POPULATION STRUCTURE OF *PHYTOPHTHORA CINNAMOMI* IN SOUTH AFRICA

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POPULATION STRUCTURE OF *PHYTOPHTHORA CINNAMOMI* IN SOUTH AFRICA

BY

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"What is now proved was once only imagined"

William Blake

CONTENTS

PREFACE

Research in this dissertation deals with the population structure of *Phytophthora cinnamomi* in South Africa. Knowledge on the population structure of *P. cinnamomi* is important and will contribute to programs aimed at breeding and selection of resistance to *P. cinnamomi* and, to the implementation of effective disease control practices.

Phytophthora cinnamomi is an extremely important plant pathogen with a wide host range. It is well-known for the devastating effects it has on the *Eucalyptus* forests and understorey vegetation in Australia. In South Africa, significant losses have been experienced with *P. cinnamomi* infection of cold tolerant *Eucalyptus* species used in commercial forestry. The aim of this study was to characterise the South African *P. cinnamomi* population to provide insight into the origin, variation in levels of pathogenicity, occurrence of sexual reproduction, and genetics of *P. cinnamomi.* This knowledge should contribute to develop and implement programs aimed at reducing the impact of root and crown diseases caused by *P. cinnamomi* in *Eucalyptus*.

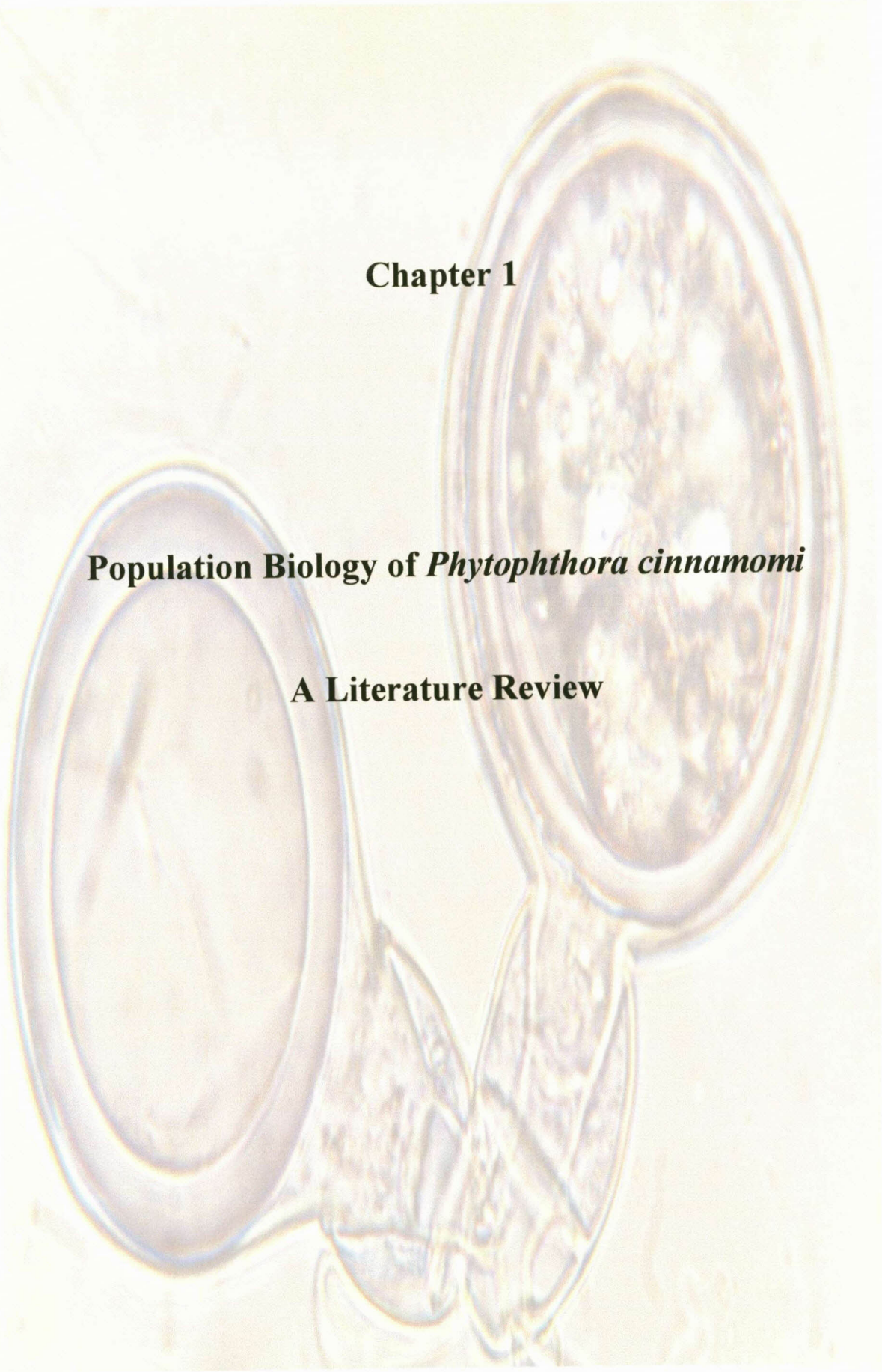
The introductory chapter presents a comprehensive review of the literature pertaining to the life cycle of *P. cinnamomi*, mechanisms of pathogenicity, and control measures. Special reference is also given to available genetic markers useful for population studies on fungal pathogens, as well as the origin and maintenance of genetic diversity in *Phytophthora* spp. Particular emphasis is given to *P. cinnamomi.*

In order for breeding and selection programs to be successful, an understanding of the pathogen population is required. The population structure of *P. cinnamomi* in South Africa as determined using isozymes is discussed in chapter two. This chapter considers levels of genetic diversity in South African *P. cinnamomi* populations. Differentiation between regional populations, stability over time, and occurrence of sexual reproduction in the South African *P. cinnamomi* population is discussed. The hypothesis that South Africa is a possible origin of *P. cinnamomi* is challenged in this chapter.

The success of breeding and selection programs against *P. cinnamomi* is dependant on the levels of pathogenicity and the variation in pathogenicity of the pathogen. In chapter three, pathogenicity tests on *Eucalyptus smithii* were conducted to assess variation in levels of pathogenicity of South African *P. cinnamomi* populations. Seasonal differences in pathogenicity, effect of culture age, and possible associations with isozyme properties and growth rate *in vitro* were examined. The influence of mating type and geographic origin of isolates on levels of pathogenicity was also explored.

Most *Eucalyptus* species used in South African forestry originates from Australia, which raises questions concerning the Australian *P. cinnamomi* population. Chapter four deals with levels of gene and genotypic diversity in South African and Australian *P. cinnamomi* populations. A few available isolates from Papua New Guinea, another hypothesised centre of origin of *P. cinnamomi*, were also included for comparative purposes. Dominant and co-dominant markers such as RAPD's and RFLP's were used to characterise these populations. The similarity of the Australian and South African *P. cinnamomi* populations, origin of *P. cinnamomi*, occurrence of sexual reproduction, and its consequences for breeding programs are considered.

Evidence for the occurrence of sexual reproduction of *P. cinnamomi in vitro* or *in vivo* is limited. It has been suggested that the two mating types are in the process of evolutionary divergence and that they apparently have lost the ability to reproduce sexually. Alternatively, sexual reproduction may take place, but loss of pathogenicity of F_1 hybrid isolates would hamper detection *in vivo*. The occurrence of sexual reproduction *in vitro*, and the ability to produce pathogenic progeny, was tested in chapter five. Sexual reproduction in *P. cinnamomi* has serious consequences for breeding and selection programs as it would change the genetic structure of the pathogen population, and result in isolates with higher levels of pathogenicity. Levels of pathogenicity of F_1 progeny and their parents is compared by means of artificial inoculation on *E. smithii* to test assumptions regarding pathogenicity of *P. cinnamomi* F_1 hybrid isolates.



Chapter 1

Population Biology of *Phytophthora cinnamomi*

A Literature Review

I. INTRODUCTION

Most of the 67 described *Phytophthora* species are important plant pathogens that cause significant production losses in a wide range of agricultural and forestry based industries in temperate and tropical areas of the world. *Phytophthora cinnamomi* Rands is a soilborne fungal plant pathogen that affects almost 1000 different plant species (Zentmyer, 1980). In South Africa, *P. cinnamomi* is particularly important in *Eucalyptus* forestry (Linde *et al.*, 1994; Wingfield and Knox-Davies, 1980) and the avocado industry. Forestry is one of the largest industries in South Africa with approximately 1.5 million ha of *Pinus* and *Eucalyptus* planted in more or less equal proportions (van der Zel, 1994). The predominant *Eucalyptus* species planted is *E. grandis* Hill ex Maid. which comprises 90% of the *Eucalyptus* plantings. In these plantings *E. grandis* is either used as seedlings, selected clones, or in crosses with other species.

Eucalyptus spp. affected by *P. cinnamomi* are mainly species planted at high elevation, namely *E. smithii* Donn. ex Smith, *E. fastigata* Deane and Maid., and *E. fraxinoides* Deane and Maid. In the late 1970's - 1980's, *E. fastigata* and *E. fraxinoides* were severely affected by *P. cinnamomi* (Wingfield and Knox-Davies, 1980), which led to the use of more resistant species. As a result, the current area planted to high elevation *Eucalyptus* spp. susceptible to *P. cinnamomi,* is only approximately 25, 000 ha. Mortalities are highest in *E. fraxinoides* (> 90%) and *E. smithii* (> 50%), whereas mortality could be as high as 40% in *E. fastigata* but rarely exceeds 20%. However, due to their wood characteristics suitable for high quality pulp production, the area planted to these species has the potential to increase significantly, if *Phytophthora* resistant planting stock were available.

P. cinnamomi was first described by Rands (1922) on cinnamon trees (*Cinnamomum burmannii* Blume) in Sumatra. Since then, it has been described as a destructive pathogen of many plant species (Zentmyer, 1980). In South Africa, it was first described in 1931 on avocado (*Persea americana* Mill.) (Doidge and Bottomley, 1931; Wager, 1931). In forestry, major outbreaks of diseases caused by *P. cinnamomi* include *Eucalyptus* die-back in Australia (Podger *et al.*, 1965; Podger and Batini, 1971) and South Africa (Wingfield and Knox-Davies, 1980), chestnut (*Castanea*) decline in the United States and Europe (Crandall *et al.*, 1945;

Grente, 1961), oak (*Quercus*) decline in Iberia (Brasier, 1992b), red oak (*Quercus rubra* L.) disease in France (Robin *et al.*, 1992), *Pinus radiata* D. Don. die-back in New Zealand (Newhook, 1959), little leaf disease of *Pinus echinata* Mill. and *Pinus taeda* L. in the south eastern United States (Lorio, 1966), and Ohia (*Metrosideros polymorpha* Gaud.) decline in Hawaii (Kliejunas *et al.*, 1977).

P. cinnamomi belongs to the class Oomycetes, order Perenosporales and family Pythiaceae and is heterothallic with two mating types, A1 and A2. Haasis and Nelson (1963) first noted that *P. cinnamomi* has two mating types, which they designated + and - isolates. Then, heterothallism in *P. cinnamomi* was confirmed by Galindo and Zentmyer (1964) who showed that the different mating types, which they called A1 and A2, participate in the sexual process. Interaction between hyphae of different mating type results in the formation of oospores. Oospore production *in vitro* is abundant and easily obtained (Galindo and Zentmyer, 1964), whereas the occurrence of oospores *in vivo* is rare. Oospores of *P. cinnamomi* have only been observed sporadically in soil and naturally infected host tissue (Mircetich and Zentmyer, 1966). Oospore germination is difficult, although germination rates between 1-45% have been reported among oospores produced *in vitro* (Ribeiro *et al.*, 1975). However, *P. cinnamomi* oospore germination studies have, as yet, failed to provide unambiguous evidence concerning their genetic make-up (outbreeding, inbreeding, or perhaps germination of parental material).

Despite the importance of *P. cinnamomi* as a plant pathogen, and the availability of various genetic markers to study populations, few population genetic studies have been conducted on *P. cinnamomi*. The only such studies pertaining to this fungus include isozyme analysis of Australian and Papua New Guinea *P. cinnamomi* populations in the 1980's (Old *et al.*, 1984, 1988). In contrast, numerous population genetic studies have been conducted on *Phytophthora infestans* (Mont.) de Bary, the late blight pathogen of potatoes (Drenth *et al.*, 1993, 1994; Fry *et al.*, 1992, 1993; Goodwin *et al.*, 1992; Spielman *et al.*, 1991; Tooley *et al.*, 1985).

In this review, the life cycle, mechanisms of pathogenesis, and available control measures for *P. cinnamomi* are only briefly described as this subject has been reviewed in detail elsewhere (Zentmyer, 1980; Weste and Marks, 1987). Available genetic markers useful for population genetic studies of fungal pathogens are described. The origin and maintenance of

genetic diversity in *Phytophthora* spp., with particular emphasis on *P. cinnamomi*, is discussed. Research concerning the genetic structure of *P. cinnamomi* populations is summarised, before the overall and specific aims of the research described in this dissertation are presented.

II. LIFE CYCLE AND MECHANISMS OF PATHOGENESIS IN *P. CINNAMOMI*

Traditionally the Oomycetes have been placed within the fungal Kingdom mainly on the basis of their somatic structures, growth, and nutritional behaviour. However, it has long been recognised that the Oomycetes are different from the higher fungi (Eumycota). Unique characteristics of the Oomycetes include; their diploid nature in the vegetative state (Brasier and Sansome, 1975), coenocytic mycelia, and cell walls that are comprised predominantly of cellulose rather than chitin as is found in higher fungi (Bartnicki-Garcia, 1968). Recent phylogenetic studies based on sequence analysis of the small subunit ribosomal DNA (Förster *et al.*, 1990), separates Oomycetes from the Ascomycetes and Basidiomycetes. In fact, Oomycetes show closer evolutionary relationship to the chrysophytes and therefore, are accommodated in the Kingdom Protista (Gunderson *et al.*, 1987; Wainright *et al.*, 1993).

Asexual life cycle

Phytophthora spp. reproduce asexually by means of sporangia that can either germinate directly or differentiate into zoospores. Zoospore differentiation occurs inside sporangia, unlike that of *Pythium* spp., another member of the Oomycetes, where zoospores are differentiated in a vesicle outside the sporangium, before release. Two flagella enable zoospores to swim to new host tissue for infection. After zoospores encyst, they can germinate directly to produce additional zoospores, or vegetative hyphae. Under conditions of high nutrition and optimum temperatures for growth, sporangia have the ability to germinate directly to give rise to additional sporangia, or vegetative hyphae. Other asexual survival structures, include chlamydospores, which are more often produced under unfavourable conditions. *In vivo* production of chlamydospores has been noted in soil, gravel, and host

tissue, under dry conditions (Weste and Vithanage, 1979). Depending on environmental conditions, chlamydospores can germinate to produce sporangia, vegetative hyphae, or additional chlamydospores. Chlamydospores are thick walled and have been shown to survive for at least six years in soil (Zentmyer and Mircetich, 1966). *P. cinnamomi* can also survive as zoospore cysts for up to 6 weeks in soil (MacDonald and Duniway, 1979), in the mycelial state (Shea *et al.*, 1980) or as zoospores, although the latter structures are the least effective for long term survival.

Sexual life cycle

P. cinnamomi is heterothallic with two mating types, A1 and A2. Sexual reproduction takes place when specialised structures (antheridia and oogonia) produced on opposite hyphae of both mating types, interact to produce oospores. In *P. cinnamomi*, the antheridia have an amphigynous configuration with respect to the oogonia. After the oogonium grows through the antheridium, the oogonium expands rapidly before the oogonial wall is penetrated and a single antheridial nucleus is deposited in the oogonium to fuse with one of the oogonial nuclei. Meiosis occurs in the multinucleate gametangia (Shaw, 1983). For *Phytophthora* species in general, optimum temperature for oospore production (15-21 C) is lower than that required for normal growth (Drenth *et al.*, 1995; Zentmyer *et al.*, 1979). Oospores are thick walled structures that enable the fungus to survive outside the host. Oospores of Oomycetes have the ability to survive for many years in soil (Duncan and Cowan, 1980; McKay, 1957). They are the most resistant structures produced and serve to provide long term survival in the absence of the host. Oospores can either germinate by forming a germtube which can initiate mycelial growth directly, or terminate into a sporangium, producing zoospores. To date, the importance of the sexual life cycle and the role of oospores in the population biology of *P. cinnamomi* is largely unknown.

Mechanisms of pathogenesis

P. cinnamomi infects its hosts via zoospores, which are chemically attracted by root exudates (positive chemotaxis), to the region of elongation at root tips (Zentmyer, 1961). *P. cinnamomi* generally infects small feeder roots, but can also infect larger roots and root collars

of *E. marginata* Donn. ex Sm. (Shearer *et al.*, 1981). On *Eucalyptus,* it causes various symptoms such as damping-off, root and root collar rot, wilting, reduction in leaf size, leaf discolouration, and death of infected plants (Marks *et al.*, 1972; Podger and Batini, 1971). *P. cinnamomi* infection of susceptible *Eucalyptus* roots is accompanied by a failure in the hydraulic conductance of roots, even when only part of the root system is infected. The failure in conductance precedes changes in xylem and leaf water potential and also precedes all secondary shoot symptoms such as wilt and die-back. This indirect response to infection is associated with a reduction in cytokynin levels (Cahill *et al.*, 1985). The enzyme N-acetylglucoseaminodase, a chitinolytic enzyme produced by *P. cinnamomi* (Hodge *et al.*, 1995), may also be important in the infection process.

Recent studies of the mechanisms of pathogenesis in *P. cinnamomi,* include a better understanding of zoosporogenesis, and its role in the infection process (Hardham, 1995; Hardham *et. al.*, 1994). Biflagellate zoospores are the major infective agents and these have a number of cell components, which play a role in the infection process. These cell components include three types of vesicles that occur in the peripheral cytoplasm. Two of these vesicle types are secretory and are thought to be responsible for the formation of the cyst coat and the deposition of adhesive material during encystment and host infection. The third vesicle type is not secreted and appears to store proteins used to support early germling growth (Dearnaley and Hardham, 1994; Dearnaley *et al.*, 1996; Hardham, 1995). All three vesicle types are formed in the Golgi apparatus in hyphae following the induction of sporulation (Chambers *et al.,* 1995; Dearnaley *et al.*, 1996). They move into sporangia where they are randomly distributed. Vesicles are also produced in oospores which were formed on chestnut roots (Chambers *et al.*, 1995), suggesting that oospores are possibly important as infection structures.

Homing responses in *Phytophthora* (host and substrate location) involves zoospore taxis, encystment, cyst adhesion, germination, and germ-tube tropism as reviewed by Deacon and Donaldson, (1993). This process is mediated by recognition of chemical diffusates and surface components of the host or substrate. Induction of encystment is a key stage in the homing sequence, as it may lead to adhesion, germination, and host penetration by an autonomous, calcium-mediated, cascade. Zoospores orientate during encystment apparently by interaction

of flagella with host surface components, so that the fixed site of germ-tube outgrowth lies next to the host. An adhesive glycoprotein is released and this interacts with calcium which is released during encystment. Then reabsorption of calcium apparently triggers germination, synergised by specific organic compounds in host exudates.

III. CONTROL OF *P. CINNAMOMI*

A number of management practices are available to control diseases caused by *P. cinnamomi*. The most common control options include *(1)* fungicides, *(2)* phosphonates, *(3)* cultural practices, *(4)* resistance breeding, and *(5)* biological control.

1. Fungicides

Fungicides such as fosetyl-Al and metalaxyl have been widely used to control *Phytophthora* diseases. Metalaxyl is a xylem-translocated phenylamide fungicide and has an upward movement in plants (Edgington and Peterson, 1977), while fosetyl-Al is phloem-translocated (Ouimette and Coffey, 1990) with both downward and upward movement in the host. This means that metalaxyl will have no effect on root diseases if applied as a foliar spray, whereas fosetyl-Al can be applied to any part of the plant. Metalaxyl is usually applied as a drench, whereas fosetyl-Al can be applied either as a drench, foliar spray, stem canker paint, or trunk injection for direct systemic control. Fungicides applied as foliar sprays and drenches are often limited in their effectiveness against *Phytophthora*. This is because fungicide uptake into the plant tissue is generally poor, fungicide activity is rapidly lost due to degradation by soil and phylloplane microbes, and fungicides are lost to the environment through leaching and wash-off. Therefore, Darvas *et al.* (1984) developed a fosetyl-Al trunk injection technique which has minimum wastage and environmental contamination, and achieves maximum persistence within the host.

Unfortunately, fungi have the ability to overcome fungicides by developing resistance, e.g. wide use of metalaxyl in Europe to control *P. infestans* has lead to the rapid acquisition of resistance in the pathogen population after only one year of use (Davidse *et al.*, 1981).

Appearance and economic losses due to fungicide resistance largely depends on; *(i)* size of affected pathogen population, *(ii)* area and intensity of the crop, *(iii)* intensity of use of a particular fungicide, and *(iv)* use of other disease management practices. Parameters such as base line sensitivity for particular fungicides in wild type pathogen populations have often been neglected, or are lacking and this is also the case in *P. cinnamomi* populations treated with fungicides. No increase in metalaxyl nor fosetyl-Al resistance has been reported in *P. cinnamomi* populations, although fosetyl-Al resistance of individual *P. cinnamomi* isolates *in vitro* has been reported (Coffey and Bower, 1984). Care must be taken when extrapolating antifungal activity of fosetyl-Al *in vitro* because of the contributing activity of aluminium ions released upon hydrolysis of fosetyl-Al (Guest and Grant, 1991). Furthermore, effective, long term survival of *P. cinnamomi* in host tissue and the ability to survive at considerable depths in soil (Hill *et al.*, 1995), precludes any attempt of rapid and effective chemical control in field situations. Future research should aim to obtain alternative, more durable, and environmentally friendly methods for control.

2. Phosphonates

The use of phosphonates (phosphonic acids) has had a substantial impact on *Phytophthora* disease control, especially in Australia (Hill *et al.*, 1995). It is now accepted that the phosphonate produced by the metabolism of the phosphonate ester provides the protection to the plant. It has been shown that potassium phosphonate is as effective as fosetyl-Al as a fungicide (Fenn and Coffey, 1984; Pegg *et al.*, 1985). Phosphorus acid is much cheaper than its relative, fosetyl-Al, and has a similar mode of action in that it is systemic and has an indirect effect by enhancing the defense mechanisms of plants (Guest, 1984). Application is, therefore, effective in that it can be used as a trunk injection, or sprayed onto plants of many plantation crops such as avocado (Guest *et al.*, 1995).

As with fungicides, the major drawback of phosponate is that it is impractical to apply on an annual basis to large areas such as in forestry. Furthermore, important questions such as its longevity, the interrelationship between the phosphate status of a plant and the effectiveness of phosphonate application, still need to be investigated. Phosphonates should also not be used as the sole means to control *Phytophthora,* although some groups firmly believe that it is

unlikely that resistance will develop against them (Guest and Grant, 1991; Guest *et al.*, 1995). This is because of their complex mode of action, which is not directed only at the pathogen itself, but also towards stimulating host defense responses (Guest and Bompeix, 1990; Guest and Grant, 1991). It is important to note that phosphonate will not eliminate disease and does not have a curative effect, but it remains an excellent, cost effective option for control of *Phytophthora* diseases in many crop plant species.

3. Cultural practices

Cultural practices, although not always completely effective, are extremely important in controlling and preventing spread of pathogens. On the other hand, *P. cinnamomi* often affects plants over a large area which, to a certain extent is a limiting factor for cultural practices as control options. Quarantine, nursery hygiene, and improved drainage are commonly used cultural practices.

i) Quarantine and nursery hygiene

Quarantine is the only means to prevent introduction of a new pathogen or new pathogen genotypes into an area. A classic example of quarantine to control *P. cinnamomi* can be found in Australian *Eucalyptus* forests, where traffic to certain areas is reduced and quarantine vehicle baths are installed to prevent spread (Shea and Broadbent, 1983). Quarantine is also extremely important in nurseries where millions of plants are produced each year, providing opportunities for rapid spread of *Phytophthora*. However, quality control and rigorous testing to ensure *P. cinnamomi* free nursery material has sometimes been ignored in the past, and needs urgent attention. The recent development of rapid DNA based diagnostic tests for many fungal pathogens has the potential to improve this situation. This includes PCR-based detection techniques for *Phytophthora* (Bonants *et al.*, 1997; Coelho *et al.*, 1997; Dobrowolski and O'Brien, 1993; Lacourt and Duncan, 1997; Lacourt *et al.*, 1996; Niepold and SchöberButin, 1997; Stammler and Seemüller, 1993), and other species specific and selective techniques (Cahill and Hardham, 1994; Gabor *et al.*, 1993; Judelson and Messenger-Routh, 1996). PCR-based techniques have the advantage of being time efficient compared to

the other techniques (Judelson and Messenger-Routh, 1996; Lacourt and Duncan, 1997) which are complex and require the use of probes.

ii) Improved drainage

Waterlogging is an important inducing factor in *P. cinnamomi* root diseases (Davison and Tay, 1987) as it predisposes plants to disease. Additionally, water stress before waterlogged conditions increases this predisposition to infection by *Phytophthora* spp. (Duniway, 1983). The process in which host plants are predisposed to infection as a result of waterlogging is still unclear. Hypotheses for such predisposition include the fact that oxygen-stressed roots "leak" or exude greater amounts of soluble metabolites, which may stimulate chemotactic movement of zoospores along the concentration gradient to the root surface (Kuan and Erwin, 1980). Chemical or physiological change in waterlogged hosts may also be responsible for the increased susceptibility of hosts to *P. cinnamomi* (Duniway, 1977). Under waterlogged conditions in *Eucalyptus*, the normal responses of the host to infection is suppressed, allowing xylem invasion by *P. cinnamomi* to become more extensive (Davison *et al.,* 1994). Improved drainage can be achieved by variations in irrigation schedules, adjustment of volumes applied, means of delivery, and features of surface and soil drainage (Shea and Broadbent, 1983).

4. Resistance breeding

Although single resistance loci relating to *P. cinnamomi*, have as yet not been identified, opportunities exist to breed and select for resistance. Resistance to *P. cinnamomi* under polygenic control has been demonstrated in *Eucalyptus* (Cahill *et al.,* 1992; Stukely and Crane, 1994), pines (Butcher *et al.,* 1984), and avocado rootstocks (Phillips *et al.*, 1987). The mechanism of this host resistance might be anatomical barriers to prevent infection (Phillips *et al.*, 1987), physical barriers such as callose (Cahill and Weste, 1983), and lignin formation (Cahill *et al.,* 1989), and/or the formation of a necrophylactic periderm (Tippett and Hill, 1984). Resistance may also be biochemically based as demonstrated in *Eucalyptus calophylla* Lindley where partial resistance was related to production of phenylalanine ammonia-lyase, and the subsequent production of lignin and specific phenolic compounds (Cahill and McComb, 1992; Cahill *et al.*, 1993).

Strong genetic control of resistance as shown in *Eucalyptus marginata* families and narrow sense heritability of the resistance characters both in individual trees and at family level (Stukely and Crane, 1994), make breeding and selection for resistance a viable and attractive option. This resistance is also maintained when trees are exposed to a wide range of *P. cinnamomi* isolates belonging to different isozyme genotypes (Dudzinski *et al.*, 1993). The fact that resistance screening of small trees (one-year-old *P. radiata* seedlings) under glass house conditions could be correlated to field resistance (Butcher *et al.*, 1984), provides a reliable, and relatively rapid, screening strategy for tree crops such as pines and eucalypts. Recent developments in molecular genetics of forest species such as pines and eucalypts provide additional avenues to develop *P. cinnamomi* resistant material. Developments in *Eucalyptus* such as micropropagation, genetic transformation (Serrano *et al.*, 1996), and the construction of detailed genetic linkage maps (Grattapaglia and Sederoff, 1994), may all provide significant improvement in the long term resistance breeding and selection programs in forestry species.

5. Biological control

Biological control is generally less effective than the use of fungicides in controlling diseases. Research on biological control all too often encompasses only large scale screening efforts without seeking further understanding of the interaction between biological control agents and *Phytophthora.* If disease management is to be heavily based on biological control, the research effort in this area will need to be significantly increased. The efficacy of biological control under field conditions requires considerably more attention, as most research has been conducted only *in vitro*. However, biological control does provide an attractive and environmentally friendly option to control or suppress the development of *Phytophthora* diseases. Recent developments in biological control include the identification of biocontrol agents such as; Actinomycetes (You *et al.*, 1996), *Trichoderma* spp., (Chambers and Scott, 1995), *Penicillium funiculosum* Thom (Fang and Tsao, 1995), *Gliocladium virens* Miller, Giddens and Foster (Chambers and Scott, 1995; Heller and TheilerHedtrich, 1994), *Bacillus subtilis* Cohn *emend.* Prazmowski (Berger *et al.*, 1996) and *Chaetomium globosum* Kze. (Heller and TheilerHedtrich, 1994). These agents have all suppressed growth of *P.*

cinnamomi, mostly by hyphal lysis, but can also promote the growth of the host (El-Tarabily *et al.*, 1996). The use of organic media (mulches, composted pine bark etc.) which have high microbial activity and low pH (Hoitink and Fahy, 1986; You and Sivasithamparam, 1995), also provide promising options to control *P. cinnamomi* in container grown plants in nurseries, but can also be extrapolated to the field, for example in the control of apple replant disease (Utkhede and Smith, 1994). Mycorrhizae can also provide biological control against *P. cinnamomi* as identified in pines (Marais and Kotzé, 1976) and pineapple (Guillemin *et al.*, 1994).

IV. MARKERS FOR POPULATION GENETIC STUDIES OF FUNGAL PATHOGENS

Knowledge of the level and distribution of genetic diversity within a pathogen population has direct value in the application of disease control measures. Fungal populations with high levels of genetic diversity are likely to adapt more rapidly to fungicides and resistant hosts, than are populations with low levels of genetic diversity. Characterising and analysing genetic diversity in fungal populations requires genetic markers that provide information about the genotype of an organism. Such markers, for example, include all characteristics used to describe those organisms, and all pathogenic markers used by plant pathologists. Genetic markers preferably should be neutral, unambiguous, numerous, and independent of tissue or developmental stage (Michelmore and Hulbert, 1987). Numerous types of markers, including (1) morphology, (2) physiology, (3) vegetative and sexual compatibility, (4) pathogenicity and virulence, (5) isozymes, and (6) DNA based markers have been used to assess levels of genetic diversity in plant pathogens.

1. Morphology

Morphological markers within a fungal species, such as spore colour forms, have been critically important in studies on the genetic basis of diversity and recombination in some ascomycetes (Fincham *et al.*, 1979). Pigment variation is also found in plant pathogens, for

example, *Puccinia graminis* Pers. (Green, 1964), *Cochliobolus heterostrophus* (Drechs.) Drechs. (Fry *et al.*, 1984), and *Magnaporthe grisea* (Hebert) Barr (Chumley *et al.*, 1986). However, these were shown to have deleterious effects such as reduced pathogenicity. Other morphological variants include spore ornamentation, and size and shape of spores (Groth and Roelfs, 1987). Although useful in laboratory studies, morphological markers are rarely observed in natural populations, have a limited number of alleles, and often have adverse phenotypic effects. Since these markers are rare in nature, mutagenesis has been used to generate morphological variants for genetic studies in a small range of haploid fungi such as *Aspergillus nidulans* Shear and Dodge (Cove, 1976) and *Neurospora crassa* (Eidam) Wint. (Leslie and Raju, 1985).

2. Physiology

Physiological markers in *Phytophthora* include growth rate, influence of temperature on growth rate or pathogenicity, nutritional requirements etc. Although characters such as nutritional requirements are extensively used with *in vitro* systems such as *nit*-mutants in *Fusarium* for the study of vegetative incompatibility groups (Correll *et al.*, 1987), application in *Phytophthora* is limited. Nevertheless, some variation in physiological characters has been noted in the past e.g. optimum temperature for growth (Grente, 1961; Mehrlich, 1936; Tucker, 1931) and growth rate at particular temperatures which varies considerably within *P. cinnamomi* (Shepherd and Pratt, 1974). This variation in growth rate at different temperatures was later associated with the source of the *P. cinnamomi* isolates tested (Shepherd and Pratt, 1974). Physiological characters typically do not give clear cut results, their numbers are rather limited, and are often qualitatively expressed. Moreover, very little is known of the genetics underlying these characters and stability over time, which severely reduces their usefulness as markers in population genetic studies.

3. Vegetative and sexual compatibility

Vegetative and sexual compatibility provide natural occurring markers for a wide variety of fungi and have been used in studies of fungal species such as *Armillaria luteobubalina* Watl. and Kile (Kile, 1983), *Heterobasidium annosum* Bref. (Stenlid, 1985), *Endothia*

parasitica (Schw.) Fr. (Anagnostakis, 1984) and *Fusarium oxysporum* Schlecht. emend. Snyd. and Hans. (Jacobson and Gordon, 1988). These markers are often easy to score in culture as the presence of clamp connections, or the presence of sexual structures. Sexual compatibility in Basidiomycetes has been shown to be determined by two or multiple alleles at one or two loci (Day, 1973; Fincham *et al.*, 1979). Vegetative incompatibility genes have been characterised in various Ascomycetes where identical alleles at several loci are required for the formation of a heterokaryon (Leslie, 1993), so that only closely related isolates are likely to anastomose (Fincham *et al.*, 1979). It has also been used extensively to asses levels of diversity in fungal populations (Glass and Kaldau, 1992; Leslie, 1993; Mena *et al.*, 1994; Stenlid, 1985). However, vegetative and sexual compatibility markers have severe drawbacks: They are not generally available for all fungal species and then only for heterothallic species and, while vegetative incompatibility is absent, mating type groupings do not necessarily reflect the underlying overall level of genetic diversity. Sexual compatibility is present in heterothallic *Phytophthora* species, but vegetative compatibility has not been identified in *P. cinnamomi* or any other *Phytophthora* species.

4. Pathogenicity and virulence

Pathogenicity reflects the ability of a fungal pathogen to infect a specific host (Shaner *et al.*, 1992). Virulence refers to the ability of a pathogen to overcome specific resistance genes present in a particular host plant species (Shaner *et al.*, 1992). Using virulence genes as markers has the advantage of direct interpretation of results which are relevant to host-pathogen interactions. However, for many host-pathogen systems, including *P. cinnamomi*, such markers are not available due to the lack of specificity towards a cultivar or variety of hosts carrying specific resistance genes. *P. cinnamomi* has a wide host range (Zentmyer, 1980), and resistance genes under simple genetic control have not been identified in any host species. In host specific *Phytophthora* spp. such as *P. infestans* and *P. sojae* Kaufm. and Gerd., resistance genes provide a valuable tool to identify different virulence genes in the pathogen population, and to determine the levels of diversity for virulence within populations. This provides a better understanding of virulence in pathogen populations. Pathogenicity in organisms like *P. cinnamomi* is also often quantitatively expressed (Dudzinski *et al.*, 1993;

Linde *et al.*, 1998, Chapter 3). In addition, genes for pathogenicity and virulence are subject to intense selection pressure by the host and the frequency of such markers in field populations is influenced to a great extent by man (Burdon, 1993). Several studies have revealed the discrepancy in estimates of genetic diversity based on virulence loci compared to neutral genetic markers such as isozymes (Burdon and Roelfs, 1985; Leung and Williams, 1986) or RFLP's (Drenth *et al.*, 1994).

5. Isozymes

Isozymes detect variation in the amino acid sequence of proteins that have the same catalytic function, thus ultimately detecting variants among DNA sequences that encode for proteins. Isozymes have been used extensively in genetic studies of pathogenic fungi including *Phytophthora* (Fry *et al.*, 1992; Old *et al.*, 1984, 1988; Oudemans and Coffey, 1991; Tooley *et al.*, 1985). The advantages of isozymes are that they are codominant, selectively neutral, relatively inexpensive, and easy to assay. On the other hand, each isozyme requires a separate optimisation of conditions for extraction and buffer systems. The amount of genetic diversity detected using isozymes is subject to strong bias. A major source of bias is that only approximately one-third of all amino acid substitutions can be detected by using electrophoresis (Lewontin, 1974). The other amino acid substitutions do not change the charge of the protein and will thus not result in separation of the isozymes in an electrical field. In addition, small differences in rate of migration are not always detectable, therefore, some amino acid substitutions that do influence the charge, are also "silent" (Murphy *et al.*, 1990; Weising *et al.*, 1995). Another common problem in the use of isozymes is the lack of different alleles for many isozyme loci in fungi, reducing the usefulness for population genetic studies. Furthermore, isozymes are not necessarily controlled by the same locus (Weising *et al.*, 1995). Allozymes, therefore, need to be established in advance for any population genetic study.

6. DNA based markers

DNA based markers have several highly desirable characters in addition to the advantages that apply to isozymes. Firstly, analysis of DNA allows assessment of the whole genome,

including variation in the non-coding regions which are not detectable at the protein level using isozymes. Secondly, DNA sequences are unlikely to be affected by the environment or the developmental stage of the organism. Thirdly, a much greater number of markers are available. The last decade has witnessed a surge in genetic diversity studies using DNA based markers, in a large variety of fungal pathogens, at all taxonomic levels. The use of DNA based markers in plant pathology has been extensively discussed in a number of reviews (Bruns *et al.*, 1991; Foster *et al.*, 1993; Hadrys *et al.*, 1992; Lynch and Milligan, 1994; McDonald and McDermott, 1993; Michelmore and Hulbert, 1987). A number of different DNA based markers have been used e.g. RFLPs (Restriction Fragment Length Polymorphisms) (Botstein *et al.*, 1980; Grodzicker *et al.*, 1974), RAPDs (Random Amplified Polymorphic DNA) (Williams *et al.*, 1990), SCAR (Sequence Characterised Amplified Regions) (Paran and Michelmore, 1993), VNTR (Variable Number of Tandem Repeats) (Jeffreys *et al.*, 1985; Nakamura *et al.*, 1987), RAMS (Random Amplified Microsatellites) (Hantula *et al.*, 1996) and DAF (DNA Amplification Fingerprinting) (Caetano-Anollés *et al.*, 1991). Other recently introduced markers used in the analysis of human, animal, plant, and fungal genomes include AFLP (Amplified Fragment Length Polymorphism) (Vos *et al.*, 1995; Van der Lee *et al.*, 1997) and SSR (Simple Sequence Repeats) (Gupta *et al.*, 1994).

V. ORIGIN AND MAINTENANCE OF GENETIC DIVERSITY IN *P. CINNAMOMI* POPULATIONS

Fungal pathogens have long been considered as highly variable organisms, apparent both in field collections (Brasier, 1970; Perkins *et al.*, 1976) and from the behaviour of single isolates in the laboratory (Caten and Jinks, 1968). Until recently, the study of fungal diversity has been largely based on morphological and physiological diversity, vegetative and sexual compatibility, and virulence and pathogenicity characteristics. However, the levels of such phenotypic diversity in a pathogen population reflects only part of the genotypic diversity present (Perkins, 1991). The mechanisms by which genetic diversity arises in fungi are diverse as a result of a varied range of reproductive strategies and life histories. The processes

involved in generating and maintaining genetic diversity include; *(1)* mutation, *(2)* sexual recombination, *(3)* heterokaryosis, *(4)* parasexuality, *(5)* cytoplasmic inheritance, *(6)* centre of origin, *(7)* migration, *(8)* genetic drift, and *(9)* selection.

1. Mutation

Mutations form the basis of all genetic diversity as it give rise to changes in the genetic make-up of an organism. Different forms of mutation may occur; base substitution, base deletion, base insertion and inversion-, duplication-, deletion-, or translocation of sections of DNA. These kinds of mutations occur at different rates and there are no constraints at the molecular level of DNA, on what mutations can occur (Bos and Stadler, 1996). Constraints on genetic diversity arise from physiology and development of an individual and not from the mutational process itself. Mutations occur at random and can either increase or decrease the fitness of an individual. It is generally accepted as a rare event and estimates of the rate of mutation based on that of specific virulence loci are between 10^{-3} and 10^{-6} (Zimmer *et al.*, 1963). While mutations are rare, there are several factors which make the contribution of mutations more significant for fungi, compared to that of other eukaryotes. Firstly, the relatively large size of fungal populations and the potential for extremely rapid asexual reproductive rates, means that mutant alleles arise continuously and may rapidly increase in frequency, despite the rarity of mutation (Person *et al.*, 1976). Secondly, the absence of a distinct germline in fungi ensures that mutations arising in virtually any tissue can be transmitted to subsequent generations, either sexually or asexually. Thirdly, for fungal life cycles which are predominantly haploid (excluding Oomycetes), new mutations are expressed immediately. There are several examples of mutation in *Phytophthora.* In *P. infestans*, mutations have been noted at fingerprint loci (Goodwin *et al.*, 1995b), mt DNA (Goodwin, 1991) and resistance to metalaxyl (Goodwin *et al.*, 1996), as well as at virulence loci (Drenth *et al.*, 1996) in *P. sojae*.

2. Sexual recombination

Phytophthora spp. posses different reproductive strategies including sexual reproduction (outbreeding or inbreeding), asexual, and/or mitotic and parasexual recombination. Mating systems occupy a unique position in that any change in the mode of reproduction will change the genetic structure of the population. Outbreeding, involves nuclei from opposite mating type isolates which contribute to the sexual process, while inbreeding involves gametangial nuclei from only one parent. Outbreeding results in higher levels of heterozygosity and genetic diversity in the progeny than inbreeding, because rearrangement of the alleles from both parents takes place, giving rise to offspring carrying new and unique allele combinations. While meiosis occurs in inbred gametangia, continued inbreeding reduces heterozygosity and may lead to a condition approaching that in asexually reproducing species (Brasier and Sansome, 1975). Inbreeding reduces the level of heterozygosity by half every generation, which effectively leaves less than 1% of the original heterozygosity after seven generations of inbreeding. Intermediate levels of heterozygosity are expected in *Phytophthora* species which show mixed outbreeding and inbreeding (Goodwin, 1997).

Both mating types of *P. cinnamomi* have a global distribution, although the A2 mating type is more frequently isolated than A1 mating type (Zentmyer, 1980, 1988). Although *P. cinnamomi* produces oospores readily and abundantly *in vitro* after pairing isolates of different mating type, information regarding sexual recombination *in vivo,* is lacking. Even the nature (inbreeding or outbreeding) of *in vitro* produced oospores is unknown. Old *et al.* (1988) found no indication of genetic recombination based on isozyme analysis of Australian populations of *P. cinnamomi* and suggested that sexual reproduction does not occur *in vivo,* even though both mating types may be present. A possible hypothesis to explain the lack of sexual recombination being detected in the field, is that oospores abort (Rutherford and Ward, 1985), preventing reproduction of non-parental genotypes as they are eliminated from the population, although this has not been experimentally tested in any *P. cinnamomi* population. High fixation indices of the Australian and to a lesser extent, Papua New Guinea *P. cinnamomi* populations, significantly deviate from random mating (Hardy-Weinberg equilibrium) populations (Goodwin, 1997). This indicates a lack of, or low levels, of sexual reproduction in these populations.

The genetic basis of mating types in *Phytophthora* is not well understood and this is a topic of much speculation. Sansome (1980) suggested that the A2 mating type is heterozygous for a gene, located on a segment of the translocation heterozygote, for which the A1 mating type is homozygous. However, segregation of mating type among progeny of crosses in *P. infestans*, provided evidence for a balanced lethal system for mating type genes, both of which are homozygous. The mating type genes are hypothesized to be situated on a single locus and to display non-Mendelian segregation (Brasier, 1992a). Segregation at the mating type locus was observed to be not significantly different to the ratio of 1:1 (A1:A2). Only homozygous A1 and homozygous A2 progeny were recovered from crosses where mating type was shown to segregate. Individuals heterozygous for the mating type locus as determined using closely linked molecular markers, were shown not to germinate or to cease growth soon after germination (Judelson *et al.*, 1995). The balanced lethal system ensures that progeny with conflicting combinations of mating type alleles, such as those simultaneously expressing A1 and A2 functions, are excluded (Judelson *et al.*, 1995). Although no genetic studies have been conducted on regarding the genetics of mating type segregation in *P. cinnamomi*, it seems likely that it will be similar to that of *P. infestans*. In general, genetic studies on *P. cinnamomi* are lacking and although crosses have been made and oospores produced, their genetic make-up has never been investigated.

3. Heterokaryosis

The combination of two unique properties of fungi, namely hyphal anastomosis and nuclear migration, suggests that there is potential for formation of heterokaryons between unrelated genotypes. The formation of heterokaryons *in vitro* was demonstrated for *P. infestans* and *P. megasperma*, in which new races, auxotrophic, or drug resistance combinations have been obtained when different genotypes of mycelium or zoospores were combined (Dyakov and Kuzovnikova, 1974; Leach and Rich, 1969; Long and Keen, 1977). These authors have suggested heterokaryosis and parasexuality as possible explanations for the development of somatic variants. Their evidence, though only in two species, indicated that hyphal fusion may occur between different genotypes (Brasier, 1983). Furthermore, the mycelia of *Phytophthora* spp. have few crosswalls and many nuclei are present in a common

cytoplasm which could provide opportunities for the formation of multiple heterokaryons. There is, however, no sound genetic evidence for the occurrence, and importance of heterokaryons in *Phytophthora*.

4. Parasexuality

Parasexuality was broadly defined by Pontecorvo (1956) as the transfer of genetic material from one organism to another without meiosis or the development of specialised sexual structures. The parasexual cycle in *Phytophthora* would consist of three events; *(i)* fusion of different diploid nuclei, *(ii)* mitotic recombination, and *(iii)* diploidisation through loss of the extra chromosomes. The parasexual cycle was suggested as a possible means whereby new pathogenic phenotypes are produced by the somatic recombination of pathogenic genotypes (Leach and Rich, 1969). The parasexual process provides fungi with an alternative, although not very effective, method of genetic recombination. There is little evidence as to the significance, importance, and frequencies of parasexuality in natural fungal populations (Caten, 1981). In *Phytophthora*, hyphal anastomosis (Stephenson *et al.*, 1974), heterokaryon formation (Long and Keen, 1977; Layton and Kuhn, 1988a), and asexual karyogamy (Layton and Kuhn, 1988b) have all been reported independently, but evidence for a complete parasexual cycle has not yet been produced.

Population genetic studies on *P. infestans* in the United States and Europe using DNA fingerprinting, revealed that clonal lines of *P. infestans,* maintain their separate identities in the absence of sexual reproduction (Goodwin *et al.,* 1994). This suggests that parasexual recombination does not take place at detectable levels in *P. infestans,* although parasexuality has been suggested to explain diversity for virulence (Leach and Rich, 1969) and vegetative hybridity (Malcolmson, 1970). Goodwin (1997) argued that there is no evidence to support the high frequency of parasexual recombination that would be necessary to explain the results of those studies.

5. Cytoplasmic inheritance

Three classes of cytoplasmic genomes have been identified in fungi; mitochondrial genomes (Tzagoloff, 1982), DNA plasmid genomes (Gunge, 1983) and, double stranded RNA

mycoviral genomes (Buck, 1980). Cytoplasmic genomes can contribute to genetic diversity through copy number differences, mutation, heteroplasmosis, and recombination (Caten, 1987). The significance of this contribution to genetic diversity is small in comparison to the nuclear genome, but may lead to subtle continuous variation which may not divide the population into discreet phenotypic classes (Fincham *et al.*, 1979). Double stranded RNA has been used in population studies of *P. infestans* (Newhouse *et al.*, 1992; Tooley *et al.*, 1989), but not in any other *Phytophthora* spp.

Variation in uninucleate, single spore cultures of *Phytophthora,* which produce variants during vegetative growth and asexual reproduction, is often attributed to cytoplasmic factors (Caten and Jinks, 1968). Although the involvement of cytoplasmic factors has not been shown for *P. cinnamomi,* extensive variation was found among first generation single-zoospore progenies of field isolates, with less variation among progenies of single sporangia, terminal hyphal cultures and second and third generation zoospore derivatives (Shepherd and Pratt, 1974). Furthermore, colony morphology varied for three to five asexual generations of selfed-oospored cultures of *P. cinnamomi* (Zheng and Ko, 1996). A cytoplasmic mechanism of inheritance was suggested to account for those variations. Whittaker *et al.*, (1994) demonstrated that mitochondrial DNA was uniparentally inherited in their crosses of *P. infestans*. However, it was not possible to elucidate which parent contributed the antheridium and oogonium in the cross. Additional genetic studies using cytoplasmic and genomic DNA based markers are needed to reveal the importance of cytoplasmic DNA in the generation of genetic diversity.

6. Centre of origin

The level of genetic diversity is often directly related to the geographic area where a species evolved over any period of time, as for example is found for *P. infestans* in central Mexico (Tooley *et al.*, 1985; Fry *et al.*, 1992). A high level of genetic diversity in the centre of origin can be explained by mutations accumulated over long periods of time and the presence of both mating types, providing opportunities for sexual reproduction and a great variety of different allele combinations.

P. cinnamomi has a cosmopolitan distribution and various hypotheses exist regarding its centre of origin (Zentmyer, 1980, 1988). In South Africa, both mating types occur together in some areas. This, and because the A1 mating type could be found in remote areas of the south-western Cape region of South Africa, led to the suggestion that *P. cinnamomi* is indigenous to South Africa (von Broembsen and Kruger, 1985). However, solely the occurrence of A1 mating type isolates in native fynbos (unique flora of the south-western Cape - South Africa) and rivers draining native fynbos mountain catchment areas (von Broembsen, 1989), is not necessarily indicative of indigenous populations. The same situation has been found in Papua New Guinea where only the A1 mating type was recovered from native areas (Arentz and Simpson, 1986). The A1 mating type has also been frequently isolated from Taiwan (Ko *et al.*, 1978) and it was suggested that *P. cinnamomi* might be indigenous in Asia. Due to little variation in morphological and physiological characteristics of *P. cinnamomi* in Shanghai (east China), it was suggested that it was recently introduced in east China (Zhou *et al.*, 1992). A New Guinea-Malaysia-Celebes region as suggested by Zentmyer (1988), is still the most likely option as centre of origin for *P. cinnamomi.* A population genetic study on a global scale using neutral genetic markers is clearly needed to accurately define the centre of origin of *P. cinnamomi.*

7. Migration

Migration of a few pathogen isolates away from the centre of origin usually leads to the establishment of a new fungal population initially with a low level of genetic diversity, due to the so-called founder effect. These founder populations have a narrow genetic base and, therefore, show low levels of genetic diversity. After some time, a degree of genetic differentiation will appear among geographically separated populations. Only continued, high migration rates, which facilitate rapid movement of new genotypes between populations, results in the continued similarity of the two populations. A good example of migration and low levels of genetic diversity can be found in *P. infestans*. Only the A1 mating type migrated to Europe from Mexico in 1845, and although it had devastating effects on potato cultivation and resulted in starvation of many people (Large, 1940; Woodham-Smith, 1962), the founder population consisted of only a few or maybe even one clonal line. Levels of genetic diversity

in the United States and Europe were extremely low compared to the centre of origin, central Mexico (Goodwin *et al.,* 1994; Tooley *et al.*, 1985). However, shortly before 1980, A2 mating type and new A1 mating type isolates migrated to Europe and displaced the old, strictly asexual population. The presence of both mating types provided opportunities for sexual reproduction and thus levels of genetic diversity increased dramatically (Drenth *et al.,* 1994; Fry *et al.*, 1992, 1993; Spielman *et al.*, 1991).

The effectiveness of migration on the genetic structure of particular geographic populations, will largely depend on the mode of dispersal of such a fungus. Species producing airborne spores such as *P. infestans*, have an obvious advantage to spread from one geographical area to another, compared to soilborne pathogens such as *P. cinnamomi*. On the other hand, quarantine measures were virtually non-existent until the 1900's, which provided multiple opportunities for *P. cinnamomi* to spread around the world with infested soil or plant material. That *P. cinnamomi* has indeed spread around the world is without doubt and can be seen by the global distribution of both mating types (Zentmyer 1980, 1988). For localised spread, *P. cinnamomi* also relies on soil movement by man, animals and free water, which is restricted by the slope of the specific area. Unfortunately, population studies on the levels of genetic diversity or similarity between populations of *P. cinnamomi* is lacking. It is, therefore, difficult to determine the effect of migration on *P. cinnamomi* populations.

8. Genetic drift

Genetic drift relates to changes in allelic frequencies resulting from change alone. Change can be either seasonal reductions in population size due to unfavorable environmental conditions, and/or founder effects. The magnitude of allele frequency change in each generation depends on the effective population size, reflected in the number of genotypically distinct individuals in the population (Futuyma, 1986). Plant pathogen populations, often introduced from other parts of the world and showing low levels of gene and genotypic diversity, have small effective population sizes and, therefore, are especially vulnerable to genetic drift. In addition to these founder effects, plant pathogen populations in agricultural and forestry systems often pass through severe bottle necks which significantly reduce the effective population size (Nei *et al.*, 1975). These human induced bottle necks involve harvest

of the crop, crop rotation, fungicide use, and deployment of resistance genes. Such practices often severely reduce the population size of the pathogen or even lead to local extinction. High rates of local extinction and subsequent recolonisation make these populations extremely vulnerable to genetic drift as noted for *P. infestans* in the Netherlands (Drenth *et al.*, 1994) and United States (Goodwin *et al.*, 1995a, 1995b). However, molecular data to estimate the full effects of genetic drift on populations of *Phytophthora* spp. are still lacking and need to be addressed.

9. Selection

Maintenance of genes in a pathogen population, whether introduced by mutation or migration, will depend on selection forces in that specific environment. Natural selection changes the frequency of alleles in a population by giving a reproductive advantage to those individuals with favoured combinations of alleles, providing them with a higher level of fitness. Depending on the fitness differences in a population and the mode of reproduction, selection has the ability to rapidly change the genetic structure of a population (Fincham, 1983). Deployment by breeders of specific resistance genes in the host, is probably a common cause of selection in which pathogen genotypes without the appropriate virulence alleles, will be eliminated from the population. In large scale, uniform, monocultured host systems, this will result in the occurrence and presence in high frequency of particular genotypes of the pathogen with specific virulence alleles.

The influence of selection on the genetic structure of *Phytophthora* populations is particularly important in host specific species such as *P. infestans* and *P. sojae*. In *P. infestans*, specific clonal lines in Europe may have been completely lost by the sequential deployment of potato cultivars with new resistance genes. Genotypes which could overcome that resistance, had by default, greater fitness and reproduced rapidly, resulting in an increased frequency of such specific clonal lines. The end result of this continuous selection process in a strictly asexual population was many different races, which possessed an identical genetic background (Drenth *et al.*, 1994). The introduction and large scale application of the fungicide metalaxyl also resulted in the selection of metalaxyl resistant genotypes of *P. infestans* by reducing or eliminating metalaxyl sensitive genotypes. As a result, the frequency of metalaxyl

resistant genotypes increased (Davidse *et al.*, 1989). In *P. sojae*, deployment of specific resistance genes in soybean lead to the selection of similar races in Australia and in the United States. However, these identical races had evolved independently in different geographical areas and had different genetic backgrounds (Drenth *et al.*, 1996). Nevertheless, the influence of selection, other than virulence and fungicide resistance, on the genetic structure of *Phytophthora* populations, is unknown. Furthermore, the effect of selection on the genetic structure of *P. cinnamomi* populations, has not been studied at all.

VI. GENETIC DIVERSITY IN *P. CINNAMOMI*

Variation in pathogenicity among different *P. cinnamomi* isolates, has been observed on different hosts (Crandall *et al.*, 1945; Manning and Crossan, 1966; Zentmyer and Guillemet, 1981). A difference in pathogenicity between isolates of different mating types has also been suggested (Galindo and Zentmyer, 1964). The authors reported that an A1 mating type isolate from Hawaii was less pathogenic to avocado roots and not pathogenic to avocado stem tissue, compared with fifteen pathogenic A2 mating type isolates tested, but no connection has been identified between growth rate and pathogenicity of *P. cinnamomi* isolates from pineapple (Mehrlich, 1936). Unfortunately, sample sizes used in these studies were extremely small making it difficult to draw reliable conclusions on variation in pathogenicity. In a more recent and complete study, significant variation in pathogenicity among Australian *P. cinnamomi* isolates to *E. marginata* was demonstrated (Dudzinski *et al.*, 1993). Pathogenicity was unrelated to mating type or isozyme properties and the authors concluded that pathogenicity is a relative stable characteristic.

Population genetic studies in *P. cinnamomi* have been limited to two isozyme studies (Old *et al.*, 1984, 1988) and a RAPD study (Chang *et al.*, 1996). The latter study hypothesised a lack of sexual reproduction in *P. cinnamomi* in Taiwan due to high levels of genetic differentiation between mating type isolates as tested in 10 isolates from the same location. The isozyme studies revealed a relatively uniform population structure with two A2 and two A1 multilocus isozyme genotypes of *P. cinnamomi* in Australia (Old *et al.*, 1984, 1988). A

small *P. cinnamomi* population from Papua New Guinea showed higher levels of genetic diversity in the A1 mating type population (seven A1 multilocus isozyme genotypes), while the A2 mating type population was resolved in only two multilocus isozyme genotypes (Old *et al.,* 1984). Overall levels of genetic diversity in populations of *P. cinnamomi* from Australia and Papua New Guinea were, lower than expected from a heterothallic, outbreeding Oomycete (Goodwin, 1997), and indicated the introduction of *P. cinnamomi* to Australia.

VII. AIMS OF RESEARCH DESCRIBED IN THIS DISSERTATION

In this review, special attention has been given to the population genetic structure of *P. cinnamomi,* and possible ways in which levels of genetic diversity are obtained and maintained. Little is known about levels of genetic diversity and genetic structure of *P. cinnamomi* populations, despite its importance as a plant pathogen. Its origin is also, still unknown. Due to the importance of *P. cinnamomi* in forestry and agricultural industries in South Africa, the overall aim of the research described in this dissertation is to determine levels of genetic diversity and the genetic structure of South African *P. cinnamomi* populations. Markers which will be used include; growth rate, pathogenicity to *E. smithii*, isozymes, RAPDs, and RFLP's. South African *P. cinnamomi* populations are also compared with an Australian *P. cinnamomi* population, which is known to be introduced and contains low levels of genetic diversity (Old *et al.,* 1984, 1988). Specific attention is also given to the possibility of sexual reproduction and the formation of oospores. Crossing experiments between different mating type isolates are conducted to confirm the compatibility and outbreeding nature of different mating type populations in addition to the viability and pathogenicity of hybrid oospores.

Specific questions which will be addressed in this dissertation include; *(i)* levels of genetic diversity in South African *P. cinnamomi* populations, *(ii)* clonal fractions of those populations, *(iii)* occurrence of particular genotypes in South Africa, *(iv)* relationship between A1 and A2 mating type populations, *(v)* mode of reproduction, *(vi)* possible changes in population structure over time, *(vii)* variation in levels of pathogenicity to *E. smithii* under

field conditions, *(viii)* comparison of the population structure of South African and Australian *P. cinnamomi* populations, *(ix)* viability of oospores, *(x)* possibility of outbreeding among different mating type populations, and *(xi)* establish that oospores form hybrid and pathogenic progeny. The assessment of levels of gene and genotypic diversity will give insight into the centre of origin, the occurrence of sexual reproduction, and the occurrence and spread of particular clonal genotypes in the population of *P. cinnamomi*. This information will be useful in breeding and selection for resistance against *P. cinnamomi*, and in the implementation of effective and durable disease control strategies.

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Chapter 2

Population Structure of *Phytophthora cinnamomi* in South Africa

Adopted from:

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ABSTRACT

Isozymes were used to analyze *Phytophthora cinnamomi* isolates collected from 1977 to 1986 and 1991 to 1993 in two regions in South Africa. A total of 135 isolates was analyzed for 14 enzymes representing 20 putative loci, of which four were polymorphic. This led to the identification of nine different multilocus isozyme genotypes. Both mating types of *P. cinnamomi* occur commonly in the Cape region, whereas predominantly the A2 mating type occurs in the Mpumalanga region of South Africa. A2 mating type isolates could be resolved into seven multilocus isozyme genotypes, compared with only two multilocus isozyme genotypes for the A1 mating type isolates. Low levels of gene (0.115) and genotypic diversity (2.4%), and a low number of alleles per locus (1.43) were observed for the South African *P. cinnamomi* population. The genetic distance between the Cape and Mpumalanga *P. cinnamomi* populations was relatively low (D_m = 0.033) and no specific pattern in regional distribution of multilocus isozyme genotypes could be observed. The genetic distance between the "old" (isolated between 1977 and 1986) and "new" (isolated between 1991 and 1993) *P. cinnamomi* populations from the Cape was low (D_m = 0.033), indicating a stable population over time. Three of the nine multilocus isozyme genotypes were specific to the "old" population and only one multilocus isozyme genotype was specific to the "new" population. Significant differences in allele frequencies, a high genetic distance (D_m = 0.117) between the Cape A1 and A2 mating type isolates, significant deviations from Hardy-Weinberg equilibrium, a low overall level of heterozygosity, and a high fixation index (0.71) all indicate that sexual reproduction occurs rarely if at all in the South African *P. cinnamomi* population.

The Oomycete *Phytophthora cinnamomi* Rands is a notorious soil-borne plant pathogen with a wide host range (Zentmyer, 1980). It was first recorded in South Africa in 1931 (Doidge and Bottomley, 1931; Wager, 1931) and led to diseases of major economic importance, e.g. root disease of *Eucalyptus* and *Pinus* species (Linde *et al.*, 1994: Wingfield and Knox-Davies, 1980) and root rot of avocado (Wager, 1942). It is also a pathogen of grapevines (Van der Merwe *et al.*, 1972), commercially cultivated *Protea* species (Von Broembsen and Brits, 1985), and native undisturbed fynbos vegetation (Von Broembsen and Kruger, 1985) which is an unique floral kingdom of the southwestern Cape, South Africa.

P. cinnamomi is diploid and heterothallic with two mating types, A1 and A2 (Galindo and Zentmyer, 1964). Sexual reproduction in heterothallic *Phytophthora* species occurs when gametangia of opposite mating type meet in host tissue, leading to the formation of oospores. Oospores can survive for long periods of time, in or outside the host. *P. cinnamomi* has a global geographical distribution in which the A2 mating type is more frequently isolated than the A1 mating type (Zentmyer, 1980, 1988). In South Africa, the A2 mating type has usually been associated with agricultural crops and cultivated forests (Von Broembsen, 1984). It was also prevalent in native forests, where both mating types sometimes occurred together at the same site. In native undisturbed fynbos vegetation and rivers draining mountain catchments, only the A1 mating type has been found (Von Broembsen, 1989). This was also the case in Papua New Guinea where the A1 mating type has been recovered exclusively from native areas (Arentz and Simpson, 1986).

The centre of origin of *P. cinnamomi* is unknown. Based on the observation that many plants in Asia are resistant to *P. cinnamomi*, Crandall and Gravatt (1967) hypothesized that *P. cinnamomi* originated there. Zentmyer (Zentmyer, 1980, 1988) suggested the New Guinea-Celebes-Malaysia region, possibly extending into north-eastern Australia. However, recent evidence indicates that *P. cinnamomi* has been introduced into east Asia (Zhou *et al.*, 1992). Furthermore, isozyme studies on *P. cinnamomi* in Australia indicated low levels of genetic diversity and the absence of sexual reproduction, indicative of an introduced pathogen (Old *et al.*, 1988; Old *et al.*, 1988). High levels of genetic diversity were found among A1 mating type isolates from Papua New Guinea, indicating a possible centre of origin for *P. cinnamomi*

(Old *et al.*, 1988). Taiwan (Ko *et al.*, 1978) and South Africa (Von Broembsen and Kruger, 1985) have also been hypothesized as having indigenous *P. cinnamomi* populations.

The level of genetic diversity in fungi is usually the highest in isolates obtained from the centre of origin. This has, for example, been revealed by an isozyme study with *Phytophthora infestans* where many isozyme alleles, the occurrence of sexual reproduction, and a high level of genetic diversity was identified for isolates from central Mexico, the presumed centre of origin of this fungus, compared to elsewhere in the world (Spielman *et al.*, 1991; Tooley *et al.*, 1985). In addition, DNA fingerprinting revealed high levels of genotypic diversity in *P. infestans* isolates from central Mexico, to such an extent that almost every isolate had an unique genotype (Fry *et al.*, 1992; Goodwin *et al.*, 1992). However, the presence of both mating types does not necessarily imply the occurrence of sexual reproduction, as low levels of genetic diversity were found among *P. infestans* isolates in northwestern Mexico where both mating types were present (Goodwin *et al.*, 1992). *Phytophthora* species can rapidly reproduce asexually through the formation of large numbers of sporangiospores, which can germinate either directly, or differentiate into motile zoospores. Asexual, or clonal reproduction, may lead to lower levels of genotypic diversity, compared to sexual reproduction.

The aims of the current investigation were to; (*i*) assess the level of gene and genotypic diversity in the South African *P. cinnamomi* population using isozymes, (*ii*) study the occurrence of particular multilocus isozyme genotypes, (*iii*) compare the A1 and A2 mating type populations, (*iv*) test for the presence of sexual reproduction in the South African *P. cinnamomi* population, and (*v*) study changes in population structure over time. The assessment of levels of gene and genotypic diversity will give insight into the centre of origin, the frequency of sexual reproduction, and the occurrence and spread of particular genotypes in the pathogen population. This information may be important for breeding and selection for resistance against *P. cinnamomi* and the implementation of effective disease control strategies.

MATERIALS AND METHODS

Sources of isolates. One hundred and thirty-five isolates of *P. cinnamomi* collected between 1977 and 1993 from the south and southwestern Cape region (collectively referred to as Cape) and from the northeastern parts of South Africa (collectively referred to as Mpumalanga) (Fig. 1) were selected for this study. These isolates were obtained from a broad variety of host plants. Isolates collected from 1977 to 1986 are referred to as "old" isolates and those collected from 1991 to 1993 as "new" isolates.

Isolations from soil were made by baiting with lupin seedlings (Chee and Newhook, 1965) and *Citrus* leaf discs (Grimm and Alexander, 1973). Direct isolations from diseased host tissue were performed onto a selective medium (Tsao and Guy, 1977). Possibly infested water was filtered through 0.3 µm Nucleopore polycarbon membrane filters (Nucleopore Corporation, Pleasanton, CA). The filters were then plated onto a selective medium (Tsao and Guy, 1977). All isolates have been maintained in sterilized water at room temperature and are part of the culture collection of the Tree Pathology Cooperative Programme, Department of Microbiology and Biochemistry, University of the Orange Free State, Bloemfontein, South Africa.

Determination of mating type. The mating type of all isolates was determined by pairing with isolates of known mating type on carrot agar plates (Ribeiro, 1978). Plates were incubated at 20°C in the dark and examined for the presence of oospores after 4 weeks.

Mycelium production and enzyme extraction. Isolates for isozyme analyses were cultured in clarified V8 broth at 25°C in the dark. After 5 days, excess liquid was drained from the resultant mycelial mats before further drying between sterile paper towels. Approximately 0.5 g of mycelial tissue was transferred to 1.5 ml microcentrifuge tubes and 0.5 ml extraction buffer (0.1 M Tris/HCL, pH 7, 20 mg/ml polyvinylpyrrolidone) were added before freezing in liquid nitrogen. The frozen mycelium was then ground with a rod in a microcentrifuge tube and the crude enzyme suspension absorbed onto paper wicks (Oudemans and Coffey, 1991). Paper wicks were stored at -70°C until use.

Isozyme analyses. Fourteen different enzymes and corresponding buffer systems were used in this study (Table 1). Procedures for starch-gel electrophoresis followed those of Oudemans and Coffey (1991). Buffers were prepared according to the following; Morpholine citrate buffers at pH 6 (MC6), pH 7 (MC7) and pH 8 (MC8) (Clayton and Tetriak, 1972), Tris-EDTA-borate buffer (TEB) (Markert and Faulhaber, 1965), and a discontinuous buffer system (LiOH) (Ridgeway *et al.*, 1970). Enzyme staining recipes were similar to those used by Oudemans and Coffey (1991), except for final volume, as agar overlays were used to reduce costs. The staining procedure for SOD was identical to that of Oudemans and Coffey (1991). Mycelium production, enzyme extraction, and isozyme analyses were repeated three times to ensure reliability of results. For comparison between gels, isolates representing the different alleles were used as standards.

Data analysis. Each band identified was treated as an allele of a specific locus. Because *Phytophthora* species are diploid (Brasier and Sansome, 1975), genetic interpretation of banding patterns was based on that of a diploid organism. Isozyme alleles of each isolate were scored and combined to generate a multilocus isozyme genotype. Isolates with the same overall multilocus isozyme genotype were considered clonal.

Genotypic diversity (Stoddard and Taylor, 1988) was calculated on the basis of the number of multilocus isozyme genotypes in the two regions studied in South Africa (Cape and Mpumalanga), as well as in the "old" and "new" *P. cinnamomi* populations within the two regions. To compare levels of genotypic diversity ($\hat{G}$) between regions, the individual diversity values were divided by the sample size of each region (McDonald *et al.*, 1994) to calculate the percentage maximum possible diversity obtained ($\hat{G}/N$ %).

Gene diversity for the four different South African *P. cinnamomi* populations was calculated using Nei's gene diversity (Nei, 1973). The probable mating system was determined by comparing the observed proportion of heterozygotes with the expected level of heterozygosity. Wright's fixation index, $F = 1 - (H_{obs} / H_{exp})$, was used for this purpose where H_{obs} is the observed mean heterozygosity per locus and H_{exp} is the expected mean heterozygosity in the population which is the same as Nei's gene diversity (Brown, 1979).

Regional and temporal differentiation between *P. cinnamomi* populations was determined using differences in allele frequencies. Based on allele frequencies of the four polymorphic

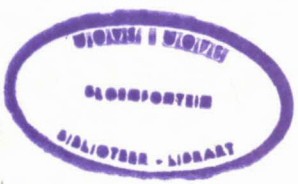
isozyme loci, population differentiation between the subpopulations from the Cape and Mpumalanga regions, between the "old" and "new" populations of the Cape, as well as between A1 and A2 mating type populations of the Cape, was determined using an unbiased minimum genetic distance (D_m) (Nei, 1978).

Frequencies of genotypes at the four polymorphic isozyme loci in the "old" and "new" populations of the Cape region were tested for deviations from expected Hardy-Weinberg equilibrium using the BIOSYS-1 Statistical Package (Swofford and Selander, 1981). No correction for clonal genotypes was conducted as this would lead to an unacceptably small population size ($n = 9$).

RESULTS

Overall diversity in South Africa. Both *P. cinnamomi* mating types were isolated from the Cape (63 A1 and 46 A2) and Mpumalanga (one A1 and 25 A2) regions. A total of 64 A1 and 71 A2 mating type isolates were obtained. The A2 mating type isolates were predominantly from commercial crops, but also from native fynbos vegetation, in contrast to A1 mating type isolates which were predominantly from native fynbos vegetation in the Cape region. Regional distribution of mating type isolates shows that the A1 mating type was primarily in the Cape region with only one A1 mating type isolate recovered from the Mpumalanga region. The A2 mating type occurred commonly in both regions.

Twenty putative loci were observed for the 14 enzymes examined. Polymorphisms were observed for only four enzymes: IDH; ACO; M6PI; and SOD with the first three enzymes each having four alleles and SOD with only two alleles (Fig. 2). The other 10 enzymes examined were all monomorphic with two putative loci observed at AK, DIA, HEX, LDH, MDH. Two putative loci were also observed at SOD, of which one locus was monomorphic (Fig. 2D). Based on the 14 different alleles of the four polymorphic loci, nine different multilocus isozyme genotypes could be distinguished among the 135 isolates analyzed (Table 2). The 71 A2 mating type isolates could be resolved into seven different multilocus isozyme genotypes with genotype 6 being the most common (Table 2). Among the 64 A1 mating type isolates analyzed, 63 were of multilocus isozyme genotype 4 and one was of multilocus



isozyme genotype 5 (Table 2). The overall levels of gene and genotypic diversity for the South African *P. cinnamomi* population were; H_{exp} = 0.115 and $\hat{G}/N$ = 2.4%, respectively (Table 3).

Regional and temporal diversity. In the Cape region, genotype 9 could be identified only among the "new" isolates whereas multilocus isozyme genotypes 2, 3, 5, and 8 could be identified only in the "old" population. In the Mpumalanga region, multilocus isozyme genotype 4 was identified only in the "old" population (Table 2).

There was no association between multilocus isozyme genotype and host. The highest number of different multilocus isozyme genotypes was recovered from *Pinus* species (7 genotypes out of 11 isolates), *Ocotea bullata* (six genotypes out of 39 isolates), and *Eucalyptus* species (5 genotypes out of 11 isolates). Two isolates recovered from the same tree represented different multilocus isozyme genotypes (1 and 2, respectively). Overall levels of genotypic diversity were low (Table 3).

Similar numbers of different alleles per locus were observed for "old" and "new" Cape and Mpumalanga *P. cinnamomi* populations. Levels of gene diversity observed for the "old" (H_{exp} = 0.072) and the "new" (H_{exp} = 0.105) *P. cinnamomi* populations from the Cape region, were also similar. The slightly higher levels of gene diversity observed for the "new" *P. cinnamomi* population from the Cape region, resulted in a lower fixation index for the "new" compared to the "old" Cape *P. cinnamomi* population. The same was observed for the Mpumalanga *P. cinnamomi* population (Table 3). Allele frequencies between regional and temporal *P. cinnamomi* populations were dissimilar (Table 4). The genetic distance between the two regions (D_m = 0.033) and between the "old" and "new" (D_m = 0.033) *P. cinnamomi* populations from the Cape region was low (Table 5).

Sexual reproduction in South Africa. A low level of heterozygosity (H_{obs} = 0.033) and a high fixation index (F = 0.71) were observed for the South African *P. cinnamomi* population (Table 3). The "old" and "new" *P. cinnamomi* populations from the Cape region were analyzed for conformance to expected Hardy-Weinberg equilibrium at the four polymorphic isozyme loci. Seven of the eight Hardy-Weinberg analyses deviated significantly from the assumption of random mating. Only allele frequencies at the SOD locus in the "old" Cape population were in equilibrium. Alleles are shared among the A1 and A2 mating type

populations only at the SOD locus (Table 4) without any polymorphism among the A1 mating type population. The genetic distance (D_m = 0.117) between the mating type populations was high (Table 5).

DISCUSSION

Our isozyme study on 135 isolates of *P. cinnamomi* from South Africa revealed lower levels of genotypic diversity than would be expected from a heterothallic, outbreeding organism. The low number of isozyme alleles per locus indicates that *P. cinnamomi* was probably introduced to South Africa. The small number of clones, significant deviation from Hardy-Weinberg equilibrium for genotype frequencies, and a high level of differentiation between the A1 and A2 mating type populations indicate that sexual reproduction probably does not play an important role in the South African *P. cinnamomi* population.

The level of isozyme gene diversity for the South African *P. cinnamomi* population (H_{exp} = 0.115) is similar to that of the Australian *P. cinnamomi* population (Old *et al.*, 1988), as reviewed by Goodwin (1997). Thus, the South African *P. cinnamomi* population is very similar to the Australian one with respect to the presence of both mating types, the apparent lack of sexual reproduction, and an introduced pathogen population.

The low level of isozyme gene diversity is also reflected in low levels of genotypic diversity for the South African *P. cinnamomi* population. Comparison of genotypic diversities revealed similarity between the two regions. The higher level of genotypic diversity observed for the Mpumalanga population might be due to the small sample size from this region. A low level of maximum genotypic diversity (2.4%) in the Cape *P. cinnamomi* population, is partially due to a high number of A1 mating type isolates of multilocus isozyme genotype 4.

There are three lines of evidence that indicate the absence or infrequent occurrence of sexual reproduction in the South African *P. cinnamomi* population. First, allele frequencies in only one of the eight tests were in Hardy-Weinberg equilibrium. Second, some alleles present in the A1 population were not found in the A2 mating type population (Table 4), and genetic distance (D_m = 0.117) was relatively high between A1 and A2 mating type populations from the Cape. This was significantly higher than the genetic distance values between regions and

among the "old" and the "new" *P. cinnamomi* population from the Cape. Infrequent genetic recombination is the most likely explanation for the observed high level of differentiation between mating type populations which apparently lack a common gene pool. Third, the low level of observed heterozygosity and the high fixation index (F = 0.71), indicate that the population is not randomly mating (Brown, 1979).

In a review by Goodwin (1997), fixation indices of various *Phytophthora* species were compared. From these it is evident that the *P. cinnamomi* population from Papua New Guinea had a high level of heterozygosity and a fixation index of 0.34, which is consistent with a random mating population. The Australian *P. cinnamomi* population had a fixation index of 0.56 which significantly deviates from that expected of a randomly mating *Phytophthora* population (1997). The South African *P. cinnamomi* population, with a fixation index of 0.71, compares well with the Australian population in this regard. Based on isozyme data, a lack of genetic recombination in the Australian *P. cinnamomi* population was demonstrated by Old *et al.* (Old *et al.*, 1984). Populations of *P. cinnamomi* in Australia and South Africa seem to approach homozygosity as experienced for homothallic *Phytophthora* species. However, more genetic markers should be employed to determine the actual levels of heterozygosity more precisely to identify the importance of sexual reproduction in populations of *P. cinnamomi*. Population size and regional distribution of isolates also should be considered.

Gene diversity in the South African *P. cinnamomi* population is fairly stable over time as indicated by the low genetic distance (D_m = 0.033) between the "old" and "new" *P. cinnamomi* populations from the Cape region. Also, the two regional populations appear to be similar as a low genetic distance (D_m = 0.033) was obtained between populations. Overall, three multilocus isozyme genotypes identified in 1977 to 1986 were not identified again in 1991 to 1993. This is also reflected in the presence of more alleles in the "old" compared to the "new" *P. cinnamomi* population in both regions. The inability to detect these specific alleles especially occurred in the Cape population, which may be due to a smaller number of isolates sampled from the "new" population. In addition, multilocus isozyme genotype 9 was identified only in the "new" Cape population. Multilocus isozyme genotype 4 could not be found in the "new" Mpumalanga population but could have escaped isolation as it was rarely encountered in the "old" Mpumalanga population.

This study does not answer the question of origin of *P. cinnamomi*. A higher level of genetic diversity would be expected in the *P. cinnamomi* population if it was indigenous to South Africa as hypothesized by von Broembsen and Kruger (1985). The low levels of gene and genotypic diversity in the South African *P. cinnamomi* population are not consistent with a South African origin. Commercial forestry in South Africa has been established in the Cape region and probably acted as a means of distribution for *P. cinnamomi* A2 mating type isolates in forestry areas. This is evident as a high number of different multilocus isozyme genotypes was observed in isolates from *Pinus* species (seven genotypes out of 11 isolates) and *Eucalyptus* species (5 genotypes out of 11 isolates) in forestry plantations. A high number of different multilocus isozyme genotypes was also observed from *Ocotea bullata* (native forest tree species) (6 genotypes out of 39 isolates). However, this may be partly due to a higher number of isolates analyzed from this host compared to *Pinus* and *Eucalyptus* species. *Ocotea* trees occur in the same areas as the commercial forest plantations and it is possible that the A2 mating type spread from the plantations to the *Ocotea*. To determine the origin of *P. cinnamomi*, a comprehensive study on a global scale is needed where populations from Papua New Guinea, South Africa, Australia, and Asia in particular are compared. Among the four geographical areas mentioned, Papua New Guinea seems the most likely option as isozyme studies have already indicated the presence of high levels of gene diversity (Old *et al.*, 1984, 1988).

The locus for the aconitase enzyme was previously reported as heterozygous for *P. cinnamomi* populations from Papua New Guinea, China, and Taiwan (Old *et al.*, 1984; Oudemans and Coffey, 1991). In this study, the ACO locus was always homozygous in the South African *P. cinnamomi* population. This may either be due to different enzyme buffer systems employed which could have resulted in the detection of different alleles, or simply due to the lack of ACO heterozygotes in the South African population. The absence of heterozygosity at the ACO locus in the South African population was also observed by Oudemans and Coffey (1991). This may indicate the introduction of only *P. cinnamomi* isolates homozygous at the ACO locus and the lack of genetic recombination between isolates homozygous for different alleles.

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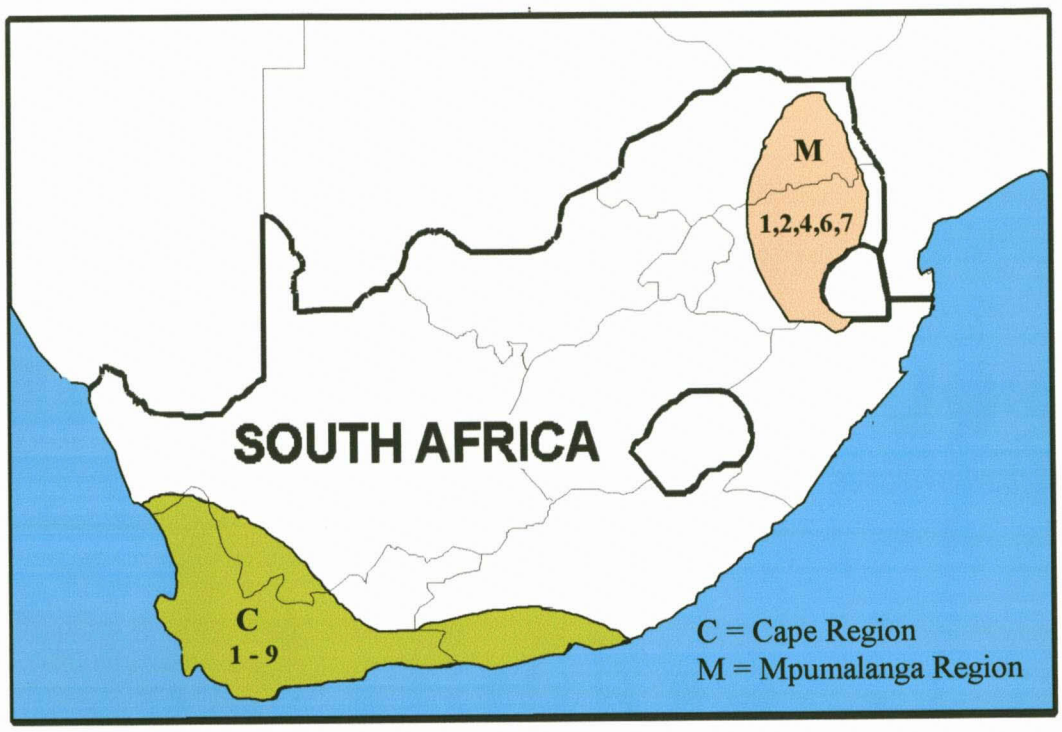

Fig. 1. Map of South Africa showing the different multilocus isozyme genotypes of *Phytophthora cinnamomi* collected from the Cape (C) and Mpumalanga (M) regions.

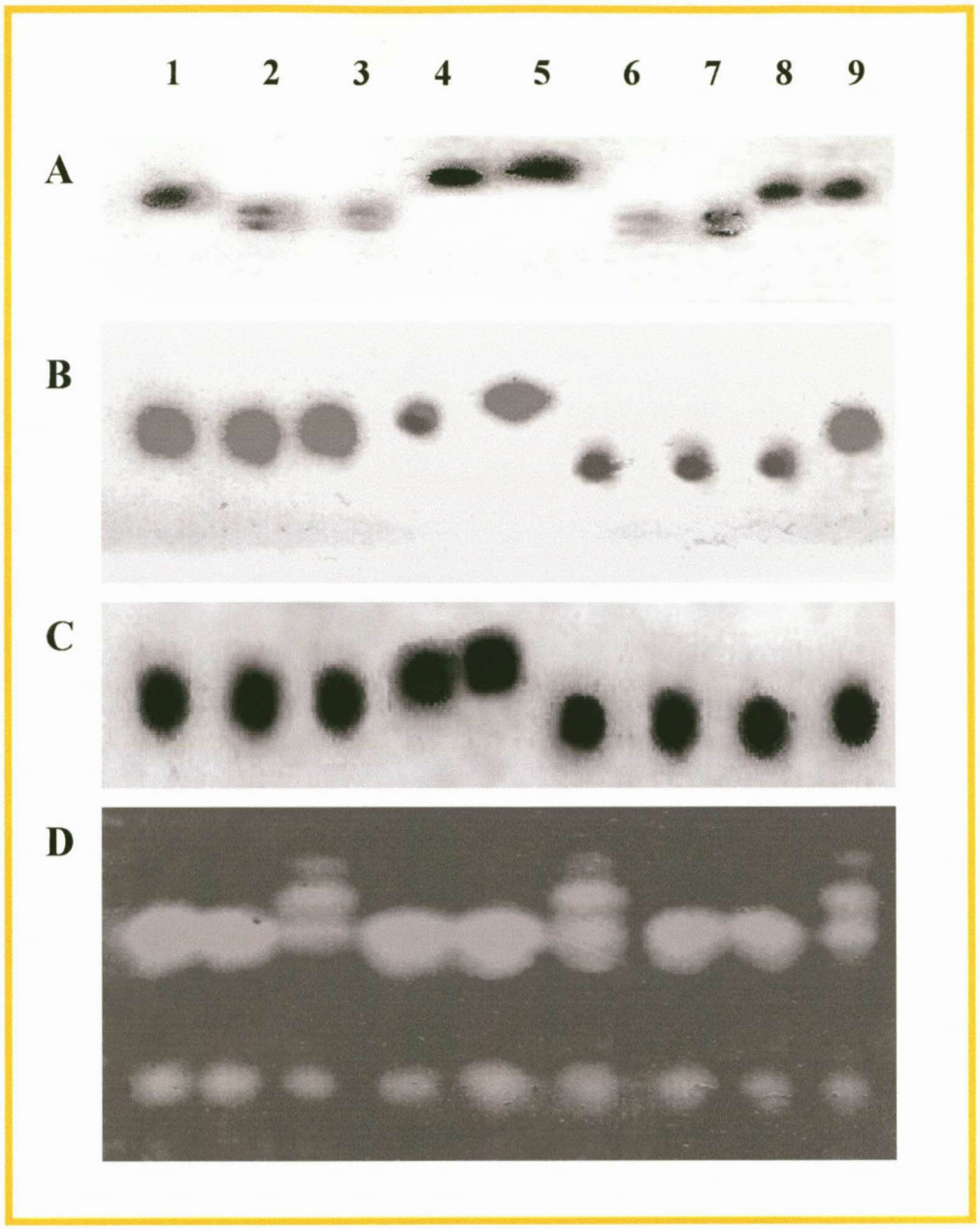

Fig. 2. Isozyme patterns for four polymorphic enzymes in South African *Phytophthora cinnamomi* isolates. Lanes 1 to 9 represent multilocus isozyme genotypes 1 to 9. **A,** Isocitrate dehydrogenase, **B,** aconitate hydratase, **C,** mannose-6-phosphate isomerase, and **D,** superoxide dismutase showing monomorphic locus at the bottom.

TABLE 1. Enzyme stains and corresponding buffer systems used for isozyme analysis of South African *Phytophthora cinnamomi* isolates

Enzyme system	EC. number	Abbreviation	Buffer[z]
Oxireductases			
Diaphorase	1.6.4.3	DIA	LiOH
Glucose-6-phosphate dehydrogenase	1.1.1.49	G6PDH	LiOH
Isocitric dehydrogenase	1.1.1.42	IDH	MC8
Lactate dehydrogenase	1.1.1.27	LDH	LiOH
Malate dehydrogenase	1.1.1.37	MDH	LiOH
Malic enzyme	1.1.1.40	ME	LiOH
Phosphogluconate dehydrogenase	1.1.1.44	6PGD	TEB
Superoxide dismutase	1.15.1.1	SOD	LiOH
Transferases			
Adenylate kinase	2.7.4.3	AK	TEB
Hexokinase	2.7.1.1	HEX	TEB
Isomerases			
Glucose-6-phosphate isomerase	5.3.1.9	GPI	MC7
Mannose-6-phosphate isomerase	5.3.1.8	M6PI	MC6
Others			
Aconitate hydratase	4.2.1.3	ACO	MC6
Fructose-1,6-diphosphatase	3.1.3.11	F16DP	TEB

[z] Morpholine citrate buffers at pH 6 (MC6), pH 7 (MC7), and pH 8 (MC8); Tris-EDTA-borate buffer (TEB); and a discontinuous buffer system (LiOH).

TABLE 2. Alleles present in South African isolates of *Phytophthora cinnamomi* in each of the nine different multilocus isozyme genotypes

Genotype	Locus[x]				Mating type	No. of isolates						
	IDH	ACO	M6P1	SOD		Cape			Mpumalanga			South Africa
						"Old"[y]	"New"[z]	Total	"Old"	"New"	Total	total
1	BB	BB	BB	AA	A2	8	2	10	4	2	6	16
2	AB	BB	BB	AA	A2	4	0	4	1	2	3	7
3	AB	BB	BB	AB	A2	1	0	1	0	0	0	1
4	CC	CC	CC	AA	A1	52	10	62	1	0	1	63
5	DD	DD	DD	AA	A1	1	0	1	0	0	0	1
6	AB	AA	AA	AB	A2	2	19	21	7	7	14	35
7	AB	AA	AA	AA	A2	2	3	5	1	1	2	7
8	BB	AA	AA	AA	A2	3	0	3	0	0	0	3
9	BB	BB	BB	AB	A2	0	2	2	0	0	0	2
No. of alleles	4	4	4	2								

[x] IDH = isocitric dehydrogenase; ACO = aconitate hydratase; M6PI = mannose-6-phosphate isomerase; and SOD = superoxide dismutase.

[y] "Old" = collected from 1977 to 1986.

[z] "New" = collected from 1991 to 1993.

TABLE 3. Summary statistics for "old" (1977-1986) and "new" (1991-1993) *Phytophthora cinnamomi* populations from the Cape and Mpumalanga regions in South Africa based on 30 isozyme alleles from 20 putative loci

	Cape			Mpumalanga			South Africa
	"Old"	"New"	Total	"Old"	"New"	Total	total
Genotypes	1-8	1,4,6,7,9	1-9	1,2,4,6,7	1,2,6,7	1,2,4,6,7	1-9
$\hat{G}$[u]	1.9	2.7	2.6	2.9	2.5	2.8	3.3
$\hat{G}/N$ (%)[v]	2.6	7.5	2.4	20.6	20.7	10.6	2.4
A[w]	1.71	1.50	1.71	1.50	1.29	1.50	1.43
H_{obs}[x]	0.008	0.060	0.025	0.050	0.071	0.063	0.033
H_{exp}[y]	0.072	0.105	0.104	0.104	0.083	0.095	0.115
F[z]	0.89	0.43	0.76	0.52	0.14	0.34	0.71

[u] $\hat{G}$ = Genotypic diversity (Stoddard & Taylor, 1988).

[v] $\hat{G}/N$ (%) = The percentage of maximum possible diversity obtained.

[w] A = Mean number of different alleles per locus.

[x] H_{obs} = Observed heterozygosity.

[y] H_{exp} = Expected heterozygosity (= Nei's gene diversity index [Nei, 1973]).

[z] F = Wright's fixation index.

TABLE 4. Allele frequencies of "old" (1977-1986) and "new" (1991-1993) and A1 and A2 mating type populations of *Phytophthora cinnamomi* from the Cape and Mpumalanga regions in South Africa

Locus[z]	Allele	Cape		Mpumalanga		Mating type	
		"Old"	"New"	"Old"	"New"	A1	A2
IDH	A	0.062	0.306	0.321	0.417	0.000	0.352
	B	0.212	0.417	0.607	0.583	0.000	0.648
	C	0.712	0.278	0.071	0.000	0.984	0.000
	D	0.014	0.000	0.000	0.000	0.016	0.000
ACO	A	0.096	0.611	0.571	0.667	0.000	0.634
	B	0.178	0.111	0.357	0.333	0.000	0.366
	C	0.712	0.278	0.071	0.000	0.984	0.000
	D	0.014	0.000	0.000	0.000	0.016	0.000
M6PI	A	0.096	0.611	0.571	0.667	0.000	0.634
	B	0.178	0.111	0.357	0.333	0.000	0.366
	C	0.712	0.278	0.071	0.000	0.984	0.000
	D	0.014	0.000	0.000	0.000	0.016	0.000
SOD	A	0.979	0.708	0.750	0.708	1.000	0.732
	B	0.021	0.292	0.250	0.292	0.000	0.268

[z] IDH = isocytric dehydrogenase; ACO = aconitate hydratase; M6PI = mannose-6-phosphate isomerasee; and SOD = superoxide dismutase.

TABLE 5. Unbiased minimum genetic distance (D_m) among *Phytophthora cinnamomi* mating type and temporal populations from the Cape region, and among regional populations in South Africa

	D_m		
Locus[y]	A1:A2	"Old":"New"[z]	Cape:Mpumalanga
IDH	0.758	0.139	0.209
ACO	0.749	0.224	0.214
M6PI	0.749	0.224	0.214
SOD	0.066	0.070	0.021
All monomorphic loci	0	0	0
Average	0.117	0.033	0.033

[y] IDH = isocytric dehydrogenase; ACO = aconitate hydratase; M6PI = mannose-6-phosphate isomerase; and SOD = superoxide dismutase.

[z] "Old" = collected from 1977 to 1986 and "new" = collected from 1991 to 1993.

Chapter 3

Variation in Pathogenicity among South African Isolates of *Phytophthora cinnamomi*

Linde, C., Kemp, G. H. J., and Wingfield, M. J. 1998. European Journal of Plant Pathology *in press*.

ABSTRACT

Phytophthora cinnamomi isolates from South Africa were evaluated for differences in growth rate *in vitro* and levels of pathogenicity towards *Eucalyptus smithii* in the field. Fifty-nine *P. cinnamomi* isolates were used to inoculate field trials in summer and winter in two subsequent years at two locations in South Africa. The isolates differed significantly in growth rate *in vitro*, as well as in levels of pathogenicity to *E. smithii* in the field. Growth rate *in vitro* was significantly influenced by interactions with culture age, geographic origin and multilocus isozyme genotype. Levels of pathogenicity in the field were influenced by season of inoculation and average minimum temperatures at trial sites. The host from which *P. cinnamomi* isolates were originally obtained did not significantly affect growth rate *in vitro* and levels of pathogenicity in the field. Culture age, determined by storage of the cultures in the laboratory, had a significant negative effect on growth rate *in vitro* and pathogenicity in the field. Significant differences in levels of pathogenicity could be found for different multilocus isozyme genotypes. Geographic origin and mating type of *P. cinnamomi* isolates had no significant effect on levels of pathogenicity in the field. A positive correlation was found between growth rate *in vitro* and levels of pathogenicity in the field. Levels of variation for pathogenicity in the field within A1 mating type isolates were significantly lower than for A2 mating type isolates. Results of this study provide valuable information on selection of *P. cinnamomi* isolates for future resistance/tolerance screening assays of *Eucalyptus* germplasm in South Africa.

INTRODUCTION

Phytophthora cinnamomi Rands is an oomycetous, soilborne fungal pathogen of many ornamental and woody plants (Zentmyer, 1980). It is heterothallic with two mating types, A1 and A2. Its geographic center of origin is unknown, although isozyme studies have indicated that *P. cinnamomi* was introduced into South Africa (Linde *et al.*, 1997, Chapter 2) and Australia (Old *et al.*, 1984, 1988) based on low levels of gene and genotypic diversity in these locations. As a pathogen of forest trees, it is probably most significant in die-back of jarrah (*Eucalyptus marginata*) in western Australia, where it was first described in 1965 (Podger *et al.*, 1965). It infects mainly roots and feeder roots and has a host range of close to a 1000 different plant species (Zentmyer, 1980). *P. cinnamomi* is also able to induce cankers in various tree species, such as stripe canker in cinnamon in the tropics (Rands, 1922) and root-collar cankers in *E. marginata* in Australia (Shearer *et al.*, 1981). In South Africa, mortality of *Eucalyptus* spp. planted in commercial forestry areas is predominantly associated with root-collar cankers (Linde *et al.*, 1994).

Commercial forestry is one of the largest sectors of the South African economy. More than 1.5 million ha of land is utilised for plantations of mainly *Pinus*, *Eucalyptus*, and *Acacia* spp. (Van der Zel., 1994). Root diseases of *Eucalyptus* and *Pinus* spp. associated with *Pythium* and *Phytophthora* spp. were most prevalent in nurseries in the late 1970s (Donald and von Broembsen, 1977). Alteration of cultivation procedures and improvement of sanitary control, resulted in a dramatic decrease in root diseases caused by Oomycetes. However, established *Eucalyptus* trees, especially cold tolerant *Eucalyptus* spp. planted in South Africa, are particularly susceptible to *P. cinnamomi*. High levels of susceptibility in *E. fastigata* and *E. fraxinoides*, have made them unsuitable for use in commercial forestry (Wingfield and Knox-Davies, 1980). Another cold tolerant species, *E. smithii*, is also susceptible to *P. cinnamomi* root disease (Wingfield and Kemp, 1994). Mortalities of these *Eucalyptus* spp. are usually restricted to one to two-year-old trees, whereas basal cankers may persist in more mature trees until harvest.

Breeding and selection programs for *P. cinnamomi* disease-resistant planting stock in South Africa focused mainly on avocados (Botha *et al.*, 1990). The importance of cold tolerant *Eucalyptus* spp. as high quality pulp producers, and a limited number of suitable

Eucalyptus spp. to plant at high elevation areas, has prompted the South African forestry industry to breed and select for resistance/tolerance to *P. cinnamomi*. Breeding and selection programs in *E. marginata* (Stukely and Crane, 1994) and *Pinus radiata* (Butcher *et al.*, 1984) carried out in Australia, have shown that resistance is under strong genetic control. However, the long term success of breeding and selection programs will be partly dependent on variation in pathogenicity of the pathogen population.

The term variation in pathogenicity could potentially be confusing as various definitions and misconcepts of the terms pathogenicity and virulence can be found in the literature. Furthermore, the definitions also differ depending on whether it is used in medicine, fungi, prokaryotes or viruses (Shaner *et al.*, 1992). To avoid any confusion in this chapter, the two terms are defined as follows. Pathogenicity; The capability to cause disease, and the amount of damage to the host (Shaner *et al.*, 1992). Virulence; The genetic ability of a pathogen race to overcome genetically determined host resistance, which is effective against other races of that pathogen, and cause a compatible (disease) interaction. Virulence is the ability of a fungal strain to overcome specific resistance genes in the host plant and is considered a qualitative character. (Shaner *et al.*, 1992). Because virulence genes could not yet be identified in *E. smithii,* the term variation in pathogenicity is preferred to virulence.

The aims of the current investigation were to; *(i)* assess variation in growth rate *in vitro* among the South African *P. cinnamomi* population, *(ii)* assess variation in levels of pathogenicity to *E. smithii* under field conditions in the South African *P. cinnamomi* population, *(iii)* investigate the influence of climate (temperature and rainfall) on pathogenicity in the field at two different localities, *(iv)* study the influence of culture age on growth rate *in vitro* and levels of pathogenicity in the field, *(v)* explore the relationship between growth rate *in vitro* and levels of pathogenicity in the field, *(vi)* compare growth rate *in vitro* and levels of pathogenicity in the field between *P. cinnamomi* populations from different geographic regions, and *(vii)* test for differences in levels of variation for growth rate *in vitro* and pathogenicity in the field between A1 and A2 mating type populations and populations with different multilocus isozyme genotype backgrounds. The assessment of variation for pathogenicity and influence of climate, region, geographic origin, mating type, and age of isolates will be important to implement efficient and reliable screening assays towards *P. cinnamomi* in germplasm of *Eucalyptus* in South Africa.

MATERIALS AND METHODS

Isolates. Fifty-nine (39 A2 and 20 A1) South African *P. cinnamomi* isolates were tested for growth rate *in vitro* and pathogenicity to *E. smithii* in the field. Isolates were collected between 1977 to 1993 from the south and southwestern Cape region (collectively referred to as Cape region) and from the northeastern parts of South Africa (collectively referred to as Mpumalanga). Isolates were divided into two subpopulations based on isolation dates. Isolates collected from 1977 to 1986 are referred to as "old" isolates (32 Cape and eight Mpumalanga isolates) and those collected from 1991 to 1993 as "new" isolates (10 Cape and nine Mpumalanga isolates). The *P. cinnamomi* isolates from Mpumalanga were collected from various hosts including avocado, *Eucalyptus,* and *Pinus* spp., whereas isolates from the Cape were collected from commercial *Eucalyptus* and *Pinus* spp., native fynbos (unique flora of the southwestern Cape), and native forest tree species. The A1 mating type isolates were predominantly obtained from native flora while A2 mating type isolates were predominantly obtained from commercial crops. Details on isolation, maintenance of cultures, determination of mating type, isozyme analysis, and definition of multilocus isozyme genotypes are provided elsewhere (Linde *et al.*, 1997).

I) Growth rate *in vitro*. Blocks (2 mm^3) were cut from margins of 3-day-old *P. cinnamomi* cultures grown on potato dextrose agar (PDA - Difco). Four blocks of each isolate were transferred to a Petri dish containing 20 ml PDA. Three Petri dishes were used for each isolate. Cultures were incubated at 25°C in the dark for 72 h before taking measurements. Petri dishes were arranged in a complete randomised block design. Growth studies were duplicated once. Colony diameter data were analysed by ANOVA (SAS Institute Inc., 1989) for possible interactions with culture age, mating type, geographic origin, and multilocus isozyme genotype background of isolates. Test statistics were approximately normal distributed. Cape *P. cinnamomi* isolates were also analysed separately by ANOVA to account for the absence of A1 isolates from Mpumalanga.

II) Assessment of pathogenicity in the field. The level of pathogenicity of each *P. cinnamomi* isolate was assessed through inoculations on established 3-year-old *E. smithii* trees at two localities, namely Piet Retief (Mpumalanga) and Richmond (KwaZulu Natal).

These two trial sites were selected because temperatures at Richmond are usually lower than those at Piet Retief. Inoculations were conducted in summer (December to February) and winter (April to June) to determine the existence of seasonal influences on levels of pathogenicity. Apart from obvious temperature differences between winter and summer, temperature differences between trial sites would possibly allow further distinction on levels of pathogenicity. Trials were conducted during 1994 and repeated in 1995. Temperature and rainfall data for the two study sites were obtained from nearby weather stations for the duration of each trial.

P. cinnamomi isolates were grown on PDA for 7 days at 25°C. Mycelial discs were used to inoculate wounds on the stem of each tree, 1.3 m above the ground, after the bark had been removed with a 10-mm diameter cork borer. In each trial, each isolate was used to inoculate 20 trees in a randomised block design. For control inoculations, twenty trees in each trial were inoculated with a sterile disc of PDA. Wounds were sealed with masking tape to prevent desiccation. Thus 160 trees for each isolate with a total of 9600 trees were inoculated. Lesion lengths in the secondary phloem (Shearer *et al.*, 1987, Tippett *et al.*, 1983) were measured 8 weeks after inoculation. Re-isolations onto a selective medium (Tsao and Guy, 1977) were made from control and inoculated trees.

Data analysis. Stem lesion data was analysed using ANOVA (SAS Institute Inc., 1989). ANOVA for lesion length data in the field was similar to that for growth rate *in vitro* as measured by colony diameter, except that additional interactions with locality and season of inoculation were included as block factors. Least significant differences (LSD) were calculated using the harmonic means of numbers on which each means was based. Test statistics were approximately normally distributed. Controls were excluded from statistical analysis as lesions did not develop in these trees. Because A1 mating type isolates from Mpumalanga were not included in this study, lesion length data for Cape *P. cinnamomi* isolates were also analysed separately to distinguish between levels of pathogenicity of mating type isolates.

Relationships between lesion length and other variables (average maximum and minimum temperatures, and rainfall) were investigated by calculating Pearson's coefficient of correlation (Snedecor and Cochran, 1980). To determine the relationship between the growth rate *in vitro* and level of pathogenicity in the field among *P. cinnamomi* isolates, a natural

growth function based on a least square analysis (Snedecor and Cochran, 1980) was used in which: *Lesion length = A + B(colony diameter)*. This is a non-linear relationship which is an asymptotic regression where A = the asymptote and the maximum value of the function, and B = the parameter that controls the rate at which the function approaches "A". A Pearson correlation coefficient (Snedecor and Cochran, 1980) was calculated for this non-linear relationship to test the significance of the influence of lesion length and growth rate *in vitro*. Bartlett's test for homogeneity of variances (Snedecor and Cochran, 1980) was conducted to test for group variances in heterogeneity of colony diameter and stem lesion data within "old" and "new", as well as within A1 and A2 mating type subpopulations of *P. cinnamomi*.

RESULTS

I) Growth rate *in vitro*.

Variation in growth rate. Significant differences ($P < 0.05$) were observed for growth rate *in vitro* among *P. cinnamomi* isolates with colony diameters for individual isolates ranging from 19.9 to 45.5 mm after 72 h. All main effects [culture age X origin X multilocus isozyme genotype (mating type)] significantly ($P = 0.0001$) affected growth rate (Fig 1).

Influence of culture age. Average colony diameters of "old" isolates were significantly ($P < 0.05$) smaller than those of "new" isolates. This applied to isolates of multilocus isozyme genotypes 1 and 4 from the Cape and multilocus isozyme genotypes 1 and 7 from Mpumalanga. Average colony diameters of "old" and "new" isolates belonging to multilocus isozyme genotype 6 from the Cape and Mpumalanga did not differ significantly from each other (Fig 1). When isolates from the Cape were analysed alone, no significant difference in average colony diameter of "old" and "new" A2 isolates could be observed. Average colony diameter of "new" A1 isolates was significantly ($P < 0.05$) greater than that of "old" A1 isolates (Table 1).

Influence of mating type. Mating type as one of the main effects, is related to genetic background as determined by multilocus isozyme genotype. Multilocus isozyme genotype 4 and 5 consist of A1 isolates and multilocus isozyme genotype 1 to 3 and 6 to 9 consist of A2 mating type isolates (Linde *et al.*, 1997, Chapter 2). Average colony diameter of "old" A1 mating type isolates (multilocus isozyme genotype 4) was significantly ($P < 0.05$) greater than

that of other "old" A2 multilocus isozyme genotypes tested (Fig. 1). Exceptions were Cape isolates belonging to multilocus isozyme genotype 7 and Mpumalanga isolates belonging to multilocus isozyme genotype 6. Colony diameter of the one isolate belonging to multilocus isozyme genotype 5 was significantly ($P < 0.05$) smaller than any other multilocus isozyme genotype tested.

Average colony diameter of "new" A1 mating type isolates did not differ significantly from A2 isolates belonging to multilocus isozyme genotypes 1, 2 and 7 from Mpumalanga. Average colony diameter of A1 mating type isolates was significantly greater ($P < 0.05$) than Cape A2 isolates belonging to multilocus isozyme genotypes 1, 6 and 9 and Mpumalanga genotype 6 (Fig. 1). When isolates from the Cape were analysed alone, average colony diameter of A1 isolates was significantly ($P < 0.05$) greater than that of A2 isolates in both "old" and "new" subpopulations (Table 1).

Influence of geographic origin. Average colony diameters of "old" Cape isolates belonging to multilocus isozyme genotype 1 and 7 were significantly greater than those of Mpumalanga isolates from the "old" population (Fig 1). Average colony diameter of Cape multilocus isozyme genotype 6 isolates did not differ significantly from that of Mpumalanga isolates within the "old" and "new" populations. In the "new" population, average colony diameter of Mpumalanga isolates (multilocus isozyme genotype 1) was significantly ($P < 0.05$) greater than that of isolates from the Cape (Fig. 1).

II) Pathogenicity in the field.

Variation in levels of pathogenicity. Significant differences ($P < 0.05$) in lesion length on *E. smithii* caused by individual *P. cinnamomi* isolates were found with lesion lengths ranging from 5.2 to 27.4 cm. Lesion length data represent lesion lengths on 160 *E. smithii* trees inoculated for each isolate and represent an average of all trials (season X locality). Successful re-isolations of *P. cinnamomi* could be made from inoculated trees.

Influence of locality and climate. Lesion lengths associated with different isolates of *P. cinnamomi* differed significantly ($P = 0.001$) for each locality and season (Fig. 2A). Lesion lengths for the Piet Retief summer inoculations (PRS 94; 95) were the longest, followed by Richmond winter inoculations (RMW 94; 95), Piet Retief winter (PRW 94; 95), and then Richmond summer inoculations (RMS 94; 95). Lesion lengths for the Piet Retief summer

inoculations in 1994 were significantly ($P < 0.05$) longer than those at the same site in 1995. Except for the summer inoculations at Piet Retief, lesion lengths from 1994 inoculations did not differ significantly from those of 1995.

Average maximum temperatures (Fig. 2B) and average minimum temperatures (Fig. 2C) varied considerably for the winter and summer inoculations. Average maximum temperatures in the winter were consistently lower at Richmond trials than at Piet Retief. In summer trials, average maximum temperatures for 1994 Richmond and Piet Retief trials were approximately the same, whereas temperatures were lower at Richmond for the 1995 trial (Fig 2B). Average lesions length and average maximum temperatures at trial sites did not correlate ($r = 0.30$) significantly. Average lesion lengths and average minimum temperature did correlate significantly ($P < 0.05$; $r = 0.42$). Rainfall was higher during summer than winter inoculation trials (Fig. 2D), but no significant correlation ($r = 0.23$) between lesion length and rainfall was found.

Influence of culture age. Average lesion lengths induced by "old" Cape and Mpumalanga *P. cinnamomi* isolates were significantly smaller ($P < 0.05$) than those for "new" isolates (Table 2). Culture age X multilocus isozyme genotype significantly ($P = 0.0002$) affected levels of pathogenicity (Fig. 3). Average lesion lengths induced by "old" isolates belonging to multilocus isozyme genotypes 1, 6, and 7 were significantly ($P < 0.05$) smaller than those from similar multilocus isozyme genotype's of "new" isolates. Average lesion lengths associated with multilocus isozyme genotypes 2 and 4 did not differ significantly between "old" and "new" isolates (Fig. 3).

Influence of mating type. Average lesion length induced by "old" A1 mating type isolates (multilocus isozyme genotype 4) was significantly ($P < 0.05$) greater than that of "old" A2 mating type isolates belonging to multilocus isozyme genotypes 1, 7 and 8, but did not differ significantly from "old" A2 mating type isolates belonging to multilocus isozyme genotypes 2 and 6. Average lesion length for "new" A1 mating type isolates (multilocus isozyme genotype 4) was significantly ($P < 0.05$) greater than that of A2 mating type isolates belonging to multilocus isozyme genotype 2, but did not differ significantly from A2 mating type isolates belonging to multilocus isozyme genotypes 1, 6, 7, and 9 (Fig. 3).

Influence of geographic origin of isolates. No significant differences in average lesion length could be found when comparing the sets of isolates from Cape and Mpumalanga (Table 2).

Host adaptation. Average lesion lengths on *E. smithii* associated with isolates from *Eucalyptus* (24.9 cm) and *Ocotea bullata* (23.7 cm) did not differ significantly from each other (Table 3).

III) Correlation between growth rate *in vitro* and level of pathogenicity in the field. Significant (P = 0.0052) correlation (r = 0.35) was found between growth rate *in vitro* of isolates and their level of pathogenicity in the field. The relationship between growth rate and level of pathogenicity was non-linear as calculated by a natural growth function and expressed as predicted lesion length (Fig. 4).

IV) Level of variation within "old" and "new" isolates of A1 and A2 mating type.

Growth rate *in vitro*. Variances within "old" and "new" as well as within A1 and A2 mating type isolates were not significantly different according to Bartlett's test for homogeneity of variances (Table 4).

Level of pathogenicity in the field. Level of variation within "old" (S_i^2 = 19.291) and "new" (S_i^2 = 17.757) *P. cinnamomi* isolates were not significantly different at 5 % level according to Bartlett's test for homogeneity of variances (χ^2 = 0.288; P = 0.592). Variances within A1 mating type isolates (S_i^2 = 17.017) differed significantly ($P < 0.05$) from that of A2 mating type isolates (S_i^2 =25.678) (χ^2 = 4.327; P = 0.038) (Table 4).

DISCUSSION

The results of this study indicate that significant variation in pathogenicity is present among South African *P. cinnamomi* isolates. Dudzinski *et al.* (1993) also showed significant differences in pathogenicity towards a susceptible *E. marginata* clone among 42 *P. cinnamomi* isolates. These results are in contrast to findings of Podger (1989) who concluded that 14 *P. cinnamomi* isolates tested on five highly susceptible host species, did not differ significantly in pathogenicity. Those isolates were obtained from 10 different species of host plants and from 14 localities across Australia, and represented three of the four multilocus

isozyme genotypes of the fungus that are known to occur in Australia (Podger, 1989). Therefore, uniformity in levels of pathogenicity cannot be attributed to isolates having the same genetic background, but may be because levels of pathogenicity were determined on highly susceptible host species. The high susceptibility of test species made it impossible to distinguish between isolates with different levels of pathogenicity. The larger sample sizes used by Dudzinski *et al.* (1993), and the more extensive experimental approach in the current study provide a finer level of resolution regarding pathogenic variation among *P. cinnamomi* isolates. Significant variation in levels of pathogenicity contrasts with the low levels of genetic diversity in the South African (Linde *et al.*, 1997, Chapter 2) and Australian (Old *et al.*, 1984, 1988) *P. cinnamomi* populations.

The influence of climate on levels of pathogenicity was inconsistent in this study. Levels of pathogenicity were not always the highest in summer as was found in Australia and France. In those studies, trunk and stem inoculations on *Eucalyptus* spp., *Banksia grandis* (Shearer *et al.*, 1988, Tippett *et al.*, 1989) and red oaks (*Quercus rubra*) (Robin *et al.*, 1994) with *P. cinnamomi*, have shown that pathogenicity as assessed by fungal growth rate in trees, increases in summer. Furthermore, average lesion lengths did not correlate with average maximum temperatures in this study, but rather correlated ($P < 0.05$; $r = 0.42$) with average minimum temperatures at trial sites. Correlation between minimum temperature data and lesion development in red oak trunks, suggested that temperature is most likely the climatological factor that limits disease in red oak forest stands (Robin *et al.*, 1994). However, the effect of minimum temperatures on pathogen growth cannot entirely account for low levels of pathogenicity during winter. This is confirmed by low levels of pathogenicity found in the summer Richmond trial, compared to winter inoculations at Piet Retief and Richmond. Furthermore, although correlation between average minimum temperature and average lesion length was significant, it was very low and did not entirely explain low levels of pathogenicity in winter.

Results of this study showed no relationship between rainfall and levels of pathogenicity. These results might have been different if the relative water content of phloem was used as a criterion, because phloem water content varies with timing of rainfall (Tippett and Hill, 1983) and site (Tippett and Shea, 1985). It has been shown that relative water content of red oak (Robin *et al.*, 1994) and *E. marginata* (Tippett *et al.*, 1987) bark is related to average length of

lesions in excised bark. However, no correlation was observed between the average relative water content and average linear lesion extension in red oak trunks (Robin *et al.*, 1994). It could also not be conclusively shown that relative water content in non-excised *E. marginata* stems is correlated with lesion development (Tippett *et al.*, 1987). This suggests that relative water content also does not explain different levels of pathogenicity found during winter in this study. Various other possible explanations exist such as the physiological and chemical changes in tree cortical tissue during the year (Kramer and Kozlowski, 1960, Srivastava, 1964).

Growth rate of *P. cinnamomi* isolates *in vitro* was positively correlated ($P = 0.0052$; $r = 0.35$) with level of pathogenicity in the field (Fig. 4). This suggests that fast growing isolates could be selected in the laboratory for utilisation in resistance assays. This might eliminate the need to inoculate trees to determine relative levels of pathogenicity. However, since this was not an absolute relationship with very poor correlation, it is suggested that fast growing isolates should be tested in the field to verify their levels of pathogenicity. Those isolates with the highest levels of pathogenicity could then be used in further screening trials. Growth rate *in vitro* was negatively affected by the length of time the isolates were maintained in pure culture. This was observed in some isolates with interactions between culture age, origin, multilocus isozyme genotype, and mating type of isolates, making it difficult to predict which isolates would be fast growing. It is common that fast growing fungal isolates are more pathogenic than slow growing isolates as in *Ophiostoma ulmi* (Brasier and Webber, 1987).

In this study, for most of the multilocus isozyme genotypes, no differences in pathogenicity were detected between the A1 and A2 mating types. In the "new" *P. cinnamomi* population, lesion length of A1 mating type isolates (multilocus isozyme genotype 4) differed significantly ($P < 0.05$) only from A2 mating type isolates belonging to multilocus isozyme genotype 2. Levels of pathogenicity for A1 and A2 mating type isolates from Australia also did not differ significantly from each other (Dudzinski *et al.*, 1993). Previous reports demonstrating variation in pathogenicity between mating types of *P. cinnamomi* to *Nothofagus* (Weste, 1975), as well as to camellia and avocado (Zentmyer and Guillemet, 1981), included only a limited number of isolates (1 or 2 isolates of each mating type), preventing meaningful statistical analysis. Thus, differences in pathogenicity observed in those studies may represent pathogenic variation among *P. cinnamomi* isolates, as was found

in the present and Australian study (Dudzinski *et al.*, 1993) and not a correlation with mating type.

Cape and Mpumalanga regional *P. cinnamomi* isolates did not show significant differences in levels of pathogenicity. This result was not surprising as low levels of genetic differentiation have been identified between these two populations (Linde *et al.*, 1997, Chapter 2). Isolates may thus be selected without considering geographic origin in South Africa, for future disease resistance/tolerance screening assays.

The relationship between pathogenicity and different multilocus isozyme genotypes in the South African *P. cinnamomi* population is difficult to simplify and shows interaction with culture age. Sample sizes of most of the multilocus isozyme genotypes tested were extremely small because only limited numbers of individuals of some of the multilocus isozyme genotypes were identified in a previous study (Linde *et al.*, 1997, Chapter 2). Small sample sizes make statistical analysis unreliable, although significant ($P < 0.05$) differences between multilocus isozyme genotype's were identified in this study. Significant differences for growth rate *in vitro* and pathogenicity in the field was observed among different multilocus isozyme genotypes. Population genetic studies have recently shown that sexual reproduction is rare, if not absent, in the south African *P. cinnamomi* population (Linde *et al.*, 1997, Chapter 2). The lack of sexual reproduction, giving rise to more or less stable clonal lines over time, provides the simplest explanation for this apparent linkage between multilocus isozyme genotype and pathogenicity.

The level of pathogenicity of South African *P. cinnamomi* isolates that were obtained from *Eucalyptus* spp. (from commercial forest plantations) was comparable with that of isolates from *Ocotea bullata* (an indigenous forest tree species). This suggests that host adaptation may not have occurred in South Africa. Ideally, these isolates also should have been inoculated onto *O. bullata* to test reciprocal pathogenicity on that host. Host specialisation to avocado and camellia, and the presence of strains or "races" in *P. cinnamomi*, were suggested previously (Zentmyer and Guillemet, 1981). However, only one isolate of each mating type was used preventing any meaningful conclusions to be drawn from that study.

Valuable conclusions can be drawn from this study regarding selection of highly pathogenic *P. cinnamomi* isolates to be used and the implementation of resistance/tolerance screening assays of *Eucalyptus* germplasm in South Africa. For future disease

resistance/tolerance screening assays, highly pathogenic *P. cinnamomi* isolates should be used, and these can be partly selected using *in vitro* assays. Geographic origin and mating type of *P. cinnamomi* isolates do not significantly influence pathogenicity, as long as isolates have not been in storage for an extended period. Disease resistance screening assays should be conducted during summer, rather than winter months in order to identify resistance/tolerance more easily.

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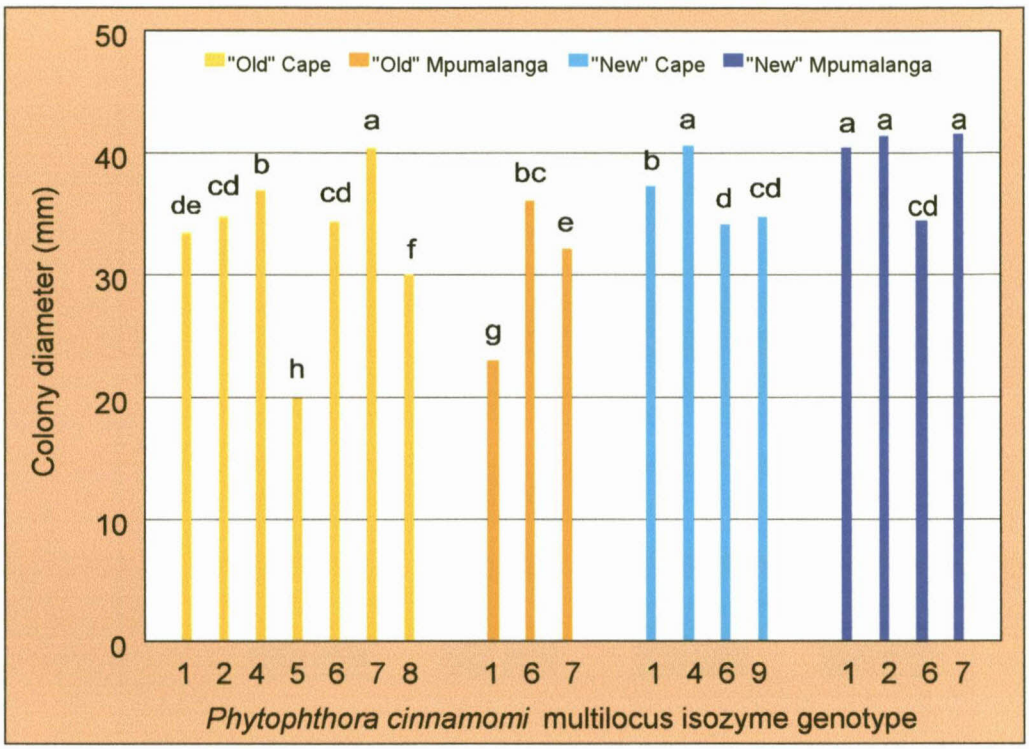

Fig. 1. Average colony diameters of "old" and "new" *Phytophthora cinnamomi* isolates from Cape and Mpumalanga areas, representing different multilocus isozyme genotypes. Bars topped by the same letter at each multilocus isozyme genotype are not significantly different ($P < 0.05$) as analysed by ANOVA.

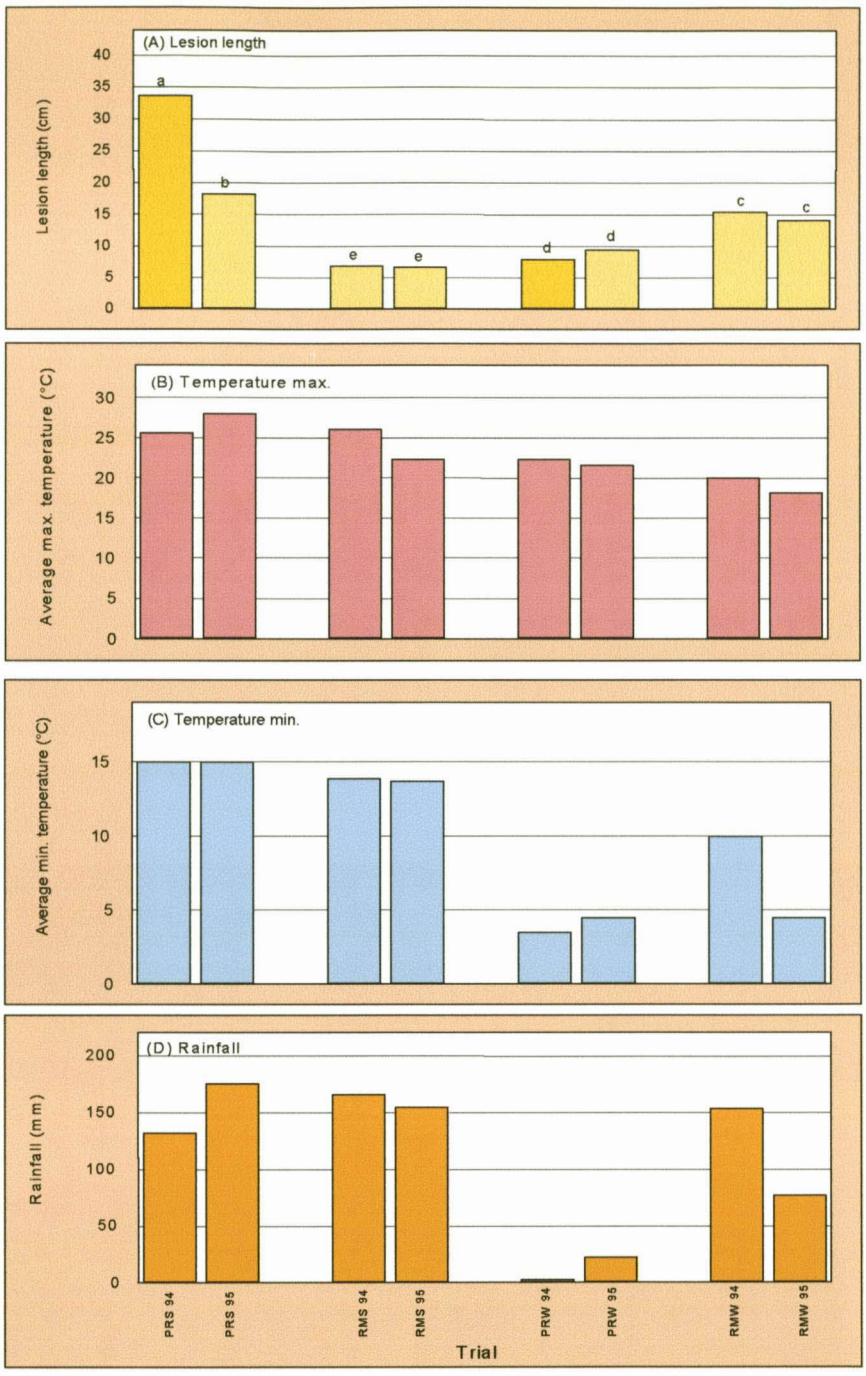

Fig. 2. Data for 3-yr-old *Eucalyptus smithii* trees inoculated with *Phytophthora cinnamomi* in winter or summer at Piet Retief and Richmond. (A) Average lesion lengths on *Eucalyptus smithii*, 8 weeks after inoculation with 59 *Phytophthora cinnamomi* isolates. Each bar represents the average lesion length of 1180 trees. Bars topped by the same letter at each trial are not significantly different ($P < 0.05$) as analysed by ANOVA, (B) average maximum temperature recorded for each locality for duration of the trial, (C) average minimum temperature recorded for each locality for duration of the trial, (D) average rainfall recorded at locality for duration of the trial. PRS = Piet Retief summer inoculation, RMS = Richmond summer inoculation, PRW = Piet Retief winter inoculation, RMW = Richmond winter inoculation, 94 and 95 indicate to inoculations conducted during 1994 and 1995 respectively.

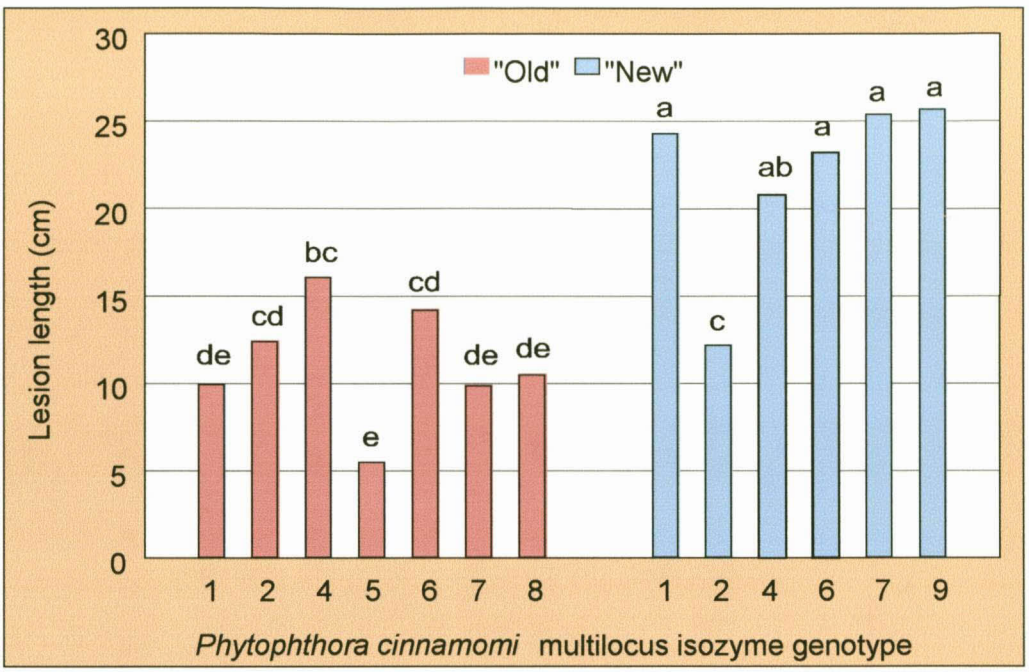

Fig. 3. Average lesion lengths on 3-yr-old *Eucalyptus smithii* trees inoculated with 59 *Phytophthora cinnamomi* isolates representing different multilocus isozyme genotypes, of "old" and "new" isolates. Data represented are averages of lesion length data obtained eight weeks after inoculations at two trial localities during winter and summer. Bars topped by the same letter at each multilocus isozyme genotype are not significantly different ($P < 0.05$) as analysed by ANOVA.

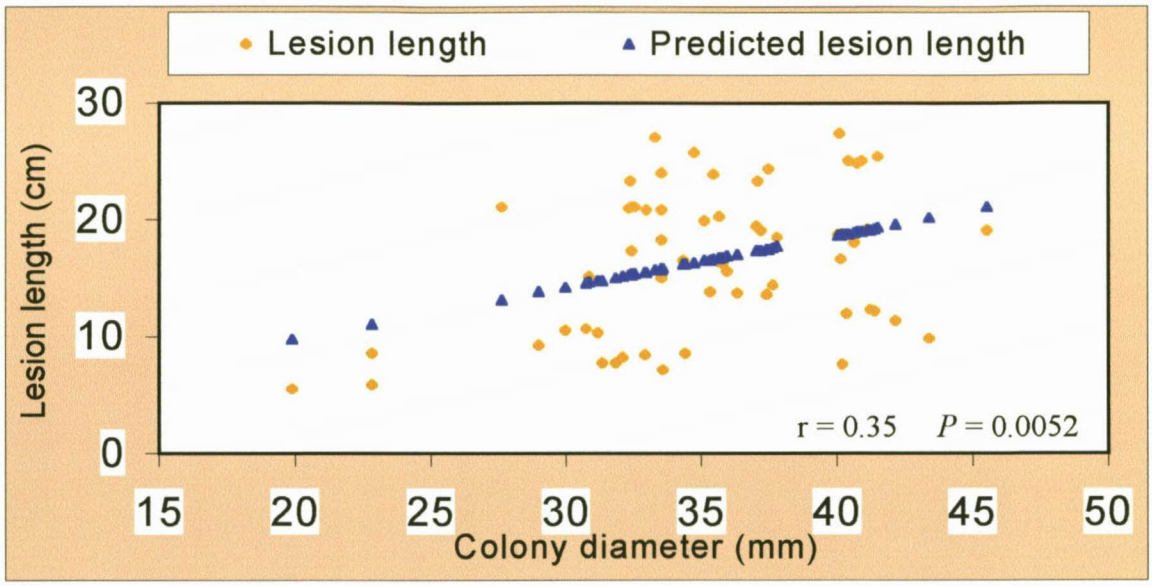

Fig. 4. Regression analysis between average lesion lengths for 59 *Phytophthora cinnamomi* isolates and colony diameter. Lesion lengths represent an average of lesion length data from inoculations at two localities during winter and summer.

TABLE 1. Average growth rate *in vitro* as measured by colony diameter of A1 and A2 mating type isolates of "old" and "new" Cape *Phytophthora cinnamomi* isolates

Culture age	Mating type	No. of isolates	Colony diameter (mm)[a]
"Old"	A1	18	35.8 b
	A2	14	34.1 c
"New"	A1	2	40.5 a
	A2	8	34.8 bc

[a] Colony diameter is an average of 12 colonies measured. Means followed by different letters were significantly different at $P < 0.05$.

TABLE 2. Average lesion lengths on *Eucalyptus smithii* in the field eight weeks after inoculation with "old" and "new" *Phytophthora cinnamomi* isolates from the Cape and Mpumalanga

Culture age	Origin	No. of isolates	Lesion length (cm)[a]
"Old"	Cape	32	13.6 b
	Mpumalanga	8	12.6 b
"New"	Cape	10	23.1 a
	Mpumalanga	9	22.3 a

[a] Lesion length is an average of lesion lengths on 160 *Eucalyptus smithii* trees inoculated in summer and winter with *Phytophthora cinnamomi* isolates at two localities, Piet Retief and Richmond. Means followed by different letters were significantly different at $P < 0.05$.

TABLE 3. Average lesion lengths of "new" *Phytophthora cinnamomi* A2 mating type isolates from *Eucalyptus* and *Ocotea bullata* hosts as tested on *Eucalyptus smithii*

Host	No. of isolates	Lesion length (cm)[a]
Eucalyptus	4	24.6 a
Ocotea bullata	8	23.7 a

[a] Lesion length is an average of lesion lengths on *Eucalyptus smithii* inoculated in summer and winter with *Phytophthora cinnamomi* isolates at two localities, Piet Retief and Richmond. In total 160 trees for each isolate were measured. Means followed by the same letters were not significantly different at $P < 0.05$.

TABLE 4. Bartlett's test for homogeneity of variances for growth rate *in vitro* and level of pathogenicity in the field within "old" and "new", and mating type *Phytophthora cinnamomi* isolates

	P. cinnamomi population	df	Variance (S_i^2)	χ^2	*P*
Colony diameter *in vitro*:	"Old"	80	1.91	2.977	0.315
	"New"	38	1.16		
	A1	40	1.29	1.662	0.197
	A2	78	1.87		
Lesion length in the field:	"Old"	273	19.29	0.288	0.592
	"New"	126	17.76		
	A1	133	17.02	4.327[a]	0.038
	A2	266	25.68		

[a] Variances differ significantly at $P < 0.05$.

Chapter 4

Gene and Genotypic Diversity of *Phytophthora cinnamomi* from South Africa and Australia

Linde, C., Drenth, A., and Wingfield, M. J. 1999. European Journal of Plant Pathology *submitted*.

ABSTRACT

Phytophthora cinnamomi isolates from South Africa and Australia were compared to assess genetic differentiation between the two populations. RAPD's (random amplified polymorphic DNA) were used to analyse these two populations for levels of phenotypic diversity and RFLP's (restriction fragment length polymorphisms) were used to analyse gene and genotypic diversity. Sixteen RAPD markers from four decanucleotide Operon primers and 34 RFLP alleles from 15 putative loci were used. A few isolates from Papua New Guinea known to posses alleles different from Australian isolates were also included for comparative purposes. South African and Australian *P. cinnamomi* populations were almost identical with an extremely low level of genetic distance between them (D_m = 0.003). Common features for the two populations include shared alleles, low levels of phenotypic/genotypic diversity, high clonality, and low observed and expected levels of heterozygosity. Furthermore, relatively high levels of genetic differentiation between mating type populations (D_m South Africa = 0.020 and D_m Australia = 0.025 respectively), negative fixation indices, and significant deviations from Hardy Weinberg equilibrium, all provided evidence for the lack of frequent sexual reproduction in both populations. The data strongly suggest that both the South African and Australian *P. cinnamomi* populations are introduced.

INTRODUCTION

Phytophthora cinnamomi Rands is a host-nonspecific, soilborne, fungal pathogen of many plant species (Zentmyer, 1980). In South Africa it is most important as a pathogen of *Eucalyptus* (Wingfield and Knox-Davies, 1980), avocado trees (Darvas *et al.*, 1984; Wager, 1942), grapevines (Van der Merwe *et al.*, 1972) and members of the Proteaceae family (Von Broembsen and Brits, 1985). It is especially important in the South African forestry industry where 90% mortality has been experienced in some of the cold tolerant *Eucalyptus* spp. such as *E. fraxinoides*. In Australia, *Eucalyptus* spp., especially *E. marginata*, are severely affected by *P. cinnamomi* (Podger *et al.*, 1965; Weste, 1994). *P. cinnamomi* infects its hosts mainly through its motile zoospores (Zentmyer, 1961) and can survive for many years in soil and plant material in the form of survival structures such as chlamydospores (Zentmyer and Mircetich, 1966) or oospores (Kassaby *et al.*, 1977).

P. cinnamomi has two mating types, A1 and A2, which can interact to produce sexual spores (oospores). Both mating types have a global distribution (Zentmyer, 1980, 1988) and both occur in South Africa (Linde *et al.*, 1997, Chapter 2; Von Broembsen, 1989), Australia (Pratt and Heather, 1973) and Papua New Guinea (Arentz and Simpson, 1986). The A2 mating type is found throughout South Africa while the A1 mating type is most commonly encountered in southern South Africa (Cape region) (Linde *et al.*, 1997, Chapter 2; Von Broembsen, 1989). The presence of both mating types in these regions may provide opportunities for sexual reproduction and thus acquisition of new allele combinations that give rise to more pathogenic genotypes.

A recent isozyme study on the South African *P. cinnamomi* population indicated low levels of gene and genotypic diversity and the absence or rare occurrence of sexual recombination (Linde *et al.*, 1997, Chapter 2). This population study showed low levels of genetic differentiation between regional and temporal *P. cinnamomi* populations. Isozyme studies on *P. cinnamomi* in Australia (Old *et al.*, 1984, 1988) also indicated low levels of genetic diversity, a high frequency of A2 and rare occurrence of A1 mating type isolates, and the absence of sexual reproduction. It was thus hypothesised that the fungus was introduced into Australia (Old *et al.*, 1984, 1988). In contrast, a high level of genetic diversity was found among *P. cinnamomi* isolates from Papua New Guinea (Old *et al.*, 1984).

When isozymes are used, population genetic studies on fungi are often limited by the number of isozyme loci available and in the number of allele's at each locus (Michelmore and Hulbert, 1987). This is in contrast to higher eucaryotic organisms where such markers have been used very successfully. These limitations can be overcome by using DNA based markers such as RAPD's (random amplified polymorphic DNA) (Williams *et al.,* 1990) and RFLP's (restriction fragment length polymorphism's) or multilocus RFLP probes (Botstein *et al.,* 1980), often referred to as DNA fingerprint probes. Such techniques offer a virtually unlimited number of selectively neutral markers from which to randomly select a sufficient number to conduct population genetic analyses. DNA fingerprint and low copy RFLP probes were used respectively, to conduct detailed genetic and population genetic studies on the heterothallic *Phytophthora infestans* (Drenth *et al.*, 1993, 1994; Fry *et al.*, 1992, 1993; Goodwin *et al.*, 1992, 1995) and the homothallic *P. sojae* (Drenth *et al.*, 1996; Förster *et al.,* 1994). Despite the importance of *P. cinnamomi* as a plant pathogen, very little effort has been made to apply DNA based markers such as RAPD's (Chang *et al.*, 1996), RFLP's, or DNA fingerprint probes to elucidate the genetic structure of the pathogen population.

The availability of DNA based genetic markers such as RAPD's and RFLP's provides multiple opportunities to accurately determine the levels of gene and genotypic diversity in *P. cinnamomi* populations. In order to directly compare the structure of the South African and Australian *P. cinnamomi* populations, both should ideally be analysed using the same set of RAPD and RFLP markers. If *P. cinnamomi* was introduced into South Africa, a similar population structure to the Australian population would be expected. Strong evidence to support the view that *P. cinnamomi* was introduced into Australia is provided by the irreversible destruction *P. cinnamomi* causes in native vegetation and the low levels of gene diversity (Shepherd, 1975; Old *et al.,* 1984, 1988).

Knowledge about the population genetics of *P. cinnamomi* will be useful for disease resistance and selection programs, and will give further information regarding the origin of *P. cinnamomi* in South Africa. Various population genetic questions pertaining to *P. cinnamomi* already partially answered in an isozyme study (Linde *et al.,* 1997, Chapter 2), will be further addressed in this study. *P. cinnamomi* populations used in this study include populations from South Africa and Australia, as well as a few isolates from Papua New

Guinea included for comparative purposes. RAPD and RFLP markers will be used to specifically address these population genetic questions.

The specific aims of the current investigation were to; *i*) identify the extent of clonality in the *P. cinnamomi* population, *ii*) assess the level of genotypic diversity in the respective *P. cinnamomi* populations, *iii*) assess the level of gene diversity in the respective *P. cinnamomi* populations, *iv*) compare A1 and A2 mating type populations from different *P. cinnamomi* populations, *v*) test for the presence of sexual reproduction in the South African and Australian *P. cinnamomi* populations, and *vi*) quantify the genetic differentiation between the South African and Australian *P. cinnamomi* populations.

MATERIALS AND METHODS

Collection of *P. cinnamomi* isolates. *P. cinnamomi* isolates were collected in South Africa between 1977 to 1986 and 1991 to 1993 from two discrete geographical regions (Cape and Mpumalanga) (Table 1). These different regional and temporal subpopulations have previously been shown to be genetically similar (Linde *et al.,* 1997, Chapter 2), and in this study they are, therefore, regarded as a single population. Isolates represent both mating types and were isolated from various host species. A2 mating type isolates were predominantly isolated from forestry species such as *Eucalyptus* and *Pinus* spp., whereas A1 mating type isolates were predominantly isolated from native vegetation. Details pertaining to isolation, maintenance of cultures, determination of mating type, and isozyme analysis have been provided elsewhere (Linde *et al.*, 1997, Chapter 2). Australian and Papua New Guinea isolates were obtained from M. J. Dudzinski, Division of forestry, CSIRO, Canberra, Australia (Table 1). RAPD's were used to analyse a total of 166 *P. cinnamomi* isolates from South Africa, Australia, and Papua New Guinea. A subset of isolates for each population was randomly chosen for RFLP analysis.

DNA extraction. Mycelium for DNA extraction was grown in clarified V8 broth in 250 ml Erlenmeyer flasks for 1 week. Mycelium was harvested through a Büchner funnel, freeze-dried, and ground into a fine powder in liquid nitrogen. DNA extraction procedures followed those used for *P. infestans* (Drenth *et al.*, 1993). DNA concentration was measured on a fluorometer and adjusted in milli-Q water to the required concentration for analysis. All DNA samples were stored at -20°C.

RAPD analysis. Each RAPD reaction was done in a total volume of 25 µl and comprised of 250 µM each dNTP's, 25 ng primer, 4 mM $MgCl_2$, 60 ng *P. cinnamomi* DNA, 1.6 units of *Tth* Plus DNA polymerase (Biotech International, Australia), 2.5 µl of 10x buffer supplied by the manufacturer, and ultra-pure water. Thermocycling was carried out at 39 cycles of 1 min at 94°C, 1 min at 37°C, and 2 min at 72°C. Then, 1 cycle of 1 min at 94°C, 1 min at 37°C, and 10 min at 72°C was performed. Each isolate was amplified with four decanucleotide primers (Table 1). The total volume of amplified DNA fragments was size fractionated on 1.5% agarose gels in 1x TBE buffer (Sambrook *et al.*, 1989) at 120 V and maximum current for 6 hours. The gels were stained in an ethidium bromide solution for 20 min, and destained in deionised water before visualisation on a UV transilluminator. Four decanucleotide primers were used to analyse the *P. cinnamomi* populations (Table 2).

RFLP analysis. Three µg of total DNA digested overnight at 37°C with 30 units of restriction enzymes *Hin*dIII, *Xho*I, and *Pst*I (Boehringer Mannheim, New England Biolabs, Beverly, MA) was used for restriction digests. Restricted DNA fragments were separated on 0.8% agarose gels for 14-16 h at 40 V (600 Vh) before alkaline transfer to Hybond N^+ (Amersham) hybridisation membrane. Membranes were prehybridised at 65°C in 15 ml of hybridisation solution (0.36 M Na_2HPO_4, 0.14 M NaH_2PO_4, 1 mM disodium ethylenediaminetetraacetic acid, 7% sodium dodecyl sulfate, pH 7.2), 50 µg salmon sperm DNA per ml, and 15 µl poly AAA for 4 h. Probes were labeled with $\alpha^{32}P$ dCTP following manufacturer's instructions (Amersham, Australia). Blots were hybridised overnight and subjected to stringency washes (twice with 5x SSPE, twice with 1x SSPE, 0.1% sodium dodecyl sulfate, and once with 0.1x SSPE, 0.1% sodium dodecyl sulfate). Filters were exposed at -70°C to Kodak X-Omat™ film. Stripping of blots for reuse was carried out at 45°C for 30 min in 0.4 M NaOH and 30 min in (0.2 M Tris-HCl pH 7.5, 0.1x SSC, 0.1x sodium dodecyl sulfate). (20x SSPE = 350.6 g NaCl, 55.2 g NaH_2PO4, 14.8 g EDTA, pH 7.4) (20x SSC = 346.6 g NaCl, 76,4 g Na_3 Citrate, pH 7.0).

Selection of RFLP probes. Fifteen probes (Table 3) were screened for single copy RFLP patterns by hybridisation to a sub-set of *Hind*III, *Xho*I, and *Pst*I digested *P. cinnamomi* DNA. On the basis of this screen, eight probes were selected to characterise *P. cinnamomi* populations. These probes were selected because they showed clearly identifiable single copy loci using *Hind*III, *Xho*I, or *Pst*I as restriction enzyme. In the case

of *pEf*1α at *Hind*III and *Pst*I, two fragments were observed that were confirmed to be allelic after simultaneous digestion with *Hind*III and *Pst*I.

Data analysis. RAPD data were scored for the absence or presence of fragments which was used to derive a multicharacter phenotype for each isolate. Isolates with the same overall multicharacter phenotype were considered clonal. The clonal fraction of each population was calculated. RFLP data were scored based on the presence of RFLP fragments with similar sequence but different size, and each fragment of a particular size was assumed to represent a specific allele at single genetic locus. A multilocus RFLP genotype was derived for each isolate studied, based on all fragments observed with eight selected RFLP probes. Isolates with the same overall RFLP genotype were considered clonal.

Genotypic diversity ($\hat{G}$) (Stoddard and Taylor, 1988) was calculated on the basis of the number of multilocus RAPD phenotypes and RFLP genotypes within each *P. cinnamomi* population studied. To compare levels of genotypic diversity ($\hat{G}/N$) between regions, diversity values of each region were corrected for sample size (McDonald *et al.*, 1994), to calculate the percentage maximum possible diversity obtained ($\hat{G}/N$ %). The significance of differences between the percentages of maximum diversity ($\hat{G}/N$) obtained with RAPD and RFLP analyses for South African and Australian populations was calculated using a *t*-test (Chen *et al.*, 1994; Stoddard and Taylor, 1988).

The mean number of alleles per locus was calculated for each population to give an estimate of the level of gene diversity. Gene diversity for each *P. cinnamomi* population studied was calculated using Nei's gene diversity (H_{exp}) (Nei, 1973). The probable mating system was determined by comparing the observed proportion of heterozygotes with the expected level of heterozygosity by Wright's fixation index, $F = 1 - (H_{obs} / H_{exp})$, where H_{obs} is the observed mean heterozygosity per locus and H_{exp} is the expected mean heterozygosity in the population which is the same as Nei's gene diversity (Brown, 1979).

Regional differentiation between *P. cinnamomi* populations was determined using differences in allele frequencies. Based on allele frequencies of the 15 RFLP loci, population differentiation between A1 and A2 mating type populations of South Africa and Australia, and between the South African, Australian and Papua New Guinea populations was determined using an unbiased minimum genetic distance (D_m) (Nei, 1978). The

occurrence of sexual reproduction in *P. cinnamomi* was tested using frequencies of genotypes in the 6 polymorphic RFLP loci in South African and Australian populations. These genotypic frequencies were tested for deviations from expected Hardy Weinberg equilibrium using the Biosys-1 Statistical Package (Swofford and Selander, 1981). No correction for clonal genotypes was conducted as this would lead to an unacceptably small population size (n=6).

RESULTS

RAPD analysis.

Overall diversity. Sixteen polymorphic RAPD fragments revealed 63 RAPD phenotypes among a total of 166 *P. cinnamomi* isolates analysed (Table 5). South African *P. cinnamomi* isolates were resolved into 48, Australian isolates into 15, and Papua New Guinea isolates into seven different RAPD phenotypes (Table 5).

Regional diversity. Only one RAPD phenotype, phenotype 3 (A2 mating type), occurred in isolates from all the countries analysed (South Africa, Australia, and Papua New Guinea). RAPD phenotypes 10 (A2 mating type), 39 (A2 mating type) and 55 (A1 mating type) occurred in both the South African and Australian populations, whereas RAPD phenotypes 7 (A2 mating type) and 38 (A2 mating type) occurred in both the South African and Papua New Guinea populations. No RAPD phenotypes other than phenotype 3 occurred in both the Australian and Papua New Guinea populations. The overall levels of phenotypic diversity as determined using RAPDs for the different *P. cinnamomi* populations were low for South Africa ($\hat{G}/N = 11.2$) and Australia ($\hat{G}/N = 8.7$), compared to the high level found in the small Papua New Guinea population ($\hat{G}/N = 100.0$) (Table 5). Genotypic diversity for South African ($\hat{G} = 13.6$) and Australian ($\hat{G} = 3.3$) *P. cinnamomi* populations did not differ significantly ($t = 0.138$) from each other.

RFLP analysis.

RFLP probe screening and assessment of fragment patterns. Eight of the 15 RFLP probes tested in the preliminary screening showed clearly identifiable polymorphisms, or monomorphic identifiable single or low copy loci, using *Hind*III, *Xho*I, or *Pst*I as restriction

enzymes. These eight probes were used in different probe enzyme combinations with the three restriction enzymes to yield a total of 15 loci for the analysis of all isolates. Eleven RFLP loci showed polymorphisms and four were monomorphic with a total of 31 different alleles among a total of 109 *P. cinnamomi* isolates analysed from South Africa, Australia, and Papua New Guinea (Table 4). Based on these analyses, a total of 13 multilocus RFLP genotypes could be identified.

Among the 65 South African *P. cinnamomi* isolates analysed, all 15 A1 mating type isolates were of multilocus RFLP genotype 3. The A2 mating type isolates could be separated into four multilocus RFLP genotypes, with genotypes 1 and 2 being the most common. The seven A1 mating type isolates analysed from Australia could be resolved into two multilocus RFLP genotypes with genotype 3 being the most common. Australian A2 mating type isolates could be resolved into five different multilocus RFLP genotypes with genotypes 1 and 2 being the most common. Six of the seven Papua New Guinea isolates could be resolved into different multilocus RFLP genotypes, with two isolates belonging to multilocus RFLP genotype 1 (Table 4). All RAPD phenotypes identified in this study represent their own specific RFLP genotype except RAPD phenotypes 3 (RFLP genotype 1 and 10) and 10 (RFLP genotype 1 and 4). Combining RAPD and RFLP data does not increase the overall number of RAPD phenotypes found.

Regional diversity. *P. cinnamomi* RFLP genotypes 1 and 2 (A2 mating type) could be identified in isolates from all three countries considered (South Africa, Australia and Papua New Guinea). RFLP genotype 3 (A1 mating type) occurred only in South Africa and Australia, whereas RFLP genotypes 4 to 13 were specific to only one country. In total, five RFLP genotypes could be identified in South Africa giving rise to a low overall level of genotypic diversity ($\hat{G}/N$ = 4.7), seven RFLP genotypes in Australia and a low level of genotypic diversity ($\hat{G}/N$ =8.9), and seven RFLP genotypes in Papua New Guinea ($\hat{G}/N$ = 77.8) (Table 5). Levels of genotypic diversity of the South African and Australian *P. cinnamomi* populations did not differ significantly (t = 0.055). Levels of gene diversity were low for South Africa (H_{exp} = 0.105) and Australia (H_{exp} = 0.118) compared to those of the Papua New Guinea isolates (H_{exp} = 0.288) (Table 5). Genetic distance between South African and Australian populations was low (D_m = 0.003). Genetic distances were larger between the South African and Papua New Guinea population (D_m = 0.022), as well as between the Australian and Papua New Guinea population (D_m = 0.020) (Table 6).

Sexual reproduction in *P. cinnamomi*. *P. cinnamomi* populations from South Africa and Australia appeared to contain a large fraction of clonal lines, as analysed using RAPD and RFLP markers. In contrast, the seven isolates analysed from Papua New Guinea were not clonal at all, with a clonal fraction of zero (Table 5). Levels of observed heterozygosity of South African (H_{obs} = 0.129) and Australian (H_{obs} = 0.155) *P. cinnamomi* populations were lower than that of Papua New Guinea (H_{obs} = 0.308). Fixation indices for all three populations were negative (Table 5). Genetic distances between A1 and A2 mating type populations were similar for South African (D_m = 0.020) and Australian (D_m = 0.025) populations, but higher for the Papua New Guinea population (D_m = 0.060) (Table 6).

The South African and Australian *P. cinnamomi* populations were analysed for conformance to expected Hardy-Weinberg equilibrium at the six polymorphic RFLP loci for each population. In the South African population, five out of six, and in the Australian population, three out of six Hardy-Weinberg analyses, deviated significantly from the assumption of random mating.

DISCUSSION

This study, using RAPD and RFLP markers, revealed low levels of gene and genotypic diversity in the South African and Australian *P. cinnamomi* populations. The levels of gene and genotypic diversity in the South African *P. cinnamomi* populations were similar to those found in a previous study using isozymes (Linde *et al.*, 1997, Chapter 2). These are lower than would be expected from a heterothallic, outbreeding organism. Similar results have been obtained for the Australian *P. cinnamomi* population, although levels of observed heterozygosity were slightly lower using isozymes (Goodwin, 1997; Old *et al.*, 1988). These differences in levels of heterozygosity may be partially attributed to the use and/or selection of different markers in this and other studies (Linde *et al.*, 1997, Chapter 2, Old *et al.*, 1988).

There are several lines of evidence that indicate the absence or rare occurrence of sexual reproduction in the South African and Australian *P. cinnamomi* populations. These include: *i*) low levels of observed heterozygosity, *ii*) negative fixation indices which seemed to be fixed for asexual reproduction of heterozygotes, *iii*) high levels of genetic distance between mating type populations which is higher than the genetic distance between South

African and Australian populations, *iv*) populations are highly clonal as determined using RAPD's, and *v*) of the six polymorphic loci tested for the South African and Australian populations, five loci in the South African and three loci in the Australian population deviated significantly from Hardy Weinberg equilibrium and represent non-randomly mating populations. In a previous study using isozymes, it was also suggested that reproduction in the South African *P. cinnamomi* population is predominantly asexual (Linde *et al.*, 1997, Chapter 2). Similar results were found for Australian *P. cinnamomi* isolates (Old *et al.*, 1984, 1988).

Negative fixation indices for the South African and Australian populations, indicate a possible excess of heterozygosity in populations. This could be either from disassortive mating or by the predominance of one, or a few, particularly fit heterozygous clonal lines. Asexual reproduction of heterozygous clonal lines is the most likely explanation for the negative fixation index, as was the case for *P. infestans* population from outside Mexico (Goodwin, 1997; Tooley *et al.*, 1985). Although the fixation index for the Papua New Guinea isolates analysed was also negative, no meaningful conclusions can be drawn from this observation, as very few isolates have been analysed.

Distribution of RFLP genotypes revealed that two A2 mating type RFLP genotypes seem to be wide-spread and that they occur in all three countries analysed. Common A1 mating type RFLP genotypes occurred only in the South African and Australian *P. cinnamomi* populations. A1 mating type isolates analysed from Papua New Guinea all represented unique RFLP genotypes. Unfortunately, very few isolates from Papua New Guinea have been analysed and it is possible that isolates representing RFLP genotype 3 also occur in Papua New Guinea.

South African and Australian *P. cinnamomi* populations show a similar structure and close relationship. Firstly, the low number of different alleles in the Australian and South African *P. cinnamomi* population found in this and previous studies (Linde *et al.*, 1997, Chapter 2; Old *et al.*, 1984, 1988), are indicative of introduced pathogen populations. Secondly, both populations are reproducing predominantly asexually. Thirdly, South African and Australian *P. cinnamomi* populations share many alleles, which is reflected in their low genetic distance (D_m = 0.003), indicating the similarity of the two populations. This is even more evident, if it is considered that the genetic distances between mating type populations within both regional *P. cinnamomi* populations is considerably higher than the

genetic distance between the populations from the two different continents. South African and Australian *P. cinnamomi* populations also show remarkable similarities when analysed using RAPD's. Low levels of phenotypic/genotypic diversity as well as the high clonal fractions were almost identical for the two populations analysed. In contrast, the seven *P. cinnamomi* isolates studied from Papua New Guinea revealed no RAPD clones.

Using RFLP's, genotypic diversity for the South African population was slightly lower, but not significantly different from that for Australia. However, genotypic diversities for both populations were significantly lower than those for Papua New Guinea. Furthermore, low observed and expected levels of heterozygosity for both regional populations were similar. Different alleles and allelic combinations were found among the seven Papua New Guinea isolates using RFLP's. This suggests that these isolates belong to a different gene pool than the South African and Australian populations. The similarity between the Australian and South African *P. cinnamomi* populations is of direct practical importance to the forestry plantation industry. Disease management by means of host resistance in the two countries, could be similar (Leung *et al.*, 1993) because pathogen populations are similar.

Based on low levels of gene and genotypic diversity, results of the RFLP analyses confirm previous reports that *P. cinnamomi* has been introduced, in recent times, into South Africa (Linde *et al.*, 1997, Chapter 2) and Australia (Shepherd, 1975; Old *et al.*, 1984). In addition, based on the striking similarity between the two populations in both countries, we speculate that a related introduction or migration events have occurred. However, populations in other parts of the world need to be analysed to confirm or reject this hypothesis in an unambiguous manner. Isolates from Papua New Guinea showed no clonality using RAPD's and high levels of gene and genotypic diversity using RFLP's. This confirms the hypothesis by Old *et al.* (1984), that Papua New Guinea is probably within the centre of origin for *P. cinnamomi*. Similarly, Shepherd (1975) suggested that the New Guinea/Celebes area is the most likely centre of origin for *P. cinnamomi,* based on mating types occurring in equal proportions and general resistance of native plants to disease. A New Guinea-Malaysia-Celebes region was then later suggested by Zentmyer (1988) as the centre of origin for *P. cinnamomi.* However, a detailed study including large population samples from the New Guinea-Malaysia-Celebes origin is necessary to gain a deeper understanding to the centre of origin for *P. cinnamomi.*

There are several reasons why it is important to know the centre of origin for plant pathogenic fungi, including Oomycetes. Ample opportunities exist to select for disease resistance in the geographical area known as the centre of origin for a particular pathogen. Such information, also provides further knowledge on quarantine issues, so that spread of a pathogen from the centre of origin should be avoided to restrict distribution of genotypes. The origin of *P. infestans* has been shown to be a single geographical area in central Mexico (Goodwin *et al.*, 1994; Tooley *et al.*, 1985). *P. infestans* has a very narrow host range and its host occurs only in a geographically restricted area in the highlands of central Mexico. In contrast, *P. cinnamomi* has a host range of almost 1000 plant species (Zentmyer, 1980). It would, therefore, be expected that *P. cinnamomi* originated within a larger geographical area, or an area representing a rather diverse flora. The New Guinea-Malaysia-Celebes region would probably be an example of such a geographical area. Detailed genetic studies on large populations from this proposed centre of origin are needed to gain insight into the centre of origin of this important plant pathogen (Milgroom and Fry, 1997).

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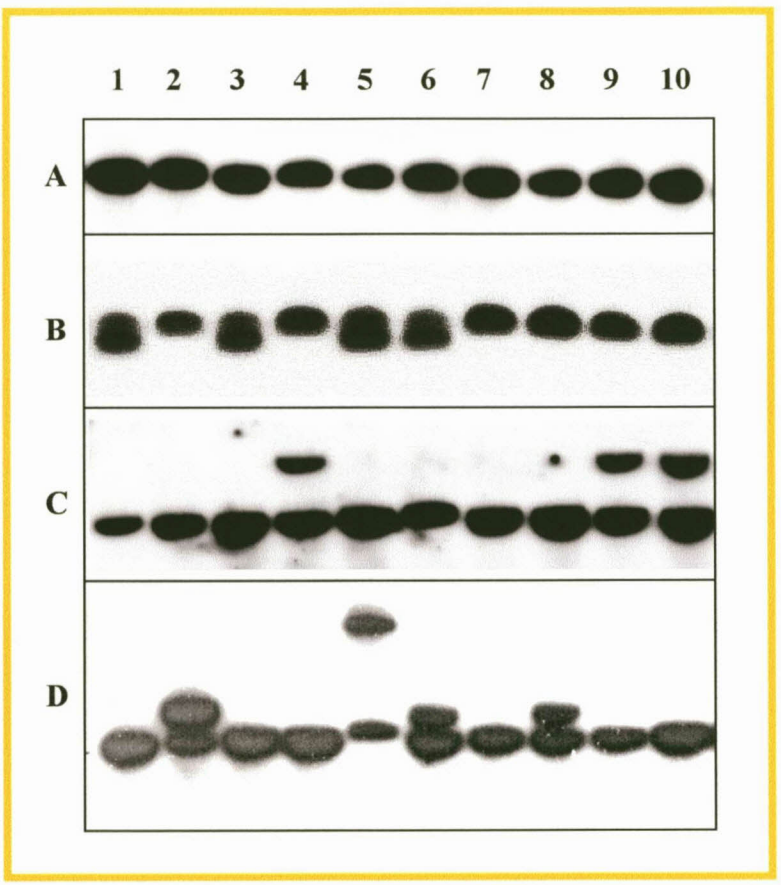


Fig. 1. Autoradiograph of Southern blots containing 10 isolates of *Phytophthora cinnamomi* in four probe/enzyme combinations. **A**, Genomic DNA cut with *Hind*III and hybridised with probe *pPi*120 **B**, Genomic DNA cut with *Pst*I and hybridised with *pPc*2 **C**, Genomic DNA cut with *Pst*I and hybridised with *pPc*3 **D**, Genomic DNA cut with *Hind*III and hybridised with *BipBh*5. Isolates represented in 1-10 in panel **A**, **B**, and **C** are identical: UQ734, T13, C504, CP80, C218, C167, T31, T33, CP517, and CP529. Isolates represented in lines 1 to 10 of **D** are: CP468, UQ835, UQ821, UQ820, UQ832, UQ831, UQ819, UQ836, T44, and CP548.

TABLE 1. Mating type, random amplified polymorphic DNA (RAPD) phenotype, and restriction fragment length polymorphic (RFLP) genotype characteristics of *Phytophthora cinnamomi* isolates from South Africa, Australia and Papua New Guinea

Isolate number	Origin	Mating type	RAPD phenotype	RFLP genotype	Host
C8	South Africa	A1	51	...	*Vitis vinifera*
C9	South Africa	A2	40	2	*Eucalyptus fraxinoides*
C11	South Africa	A1	54	...	*Leucadendron argenteum*
C19	South Africa	A2	47	1	*Leucadendron rubrum*
C44	South Africa	A2	14	1	*Leucospermum reflexum*
C69	South Africa	A1	53	...	*Leucospermum cordiifolium*
C73	South Africa	A2	3	1	*Leucadendron argenteum*
C161	South Africa	A1	51	...	*Leucadendron laureolum*
C167	South Africa	A2	24	1	*Leucadendron argenteum*
C168	South Africa	A1	53	...	*Leucospermum* sp.
C174	South Africa	A1	51	3	*Hakea sericeae*
C177	South Africa	A1	53	...	*Protea* sp.
C180	South Africa	A2	3	1	*Leucadendron argenteum*
C196	South Africa	A2	45	2	*Cryptomeria* sp.
C202	South Africa	A1	53	...	*Eucalyptus fraxinoides*
C208	South Africa	A2	60	1	*Widdringtonia nodiflora*
C210	South Africa	A1	57	3	*Protea magnifica*
C213	South Africa	A2	15	...	*Widdringtonia cypressoides*
C214	South Africa	A1	54	...	*Erica patersonia*
C215	South Africa	A1	61	3	*Leucospermum cordiifolium*
C218	South Africa	A2	23	1	*Araucaria angustifolia*
C220	South Africa	A1	53	...	*Leucadendron argenteum*
C223	South Africa	A2	42	1	*Leucospermum patersonii*
C226	South Africa	A2	7	...	*Pinus pinaster*
C227	South Africa	A2	19	1	*Pinus pinaster*
C228	South Africa	A2	20	2	*Eucalyptus* sp.
C274	South Africa	A2	25	1	*Pinus pinaster*
C284	South Africa	A1	54	...	*Leucadendron tinctum*
C371	South Africa	A1	52	3	*Serruria florida*
C403	South Africa	A1	53	...	*Mimetus capitulatus*
C410	South Africa	A1	55	...	*Leucospermum pluridens*
C411	South Africa	A2	59	5	*Pinus radiata*
C412	South Africa	A2	3	...	*Pinus radiata*
C414	South Africa	A1	51	...	*Leucospermum conocarpodendron*
C415	South Africa	A1	54	...	*Leucospermum praecox*
C418	South Africa	A1	62	3	*Cunninghamia casuarina*
C419	South Africa	A1	54	...	*Banksia burdenii*
C432	South Africa	A1	53	...	Water
C435	South Africa	A2	10	1	*Pinus radiata*
C453	South Africa	A2	8	1	*Pinus patula*
C504	South Africa	A2	4	1	*Pinus radiata*
CP80	South Africa	A2	37	2	*Persea americana*
CP81	South Africa	A2	43	...	*Persea americana*
CP227	South Africa	A1	51	...	*Casuarina* sp.

Isolate number	Origin	Mating type	RAPD phenotype	RFLP genotype	Host
CP233	South Africa	A1	62	3	*Casuarina* sp.
CP467	South Africa	A2	45	2	*Eucalyptus fastigata*
CP468	South Africa	A2	44	2	*Eucalyptus fastigata*
CP470	South Africa	A2	15	...	*Eucalyptus smithii*
CP477	South Africa	A2	16	1	*Eucalyptus smithii*
CP481	South Africa	A2	15	1	*Eucalyptus smithii*
CP488	South Africa	A2	45	...	*Eucalyptus macarthurii*
CP490	South Africa	A2	3	1	*Pinus radiata*
CP491	South Africa	A2	3	1	*Pinus radiata*
CP492	South Africa	A2	3	...	*Pinus patula*
CP494	South Africa	A2	17	1	*Pinus patula*
CP499	South Africa	A2	12	2	*Persea americana*
CP503	South Africa	A2	38	2	*Ocotea bullata*
CP504	South Africa	A2	6	1	*Prunus* sp.
CP506	South Africa	A2	34	...	*Ocotea bullata*
CP507	South Africa	A2	36	2	*Ocotea bullata*
CP508	South Africa	A2	43	2	*Ocotea bullata*
CP509	South Africa	A2	43	...	*Ocotea bullata*
CP510	South Africa	A2	49	2	*Ocotea bullata*
CP511	South Africa	A2	29	2	*Ocotea bullata*
CP513	South Africa	A1	53	...	*Ocotea bullata*
CP514	South Africa	A2	37	2	*Ocotea bullata*
CP517	South Africa	A2	35	2	*Ocotea bullata*
CP518	South Africa	A1	58	3	*Ocotea bullata*
CP522	South Africa	A2	3	1	*Ocotea bullata*
CP525	South Africa	A2	38	2	*Ocotea bullata*
CP526	South Africa	A2	38	...	*Ocotea bullata*
CP527	South Africa	A1	53	...	*Ocotea bullata*
CP528	South Africa	A2	35	...	*Ocotea bullata*
CP529	South Africa	A2	11	2	*Ocotea bullata*
CP530	South Africa	A2	34	2	*Ocotea bullata*
CP531	South Africa	A1	53	...	*Ocotea bullata*
CP532	South Africa	A1	54	...	*Ocotea bullata*
CP533	South Africa	A1	53	...	*Ocotea bullata*
CP534	South Africa	A2	40	7	*Ocotea bullata*
CP537	South Africa	A2	39	2	*Ocotea bullata*
CP538	South Africa	A2	39	2	*Ocotea bullata*
CP541	South Africa	A2	35	2	*Ocotea bullata*
CP542	South Africa	A2	32	2	*Ocotea bullata*
CP544	South Africa	A2	35	...	*Ocotea bullata*
CP545	South Africa	A2	38	...	*Ocotea bullata*
CP546	South Africa	A2	35	...	*Ocotea bullata*
CP548	South Africa	A2	3	1	*Ocotea bullata*
CP550	South Africa	A2	3	...	*Ocotea bullata*
CP551	South Africa	A1	54	...	*Ocotea bullata*
T2	South Africa	A2	17	1	*Ananas comosum*
T3	South Africa	A1	54	...	*Leucadendron argenteum*

Isolate number	Origin	Mating type	RAPD phenotype	RFLP genotype	Host
T4	South Africa	A2	26	1	*Leucospermum cordiifolium*
T5	South Africa	A1	53	...	*Leucospermum comosum*
T6	South Africa	A2	31	2	*Persea americana*
T7	South Africa	A1	53	...	*Vitis vinifera*
T8	South Africa	A2	7	1	*Eucalyptus fraxinoides*
T11	South Africa	A1	53	3	*Serruria krausii*
T12	South Africa	A1	53	...	*Priestleya* sp.
T13	South Africa	A1	55	3	*Leucospermum reflexum*
T14	South Africa	A1	54	3	*Orothamnus zeiheri*
T16	South Africa	A1	54	...	*Hakea sericea*
T17	South Africa	A1	53	...	*Protea* sp.
T18	South Africa	A1	53	...	*Mimetus splendidus*
T19	South Africa	A2	29	...	*Cryptomeria liebertiana*
T21	South Africa	A1	53	3	Water
T22	South Africa	A1	54	...	Water
T24	South Africa	A1	53	...	Water
T25	South Africa	A1	53	...	Water
T26	South Africa	A1	53	...	Water
T27	South Africa	A1	53	3	Water
T28	South Africa	A1	53	...	Water
T30	South Africa	A1	53	...	*Mimetus splendidus*
T31	South Africa	A1	13	3	*Ocotea bullata*
T32	South Africa	A1	53	...	*Ocotea bullata*
T33	South Africa	A1	55	3	*Ocotea bullata*
T34	South Africa	A1	53	3	*Cunnonia capensis*
T35	South Africa	A1	54	...	*Ocotea bullata*
T36	South Africa	A2	40	...	*Ocotea bullata*
T37	South Africa	A1	51	...	*Ocotea bullata*
T38	South Africa	A2	28	2	*Protea roupelliae*
T44	South Africa	A2	5	1	*Ocotea bullata*
UQ629	Australia	A2	39	2	*Telopea* sp.
UQ633	Australia	A1	55	3	unknown
UQ640	Australia	A2	3	1	*Lychee chinensis*
UQ642	Australia	A2	39	2	*Oryza sativa*
UQ665	Australia	A2	3	10	*Leucospermum* sp.
UQ732	Australia	A2	3	10	*Banksia* sp.
UQ733	Australia	A2	3	1	*Darwinia oxylepis*
UQ734	Australia	A2	3	1	*Allocasuarina fraseriana*
UQ735	Australia	A2	3	10	*Pinus radiata*
UQ736	Australia	A2	3	10	*Adenanthos* sp.
UQ737	Australia	A2	33	2	*Hibbertia subvaginata*
UQ738	Australia	A2	3	1	*Banksia grandis*
UQ739	Australia	A2	3	1	*Banksia* sp.
UQ740	Australia	A2	3	1	*Banksia quercifolia*
UQ741	Australia	A2	3	1	*Pinus radiata*
UQ742	Australia	A2	1	1	*Xanthorrhoea preissii*
UQ743	Australia	A2	3	1	*Myrtaceae*

Isolate number	Origin	Mating type	RAPD phenotype	RFLP genotype	Host
UQ771	Australia	A2	46	1	*Persea americana*
UQ787	Australia	A2	63	2	*Eucalyptus globoidea*
UQ788	Australia	A2	3	1	*Castenea sativa*
UQ789	Australia	A1	55	3	*Eucalyptus gummifera*
UQ790	Australia	A1	55	3	*Pinus radiata*
UQ791	Australia	A2	10	4	*Banksia marginata*
UQ792	Australia	A1	55	3	*Pinus elliottii*
UQ794	Australia	A2	3	1	*Persea americana*
UQ795	Australia	A1	56	3	Soil
UQ817	Australia	A2	30	2	*Aotus ericoides*
UQ818	Australia	A2	22	9	*Dillwynia floribunda*
UQ822	Australia	A2	3	1	Soil
UQ823	Australia	A2	3	1	*Monotoca glauca*
UQ824	Australia	A2	21	2	Soil
UQ827	Australia	A1	27	6	*Allocasuarina littoralis*
UQ828	Australia	A1	50	3	unknown
UQ871	Australia	A2	2	1	*Castanea* sp.
UQ873	Australia	A2	3	1	Soil
UQ877	Australia	A2	3	1	Soil
UQ879	Australia	A2	3	...	Soil
UQ891	Australia	A2	3	1	*Persea americana*
UQ835	Papua New Guinea	A1	41	13	Soil
UQ821	Papua New Guinea	A2	7	1	Soil
UQ820	Papua New Guinea	A2	3	1	Soil
UQ832	Papua New Guinea	A2	18	11	Soil
UQ831	Papua New Guinea	A1	48	8	Soil
UQ819	Papua New Guinea	A2	38	2	Soil
UQ836	Papua New Guinea	A1	9	12	Soil

TABLE 2. Nucleotide sequence of decanucleotide Operon primers used to characterise *Phytophthora cinnamomi* populations and the number of polymorphic polymerase chain reaction (PCR) fragments analysed for each primer

Primer	Nucleotide sequence	No. of polymorphic fragments
OPAC-01	TCCCAGCAGA	3
OPM-10	TCTGGCGCAC	6
OPT-07	GGCAGGCTGT	5
OPZ-04	AGGCTGTGCT	2

TABLE 3. Fifteen probes screened for restriction fragment length polymorphisms (RFLP's) of *Phytophthora cinnamomi* populations

Probe			Restriction enzyme[z]		
Name	Species of origin	Obtained from	*Hind*III	*Xho*I	*Pst*I
ß-tubilin	*P. cinnamomi*	Lehnen and Hardham, unpubl.	-	+	+
BipBh5	*P. cinnamomi*	Lehnen and Hardham, unpubl.	+	+	-
LPV18	*P. cinnamomi*	Marshall and Hardham, unpubl.	-	-	-
pADACT	*P. infestans*	Unkles *et al.* 1991	-	+	-
pEf1α[y]	*P. infestans*	Pieterse *et al.* 1993	++	+	++
pNia7-Ss	*P. infestans*	Pieterse *et al.* 1995	-	-	-
pPc2	*P. cinnamomi*	Marshall and Hardham, unpubl.	-	-	+
pPc3	*P. cinnamomi*	Marshall and Hardham, unpubl.	-	-	+
pPc5	*P. cinnamomi*	Marshall and Hardham, unpubl.	+	+	-
pPc7	*P. cinnamomi*	Marshall and Hardham, unpubl.	-	-	-
pPc9	*P. cinnamomi*	Marshall and Hardham, unpubl.	-	-	-
pPc10	*P. cinnamomi*	Marshall and Hardham, unpubl.	-	-	+
pPc11	*P. cinnamomi*	Marshall and Hardham, unpubl.	-	-	-
pPc14	*P. cinnamomi*	Marshall and Hardham, unpubl.	-	-	-
pPc15	*P. cinnamomi*	Marshall and Hardham, unpubl.	-	-	-

[y] In the case of *pEf1α* at *Hind*III and *Pst*I, two fragments were observed that were confirmed to be not allelic after simultaneous digestion with *Hind*III and *Pst*I. Therefore, two loci *pEf1α*-1 and *pEf1α*-2 were scored when genomic *P. cinnamomi* DNA was digested with *Hind*III and *Pst*I.

[z] (+) = polymorphic; (-) = monomorphic, or (++) = multiple.

TABLE 4. Alleles present in South African, Australian and Papua New Guinea isolates of *Phytophthora cinnamomi* in each of 13 different restriction fragment length polymorphism (RFLP) genotypes

	Locus																No. of isolates		
	*Hind*III				*Xho*I					*Pst*I									
Genotype	*BipBh5*	*pPc5*	*pEfl α*-1	*pEfl α*-2	*BipBh5*	*β*-tubilin	*pEfl α*	*pADACT*	*pPc5*	*β*-tubilin	*pPc3*	*pPc2*	*pEfl α*-1	*pEfl α*-2	*pPc10*	Mating type	SA[x]	Aus[y]	PNG[z]
1	AA	AA	AA	BB	AA	AA	AA	AB	AA	AA	AA	AB	AA	BB	AB	2	25	18	2
2	AA	AA	AA	BB	AB	AA	AA	BB	AA	AA	AB	AA	AA	BB	AA	2	23	6	1
3	AA	AA	AA	BB	AA	AA	AA	BB	AA	AA	AA	AA	AA	BB	AA	1	15	6	0
4	AA	AA	AA	BB	AA	AA	AA	AA	AA	AA	AA	AB	AA	BB	AB	2	0	1	0
5	AA	AA	AA	BB	AA	AA	AA	AB	AA	AA	AA	AB	AA	BC	AB	2	1	0	0
6	AA	AA	AA	BB	AA	AA	AA	BB	AA	AA	AA	BB	AA	BC	AA	1	0	1	0
7	AA	AA	AA	BB	AA	AA	AA	BB	AA	AA	AB	AA	AA	BB	AA	2	1	0	0
8	AB	AA	AA	BB	AA	AB	AA	BB	AA	AA	AB	AB	AA	BB	AB	1	0	0	1
9	AA	AA	AA	BB	AB	AA	AA	BB	AA	AA	AA	AA	AA	BB	AA	2	0	1	0
10	AA	AA	AA	BB	AC	AA	AA	AB	AA	AA	AA	AB	AA	BB	AB	2	0	4	0
11	CD	BB	AA	BC	AC	CD	AA	BB	BC	AA	AA	AB	AA	BC	AB	2	0	0	1
12	AB	AB	AA	BB	AC	BC	AA	BB	AA	AA	AB	AB	AA	BB	AB	1	0	0	1
13	AB	AA	AA	BB	AC	BB	AA	BB	AA	AA	AB	AB	AA	BB	AB	1	0	0	1
																Total no. of isolates:	65	37	7

[x] SA = South Africa

[y] Aus = Australia

[z] PNG = Papua New Guinea

TABLE 5. Summary statistics for *Phytophthora cinnamomi* populations from South Africa, Australia and Papua New Guinea, based on 16 random amplified polymorphic DNA (RAPD) markers and 34 restriction fragment length polymorphism (RFLP) alleles from 15 putative loci

	South Africa	Australia	Papua New Guinea
RAPD's			
No. of isolates	121	38	7
No. of phenotypes	48	15	7
$\hat{G}$ [t]	13.6	3.3	7.0
$\hat{G}/N$ (%)[u]	11.2	8.7	100.0
Clonal fraction	60.3	60.5	0
RFLP's			
No. of isolates	65	37	7
Genotypes	1-3,5,7	1-4,6,9,10	1,2,8,11-13
$\hat{G}$ [t]	3.1	3.3	5.5
$\hat{G}/N$ (%)[u]	4.7	8.9	77.8
A[v]	1.4	1.5	2.1
H_{obs}[x]	0.129	0.155	0.308
H_{exp}[y]	0.105	0.118	0.288
F[z]	-0.229	-0.313	-0.069

[t] $\hat{G}$ = Phenotypic/genotypic diversity (Stoddard and Taylor, 1988).

[u] $\hat{G}/N$ (%) = The percentage of maximum possible diversity obtained.

[v] A = Mean number of alleles per locus.

[x] H_{obs} = Observed heterozygosity.

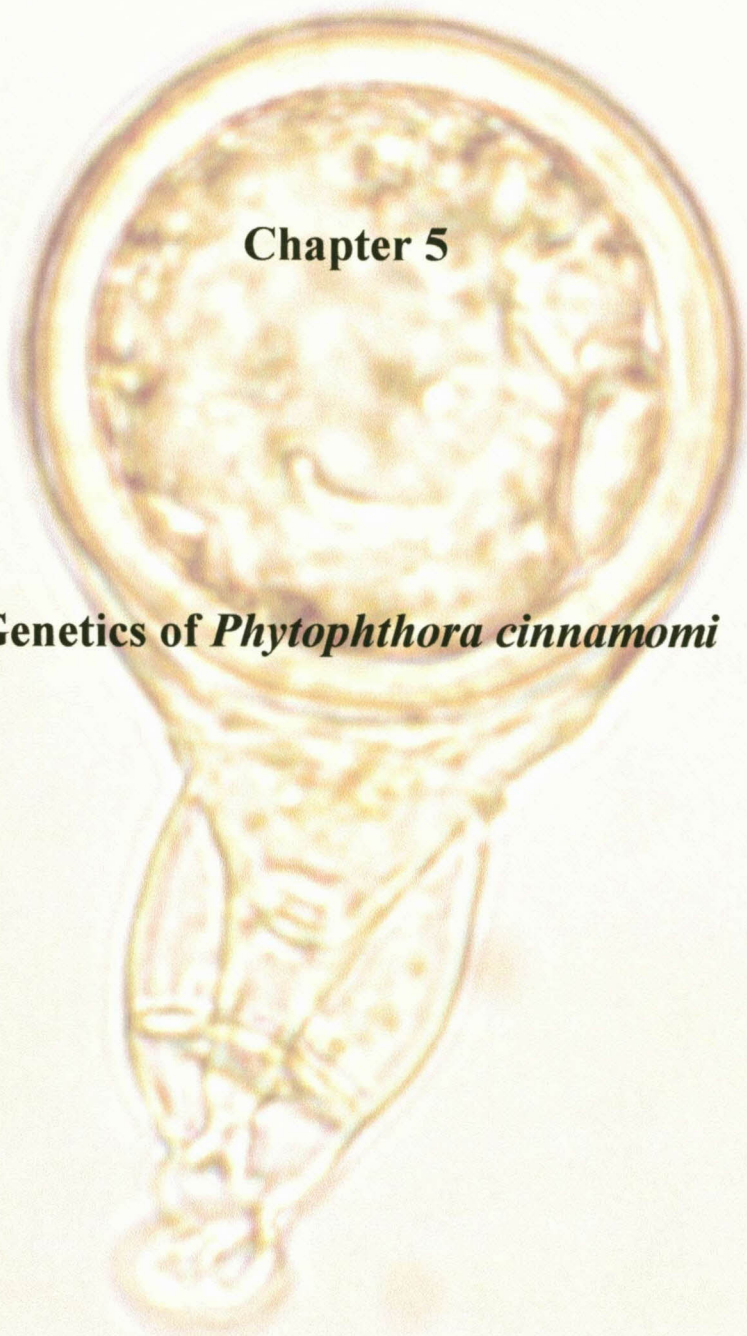
[y] H_{exp} = Expected heterozygosity (= Nei's gene diversity index [Nei, 1973]).

[z] F = Wright's fixation index.

TABLE 6. Unbiased minimum genetic distance (D_m) among *Phytophthora cinnamomi* mating type and regional populations from South Africa, Australia, and Papua New Guinea

Region	Population	D_m [z]
South Africa	A1:A2	0.020
Australia	A1:A2	0.025
	SA:AUS	0.003
	SA:PNG	0.022
	AUS:PNG	0.020

[z] Genetic distance (Nei, 1978) calculated including all 15 restriction fragment length polymorphism (RFLP) loci.



Chapter 5

Genetics of *Phytophthora cinnamomi*

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ABSTRACT

In this study, South African *P. cinnamomi* A1 and A2 mating type isolates were paired, oospores were produced, and 50 oospores were successfully germinated. Forty-nine progeny were identified as F_1 hybrids using 16 RAPD fragments. One of the progeny was a selfed isolate of the A1 mating type parent. Among the 49 hybrid F_1 isolates identified, 24 were of the A1 mating type, and 25 of the A2 mating type. Pathogenicity tests using parental and hybrid F_1 mating type isolates were conducted on *Eucalyptus smithii* to demonstrate the pathogenicity of hybrid F_1 isolates. The average levels of pathogenicity of hybrid F_1 isolates, measured as lesion length on *E. smithii*, were significantly lower than that of their parents. No significant differences in levels of pathogenicity between A1 and A2 mating type F_1 hybrid isolates was identified. This is the first report which demonstrates sexual recombination in *P. cinnamomi*. Although sexual recombination of *P. cinnamomi* is possible *in vitro,* it has not been demonstrated under field conditions. Sexual reproduction may play a significant role in long term survival as oospores in the absence of suitable host plants, and in generating and maintaining genetic diversity to ensure its continual survival.

INTRODUCTION

Phytophthora cinnamomi Rands is a soilborne fungus which is particularly important as a pathogen of *Eucalyptus* in South Africa (Linde *et al.,* 1994; Wingfield and Knox-Davies, 1980). *P. cinnamomi* belongs to the class Oomycetes which are diploid in the vegetative stage (Brasier and Sansome, 1975). *P. cinnamomi* is heterothallic with mating types first noted by Haasis and Nelson (1963) which they designated + and - isolates. Then, Galindo and Zentmyer (1964) showed that the different mating types, which they called A1 and A2, participate in the sexual process. Sexual reproduction takes place when specialised structures (antheridia and oogonia) produced on opposite thalli of both mating types, interact to produce an oospore. Meiosis occurs in the multinucleate antheridia and oogonia (Shaw, 1983) before a single antheridial nucleus migrates to the oogonium where it fuses with one of the oogonial nuclei to form an oospore. Oospores are thick walled structures which enable *Phytophthora* spp. to survive many years outside the host (Duncan and Cowan, 1980).

Both mating types of *P. cinnamomi* have been introduced into South Africa (Linde *et al.,* 1997, Chapter 2; Chapter 4) and opportunities for sexual reproduction exist. Population analysis using isozymes, indicated the lack of, or rare occurrence of sexual reproduction in Australian (Old *et al*, 1984, 1988) and South African *P. cinnamomi* populations (Linde *et al.,* 1997, Chapter 2). Significant genetic differentiation identified between the South African *P. cinnamomi* mating type populations, provides evidence for the lack of sexual reproduction (Linde *et al.*, 1997, Chapter 2). Various hypothesis for the apparent lack of sexual reproduction in *Phytophthora* populations have been put forward. These include: *(i)* inability to mate and produce viable oospores because of genetic barriers between mating types such as different ploidy levels or chromosome numbers (Whittaker *et al.*, 1991), *(ii)* oospore abortion preventing reproduction of non-parental genotypes (Old *et al.*, 1988; Rutherford and Ward, 1985), *(iii)* F_1 hybrids are non-pathogenic and, therefore, never make up a significant part of the population and thus escape detection, and *iv)* failure of oospores to germinate (Chang and Ko, 1991; Chang *et al.,* 1996). Because of the lack of genetic studies in *P. cinnamomi*, unambiguous evidence to test these hypotheses is, unfortunately, not available.

Despite the importance of *P. cinnamomi* as a plant pathogen (Zentmyer, 1980), the importance of sexual reproduction in providing genetic diversity in *P. cinnamomi* populations, coupled with the importance of the formation of long term survival structures in the form of oospores, have not been determined. *In vitro* oospore production when pairing opposite mating types of *P. cinnamomi* cultures is abundant (Galindo and Zentmyer, 1964; Ribeiro *et al.*, 1975), whereas the occurrence of oospores *in vivo* is rare and has been observed only sporadically in soil and naturally infested host tissue (Mircetich and Zentmyer, 1966). Oospore germination is difficult, although germination rates between 1-45% have been reported among oospores produced *in vitro* (Ribeiro *et al.*, 1975). However, *P. cinnamomi* oospore germination studies as yet failed to provide unambiguous evidence concerning the genetic make-up of the generated oospores.

Sexual reproduction in *Phytophthora* species has a significant influence on the levels of gene and genotypic diversity in pathogen populations. For example, the introduction of the A2 mating type of *Phytophthora infestans* into Europe, resulted in sexual recombination in the *P. infestans* populations, increased gene and genotypic diversity, and rapid development of more complex virulent races able to overcome combinations of resistance genes in existing potato cultivars (Drenth *et al.*, 1994). Hence, in breeding and selection programs for resistance against *P. cinnamomi* in *Eucalyptus*, it is important to determine whether *P. cinnamomi* is able to reproduce sexually and yield viable, pathogenic progeny.

The aims of the current investigation were to: *(i)* demonstrate *in vitro* sexual reproduction in *P. cinnamomi* and determine mating type segregation among F_1 hybrids, *(ii)* determine the hybrid nature of F_1 progeny using genetic markers, and *(iii)* determine the pathogenicity of F_1 hybrids as compared to the pathogenicity levels present in the parental isolates. This information is vital to demonstrate the ability of *P. cinnamomi* to outbreed and produce viable hybrid progeny. Levels of pathogenicity in the progeny compared to the parents will provide valuable information regarding the ability of hybrids to cause disease and provide insight into the possibility of sexual recombination of *P. cinnamomi* in the field.

MATERIALS AND METHODS

***P. cinnamomi* isolates**. Initially, 15 crosses using South African *P. cinnamomi* isolates were attempted over a period of 2 years, using different parents for each cross. All the crossings produced oospores but the oospores failed to germinate. Then a cross was repeated between *P. cinnamomi* isolates CP513 (A1 mating type) and CP508 (A2 mating type) from *Ocotea bullata*, a native South African forest tree species and these provided viable oospores. These isolates are in the culture collection of Infruitec: Plant Biotechnology and Pathology, Stellenbosch, South Africa and deposited as UQ2923 and UQ2919 in the culture collection of the Cooperative Centre for Tropical Plant Pathology, the University of Queensland, Australia. It was already shown in Chapter 4 that RAPD and RFLP profiles of these parent isolates differ.

Crosses, isolation and analysis of F_1 progeny. Mycelial discs from both parents were placed on opposite sides of five plates of carrot agar (Ribeiro, 1978). Plates were incubated at 20°C in the dark for 3 months. Oospores were harvested and isolated by maceration of agar strips in a cooled domestic blender at full speed for 10 minutes. The oospore suspension was sieved through a nylon mesh (75 μm), plated on 1.5 % water agar plates and allowed to germinate in the dark at 25°C. Germinating oospores were obtained after 3 days and using microdissection transferred to 20 % clarified V8 agar plates (Ribeiro, 1978) and incubated in the dark at 25°C. To ensure single colonies, hyphal tipping was performed before further analysis of F_1 progeny. Mycelia scraped from the surface of each hyphal tipped isolate was inoculated into 2 plates containing 20 % clarified V8 broth and incubated in the dark at 25°C for 5-6 days. Mycelium was harvested using a Büchner funnel and freeze-dried for DNA extraction according to Drenth *et al.*, (1993). A total of 55 RAPD decanucleotide primers were screened from Operon kits OPG, OPS, and OPX to distinguish hybrids and selfs from parental isolates. Seven primers were selected (Table 1) to identify F_1 individuals. RAPD procedures were as described previously (Chapter 4), except 30 ng instead of 60 ng of DNA, and 1% instead of 1.5% agarose gels were used.

Determination of mating type. Mating type of all F_1 isolates was determined by pairing with isolates of known mating type on carrot agar plates (Ribeiro, 1978). Plates were incubated at 20°C in the dark and examined for the presence of oospores after 4 weeks.

Pathogenicity tests. The parental *P. cinnamomi* isolates are both pathogenic on *Eucalyptus smithii* as tested previously (Linde *et al.*, 1998, Chapter 3). The parental isolates and all hybrid F_1 isolates were artificially inoculated on 1-year-old *E. smithii* seedlings in the greenhouse. Eight trees per isolate were artificially inoculated in a complete randomised block design. *P. cinnamomi* isolates were grown on potato dextrose agar (PDA) for 7 days at 25°C. Mycelial discs were inoculated into 10 mm diameter wounds made with a corkborer in the stem of each tree. For control inoculations, eight trees were inoculated with a sterile disc of PDA. Wounds were sealed with parafilm to prevent desiccation. Lesion lengths in the secondary phloem (Shearer *et al.*, 1987; Tippett *et al.*, 1983) were measured 2 weeks after inoculation. Re-isolations onto a selective medium (Tsao and Guy, 1977) were made from control and inoculated trees. To prevent the release of *in vitro* produced hybrid F_1 isolates, seedlings and seedling pots used in pathogenicity tests were autoclaved after measurements were made. Stem lesion data were analysed using ANOVA (Snedecor and Cochran, 1980). Contrasts for differences in lesion lengths were specified between F_1 hybrid and parental isolates, and between F_1 hybrid isolates of different mating type (Snedecor and Cochran, 1980).

RESULTS

Identification of F_1 hybrids. Oospores were produced 3-4 weeks after incubation (Fig. 1). Germinated oospores (Fig. 2) were observed 3-18 days after harvesting. The highest rate of germination was 5-10 days after harvesting. Sixty-seven germinated oospores were obtained, and after hyphal tipping, 50 were screened with RAPD primers to verify their hybrid nature. Among the 50 progeny analysed, 49 contained one or more RAPD fragments from each parent indicating that they were true hybrids. One of the progeny, S14, was a selfed isolate.

Mating type. Among the 49 F_1 hybrids identified, 24 were of the A1 mating type and 25 of the A2 mating type. The one selfed isolate (S14) was a self of the A1 mating type parent CP513 and had the same mating type (Table 2).

Pathogenicity tests. All 49 F_1 hybrids and both parental isolates were pathogenic towards *E. smithii* (Table 3). Lesion lengths of *P. cinnamomi* isolates inoculated on *E. smithii,* differed significantly (P = 0.0001) from each other (Table 4). Contrast analysis showed that the average lesion length of F_1 hybrid isolates as a group, was significantly (P = 0.0001) lower than both parental isolates (Table 4). However, the average lesion lengths of some F_1 hybrid progeny did not differ significantly from that of the parents (Table 3). Average lesion lengths of A1 and A2 mating type F_1 *P. cinnamomi* isolates did not differ significantly from each other (Table 4). Inoculated *P. cinnamomi* isolates were successfully re-isolated from all inoculations. Control inoculations did not develop any lesions.

DISCUSSION

This study provides the first unequivocal evidence for sexual recombination between A1 and A2 mating type isolates of *P. cinnamomi*. Previous studies aimed at constructing crosses between different mating type populations of various *Phytophthora* species, often failed to provide unambiguous evidence concerning the hybrid nature of the progeny isolated. The use of genetic markers in our study shows the hybrid nature of progeny and distinguishes hybrids from selfs. The hybrid nature of the oospores was unambiguously verified using RAPDs. RAPD and other markers have also been used successfully to construct crosses among isolates of homothallic Oomycetes such as *Pythium ultimum* (Francis and St. Clair, 1993) and *Phytophthora sojae* (Whisson *et al.*, 1994), and in the heterothallic *P. infestans* (Judelson *et al.,* 1995; Shattock *et al.*, 1986).

Mating type segregated in our cross in a close to perfect 1:1 ratio. Mating type in Oomycetes and in *P. infestans* in particular, is hypothesised to be controlled by one locus. The A1 type being determined by heterozygosity *Aa* and the A2 type by homozygosity *aa* (Judelson, 1996). However, mating type segregation in *P. infestans* frequently is not

Mendelian (Judelson *et al.,* 1995) and the same could well be observed in more crosses of *P. cinnamomi.*

The inability to germinate oospores in the first fifteen attempts could either be due to genetic barriers between the parents used, and/or unfavourable conditions for germination of oospores. Oospores are believed to have some form of dormancy, and although conditions for oospore germination have been investigated in great detail (Erwin and Ribeiro, 1996), no reliable triggers to obtain high germination rates have been found. The parents in our last and successful cross had been used once before, without obtaining any germinating oospores. Our results indicate that there are probably no genetic barriers to mating but that the inability to select the correct biologically based time for preparation of oospores and the conditions for germination are the main sources of failure to obtain germinating oospores and establish hybrid F_1 cultures. Under natural conditions in infected plant material, this might well be different.

Levels of pathogenicity of F_1 hybrid isolates were similar to, or lower than that of the parental isolates. In host specific *Phytophthora* species such as *P. sojae* (Whisson *et al.*, 1994) and *P. infestans* (Al-Kherb *et al.*, 1995), genes for avirulence are often dominant to virulence. Although the genetics of pathogenicity in *P. cinnamomi* is unknown, recessivenes of some of the pathogenicity genes required to show pathogenicity to *E. smithii*, could provide an explanation for F_1 isolates with levels of pathogenicity lower than that of their parents. Since pathogenicity has been defined only in terms of growth rate on *E. smithii*, in constitutes to only one factor among many which determine the ultimate fittness of an isolate. Experiments in the greenhouse with mixtures of clonal lines and F_1 hybrids are needed over a time frame of a few years, to assess the comparative fitness of hybrids versus established clonal lines.

Generation and maintenance of genetic diversity for plant pathogens is important for their adaptability and continued survival. In heterothallic Oomycetes, two mating types are required for sexual reproduction which will ensure outcrossing, although inbreeding has also been observed in *P. infestans* (Goodwin *et al.*, 1992), *Phytophthora parasitica* (Förster and Coffey, 1990) and *Pythium sylvaticum* (Martin, 1989). In homothallic Oomycetes, only a single isolate is necessary for sexual reproduction, but outcrossing also occur at low frequencies in mixed cultures of *P. sojae* (Whisson *et al.*, 1994) and *Pythium ultimum* (Francis and St. Clair,

1993). In the homothallic *P. ultimum*, isolates showing unusual hyphal swellings are incapable of forming oospores in pure culture (Francis and St. Clair, 1993; Martin, 1990). These secondary homothallic isolates act as males in crosses and may provide a mechanism to force outcrossing and generate genetic diversity. Occasional outcrossing in homothallic species probably contributes to the development of new races (Förster *et al.*, 1994) and play an important role in generating and maintaining genetic diversity in populations (Whisson *et al.*, 1994). Inbreeding in heterothallic species is expected not to have a significant effect on the genetic diversity of populations, because of the availability of outcrossings as means of maintaining genetic diversity.

Sexual recombination in *P. cinnamomi* populations could have serious consequences for breeding and selection programs, not only for *Eucalyptus* breeding in South Africa, but for any other plant breeding program for *P. cinnamomi* resistance. Sexual recombination in *P. cinnamomi* populations would provide high levels of gene and genotypic diversity and new combinations of alleles could result in new and more pathogenic isolates. This may prove breeding for resistance in a long term crop such a *Eucalyptus,* considerably more difficult. Although resistance in *Eucalyptus marginata* was maintained when trees were exposed to a wide range of *P. cinnamomi* isolates belonging to different isozyme genotypes (Dudzinski *et al.*, 1993), it is possible that resistance could be overcome when new *P. cinnamomi* genotypes are produced as a result of sexual recombination. However, resistance to *P. cinnamomi* in *E. marginata* families has been found to be under strong genetic control (Stukely and Crane, 1994), making breeding for resistance still a viable option.

In addition to generating genetic diversity, oospores are also formidable survival structures. They are the only structures which can survive in the absence of the host plant for long periods of time (Duncan and Cowan, 1980). Oospores of *P. infestans* have been shown to survive for over a year (Drenth *et al.*, 1995; Pittis and Shattock, 1994), while oospores of *Peronospora destructor* have been shown to survive for over 25 years in soil (McKay, 1957).

Our pathogenic F_1 hybrids show that there is no genetic barrier between A1 and A2 mating type populations in South Africa which prevent sexual recombination. The discovery of sexual recombination *in vitro* in *P. cinnamomi* certainly provides ample opportunities for further genetic and population genetic studies to determine the importance of the formation of

oospores and outbreeding under field conditions. There is a need to determine to what extent oospores contribute to disease and which factors influence production, survival, germination and infectivity of oospores.

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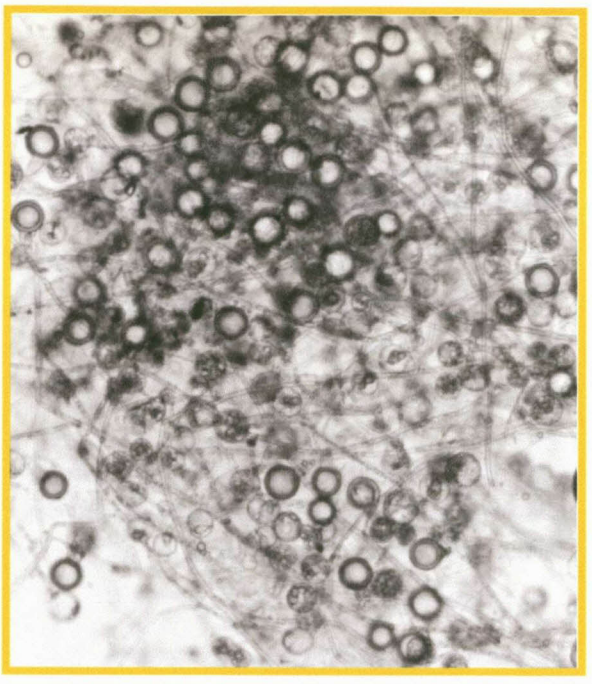

Fig. 1. *Phytophthora cinnamomi* oospores produced *in vitro.*

Fig. 2. Germination of *Phytophthora cinnamomomi* oospore *in vitro.*

TABLE 1. RAPD markers used to identify F_1 hybrid progeny by the presence of fragments in the parental South African *Phytophthora cinnamomi* isolates CP513 and CP508

RAPD Primer [a]	Sequence	Marker	Fragment presence	
			CP513	CP508
OPS-14	AAAGGGGTCC	S14-1	0	1
OPS-20	TCTGGACGGA	S20-1	0	1
		S20-2	1	0
OPX-12	TCGCCAGCCA	X12-2.1	1	0
		X12-2.2	1	0
OPG-16	AGCGTCCTCC	G16-1	0	1
		G16-2	1	0
OPG-15	ACTGGGACTC	G15-1	0	1
		G15-2.1	1	0
		G15-2.2	1	0
		G15-2.3	1	0
OPG-14	GGATGAGACC	G14-1	0	1
		G14-2.1	1	0
		G14-2.2	1	0
OPG-11	TGCCCGTCGT	G11-2.2	1	0
		G11-2.2	1	0

[a] Operon Technologies.

TABLE 2. Mating type segregation and identity of South African *Phytophthora cinnamomi* F_1 progeny as determined with RAPD markers

Cross	
CP513 x CP508	Number of isolates
Hybrid	
A1 (CP513)	24
A2 (CP508)	25
Selfs	
A1 (CP513)	1
A2 (CP508)	0
Total number of progeny analysed	50

TABLE 3. Comparison of lesion lengths (mm) induced by parental and F_1 hybrid progeny of A1 and A2 mating type of *Phytophthora cinnamomi* isolates, two weeks after stem inoculation on *Eucalyptus smithii*

Isolate	Lesion length (mm)[x]	Isolate	Lesion length (mm)[x]
CP513 A1	170	CP508 A2	110
A1 Hybrids		**A2 Hybrids**	
S2	73	S1	53
S5	55	S3	55
S6	98	S4	53
S11	32	S7	82
S22	80	S8	50
S24	81	S10	68
S28	48	S13	134
S29	88	S15	37
S30	21	S16	106
S33	30	S17	57
S34	71	S18	59
S37	50	S20	48
S42	56	S21	53
S45	34	S25	72
S46	107	S26	40
S48	40	S31	55
S51	80	S35	54
S52	61	S36	82
S53	51	S38	46
S55	49	S39	69
5S7	37	S40	62
S58	71	S41	40
S59	62	S44	40
S60	41	S47	33
		S54	94
Average of F_1 A1 mating type isolates	59	**Average of F_1 A2 mating type isolates**	62

[x] Least significant difference (LSD) = 27.739; $P < 0.05$.

TABLE 4. Analysis of variance for the variation in pathogenicity among F_1 hybrid isolates, and contrasts between F_1 hybrid versus the parents, and between A1 and A2 F_1 hybrid mating type populations

Source of variation	Degrees of freedom	Mean square	*P*
Isolates	52	6494.9	0.0001
Contrasts:			
F_1 vs Parents[x]	1	96523.4	0.0001
A1 vs A2[y]	1	663.6	0.3618

[x] Contrasts were specified between F_1 hybrid isolates and parental isolates (CP508 and CP513).

[y] Only F_1 hybrid isolates were used to specify contrasts.

SUMMARY

Research presented in this dissertation describes the population structure of *Phytophthora cinnamomi* in South Africa. Special emphasis is given to the practical implications of population diversity issues for breeding and selection programs against *P. cinnamomi*. Levels of gene and genotypic diversity of *P. cinnamomi* populations, their origin, stability over time, variation in levels of pathogenicity and occurrence of sexual reproduction *in vivo* and *in vitro,* are considered.

The first chapter represents a literature review on the population biology of *P. cinnamomi*. Special reference is given to the life-cycle of *P. cinnamomi*, mechanisms of pathogenicity, and control measures. Available genetic markers useful for population genetic studies of fungal pathogens are described. The origin and maintenance of genetic diversity in fungi, with particular emphasis on *P. cinnamomi*, is discussed. Research concerning the genetic structure of *P. cinnamomi* populations is summarised, before the overall and specific aims of the research described in this dissertation are presented. This literature review also emphasises the lack of, or limited number of studies pertaining to the population structure of *P. cinnamomi*.

Chapter two considers levels of genetic diversity as determined in populations of *P. cinnamomi* in South Africa, using isozymes. Differentiation in regional *P. cinnamomi* populations, stability over time, and occurrence of sexual reproduction in the South African *P. cinnamomi* population is discussed. Evidence is presented suggesting that *P. cinnamomi* is introduced into South Africa and has low levels of gene and genotypic diversity. There is no genetic differentiation between regional *P. cinnamomi* populations and the populations are stable over time. It is also shown that *P. cinnamomi* rarely reproduces sexually, if at all.

The success of breeding and selection programs against *P. cinnamomi* is dependant on the levels of variation in pathogenicity of the pathogen. In chapter three, significant differences in levels of pathogenicity to *E. smithii* in the field, were found. Various factors are shown to affect levels of pathogenicity. These include growth rate *in vitro* of *P. cinnamomi,* culture age, season of inoculation, and multilocus isozyme genotype characteristics. On the other hand, mating type and geographic origin of *P. cinnamomi* isolates did not affect the level of pathogenicity. Significantly more variation was found among A2 than A1 mating type isolates, demonstrating the clonality of A1 mating type isolates. Results of this study provide valuable information on selection of *P. cinnamomi* isolates for future resistance/tolerance screening assays, of *Eucalyptus* germplasm in South Africa.

Chapter four deals with levels of gene and genotypic diversity in South African and Australian *P. cinnamomi* populations, using RAPD and RFLP markers. The high degree of similarity between the South African and Australian *P. cinnamomi* populations is demonstrated. A low level of genetic differentiation (D_m = 0.003) between the populations provides the most striking evidence of similarity in these two populations. A few isolates from Papua New Guinea, possessed unique alleles not found in the South African or Australian *P. cinnamomi* populations. The similarity of the South African and Australian *P. cinnamomi* populations provide opportunities to breed and select for resistance/tolerance in *Eucalyptus,* using either *P. cinnamomi* population.

Evidence for the occurrence of sexual recombination of *P. cinnamomi in vitro* and *in vivo*, is limited. The occurrence of sexual recombination *in vitro* is investigated in chapter five and the first unambiguous evidence for sexual recombination in *P. cinnamomi* is provided. This has important consequences for breeding and selection programs, as it could change the genetic structure of the population. Levels of pathogenicity of the F_1 progeny was on average lower than that of parental *P. cinnamomi* isolates. Other factors concerning fitness of the progeny should be investigated to explain the apparent lack of sexual reproduction *in vivo* in *P. cinnamomi.*

All chapters of this dissertation deal with some aspects of the population structure and genetic diversity within *P. cinnamomi* populations from South Africa. This is the first comprehensive study on the genetic diversity of *P. cinnamomi* populations. It sheds light on the origin, occurrence of sexual reproduction, variation in levels of pathogenicity amongst *P. cinnamomi* isolates, similarity with the Australian population, and the occurrence of genetic recombination *in vitro*. It also provides important information for the South African forestry industry, particularly in their efforts to breed and select cold tolerant *Eucalyptus* spp. that are resistant/tolerant to *P. cinnamomi.*

OPSOMMING

Navorsing in hierdie proefskrif beskryf die populasie struktuur van *Phytophthora cinnamomi* in Suid Afrika. Spesiale vermelding word gemaak van die praktiese implikasies wat populasie diversiteits aspekte het vir die teling en seleksie programme teen *P. cinnamomi*. Populasie aspekte wat ondersoek word sluit in, vlakke van geen en genotipiese diversiteit in *P. cinnamomi* populasies, hul oorsprong, stabiliteit, variasie in patogenisiteit, en die voorkoms van geslagtelike voortplanting *in vivo* en *in vitro*.

Die eerste hoofstuk is 'n literatuur oorsig wat die populasie biologie van *P. cinnamomi* beskryf. Vermelding word gemaak van die lewenssiklus van *P. cinnamomi*, patogenisiteits meganismes, and beheer praktyke. Genetiese merkers wat gebruik kan word vir populasie genetika studies van swam patogene word ook beskryf. Verder word die oorsprong en instandhouding van genetiese diversiteit in swamme, en spesifiek in *P. cinnamomi*, bespreek. 'n Belangrike aspek wat uitgelug word is die klein hoeveelheid studies wat tot dusver bygedra het tot die populasie struktuur van *P. cinnamomi*. Ten slotte word die navorsing wat betrekking het op die genetiese struktuur van *P. cinnamomi* populasies saamgevat, en die spesifieke doelwitte van die proefskrif opgesom.

Navorsing gedoen in hoofstuk twee beskryf die vlakke van genetiese variasie in Suid Afrikaanse *P. cinnamomi* populasies soos bepaal met isoensieme. Uit hierdie data blyk dit dat *P. cinnamomi* nie sy oorsprong in Suid Afrika het nie. Die vlakke van geen en genotipiese variasie is ook baie laag. Daar is nie genetiese differensiasie tussen *P. cinnamomi* populasies met verskillende geografiese oorspronge nie, en die populasies is stabiel oor tyd. Dit word ook gewys dat voortplanting in *P. cinnamomi* hoofsaaklik ongeslagtig is.

Verskille in patogenisiteits vlakke van *P. cinnamomi* bepaal deels die sukses van teel en seleksie programme daarteen. Resultate van hoofstuk drie wys dat isolate van *P. cinnamomi* wel betekenisvol verskil in patogenisiteits vlakke op *Eucalyptus smithii*. Verskeie faktore het die vlakke van patogenisiteit beïnvloed. Dit sluit in groei tempo *in vitro* van *P. cinnamomi*, ouderdom van kulture, seisoen van inokulasie, en multigeniese isoensiem eienskappe. Paringstipe en geografiese oorsprong van *P. cinnamomi* isolate het geen invloed op patogenisiteits vlakke nie. Daar was betekenisvol meer variasie binne A2 as binne A1 paringstipe isolate. Dit dui ook op die klonaliteit van A1 paringstipe isolate. Hierdie resultate verskaf belangrike inligting ten opsigte van die seleksie van *P. cinnamomi* isolate vir toekomstige weerstands/toleransie toetse van *Eucalyptus* in Suid Afrika.

Hoofstuk vier handel oor die vlakke van geen en genotipiese diversiteit in die Suid Afrikaanse, vergeleke met 'n Australiaanse *P. cinnamomi* populasie, soos bepaal met RAPD en RFLP merkers. Beide populasies het lae vlakke van genotipiese diversiteit, plant hoofsaaklik ongeslagtelik voort, het 'n hoë klonale fraksie, en het ooreenstemmende allele. Lae vlakke van genetiese differensiasie (D_m = 0.003) tussen die twee populasies dui ook sterk op die ooreenkoms tussen die twee populasies. Inteenstelling hiermee, het die isolate vanaf Papua New Guinea unieke allele wat nie in die ander populasies voorkom nie.

Hoofstuk vyf bied die eerste bewyse vir geslagtelike rekombinasie *in vitro* in *P. cinnamomi*. Nege-en-veertig F_1 hibriede en een self van die A1 paringstipe ouer is geïdentifiseer met RAPD's. F_1 hibriede het gesegregeer in 'n 1:1 verhouding vir paringstipe. Patogenisiteit van F1 hibriede is gemiddeld laer as dié van hul ouers. Dit dui daarop dat faktore buiten die verlies in patogenisiteit, ondersoek moet word om die afwesigheid van geslagtelike voortplanting *in vitro* te verklaar. Geslagtelike rekombinasie het belangrike gevolge vir teel en seleksie programme aangesien dit die genetiese struktuur van die populasie kan verander.

Al die hoofstukke in hierdie proefskrif beskryf sekere aspekte van die populasie struktuur van *P. cinnamomi* in Suid Afrika. Meer inligting is nou beskikbaar aangaande die oorsprong van *P. cinnamomi*, geslagtelike voortplanting, variasie in vlakke van patogenisiteit tussen *P. cinnamomi* isolate, ooreenkoms met die Australiaanse populasie, en die moontlikheid van geslagtelike rekombinasie. Dit bied ook belangrike inligting vir die Suid Afrikaanse bosbou industrie met betrekking tot die teel en seleksie van koue tolerante *Eucalyptus* spp. wat weerstandbiedend is teen *P. cinnamomi*.

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