leaf discs that become infected developed blackened areas, whereas cotyledons that become infected turned chlorotic. Infected baits and bleached *E. sieberi* cotyledons were removed from the water, blotted dry and plated onto NARPH culture growth medium selective for *Phytophthora*. NARPH is made up of Corn Meal Agar (CMA) 17

g^{-1} amended with 50 µg/ml^{-1} nystatin, 200 µg ml^{-1} ampicillin, 10 µg ml^{-1} rifampicin, 25 µgml^{-1} pentacloronitrobenzene (PCNB), and 50 µg ml^{-1} hymexazol (Hüberli *et al*. 2000).

Bark from basal cankers adjacent to the root collar region was removed using a scalpel and cut into smaller pieces with diameter ≤ 5 mm and placed onto the surface of NARPH growth medium in Petri dishes. Petri dishes were then incubated in the dark at room temperature and examined using a compound microscope, daily after two days. All isolates with typical *Phytophthora* growth characteristics were sub-cultured on 10 % V8 agar plates for identification (Erwin & Riberio 1996). Cultures were stored in sterile distilled water at room temperature (Ko 2003) and are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI). Details of all isolates used in this study are presented in (Table. 1).

2.2 Identification of isolates

2.2.1 Morphology

All isolates sub-cultured on to V8 agar were separated into groups based on culture morphology since most *P. cinnamomi* and *P. nicotianae* have distinct rosaceous or stoloniferous colony growth, respectively. Zoosporangia were produced using a modification of the method of Byrt & Grant (1979). Single spore cultures from each sampling site were obtained using the method described by Wang-Ching & Wen-Hsiung (1997). All isolates were identified with the aid of taxonomic keys (Stamps *et al.* 1990). The characters used to identify *P. cinnamomi* included coralloid hyphae, grapelike hyphal swellings, numerous chlamydospores, and absence of zoospores in solid media or oospores in single cultures. Features used to identify *P. nicotianae* isolates included papillate zoosporangia with various shapes and their unique ability to survive at 35 °C. A sub-set of six isolates of *P. nicotianae* and *P. cinnamomi* were used for morphological examination. Dimensions of 50 randomly selected reproductive structures were measured and recorded using light microscopy with complimentary Axiovision® 3.1 software and HRc Axiocam digital camera (Axioskop, Carl Zeiss, München, Germany).

2.2.2 DNA sequence comparisons

Clarified V8 broth was seeded with small mycelial plugs of isolates derived from single hyphal tips. Broth cultures were incubated at room temperature. Mycelium of the isolates was harvested, freeze-dried and stored at–20 °C until isolation of DNA. Lyophilised mycelium was pulverised using a mortar and pestle and 10–20 ng was transferred to Eppendorf tubes. Extracted DNA was isolated using the modified phenol-chloroform extraction method as described by Al-Samarrai & Schmid (2000). DNA (5 µl) was run on 1 % agarose gel, stained with 2 µl of ethidium bromide and visualised by UV

illumination. The concentration of the DNA was estimated using 1 kb molecular weight marker. The remaining DNA was suspended in sabax water and stored at –20 °C.

To confirm the identity of *Phytophthora* isolates used in this study, the Internal Transcriber Spacer (ITS) regions of the rDNA were amplified and sequenced using ITS 4 (5′ TCC TCC GCT TAT TGA TAT GC 3′) and ITS 6 (5′GAA GGT GAA GTC TAA CAA GG 3′) primers as described by Cooke & Duncan 1997. Each PCR reaction mixture (50 µl) consisted of 50-90 ng of DNA template, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 µM of dNTP's (Fermentas, UAB, Luthuania). PCR amplification conditions were 1 cycle at 96 °C for 2 min (initial denaturation), followed by 35 denaturation cycles at 96 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension 72 °C for 1 min, and final cycle at 72 °C for 10 minutes. Resulting amplified PCR products were separated on 1, 5 % agarose gel in a 1 × Tris–acetate / EDTA buffer. The PCR products were purified with the aid of a purification kit (Roche Diagnostics) and used as for sequencing reactions on an ABI automated 3100 DNA sequencer.(Perkin Elmer, Norwalk, CON). The same ITS primers used for the amplification reactions were used for the sequencing reaction conducted using the Big Dye Terminator Cycle sequencing reaction kit (Perkin Elmer, Applied Biosystems).

Raw sequence data was trimmed and aligned by using Sequencher software (ver. 4.5 Gene Codes, USA) and optimized by automatic alignment using MUSCLE software (Edgar 2004). A BLAST search of the GenBank database was conduced using the resulting consensus sequences and sdata for other *Phytophthora* spp. was added. Sequence data were analyzed using the maximum parsimony (MP) method in PAUP software version 4. The ends of aligned sequences were trimmed and uninformative characters removed. The most parsimonious phylogenetic trees were generated using the heuristic search with random stepwise addition of 100 replicates with tree bisection-reconnection branch-swapping option on and the steepest-descent option disabled. Maxtrees and branches of zero length were collapsed and the most parsimonious trees saved. Strict consensus and 50 % majority-rule trees were computed using *Pythium irregulare* (AY907917) as an out group. The confidence intervals of the branch nodes for the consensus tree were evaluated by bootstrap analysis of 1000 replications (Felsenstein 1985)

2.3 Mating type determination

The mating studies were determined using a method described by Erwin & Ribeiro (1996). All isolates used for the mating tests were cultured in 65 mm Petri dishes containing carrot agar (Brasier & Kirk 2004). Known A_1 (CMW21989, CMW21920) and A_2 (CMW21993; CMW21959) mating isolates of *P. nicotianae* and *P. cinnamomi* respectively, were used as testers. A mycelial plug (5 mm in diam) cut from the edges of actively growing colonies of known mating type isolates of *P. nicotianae* and *P. cinnamomi* were paired side by side with 30 *P. nicotianae* and 10 *P. cinnamomi* unknown isolates

respectively. All isolates were paired in all possible combinations, including positive and negative controls. Thereafter, Petri dishes were sealed with parafilm and incubated in the dark at room temperature for 15–30 days and examined for the production of sexual using a stereomicroscope (× 100 magnification). A polycarbonate membrane technique of Ko (1978), was also used to determine which isolates produced gametangia in compatible crosses. A sterile polycarbonate membrane (47 mm diam, 0.2µm pore size; Millipore) was sandwiched between a two mycelium plugs (10 mm) paired with one another. The cultures were incubated in the dark, at room temperature for 15–30 days and examined for oogonia or antheridia formation.

2.4 Pathogenicity tests

A subset of thirty *P. nicotianae* and ten *P. cinnamomi* isolates collected from various areas were used to inoculate one-year-old *E. smithii* trees in the field. All isolates were inoculated into *E. smithii* saplings in glasshouse pilot test prior to use in these experiments to ensure that they had not lost pathogenicity. The pathogenicity trial was located in a commercial stand at Sutton (29° 58´S, 30° 08´E), near Ixopo in the KwaZulu -Natal Province of South Africa. A complete random block design was used for field trial. Mean lesion lengths (mm) developed after 6 weeeks artificial inoculation of one-year-old *E. smithii* trees in the field were used to rank the aggressiveness of the *Phytophthora* isolates. A modification of the wounded bark inoculation technique described by Dixon *et al.* (1984) was used to inoculate side branches, roughly at the middle of each branch. A sterile blade was used to cut a flap (dimensions of 2 × 1 cm) through the outer bark. A 4 mm disc diameter mycelium plug from week-old cultures of *P. nicotianae* or *P. cinnamomi* grown on PDA was inserted under the flap and covered with masking tape. In total, 410 side branches (10 replicate trees per isolate) including controls were inoculated with each isolate of *P. nicotianae* and *P. cinnamomi*. Control side branches were inoculated with sterile agar plugs only.

Six weeks after the inoculation, labelled side branches were harvested and placed into plastic bags. These were transported to the laboratory for analysis. Lesion lengths extending above and below the inoculation points were measured. Lesion development in the inner bark was used as a measure of pathogenicity. Tissue cut from three randomly chosen side branches was plated onto selective media, as described previously, in order to ensure that the inoculated fungi could be re-isolated from the lesion and thus satisfying the Koch's postulates. The experiment was established in Summer (February) and repeated in Spring (October) to evaluate the influence of temperature on lesion development. Data were analysed using STATISTICA, a data analysis software system, version 6 (StatSoft Inc. 2001). Analysis of variance (ANOVA) was used to compare the pathogenicity between *P. nicotianae* and *P. cinnamomi* isolates used in this study. Pearson correlation coefficient *r* between the summer and spring trials was also calculated.

3. Results

3.1 Collection of samples

In total, 220 *Phytophthora* isolates were retrieved from 564 diseased trees (39 %) and only 48 isolates were obtained from the soil samples (15 %). In most cases, *Phytophthora* spp. were readily isolated from infected root collars compared to soil samples taken from the rhizosphere. The occurrence and distribution of *Phytophthora* spp. retrieved from soil and diseased plant samples varied from stand to stand. The pathogen population levels fluctuated considerably among the sampled stands throughout the duration of this study. The highest levels of tree decline and pathogen retrieval levels were observed during late summer (January and February) and early autumn months (March and April). A summary of the retrieval of *Phytophthora* spp. from different hosts is shown in Table 2.

3.2 Identification of isolates

3.2.1 Morphology

Phytophthora nicotianae and *P. cinnamomi* isolates typically produced stoloniferous and rosaceous (petaloid) growth pattern on V8 and carrot agar, respectively. However, some isolates displayed a variation in growth patterns. Some *P. nicotianae* and *P. cinnamomi* isolates did not produce any defined colony growth pattern on the media used. Measurements of asexual and sexual reproductive structures for *P. nicotianae* and *P. cinnamomi* were, therefore, also important (Table 3). *P. nicotianae* was the most common species retrieved from soil and diseased plant material collected from the sampling sites. The fungus was widely distributed with no obvious trend in geographic distribution observed. In total, 132 isolates (56 %) from both soil and diseased plant material were identified as *P. nicotianae*. Both mating types A1 and A2 of *P. nicotianae* were retrieved from both soil and diseased plant material. The A2 mating type (82 %) was more prevalent than the A1 mating type (18 %).

Phytophthora cinnamomi was the second most prevalent species retrieved from cold-tolerant eucalypts at the sites investigated. The percentage retrieval of *P. cinnamomi* from soil and diseased plant material was 24 % (54 isolates). *P. nicotianae* was more readily isolated from soil when compared to *P. cinnamomi.* However, at some sites both pathogens were isolated from dying plants. Of the *P. cinnamomi* isolates, 24 % were of mating type A1 and 76 % were of the A2 mating type. *Phytophthora boehmeriae* (4 %) and two newly described species, *P. alticola* (7 %), and *P. frigida* (9 %), were also retrieved occasionally.

3.2.2 DNA sequence comparisons

The amplification of the ITS region resulted in a single band of 900bp for all isolates examined. The final sequence data matrix consisted of 34 taxa with 865 characters of which 631 were excluded and 234 characters were included in the analysis. A significant phylogenetic signal of ($P < 0.01$, g1 = -0.68) was obtained after evaluating 1000 random trees. The heuristic search resulted in 2 most parsimonious trees (CI = 0.743, RI = 0.923, RC = 0.686, HI = 0.257), which differed only in the lengths of the branches.

The ITS rDNA sequence data set consisted of two major clades comprising ITS clade 1–10 as described by Cooke *et al*. (2000). The isolates sequenced in this study fell into two distinct groups together with authentic isolates of *P. nicotianae* and *P. cinnamomi.* The first clade consisted of the larger *Phytophthora* clade with species belonging to the ITS clades 1, 2, 6 and 7 (Cooke *et al*. 2000) and the second clade consisted of *P. boehmeriae* which belongs to the ITS 8 clade of (Cooke *et al*. 2000; Kroon *et al*. 2004). The *P. nicotianae* and *P. cinnamomi* isolates used in this study grouped in clade 1 and clade 7, respectively (Fig 3).

3.2 Pathogenicity tests

Discoloured lesions were observed on the green inoculated side branches above and below the points of inoculation after 6 weeks. When the bark was scrapped with a scalpel blade, distinct brown lesions were observed and whereas the control side branches did not display lesions (Fig 4). *P. cinnamomi* and *P. nicotianae* were re-isolated from up to 10 mm beyond the visible lesions when plated directly on selective culture medium but could not be reisolated from control branches.

All *Phytophthora* isolates inoculated on one-year old *E. smithii* branches in the field displayed a range in aggressiveness levels. However, side branches inoculated with *P. cinnamomi* isolates consistently had a rapid rate of lesion development and produced significantly ($P < 0.01$) longer lesions relative to those inoculated with *P. nicotianae*. The results of the mean lesion lengths produced by *P. cinnamomi* and *P. nicotianae* isolates are shown in Fig 5. *P. cinnamomi* isolates produced mean lesions length ranging between 19 mm to 59 mm in spring and between 33 mm and 77 mm in summer. The three most aggressive *P. cinnamomi* isolates on *E. smithii* were CMW21959, CMW21979, and CMW21983. A considerable variation in aggressiveness amongst the 30 *P. nicotianae* isolates was observed. Lesions produced by *P. nicotianae* isolates were between 7 mm to 23 mm long in spring and between 13 mm to 38 mm in summer. Isolates (CMW20326, CMW21972, and CMW22038 were the most aggressive *P. nicotianae* isolates.

The analysis of variance (ANOVA), revealed highly significant differences (P<.0001) in mean lesion lengths produced by the different *Phytophthora* isolates (Table 4). When the season and individual *Phytophthora* isolates were analysed as variables for the pathogenicity tests highly significant differences (F = 389.34, P <.0001, F = 58.37, P <.0001) were obtained. However, the correlation coefficient (r = 0.74) obtained between the spring and summer data sets indicate presence of seasonal interaction. Thus, the ranking of the mean lesion length produced by the different *Phytophthora* isolates was not always the same during the different seasons as illustrated in a scattergram (Fig 6). The overall summer lesion least squares means mean was 17.5 cm, the standard error was 0.36, the confidence limits (0.95) was 16.76. For the Spring inoculation spring, the average lesion length was 27.3 cm, standard error of 0.34 and the confidence limits (0.95) of 26.6.

4. Discussion

The aim of this study was to verify the identity of the *Phytophthora* spp. responsible for recent outbreaks of root and collar rot on several cold-tolerant *Eucalyptus* plantations in the Mpumalanga and KwaZulu-Natal Provinces of South Africa. Results confirmed the presence of both *P. nicotianae* and *P. cinnamomi* on cold-tolerant *Eucalyptus* stands as previously reported by Linde *et al*. (1994). However, they indicated that *P. nicotianae* rather than *P. cinnamomi* is the most common pathogen associated with diseased plant samples and soil. The consistent isolation of *P. nicotianae* from soil and diseased plant material was unexpected since *P. cinnamomi* has typically been seen as the dominant species affecting cold- tolerant *Eucalyptus* spp. in South Africa.

Very little is known about the role of *P. nicotianae* in die-back of cold-tolerant *Eucalyptus* spp. This is despite the abundance of *P. nicotianae* in soil and diseased *Eucalyptus* observed in this study. *P. nicotianae* has a wide host range (Erwin & Riberio 1996) and is associated with black-butt disease of *A. mearnsii* in South Africa (Zeijlemaker 1971). Planting of *Eucalyptus* spp. on sites previously planted to *A. mearnsii* may explain the high levels of *P. nicotianae* found in this study.

The relatively low level of recovery of *Phytophthora* spp., especially *P. cinnamomi* observed in this study, has also been found in *Eucalyptus* forest soils in Australia. (Marks *et al*. 1975, Weste & Ruppin 1977; Davison & Tay 2005). However, the consistent association of *P. cinnamomi* and *P. nicotianae* with infected root collars provides good anecdotal evidence that they are responsible for the root and root collar disease problem observed in plantations.

The morphology and ITS sequence data results confirmed the identity of both *P. nicotianae* and *P. cinnamomi* as well as *P. boehmeriae* and two newly named species (Maseko *et al*. 2007). These results are similar to those of an earlier report (Linde *et al.* 1994). Results of this study, strongly suggest that

through the routine use of DNA sequencing techniques, new records and possibly new *Phytophthora* spp. are likely to be encountered. Furthermore, the results revealed that the A2 mating type is the dominant form for both *P. nicotianae* and *P. cinnamomi. This* suggests that both pathogens predominantly reproduce asexually as reported in a previous study (Linde *et al*. 1999a)

Pathogenicity tests clearly illustrated that both *P. nicotianae* and *P. cinnamomi* are capable of causing disease in *Eucalyptus*. In a previous study (Linde *et al*. 1994), the pathogenicity of *P. nicotianae* and *P. cinnamomi* on *E. grandis* was demonstrated. The small numbers of *P. nicotianae* isolates included in that study were more aggressive than *P. cinnamomi*. In contrast, pathogenicity tests in the present study indicate that *P. cinnamomi* isolates are more virulent than those of *P. nicotianae* on *E. smithii.* In this study, highly significant differences in mean lesion length were found for different *P. nicotianae* and *P. cinnamomi* isolates inoculated onto *E. smithii*. Such variable levels of pathogenicity amongst isolates have previously been noted for *P. nicotianae* and *P. cinnamomi* (Linde *et al*. 1999b).

Likewise, variation in pathogenicity amongst different Australian isolates of *P. cinnamomi* inoculated onto *E. marginata* has been reported (Dudzinski *et al*. 1993). Season had a significant effect on the pathogen retrieval levels as well as on the development of lesion caused by the different *Phytophthora* isolates. Lesions produced by the different *Phytophthora* isolates were generally larger in the summer trial. The levels of tree decline pathogen retrieval levels were late summer and early autumn months.

Results of this study indicate that *Phytophthora* spp. are prevalent and important pathogens on cold-tolerant *Eucalyptus* spp. in South Africa. *Phytophthora nicotianae* is the most frequently recovered species and has the widest distribution. It was retrieved from declining trees and rhizosphere soil, which supports our hypothesis, that this pathogen rather than *P. cinnamomi* is the key causal agent of recent disease outbreaks. Thus, *P. nicotianae* should also be included in future disease-screening assays of cold-tolerant *Eucalyptus* seedlings in South Africa

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Table. 1 Isolates examined and description of the sites sampled for the presence of *Phytophthora* spp.

Isolate Number	Species identity	Host	Mating Type	Origin	Latitude	Longitude
CMW21959	*P. cinnamomi*	*E.nitens*	A2	Hlelo	30° 65´S	26° 09´ E
CMW21960	*P. cinnamomi*	*E. fastigata*	A1	Lothair	30° 44´ S	26° 28´ E
CMW21968	*P. cinnamomi*	*E .smithii*	A2	Shafton	30° 13´S	29° 19´ E
CMW21976	*P. cinnamomi*	*E .smithii*	A2	Hodgsons	30° 40´S	29° 24´E
CMW21920	*P. cinnamomi*	*E .smithii*	A2	Sutton	29° 58´S	30° 08´E
CMW21979	*P. cinnamomi*	*E .smithii*	A2	Epson	29° 47´S	29° 54´E
CMW21982	*P. cinnamomi*	*E. nitens*	A1	Lothair	30° 44´ S	26° 28´ E
CMW21983	*P. cinnamomi*	*E .smithii*	A2	Demagtenburg	30° 20´S	29° 33´E
CMW21984	*P. cinnamomi*	*E .smithii*	A2	Sutton	29° 58´S	30° 08´E
CMW22003	*P. cinnamomi*	*E. smithii*	A2	Spitzkop	30° 76´S	27° 41´E
CMW20203	*P. nicotianae*	*E. smithii*	A2	Enon	30° 24´S	29° 82´E
CMW20204	*P. nicotianae*	*E. smithii*	A2	Hlelo	30° 65´S	26° 09´ E
CMW20205	*P. nicotianae*	*E. smithii*	A2	Glenbain	30° 03´S	30° 00´E
CMW20297	*P. nicotianae*	*E. smithii*	A2	Shafton	30° 13´S	29° 19´ E
CMW20326	*P. nicotianae*	*E. smithii*	A2	Hodgsons	30° 40´S	29° 24´E
CMW21965	*P. nicotianae*	*E. dunnii*	A2	Pinewoods	30° 10´S	29° 64´E
CMW21970	*P. nicotianae*	*E. macarthurii*	A2	Demagtenburg	30° 20´S	29° 33´E
CMW21972	*P. nicotianae*	*E. smithii*	A2	Ingwe	30° 18´S	29° 49´E
CMW21989	*P. nicotianae*	*E. smithii*	A1	Hodgsons	30° 40´S	29° 24´E
CMW19442	*P. nicotianae*	*E. smithii*	A2	Spitzkop	30° 76´S	27° 41´E
CMW19443	*P. nicotianae*	*E. smithii*	A2	Shafton	30° 13´S	29° 19´ E
CMW21993	*P. nicotianae*	*E. smithii*	A2	Hodgsons	30° 40´S	29° 24´E
CMW22000	*P. nicotianae*	*E. smithii*	A2	Hodgsons	30° 40´S	29° 24´E
CMW22001	*P. nicotianae*	*E. smithii*	A1	Hodgsons	30° 40´S	29° 24´E
CMW22004	*P. nicotianae*	*E. smithii*	A2	Sutton	29° 58´S	30° 08´E
CMW22009	*P. nicotianae*	*E. macarthurii*	A2	Demagtenburg	30° 20´S	29° 33´E
CMW22010	*P. nicotianae*	*E. smithii*	A2	Sutton	29° 58´S	30° 08´E
CMW22011	*P. nicotianae*	*E. smithii*	A2	Sutton	29° 58´S	30° 08´E
CMW22016	*P. nicotianae*	*E. dunnii*	A2	Winterton	29° 48´S	29° 03´E
CMW22020	*P. nicotianae*	*E. smithii*	A2	Sutton	29° 58´S	30° 08´E
CMW22022	*P. nicotianae*	*E. macarthurii*	A2	Winterton	29° 48´S	29° 03´E

Table. 1 Cont. Isolates examined and description of the sites sampled for the presence of *Phytophthora* spp.

Isolate Number	Species identity	Host	Mating Type	Origin	Latitude	Longitude
CMW22037	*P. nicotianae*	*E. smithii*	A1	Sutton	29° 58´S	30° 08´E
CMW22038	*P. nicotianae*	*E. smithii*	A2	Ingwe	30° 18´S	29° 49´E
CMW22039	*P. nicotianae*	*E. smithii*	A2	Geelhoutboom	30° 14´S	29° 50´E
CMW22040	*P. nicotianae*	*E. smithii*	A2	Geelhoutboom	30° 14´S	29° 50´E
CMW22041	*P. nicotianae*	*E. smithii*	A2	Sutton	29° 58´S	30° 08´E
CMW22042	*P. nicotianae*	*E. dunnii*	A2	Tamboekiesbult	30° 52´S	27° 47´ E
CMW22043	*P. nicotianae*	*E. smithii*	A2	Ingwe	30° 18´S	29° 49´E
CMW22044	*P. nicotianae*	*E. dunnii*	A2	Tamboekiesbult	30° 52´S	27° 47´ E
CMW22045	*P. nicotianae*	*E. smithii*	A2	Sutton	29° 58´S	30° 08´E

Table 2. Isolation frequency of *Phytophthora* sp from cold-tolerant *Eucalyptus* stands surveyed in this study

Species	Host species	No. of isolates	No. of symptomatic trees	No. of soil samples	Total retrieval frequency (%)
P. cinnamomi	*E. dunnii, E. elata, E. fastigata, E. nitens, E. smithii*	54	172	115	24
P. nicotianae	*E. dunnii, E. elata, E. fastigata, E. nitens, E. smithii*	123	280	152	56
P. boehmeriae	*E. macarthurii, E. smithiii*	8	24	15	4
P. frigida	*E. dunnii, E. smithii*	20	47	20	9
P. alticola.	*E. badjensis, E. dunnii, E. macarthurii*	15	41	18	7
Total		**220**	**564**	**320**	**100**

Table 3. Measurements for the asexual and sexual structures of *Phytophthora* spp. isolated from *Eucalyptus* spp. in South Africa

Identity	Culture No.	Sporangia Measurements L×B (µm)	Oogonium (µm)	Oospore (µm)	Antheridia (µm)	Chlamydospore (µm)
P. nicotianae	CMW21970	(33–)35(–40)× (22–)30 (–33)	(23–)20 (–25)	(20–)22(–26)	(13–)19(–25)×(10–)15(–24)	(20–)22(–34)
P. nicotianae	CMW21993	(35–)48(–60)× (28–)36 (–55)	(18–)20 (–22)	(15–)18(–20)	(13–)19(–25)×(10–)15(–24)	(25–)30(–45)
P. nicotianae	CMW22000	(40–)49(–74)× (30–)35 (–50)	(16–)18 (–26)	(14–)16(–20)	(13–)19(–25)×(10–)15(–24)	(24–)28(–32)
P. nicotianae	CMW22001	(30–)38 (–43)× (25–)32 (–58)	(24–)20(–28)	(18–)20(–24)	(13–)19(–25)×(10–)15(–24)	(18–)20(–28)
P. nicotianae	CMW22009	(42–)52(–62)× (36–)42 (–47)	(19–)21(–25)	(16–)22(–28)	(13–)19(–25)×(10–)15(–24)	(28–)34(–45)
P. nicotianae	CMW22016	(25–)45(–60)× (19–)29 (–37)	(19.8–)20(–23)	(15–)18(–20)	(13–)19(–25)×(10–)15(–24)	(26–)30(–44)
P. cinnamomi	CMW21960	(55–)75(–95)× (35–)41 (–45)	(22–)34(–40)	(18–)28(–34)	(12–)16(–24)×(10–)14(–18)	(25–)42(–54)
P. cinnamomi	CMW21979	(55–)76(–100)× (30–)40 (–56)	(25)30(–35)	(22–)26(–28)	(14–)16(–26)×(12–)12(–16)	(34–)40(–52)
P. cinnamomi	CMW21959	(45–)67(–95)× (30–)38 (–49)	(32)40(–48)	(18–)24 (–34)	(16–)20(–30)×(14–)16(–22)	(31–)37(–50)
P. cinnamomi	CMW21985	(49–)58(–66)× (39–)48 (–57)	(25–)34(–50)	(15–)30(–49)	(13–)19(–25)×(10–)15(–24)	(26–)38(–55)
P. cinnamomi	CMW21977	(40–)51(–64)× (26–)32 (–40)	(30–)40(–50)	(23–)36(–48)	(15–)18(–24)×(13–)16(–22)	(26–)38(–55)
P. cinnamomi	CMW21983	(35–)65(–80)× (24–)30 (–39)	(23–)36(–45)	(20–)32(–40)	(15–)20(–28)×(13–)17(–20)	(27–)44(–56)

Table. 4. Summary of the data analysis of the lesion lengths caused by *P. nicotianae* and *P. cinnamomi* in summer and spring when inoculated into *Eucalyptus smithii*

Variable	df	SS	MS	F Value	Pr > F	R2	Coeff Var	Root MSE
Model	79	140102.9	1773.5	37.67	<.0001	0.81	30.3	6.861840
Error	682	32111.9	47.08					
Corrected	761	172214.8						
Season	1	18458.44	18458.4410	392.03	<.0001			
Isolate	39	110303.40	2828.2923	60.07	<.0001			
Season × isolate	39	110303.40	290.7971	6.18	<.0001			

Fig 1 – Disease symptoms associated with *Phytophthora* infection on cold tolerant *Eucalyptus* spp. A. *Phytophthora* dieback in an affected stand, B. Canker at the base of the tree showing kino exudations, C. Lesion on wood following removal of the bark. Photo courtesy of J Roux, FABI, UP

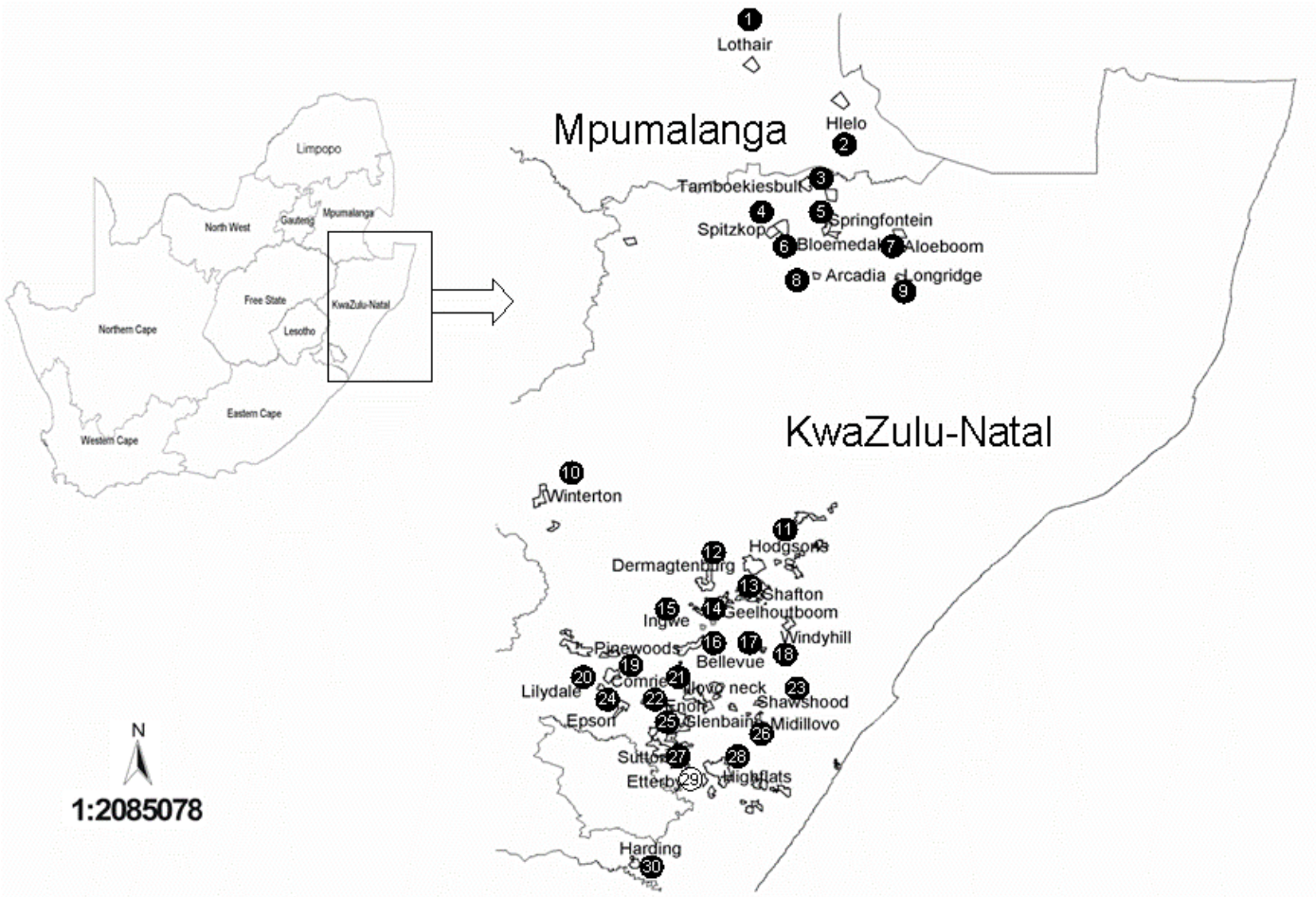

Fig 2 – Map showing the location and distribution of the 31 *Eucalyptus* stands sampled for the presence of *Phytophthora* spp. Insert map showing the location of the Mpumalanga and KwaZulu-Natal Provinces within the Republic of South Africa, where samples were taken.

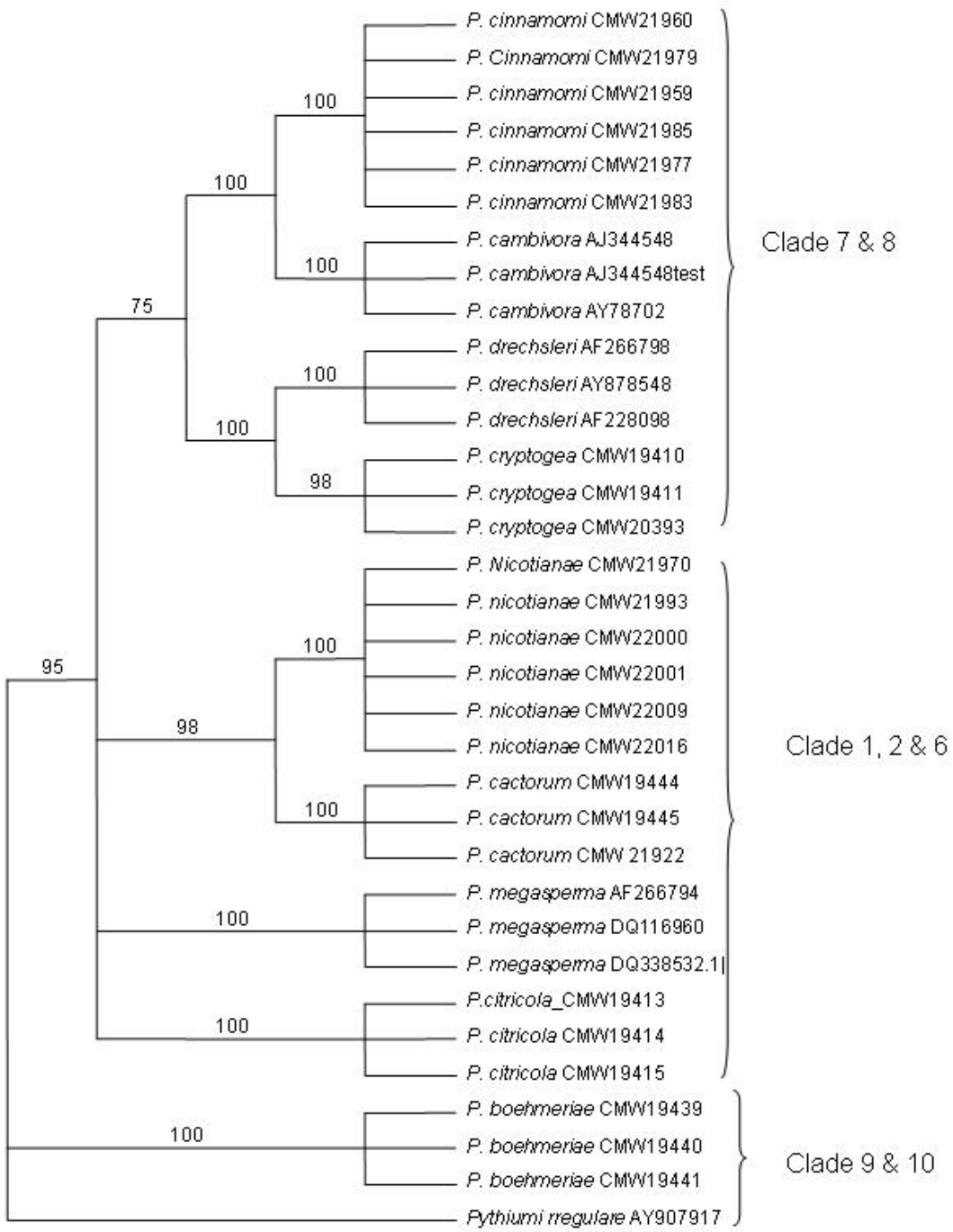

Fig – 3 ITS cladogram of *Phytophthora* spp. known to infect *Eucalyptus* spp. One of four most parsimonious trees (length=444, CI=0.743, RI=0.923, HI=0.257) from heuristic searches in PAUP (ver 4.0b1. Bootstrap support values of 1000 replicates

Fig – 4 Response of year old side branches of *Eucalyptus smithii* in the inoculated with *Phytophthora cinnamomi* and *Phytophthora nicotianae* isolates

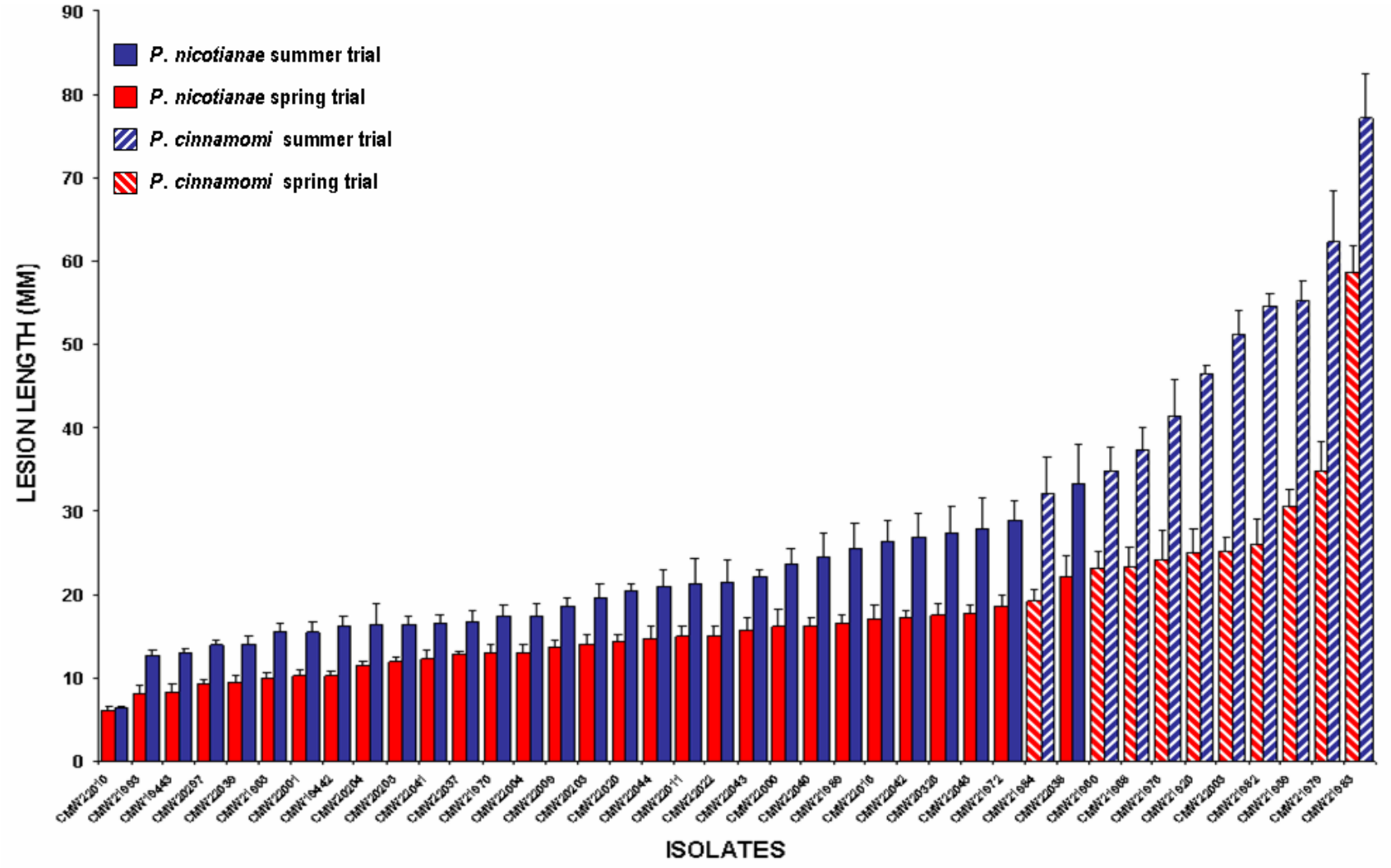

Fig 5– Ranked mean lesion length (mm) on year old side branches of *E. smithii* inoculated with 10 *P. cinnamomi* and 30 *P. nicotianae* isolates for six weeks in spring and summer trials. Error bars represent ± standard error.

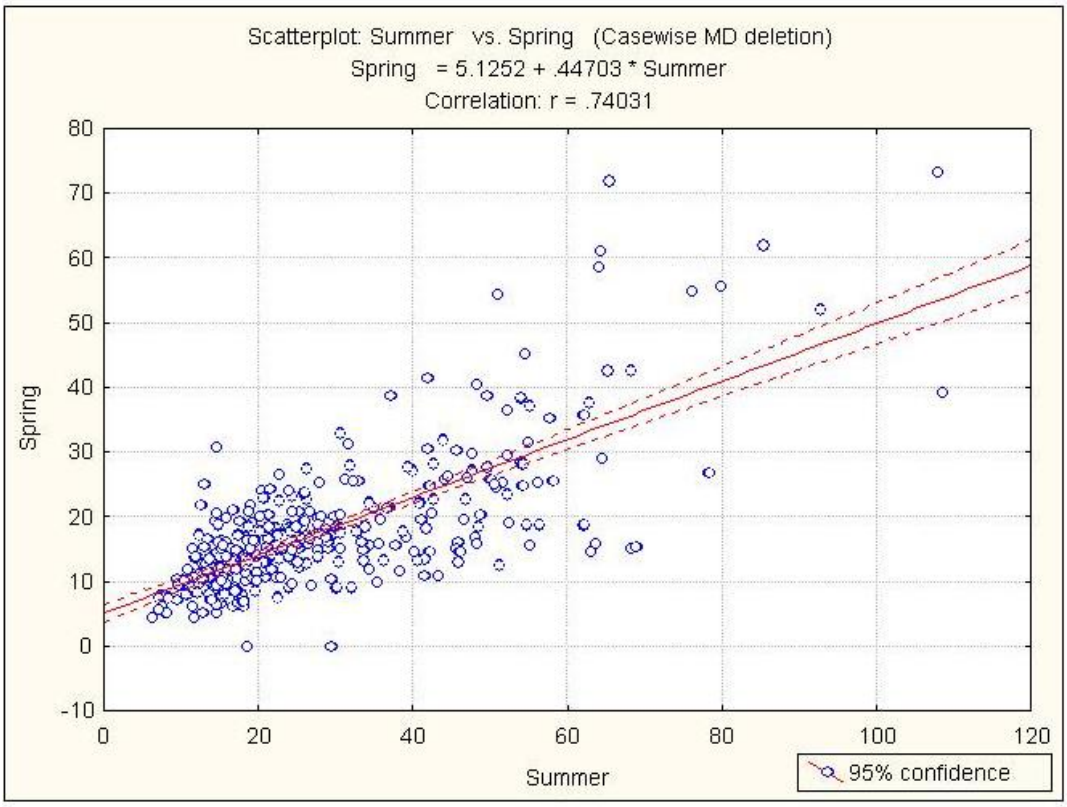

Fig 6–. Scattergram showing the correlation of the mean lesion length produced by the different *Phytophthora* isolates during the spring and summer.

Chapter Three

Role of *Phytophthora* root disease in the poor establishment of *Eucalyptus smithii* in South Africa

ABSTRACT

Eucalyptus smithii is one of the important commercial tree species cultivated in higher altitude summer rainfall regions of South Africa, but is notorious for high seedling mortality during establishment. The aim of this study was investigate the effects of site preparation and role of root disease in the poor establishment of *E. smithii*. Five study sites of standard quadrat design (8 × 12 × 4 trees per quadrat, 4 replications per study site) were established in two commercial *E. smithii* stands near Ixopo in the KwaZulu-Natal Province. Seedling mortality was monitored and recorded over 12 months following establishment. Soil samples were taken from each study site and diseased seedlings examined to determine the cause of death. Seedling mortality rates varied significantly between and within the sites throughout the monitoring period. Three *Phytophthora* spp. were detected from re-established stands and included *P. nicotianae*, *P. boehmeriae,* and the recently described *P. frigida*. None of these *Phytophthora* spp. were recovered from the grassland site. However, 20 % seedling mortality was recorded at this *Phytophthora*-free site and mortality did not differ significantly from the three re-established stands investigated. The highest mortality (47 %) was found on one re-established site that was later replanted with alternative species. The death of seedlings, together with the absence of *Phytophthora* spp. on the grassland site highlights the involvement of factors other than pathogens in the poor establishment of *E. smithii* on the stands investigated.

Key words: *Eucalyptus* establishment, seedling mortality, *Phytophthora* dieback

1 Introduction

Eucalyptus spp. are planted extensively in South Africa for the production of pulp and paper and various forest products (Owen & van der Zel 2000). Improved varieties and clones of *E. grandis* are the most commonly planted exotic tree species (Schönau 1994). Over the past few decades, *Eucalyptus* fibre plantations have expanded towards marginal areas that are inordinately cold and dry for *E. grandis* (Darrow 1996; Louw 2006). This has prompted the South African forestry industry to introduce alternative species to increase the pulp and fibre supply under such environmental conditions (Clarke 1995).

Eucalyptus smithii is one of the newly introduced species that has become an economically important and is planted on a moderate scale in the colder, higher altitude areas of South Africa (Herbert 2000). It is usually plated in the Mpumalanga and KwaZulu-Natal provinces of South Africa on sites with altitude above 1150 m, rainfall less than 900 mm and soil depth greater than 600 mm (Swain & Gardner 2003). This species is drought tolerant, grows rapidly and has superior pulping qualities compared to other commercially grown *Eucalyptus* spp. (Clarke *et al*. 1999). *Eucalyptus smithii* is notorious for high levels of post-establishment mortality that often leads to poor stocking (Swain *et al.* 2000). However, even with poor stocking, biomass production over a rotation is still high compared to other species (Clarke 1999). The post-planting mortality of *E. smithii* seedlings is most severe during the first year of establishment, but subsides as the trees mature (Swain *et al*. 2000).

The majority of plantations in South Africa are re-established on sites previously planted to either to eucalypt, wattle or pine and various silvicultural methods are used to promote survival and growth of the seedling (Smith *et al*. 2001). Poor establishment of eucalypts is often attributed to a number of factors such as poor seedling quality, damage during planting and suboptimal planting conditions, which often increase the vulnerability of seedlings to insect pests and soil borne pathogens (Caulfield *et al*. 1992). *E. smithii* is known for poor survival, particularly when planted on re-established sites (Jarvel 1998). The focus of previous studies was mainly on seedling quality and planting practice to improve post-planting survival and growth of *E. smithii* (Zwolinski & Bayley 2001). However, very little has been published on the effects of site treatment in predisposing *E. smithii* seedlings to soil-borne root pathogens. Linde *et al*. (1994) reported that *E. smithii* seedlings planted on water gaining sites are more susceptible to root and collar rot disease mainly caused by *P. cinnamomi*. Site treatment during establishment is important for the survival of eucalypt transplants (Pallett & Sale 2004). The aim of this study was investigate the effects of site preparation and the incidence of root disease caused *Phytophthora* spp. on establishment of *E. smithii*. Accurate identification of *Phytophthora* spp.

involved in early death of *E. smithii* seedlings and understanding the underlying causes of seedling mortality are key in reducing the risk of *E. smithii* seedling mortality in the future.

2 Materials and Methods

2.1 Study area and experimental design

This study was conducted on two *Eucalyptus* plantations located in the midlands region of KwaZulu-Natal Province, one of the major forest plantation areas of South Africa. It is situated in the summer rainfall region characterized by cold and dry winters. To investigate the role of various site treatments on poor plantation survival of *E. smithii* seedlings, five experimental sites were set up within two newly established commercial plantations stands located at Sutton (29° 58′, 30° 08′E) and Lilydale (30° 18′S, 29° 49′E). These plantations are separated by a distance of approximately 40 km. For each study site and factor investigated, experiments were deliberately set up on high riks sites prone to flooding and waterlogging and conducive to *Phytophthora* growth and survival and low risk sites with well-drained soil.

Details of the experiment sites are summaried in Table 1, and a brief explanation of the various land preparation treatments referred to in this study is provided below. Treatments investigated in this study included, various clearing methods (soil ripping, hot or cool burn), previous crop (reestablishment sites or grassland), development of competing weeds and water drainage. Soil ripping refers to ripping with a single tine mounted on a tractor. The removal of plantation residues using fire prior to treatment application can be either be cool or hot burn. Cool burn refers to controlled burning of plantation residues aimed at stimulating the germination of forest trees and hot burn is uncontrolled burning which lead to soil water-repellency.

Standard silvicultural land treatments treatment procedures such as pitting, watering the pit, application of fertiliser (100g super phosphate) and weeding were followed as described by du Toit (1995). Seedlings were planted into the centre of each pit (depth of 15 cm) at a spacing of 2 × 3 m. However, the Lilydale site was a new forest land site established on grassland and had been subjected to a soil ripping as a pre-planting treatment. This study site comprised of four quadrats (96 trees / per quadrat planted at 2 × 4 m spacing) and was regarded as a control site, since it has not yet been forested and most likely the soil was free of *Phytophthora*.

Four study sites each with four quadrats (96 trees/quadrat: 384 trees/study site) were set up on newly established *E. smithii* compartments at Sutton within commercial compartments D7 and D9 (two study sites in each compartment) (Fig 1). Compartment D7 and D9 at Sutton were re-establishment stands and had been subjected to a slash and cool burn pre-planting treatment. Two sites were established in compartment D7 and were designated D7a and D7b, respectively. Study site D7a was established on a well-drained and relatively flat surface while D7b was established along a flood plain, considered favourable for *Phytophthora* spp. The remaining two sites, namely D9a and D9b, were established in compartment D9. Study site D9a was established on an area with no weeds while D9b had heavy weed cover (Fig 1).

2.2 Sampling, isolation, and assessment

Seedlings were planted in December 1999, 2 months before the first assessment was conducted. Assessments were undertaken during summer (January–February), autumn (March–April) and spring months (October–November) of 2000 and 2001. No assessments were done in winter since *Phytophthora* spp. are likely to be inactive as this is a summer rainfall region and winter conditions are very cold and dry.. Each quadrat was subdivided into eight blocks containing 12 seedlings to facilitate easy sampling and mapping of sites. In order to collect data on the distribution of diseased seedlings and seeding mortality, each block was assigned a letter (A–H) and the position each seedling was mapped using a ground-based identification method which entailed walking parallel transects in 2 m × 3 m grid system.

During each evaluation, the number and position of dead and replacement seedlings planted in the position of the dead seeding was recorded. All dying seedlings within quadrats were recorded, removed, and analysed to determine the cause of death. The number of dying seedlings from which *Phytophthora* spp. were recovered in each quadrat was also recorded. Segments of the diseased plant tissue and surface disinfested roots were plated directly on selective media for (NARPH) the isolation of *Phytophthora* (Difco, Corn Meal Agar (CMA), 17 g/l^{-1} amended with 50 $\mu g/ml^{-1}$ nystatin, 200 $\mu g\ ml^{-1}$ ampicillin, 10 $\mu g\ ml^{-1}$ rifampicin, 25 μgml^{-1} pentacloronitrobenzene (PCNB), and 50 $\mu g\ ml^{-1}$ hymexazol 3 hydroxy-5-methylisoxazole, Sigma-Aldrich, St. Louis] (Hüberli *et al*. 2000). Plates were then incubated at room temperature for a period 2–3 days.

Rhizosphere soil samples were collected from each block in each quadrat at all sites to test for the presence of *Phytophthora* spp. All samples were collected and tested during the spring and summer months when soil moisture and temperature were conducive for the isolation of *Phytophthora* spp. To determine the presence or absence of *Phytophthora* spp, four sub-samples of soil were randomly collected from each block and were mixed and pooled into one sample of approximately 250 g,

resulting in 32 soil samples per site. In total, 160 soil samples were collected from all quadrats during each visit and each soil sample was placed in a sealed plastic bag. Soil samples were baited using the host and citrus leaf pieces as described by Grimm & Alexander (1973). Baited leaf pieces were harvested and plated on to a modified selective media for *Phytophthora* as described above.

Phytophthora isolates retrieved from the soil and from diseased plant material were sub- cultured on to clarified V8 juice agar for further identification and maintenance. Sporulation of *Phytophthora* isolates was induced by mineral salt solution (Erwin & Riberio 1996). Isolates were stored in sterile distilled water at room temperature (Ko 2003). All isolates obtained in this study were deposited in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

2.3 Analysis of results

The severity of the disease within each quadrat, measured in terms of seedling mortality or survival, was recorded, at least twice per season, during the 12 months after establishment. The total number of dead seedlings was used as a measure of the rate of mortality over time. The mortality of the seedlings was expressed as the percentage of dead seedlings at the end of each sampling period, relative to the number of seedlings at the beginning of sampling period.. A generalized linear model was used to analyse the relationship between seedling mortality with different variables including study sites, season, presence, or absence of *Phytophthora* spp. in diseased seedlings and rhizosphere soil. All statistical analyses were conducted using the SAS/STAT® software, version 8.02 (SAS Institute, Inc., Cary, North Carolina).

2.4 Identification of *Phytophthora* isolates

Phytophthora isolates isolated retrieved from roots and infected collars from symptomatic seedlings collected from the study sites were identified with the aid of keys in Stamps *et al*. (1990). Characteristics used to group isolates were the sporangia, growth patterns in culture, morphology of the hyphae oogonia and antheridia. The identity of three representative isolates for each species were confirmed using comparisons of ITS sequences as described previously (Maseko *et al*. 2007)

3 Results

3.1 Seedling mortality

Result of analysis of variance (ANOVA) showed highly significant differences in mortality levels on all study sites ($P < 0.001$, $F = 7.9$, $R^2 = 0.56$). Highly significant differences in seeding mortality were also found between study sites ($P < 0.001$, $F = 149.32$), within the different quadrats in sites ($P < 0.001$, $F = 4.34$) and between quadrats over time ($P< 0.01$, $F = 1.5$). A summary of the result analysis is presented in Table 2. First-year seedling mortality data for all study sites is illustrated in Fig 2. It is evident from the graph that seedling mortality rates varied between and within the sites throughout the monitoring period. The highest seedling mortality was recorded at site D9b in Sutton. Seedling mortality occurred shortly after planting and it progressed gradually during the first growing season.

At the end of the monitoring period the total cumulative mean mortality on all five study sites was 24 %. However, the total cumulative mean seedling mortality on four of the five study sites was below 20 % (D7a = 16 %, D7b = 18 %, D9a = 14 %, Lilydale = 19 %). The exception was site D9b at Sutton, which had total cumulative seedling mortality of 47 %.

3.2 Seedling mortality due to *Phytophthora*

Due to the challenge associated with distinguishing between seedling mortality caused by *Phytophthora* and other causes. Only if *Phytophthora* was re-isolated from the infected seedling was death attributed to *Phytophthora* being the main cause of seeding death. The mean percentage recovery of *Phytophthora* spp. from diseased seedlings was lowest (29 %) during the drier autumn month of April and highest during the wet summer month of January (58 %). A significant difference ($P < 0.01$) in the recovery of *Phytophthora* from diseased plants was observed within and between the four study sites as well as during the different sampling periods. The highest pathogen recovery and seedling mortality was recorded on theD9b study site. The recovery of *Phytophthora* spp. from study sites, D7a, D7b, D9a, and D9b is shown in Fig 3. Dying seedlings appeared withered especially during autumn months (March and April) when soil conditions where dry. The mean monthly rainfall data from the weather station, Ixopo located near the experimental site is illustrated in Fig 4. All diseased seedlings assayed had noticeable lesions on the upper root, collar, and the lower stem regions. In general, disease symptoms were observed either on single seedlings scattered on each quadrat or in small patches in areas prone to water-logging. No seedling mortality was attributed to *Phytophthora* from Lilydale (control site) since *Phytophthora* spp. were could not be retrieved from either soil or seedlings. Instead, seedling damage due to cutworms was observed at the Lilydale study sites.

3.3 Recovery of *Phytophthora* spp. from soil

A total of 800 soil samples were collected from all experimental sites for the duration of this study. *Phytophthora* spp. were only retrieved from 9 % (71 out of 800) of the soil samples assayed. A summary of the results for isolation of *Phytophthora* spp. from rhizosphere soil is given in Table. 3. *Phytophthora* spp. were isolated only from re-established sites (D7a, D71b, D9a and D9b) and not from the grassland site. Isolation of *Phytophthora* spp. varied from site to site during the monitoring period and it had a similar pattern with the mean monthly rainfall in the area (Fig 4). The highest recovery of *Phytophthora* spp. was recorded during the warm, rainy months from October to January and the lowest during February to April. Significant differences in the recovery rate of *Phytophthora* spp. were found for different sites (F-ratio 3.27, $P < 0.05$). Study site D9a had significantly higher pathogen levels in the soil relative to the other re-establishment sites investigated.

3.4 Identification of *Phytophthora* isolates

Three *Phytophthora* spp., namely *P. nicotianae*, *P. boehmeriae* and *P. frigida* were retrieved from the soil samples. *Phytophthora nicotianae* and the newly described *P. frigida* were isolated most frequently and were recovered from all re-establishment sites. *Phytophthora boehmeriae* was recovered only occasionally and only from quadrat D7 b (Table 3). The identity of the *P. nicotianae*, *P. boehmeriae* and *P. frigida* was determined based on morphological characteristics and further confirmed using DNA sequence comparisons.

4 Discussion

In general, the results of this study indicate that the diffeent land preparation treatments on re-established and on sites that have not yet been forested have little effect on the final survival of *E.smithii* in the field. However, the effect of the land preparation treatment was only significant ($P<$ 0.01) at only one of the reestablsihement site. In particular, study D9b had a highest mortality levels and *Phytophthora* species is most likely playing an important role but other factors such as cut worm, poor drainage contribute to seedting mortality. Site D9a appeared to be the best with lower mortality but *Phytophthora* was reisolated from diseased plant material and sol. Not significant difference in mortality levels was found at site D7a and D7b but a proposition of death due to *Phytophthora* was recorded. The Lilyadale site had a modate level of mortality. This was enexpected and interesting as was considered a disease free site and control. Quite a high proposion of the trees that died has cutworm damage and this togheter with normal transport, stock seedlings mortality gave a higher that expected numbers of deaths.

In view of the outcome of Koch's postulates Results of this study show that *Phytophthora* spp. are involved in the death of *E. smithii* seedlings but that a proportion of seedling death is caused by other agents. *Eucalyptus smithii* is well-known susceptible afforestation species with specific planting requirements (Swain *et al*. 2000). Unsuitable planting sites results in poor establishment and this is often aggravated by the susceptibility of the species to the *Phytophthora* root and collar rot (Wingfield & Roux 2000).

Previous reports attribute poor survival of *E. smithii* to various factors including seedling age, land-use history of regeneration sites, planting methods and *Phytophthora* root and collar rot Bayley & Snell (1997) and Jarvel 1998 and Herbert 2000 reported *E. smithii* seedling mortality levels ranging from between 31 % (survival 69.3 %) and 41 % (survival 59.5 %). In this study, the total mortality assessed after a period of 12 months was below 20% at four sites and 43 % on one other site. No significant difference in seedling mortality was found amongst the four sites (D7a D7b, D9a, Lilydale), which were subjected different pre-planting treatments. This observation indicates that pre-planting treatments and land-use history of a site did not contribute to the poor survival of *E. smithii* seedlings. Results obtained in this study indicate that seedling mortality due to undetermined causes, but propably including transplant shock, J rooting, wounding and localized drought occurs shortly after planting and gradually increase up to 20 %. Similarly, Bayley & Snell (1997) reported that most seedling mortality occurred within the first month after planting and the seedling age was found to be the main course of seedling failure.

In this study, recovery of *Phytophthora* spp.from diseased seedlings followed a clear seasonal pattern. The recovery of *Phytophthora* spp. from diseased seedlings was low (29 %) during Autumn and high during summer (58 %). In addition, significant differences ($P < 0.01$) in the recovery of *Phytophthora* spp. from diseased plants were observed within, between the four reforestation study sites (D7a, D7b, D9a, and D9b), and during the different sampling periods. The high level of recovery of *Phytophthora* spp. from diseased plants and high seedling mortality recorded at the D9b study site, suggest that root rot is a key factor contributing to seedling death in that situation.

Seedling death was more apparent during February and March when soil conditions where dry. Previous studies have shown that drought stress enhances the development of *Phytophthora* within the infected eucalypts tissue is enhanced (Cahill *et al*. 1985; Shearer & Tippett 1989). In South Africa, dieback of *E. smithii* associated with *Phytophthora* spp. is prevalent on plantation sites prone to both waterlogging and drought stress (Herbert 2000). It is known that soil moisture also plays an important role in the development of *Phytophthora* diseases, because zoospores require free water and warm soil conditions for dispersal (Erwin & Ribeiro 1996). Thus, it is likely that the seedlings were infected

spring and summer, when there were favourable temperature and moisture in the soil for infection and that the symptoms were observed somewhat later. Seedling mortality on the control grassland site could not be attributed to *Phytophthora* as these pathogens were not retrieved from either soil or dying seedlings.

Poor recovery of *Phytophthora* sp from the soil in re-establishment sites observed in this study is consistent with results of previous studies (Erwin & Ribeiro 1996; McDougall *et al*. 2002). Recovery of *Phytophthora* spp. improved when diseased plant material was plated directly into selective media. This observation suggests strongly that plant tissue most probably acts as a reservoir for *Phytophthora* inoculum. Observations from this study also indicate that *Phytophthora* spp. have an uneven distribution within the quadrats that tested positive for the presence of this pathogen. This observation is consistent with other reports showing uneven distribution of *Phytophthora* inoculum in soil (Marks *et al*. 1975; Shearer & Shea 1987).

Phytophthora nicotianae, *P. boehmeriae* and *Phytophthora frigida* were retrieved from the soil and diseased plant samples from re-establishment sites. These results suggest that more than one *Phytophthora* sp. is responsible for the *Phytophthora*-related deaths of *E. smithii* seedlings at the sites investigated. *Phytophthora cinnamomi* was not retrieved from any of the study sites although it has previously been reported as the most common pathogen associated with root and collar rot of cold tolerant eucalypts in South Africa (Lundquist & Baxter 1985). *Phytophthora nicotianae* and *P. boehmeriae* have also been reported as pathogens of *Eucalyptus* spp (Sankaran *et al*. 1995; Linde *et al*. 1994). Pathogenicity tests conducted in South Africa have shown that *P. nicotianae* and *P. boehmeriae* are pathogenic to *Acacia mearnsii* and *Eucalyptus* spp. (Linde *et al*. 1994, Maseko *et al*. 2001; Roux & Wingfield 1997). The role of *Phytophthora frigida* as a pathogen requires further investigation. In conclusion, five study sites were established on newly established *E. smithii* stands to investigate the effects of site preparation and role of root disease in the poor establishment of *E. smithii*. Our results indicate that a number of factors contribute to the poor establishment of *E. smithii* and at least three *Phytophthora* spp. are involved in the early death of seedlings.

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Table 1 Details of compartments used in this study.

Plantation compartment details						Land preparation Method		
Plantation Name	Stand No (Size)	Quadrat designation	Species	Previous crop	Month of establishment	Clearing method	Site problems Recorded	*Rating
Sutton	D7 (33.6 ha)	D7a	*E. smithii*	Pine	Jan 2000	slash & burn	None	Low Risk
Sutton	D7 (33.6 ha)	D7b	*E. smithii*	Pine	Jan 2000	slash & burn	Flood plain	High Risk
Sutton	D9 (36 ha)	D9a	*E. smithii*	Pine	Nov 1999	slash & burn	None	Low Risk
Sutton	D9 (36 ha)	D9b	*E. smithii*	Pine	Jan 2000	slash & burn	Weeds & Cutworms	High Risk
Lilydale	Lilydale	Control	*E. smithii*	Grass	Nov 1999	soil ripping	None	Low Risk

*Rating based on results reported by Jarvel, 1998

Table 2 Summary of the generalized linear model analysis for mortality

Variable	***df***	***SS***	***MS***	***F Value***	***Pr > F***	***R^2***	***Coeff Var***	***Root MSE***	***Mortality Mean***
Model	99	156904.63	1584.9	7.86	0.0001	0.6	60.5	14.2	23.5
Error	620	124986.0	201.6						
Corrected Total	719	281890.67							
			Summary of mortality analysis between study sites quadrats and time						
Site	4	120402.6	30100.7	149.3	0.0001				
Quadrat (site)	15	13125.68	875.04	4.34	0.0001				
Time (site ×quadrat)	80	23376.3	292.2	1.45	0.0092				

Table 3: Summary of the results for the recovery of *Phytophthora* spp. from rhizosphere soil

Site	Mean percentage recovery of *Phytophthora* spp. from soil (N =32)					Total mean frequency of recovery (%) of *Phytophthora* spp. from soil sampling (160 samples)	*Phytophthora* spp. recovered
	February	March	April	October	January		
D7a	9.4	6.3	3.1	13	19	10	*P. nicotianae, P. frigida*
D7b	13	9.4	3.1	9.4	22	11	*P. nicotianae, P. boehmeriae*
D9a	9.4	6.3	3.1	6.3	16	8	*P. nicotianae, P. frigida*
D9b	16	9.4	6.3	16	28	15	*P. nicotianae. P. frigida*
Lilydale	0	0	0	0	0		None

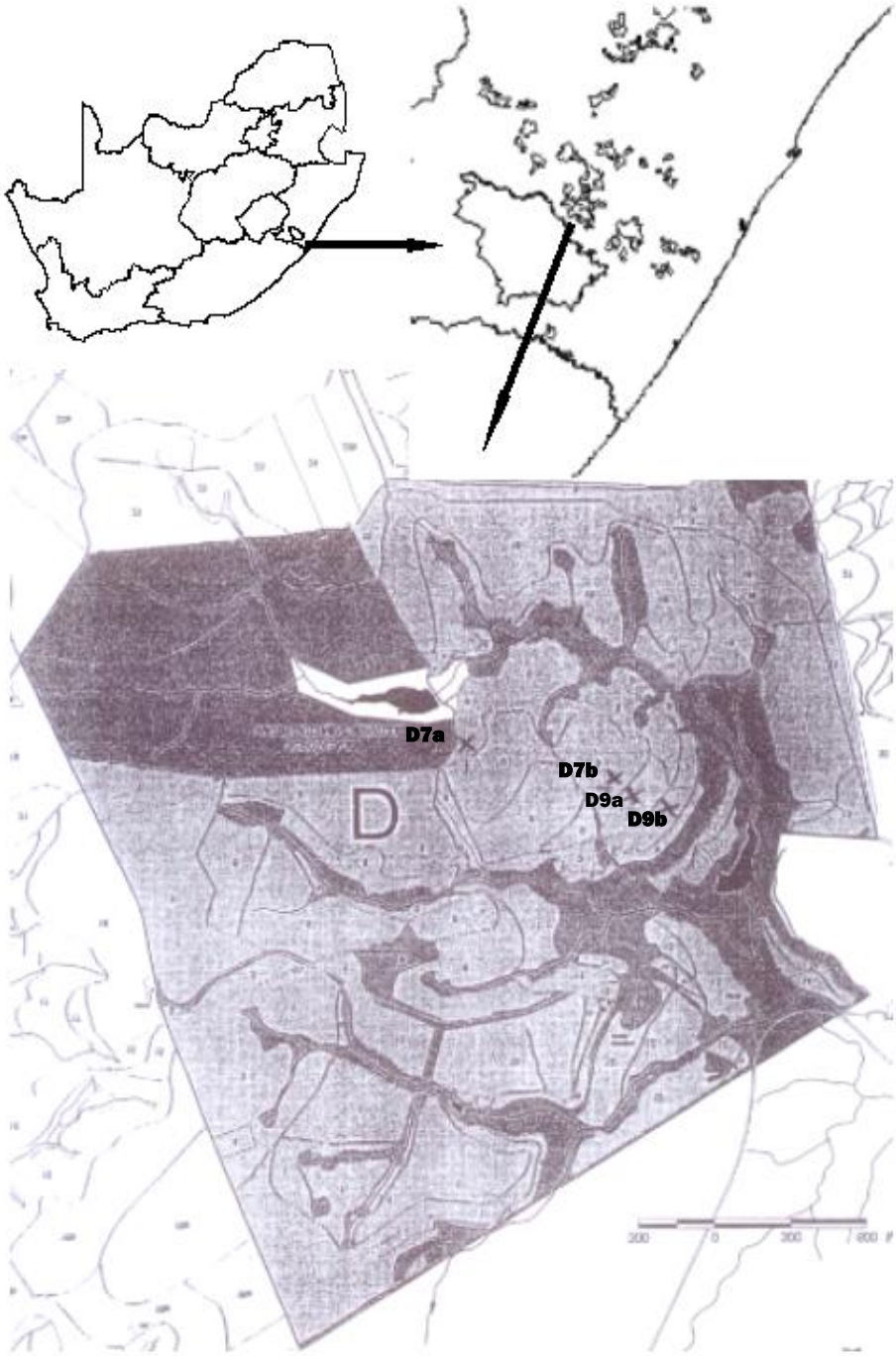

Fig 1 – Map of showing location of the experimental plot at Sutton plantation, in the KwaZulu-Natal Province of South Africa. The crosses (×) on the map indicate the location of four quadrats (D7a, D7b, D9a and D9b).

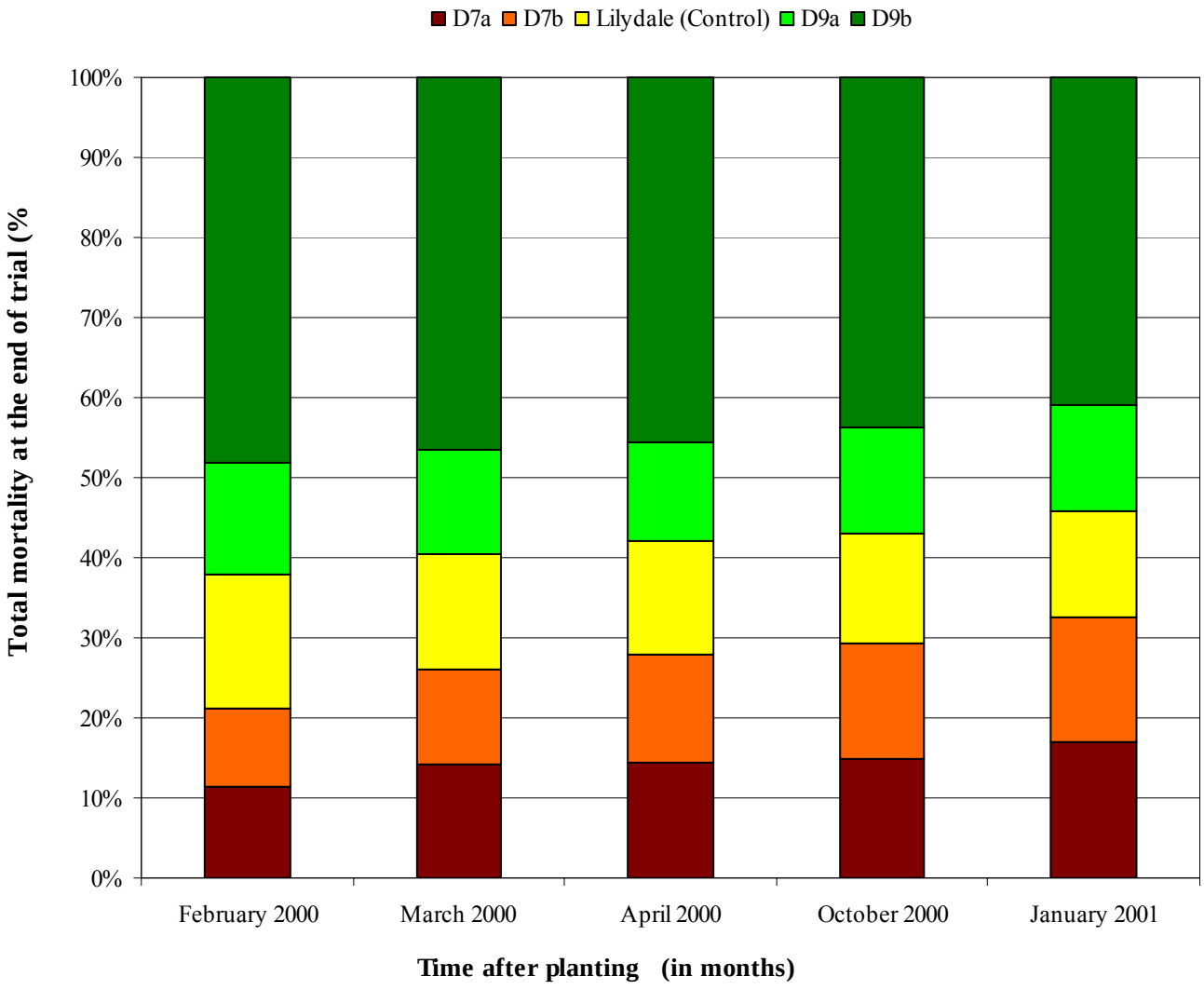

Fig 2 –Mortality levels (%) of *E.smithii* seedlings at the end of trial

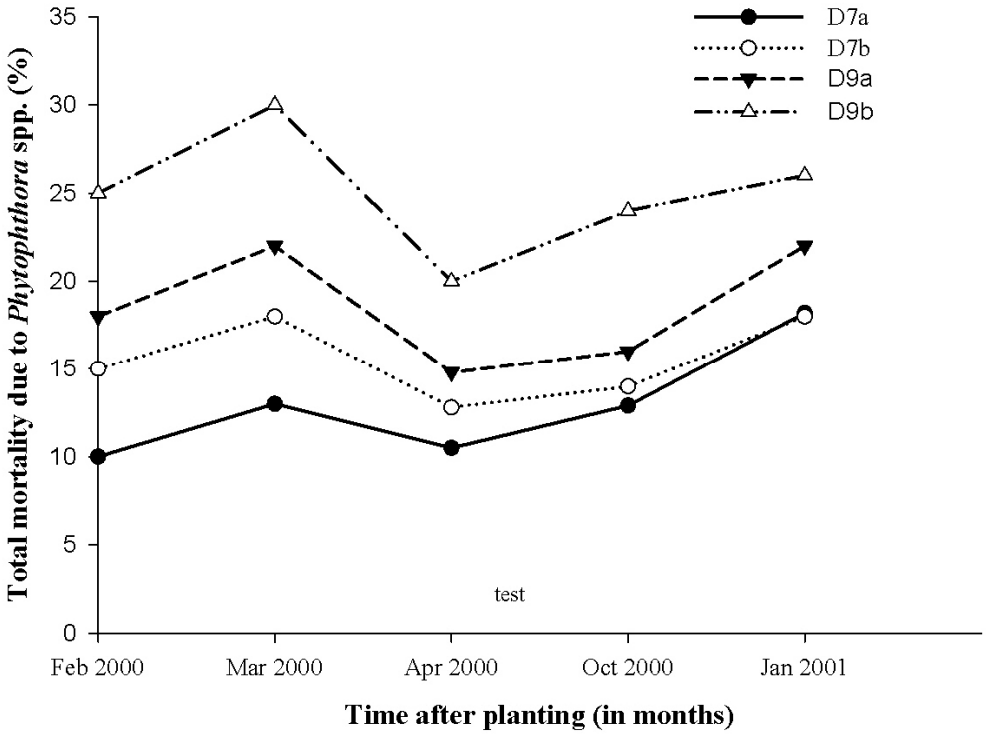

Fig 3 –Total mortality levels of *E.smithii* seedlings associated with *Phytophthora* dieback at the end of trial

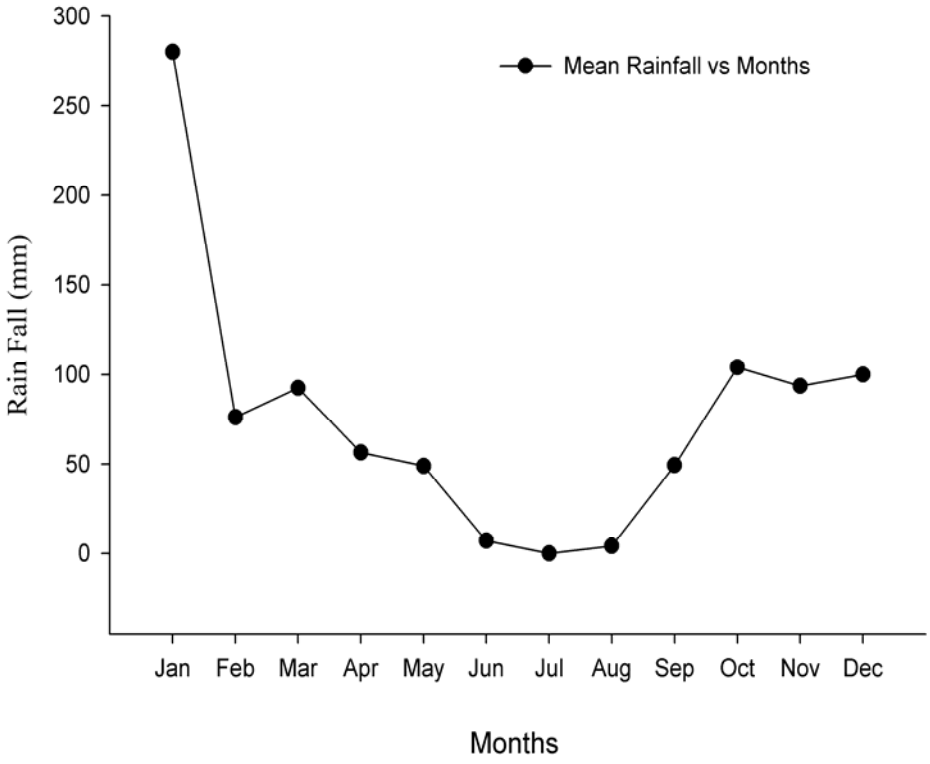

Fig. 4 Mean Monthly Rain (mm) Data for station [0210099 2] - IXOPO

Chapter Four

Screening of *Eucalyptus smithii* half-sib families for tolerance to infection by *Phytophthora cinnamomi* and *P. nicotianae*

ABSTRACT

Phytophthora root and collar rot caused by *Phytophthora cinnamomi* and *P. nicotianae* is associated with severe disease and mortality of *Eucalyptus smithii* in forest nurseries and plantations in South Africa. Variation in mortality levels has been observed between *E. smithii* genotypes in three different breeding trials in Kwa-Zulu-Natal. Twelve half-sib *E. smithii* families were screened for tolerance using two rapid screening techniques. A large number of one-year-old seedlings were stem inoculated using pre-selected virulent isolates of *P cinnamomi* and *P. nicotianae* to determine their susceptibility under greenhouse conditions. Total soluble phenolic compounds were extracted from young, fully expanded leaves of plants representing 12 half sib *E. smithii* families. Concentrations of the resulting crude extracts were analysed using High Performance Liquid Chromatography. *P. cinnamomi* resulted in rapid lesion development and produced significantly longer lesions relative to those inoculated with *P. nicotianae*. The disease severity ratings for the *E. smithii* families based on the inoculations correlated with field observations for susceptibility. The mean total soluble leaf phenolic concentration was found to be higher in tolerant than in susceptible *E. smithii* genotypes. Screening using inoculation and assessment of total soluble leaf phenolics may provide a rapid means to screen *E smithii* for resistance to infection by *P. cinnamomi* and *P. nicotianae*

Keywords: *Phytophthora cinnamomi*; *P. nicotianae*; disease tolerance, stem inoculation

1 Introduction

Eucalyptus smithii was first introduced into South Africa during the early 1900s, chosen for its high leaf cineole oil content (Poynton 1979). It is tolerant to drought, snow and frost (Gardner & Swain 1996), grows vigorously, and has outstanding pulping properties in comparison to other eucalypts grown in South Africa (Clarke *et al*. 1997; 1999). As a result, *E. smithii* is an ideal species for establishment in frost and drought prone areas situated above an altitude of 1000 m (Darrow 1996; Swain & Gardner 2003).

Eucalyptus smithii is well known to suffer from high seedling mortality in nurseries and plantations sites, leading to poor establishment and stocking (Bayley & Snell 1997). Poor establishment of *E. smithii* in plantations is due to a number of factors including transplanting over aged seedlings, usuitable planting sites and root and collar rot (Swain *et al*. 2000). Of these, root and collar rot disease is the single most limiting factor for large scale planting of *E. smithii* in South Africa (Herbert 1994). The introduced soil borne pathogen *P. cinnamomi* is a well known causal agent of root and collar rot disease of *E. smithii* (Linde *et al*. 1999). However, recently *P. alticola, P. frigida* and *P. nicotianae* have also been reported to be cause this disease of *E. smithii* (Maseko *et al*. 2001; 2007).

Breeding and selection for disease tolerant planting stock is an important part of an integrated strategy to reduce losses caused by fungal diseases of non-native trees in plantations in South Africa (Wingfield & Roux 2000; Wingfield *et al*. 2004). It also is the most economical and practical method for the long-term management of *Phytophthora* root and collar rot. Based on field reports, the susceptibility of *E. smithii* to *Phytophthora* spp. is worst during the first year of establishment, but subsides considerably as the trees mature (Bayley & Snell 1997, Jarvel 1998). Some individual trees within *E. smithii* half-sib families planted on sites with a history of *Phytophthora* root and collar rot survive despite exposure to *Phytophthora* inoculum. This suggests that there is some level of genetically controlled disease tolerance amongst the half- sib families. Tolerance to *P. cinnamomi* has been reported for some forest species such as *Pinus radiata, E. marginata* and *E. regnans* in Australia (Butcher *et al*. 1984; Harris *et al*. 1985; Stukely & Crane 1994). In contrast, there is limited information available regarding the susceptibility of plantation trees to *P. cinnamomi* in South Africa (Wolfaardt *et al*. 1997; Maseko 1999).

Genetic improvement programs for *E. smithii* and development of rapid and reliable disease screening methods against root pathogens are two strategies that can be used to achieve higher yields in intensively managed forest plantations. The stem inoculation method has been used successfully for selecting dieback resistant *E. marginata* and *P. radiata* seedlings in Australia (Butcher *et al*. 1984; Stukely & Crane 1994). This method is relatively simple and cost effective to screen large number of genotypes. In South Africa this method was successfully used to screen *E. fraxinoides* and *Pinus*

hybrids under field conditions (Wolfaardt *et al.* 1997; Roux *et al.* 2007). In this study, large numbers of half-sib seedlings of *E. smithii* families were screened for susceptibility to *P. cinnamomi* and *P. nicotianae*. This was achieved by using stem inoculation technique. Results were further correlated with the total leaf phenolic content of the inoculated families. The results obtained were then compared with the available field data relating to susceptibility of *E. smithii* to root rot.

2 Materials and Methods

2.1 Plant material

Twelve half-sib *E. smithii* families were used in this study and these represented a portion of the progeny selected from a collection of 50 open-pollinated parent trees that had been established in a provenance trial. Three trial sites were established on stands with a history of *Phytophthora* root rot. These trial sites were located at Gourock (30^{o} 08´S, 27^{o} 30´E), Palerang (30^{o} 40´S, 29^{o} 24´E) and Mullon (29^{o} 47´S, 29^{o} 54´E) in Kwa-Zulu-Natal Province.

Twelve half-sib *E. smithii* families were selected to represent the six most tolerant and susceptible families based on their performance in the provenance trials. Seeds were sown in seedling trays (340 × 340 mm; consisting of 49 cavities with 80 ml vol) containing composted bark medium. Seedlings were re-potted into 2 L black plastic pots containing a river sand potting mix. Seedlings were grown in a shade house, watered daily and fertilised weekly. During this period, the seedlings were pruned periodically to retain a single stems of 5-10 mm in diameter. A month prior to the commencement of the inoculation experiment, seedlings were moved to a greenhouse with an ambient temperature (25 ± 5 °C) in order to acclimatize them to these conditions.

2.2 Inoculum preparation

Two of the single most aggressive isolates of *P. cinnamomi* (CMW19408, isolated from *E. fraxinoides*) and *P. nicotianae* (CMW19444, isolated from *E. smithii*) were selected based on previous results from an inoculation trial on 1-year-old *E. smithii* (Maseko unpublished data). The inoculum for the trial was prepared by cutting mycelium-covered plugs from the edges of an actively growing colony and transferring them to Petri dishes (90 mm) containing half strength Potato Dextrose Agar (Biolab Agar; 20 gl^{-1} Biolab, Johannesburg). Plates were sealed with cling plastic wrap and incubated for 5 days in the dark at 25 °C.

2.3 Inoculation procedure

Inoculations were first conducted in October 2001 and these were repeated in September 2002. Individual trees representing the twelve half-sib *E. smithii* families were inoculated using a 4 mm

(diam) cork borer to remove a piece of bark more or less at the midpoint of the stems. A 4 mm^2 agar disc colonised by one of the two *Phytophthora* species was inserted into the wound and sealed with Parafilm (M, Pechiney Plastic Packaging) to restrict desiccation. Control seedlings were inoculated with sterile agar disks.

In total, 1920 half-sib seedlings were used for the experiment and there were 40 replicates per plant family. Due to uneven growth of seedlings the stem diameters and heights of the inoculated seedlings were measured. Each family was then divided into two random blocks consisting of 20 thicker (10 mm, diam) and taller (90–110 cm) and 20 thinner (8 mm, diam) and shorter (65–80 cm) stems. Each block included two control plants for each half-sib family. All of the *E. smithii* families were arranged using a completely randomised design in a greenhouse. After 3 weeks, the bark around the points of inoculation was carefully scraped with a scalpel blade to expose the wound. Lesion lengths were measured above and below the inoculation points on the surface of the stems. Small pieces of the host tissue cut from the margins of lesions were plated onto selective medium to re-isolate the pathogens used for the inoculations. The identity of the recovered *P. cinnamomi* and *P. nicotianae* isolates was confirmed using light microscopy.

2.4 Statistical analysis

Analysis of variance of resulting mean lesion lengths measured after 3 weeks was computed and compared between sources of variance (family genotypes and pathogen) and co variance (height and diameter) using the generalized linear model (GLM) procedure and ANOVA. Data sets were checked for normal distribution and equal variance and transformed using log transformation. Results of the September and October trials were analysed separately. Analyses of all data were performed using the SAS/STAT® Software, Version 8.02 (SAS Institute, Inc., Cary, North Carolina).

2.5 Extraction and determination of total phenolics

Young and fully expanded leaves (3-5 per seedling) were excised from 10 randomly selected seedlings representing the 12 half-sib families used in the inoculation trials. These thus represented six families known to be susceptible to *P. cinnamomi* and six considered more resistant to the pathogen. Pooled leaf samples from each genotype were washed; air-dried and sealed in plastic bags, stored at -70°C and lyophilized in a freeze dryer 48 hours before use. Leaves were crushed into a fine powder using a mortar and pestle and passed through a fine steel sieve (20 mesh screen). The homogenised fine leaf powder was weighed and 0.05g was transferred into 1.5 ml Eppendorf tubes. The experiment was done in triplicate for each of the plants representing the 12 half-sib families.

Total phenolics were extracted using a modification of the method described by Cork & Krockenberger (1991). Phenolics were first extracted by adding 1 ml methanol-acetone-water (7:7:1

v/v) solvent solution to each tube. The mixture was incubated overnight on a rotary shaker (set at 200 rpm, 25 °C). The tubes were then centrifuged at 10 000 rpm for 1 min and the supernatant was collected and transferred into new 1.5 ml Eppendorf tubes. The pellets were sequentially extracted three times using the same volume of methanol-acetone-water (7:7:1 v/v) followed by a 30 min incubation on a rotary shaker (200 rpm, 25 °C). The resulting supernatants were mixed and concentrated to 1 ml under vacuum. Deionized water was added to the supernatant and the tubes were centrifuged at 10 000 rpm for 1 min. This step was repeated three times to remove chlorophyll and the resulting upper phase supernatant was adjusted to 1 ml for further analysis using High Performance Liquid Chromatography (HPLC) and the results were analyzed statistically.

In total, 20 µl of plant extract from each of the ten plants representing the 12 half-sib families was injected into the HPLC. The HPLC gradient elution was performed using a linear gradient of 10% acetonitrile in water for 1 minute. The concentration of acetonitrile was increased linearly to 55% acetonitrile over 15 minutes and the flow rate of 2 ml/min maintained. The chromatographic apparatus consisted of three phase high-pressure solvent delivery pumps (Varian, Model 9012). Ferrulic (FA) and p caumaric (PCA) acids were also used for comparative purposes.

3 Results

3.1 Greenhouse trials

E. smithii plants inoculated with *P. cinnamomi* and *P. nicotianae* developed necrotic lesions within three weeks and control plants showed a wound response only (Fig 1). There were significant differences in the response of the plants representing the 12 half-sib *E. smithii* families to the different *Phytophthora* spp. Lesions produced by *P. cinnamomi* were significantly longer ($P < 0.001$) and developed more rapidly than those inoculated with *P. nicotianae* in both trials. When these results were presented graphically, the mean lesion lengths for each family showed a range of disease tolerance to both pathogens (Fig 2). Three half-sib families (SN21, SN30 and SN37) consistently developed longer lesions (> 25 mm) when inoculated with both *P. cinnamomi* and *P. nicotianae* and were considered susceptible. Families (SN17, SN45 and SN47) produced shortest mean lesion lengths (8 – 11 mm) and were considered disease tolerant.

Analysis of variance showed significant main effects of half-sib families ($P < 0.001$), the pathogens ($P < 0001$) and the interaction ($P < 0.001$) between these factors (Table 1). The data revealed significant effects ($P < 0.05$) relating to stem diameter and height as covariate factors. In both trials, significant differences in lesion length were found among individual seedlings and between the half-sib families following inoculation with *P. cinnamomi* and *P. nicotianae*. The mean lesion lengths induced by isolates of *P. cinnamomi* ($r^2 = 0.9983$, $P < 0.0001$) and *P. nicotianae* ($r^2 = 0.9965$, $P < 0.0001$) were significant and showed a strong positive correlation for lesions length for both trials (Fig 3).

3.2 Comparison between field survival and green house inoculations data

Survival of *E. smithii* families planted on *Phytophthora*-infested sites ranged between 41 % and 86 % in three trial sites (Jones unpublished data). Based on field survival data, six half-sib families (SN8, SN21, SN24, SN26, SN30 and SN37), were considered susceptible while six half-sib families (SN2, SN6, SN14, SN17 SN45 and SN47) were considered tolerant (Table 2). These field survival results were used to distinguish between susceptible and tolerant families. The results of the greenhouse inoculation trials indicated that only three of 12 families (SN17, SN45 and SN47) had a significantly higher level of tolerance to *P. cinnamomi* and *P. nicotianae*. Half-sib families SN21, SN30 and SN37 that were highly susceptible in the field were also highly susceptible in the greenhouse inoculation tests.

3.3 Total phenolic content

The HPLC chromatograms of crude leaf extracts for plants representing the 12 half-sib *E. smithii* families had similar patterns with one major peak and five minor peaks. Results showed that the mean phenolic content in the leaves for each of the twelve half-sib *E. smithii* families varied significantly ($P < 0.05$) (Fig 4). The highest, medium and lowest mean leaf tannin content for the 12 half-sib *E. smithii* families ranged between 345 – 360 mg^{-g} dry wt, 200 – 298 mg^{-g} dry wt and 148 – 180 mg^{-g} dry wt, respectively. The results show that the three families, SN17, SN45 and SN47, which had low percentage field mortality and produced significantly shorter lesion in the greenhouse trial had higher leaf phenolic concentrations.

4 Discussion

Results of this study showed that it was possible to use rapid screening techniques to obtain a statistically reliable estimate of susceptibility and tolerance of *E. smithii* breeding stock to *P. cinnamomi* and *P. nicotianae*. The two techniques employed are relatively simple and cost effective and could be used to screen large numbers of genotypes relatively rapidly. Three tolerant and three susceptible *E. smithii* half-sib families were identified using the stem inoculation technique. In addition, we found that field tolerant genotypes had higher concentrations of leaf phenolic compounds than susceptible genotypes. Results obtained using the two techniques compared well and reflected field performance relating to *Phytophthora* root rot.

The *Phytophthora cinnamomi* isolates used in this study consistently produced significantly larger lesions than those of *P. nicotianae* in both trials. Mean lesion lengths that developed on the different host genotype covered showed a range of disease tolerance and susceptibility to both pathogens, which

indicates genetic variation among the half-sib families. This genetic variation to *P. cinnamomi* is consistent with results of similar studies on *P. radiata* and *E. marginata* (Butcher *et al*. 1984; Stukely & Crane, 1994). Our study further showed a strong positive correlation between lesion length for both pathogens in two different trials. However, the family ranking in the two trials was not always consistent in both trials this was probably due to low levels of within family genetic variation.

Results of this study, demonstrated that susceptibility of *E. smithii* to *P. cinnamomi* and *P. nicotianae* is transmitted consistently to the progeny of parent plants. In this regard, greenhouse screening using stem inoculation validated the field performance of these genotypes. Results of this study are consistent with those of similar studies conducted in Australia where stem inoculation provides a reliable method to screen a large number seedlings (Stukely *et al*. 2007). Furthermore they confirm that mean lesion length can be useful in assessing susceptibility amongst half-sib families.

Total leaf phenolic content varied considerably within each of the 12 half-sib families investigated in this study. Our results showed that the phenolic compounds are present in greater amounts in field tolerant than in susceptible genotypes of *E. smithii*. These results also support the fact that phenolics play a role in disease tolerance (Hahlbrock & Scheel 1989). A similar technique has been used to screen cacao genotypes for resistant to *P. palmivora* (Okey *et al*. 1997; Tahi *et al*. 2000) in the past and it could provide a general view of the likely susceptibility of *E. smithii* planting stock to infection by *P. cinnamomi*.

Breeding and selection for disease tolerant planting stock is part of an integrated disease management strategy to manage root and collar rot of cold tolerant eucalypts in South Africa. However, root and collar rot disease predominantly caused by *P. cinnamomi* and *P. nicotianae* remains the single most common disease problem amongst cold tolerant eucalypts, including *E. smithii*. The development of genetically tolerant planting material is the most sustainable long-term solution to minimize yield loss due to *Phytophthora* root and collar in nursery and plantations. This study provides promising results using two screening methods that could be useful in assessing the susceptibility and tolerance of a large number of half-sib *E. smithii* families to root and collar rot prior to large scale planting.

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Table 1. Summary of the analysis of variance of mean lesion length (mm) between and within the 12 half-sib families in response to inoculation with virulent isolates of *P. nicotianae* and *P. cinnamomi*

	Lesion length		
Effect	**DF**	***F* Value**	***Prob* > *F***
Family	11	24.64	< 0.001
Pathogen	1	55.47	< 0.001
Family* Pathogen	11	3.54	< 0.001
Height	1	0.55	< 0.04
Diameter	1	5.76	< 0.05

Table 2. Summary of field survival and total phenolic leaf content of the 12 half-sib *E. smithii* families investigated in this study

Family	Survival %	Field ranking	Glasshouse ranking	Phenolic ranking
SN30	41 %	Susceptible	Moderately Tolerant	high
SN37	46 %	Susceptible	Susceptible	low
SN21	47 %	Susceptible	Susceptible	low
SN24	47 %	Susceptible	Susceptible	low
SN26	47 %	Susceptible	Moderately Tolerant	low
SN8	48 %	Susceptible	Moderately Tolerant	medium
SN14	80 %	Tolerant	Moderately Tolerant	medium
SN17	81 %	Tolerant	Highly Tolerant	high
SN47	81 %	Tolerant	Resistant	high
SN45	84 %	Tolerant	Highly Tolerant	high
SN2	85 %	Tolerant	Moderately Tolerant	medium
SN6	86 %	Tolerant	Moderately Tolerant	medium

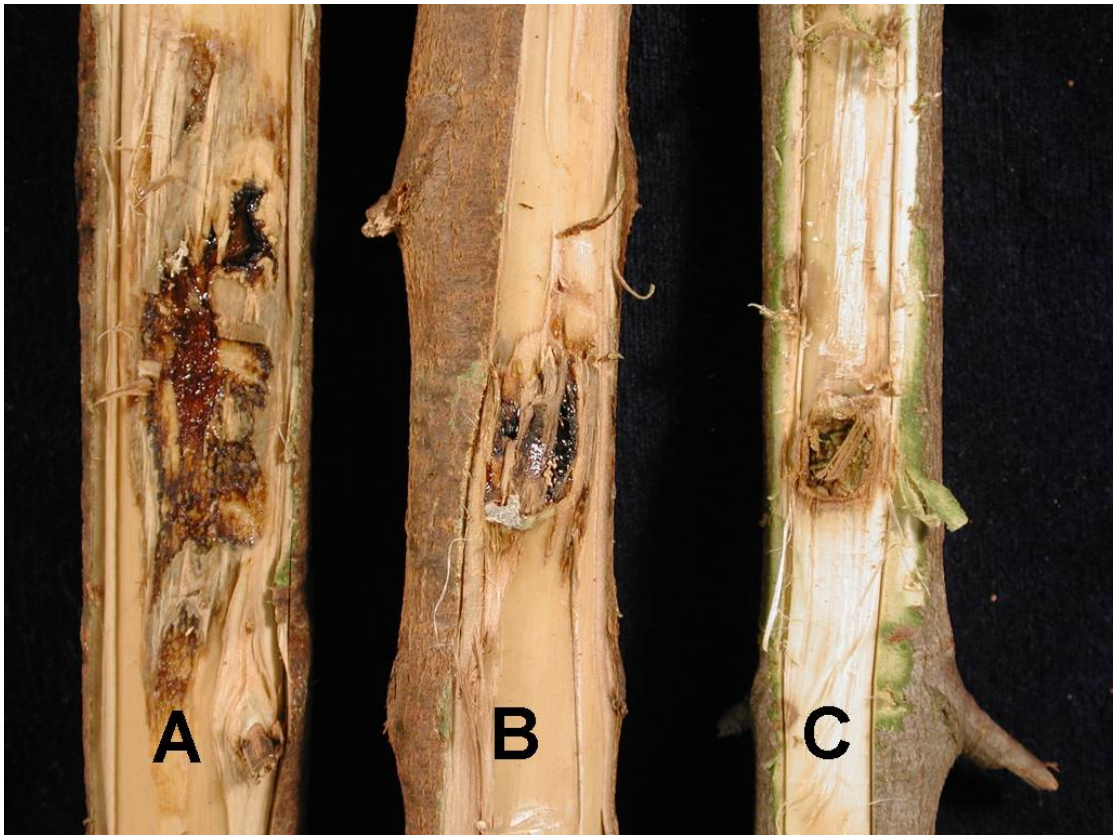

Fig. 1 Lesions produced on *E. smithii* three weeks following stem inoculation with virulent strains of *P. cinnamomi* and *P. nicotianae*. **(A)** lesion caused by *P. cinnamomi* lesion, (B**)** = lesion caused by *P. nicotianae*, **(C)** = Control.

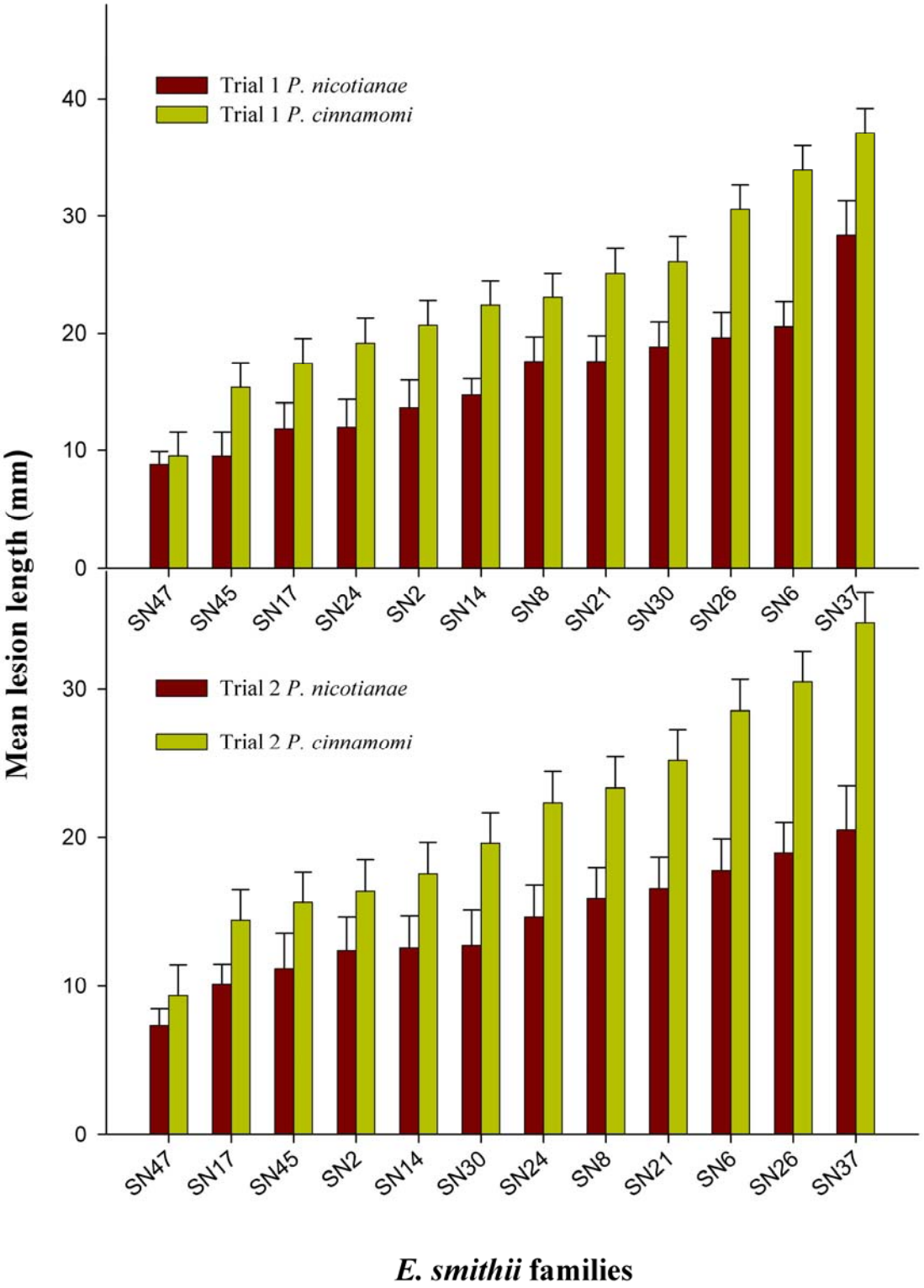

Fig. 2 Mean lesion lengths (mm) in stems of 12 half-sib *E. smithii* families measured three weeks after inoculation with virulent isolates of *P. cinnamomi* and *P. nicotianae* in two trials. Bars indicate standard errors

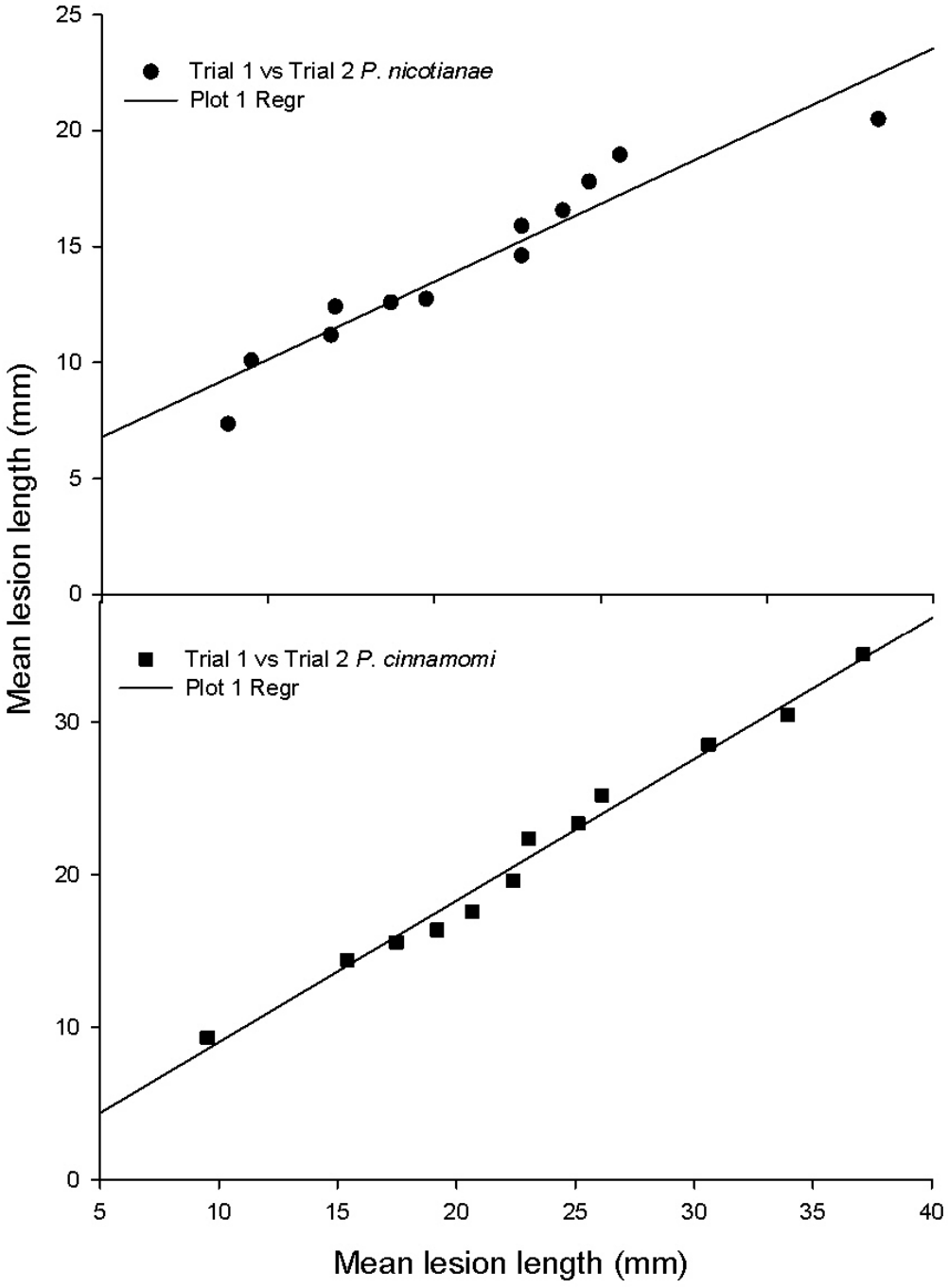

Fig. 3 Scatter plot illustrating the positive correlation between measurement of lesions lengths produced by virulent isolate of *P. cinnamomi* and *P. nicotianae* in two trials.

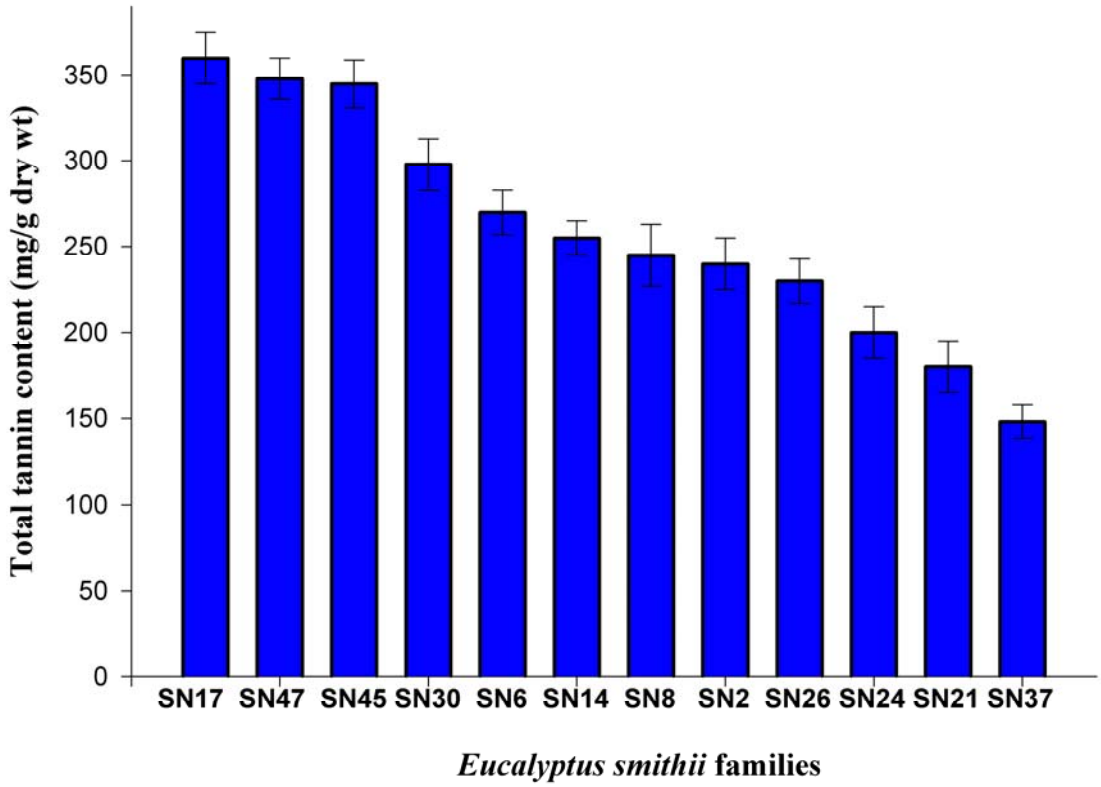

Fig. 4 Mean total phenolic leaf content expressed as mg tannic ac./g DW of 12 half-sib *E. smithii* families. Error bars represent ± standard error

Chapter Five

Two new *Phytophthora* species from South African *Eucalyptus* plantations

This chapter published as follows:

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ABSTRACT

A recent study to determine the cause of collar and root rot disease outbreaks of cold-tolerant *Eucalyptus* species in South Africa resulted in the isolation of two putative new *Phytophthora* species. Based on phylogenetic comparisons using the ITS and β-tubulin gene regions, these species were shown to be distinct from known species. These differences were also supported by robust morphological characteristics. The names, *Phytophthora frigida* sp. nov. and *Phytophthora alticola* sp. nov. are thus provided for these taxa, which are phylogenetically closely related to species within the ITS clade 2 (*P. citricola*, *P. tropicali* and *P.multivesiculata*) and 4 (*P. arecae* and *P. megakarya*), respectively. *Phytophthora frigida* is heterothallic, and produces stellate to rosaceous growth patterns on growth medium, corraloid hyphae, sporangia with a variety of distorted shapes and has the ability to grow at low temperatures. *Phytophthora alticola* is homothallic and has a slower growth rate in culture. Both *P. frigida and P. alticola* are pathogenic to *Eucalyptus dunnii*. In pathogenicity tests, they were, however, less pathogenic than *P. cinnamomi*, which is a well-known pathogen of *Eucalyptus* in South Africa.

Keywords: Oomycetes, phylogeny, taxonomy, root and collar rot

1 Introduction

Cold-tolerant *Eucalyptus* spp. are grown extensively for pulpwood production in summer rainfall areas of South Africa with an altitude above 1150m (Swain & Gardner 2003). During the mid-1980s, an increased demand for pulpwood led to the expansion of cold-tolerant *Eucalyptus* plantations (Darrow 1996). This period also marked the beginning of breeding programmes for cold-tolerant *Eucalyptus* spp. and the introduction of several alternative *Eucalyptus* spp. from seeds collected in natural stands in Australia (Darrow 1994; Swain & Gardner 2003). Several cold-tolerant species with high commercial potential have since been reported (Clarke *et al*. 1999; Little & Gardner 2003). However, some species such as *E. fastigata* and *E. fraxinoides* are well known for their susceptibility to infection by *Phytophthora cinnamomi* (Wingfield & Kemp 1994), which is considered to be an introduced pathogen in South Africa (Linde *et al*. 1999).

Phytophthora collar and root rot is a widespread disease affecting a number of cold-tolerant *Eucalyptus* spp. in South Africa (Linde *et al*. 1994a,b). This disease hampers progress towards introducing alternative *Eucalyptus* species yielding high pulp volumes. The most common disease symptom is progressive wilting of the leaves due to the girdling of the root collars. When the bark is removed, brown lesions extending from the roots are typically observed. Other disease symptoms include root disease, bleeding lesions from diseased stem tissue, and the formation of epicormic shoots on the stems of dying trees. Dying trees are usually present in small patches throughout the plantations, especially in areas prone to water-logging during the rainy seasons.

Phytophthora spp. known to be associated with collar and root rot of *Eucalyptus* spp. in South Africa include *P. boehmeriae*, *P. cinnamomi*, and *P. nicotianae* (Linde *et al*. 1994b). In 2001, *P. nicotianae* caused disease outbreaks on several cold-tolerant *Eucalyptus* spp. in South Africa (Maseko *et al*. 2001). This was particularly interesting as *P. cinnamomi*, rather than *P. nicotianae*, has typically been associated with mortality of cold tolerant *Eucalyptus* spp. (Linde *et al*. 1994b). During the same period, new and invasive *Phytophthora* spp. such as *P. ramorum* and *P. quercina* were emerging as important pathogens in Europe and North America (Jung *et al*. 1999; Werres *et al*. 2001; Rizzo *et al*. 2002). This prompted extensive surveys of cold-tolerant *Eucalyptus* stands to assess the presence of *P. nicotianae* and other possible invasive *Phytophthora* spp. that might be present on *Eucalyptus* spp. in South Africa.

Isolations of *Phytophthora* spp. during surveys of cold-tolerant *Eucalyptus* spp. yielded two groups of isolates that could not be assigned to known species. The aim of this study was to characterise these new *Phytophthora* spp. based on comparisons of DNA sequence data and morphology. Pathogenicity

tests were also conducted with isolates representing the two unknown species, as well as *P. cinnamomi*, which was included for comparative purposes.

2 Material and Methods

2.1 Sampling and isolation of isolates

Between 2000 and 2004, *Phytophthora* root rot was recorded in several plantations of cold-tolerant *Eucalyptus* spp. in KwaZulu-Natal Province (Swain *et al*. 2000). In particular, three areas severely affected by this disease were located in Sutton plantation near Ixopo (29° 58´S, 30° 08´E), Mid-Illovo (29° 53´S, 30° 24´E), and Paulpietersburg (27° 31´S, 30° 47´E) provenance/progeny trials. Four soil samples from the top 10 cm at the bases of dying trees were pooled in a single plastic bag. In addition, plant tissue was collected from infected root collars. Isolation from soil and diseased plant samples was performed within 48 h of collection. A total of 368 diseased trees and 240 soil samples were collected and assayed for the presence of *Phytophthora* spp.

Soil samples were flooded with distilled water and baited using citrus leaf discs (5 mm, diam) or *Eucalyptus sieberi* cotyledons as described by Grimm & Alexander (1973) and Marks & Kassaby (1974), respectively. After incubation at room temperature in the dark for 2–3 d, the leaf discs or cotyledons were plated on modified selective NARPH agar [Difco, Detroit, MI, corn meal agar (CMA), 17 g/l^{-1} amended with 50 µg/ml^{-1} nystatin, 200 µg ml^{-1} ampicillin, 10 µg ml^{-1} rifampicin, 25 µgml^{-1} pentacloronitrobenzene (PCNB), and 50 µg ml^{-1} hymexazol 3 hydroxy-5-methylisoxazole, Sigma-Aldrich, St. Louis] (Hüberli *et al*. 2000). Small pieces of diseased plant tissue were plated directly on NARPH. Petri dishes were incubated at room temperature in the dark and examined after 2–3 d using a compound microscope. Hyphal tips were cut from the edges of growing colonies and subcultured onto clarified V8 juice agar (V8A, Campbell's V8 juice 340 ml, 5 g CaCO3, 15 g agar and 900 ml distilled water) and CMA for further study and storage. Isolates could be divided into four groups based on colony morphology. These corresponded to *P. cinnamomi* or *P. nicotianae* and two unknown groups (unpublished data). Single zoospore cultures for isolates residing in each of these two groups, tentatively treated as *Phytophthora* sp. A, and *Phytophthora* sp. B, were made using the method described by Wang-Ching & Wen-Hsiung (1997). Ten isolates were randomly selected from each of the unknown *Phytophthora* spp. for more detailed study.

All but one of the isolates of *Phytophthora* sp. A were from dying *Eucalyptus smithii* in Sutton plantation after extensive sampling during 2000 and 2001 (unpubl. data). The only exception was an

isolate (CMW 19428) from *Acacia decurrens*, which was received by the diagnostic clinic of the Tree Protection Cooperative Programme (http://www.fabinet.up.ac.za/tpcp). Six isolates representing *Phytophthora* sp. B were from diseased *E. bajensis* (CMW19416–21) in provenance/progeny trials at Mid-Illovo and Paulpietersburg in the KwaZulu-Natal Province. Four additional isolates included in this study were from diseased *E. dunnii* (CMW19422–24) and *E. macarthurii* (CMW20393) samples submitted to the diagnostic clinic. Cultures of *P. arecae*, *P. colocasiae*, *P. multivesiculata*, and *P. nicotianae,* included in this study for comparative purposes, were obtained from the Centraalbureau voor Schimmelcultures (CBS; Table 1). In addition, isolates of several *Phytophthora* spp. found in South Africa were also included (Table 1). All isolates used are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) and representative isolates of the new taxa have been deposited with the Centraalbureau voor Schimmelcutlures (CBS), Utrecht, The Netherlands.

2.2 DNA isolation, PCR reactions, and sequencing

An agar block derived from a single zoospore culture for each of the 20 isolates representing the two unknown *Phytophthora* spp. was grown in 50 ml of 25 % clarified V8 broth at room temperature for 3–5 d. After harvesting, mycelium was freeze-dried and stored in Eppendorf tubes at room temperature. DNA was extracted using a phenol–chloroform DNA extraction method slightly modified from that described by Al-Samarrai & Schmid (2000). The ITS regions of the rDNA gene repeat for the unknown *Phytophthora* spp. was amplified using the forward ITS 6 (5′GAA GGT GAA GTC TAA CAA GG 3′) and reverse ITS 4 (5′ TCC TCC GCT TAT TGA TAT GC 3′) primers (Cooke & Duncan 1997). Amplification of the β-tubulin gene was done using the Oom-β-tub-up415 F (5′ CGC ATC AAC GTG TAC TAC AA 3′) and Oom-β tub1o1401 R (5′CGC TTG AAC ATCTCC TGG 3′) universal primers and PCR protocol of Bilodeau *et al*. (2007). The PCR reaction mixture (50 µl) contained DNA template (50–90 ng) 10mM Tris–HCl (pH 8.3), 1.5mM $MgCl_2$, 50mM KCl, 200 µM of each deoxynucleotide triphosphate, 150 nM of each primer and 1.25 U of Taq polymerase (Fermentas, UAB, Lithuania). The PCR conditions included an initial DNA template denaturation at 96 °C for 2 min, followed by 30 cycles of denaturation at 96 °C for 30 s, annealing at 55 °C for 30 s, extension 72 °C for 1 min, and final cycle at 72 °C for 10 min. A negative control consisting of all ingredients excluding template was also included. Amplicons were visualised on 1 % agarose gel stained with ethidium bromide and visualised under UV light. Resulting band size estimates were achieved using GeneRuler™ 100 bp DNA ladder (Fermentas). The PCR products were purified using a PCR products purification kit (Roche Molecular Biochemicals, Almeda, CA). They were then sequenced using the forward and reverse primers used in the amplification of the ITS and β-tubulin gene regions. Reactions were performed using the ABI PRISM™ Big dye terminator sequencing

reaction kit according to the manufacture's instructions (Perkin-Elmer Applied BioSystems, Foster City, CA). Sequencing was done using an ABI 3100™ automated DNA sequencer.

2.3 Phylogenetic analysis of the sequence data

In order to compare the new *Phytophthora* spp. from this study with other closely related species, additional sequences of representative species from Cooke *et al*. (2000) were obtained from GenBank. Phylogenetic analyses were done using MP methods in PAUP software version 4.0b10 (Swofford 2003) and Bayesian analysis (Ronquist & Heuelsenbeck 2003).

The initial analysis was performed on an ITS dataset alone and subsequent analyses were performed on a combined dataset of ITS and β-tubulin sequence, after a partition homogeneity test (PHT) had been performed in PAUP to determine whether sequence data from the two separate gene regions were statistically congruent (Farris *et al*. 1994; Huelsenbeck *et al*. 1996). The most parsimonious trees were obtained using heuristic searches with random stepwise addition in 100 replicates, with the tree bisection-reconnection branch swapping option on and the steepest-descent option, off. Max-trees were unlimited, branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices) were determined (Hillis & Huelsenbeck 1992). Characters were unweighted and unordered, branch and branch node supports were determined using 1 K BS replicates (Felsenstein 1985).

Bayesian analysis was conducted on the same aligned combined dataset. First Mr Modeltest v2.2 (Nylander 2004) was used to determine the best nucleotide substitution model. Phylogenetic analyses were performed with MrBayes 3.1 applying a general time reversible (GTR) substitution model with a gamma (G) and proportion of invariable site (I) parameters to accommodate variable rates across sites. The MCMC analysis of four chains started from random tree topology and lasted 2 M generations. Trees were saved, each resulting in 1 K trees. Burn-in was set at 200 K generations after which the likelihood values were stationary, leaving 950 trees from which the consensus trees and PPs were calculated. PAUP 4.0b10 was used to construct the consensus tree and maximum PPs assigned to branches after 50 % majority rule consensus tree was constructed from the 950 sampled trees.

2.4 Morphological, cultural, and physiological characteristics

Starter cultures (five per taxon examined) were grown on V8A incubated at room temperature for 5 d. A 4 mm cork borer was used to cut agar discs from the colony edges and these were placed at the centre of clarified V8 juice agar (Erwin & Riberio 1996), carrot agar (CA) (Erwin & Riberio 1996), CMA, potato dextrose agar (Difco; 24 gl^{-1}), and malt extract agar (MEA; 20 gl^{-1} Biolab, Johannesburg). Two lines intersecting at right angles at the centre of a 90mm Petri dish were drawn on

the outside of Petri dishes as reference growth points for each isolate. Five isolates of each taxon were transferred to the different media in triplicate and incubated at temperatures ranging from 5–35 °C, at 5 °C intervals. The colony diameters were measured daily with electronic digital callipers until the colonies reached the edges of the Petri dishes.

Sporulation on solid media was induced by adding Petri's mineral salt solution to cultures growing on V8A or CMA (Ribeiro 1978). Sporangial production and release of zoospores into liquid media was achieved using a modified mycelial matt method (Chen & Zentmyer 1970). Agar blocks bearing mycelium were cut from the edges of colonies and transferred into 20 ml clarified V8 juice broth and incubated at room temperature for 4 d. Mycelial mats were harvested, rinsed twice with sterile distilled water and Petri's mineral salt solution was added. Sporangia were produced after 2–3 d incubation in the dark and zoospore release was achieved by chilling the Petri dishes at 10 °C for 30 min and then returning them to 25 °C.

Sexual structures of *Phytophthora* sp. B were induced by growing ten test isolates on 10 % V8 agar and MEA at room temperature for 10–15 d. Single zoospore isolates produced oospores independently without crossing and were thus considered homothallic. Isolates of *Phytophthora* sp. A did not produce oospores independently, and thus, were tested for their ability to cross using the method outlined in Erwin & Riberio (1996). Matings were performed by pairing all ten *Phytophthora* sp. A isolates with known A1 and A2 (CMW21989, CMW21993) strains of *P. nicotianae* on 65 mm Petri dishes containing CA. In order to determine which isolates produced the oogonia in compatible pairings, a sterile polycarbonate membrane (47mm diam, 0.2 µm pore size; Millipore) was used as described by Ko (1978).

A light microscope was used to examine the reproductive structures and to compare the morphology of isolates with the aid of the revised tabular key of Stamps *et al*. (1990). For detailed microscopic examinations and measurements, sporulating mycelium was mounted on glass slides in lactophenol. Measurements of 50 randomly selected sporangia, oogonia, antheridia, chlamydospores, and hyphae were made for a single representative isolate of each of the two unknown *Phytophthora* spp. In addition, 20 of the above-mentioned structures were measured for each of the remaining nine isolates of each species. The mean ranges and confidence limits for all taxonomically relevant structures were recorded and are presented as (min–) (0.95 lower conf limit– 0.95 upper conf limit) (–max). Photographs captured with a HRc Axiocam (Carl Zeiss, München) digital camera and complementary Axiovision® 3.1 software were used to measure all morphological characters.

Petri dishes containing CMA and amended with different concentrations of hymexazol were prepared to give final concentrations between 10 and 50 $\mu g ml^{-1}$ at 10 $\mu g ml^{-1}$ increments. A similar set of Petri dishes was amended with malachite green (125 $\mu g ml^{-1}$). Small (5mm diam) agar discs bearing

mycelium of each of the ten test isolates of each unknown species were placed on the surface of the amended agar and incubated at 20 °C in the dark for 5 d (Shepherd 1976; Kennedy & Duncan 1995). Three replicate Petri dishes for each of the ten isolates were used and the sensitivity of the isolates to the test compounds was expressed as percentage growth rate versus that of isolates on control Petri dishes that were free of the test compounds.

Ten isolates of each of the unknown *Phytophthora* spp. were tested for their ability to utilise nitrate as a sole nitrogen source. Agar discs (5mm diam) were placed on the surface of agar growth media containing L-asparagine (P3) and nitrate (P4) as described by Hohl (1975). After incubation for 5 d at 20 °C, the colony growth was measured and expressed as increase in colony diameter in millimetres per day (mm d^{-1}). Pigment production of the test isolates was assessed on casein hydrolysate tyrosine (CHT) agar (Shepherd 1976). Petri dishes were incubated at 20 °C the dark for 15–20 d. The resulting extent of pigmentation was compared with control cultures grown on casein hydrolysate agar (casein hydrolysate broth 29.33 gl^{-1}, Sigma-Aldrich, St. Louis, Biolab, JHB agar 15 gl^{-1}). Petri dishes were examined on a light box and scored as having no (0), slight (1), moderate (2) or abundant (3) pigment (Shepherd 1976).

2.5 Pathogenicity tests

Ten isolates of each of the unknown *Phytophthora* spp. and five isolates of *P. cinnamomi* were used to inoculate one-year-old *Eucalyptus dunnii* trees in the field (Table 1).The pathogenicity trial was located in a commercial stand of trees at Sutton plantation, near Ixopo in the KwaZulu-Natal Province, South Africa. *E. dunnii* trees were inoculated using a 9 mm diam cork borer to remove the bark from each tree at breast height in March and November 2002. An agar plug removed from a one-week old PDA culture of each of the test isolates, was inserted into the wound and sealed with masking tape to reduce desiccation. Controls were included by inoculating trees with sterile PDA plugs. Ten trees were inoculated for each of the 25 test isolates and five trees were used as controls. A completely randomised block design was used for the inoculations and the entire trial was repeated in November during the summer season. Lesion lengths on the inner bark of trees were measured six weeks after inoculation. Lesion lengths were compared and analysed using one way analysis of variance (ANOVA), and the inoculations tests were also compared with each other using ANOVA and the STATISTICA (version 6), data analysis software.

3 Results

3.1 DNA amplification and sequence data analysis

The PCR product of the ITS-rDNA regions yielded a single band of approximately 900 bp for all the undescribed *Phytophthora* isolates used in this study. The aligned dataset consisted of 885 characters of which 381 were parsimony informative. These data contained significant phylogenetic signal ($P<$ 0.01; gl = –0.627) to allow for meaningful analysis. Initial heuristic searches of unweighted characters in PAUP resulted in two most parsimonious trees of 900 steps (CI=0.627, RI=0.90). The two new *Phytophthora* species formed well-supported terminal clades (Fig 1, TreeBASE= SN3042). *Phytophthora* sp. A grouped together with an undescribed *Phytophthora* sp. Associated with Rosaceae hosts (GenBank AF408625) of Abad *et al*. (2001) (Fig 1). *Phytophthora* sp. B isolates grouped together with *P. arecae*, *Peronophythora litchii*, *Phytophthora megakarya*, and *Peronospora sparsa* (Fig 1). However, *Phytophthora* sp. B isolates were closest to an isolate of another undescribed species from the US oak forests namely, *Phytophthora* sp.MD92, GenBank DQ 313223).

PCR amplification of the β-tubulin regions yielded a single band (ca 900 bp) for *Phytophthora* isolates examined in this study. The aligned dataset for the combined ITS and β-tubulin sequences consisted of 1689 characters, of which 318 were parsimony informative and were included in analysis. The partition homogeneity test showed no significant difference (P = 0.91) between the data from the different gene regions (sum of lengths of original partition was 778, range for 1000 randomisations was 771–779).

Phytophthora sp. A and *Phytophthora* sp. B also formed highly supported terminal clades in the combined ITS and β –tubulin tree (not shown; Tree BASE = SN3042). However, they grouped in an unresolved clade, but remained within their respective sub-groups with closely related species as the ITS tree. Based on sequence data comparisons for the combined ITS-rDNA and β–tubulin gene regions, isolates of *Phytophthora* sp. A and *Phytophthora* sp. B from cold-tolerant *Eucalyptus* spp. represent undescribed taxa. Descriptions for these new species are given below.

4 Taxonomy

Phytophthora frigida Maseko, Coutinho & M.J Wingf., sp. nov (Figs 2–4).

MycoBank no.: MB511178

Etym.: ‘frigida’ refers to the fact that this species is cold tolerant.

Phytophthora frigida sp. nov. crescit stellater vel rosaceiter in mediis plurimis, et potest in frigidis crescere (ita nomen), heterothalla, sporangiis ovoideo-obpyriformibus papillatis. Inter Phytophthoris aeriis typica est, sporangiis caducis et chlamydosporis permultis.

Typus: **Republic of South Africa**: Natal Province: Sutton plantation, Ixopo, *Eucalyptus smithii*, Feb. 2001, B.O.Z Maseko, (PREM 59222 – **holotypus**. dried culture with asexual structures on CMA with a corresponding microscope slide; ex-type culture CMW20311; **paratypes** PREM, (59218, 59218, 59220, 59221), dried cultures with asexual structures on CMA with matching microscope slides; ex-paratype cultures CMW 19426 CMW 19428, CMW 19433, CMW 19435)

Primary hyphae coralloid, irregular, and sympodially branched, fairly uniform in width measuring up to 5 µm, (3.5–) 4–5 µm(mean 4.5 µm) (Fig 2A–B). Hyphal swellings globose and intercalary (Fig 2C–D). Chlamydospores terminal, globose,n(20–) 24–26 (–35) diam, (mean 25 µm), thin or thick-walled and brown (Figs 2E–F, 3D–F). Sporangiophores thin branches, arising near or directly from hyphal swellings. Sporangia terminal or sometimes intercalary, readily produced in solid or liquid media, conspicuously papillate, exit pore (3–) 5–6 (7–) mm, (mean 5 µm), ovoid, obpyriform or irregular shaped, (*l* × *b*) (24–) 31–34 (–40) × (20–) 26–28 (–33) (mean 33 × 27 µm), caducous with short pedicels (Fig 4A–L). Oogonia produced only in dual cultures, terminal, spherical with smooth walls, often thicker and golden brown with age and mostly (25–) 31–34 (42) (mean 33 µm) diam (Fig 3A–C). Oospores aplerotic (19–) 26–30 (–38) µm diam (mean 28 µm), wall 1.5–2 (–3) µm thick, often light yellow or colourless. Antheridia amphigynous (95 %), elongated, cylindrical or spherical to ellipsoidal (Fig 3B).

Cultural characterisitics: Phytophthora frigida produces stellate to rosaceous colony types (5 d at 20 °C in darkness) on V8A, CA, MEA, CMA, and PDA (10 d at 20 °C in darkness). Cottony colonies with irregular growth patterns are produced on V8A, CA, MEA, and PDA. Submerged colonies with only sparse aerial mycelium were produced on CMA (Fig 5). Primary hyphae corraloid measuring 5 mm in width (3.5–) 4–5 µm (mean 4.5; Fig 2). Hyphal swellings intercalary and globose in solid and water media. Sporangiophores branched in sympodia arising near or directly from hyphal swellings. The cardinal temperatures for *P. frigida* isolates examined were 10 °C (mean 2mm d^{-1}) and 30 °C (mean 6mm d^{-1}). None of the *P. frigida* isolates grew at the lowest (5 °C) or highest (35 °C) temperatures. The mean growth rates on five test media at 20 °C for all ten *P. frigida* isolates are presented in Table 2. The growth temperature relationships of *P. frigida* on V8 and CA are illustrated in Fig 6. All *P. frigida* isolates examined in this study were able to utilise nitrate as sole nitrogen source. Isolates produced a black pigment on CHT agar within two to three weeks. None of *P. frigida* isolates grew on a medium containing malachite green. All isolates examined in this study were tolerant to hymexazol (Table 2).

Asexual structures: Sporangia readily produced in solid and liquid media, caduceus with short pedicels, terminal, and intercalary sporangia present. Sporangiophores irregular branched and with lax sympodia. Sporangia papillate often with various distorted shapes including bipapillate, elongated necks with three apices (observed in some isolates), conspicuous basal plugs, distinctly curved apices and lateral displacement of the papilla (Fig 4). Sporangia primarily ovoid-obpyriform, however, irregular shaped sporangia with variable shapes and sizes observed in liquid media. The size range of sporangia (*l* × *b*) (24–)31–34(–40) × (20–)26–28(–33) (mean 33 × 27 mm). The exit pores range between (3–)5–6(7–) mm (mean 5 µm).

Chlamydospores: Numerous thin-walled chlamydospores are produced in liquid and on solid media. Round thin-walled chlamydospores are produced terminally with diameters ranging between (20–)24–26(–35) mean 25 µm. Characteristic thick-walled chlamydospores, producing sporangia were observed on solid media. These thick-walled chlamydospores could easily be confused with oogonia (Fig 3D–F). However, they did not have antheridia. Occasionally, direct sporangial germination from thick-walled chlamydospores observed (Fig 3E–F). The *P. multivesiculata* isolates examined did not produce chlamydospores as readily as *P. frigida*, although some isolates produced chlamydospores after long storage.

Sexual structures: Oogonia were produced only through pairing of opposite mating isolates, suggesting that the species is heterothallic. Isolates produced terminal oogonia, with spherical and smooth walls with diameters ranging between (25–)31–34(–42) (mean 33 µm). Oospores had thick inner walls and were aplerotic with diameters ranging between (19–)26–30(–38) µm, with mean of 28 µm. Antheridia were elongated, cylindrical and amphigynous (95 %) and spherical to ellipsoidal in shape (Fig 3A–C).

There are many key features that distinguish *P. frigida* from *P. multivesiculata* that is most closely related to it. In terms of mating *behaviour*, *P. frigida* is heterothallic whereas *P. multivesiculata* is homothallic. *P. frigida* has papillate sporangia rather than the semi-papillate sporangia and *P. frigida* has corraloid hyphae rather than coiled hyphae and large spherical hyphal swellings rather than catenulate hyphal swellings found in *P. multivesiculata*.

Phytophthora alticola Maseko, Coutinho & M.J Wingf., sp nov. (Figs 8–9)
MycoBank no.: MB511177
Etym: Latin. The name refers to the fact that this fungus was first reported from high altitude sites. *Phytophthora alticola* sp. nov. crescit lente sine ordinatione proprio incrementi; phylogenetice P. arecae persimilis sed homothalla, sporangiis ovoideis papillatis vel bipapillatis, saepe forma distorta, differt.

Typus: **Republic of South Africa**: *Natal Province*: Mid-illovo provenance/ progeny trials, Richmond *Eucalyptus badjensis*, Mar. 2002, B.O.Z Maseko, (PREM 59215– holotypus; dried culture with asexual and sexual structures on CMA with a corresponding microscope slide; ex-type culture CMW 19417; paratypes PREM 59214, PREM 59216, PREM 59217), dried cultures with asexual structures on CMA with matching microscopes slides, exparatype cultures CMW 19416, CMW 19424, CMW 19425).

Primary hyphae (5–)4–6 µm (mean 5 µm) wide. Sporangia papillate, occasionally bipapillate, variable size and shape. Other sporangial shapes include ovoid, globose, obturbinate, limoniform and various distorted shapes. Terminal sporangia, caducous, short pedicel, conspicuous basal plugs. Sporangia (30–)33–36(45–) × (20–)26–29(35–) (mean 36 × 28 µm), length: breadth ratio range, 1:2 and 1:4 (mean 1.4). Exit pores (4–) 5–7 (8–) µm (mean 6 µm) diam. Chlamydospores, rarely produced, terminal and spherical, shape, between 20 and 35 µm (mean 28 µm; Fig 7). Oospores produced in single cultures, with thick inner walls, markedly aplerotic, diameters between 24–36 µm, with a mean of 28.3 × 30.5 µm. Antheridia mainly amphigynous, paragynous antheridia also present.

Cultural characteristics: *Phytophthora alticola* has smooth colonies with no distinctive growth pattern on V8A, CA, MEA, CMA, or PDA after 5 d incubation at 20 °C. Mycelium dome shaped and fluffy with scant to moderate aerial mycelium on V8A, CMA, and MEA. However, colonies tend to be appressed with thinly spread aerial mycelium on CMA (Fig 5). The optimum growth temperature on V8 agar for the ten *P. alticola* isolates examined was 25–30 °C. The cardinal temperatures for *P. alticola* isolates examined were 15 °C (mean 1mm d^{-1}) and 30 °C (mean 4mm d^{-1}). None of the isolates examined grew at low temperatures (below 10 °C) or at high temperature (above 30 °C). *P. alticola* isolates examined grew slowly on all growth media tested at 20 °C. The mean growth rates for the isolates are listed in Table 2 and the growth temperature relationship on V8A and CA in Fig 6. All of the isolates examined were able to utilise nitrate as sole nitrogen source and did not produce pigment on CHT agar. None of the isolates were able to grow on malachite green media. All *P. alticola* isolates were sensitive hymexazol (Table 2).

Primary hyphae in *P. alticola* were smooth, with irregular hyphal swellings, in liquid media (5–)4–6 µm (mean 5 µm) wide. Sympodially branched hyphae and irregular hyphal swellings were present. *P. arecae*, which is closely related to *P. alticola*, did not produce distinctive growth patterns but in that species, colonies were cottony, slightly radial and with abundant aerial mycelia on V8A, CA, MEA, PDA, and appressed colonies on CMA. *P. arecae* hyphae were smooth with no hyphal swelling and measured (3–)3.5–4.5 µm (mean 4 µm). There was a marked variation between isolates examined and the two species could not be readily distinguished from each other based on growth patterns in culture.

Asexual structures: All isolates of *P. alticola* examined produced sporangia on agar as well as in liquid media. Sporangia were conspicuously papillate and occasionally bipapillate (Fig 8), with variable sizes and shapes including ovoid, obpyriform, and various distorted shapes. Sporangia were terminal, caducous with short pedicels (Fig 8E). Sporangia were (30–)33–36 (–45) × (20–) 26–29 (–35) (mean 36 × 28 µm) in size and had a length:breadth ratio ranging between 1:2 and 1:4 (mean 1.4). The mean zoospores exit pore width was 6 mm. Spherical and terminal chlamydospores were produced in some isolates and their diameter range between (22–) 25–45 (mean 35 µm). The shapes of the sporangia of the closely related *P. arecae* vary from ovoid, obturbinate, elongated and ellipsoidal, and measured (35–) 40–44 (–60) × (25–) 28–30 (–35) (mean 42 × 30 mm) with mean length:breadth ratio (1:3–) 1.4 (–1.6). Round and terminal chlamydospores produced in older cultures and their diameter measuring (14–) 19–40 mean (30 µm) for *P. arecae*.

Sexual structures: All isolates of *P. alticola* examined in this study were homothallic. Oogonia formed readily formed in solid and liquid media. Oogonia were terminal with tapered stalks and were smooth-walled with diameters ranging between (24–) 26–28 (–31) (mean 26 µm). Oospores had thick inner walls with diameters ranging between (14–) 20–22 (mean 22 µm). Antheridia were predominantly amphigynous but paragynous antheridia were also observed in some isolates. Antheridia had a tendency to detach from the oogonia as illustrated in Fig 9. The obvious distinguishing feature between *P. alticola* and *P. arecae* is that the latter species is heterothallic.

4.1 Distribution and ecology

Phytophthora frigida was first isolated from diseased plant material and rhizosphere soil samples taken around declining *Eucalyptus smithii* trees at Sutton during the spring of 1999. Since then, *P. frigida* has been associated with root and collar rot disease of *E. dunnii*, *E. smithii*, *Acacia mearnsii* and *A. decurrens* in several forest plantations. Although, *P. frigida*, is well adapted for wind or splash dispersal, it has not been associated with shoot dieback of the above forest tree species. *P. frigida* is occasionally recovered from baited soil samples of cold-tolerant *Eucalyptus* species during routine disease monitoring. In past surveys conducted during 1999–2003 on several *E. smithii* stands, *P. frigida* was found to have a wide distribution in the Mpumalanga and KwaZulu-Natal provinces of South Africa. To date, *P. frigida* is predominantly associated with root and collar rot disease of *E. smithii* and is less prevalent on other forest tree species.

P. alticola was first recovered in 2004 from dying *E. bajensis* in a mixed provenance/progeny trial at Mid-illovo and Paulpietersburg in the KwaZulu-Natal province. Subsequently, it was isolated from soil and diseased *E. dunnii* samples, established in a previous provenance/progeny trial with a history of site dieback in Paulpietersburg. In 2005, *P. alticola* was isolated from a stem canker of dying *E.*

macarthurii in a plantation forest in a neighbouring country, Swaziland. The distribution of *P. alticola* is limited to provenance/progeny trials and single outlying plantations stands in the Mpumalanga and KwaZulu- Natal.

4.2 Pathogenicity tests

All isolates inoculated on one-year-old *Eucalyptus dunnii* trees in the field were pathogenic and were consistently re-isolated from the resulting lesions. Discoloured lesions extending from the point of inoculation were produced in all inoculated trees. *P. cinnamomi* isolates were more aggressive than either *P. frigida* or *P. alticola* isolates (Fig 10). The mean lesion leng produced by *P. cinnamomi* isolates was 12.7 cm compared with 7.7 and 3.8 cm produced by *P. frigida* and *P. alticola*, respectively. Control inoculations did not produce lesions. Mean lesion lengths for the different *Phytophthora* spp. compared were significant ($P < 001$) and different to each other and to those of the controls.

5 Discussion

Two previously unknown *Phytophthora* spp. consistently associated with collar and root disease outbreaks on non-native cold-tolerant eucalypts in South Africa were identified in this study. Phylogenetic analyses of the DNA sequence data for the ITS regions of rRNA and β-tubulin region showed that these two taxa are distinct from all known species of *Phytophthora.* A number of unique morphological characteristics in these two species also support this view and we have thus described them as *P. frigida* and *P. alticola*.

The ITS phylogeny produced in this study showed that *P. frigida* is related to species within the ITS clade 2 of Cooke *et al*. (2000), and that *P. multivesiculata* was one of the species most closely related to *P. frigida*. However, *P. frigida* shares 95 % homology with an undescribed *Phytophthora* sp., which was isolated from raspberry, rose, and strawberry in 2001. At the time of the current study this undescribed *Phytophthora* sp. was not available for morphological comparison. Both *P. frigida* and the undescribed *Phytophthora* sp. of Abad *et al*. (2001) belong to a separate sub-group within the ITS clade 2 of Cooke *et al*. (2000), but the significant genetic distance between the taxa provide good evidence that they are different species. The results of this study also show that the ITS clade 2may include a greater number of sub-groups than previously reported by Cooke et al. (2000).

The distinctive morphological features of *P. frigida*, which include papillate and caducous sporangia, indicate that it is adapted for wind or splash dispersal. *P. frigida* is homothallic in culture and thus likely to be an inbreeding species. *P. frigida* has predominantly been found on *E. smithii*, planted in areas with an altitude above 1150 m in South Africa. However, its host range could possibly include

Acacia decurrens, because a few isolates of *P. frigida* were recovered from soil collected from around diseased *A. decurrens* trees. The ability to grow at temperatures lower than 15 °C indicates adaptation to a cool temperate climate. Distinctive morphological characteristics include a stellate to petalloid growth pattern on all five media tested, and the ability to utilise L-asparagine better than nitrate as sole nitrogen source. Our observations for *P. multivesiculata*, the species most closely related to *P. frigida*, are generally consistent with those reported by Ilieva *et al*. (1998). However, these authors reported a maximum growth temperature for *P. multivesiculata*, which is higher than those emerging from the present study.

The ITS sequence data presented in this study have shown that *P. alticola* clusters with taxa in ITS clade 4 of Cooke *et al*. (2000). *P. arecae*, which is conspecific with *P. palmivora* (Mchau & Coffey 1994) and *Peronophythora litchii* (Riethmüller *et al*. 2002) are the species most closely related to *P. alticola*. A single undescribed species listed in GenBank as *Phytophthora* sp. MD 92 (GenBank DQ313223) and reported as coming from eastern US oak forests is phylogenetically closely related to *P. alticola* and could represent another host and location for this species.

P. alticola is a heterothallic species with ovoid-obpyriform conspicuously papillate sporangia. Consequently, it is in group II of the taxonomic scheme of Waterhouse (1963). In terms of DNA sequence data for the ITS region, it is phylogenetically placed in clade 4 of Cooke *et al*. (2000) and is related to *P. megakarya* and *P. arecae*. Superficially, *P. alticola* shares a number of morphological features with *P. arecae* and the two species could be confused. However, *P. alticola* isolates produce smooth, dome-shaped cultures with moderate aerial mycelium on V8A and MEA, with faint stellate growth patterns on CA, PDA and submerged colonies on CMA. In contrast, *P. arecae* produces smooth colonies with fluffy aerial mycelium with faint stellate growths on V8A, CA, MEA, PDA, and submerged, thin mycelial growth with no obvious patterns on CMA. The most obvious differences distinguishing *P. alticola* from *P. arecae* include significantly slower growth rates in culture, irregular rather than absent hyphal swellings, ovoid-obpyiform rather than ellipsoid to obturbanate sporangia; large terminal chlamydospores in the former and no chlamydospores in the latter and oogonia produced abundantly rather than rarely in the latter.

P. frigida and *P. alticola* were consistently isolated from diseased plant material and from rhizosphere soil associated with dying trees. Inoculation experiments conducted on one-year-old *E. dunnii* in the field confirmed that both species are pathogenic. We thus believe that they are agents of the dieback and early death of the affected cold-tolerant *Eucalyptus* spp. in South Africa. These two new species have thus far been recovered from *A. decurrens* and non-native cold-tolerant eucalypts planted in high altitude areas. This is in contrast with previous reports that only three *Phytophthora* spp. are associated with die-back of cold-tolerant eucalypts in South Africa (Linde *et al*. 1994c). However, *P. frigida* and

P. alticola were substantially less pathogenic than *P. cinnamomi*, and their relative importance as tree pathogens will need to be determined.

Although various studies on *Phytophthora* spp. have been conducted, there has never been a detailed survey of these pathogens in South Africa. As many *Phytophthora* species are a threat to agricultural crops, forest trees species and native vegetation, such surveys would be valuable and should be encouraged. The discovery of two new pathogenic *Phytophthora* spp. in this study provides a strong indication that other new species of *Phytophthora* await discovery in South Africa. Examples include the recently discovered, *P. captiosa* and *P. fallax* (Dick *et al*. 2006) from exotic *Eucalyptus* species in New Zealand.

Supplementary material

Supplementary (Fig_D06_00234) associated with this article can be found at doi: 10.1016/j.mycres.2007.08.011

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Table 1 – Species and isolates of *Phytophthora* species examined in this study

Isolate number	Species	[a]Group	[b]Clade	Host	Location	ITS	β-tubulin
CMW21922	*Phytopthhora cactorum*	I	1a	Apple rootstock	Stellenbosch, W. Cape	DQ988205	DQ988244
CMW19445	*P. cactorum*	I	1a	Apple rootstock	Stellenbosch, W. Cape	DQ988206	DQ988245
CMW19442	*P. nicotianae*	II	1b	*Citrus jambhiri*	Tzaneen, Limpopo	DQ988174	DQ988213
CMW19443	*P. nicotianae*	II	1b	*Acacia mearnsii*	Lions River, KZN	DQ988175	DQ988214
CMW19444	*P. nicotianae*	II	1b	*E. smithii*	Hodgsons, KwaZulu-Natal	DQ988176	DQ988215
CMW19441	*P. boehmeriae*	II	9 & 10	*E. smithii*	Ixopo, KwaZulu-Natal	DQ988207	DQ988246
CMW19440	*P. boehmeriae*	II	9 &10	*E. smithii*	Howick, KwaZulu-Natal	DQ988208	DQ988247
CMW19439	*P. boehmeriae*	II	9 &10	*E. smithii*	Ixopo, KwaZulu-Natal	DQ988209	DQ988248
CBS 305.62	*P. arecae*	II	4	*Areca catechu*	India	DQ988202	DQ988241
CMW19437	*P. arecae*	II	4	unknown	Stellenbosch, W. Cape	DQ988203	DQ988242
CMW19436	*P. arecae*	II	4	unknown	Stellenbosch, W. Cape	DQ988204	DQ988243
CMW19425	*P. alticola*	II	4	*E. dunnii*	PaulPetersburg, KZN	DQ988196	DQ988235
CMW19424	*P. alticola*	II	4	*E. macarthurii*	Midillovo, KwaZulu-Natal	DQ988197	DQ988236
CMW19423	*P. alticola*	II	4	*E. dunnii*	Paulpetersburg, KZN	DQ988198	DQ988237
CMW19422	*P. alticola*	II	4	*E. dunnii*	Paulpetersburg, KZN	DQ988199	DQ988238
CMW19421	*P. alticola*	II	4	*E. dunnii*	Paulpetersburg, KZN	DQ988200	DQ988239
CMW19420	*P. alticola*	II	4	*E. badjensis*	Midillovo, KwaZulu-Natal		
CMW19419	*P. alticola*	II	4	*E. badjensis*	Paulpetersburg, KZN	DQ988201	DQ988240
CMW19418	*P. alticola*	II	4	*E. badjensis*	Midillovo, KwaZulu-Natal		
CMW19417	*P. alticola*	II	4	*E. badjensis*	Midillovo, KwaZulu-Natal		
CMW19416	*P. alticola*	II	4	*E. badjensis*	PaulPetersburg, KZN		
CMW20206	*P. citrophthora*	II	2	*Citrus* rootstock	W. Cape	DQ988186	DQ988225
CMW20204	*P. citrophthora*	II	2	*Citrus* rootstock	W. Cape	DQ988187	DQ988226
CMW20198	*P. citrophthora*	II	2	*Citrus* rootstock	W. Cape	DQ988188	DQ988227
CMW19415	*P. citricola*	III	2	*Citrus limonia*	W. Cape	DQ988183	DQ988222
CMW19414	*P. citricola*	III	2	*Citrus limonia*	W. Cape	DQ988185	DQ988224
CMW19413	*P. citricola*	III	2	*Citrus limonia*	W. Cape	DQ988184	DQ988223

Table 1. Cont Species and isolates of *Phytophthora* species examined in this study

Isolate number	Species	[a]Group	[b]Clade	Host	Location	ITS	β-tubulin
CMW19435	*P. frigida*	IV	2	*E. smithii*	Ixopo, KwaZulu-Natal	DQ988177	DQ988216
CMW19434	*P. frigida*	IV	2	*E. smithii*	Pietermaritzburg, KZN	DQ988178	DQ988217
CMW19433	*P. frigida*	IV	2	*E. smithii*	Lions River, KZN	DQ988179	DQ988218
CMW19432	*P. frigida*	IV	2	*E. smithii*	Ixopo, KwaZulu-Natal	DQ988180	DQ988219
CMW19431	*P. frigida*	IV	2	*E. smithii*	Pietermaritzburg, KZN	DQ988181	DQ988220
CMW20311	*P. frigida* (ex-type)	IV	2	*E. smithii*	Ixopo, KwaZulu-Natal	DQ988182	DQ988221
CMW19429	*P. frigida*	IV	2	*E. smithii*	Ixopo, KwaZulu-Natal		
CMW19428	*P. frigida*	IV	2	*Acacia decurrens*	Seven Oaks, KZN		
CMW19427	*P. frigida*	IV	2	*E. smithii*	Bloemendal, KZN		
CMW19426	*P. frigida*	IV	2	*E. smithii*	Shafton, KwaZulu-Natal		
CBS545.96	*P .multivesiculata*	IV	2	*Cymbidium sp*	Netherlands	DQ988192	DQ988231
CBS 955.87	*P. colocasiae*	IV	2	*Colocasia esculenta*	India		
CMW22018	*P. colocasiae*	IV	2	*Protea sp*	W. Cape	DQ988191	DQ988230
CMW19410	*P. cryptogea*	VI	8a	*Vitis vinifera*	W. Cape	DQ988194	DQ988233
CMW19411	*P. cryptogea*	VI	8a	*Vitis vinifera*	W. Cape	DQ988193	DQ988232
CMW20393	*P. cryptogea*	VI	8a	*Vitis vinifera*	W. Cape	DQ988195	DQ988234
CMW19408	*P. cinnamomi*	VI	7a	*E. fraxinoides*	Hodgsons, KwaZulu-Natal	DQ988171	DQ988210
CMW19406	*P. cinnamomi*	VI	7a	*E. dunnii*	Ixopo, KwaZulu-Natal		
CMW19405	*P. cinnamomi*	VI	7a	*E. dunnii*	Piet Retief , Mpumalanga	DQ988172	DQ988211
CMW19404	*P. cinnamomi*	VI	7a	*E. smithii*	Hodgsons, KwaZulu-Natal	DQ988173	DQ988212
CMW19403	*P. cinnamomi*	VI	7a	*E. elata*	Piet Retief Mpumalanga		

[a] Groups according to Waterhouse (1963).[b] Clades according to Cooke *et al*. (2000); Kroon *et al*. (2004)

Table 2 – A comparison of morphological characteristics of *Phytophthora* isolates

Character	***Phytopthhora frigida sp.nov***	***Phytopthhora multivesiculata***	***Phytopthhora alticola sp.nov***	***Phytopthhora arecae***
No. of isolates	10	2	10	3
Main hyphae	Coralloid	Coiled	Smooth	Smooth
Hyphal swellings	Spherical	Catenulate	Irregular	Absent
Av. width (µm)	4.5	6	5	4
Sporangia	Papillate	Semi-papillate	Papillate	Papillate
Range (µm)	24–40 × 20–33	30–60 × 20–41	(30–45) × (20–35)	35–60 × 25–35
l × b ratio (mean)	<1.6	<1.6	<1.6	<1.6
Shapes observed	Ovoid-obpyriform	Ovoid, obpyriform	ovoid, ellipsoidal obturbinate	ellipsoidal to obturbinate
Distorted shapes	+	+		+
Caducity	Caducous	Caducous	Caducous	Caducous
Pedicel (µm)	Short (<5 µm)	Short (<5 µm)	Short (<5 µm)	Short (<5 µm)
Sporangiophores	Simple sympodium	Simple and twisted sympodium	Simple or branched sympodium	Simple or branched sympodium
Exit pore (µm)	Broad (5–10)	Broad (8–12)	Narrow(<7 µm)	Narrow(<7 µm)
Chlamydospores	Abundant	In some isolates only	In some isolates only	Absent
Mean diam (µm)	<35 µm		<35 µm	
Oogonia				
Mean diam (µm)	38 µm	41 µm	30	32
Range diam (µm)	24–48	28–50	20–35	23–44
Oospore				
Mean diam (µm)	33	40	28	30
Range (µm)	25–42	28–46	24–36	25–40
Antheridia	Amphigynous	Amphigynous	Amphigynous	Amphigynous
Sex	Heterothallic	Homothallic	Homothallic	Heterothallic

Table 2 Cont – A comparison of morphological characteristics of *Phytophthora* isolates

Growth media	Colony and Cultural characteristics			
	Phytopthhora frigida sp.nov	***Phytopthhora multivesiculata***	***Phytopthhora alticola sp.nov***	***Phytopthhora arecae***
V8A (a)	Stellate-petaloid	Smooth	Smooth	Smooth
(b)	Moderately fluffy	Cottony	Fluffy	Fluffy
CA (a)	Stellate-petaloid	Smooth	Smooth	Smooth
(b)	Moderately fluffy	Cottony	Fluffy	Fluffy
MEA (a)	Stellate-petaloid	Smooth	Smooth	Smooth
(b)	Moderately fluffy	Cottony	Fluffy	Fluffy
CMA (a)	Slight stellate	Smooth	Smooth	Smooth
(b)	Appressed	Appressed	Appressed	Appressed
PDA (a)	Stellate-petaloid	Smooth	Smooth	Smooth
(b)	Moderately fluffy	Cottony	Fluffy	Fluffy
	Mean growth rate mm d^{-1} at 20 °C			
V8A	5	5.8	3	6.2
CA	4.8	5	3.8	5.3
CMA	4.6	5.2	2.4	5.5
MEA	4	4.5	2	5
PDA	3	2.6	1.5	4.2
P3 (asparagines)	1.5	2.5	1	3.0
P4 (nitrate)	0.6	1.5	0.2	2.0
Sensitivity malachite green	No growth	No growth	No growth	No growth
Percentage growth rate on CMA amended with different concentration of hymexazol, isolate ranges in brackets				
10 µg ml^{-1}	92 (90–100)	95 (90–100)	80 (90–100)	85 (90–100)
50 µg ml^{-1}	70 (60–90)	75 (45–96)	55 (51–65)	60 (40–65)
CHT Agar	Abundant (3)	Moderate (2)	None (0)	slight (1)

V8A, V8 juice agar; CA, carrot agar; CMA, corn meal agar; MEA malt-extract agar; PDA, potato dextrose agar; P3, agar growth media containing L-asparagine (P3); P4, agar growth media containing nitrate (P4); CHT agar, casein hydrolysate tyrosine.

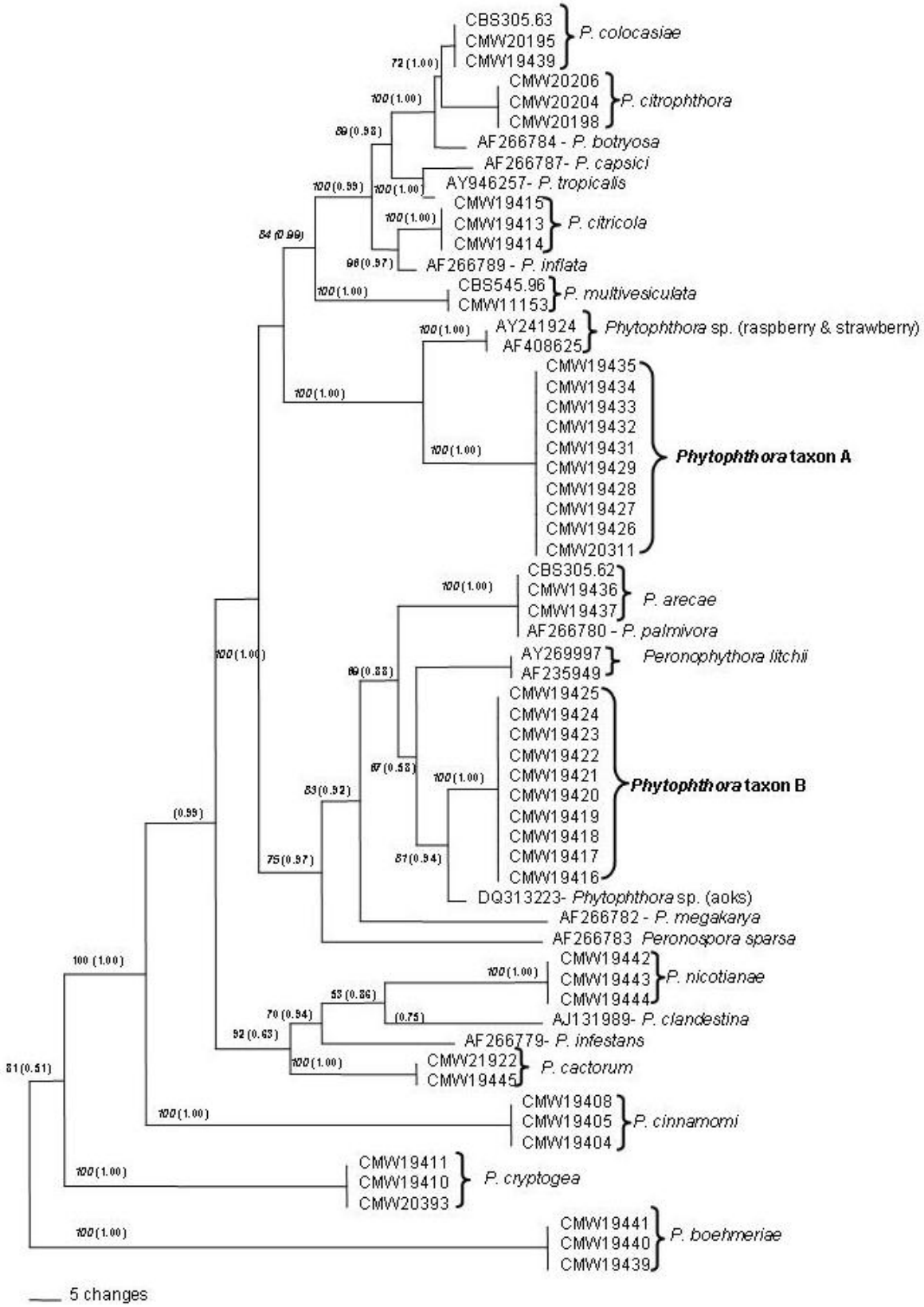

Fig 1 – One of two most parsimonious phylogenetic trees of 900 steps obtained from analysis of ITS sequence data. Branch support (BS value) is given above the branches and PP from the Bayesian analysis in brackets.

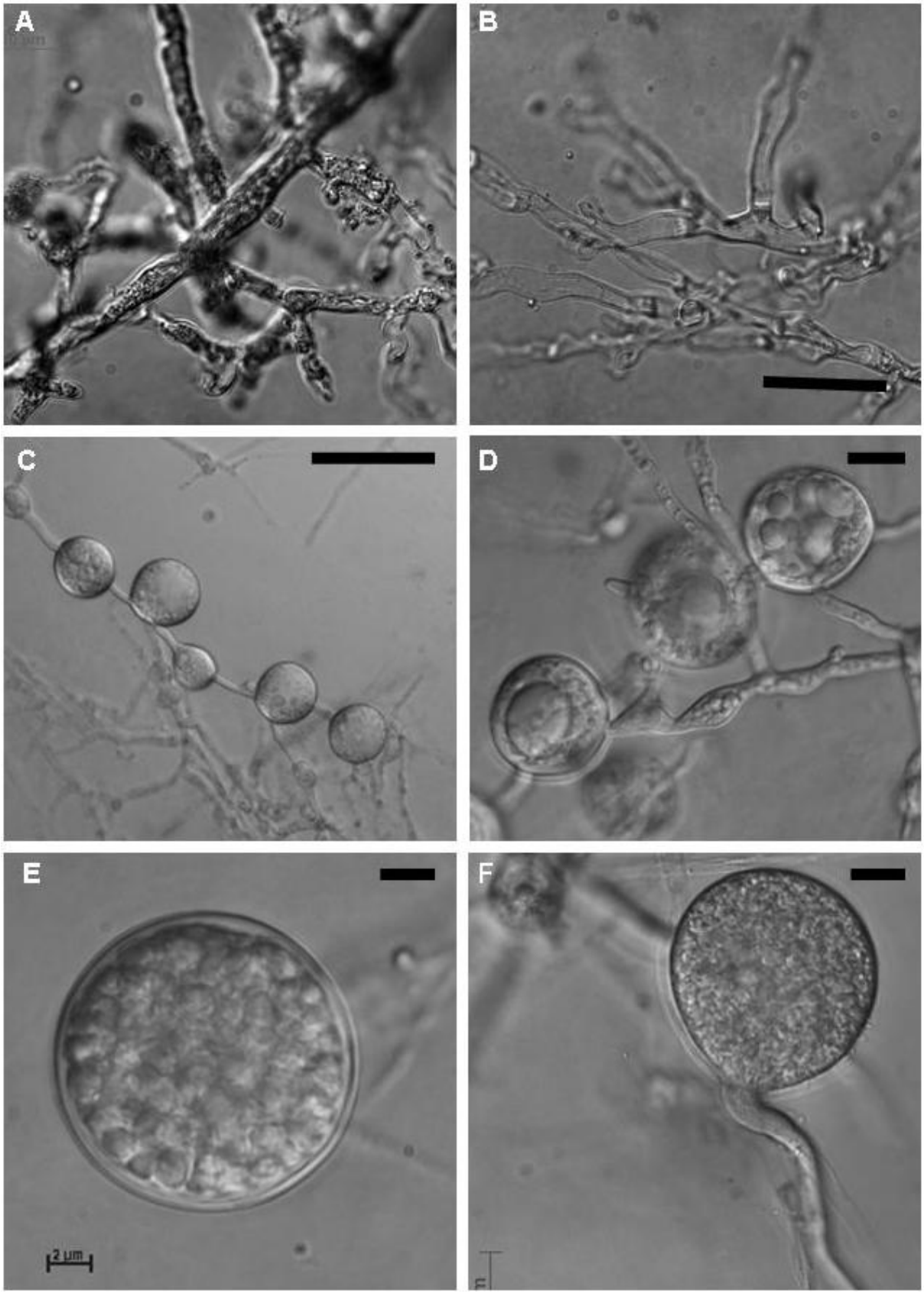

Fig 2 – Primary hyphae and chlamydospores of *Phytophthora frigida*: (A–B) Coralloid mycelia with sympodial branching on solid media. (C–D) Intercalary globose hyphal swellings. (E) Thin-walled chlamydospores. (F) Germinating sporangium. Bars =10 μm, except (E)= 2 μm.

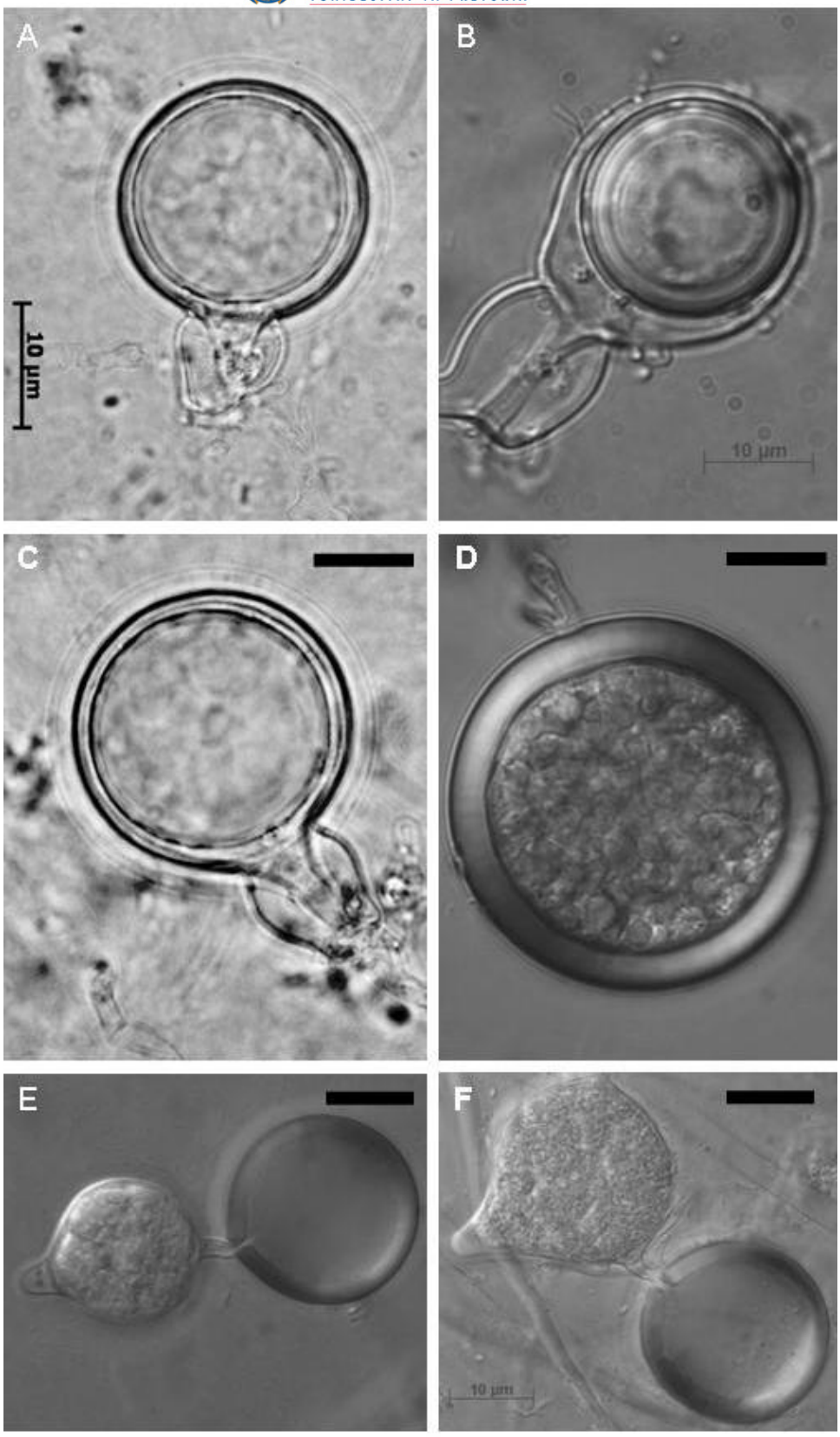

Fig 3 – (A–C) Oogonia and oospores of *Phytophthora frigida* showing amphigynous antheridial attachment. (C–D) Thick-walled chlamydospores produced abundantly on liquid MEA. (E–F) Sporangium release from chlamydospore (rarely) observed. Bars = 10 µm.

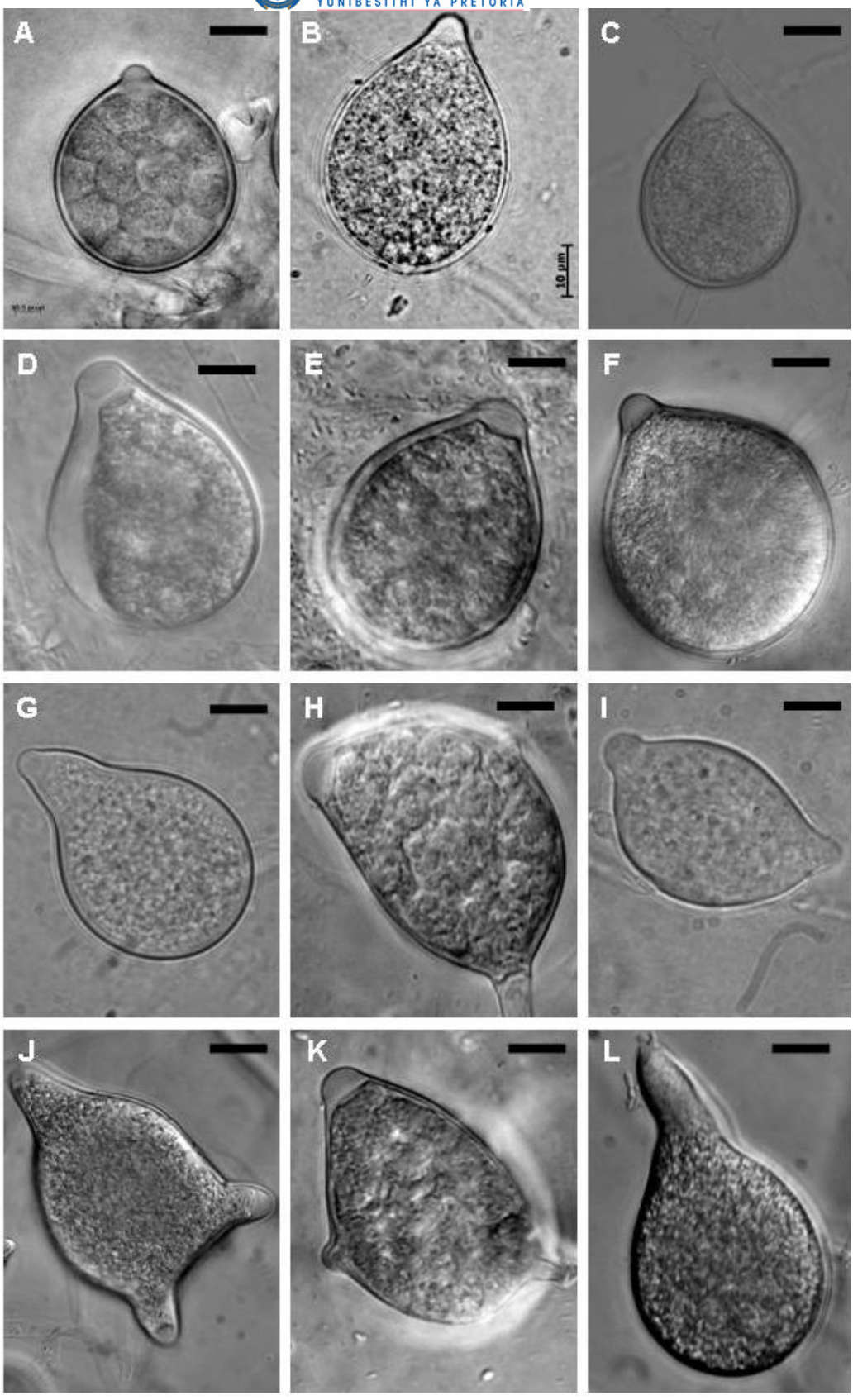

Fig 4 – Sporangia of *Phytophthora frigida*. (A–F) Papillate and semi-papillate, ovoid-obpyriform, lateral attached and caducous sporangia. (G–L) Various distorted shapes observed in liquid media. (H) Papillate sporangium with conspicuous basal plug. (J, L) sporangia with elongated necks sometimes with three apices. Bars =10 µm.

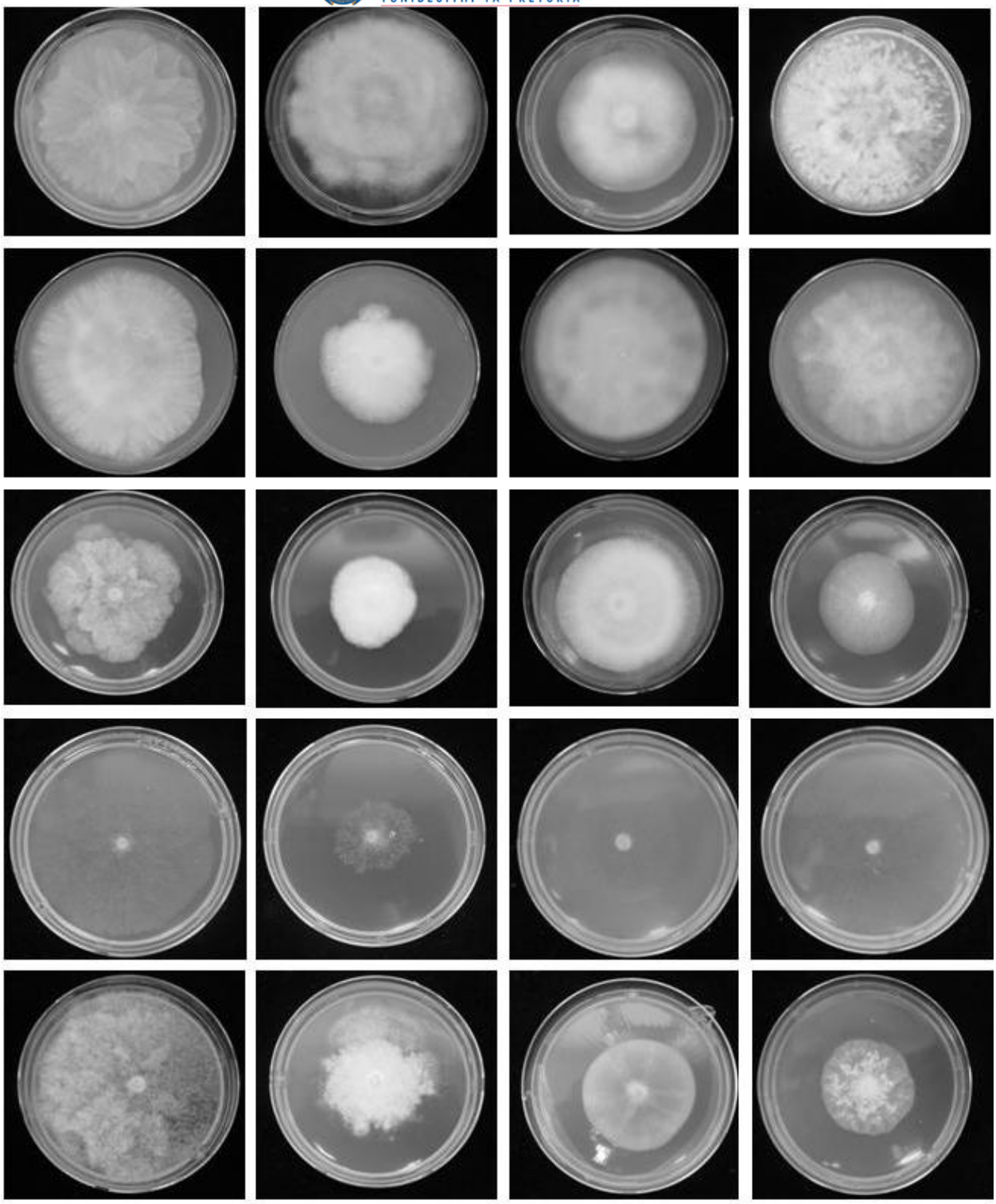

Fig 5 – Colony types of *Phytophthora* species grown on V8A, CA, MEA, CMA after 5 d at 20 °C and PDA after 10 d. *P. frigida* (column 1; left, top to bottom), *P. multivesiculata* (column 2), *P. alticola* (column 3), *P. arecae* (column 4).

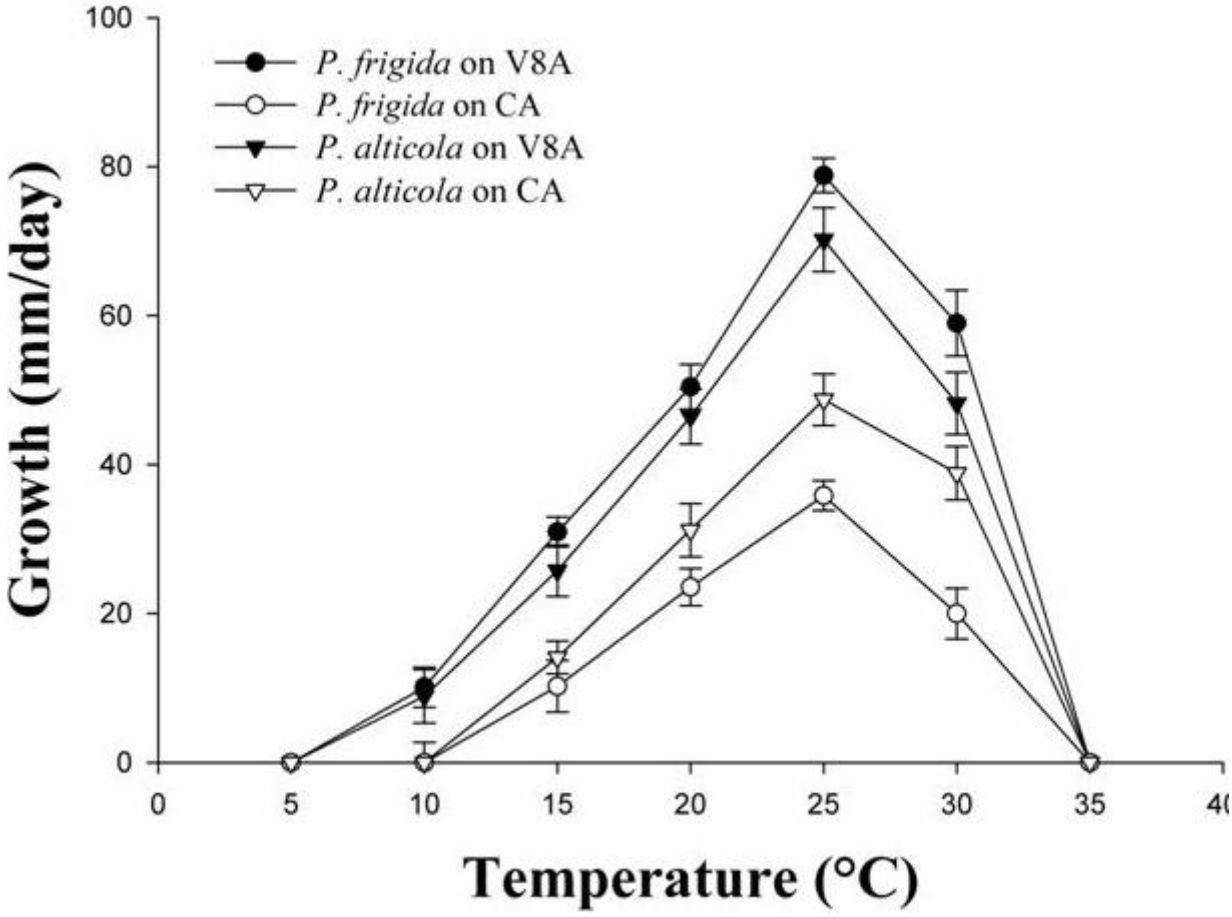

Fig 6 – Growth–temperature graph of *Phytophthora frigda* and *P. alticola* on V8A and CA at temperatures ranging from 5–35 °C for 10 d.

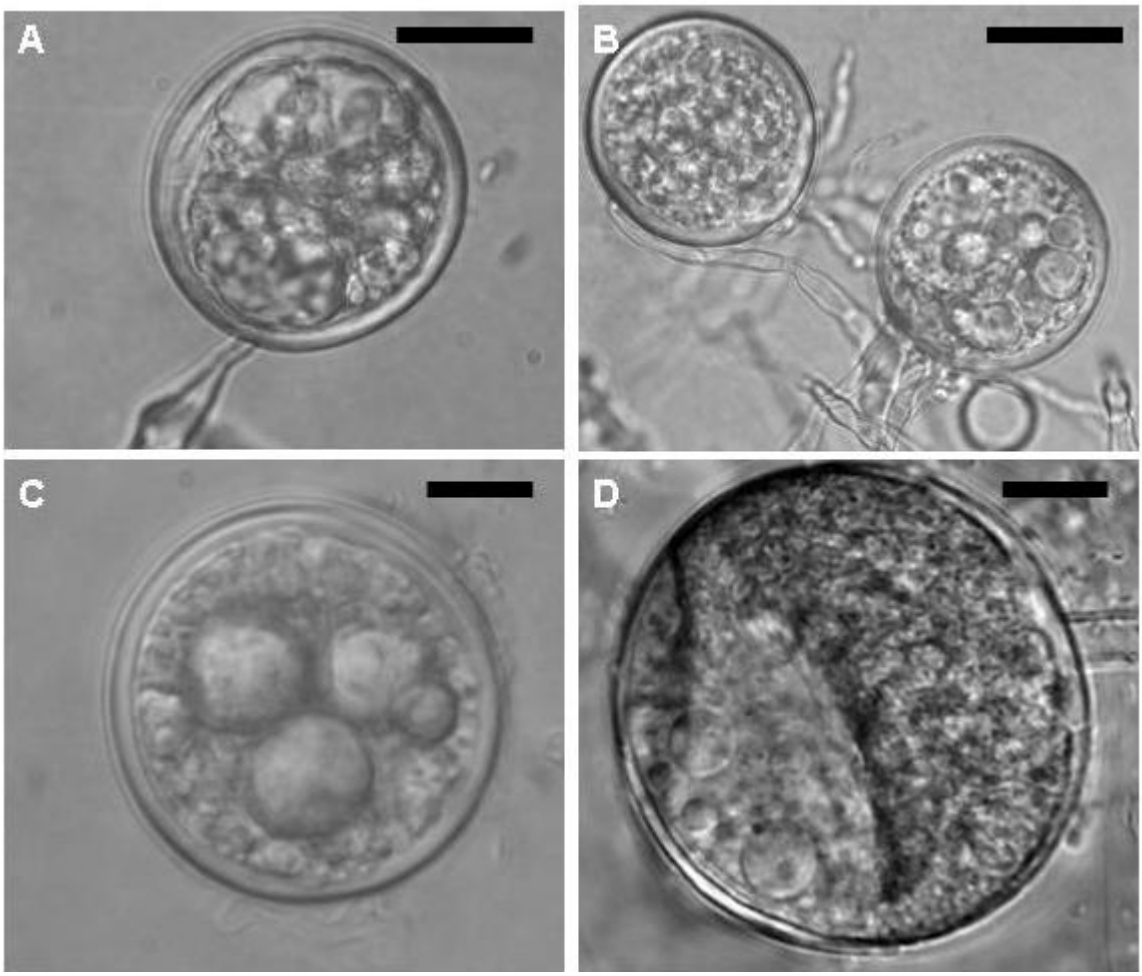

Fig 7 – Chlamydospores of *Phytophthora alticola* on V8 Agar. (A–D) Terminal chylamydospores, large and spherical. Bars = 10 µm.

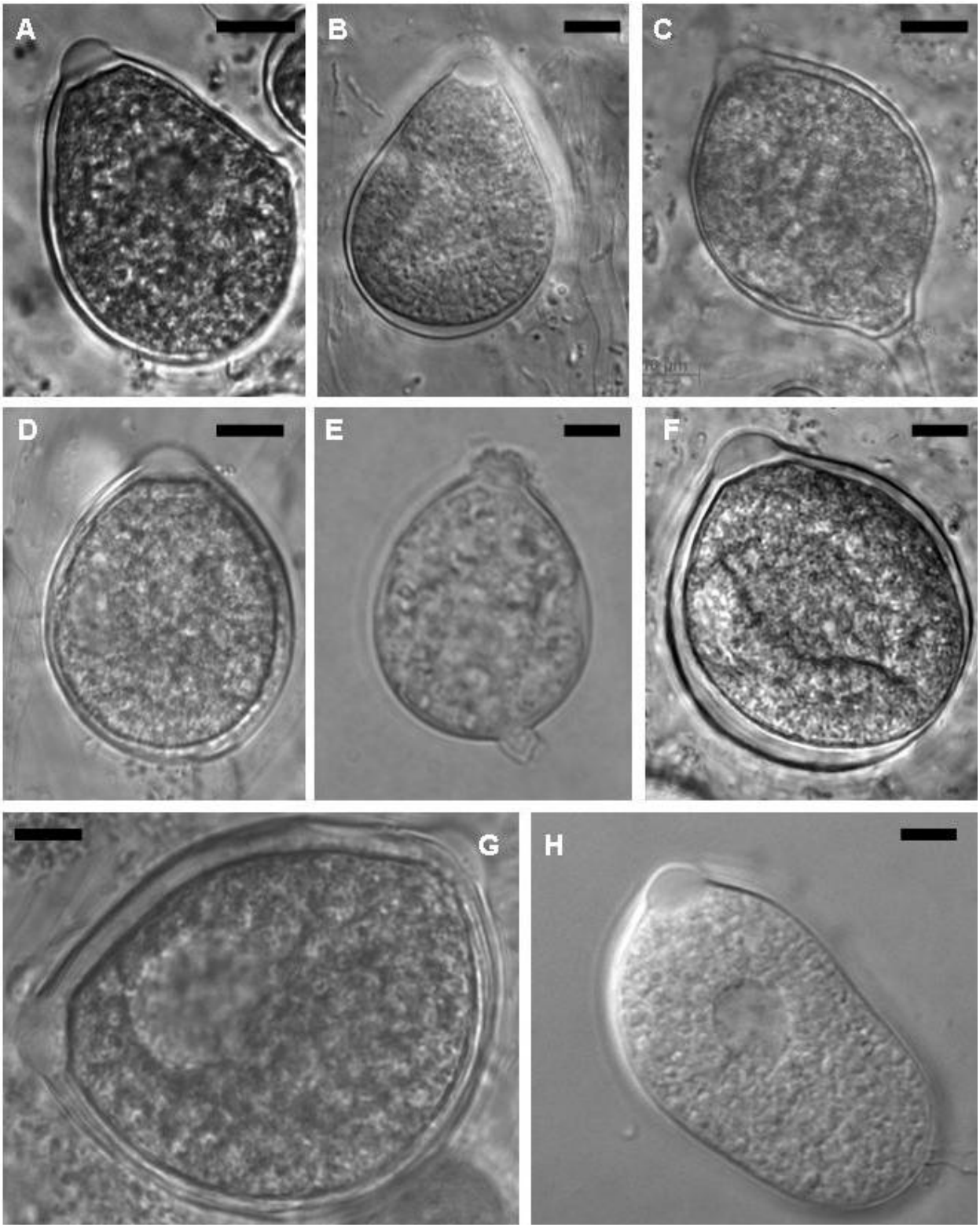

Fig 8 – Sporangia of *Phytophthora alticola*. (A–C) Papillate, ovoidobpyriform, terminal attached and caducous sporangia. (D–F) Ovoid and papillate sporangia. Distorted shapes. (G) Bipapillate sporangium. (H) Peanut-shaped sporangium. Bars =10 µm.

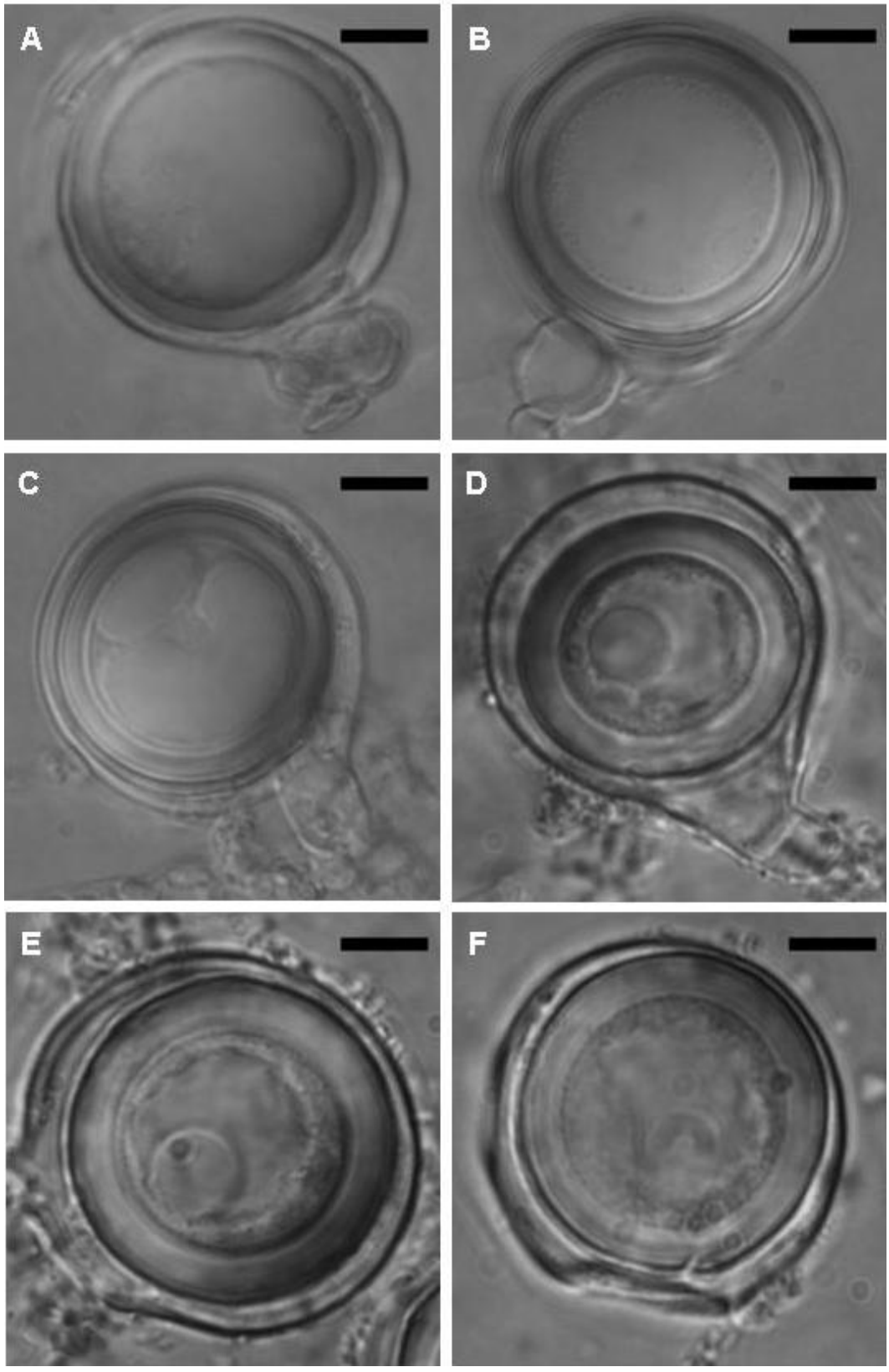

Fig 9 – Oogonia, antheridial and oospores characteristics of *Phytophthora alticola*. (A) Spherical thick-walled oogonium with tapered base. (B–F) markedly aplerotic oospores, amphigynous antheridia often breaking-off from oogonia or attached to tapered oogonial stalk. Bars= 10 µm.

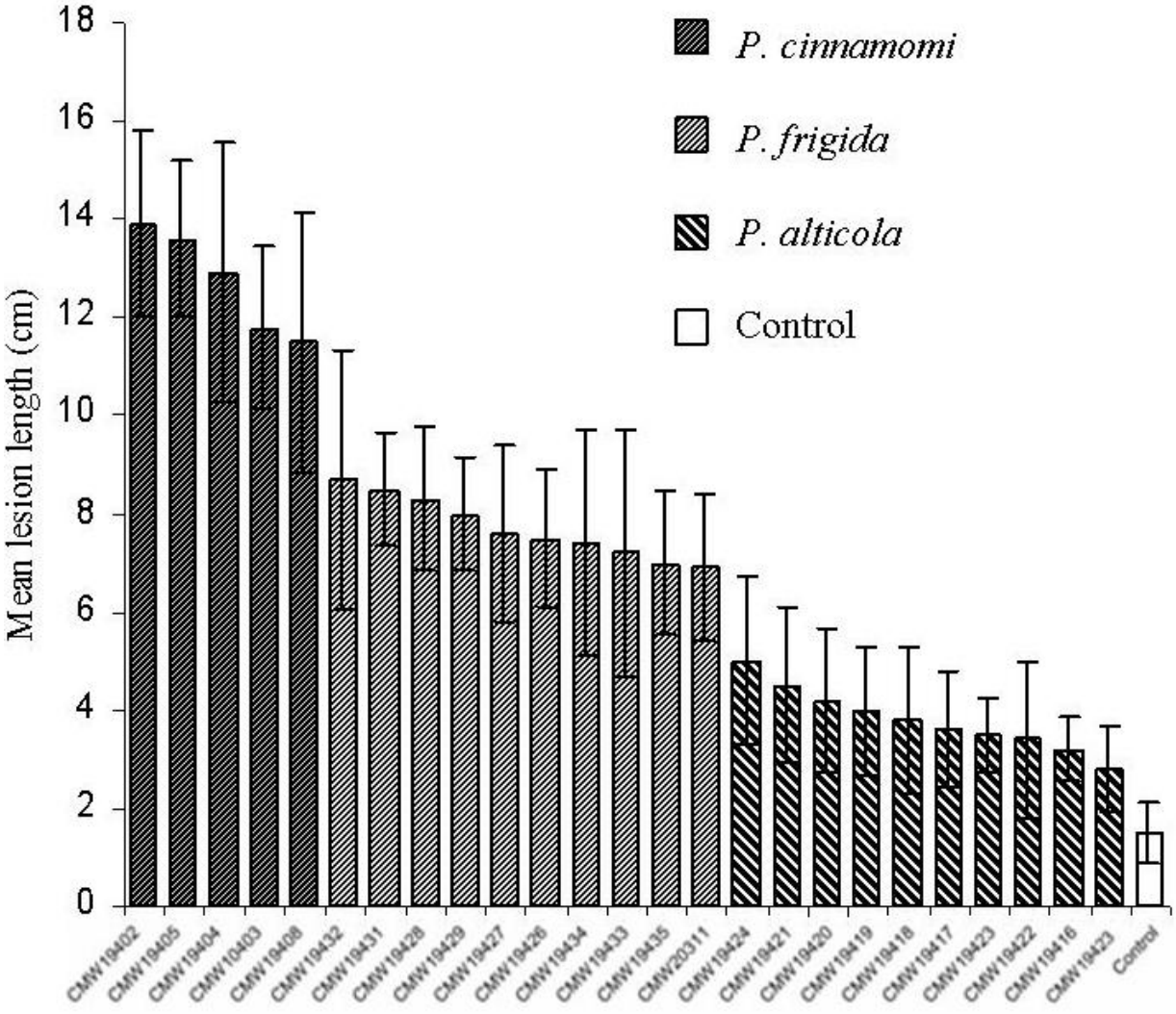

Fig 10 – Mean lesion length of selected isolates of *Phytophthora alticola*, *P. frigida* and *P. cinnamomi*, 36 d after under-bark inoculation of 12-m-old *Eucalyptus dunnii* in the field.

Supplementary Figure A–G Above ground and below ground disease symptoms of *Phytopthhora* root and collar rot in cold tolerant *Eucalyptus* spp. A. Young *E. badjensis* killed by *P. alticola* in Midillovo progeny trial. B C & G. Collar rot symptoms in *E. badjensis*, *E nitens* and *E. macarthurii* caused by *P. alticola*. D–F. Root and collar rot symptoms on young seedlings of *E. saligna* , and *E. nitens* .

Summary

This thesis deals with the dieback of cold tolerant eucalypts associated with *Phytophthora* spp. in forest plantations of South Africa. The focus of the research in this thesis is mainly on the taxonomy of *Phytophthora* spp. and their pathogenic interactions with eucalypt hosts.

The first chapter gives a broad review of the literature, which includes the taxonomy of the genus *Phytophthora*, an overview and impact of root and collar rot of cold tolerant eucalypts in South Africa as well as methods currently used to control and identify *Phytophthora* spp. This section identifies various gaps in research on this broad topic, especially on the role and potential threat of *Phytophthora* to plantations and native forests in South Africa. Some of these critical questions form the basis of the research undertaken in subsequent chapters.

Chapter two deals a survey undertaken in several plantations in the Mpumalanga and KwaZulu-Natal Provinces of South Africa. This survey was prompted by the unusual mortality of cold tolerant eucalypts and the discovery of destructive and invasive *Phytophthora* spp. in many parts of the world. *P. nicotianae* rather than *P. cinnamomi* was consistently isolated from the rhizosphere soil and from infected root collars of cold tolerant *Eucalyptus* spp. and was more frequently isolated from soil and diseased trees than *P. cinnamomi*. However, pathogenicity tests showed that *P. nicotianae* isolates were less pathogenic than *P. cinnamomi*.

Chapter three focused on the possible role of various site preparation methods on the mortality of *E. smithii* seedlings, following establishment. Results obtained indicated that at least three *Phytophthora* spp. are involved in the poor establishment of *E. smithii* in the areas investigated. However, the results also indicated the involvement of site related factors other than pathogens in the poor establishment of *E. smithii.*

Chapter four is about the development of rapid and reliable methods for screening a large number of half sib seedlings of *E. smithii* families for susceptibility to *P. cinnamomi* and *P. nicotianae*. The disease severity ratings for the *E. smithii* families based on the inoculations correlated with field observations for susceptibility and the mean total soluble leaf phenolic concentration was found to be higher in tolerant than in susceptible genotypes.

The last chapter of this thesis is the taxonomic description of two previously undescribed species namely *P. frigida* and *P. alticola* associated with dieback of *Eucalyptus* in South Africa. Prior to this study, only *P. boehmeriae*, .*P. cinnamomi* and *P. nicotianae* identified using traditional methods were associated with dieback *Eucalyptus* species in South Africa. Very little research was conducted on the identification of other possible *Phytophthora* spp. associated with *Eucalyptus* species and their role in

the poor establishment of this host. This is the first study which used DNA based methods to identify *Phytophthora* spp. associated with eucalypts in South Africa.

The results of this study illustrate that potentially many new species of *Phytophthora* still remain undetected in plantations and natural forest stands. Surveys in such ecosystems could result in the discovery of many new species which could cause devastating losses to the hosts they infect.