Studies on fungi associated with dying *Schizolobium parahybum* in Ecuador

Maria M. Geldenhuis

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Studies on fungi associated with dying *Schizolobium parahybum* in Ecuador

BY

Maria M. Geldenhuis

B. Sc. Hons. (U.P.)

Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae

in the faculty of Natural and Agricultural Sciences, Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa

April 2005

Supervisor:	Prof. M. J. Wingfield
Co-supervisors:	Dr. J. Roux
	Prof. B. D. Wingfield

Declaration

I, the undersigned, hereby declare that the dissertation herewith submitted for the degree Magister Scientiae *to the University of Pretoria, contains my own independent work and has hithertho not been submitted for any degree at any other university, and furthermore feed copyright of this dissertation in favour of the University.*

_____

Maria M. Geldenhuis

April 2005

TABLE OF CONTENTS

CHAPTER 5

Clonality in South African isolates and evidence for European origin of the root pathogen, *Thielaviopsis basicola*

AKNOWLEDGEMENTS

I would like to thank some special people who made this thesis possible:

- My supervisors, Prof. Mike Wingfield, Dr. Jolanda Roux and Prof. Brenda Wingfield ~ for their guidance, encouragement and continuous support
- Me. Riana Jacobs ~ for all her help and support
- Dr. Treena Burgess and Me. Irene Barnes ~ for their help in designing the polymorphic markers and assistance with the analysis of the microsatellite data
- Dr. Ben Eisenberg ~ for assistance with the statistical analysis
- Mr. Fernando Montenegro ~ for his help in obtaining the *Schizolobium parahybum* samples in Ecuador
- Mr. André Cilliers and Mr. Barry Landman ~ for providing samples of diseased groundnut and chicory
- TPCP and FABI ~ for financial support and facilities to undertake the studies, the UP for facilities and equipment.
- My parents and Edzard ~ for always believing in me
- My heavenly Farther ~ for a miracle

PREFACE

Species of *Ceratocystis*, *Thielaviopsis*, *Graphium*, *Pesotum* and *Ophiostoma* were previously collectively referred to as belonging to the Ophiostomatoid fungi. This economically important group of fungi has a world-wide distribution and includes many important plant pathogens. Species from these genera were isolated from diseased *Schizolobium parahybum* in Ecuador, initiating further study of them. The purpose of this dissertation was to investigate the possible role Ophiostomatoid fungi might play in disease development of *S. parahybum*.

The isolation of one particular species from diseased *S. parahybum* was intriguing. *Thielaviopsis basicola* is a root pathogen of predominantly agricultural crops and is not generally considered a tree pathogen. The isolation of this fungus directed the way to the second and major part of this dissertation that mainly deals with *T. basicola*. Previous work on *T. basicola* focused on biology, ecology, pathogenicity and disease management, while very little is known about its population biology. The second part of this thesis thus deals with the development of polymorphic markers for *T. basicola* and a population study of this fungus from various hosts. This revealed that the *T. basicola* isolates from *S. parahybum* did not originate from this tree but from the carrots that was used as bait to isolate them.

Chapter 1 represents an overview of Ophiostomatoid fungi, particularly *Ceratocystis* and *Ophiostoma* species. It then focuses on the taxonomy, ecology and disease management strategies of *T. basicola*. The last section of this chapter deals with the important role molecular studies have played in taxonomical and population biology studies of fungi and the techniques to achieve this information is discussed.

Schizolobium parahybum is a native tree in Ecuador, South America, that suffers from a serious die-back disease. Possible fungal pathogens were isolated from machete wounds on diseased trees. Chapters 2 and 3 describe a disease survey of machete wounds from diseased *S. parahybum* and the identification of some of the isolated fungi. The possibility that these fungi may be pathogenic to *S. parahybum* was also investigated.

Several different molecular techniques are available to study the population biology of a pathogen. The development of polymorphic markers from regions that are rich in microsatellites are a method of choice because these regions are often highly polymorphic. These markers are easy to use, reliable, exhibit co-dominance and can be used to screen a large number of samples. The fourth chapter describes the development of polymorphic markers for *T. basicola* from microsatellite regions. These markers were tested on 16 different *T. basicola* isolates from eight different countries. Polymorphic markers developed for *C. fimbriata*, a species phylogenetically closely related to *T. basicola*, were also tested.

Population biology studies provide important information for the development of successful management strategies of pathogens and contribute to knowledge regarding the possible origin, spread, structure and mode of reproduction of a pathogen. Chapter 5 represents a population study of *T. basicola* from groundnuts and chicory in South Africa using the polymorphic markers developed in Chapter 4. These markers were also used to compare the isolates from groundnut and chicory with isolates from several other hosts and geographical regions around the world, including isolates from carrots, obtained in the *S. parahybum* isolations.

Chapter 1

An overview of the Ophiostomatoid fungi, with particular reference to the taxonomy, ecology and management of the root pathogen *Thielaviopsis basicola*

1.0 INTRODUCTION

Species of *Ophiostoma* H. & P. Sydow and *Ceratocystis* Ellis & Halsted are often collectively referred to as the Ophiostomatoid fungi. This is primarily based on the morphological similarity of their sexual fruiting structures. In reality, species of the two genera are only very distantly related, as has recently been shown based on DNA phylogenies (Spatafora & Blackwell 1994, Wingfield *et al*. 1999). One morphological clue to their differences is their anamorphs. While *Ophiostoma* spp. have a diversity of anamorphs in genera such as *Sporothrix* Hektoen & Perkins: Nicot & Mariat, *Leptographium* Lagerberg & Melin and *Pesotum* Crane & Schocknecht, *Ceratocystis* spp. typically have *Thielaviopsis* Went. emend. Paulin, Harrington et, McNew anamorphs (Wingfield *et al*. 1993). The latter group of fungi includes important plant pathogens such as *Thielaviopsis basicola* (Berk. & Broome) Ferraris (syn. *Chalara elegans* Nag Raj & Kendrick), which is the primary topic of this review.

The rationale in developing this dissertation emerged from a wish to better understand the tree disease that has been referred to as Pachaco die-back in Ecuador. Pachaco is the common name for the Amazonian tree, *Schizolobium parahybum* (Vell.) Blake and is also known as Brazilian fire tree, tower tree, reach for the sky, fern tree, bacurubu, quapuruvu wood and yellow jacaranda. *Schizolobium parahybum* is native to Central and South America and belongs to the family Leguminosae. It is characterised by yellow flowers, large compound leaves, few branches and a tall straight growth form. This tree is fast growing and produces a light coloured wood that makes it popular for veneer production. *Schizolobium parahybum* suffers, however, from a serious die-back disease that restrain plantation development of this tree in Ecuador.

Roux *et al*. (2000), investigated the possible cause of *S. parahybum* die-back and found *Ceratocystis fimbriata* Ell. & Halst and *T. basicola*, associated with disease symptoms.

These observations were, however, based on a limited number of isolates. This thesis reports on detailed studies on some of the fungi that have been found associated with die-back of *S. parahybum*. The dissertation especially includes a number of studies on *T. basicola*, which was one of the fungi isolated from dying trees. In this review, I present a short commentary on the genera of *Ceratocystis* and *Ophiostoma* as these fungi included those encountered on *S. parahybum*. However, the bulk of this review deals with the taxonomy, ecology and management of *T. basicola*. The decision to take this approach is based on the fact that as it developed, the bulk of the thesis concerned this important root pathogen. This is despite the fact that it was discovered that the fungus is probably not involved in Pachaco die-back.

2.0 *CERATOCYSTIS* AND *OPHIOSTOMA* SPECIES

Species of *Ceratocystis* and *Ophiostoma* are ascomycete fungi that are well adapted to dispersal by insects (Malloch & Blackwell 1993). They include many important crop and forest pathogens as well as several sap staining agents (Wingfield *et al*. 1993). These include *O. ulmi* (Buisman) Nannfeldt and *O. novo-ulmi* Brasier, the causal agents of Dutch elm disease (Gibbs 1974, Brasier 1990, Brasier 1991) and *C. fagacearum* (Bretz) Hunt, the devastating oak wilt pathogen (Bretz 1952, Jewell 1956, Kile 1993, McDonald *et al*. 1998).

The taxonomy of *Ceratocystis* and *Ophiostoma* species was confused and controversial for decades. For many years *Ceratocystis* and *Ophiostoma* were considered to represent a single genus (Hunt 1956, Wright & Cain 1961, Griffin 1968, Olchowecki & Reid 1974, Upadhyay & Kendrick 1975, Upadhyay 1981, Hutchison & Reid 1988, Upadhyay 1993). The main reason for this is the morphological similarities between the two genera, especially in their sexual structures (Upadhyay 1993, Wingfield *et al*. 1993). Both these genera have characteristic long necked ascomata with globose bases and hyaline ascospores lacking pores or slits that are extruded in sticky droplets at the apices necks.

Based on biochemical and phylogenetic evidence, its is now widely accepted that *Ceratocystis* and *Ophiostoma* represent two distinct and very distantly related genera (De Hoog 1974, De Hoog & Scheffer 1984, Harrington 1987, Wingfield *et al*. 1988, Hausner *et al*. 1993). It has, furthermore, been shown, that these two genera reside in two entirely different orders. *Ceratocystis* resides with the Microascales while *Ophiostoma* resides

with the Ophiostomatales (Spatafora & Blackwell 1994, Wingfield *et al*. 1999). *Ceratocystis* species can also be distinguished from *Ophiostoma* based on its lack of tolerance to the antibiotic cycloheximide (Harrington 1981) and lack of cellulose and rhamnose in their cell walls (Spencer & Gorin 1971, Jewell 1974, Weijman & De Hoog 1975). Species in the genus *Ophiostoma* are tolerant to high concentrations of cycloheximide (Harrington 1981) and are characterised by the presence of cellulose and rhamnose in their cell walls (Spencer & Gorin 1971, Jewell 1974, Weijman & De Hoog 1975).

Ceratocystis species are characterised by the anamorph genus *Thielaviopsis* (De Hoog & Scheffer 1984, Wingfield *et al*. 1993, Paulin-Mahady *et al*. 2002) that are distinguished by enteroblastic conidiogenesis (Minter *et al*. 1983). The genus *Ophiostoma* includes anamorphs genera such as *Leptographium*, *Pesotum*, *Sporothrix* and *Hyalorhinocladiella* Upadhyay & Kendrick (Wingfield *et al*. 1993, Okada *et al*. 1998, Hausner *et al*. 2000) with holoblastic conidiogenesis (De Hoog 1974, Wingfield *et al*. 1993).

3.0 THE GENUS *THIELAVIOPSIS*

All anamorphs of *Ceratocystis* spp. have recently been transferred from *Chalara* (Corda) Rabenh. to *Thielaviopsis* (Paulin-Mahady *et al*. 2002). The type species, *T. paradoxa* (de Seynes) Höhnel, is the anamorph of *C. paradoxa* (Dade) Moreau that is generally considered a weak pathogen infecting parenchymatous tissue of primarily stressed monocotyledonous plants (Kile 1993). Many *Thielaviopsis* spp. and their associated teleomorphs are, however, of great economical importance as pathogens of a wide range of hosts. *Thielaviopsis quercina* (Henry) Paulin, Harrington et McNew, for example, is the anamorph of the oak wilt pathogen *C. fagacearum*, while *T. neocaledoniae* (Kiffer & Delon) Paulin, Harrington et McNew causes wilt diseases of coffee and guava (Kile 1993). In Australia *T. australis* (Kile) Paulin, Harrington et McNew causes a serious wilt disease of *Nothofagus cunninghamii* (Hook.) Oerst (Kile & Walker 1987) and *T. basicola* is an important pathogen of many herbaceous plants world-wide (Nag Raj & Kendrick 1975, Wingfield *et al*. 1993).

3.1 *THIELAVIOPSIS BASICOLA*

Thielaviopsis basicola (Berk. et Br.) Ferr., Flora Italica Cryptogama. Pars. I: Fungi, Hyphales, Tubercullariaceae-Stilbaceae. Fasc. 6: 113. 1910.

= *Torula basicola* Berk. et. Br., Ann. Mag. Nat. Hist., ser. 2, 13: 456. 1854.

= *Chalara elegans* Nag Raj et Kend., A monograph of *Chalara* and Allied Genera. p. 111. 1975.

Thielaviopsis basicola was first described in 1854 by Berkeley and Broome under the name *Torula basicola* Berk. & Br. (Smith 1960). This name resulted in confusion because *Torula basicola* was regarded as the asexual stage of the ascomycete, *Thielavia basicola* Zopf. Therefore, Ferraris renamed it in 1912 as *Thielaviopsis basicola* (Smith 1960). In 1975, Nag Raj and Kendrick reduced *Thielaviopsis* to synonymy with *Chalara* and they renamed *Thielaviopsis basicola* as *Ch. elegans*. No sexual state is known for this fungus, but Paulin & Harrington (2000) showed with rDNA sequence analysis that *Ch. elegans* has affinities with the genus *Ceratocystis*. In 2002, Paulin-Mahady *et al*. transferred *Chalara elegans* back to *Thielaviopsis basicola*.

3.1.1 Morphology

Species of *Thielaviopsis* are characterised by the presence or absence of thick, dark chlamydospores that can be produced either singly or in chains. Endoconidia are produced within phialides through ring wall building. These structures are cylindrical, extruded in chains and hyaline but may become dark (Paulin-Mahady *et al*. 2002).

Thielaviopsis basicola is distinguished from the other species in the genus by unicellular, hyaline, cylindrical endoconidia that are extruded in long chains and measure 7.5-19 (13) x 3-5 (4.2) µm. The thallic, dark brown chlamydospores are a rectilinear series of 5-7 segments and measures 6.5-14 (10) x 9-13 (11) µm (Nag Raj & Kendrick 1975).

***Structure of the hyphae and spores*:** The endoconidia are formed in hyphal tubes and their cell wall consists of a thin electron transparent layer (Delvecchio *et al*. 1969, Dumas-Gaudot & Tahiri-Alaoui 1992). Chlamydospores comprise of thick walled individual cells, surrounded by a distinct outer wall (Delvecchio *et al*. 1969). This outer wall consists of four layers (Hawthorne & Tsao 1969) and is an extension of the basal cell walls of the hyphae from which the spores was formed (Tsao & Tsao 1970). The individual cells of the

chlamydospores form a chain and are separated from each other by a thin electron transparent binding layer (Christias & Baker 1970). The protoplasts of two adjacent cells are connected through septal pores (Delvecchio *et al*. 1969, Christias & Baker 1970). A lid or operculum is associated with the septal pores on both sides of the cell (Patrick *et al*. 1965). The cell wall of the hyphae consists of at least three layers that differ in their electron opacity (Dumas-Gaudot & Tahiri-Alaoui 1992). Both the chlamydospore outer wall and the cell wall of the hyphae have previously been reported to consist of only two layers (Delvecchio *et al*. 1969, Christias & Baker 1970, Tsao & Tsao 1970).

***Cultural variation*:** *Thielaviopsis basicola* colonies are effuse with a white colour when young, older cultures become brownish black and powdery (Nag Raj & Kendrick 1975). Cultural variability of *T. basicola* has been observed in isolates from different hosts and areas (King & Presley 1942, Bottacin *et al*. 1994), on different media (Johnson & Valleau 1935, King & Presley 1942, Maier & Staffeldt 1960, Huang & Patrick 1971). Wild biotypes of this fungus often give rise to cultural variants. Variation in culture is usually found when the isolates have been maintained for some time. The older the culture, the more variation occurs (Johnson & Valleau 1935, Keller & Shanks 1955, Huang & Patrick 1971). Variants also formed from different chlamydospores (Johnson & Valleau 1935) and even from different cells within a chlamydospore (Huang & Patrick 1971). This suggests that these cells are not genetically identical biotypes (Huang & Patrick 1971). Changes occur in the morphology and the colour of the colony can differ from grey to brown. Differences are also observed in sporulation, shape and number of the chlamydospores, pathogenicity and growth rate (Johnson & Valleau 1935, Maier & Staffeldt 1960, Huang & Patrick 1971).

According to Huang & Patrick (1971) cultural variability of *T. basicola* is probably the result of mutation. Because the sexual state of this fungus is unknown the changes can most likely not be attributed to sexual hybridization. Heterokaryosis, somatic recombination and cytoplasmic inheritance are also named as possible causes of variation (Huang & Patrick 1971). Another possibility that cannot be ruled out is the possible presence of double stranded RNA that could also influence culture variability. The occurrence of dsRNA in *T. basicola* is common and widespread (Punja *et al*. 1993, Bottacin *et al*. 1994). Its presence or absence in *T. basicola* can influence morphology,

growth rate, survival and virulence of the pathogen (Punja *et al*. 1993, Bottacin *et al*. 1994, Punja 1995).

3.1.2 Physiology

Optimum pH and temperature: *Thielaviopsis basicola* grows the best on acid media, with little or no growth on neutral and alkaline media (Bateman 1962, Punja 1993a). The optimum pH for growth in culture depends on temperature (Bateman 1962) and ranges from pH 4 to pH 6.5 (Bateman 1962, Punja 1993a).

The optimum pH for growth of *T. basicola* in culture does not always correlate with the optimum pH for disease development. Disease incidence on poinsettia was reduced when plants were grown at pH 5.5 or below (Keller & Shanks 1955, Bateman 1962), while neutral and alkaline soil favoured disease development (Bateman 1962). A neutral pH was also conducive for disease development on citrus (Graham 1991). The reason for this may be that pH has an indirect effect on disease development and the hydrogen-ion concentration itself is not the factor of greatest importance (Bateman 1962).

Temperature is important for infection, disease development and reproduction of *T. basicola* (Rothrock 1992). The optimum temperature for growth of *T. basicola* in culture is 20-25 °C (Lloyd & Lockwood 1963, Punja 1993a). Reduced or no growth is obtained at temperatures higher than 32 °C (Bateman & Dimock 1959, Lloyd & Lockwood 1963, Punja 1993a). Optimum temperature for disease development differs for different crops (Lloyd & Lockwood 1963, Graham 1991, Prinsloo *et al*. 1992, Rothrock 1992). It is reported for example that cool temperatures and high moisture favour disease on beans and poinsettia (Keller & Shanks 1955, Nash 1965), but warm and dry conditions favours disease on peas (Bodker *et al*. 1993). According to Lloyd & Lockwood (1963) the optimum temperature for fungal growth in culture is not a factor in pathogenesis. This may explain the inconsistency in optimum temperatures for disease development. Temperatures unfavourable for the host plant promote growth of the pathogen. The plant is infected when it is under stress and not growing under optimum conditions. Tobacco grows best at high temperatures and normally becomes diseased at low temperatures. Pea is a low temperature plant and is infected when it is growing at inordinately high temperatures (Lloyd & Lockwood 1963).

***Spore survival in soil*:** Chlamydospores are the major soil-borne propagules and responsible for long-term survival in soil (Patrick *et al*. 1965, Tsao & Bricker 1966). Each of the individual cells that make up the chlamydospores is able to germinate and this increases the number of effective propagules in the soil. Being a saprophyte, *T. basicola* is able to survive and multiply on decomposing plant material (Gayed 1972). Bateman (1963), however, reported that *T. basicola* cannot grow as a saprophyte in soil, according to this author the high population of spores in the soil is due to chlamydospore formation on the host surface and not due to active growth in the soil. He concluded that a host plant is necessary for increase in the population of *T. basicola* in soil. This view was supported by Rothrock (1992). However, Chittaranjan & Punja (1994) observed survival of endospores for extended periods in fallow organic soils and they concluded that the presence of the host plant is not necessarily required.

The role of endoconidia in survival of the fungus in soil should not be underestimated. Although it has previously been reported that endospores survive only 1-3 days in sandy loam soil (Martinson 1967), they have also been reported to survive even after 5-8 months under both dry and wet soil conditions when the temperature is low (Punja & Chittaranjan 1993, Papavizas & Lewis 1971, Chittaranjan & Punja 1994). Survival of the pathogen is best at low soil temperature (Martinson 1967, Rothrock 1992, Punja & Chittaranjan 1993).

3.1.3 Reproduction

Thielaviopsis basicola is an asexual fungus with no known teleomorph. Analysis of the 18S and 28S rDNA operon revealed that *T. basicola* showed phylogenetic affinities with the teleomorph genus *Ceratocystis* (Paulin & Harrington 2000, Paulin-Mahady *et al*. 2002). Asexual fungi reproduce through the production of conidia and no sexual recombination of genes takes place. The mode of reproduction determines how effective and rapidly a pathogen can adapt to new environmental conditions (Burdon & Roelfs 1985a, McDonald & McDermott 1993). Sexual reproduction leads to new gene combinations that enable the pathogen to evolve more easily. Thus, asexual fungi will generally adapt more slowly to changes in environmental conditions. Examples of environmental changes are crop rotation, irrigation, resistant varieties and application of fertilisers or fungicides (McDonald 1997).

3.1.4 Distribution and host range

Thielaviopsis basicola was first discovered on *Pisum* sp. and *Nemophila auriculata* in England (Nag Raj & Kendrick 1975). Since then, it has been reported to have a world-wide distribution and has been found in association with more than 137 plant genera (Yarwood 1974). *Thielaviopsis basicola* has been reported from countries such as the United States, Germany, Russia, Belgium, Italy, Korea (Rosenbaum 1912), Canada (Patrick & Koch 1963) and South Africa (Prinsloo 1980) on many different plant families (Rosenbaum 1912).

Thielaviopsis basicola causes disease on many agricultural crops including pea (Lloyd & Lockwood 1961, Burke & Kraft 1974, Blume & Harman 1979, Tabachnik *et al*. 1979, Bodker *et al*. 1993), chicory (Prinsloo *et al*. 1991, Prinsloo *et al*. 1992), beans (Gayed 1972, Burke & Kraft 1974, Yarwood & Karayiannis 1974, Tabachnik *et al*. 1979, Yarwood 1981), cotton (King & Presley 1942, Staffeldt 1959, Mathre *et al*. 1966, Tabachnik *et al*. 1979, Yarwood 1981), soybean (Lockwood *et al*. 1970, Yarwood & Karayiannis 1974, Tabachnik *et al*. 1979), groundnut (Tabachnik *et al*. 1979, Prinsloo 1980), cowpea (Gayed 1972, Yarwood & Karayiannis 1974), tobacco (Gayed 1972, Yarwood 1981), and tomato (Koike & Henderson 1998). It is also a root pathogen of citrus seedlings (Tsao & Gundy 1962, Graham 1991), is associated with cankers on the stems of ginseng (Rosenbaum 1912) and is known for post harvest disease of fresh market carrots (Punja 1990, Punja 1993b). The fungus often forms part of a disease complex were it plays a major role in disease development. It is for example part of the black root rot disease complex of poinsettia (Keller & Shanks 1955) and causes black root rot of pea (*Pisum sativum* L.) and bean (*Phaseolus vulgaris* L.) together with *Fusarium solani* f. sp. *phaseoli* (Burkholder) W.C. Snyder & H.N. Hans, *F. solani* f. sp. *pisi* (F.R. Jones) W.C. Snyder & H.N. Hans, *Pythium ultimum* Trow and *Rhizoctonia solani* Kühn (Burke & Kraft 1974, Blume & Harman 1979, Bodker *et al*. 1993). In South Africa *T. basicola* is the causal agent of black pod rot of groundnuts (*Arachis hypogaea* L.) (Prinsloo 1980) and is also the cause of root disease of chicory (*Cichorium intybys* L.) (Prinsloo *et al*. 1991) and other hosts (Table 1).

There is evidence for some degree of host specificity for isolates of *T. basicola*. Keller & Shanks (1955) reported that isolates from poinsettia were not able to cause disease of tobacco and *vice versa*. Similarly, isolates pathogenic to pea and bean were not pathogenic to tobacco (Gayed 1969). Host specificity is restricted to different strains of the pathogen

as tobacco isolates were not pathogenic on pea in one study (Lloyd & Lockwood 1963) and pathogenic to pea in a different study (Gayed 1969). Punja & Sun (1999), found with randomly amplified polymorphic DNA (RAPD) analysis that *T. basicola* isolates from the same host tend to group together. They concluded that the host plant might play an important role in selective pressure on *T. basicola* populations.

3.1.5 Disease symptoms

Typical symptoms of root rot of pea, caused by *T. basicola* are black-brown discolouration of the lateral and tap roots as well as the stem base. Severe infection will result in plant stunting and wilting of the leaves. Masses of chlamydospores are often found on diseased tissue, causing the black discolouration (Lloyd & Lockwood 1961, Blume & Harman 1979, Bodker *et al*. 1993). The same disease symptoms occur on tobacco (Gayed 1969), cotton (Mathre *et al*. 1966), tomato (Koike & Henderson 1998), poinsettia (Bateman & Dimock 1959), citrus seedlings (Tsao & Gundy 1962), chicory (Prinsloo *et al*. 1991) and soybean (Lockwood *et al*. 1970). In soybean, however, wilting or death of the foliage is seldom observed (Lockwood *et al*. 1970). Pods of infected groundnuts have small black lesions at first and eventually the pod turns completely black. In advanced stages black-brown discolouration can be observed on the testae as well as insides of the pods (Prinsloo 1980). Rot of the roots and stems are seldom observed (Labuschagne *et al*. 1980).

3.1.6 Dissemination

Thielaviopsis basicola spores spread slowly in soil (King & Presley 1942), but transfer of infected soil or plant material can be a source of inoculum into new areas. Graham (1991) repeatedly recovered *T. basicola* from air samples and it is possible that the fungus spreads in the greenhouse through airborne propagules, presumably endospores. Stanghellini *et al*. (1999) reported that adult shore flies are the possible vectors of *T. basicola* on corn salad in hydroponic nurseries. Chlamydospores were observed in the frass excreted by the adult insects as well as the larvae that were in immediate vicinity of diseased plants. They were also observed in the intestinal tract of the larvae. When infected insects were placed among healthy plants, disease developed after two to three weeks (Stanghellini *et al*. 1999). Adult shore flies can thus function as agents enabling spores to move through the air. Labuschagne & Kotze (1991) investigated seed transmission in black pod rot disease of groundnuts caused by *T. basicola* in South Africa and showed that it could be successfully introduced into disease-free soil by infected seed.

3.1.7 Management

Management of diseases caused by *T. basicola* is difficult and involves several different strategies. These include chemical control, flooding, crop rotation, soil amendments and breeding for resistance.

Cultural practices: Chemical control has not been effective in controlling this pathogen on various crops (Clayton 1953, Lockwood 1961, Baard 1988,). Summer flooding and crop rotation are possible means of control (Snyder *et al*. 1959, Hsi 1967, Chittaranjan & Punja 1994) and soil amendments seem to be successful in some cases (Adams & Papavizas 1969, Punja *et al*. 1993).

Resistance: Breeding for resistant cultivars is the most practical means of control against *T. basicola*, although this too is not always successful. Clayton (1953) recommended that resistance is supported by other control measures. Most of the work in resistance breeding against this pathogen has been done on tobacco. Tobacco varieties with a high degree of resistance have been developed, but it is commonly found that *T. basicola* is able to overcome this resistance (Patrick & Koch 1963). Different environmental conditions and introduction of new strains with different pathogenicities may be some of the reasons for this. The presence of phytotoxins in the soil that enhance infection of the roots in both resistant and non-resistant plants may also contribute to breakdown of resistance (Patrick & Koch 1963).

Breeding for resistance was shown to be an effective and durable means of control against black pod rot of groundnuts in South Africa (Baard 1988, Van der Merwe & Van der Merwe 1988, Jones 1990). Selection for resistant groundnut cultivars began soon after the appearance of the disease (Van der Merwe *et al.* 1983, Van der Merwe *et al.* 1993) and in the late 1980's resistant cultivars were released (Van der Merwe & Van der Merwe 1988). Resistance of these cultivars declined over the years and new improved cultivars needed to be developed (Cilliers 2001).

4. TOOLS FOR STUDYING PHYLOGENETICS AND DIVERSITY OF *THIELAVIOPSIS BASICOLA*

The accurate identification of plant pathogens is a crucial step in the process of disease management and the effective application of quarantine regulations. Traditionally, use has been made of only morphological, cultural and physiological characters for identification. This, however, has commonly resulted in incorrect identification of pathogens. Furthermore, morphology provides no information on genotypes, as they cannot be distinguished based on morphology. Today the traditional phenotypic approach is supplemented by comparisons of DNA sequence data and other rapid DNA based identification tools.

Sequences for part or all of the ribosomal DNA operon has been most commonly used for phylogenetic studies on fungi. This gene is tandemly repeated throughout the genome (Hillis & Davis 1986, Watson *et al*. 1992), present in a high copy number and can be easily detected (Hillis & Davis 1986, Dowling *et al*.1990). The rDNA repeat unit of eukaryotes consists of a small subunit (18S), 5.8S and large subunit (28S) gene that are separated by internal transcribed spacer regions (ITS1 and ITS2) (Dowling *et al*. 1990, Mitchell *et al*. 1995). The 18S rDNA gene is often used to classify distantly related organisms (White *et al.* 1990) and to distinguish between genera (Sparafora & Blackwell 1994), while the ITS regions distinguishes between closely related species (White *et al.* 1990).

Ribosomal DNA sequence analysis has been successfully used in distinguishing species of *Ophiostoma*. For example, molecular analysis of 18S rDNA sequences revealed that *Graphium putredinis* (Corda) Hughes and *G. penicillioides* Corda were related to the Microascales and did not belong to the Ophiostomatales as previously believed (Okada *et al*. 1998). Harrington *et al*. (2001) used the ITS region of the rDNA operon to distinguish between species of the *O. piceae* (Münch) H. & P. Sydow complex.

Management of plant diseases today relies strongly on integrated management systems. This relies not only on traditional epidemiological and cultural studies, but on modern techniques to provide information on the genetic diversity and origin of the pathogen. The amount of genetic diversity in fungal populations provides valuable information on the ability of a pathogen to evolve and overcome host resistance and other management

strategies (McDonald & McDermott 1993). A population with high population diversity will more easily adapt to environmental changes and management strategies will be less viable and sustainable (McDonald & McDermott 1993). Population diversity studies also give insight into the possible origin and migration of a pathogen and enable more effective quarantine measures. Genetic diversity of a pathogen is expected to be higher in its country of origin, because the population had more time to develop diversity (McDonald 1997).

The degree of genetic diversity of a fungal population is an indication of the possible mode of reproduction of the pathogen. The degree of genetic diversity of an asexual fungus is assumed to be low (Gordon *et al*. 1996), while a high population diversity would indicate that sexual reproduction might occur in nature (Goodwin *et al*. 1992, Burdon & Silk 1997). Sexual reproduction leads to the recombination of genes and increases genetic diversity. High levels of genetic diversity could also indicate that the fungus has been introduced a number of times and from different sources (Burdon & Roelfs 1985b, Wikler & Gorden 2000). Low levels of genetic diversity will reflect recent colonisation (Milgroom & Lipari 1995, Gordon *et al*. 1996).

Various techniques are used for genetic diversity studies. These include random amplified polymorphic DNA (RAPDs) (Williams *et al*. 1990), restriction fragment length polymorphisms (RFLP's) (Bruns *et al*. 1991), amplified fragment length polymorphisms (AFLPs) (Majer *et al*. 1996), randomly amplified microsatellites (RAMS) (Hantula *et al*. 1996) and microsatellite DNA (Engel *et al*. 1996).

Microsatellites, or simple sequence repeats (SSR), are made up of highly repetitive DNA sequences of two to ten or more bases that occur throughout eukaryotic genomes (Hamada *et al.* 1982, Tautz 1989). These sequences can be highly polymorphic (Groppe *et al*. 1995, Sun *et al*. 1999) due to variation in the number of repeat units (Weber & May 1989, Messier *et al.* 1996), probably the result of replication slippage (Messier *et al*. 1996). Repeats can be perfect or imperfect (Weber 1990). The latter are generally less informative than perfect repeats (Weber 1990, Dusabenyagasani *et al*. 1998) although it has been found that imperfect repeats are also useful in detecting variation between individuals (Thomas & Scott 1993).

Using microsatellites as genetic tools involves the development of specific primers flanking microsatellite regions. These loci are subsequently amplified and analysed for variation in length. Information derived from microsatellite loci can be used for identification of individuals (Thomas & Scott 1993, Moon *et al*. 1999), paternity testing (Jeffreys *et al*. 1985, Smith & Devey 1994), gene mapping (Senior & Heun 1993, Morgante *et al*. 1994), gene flow (Groppe *et al*. 1995) and population studies of various organisms (Scribner *et al*. 1994, Djè *et al.* 1999, Perera *et al*. 2000), including fungi (Groppe *et al*. 1995, Zhou *et al*. 2001).

Some of the main advantages of microsatellite markers are their high information content, co-dominance, PCR-based detection, Mendelian inheritance and high polymorphism (Queller *et al*. 1993, Haymer 1994, Engel *et al*. 1996, Perera *et al*. 2000,). The main drawback of this technique is the effort, time and money needed to find the microsatellite loci and develop the primers. It will be a huge advantage if primers developed for one species can be used for other species as well. This is a plausible approach as primers designed for one species in artiodactyls were able to amplify other species in this group (Engel *et al*. 1996).

Very little is known regarding the population biology of *T. basicola* in South Africa, or elsewhere in the world. Although many studies have been conducted on the variation in cultural characteristics (Johnson & Valleau 1935, King & Presley 1942, Staffeldt 1960, Huang & Patrick 1971, Maier & Bottacin *et al*. 1994), very little molecular work has been done on this fungus. Recently Punja & Sun (1999) used six RAPD primers and revealed a high degree of genetic diversity between 50 *T. basicola* isolates from different hosts and geographical regions. UPGMA analysis revealed distinct clusters that generally represent the same morphological group and host or geographical region (Punja & Sun 1999).

5.0 CONCLUSIONS

Research chapters that follow this review all deal with fungi that have been isolated from wounds on dying *S. parahybum* in Ecuador. Two of these treat *C. fimbriata*, *C. moniliformis* (Hedgc.) Moreau, *Graphium penicillioides*, *Ophiostoma quercus* (Georgévitch) Nannfeldt and a *Pesotum* spp. Because *T. basicola* was encountered in isolations from wounds, the remaining two chapters deal with the population biology of

this fungus. This includes collections from various parts of the world and most importantly, from two important agronomic crops in South Africa.

Thielaviopsis basicola is an enormously important pathogen of a wide variety of crop plants, world-wide. It is thus interesting that so little has been known regarding its taxonomy until relatively recently. It has indeed been through studies on *Ceratocystis* and *Ophiostoma*, at the DNA level that the taxonomy and particularly phylogenetic placement of *T. basicola* has been resolved.

There is a relatively large body of knowledge relating to the ecology and epidemiology of *T. basicola*. Work has mainly focussed on issues pertaining to disease management. It is thus rather ironic hat almost no focus has been given to the possible origin of this pathogen, that has clearly emerged in almost every country where crops such as tobacco, cotton and carrots are grown.

Modern DNA-based tools have made it possible to understand the population diversity and consider the origins of pathogens such as *T. basicola*. One of the primary aims of this dissertation was to consider aspects of the population structure of this pathogen. Interest emerged primarily from its occurrence on *S. parahybum*, but early results promoted a study considering a much wider focus.

The chapters of the following thesis are somewhat disparate and deal with a diversity of fungi, without clear relatedness. Although the depth of studies on the different fungi is perhaps not equal in all chapters, an attempt has been made to better understand some of the fungi most consistently isolated from wounds and from dying *S. parahybum* in Ecuador.

6.0 REFERENCES

Adams, P.B. & Papavizas, G.C. (1969) Survival of root-infecting fungi in soil: X. Sensitivity of propagules of *Thielaviopsis basicola* to soil. *Phytopathology* **59**: 135-138.

Baard, S.W. (1988) Effect of fungicides, soil acidification and cultural practices on blackhull of groundnuts. *Phytophylactica* **20**: 121-123.

Bateman, D.F. (1962) Relation of soil pH to development of poinsettia root rots. *Phytopathology* **52**: 559-566.

Bateman, D.F. (1963) Influence of host and nonhost plants upon populations of *Thielaviopsis basicola* in soil. *Phytopathology* **53**: 1174-1177.

Bateman, D.F. & Dimock, A.W. (1959) The influence of temperature on root rots of poinsettia caused by *Thielaviopsis basicola*, *Rhizoctonia solani,* and *Pythium ultimum*. *Phytopathology* **49**: 641-647.

Blume, M.C. & Harman, G.E. (1979) *Thielaviopsis basicola*: A component of the pea root rot complex in New York State. *Phytopathology* **69**: 785-788.

Bodker, L., Leroul, N. & Smedegaard-Petersen, V. (1993) The occurrence in Denmark of black root rot of pea caused by *Thielaviopsis basicola*. *Plant Pathology* **42**: 8220-8230.

Bottacin, A., Lévesque, C.A. & Punja, Z.K. (1994) Characterisation of dsRNA in *Chalara elegans* and effects on growth and virulence. *Phytopathology* **84**: 303-312.

Brasier, C.M. (1990) China and the origins of Dutch elm disease: an appraisal. *Plant Pathology* **39**: 5-16.

Brasier, C.M. (1991) *Ophiostoma novo-ulmi* sp. nov., causative agent of current Dutch elm disease pandemics. *Mycopathologia* **115**: 151-161.

Bretz, T.W. (1952) The ascigerous stage of the oak wilt fungus. *Phytopathology* **42**: 435-437.

Bruns, T.D., White, T.J. & Taylor, J.W. (1991) Fungal molecular systematics. *Annual Review of Ecological Systematics* **22**: 525-564.

Burdon, J.J. & Roelfs, A.P. (1985a). The effect of sexual and asexual reproduction in the isozyme structure of populations of *Puccinia graminis*. *Phytopathology* **75**: 1068-1073.

Burdon, J.J. & Roelfs, A.P. (1985b) Isozyme and virulence variation in asexual reproducing populations of *Puccinia graminis* and *P. recondita* on wheat. *Phytopathology* **75**: 907-913.

Burdon, J.J. & Silk, J. (1997) Sources and patterns of diversity in plant-pathogenic fungi. *Phytopathology* **87**: 664-669.

Burke, D.W. & Kraft, J.M. (1974) Response of beans and peas to root pathogens during monoculture of each crop species. *Phytopathology* **64**: 546-549.

Chittaranjan, S. & Punja, Z.K. (1994) Factors influencing survival of phialospores of *Chalara elegans* in organic soil. *Plant Disease* **78**: 411-415.

Christias, C. & Baker, K.F. (1970) Ultrastructure and cleavage of chlamydospore chains of *Thielaviopsis basicola*. *Canadian Journal of Botany* **48**: 2305-2308.

Cilliers, A.J. (2001) Resistance in new groundnut breeding lines to black pod rot caused by *Chalara elegans*. *Suid-Afrikaanse Tydskrif vir Plant en Grond* **18**: 174-175.

Clayton, R.E. (1953) Control of tobacco diseases through resistance. *Phytopathology* **43**: 239-244.

Crous, P.W., Philips, A.J.L. & Baxter, A.P. (2000) *Phytopathogenic fungi from South Africa*. Department of Plant Pathology Press. Stellenbosch, South Africa.

De Hoog, G.S. (1974) *Blastobotrys, Sporothrix, Calcarisporium* and *Calcarisporiella* gen. nov. *Studies in Mycology* **7**: 1-88.

De Hoog, G.S. & Scheffer, R.J. (1984) *Ceratocystis* versus *Ophiostoma*: a reappraisal. *Mycologia* **76**: 292-299.

Delvecchio, V.G., Corbaz, R. & Turian, G. (1969) An ultrastructural study of the hyphae, endoconidia and chlamydospores of *Thielaviopsis basicola*. *Journal of General Microbiology* **58**: 23-27.

De Villiers, D.A. (1987) Alternatiewe gashere vir die tabak-swartwortelvrotswam *Thielaviopsis basicola*. *Phytophylactica* **19**: 347-348.

Djè, Y., Forcioli, D. & Ater, M. (1999) Assessing population genetic structure of sorghum landacers from Norh-western Morocco using allozyme and microsatellite markers. *Theoretical and Applied Genetics* **99**: 157-163.

Doidge, E.M. & Bottomley, A.M. (1931) A revised list of diseases occurring in South Africa. *Botanical Survey of South Africa. Memoir* No. **11**: 1-78.

Dowling, T.E., Moritz, C. & Palmer, J.D. (1990) Nucleic acids II: restriction site analysis. In *Molecular systematics* (D.M. Hillis & C. Morits, eds): 250-317. Sinauer Associates, inc. Publishers, Sunderland, Massachusetts, USA.

Dumas-Gaudot, E. & Tahiri-Alaoui, A. (1992) Cytochemical localisation of some polysaccharidic components in the cell walls of *Chalara elegans* during its life cycle. *Canadian Journal of Microbiology* **38**: 828-837.

Dusabenyagasani, M., Lecours, N. & Hamelin, R.C. (1998) Sequence-tagged sites (STS) for studies of molecular epidemiology of scleroderris canker of conifers. *Theoretical and Applied Genetics* **97**: 789-796.

Engel, S.R., Linn, R.A., Taylor, J.F. & Davis, S.K. (1996) Conservation of microsatellite loci across species of artiodactyls: implications for population studies. *Journal of Mammalogy* **77**: 504-518.

Gayed, S.K. (1969) The relation between tobacco leaf and root necrosis induced by *Thielaviopsis basicola* and its bearing on the nature of tobacco resistance to black root rot. *Phytopathology* **59**: 1596-1600.

Gayed, S.K. (1972) Host range and persistence of *Thielaviopsis basicola* in tobacco soil. *Canadian Journal of Plant Science* **52**: 869-873.

Gibbs, J.N. (1974) Biology of Dutch elm disease. *Forest Record* **94**: 1-9.

Goodwin, S.B., Spielman, L.J., Matuszak, J.M., Bergeron, S.N. & Fry, W.E. (1992) Clonal diversity and genetic differentiation of *Phytophthora infestans* populations in Northern and Central Mexico. *Phytopathology* **82**: 955-961.

Gordon, T.R., Storer, A.J. & Okamoto, D. (1996) Population structure of the pitch canker pathogen, *Fusarium subglutinans* f. sp. *pini*, in California. *Mycological Research* **100**: 850-854.

Graham, J.H. (1991) Peat-based media as a source of *Thielaviopsis basicola* causing black root rot on citrus seedlings. *Plant Disease* **75**: 1246-1249.

Griffin, H.D. (1968) The genus *Ceratocystis* in Ontario. *Canadian Journal of Botany* **46**: 689-718.

Groppe, K., Sanders, I., Wiemken, A. & Boller, T. (1995) A microsatellite marker for studying the ecology and diversity of fungal endophytes (*Epichloë* spp.) in grasses. *Applied and Environmental Microbiology* **61**: 3943-3949.

Hamada, H.-J., Petrino, M.G. & Kakanuga, T. (1982) A novel repeated element with Z-DNA-forming potential is widely found in evolutionary diverse eukaryotic genomes. *Proceedings of the National Academy of Sciences* **79**: 6465-6469.

Hantula, J., Dusabenyagasani, M. & Hamelin, R.C. (1996) Random amplified microsatellites (RAMS)- a novel method for characterising genetic variation with fungi. *European Journal for Forest Pathology* **26**: 159-166.

Harrington, T.C. (1981) Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. *Mycologia* **73**: 1123-1129.

Harrington, T.C. (1987) New combinations in *Ophiostoma* of *Ceratocystis* species with *Leptographium* anamorphs. *Mycotaxon* **28**: 39-43.

Harrington, T.C., McNew, D., Steimel, J., Hofstra, D. & Farrell, R. (2001) Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi. *Mycologia* **93**: 111-136.

Hausner, G., Reid, J. & Klassen, G.R. (1993) On the subdivision of *Ceratocystis* s.l., based on partial ribosomal DNA sequences. *Canadian Journal of Botany* **71**: 52-63.

Hausner, G., Reid, J. & Klassen G.R. (2000) On the phylogeny of members of *Ceratocystis* s. s. and *Ophiostoma* that possess different anamorphic states, with emphasis on the anamorph genus *Leptographium*, based on partial ribosomal DNA sequences. *Canadian Journal of Botany* **78**: 903-916.

Hawthorne, B.T. & Tsao, P.H. (1969) Influence of spore chain breakup, age, nutrients, and soil on germination of chlamydospores of *Thielaviopsis basicola*. *Phytopathology* **59**: 12 (Abstr.).

Haymer, D.S. (1994) Random amplified polymorphic DNAs and microsatellites: What are they, and can they tell us anything we don't already know? *Annals of the Entomological Society of America* **87**: 717-722.

Hillis, D.M. & Davis, S.K. (1986) Evolution of ribosomal DNA: Fifty million years of recorded history in the frog genus *Rana*. *Evolution* **40**: 1275-1288.

Hsi, D.C.H. (1967) Relationship between crop sequences and several diseases of *Valencia* peanuts. *Phytopathology* **57**: 461 (Abstr.).

Huang, H.C. & Patrick, Z.A. (1971) Variability of *Thielaviopsis basicola* in culture. *Canadian Journal of Botany* **49**: 1041-1047.

Hunt, J. (1956) Taxonomy of the genus *Ceratocystis*. *Lloydia* **19**: 1-59.

Hutchison, L.J. & Reid, J. (1988) Taxonomy of some potential woodstaining fungi from New Zealand 1. Ophiostomataceae. *New Zealand Journal of Botany* **26**: 63-81.

Jeffreys, A.J., Wilson, V. & Thein, S.L. (1985) Individual-specific 'fingerprints' of human DNA. *Nature* **316**:76-79.

Jewell, F.F. (1956) Insect transmission of oak wilt. *Phytopathology* **46**: 244-257.

Jewell, T.R. (1974) A qualitative study of cellulose distribution in *Ceratocystis* and *Europhium*. *Mycologia* **66**: 139-146.

Johnson, E.M. & Valleau, W.D. (1935) Cultural variations of *Thielaviopsis basicola*. *Phytopathology* **25**: 1011-1018.

Jones, B.L. (1990) Selection of resistance in peanuts against black pod rot caused by *Chalara elegans*. *Phytophylactica* **22**: 139-140.

Keller, J.R. & Shanks, J.B. (1955) Poinsettia root rot. *Phytopathology* **45**: 552-558.

Kile, G.A. (1993) Plant diseases caused by species of *Ceratocystis sensu stricto* and *Chalara*. In Ceratocystis *and* Ophiostoma*: Taxonomy, Ecology and Pathogenicity* (M.J. Wingfield, K.A. Seifert & J.F. Webber, eds): 173-183. APS Press, St. Paul, Minnesota.

Kile, G.A. & Walker, K. (1987) *Chalara australis* sp. nov. (Hyphomycetes), a vascular pathogen of *Nothofagus cunninghamii* (*Fagaceae*) in Australia and its relationship to other *Chalara* species. *Australian Journal of Botany* **35**: 1-32.

King, C.J. & Presley, J.T. (1942) A root rot of cotton caused by *Thielaviopsis basicola*. *Phytopathology* **32**: 752-761.

Koike, S.T. & Henderson, D.M. (1998) Black root rot, caused by *Thielaviopsis basicola*, on tomato transplants in California. *Plant Disease* **82**: 447 (Abstr.).

Labuschagne, N. & Kotzé, J.M. (1991) Incidence of *Chalara elegans* in groundnut seed samples and seed transmission of blackhull. *Plant Pathology* **40**: 639-642.

Labuschagne, N., Kotzé, J.M. & Wehner, F.C. (1980) *Thielaviopsis basicola* infections of groundnuts in South Africa. *Phytophylactica* **12**: 177-180.

Lloyd, A.B. & Lockwood, J.L. (1961) Pathogenicity of *Thielaviopsis basicola* on peas. *Plant Disease Reporter* **45**: 422-424.

Lloyd, A.B. & Lockwood, J.L. (1963) Effect of soil temperature, host variety and fungus strain on *Thielaviopsis* root rot of peas. *Phytopathology* **53**: 329-331.

Lockwood, J.L. (1961) Soil fungicides for control of pea root rot in greenhouse tests. *Plant Disease Reporter* **45**:569-571.

Lockwood, J.L., Yoder, D.L. & Smith, N.A. (1970) *Thielaviopsis basicola* root rot of soybeans in Michigan. *Plant Disease Reporter* **54**: 849-850.

Maier, R.C. & Staffeldt, E.E. (1960) Cultural variability of selected isolates of *Rhizoctonia solani* and *Thielaviopsis basicola*, and the variability in their pathogenicity to Acala and Pima cotton, respectively. *Plant Disease Reporter* **44**: 956-961.

Majer, D., Mithen, R. Lewis, B.G., Vos, P. & Oliver, R.P. (1996) The use of AFLP fingerprinting for the detection of genetic variation in fungi. *Mycological Research* **100**: 1107-111.

Malloch, D. & Blackwell, M. (1993) Dispersal biology of the Ophiostomatoid fungi. In Ceratocystis *and* Ophiostoma*: Taxonomy, Ecology and Pathogenicity*. (M.J. Wingfield, K.A. Seifert & J.F. Webber, eds): 195-206. APS Press, St. Paul, Minnesota.

Martinson, C.A. (1967) Passive survival of endoconidia and chlamydospores of *Thielaviopsis basicola* in soil. *Phytopathology* **57**: 821(Abstr.).

Mathre, D.E., Ravenscroft, A.V. & Garber, R.H. (1966) The role of *Thielaviopsis basicola* as a primary cause of yield reduction in cotton in California. *Phytopathology* **56**: 1213-1216.

McDonald, B.A. (1997) The population genetics of fungi: Tools and techniques. *Phytopathology* **87**: 448-453.

McDonald, B.A. & McDermott, J.M. (1993) Population genetics of plant pathogenic fungi. *Bioscience* **43**: 311-319.

McDonald, B.A., Bellamy, B.K., Zhan, J. & Appel, D.N. (1998) The effect of an oak wilt epidemic on the genetic structure of a Texas live oak population. *Canadian Journal of Botany* **76**: 1900-1907.

Messier, W., Li, S-H. & Stewart, C-B. (1996) The birth of microsatellites. *Nature* **381**: 483.

Milgroom, M.G. & Lipari, S.E. (1995) Population differentiation in the Chestnut blight fungus, *Chryponectria parasitica*, in Eastern North America. *Phytopathology* **85**: 155-160.

Minter, D.W., Kirk, P.M. & Sutton, B.C. (1983) Thalic phialides. *Transactions of the British Mycological Society* **80**: 39-66.

Mitchell, J.I., Roberts, P.J. & Moss, S.T. (1995) Sequence or structure? A short review of the replication of nucleic acid sequence information to fungal taxonomy. *Mycologist* **9**: 67-75.

Moon, C.D., Tapper, B.A. & Scott, B. (1999) Identification of epichloë endophytes in plants by microsatellite-based PCR fingerprinting assay with automated analysis. *Applied and Environmental Microbiology* **65**: 1268-1279.

Morgante, M., Rafalski, A., Biddle, P., Tingey, S. & Olivier, A.M. (1994) Genetic mapping and variability of seven soybean simple sequence repeat loci. *Genome* **37**: 763-769.

Nag Raj, T.R. & Kendrick, B. (1975) *A monograph of* Chalara *and allied genera*. Wilfred Laurier University Press. Waterloo, Ontario, Canada.

Nash, S. (1965) Relative frequency of lesions on beans incited by common soil borne pathogens. *Phytopathology* **55**: 126 (Abstr.).

Okada, G., Seifert, K.A., Takematsu, A., Yamaoka, Y., Miyazaki, S. & Tubaki, K. (1998) A molecular phylogenetic reappraisal of the *Graphium* complex based on 18S rDNA sequences. *Canadian Journal of Botany* **76**: 1495-1506.

Olchowecki, A. & Reid, J. (1974) Taxonomy of the genus *Ceratocystis* in Manitoba. *Canadian Journal of Botany* **52**: 1675-1711.

Papavizas, G.C. & Lewis, J.A. (1971) Survival of endoconidia and chlamydospores of *Thielaviopsis basicola* as affected by soil environmental factors. *Phytopathology* **61**: 108-113.

Patrick, Z.A. & Koch, L.W. (1963) The adverse influence of phytotoxin substances from decomposing plant residues on resistance of tobacco to black root rot. *Canadian Journal of Botany* **41**: 747-758.

Patrick, Z.A., Toussoun, T.A. & Thorpe, H.J. (1965) Germination of chlamydospores of *Thielaviopsis basicola*. *Phytopathology* **55**: 466-470.

Paulin, A.E. & Harrington, T.C. (2000) Phylogenetic placement of anamorphic species of *Chalara* among *Ceratocystis* species and other ascomycetes. *Studies in Mycology* **45**: 209-222.

Paulin-Mahady, A.E., Harrington, T.C. & McNew, D. (2002) Phylogenetic and taxonomic evaluation of *Chalara*, *Chalaropsis*, and *Thielaviopsis* anamorphs associated with *Ceratocystis*. *Mycologia* **94**: 62-72.

Perera, L., Russell, J.R., Provan, J. & Powell, W. (2000) Use of microsatellite DNA markers to investigate the level of genetic diversity and population genetic structure of coconut (*Cocos nucifera* L.). *Genome* **43**: 15-21.

Prinsloo, G.C. (1980) *Thielaviopsis basicola* associated with a pod disease of groundnuts in South Africa. *Phytophylactica* **12**: 25- 26.

Prinsloo, G.C., Baard, S.W. & Ferreira, J.F. (1991) Organisms associated with black root rot of chicory in South Africa. *Phytophylactica* **23**: 59-67.

Prinsloo, G.C., Baard, S.W. & Feirreira, J.F. (1992) Biotic and abiotic causes of stubby root rot in chicory. *Phytophylactica* **24**: 199-205.

Punja, Z.K. (1990) Development of black root rot (*Thielaviopsis basicola*) as a post-harvest disease on fresh market carrots and strategies for disease control. *Phytopathology* **80**: 1027 (Abstr.).

Punja, Z.K. (1993a) Influence of culture conditions on mycelial growth and phialospore production and germination in *Chalara elegans*. *Canadian Journal of Botany* **71**: 447-456.

Punja, Z.K. (1993b) Influence of postharvest handling practices and dip treatments on development of black root rot on fresh market carrots. *Plant Disease* **77**: 989-995.

Punja, Z.K. (1995) Influence of double-stranded RNAs on growth, sporulation, pathogenicity, and survival of *Chalara elegans*. *Canadian Journal of Botany* **73**: 1001-1009.

Punja, Z.K., Bottacin, A.M. & Lévesque, C.A. (1993) Double-stranded RNAs in *Chalara elegans* (*Thielaviopsis basicola*) - Distribution and significance. *Phytopathology* **83**: 693 (Abstr.).

Punja, Z.K. & Chittaranjan, S. (1993) Influence of moisture level, soil amendments, cropping sequence and temperature on survival of *Chalara elegans* (*Thielaviopsis basicola*) in organic soil. *Phytopathology* **83**: 693 (Abstr.).

Punja, Z.K. & Sun, L-J. (1999) Morphological and molecular characterisation of *Chalara elegans* (*Thielaviopsis basicola*), cause of black root rot on diverse plant species. *Canadian Journal of Botany* **77**: 1801-1812.

Queller, D.C., Strassmann, J.E. & Hughes, C.R. (1993) Microsatellites and kinship. *Tree* **8**: 285-288.

Rosenbaum, J. (1912) Infection experiments with *Thielavia basicola* on ginseng. *Phytopathology* **2**: 191-196.

Rothrock, C.S. (1992) Influence of soil temperature, water, and texture on *Thielaviopsis basicola* and black root rot of cotton. *Phytopathology* **82**: 1202-1206.

Roux, J., Wingfield, M.J., Montenegro, F., Geldenhuis, N. & Wingfield, B.D. (2000) *Ceratocystis* diseases of *Schizolobium parahybum* in Ecuador. *Proceedings of the 38th Congress of the Southern African Society for Plant Pathology, Grahamstown*, 26-28 January 2000.

Scribner, K.T., Arntzen, J.W. & Burke, T. (1994) Comparative analysis of intra- and interpopulation genetic diversity in *Bufo bufo*, using allozyme, single-locus microsatellite, minisatellite, and multilocus minisatellite data. *Molecular Biology and Evolution* **11**: 737-748.

Senior, M.L. & Heun, M. (1993) Mapping maize microsatellites and polymerase chain reaction confirmation of the targeted repeats using a T primer. *Genome* **36**: 884-889.

Smith, J.G. (1960) The influence of antagonistic fungi on *Thielaviopsis basicola* (Berk. et Br.) Ferraris. *Acta Botanica Neerlandica* **9**: 59-118.

Smith, D.N. & Devey, M.E. (1994) Occurrence and inheritance of microsatellites in *Pinus radiata*. *Genome* **37**: 977-983.

Snyder, W.C., Schroth, M.N. & Christou, T. (1959) Effect of plant residues on root rot of bean. *Phytopathology* **49**: 755-756.

Spatafora, J.W. & Blackwell, M. (1994). The polyphyletic origins of Ophiostomatoid fungi. *Mycological Research* **98**: 1-9.

Spencer, J.F.T. & Gorin, P.A.J. (1971) Systematics of the genera *Ceratocystis* and *Graphium*. Proton magnetic resonance spectra of the mannose containing polysaccharides as an aid in classification. *Mycologia* **63**: 387-402.

Staffeldt, E.E. (1959) *Thielaviopsis basicola*, a part of the cotton (*Gossypium hirsutum*) seedlings disease complex in New Mexico. *Plant Disease Reporter* **43**: 506-508.

Stanghellini, M.E., Rasmussen, S.L. & Kim, D.H. (1999) Aerial transmission of *Thielaviopsis basicola*, a pathogen of corn-salad, by adult shore flies. *Phytopathology* **89**: 476-479.

Sun, G-L., Diaz, O., Salomon, B. & Von Bothmer, R. (1999) Genetic diversity in *Elymus caninus* as revealed by isozyme, RAPD, and microsatellite markers. *Genome* **42**: 420-431.

Tabachnik, M., Devay, J.E., Garber, R.H. & Wakeman, R.J. (1979) Influence of soil inoculum concentrations on host range and disease reactions caused by isolates of *Thielaviopsis basicola* and comparison of soil assay methods. *Phytopathology* **69**: 974-977.

Tautz, D. (1989) Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Research* **17**: 6463-6471.

Thomas, M.R. & Scott, N.S. (1993) Microsatellite repeats in grapevine reveal DNA polymorphisms when analysed as sequence-tagged sites (STSs). *Theoretical and Applied Genetics* **86**: 985-990.

Tsao, P.H. & Bricker, J.L. (1966) Chlamydospores of *Thielaviopsis basicola* as surviving propagules in natural soils. *Phytopathology* **56**: 1012-1014.

Tsao, P.H. & Gundy, S.D. (1962) *Thielaviopsis basicola* as a citrus root pathogen. *Phytopathology* **52**: 781-786

Tsao, P.W. & Tsao, P.H. (1970) Electron microscopic observations on the spore wall and "opperculum" formation in chlamydospores of *Thielaviopsis basicola*. *Phytopathology* **60**: 613-616.

Upadhyay, H.P. (1981) *A monograph of* Ceratocystis *and* Ceratocystiopsis. University of Georgia Press, Athens, GA.

Upadhyay, H.P. (1993) Classification of the Ophiostomatoid fungi. In Ceratocystis *and* Ophiostoma*: Taxonomy, Ecology and Pathogenicity* (M.J. Wingfield, K.A. Seifert & J.F. Webber, eds): 7-14. APS Press, St. Paul, Minnesota.

Upadhyay, H.P. & Kendrick, W.B. (1975) Prodromus for a revision of *Ceratocystis* (Microascales, Ascomycetes) and its conidial states. *Mycologia* **67**: 798-805.

Van der Merwe, P.J.A., Mclean, N.W. & Van der Merwe, L.P. (1983) Resistance of groundnuts on black hull in South Africa. *Phytophylactica* **15**: 7-9.

Van der Merwe, P.J.A. & Van der Merwe, L.P. (1988) Harts: a groundnut cultivar with resistance to black pod rot (*Chalara elegans*). *Applied Plant Science* **2**: 81-84.

Van der Merwe, P.J.A., Van der Merwe, L.P. & Joubert, H.L.N. (1993) Implications of *Chalara elegans* resistance in groundnut breeding. *South African Journal of Plant and Soil* **10**: 91-94.

Watson, J.D., Gilman, M., Witkowski, J. & Zoller, M. (1992) *Recombinant DNA*. W.H. Freeman & Company, New York, USA.

Weber, J.L. (1990) In formativeness of human $(dC\text{-}dA)_n$ $(dG\text{-}dT)_n$ polymorphisms. *Genomics* **7**: 524-530.

Weber, J.L. & May, P.E. (1989) Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *American Journal of Human Genetics* **44**: 388-396.

Wehner, F.C., Combrink, H. & Kotzé, J.M. (1986) Root pathogens in South African *Citrus* nurseries. *Phytophylactica* **19**: 107-108.

Weijman, A.C.M. & De Hoog, G.S. (1975) On the subdivision of the genus *Ceratocystis*. *Antonie van Leeuwenhoek* **41**: 353-360.

White, T.J., Bruns,. T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR protocols: a guide to methods and applications* (M.A Innis, D.H. Gelfand, J.J. Sninsky & T.J. White, eds): 315-322. Academic Press, Inc, San diego, California, USA.

Wikler, K. & Gordon, T.R. (2000) An initial assessment of genetic relationships among populations of *Fusarium circinatum* in different parts of the world. *Canadian Journal of Botany* **78**: 709-717.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. & Tingey, S.V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Research* **18**: 6531-6535.

Wingfield, B.D., Viljoen, C.D. & Wingfield, M.J. (1999) Phylogenetic relationships of ophiostomatoid fungi associated with *Protea* infructescences in South Africa. *Mycological Research* **103**: 1616-1620.

Wingfield, M.J., Seifert, L.A. & Webber, J.F. (1993) Ceratocystis *and* Ophiostoma*: Taxonomy, Ecology and Pathogenicity*. APS Press, St. Paul, Minnesota

Wingfield, M.J., Van Wyk, P.S. & Marasas, W.F.O. (1988) *Ceratocystiopsis proteae* sp. nov., with a new anamorph genus. *Mycologia* **80**: 23-30.

Wright, E.F. & Cain, R.F. (1961) New species of the genus *Ceratocystis*. *Canadian Journal of Botany* **39**: 1215-1230.

Yarwood, C.E. (1974) Habitats of *Thielaviopsis* in California. *Plant Disease Reporter* **58**: 54-56.

Yarwood, C.E. (1981) The occurrence of *Chalara elegans*. *Mycologia* **73**: 524-530.

Yarwood, C.E. & Karayiannis, I. (1974) *Thielaviopsis* may increase plant growth. *Plant Disease Reporter* **58**: 490-492.

Zhou, Z., Miwa, M. & Hogetsu, T. (2001) Polymorphism of simple sequence repeats reveals gene flow within and between ectomycorrhizal *Suillus grevillei* populations. *New Phytologist* **149**: 339-348.

Table 1: Host range and diseases caused by *Thielaviopsis basicola* in South Africa as extracted from Crous *et al*. (2000).

Host	Common Name	Family	Disease	Reference
Cichorium intybus L.	Chicory	Asteraceae	Root rot	Prinsloo *et al*. (1991)
Arachis hypogaea L.	Groundnut	Fabaceae	Black hull	Prinsloo (1980)
Crotalaria juncea L.	Sunn hemp	"	Black root rot	De Villiers (1987)
Pisum sativum L.	Pea	"	"	"
Gossypium hirsutum L.	Cotton	Malvaceae	"	"
Avena sativa L.	Oats	Poaceae	"	"
Cenchrus ciliaris L.	Blue buffalo grass	"	"	"
Pennisetum clandestinum Chiov.	Kikuyu	"	"	"
Triticum aestivum L.	Wheat	"	"	"
Citrus spp.	Lemon trees	Rutaceae	Root rot	Wehner *et al*. (1986)
Capsicum frutescens L.	Sweet pepper	Solanaceae	Black root rot	De Villiers (1987)
Lycopersicon esculentum Mill.	Tomato	"	"	"
Nicotiana tabacum L.	Tobacco	"	"	Doidge & Bottomley (1931)

Chapter 2

Ceratocystis fimbriata and *C. moniliformis* associated with machete wounds on dying S*chizolobium parahybum* in Ecuador

Schizolobium parahybum is native to South America where it is a preferred timber species due to its excellent timber properties and fast growth. However, these trees suffer from a serious die-back disease. Die-back is often associated with streaks in the xylem originating from machete wounds at the bases of the trees. The objective of this study was to investigate the possible role that *Ceratocystis* spp., infecting machete wounds, may play in *S. parahybum* die-back in Ecuador. This was achieved through the isolation of fungi from machete wounds on diseased trees and testing their pathogenicity in greenhouse inoculation trials. Isolates were identified as *Ceratocystis fimbriata*, *C. moniliformis* and *Thielaviopsis basicola*. Greenhouse pathogenicity trials showed that *C. fimbriata* and *C. moniliformis* caused noticeable lesions on *S. parahybum*. Results suggest that both these fungi may play a role in the die-back of *S. parahybum*.

INTRODUCTION

Schizolobium parahybum (Vell.) Blake is endemic to Ecuador and is widely distributed in South America from the Pacific Coast and through the Amazon Basin. This fast growing tree with its light-coloured wood is highly desirable for veneer production. In 1982, Fundacion Forestal Manuel Durini (FFMD) initiated the planting of *S. parahybum* in the Los Sachas area of the Amazon. Five years later, serious die-back of these trees was observed. This disease, known as Pachaco die-back, has hindered the successful establishment of plantations of *S. parahybum* in Ecuador.

The disease of *S. parahybum* is characterised by the general die-back of the tree crowns. Die-back starts at the growing tips and proceeds down the stems, resulting in the production of epicormic shoots on stems, leaf loss and eventually tree death. Larger trees die slowly over a number of years. Another distinctive symptom of this disease is the discolouration and decay of the pith and wood surrounding the pith.

An important observation on diseased and dying trees is the presence of machete wounds on the stems. Foresters clear the undergrowth with machetes when they work in the plantations. This often results in damage to the bases of the trees. Discolouration of the xylem surrounding these wounds is often observed, indicating the possible presence of fungal infection. Many fungi require wounds for infection and machete wounds are common sources of such wounds. In Colombia (a neighbouring country to Ecuador) *Ceratocystis fimbriata* Ell. & Halst., for example, commonly result in a disease of coffee (*Coffea arabica* L.), as a result of stem wound infection (Pontis 1951, Marin *et al*. 2003).

Ophiostomatoid fungi including species of *Ophiostoma* H.& P. Sydow, *Ceratocystis* Ellis & Halsted, *Ceratocystiopsis* Upadhyay & Kendrick (Upadhyay 1981, De Hoog & Scheffer 1984, Wingfield *et al*. 1993), *Gondwanamyces* Marais & Wingfield (Marais *et al*. 1998), and *Cornuvesica* Viljoen & Wingfield (Viljoen *et al*. 2000) commonly colonise wounds on trees and they include some important plant pathogens. *Ophiostoma ulmi* (Buisman) Nannfeldt and *C. fagacearum* (Bretz) Hunt, causing Dutch elm disease and oak wilt respectively, are two of the best known pathogens in this group. Both these fungi as well as the other *Ceratocystis* and *Ophiostoma* spp. require wounds for infection (Jewell 1956,

Grisold 1958, Kile 1993, Upadhyay 1993). Many *Ceratocystis* and *Ophiostoma* spp. are transmitted through insect vectors that are attracted to fresh wounds (Jewell 1956, Wingfield *et al*. 1993). Such wounds are often caused by pruning or other agricultural and sylvicultural practices, but can also result from adverse weather conditions or animal damage.

In a recent study various *Ophiostoma* and *Graphium* spp. were identified from machete wounds on *S. parahybum* (Geldenhuis *et al*. 2004). The objective of the present study was to identify *Ceratocystis* spp. isolated from similar wounds. The possible role these fungi might play in disease development was investigated in greenhouse inoculations.

MATERIALS and METHODS

Survey and identification

A total of one hundred wood samples were taken from machete wounds and lesions associated with these wounds on diseased *S. parahybum* trees in Ecuador. Isolations were made from the outer margin of lesions by cutting small pieces of wood from them and placing them onto 2% malt extract agar (MEA) (20g/l malt extract and 20g/l agar, Biolab Diagnostics Ltd, Midrand, South Africa) containing streptomycin sulphate (0.4g/l) (SIGMA). The MEA plates were incubated at 25 ºC until fungal growth was observed. Each colony was transferred to fresh MEA to purify the cultures. Wood samples were also placed in moist chambers to induce formation of fungal fruiting bodies. After one week, spore masses from fungal fruiting structures on the wood were isolated and placed on 2% MEA. In addition, carrots were used as bait for possible *Ceratocystis* spp. or their *Thielaviopsis* Went anamorphs (Moller & De Vay, 1968). A piece of discoloured wood was wrapped between two sliced carrots (~5mm thickness) using masking tape and incubated at 25 ºC in plastic bags. After 6-10 days the carrots were investigated for fungal growth and possible *Ceratocystis* and *Thielaviopsis* spp. transferred to fresh MEA containing streptomycin.

The isolated fungi were examined using a Zeis Axioskop light microscope. *Ceratocystis* and *Thielaviopsis* species were identified using standard mycological keys (Nag Raj & Kendrick 1975, Upadhyay 1981).

Greenhouse pathogenicity trials

Two greenhouse pathogenicity trials were conducted. The first inoculation trial included *T. basicola* (Berk. & Broome) Ferraris (syn. *Chalara elegans* Nag Raj & Kendrick) (CMW 4684), *C. fimbriata* (CMW 4492) and *C. moniliformis* (Hedgc.) Moreau (CMW 5454). The second trial included three *C. fimbriata* isolates (CMW 4492, CMW 4682, CMW 4688) and three *C. moniliformis* isolates (CMW 961, CMW 4375, CMW 5454).

Each isolate was inoculated into 20 trees (~1-2 cm diameter) and 20 trees served as controls. To inoculate the trees a piece of bark was removed from the stems of the trees with a 9 mm cork borer to expose the cambium. A mycelial plug of equal size, taken from the margin of an actively growing culture (10 days old), was placed in each wound with the mycelium facing the xylem. Sterile MEA plugs were used as controls. The plugs and wounds were then sealed with Parafilm (Pechiney Plastic Packaging, Chicago) to prevent desiccation. Six weeks after inoculation, the outer bark was removed to expose the cambium and the length of the lesions were measured. Small pieces of wood were then cut from the streaks in the xylem and placed on 2% MEA at 25°C to test whether the inoculated fungi could be re-isolated.

Statistical analysis of data

One-way analysis of variance (ANOVA) in SAS (version 8, SAS Institute Inc., Cary, NC) was used to analyze differences in lesion length. Data were log transformed where they did not meet the assumptions of ANOVA.

RESULTS

Survey and identification

A total of 17 isolates of *Ceratocystis* spp. or their anamorphs were obtained. Nine of these were identified as *T. basicola* (CMW 4098-4100, CMW 4381, CMW 4457, CMW 4684-4686, CMW 4689), based on their hyaline cylindrical endoconidia and dark, thick-walled segmented chlamydospores (Nag Raj & Kendrick 1975). Three isolates were identified as *C. fimbriata* (CMW 4682, CMW 4688, CMW 4492) based on their hat-shaped ascospores and dark ascomata without ornamentation, and five as *C. moniliformis* (CMW 961-962, CMW 4375, CMW 5302, CMW 5454) based on their hat-shaped ascospores and dark ascomata with conical spines (Upadhyay 1981).

Greenhouse pathogenicity trials

Inoculation with *C. moniliformis*, *C. fimbriata* and *T. basicola* in the first trial resulted in lesions that differed significantly from the controls (P=0.004). The average lesion length for *C. moniliformis*, *C. fimbriata*, *T. basicola* and the control in the first trial were 19.8 mm (CMW 5454), 22.9 mm (CMW 4492), 22.45 mm (CMW 4684) and 12.4 mm respectively. None of the fungal isolates differed significantly from each other. Analysis of the 95% confidence levels revealed that the confidence levels did not overlap with the control (Fig.1).

The second trial included only *C. fimbriata* and *C. moniliformis*. All isolates except *C. fimbriata* (CMW 4682, P=0.124) developed lesions that differed significantly from the control. Lesion length averages for the three *C. fimbriata* isolates were 47.7 mm (CMW 4492), 27.5 mm (CMW 4682) and 39.8 mm (CMW 4688). The average lesion length for the *C. moniliformis* isolates was 46.7 mm (CMW 961), 34.9 mm (CMW 4375) and 36.6 mm (CMW 5454). The control inoculations had an average lesion length of 15.25 mm. None of the fungal isolates differed significantly from each other. Analysis of the 95% confidence levels revealed that the confidence levels for two of the *C. moniliformis* isolates (CMW 4375 and CMW 5454) overlapped with the control, indicating that they do not differ significantly from the control at the 95% confidence level, but more likely at the 90% confidence level (Fig.2).

In general, both *C. fimbriata* and *C. moniliformis* isolates inoculated into *S. parahybum* were able to cause significant lesions on inoculated trees (Fig. 1 and 2). Lesions were, however, not consistently produced and lesion lengths ranged from 10 mm to 170 mm for *C. fimbriata* and from 11 mm to 120 mm for *C. moniliformis*. Average lesion development caused by the same isolates (*C. moniliformis* CMW 5454, *C. fimbriata* CMW 4492) was much larger in the second trial (36.6 mm, 47.7 mm) than in the first (19.8 mm, 22.9 mm). The average lesion length of all the *C. fimbriata* isolates was the same as those caused by the *C. moniliformis* isolates (Ave. 34.5mm). Test fungi were consistently re-isolated from discoloured xylem.

DISCUSSION

In this study we identified two fungi that may be involved in the die-back disease affecting *S. parahybum* trees in Ecuador. Infection by these fungi was, however, not consistently associated with machete wounds on dying trees. Although the fungi displayed some level of pathogenicity in greenhouse trials, their lack of consistent association with die-back symptoms or machete wounds, suggests that they do not play a significant role in *S. parahybum* die-back in Ecuador.

Ceratocystis fimbriata has a world-wide distribution but has primarily been reported from Central and South America (McCracken & Burkhardt 1977) where it is the causal agent of root disease and cankers of important crops such as sweet potato, coffee and cacao (Kile 1993). It has also been reported from *Eucalyptus* in Brazil, Central and East Africa (Roux *et al*. 1999, 2001), Uruguay (Barnes *et al*. 2003) and South Africa (Roux *et al*. 2004). Wounds, caused by agricultural practices (Teviotdale 1991, Marin *et al.* 2003) or insects (Hinds 1972), play an important role in the infection of this fungus. It was, therefore, not unusual to find *C. fimbriata* associated with machete wounds on wounded *S. parahybum* trees.

Ceratocystis moniliformis, like *C. fimbriata*, has a world-wide distribution and has previously been isolated in association with wounds on trees. It has been isolated, together with *C. fimbriata*, from insects visiting wounds on *Populus tremuloides* Michx (Hinds 1972) and from wounds on *E. grandis* Hill ex Maiden in South Africa (Roux *et al*. 2004). Belezaca & Suárez (2001) isolated *C. moniliformis* from *S. parahybum* in Ecuador and found that is played a role in disease development. This fungus is, however, not regarded to be a plant pathogen. The fact that it caused lesions in pathogenicity tests in this study and those done by Belezaca & Suárez (2001) is unexpected. This might suggest that the role of the fungus as a plant pathogen deserves further study.

Two greenhouse trials, with single repetitions, were conducted in this study. The first trial included *T. basicola* that was thought to be isolated from *S. parahybum*. The second trial did not include this fungus because later studies (Chapter 5) revealed that isolates of this fungus originated from carrots used for baiting purposes and not from machete wounds on

S. parahybum. *Thielaviopsis basicola* is not a known tree pathogen although the fact that *T. basicola* were able to cause lesions on *S. parahybum* is intriguing.

Inoculation trials in this study clearly showed that both *C. fimbriata* and *C. moniliformis* are able to cause lesions on *S. parahybum*. Differences in lesion length caused by *C. fimbriata* between the first and second trials may have resulted from differences in the age of the trees. The trees in the first trial were more than two years old, while those in the second trial were only eighteen months. Pontis (1951), observed that *C. fimbriata* cankers on coffee trees developed only after the trees were more than two- years- old. The great variation in the range of lesion lengths may also be explained by possible differences in susceptibility of the trees. The fact, however, remains that although both caused lesions on trees, they were not consistently associated with *S. parahybum* die-back, and are probably not an important component of this disease.

REFERENCES

Barnes, I., Roux, J., Wingfield, B.D., O'Neill, M. & Wingfield, M.J. (2003) *Ceratocystis fimbriata* infecting *Eucalyptus grandis* in Uruguay. *Australasian Plant Pathology* **32**: 361-366.

Belezaca, C. & Suárez, C. (2001) Agentes Bióticos que causan la muerte de *Schizolobium parahybum* (pachaco) en la zona central del Litoral Ecuatoriano. *Memorias de XI Seminario Nacional de Sanidad* Vegeta. p 212-22l. Universidad Técnica de Babahoyo. Babahoyo, Ecuador.

De Hoog, G.S. & Scheffer, R.J. (1984) *Ceratocystis* versus *Ophiostoma*: a reappraisal. *Mycologia* **76**: 292-299.

Geldenhuis, M.M., Roux, J., Montenegro, F., De Beer, Z.W., Wingfield, M.J. & Wingfield, B.D. (2004) Identification and pathogenicity of *Graphium* and *Pesotum* species from machete wounds on *Schizolobium parahybum* in Ecuador. *Fungal Diversity* **15**: 137-151.

Grisold, C.L. (1958) Transmission of the oak wilt fungus by certain woodland-inhabiting *Drosophilidae*. *Journal of Economical Entomology* **51**: 733-735.

Hinds, T.E. (1972) Insect transmission of *Ceratocystis* species associated with aspen cankers. *Phytopathology* **62**: 221-225.

Jewell, F.F. (1956) Insect transmission of oak wilt. *Phytopathology* **46**: 244-257.

Kile, G.A. (1993) Plant diseases caused by species of *Ceratocystis sensu stricto* and *Chalara*. In Ceratocystis *and* Ophiostoma*: Taxonomy, Ecology and Pathogenicity* (M.J. Wingfield, K.A. Seifert & J.F. Webber, eds): 173-183. APS Press, St. Paul, Minnesota.

Marais, G.J., Wingfield, M.J., Viljoen, C.D. & Wingfield, B.D. (1998) A new ophiostomatoid genus from *Protea* infructescences. *Mycologia* **90**: 136-141.

Marin, M., Castro, B., Gaitan, A., Preisig, O., Wingfield, B.D. & Wingfield M.J. (2003) Relationships of *Ceratocystis fimbriata* isolates from Colombian coffee-growing regions based on molecular data and pathogenicity. *Journal of Phytopathology* **151**: 1-11.

McCracken, F.I. & Burkhardt, E.C. (1977) Destruction of sycamore by canker stain in the midsouth. *Plant Disease Reporter* **61**: 984-986.

Moller, W.J. & De Vay, J.E. (1968) Carrots as a species-selective isolation media for *Ceratocystis fimbriata*. *Phytopathology* **58**:123-126.

Nag Raj, T.R. & Kendrick, B. (1975) *A monograph of* Chalara *and allied genera*. Wilfred Laurier University Press, Waterloo, Ontario, Canada. 200pp.

Pontis, R.E. (1951) A canker disease of the coffee tree in Colombia and Venezuela. *Phytopathology* **49**: 179-184.

Roux, J., Coutinho, T.A., Mujuni Byabashaija, D. & Wingfield, M.J. (2001) Diseases of plantation *Eucalyptus* in Uganda. *South African Journal of Science* **97**: 16-18.

Roux, J., Van Wyk, M., Hatting, H. & Wingfield, M.J. (2004) *Ceratocystis* species infecting stem wounds on *Eucalyptus grandis* in South Africa. *Plant Pathology* **53**: 414-421.

Roux, J., Wingfield., M.J., Wingfield, B.D., Bouilett, J.P. & Alfenas, A.C. (1999) A serious new disease of *Eucalyptus* caused by *Ceratocystis fimbriata* in Central Africa. *Forest Pathology* **30**: 175-184.

Teviotdale, B.L. (1991) Infection of pruning and small bark wounds in almond by *Ceratocystis fimbriata*. *Plant Disease* **75**: 1026-1030.

Upadhyay, H.P. (1981) *A monograph of* Ceratocystis *and* Ceratocystiopsis. The University of Georgia Press, Athens. GA. 176pp.

Upadhyay, H.P. (1993) Classification of the Ophiostomatoid fungi. In Ceratocystis *and* Ophiostoma*: Taxonomy, Ecology and Pathogenicity* (M.J. Wingfield, K.A. Seifert & J.F. Webber, eds): 7-13. APS Press, St. Paul, Minnesota.

Viljoen, C.D., Wingfield, M.J., Jacobs, K. & Wingfield, B.D. (2000) *Cornuvesica*, a new genus to accommodate *Ceratocystiopsis falcata*. *Mycological Research* **104**: 365-367.

Wingfield, M.J., Seifert, L.A. & Webber, J.F. (1993) Ceratocystis *and* Ophiostoma*: Taxonomy, Ecology and Pathogenicity*. APS Press, St. Paul, Minnesota

Figure 1. Mean lesion lengths after inoculation of *S. parahybum* with *C. fimbriata* (CMW 4492), *C. moniliformis* (CMW 5454) and *T. basicola* (CMW 4684). Inoculation with all three isolates resulted in lesions that differed significantly from the control (P=0.004). Bars represent mean (Least Squares Means) lesion lengths with 95% confidence levels for each isolate.

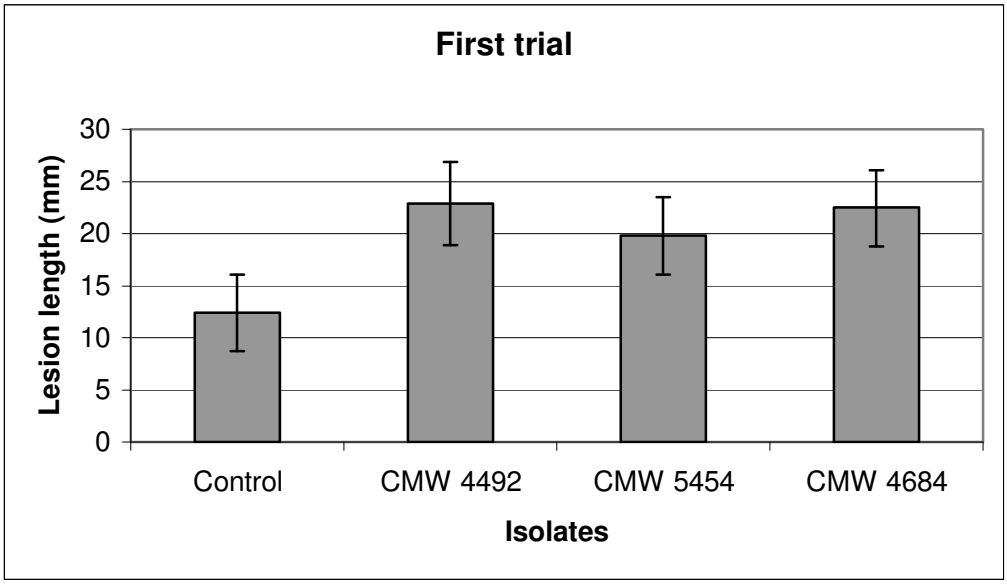
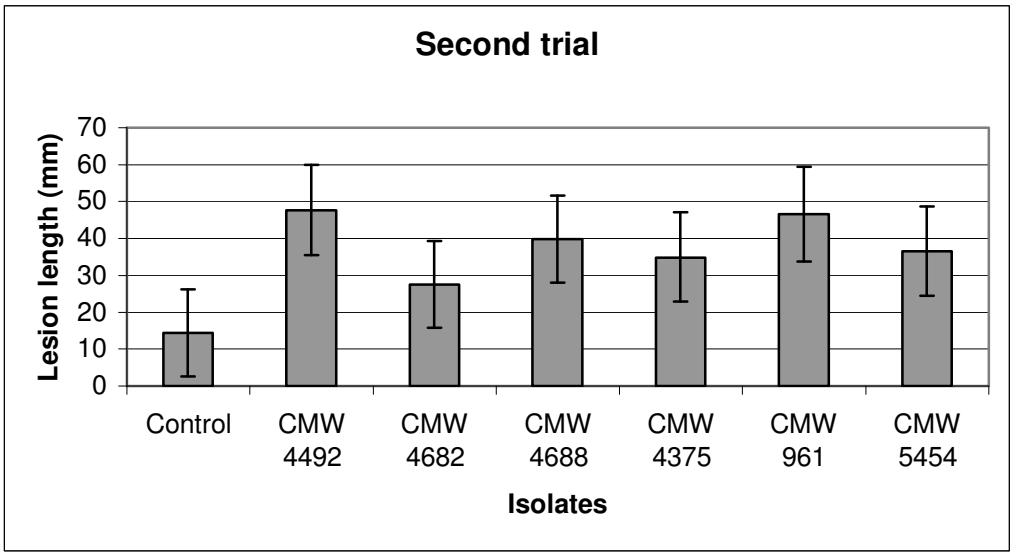

First trial
Lesion length (mm)
30
25
20
15
10
5
0
Control
CMW 4492
CMW 5454
CMW 4684
Isolates

Figure 2. Mean lesion length after inoculation of *S. parahybum* with *Ceratocystis fimbriata* (CMW 4492, CMW 4682, CMW 4688) and *C. moniliformis* (CMW 961, CMW 4375, CMW 5454). All isolates except one (*C. fimbriata*; CMW 4682; P=0.124) caused lesions that differed significantly from the control (P=0.0024). Bars represent mean (Least Squares Means) lesion lengths with 95% confidence levels for each isolate. According to the confidence levels two of the *C. moniliformis* isolates (CMW 5454 and CMW 4375) differ from the control not on the 95% confidence level, but on the 90% level.


Second trial
Lesion length (mm)
70
60
50
40
30
20
10
0
Control
CMW 4492
CMW 4682
CMW 4688
CMW 4375
CMW 961
CMW 5454
Isolates

Chapter 3

Identification and pathogenicity of *Graphium* and *Pesotum* species from machete wounds on *Schizolobium parahybum* in Ecuador*

Schizolobium parahybum is native to Ecuador and is widely distributed throughout South America. This tree has ideal timber properties and is a favoured species for plantation development. *Schizolobium parahybum* trees, however, suffer from a serious disease that causes substantial losses to plantations in Ecuador. Most diseased trees have been regularly wounded with machetes and it has been suggested that these wounds might provide entry portals for pathogens. To determine the possible role that fungi associated with machete wounds might play in disease development, wood samples were taken from these wounds and screened for possible pathogens. A number of potential pathogens were identified, including *Ceratocystis fimbriata*, *C. moniliformis*, *Graphium* spp. and *Pesotum* spp. The objective of this study was to identify the twenty-one synnematous *Hyphomycetes,* from wounds on *S. parahybum*, using small subunit (SSU) and internal transcribed spacer (ITS) sequence data from the ribosomal RNA operon. We also investigated the possible role of these species in disease development in a greenhouse inoculation trail. Results showed that fifteen isolates reside in the *Graphium penicillioides* complex (Order: Microascales). Four isolates resided in the Ophiostomatales and represent the *Pesotum* anamorph of *Ophiostoma quercus*. The remaining two isolates were unidentified *Pesotum* anamorphs of *Ophiostoma*. None of the three species produced significant lesions in a greenhouse inoculation trail and we do not consider them pathogens of *S. parahybum*.

* This chapter was published as: Geldenhuis, M.M., Roux, J., Montenegro, F., De Beer, W.Z., Wingfield, M.J. & Wingfield, B.D. (2003) Identification and pathogenicity of *Graphium* and *Pesotum* species from machete wounds on *Schizolobium parahybum* in Ecuador. *Fungal diversity* **15**: 137-151.

INTRODUCTION

Schizolobium parahybum (Vell.) Blake is a native tree species in South America, where it occurs in Ecuador and the Amazon Basin. Its timber is highly prized for production of a clear light-coloured veneer. *Schizolobium parahybum* has thus been used to establish plantations in Ecuador, where the tree is commonly known as "pachaco". In 1982, plantations of *S. parahybum* were established in the Los Sachas area of the Ecuadorian Amazon. However, trees soon developed a serious die-back disease known as Pachaco die-back.

Observations of diseased trees in Ecuador have indicated that pathogens are probably introduced into the trees through mechanical damage caused by machetes. Preliminary isolations from machete wounds led to the isolation of the well-known tree pathogen, *Ceratocystis fimbriata* Ellis & Halsted, as well as several other fungi. These included *C. moniliformis* (Hedgc.) Moreau and a collection of isolates of synnematous *Hyphomycetes* tentatively identified as belonging to the *Graphium sensu lato* complex (Roux *et al.* 2000, Geldenhuis *et al*. 2001, Chapter 2).

Species of *Graphium sensu lato*, including *Graphium* Corda, *Pesotum* Crane & Schoknecht and *Phialographium* Upadhyay & Kendrick are typified by well-developed, generally dark synnemata, producing single-celled conidia in slimy masses at their apices. The genera in this group were treated collectively for many years but were later separated based on their respective modes of conidial development. *Graphium* was first described in 1837 for five species, including *G. penicillioides* Corda (Corda 1837), later named the lectotype species for the genus, which was defined as having percurrent conidial development (Hughes 1958). Münch (1907) assigned the synnematous anamorphs of *O. piceae* (Münch) H. & P. Sydow and *O. canum* (Münch) H. & P. Sydow to *Graphium*. Crane & Schoknecht (1973) however, showed that conidiogenesis in *G. penicillioides* differs from that of the synnematous anamorphs of *O. piceae* and *O. ulmi* (Buisman) Nannfeldt, and they transferred the anamorph of the latter species, *Graphium ulmi* Schwarz, to the new genus, *Pesotum*, with *Pesotum ulmi* (Schwarz) Crane & Schoknecht as the type species. Subsequently a number of other genera were described based on patterns of conidiogenesis, including *Phialographium* (Upadhyay & Kendrick 1974) with the anamorph of *O. sagmatospora* (Wright & Cain) Solheim as the type species.

Wingfield *et al.* (1991) reassessed *Graphium*, *Pesotum* and *Phialographium*, and found that there is more than one mode of conidiogenesis present in both *Pesotum* and *Phialographium*. This suggested that differences in conidiogenesis do not provide a reliable characteristic to distinguish between these genera. They, therefore, transferred species of *Pesotum* and *Phialographium* back to *Graphium*. Seifert & Okada (1993) concluded that *Graphium* should be restricted to anamophs of the Ophiostomataceae. However, small subunit (18S) sequences showed that *G. penicillioides*, the lectotype species of *Graphium*, is not phylogenetically related to anamorphs of *Ophiostoma* (Okada *et al.* 1998). Results of their study, furthermore, showed that *G. penicillioides* includes several different taxa and it should be considered a species aggregate. The *G. penicillioides* aggregate forms a monophyletic group within the order Microascales, while the synnematous anamorphs of *O. piceae* and *O. ulmi* group with the Ophiostomatales. This classification was supported by Okada *et al.* (2000) who, based on 18S rDNA sequence data, showed that *Graphium*-like synnematous fungi currently occur in the Microascales (*Graphium*), Ophiostomatales (*Pesotum*) and Chaetothyriales (*Exophiala*). They also introduced a fourth phylogenetic group for an undescribed genus (or genera) belonging to the Erysiphales. Most recently, Harrington *et al*. (2001) recommended that the name *Pesotum* should be restricted to anamorphs related to the *O. piceae* complex, within the Ophiostomatales.

One means to distinguish between species of *Graphium* and *Pesotum* is to assess their ability to grow on media containing cycloheximide. *Ophiostoma* spp. are well known to be able to tolerate high levels of this antibiotic substance and it is commonly used in selective media for these fungi (Harrington 1981, Jacobs & Wingfield 2001, Zhou *et al*. 2001). However, cycloheximide tolerance is not sufficiently robust to group *Graphium* and *Pesotum* (Harrington *et al*. 2001). Currently the only definitive method to determine whether fungi loosely resembling species of *Graphium* reside in this or other genera is to compare DNA sequence data for them (Okada *et al*. 1998).

Several *Pesotum* species are important plant pathogens. Most notable of these is *Pesotum ulmi* (Schwarz) Crane & Schoknecht, which is the anamorph of *Ophiostoma ulmi*. This fungus together with *O. novo-ulmi* Brasier are the causal agents of Dutch elm disease (Brasier 1990). The genus *Graphium* does not include important pathogens, but it

commonly occurs in niches together with *Pesotum* spp. such as bark beetle galleries in trees killed by Dutch elm disease (Webber & Brasier 1984).

The objective of this study was to identify isolates loosely assigned to *Graphium* that had been collected from wounds on diseased *S. parahybum* trees in Ecuador. This was accomplished by sequencing the small subunit (SSU) and internal transcribed spacer (ITS) regions (including the 5.8S gene) of the ribosomal RNA operon. The possible role of these fungi in die-back of *S. parahybum* was also considered by conducting a preliminary pathogenicity test under greenhouse conditions.

MATERIALS and METHODS

Isolates

One hundred wood samples associated with machete wounds on different *S. parahybum* trees in Ecuador were collected. These samples were placed in moist chambers at 25 ºC to induce fungal growth and sporulation. Synnematal anamorphs loosely assigned to the genus *Graphium* were commonly found sporulating on the wood surface after 8-10 days. These fungi were isolated by transferring masses of conidia from individual synnemata to 2% malt extract agar (MEA) (20g/l malt extract and 20g/l agar, Biolab Diagnostics Ltd, Midrand, South Africa). The single spore drops were allowed to grow for 10 days at 25 ºC before they were grouped into morphotypes according to differences in colony colour (Rayner 1970), fruiting structures and hyphal growth characteristics on MEA (Lacap *et al*. 2003). All isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA isolation

Two isolates loosely identified as *Graphium* spp., were randomly selected from each morphological group (Table 1) and grown in 2% malt extract broth (20 g/l malt extract, Biolab) at 25 ºC. After 10 days, mycelium was harvested by filtration through sterile filter paper. DNA was extracted using a modification of the DNA extraction procedure described by Raeder & Broda (1985). The mycelium was ground to a fine powder using liquid nitrogen and a mortar and pestle, transferred to Eppendorf tubes and suspended in 800 µl DNA extraction buffer (200 mM Tris-HCl pH 8, 25 mM EDTA pH 8, 150 mM NaCl, 0.5% SDS). After addition of phenol:chloroform (5:3, v/v), the mixture was

centrifuged (10 000 rpm, 60 min) to remove cell debris. This was followed by a series of phenol:chloroform (1:1, v/v) extractions on the upper aqueous layer until the interphase was clean. Excess phenol was removed by a final chloroform extraction. Nucleic acids were allowed to precipitate overnight at –20 ºC in cold 100% ethanol (2:1, v/v). The DNA was collected by centrifugation (10 000 rpm, 30 min), washed in 70% ethanol and resuspended in 50µl sterile water. RNA was removed through addition of Rnase-A (Roche Molecular Biochemicals, Germany) and incubation at 37 ºC for 60 min. The DNA solution was stored at –20 ºC.

DNA amplification

The polymerase chain reaction (PCR) was used to amplify specific regions of the ribosomal RNA operon. The SSU gene of all representative isolates (Table 2) was amplified using the primer pair 2F (5`-ATCTGGTTGATCCTGCCAGTAG-3`) and 1794R (5`-GATCCTTCCGCAGGTTCACC-3`) (Okada *et al*. 1997). Small subunit sequences revealed the identity of only two of the six isolates. Thus, the internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S gene of the other four isolates (CMW 5565, CMW 5568, CMW 5564, CMW 5573) were amplified using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3`) (White *et al*. 1990). PCR reactions were carried out in a total volume of 50 µl and included 1 ng DNA template, Expand HF buffer containing 1.5 mM $MgCl_2$ (supplied with the enzyme), 0.2 µM of each primer, 200 µM of each dNTP and Expand^{TM} High Fidelity *Taq* polymerase mixture (1.75 U) (Roche Pharmaceuticals, Germany). PCR conditions described by Okada *et al*. (1997) were followed to amplify the SSU and ITS regions. PCR products were visualized under UV illumination after electrophoreses in a 1% agarose (Sigma) gel, stained with ethidium bromide to determine the success of the reactions.

DNA sequencing

PCR fragments were purified using a High Pure^{TM} PCR Product Purification Kit (Boehringer Mannheim) and sequenced using a ABI PRISM^{TM} Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq®DNA Polymerase, FS (Perkin-Elmer, Warrington, U.K.) following the manufacturers protocol on a ABI 377 Autosequencer (Applied Biosystems). The purified amplification products were sequenced in both directions with the same primers used for PCR. Additional internal primers 404F (5`-

GCTACCACATCCAAGGAAGG-3`) and 581R (5`-ATTACCGCGGCTGCTGGC-3`) (Okada *et al*. 1997) were used for sequencing of the SSU.

Sequence analysis

Sequence data were manually aligned by inserting gaps that were treated as missing data. Ambiguously aligned sequences were not included in the analysis. DNA sequences were analyzed using PAUP 4.0* (Phylogenetic Analysis Using Parsimony* and other Methods version 4) (Swofford 1998). Heuristic searches were done with stepwise addition (random sequence addition), branch swapping (tree bisection reconnection) and MULPAR effective with MaxTrees set to auto-increase. The tree length distributions over 100 randomly generated trees were evaluated to access the phylogenetic signal in the data sets ($g1$) (Hilles & Haulsenbeck 1992). Statistical support for internal branches of the most parsimonious SSU and ITS trees were estimated by bootstrap analysis of a 1000 replicates. Sequences for other species included in the SSU and ITS analysis were obtained from GenBank (Table 2). *Neurospora crassa* Shear & B.O. Dodge was used as monophyletic outgroup for the SSU data and *O. ulmi* for ITS sequence analysis. A BLAST search was done with ITS sequences that could not be included in the ITS analysis, due to unambiguous alignment.

Pathogenicity

A preliminary greenhouse inoculation trial was conducted with one randomly selected isolate of each of the species identified in this study (Table 2). The isolates used in the trial were CMW 5554, CMW 5568 and CMW 5573 representing *G. penicillioides*, *O. quercus* (Georgévitch) Nannfeldt and the *Pesotum* sp. respectively. The isolates were transferred to fresh MEA and incubated at 25 ºC. After 8 days, each isolate was inoculated into 20 trees (~1-2 cm diam.) and an equal number of trees served as controls. The inoculations were carried out using the technique described by Zhou *et al*. (2002). After six weeks, the outer bark was removed with a scalpel and the length of the inner lesions, measured. Pieces of wood (2 mm x 2 mm) were cut several distances from the lesions and placed on MEA to determine whether the inoculated fungi were present in the wounds.

Differences in lesion lengths were analysed using one-way analysis of variance (ANOVA) with SAS (version 8, SAS Institute Inc., Cary, NC). Data were log transformed where they did not meet the assumptions of ANOVA.

RESULTS

Isolates

A total of 21 *Graphium*-like isolates were obtained from the wood samples. Each of these isolates was specifically from a different tree. These isolates could be placed in three morphotypes, based on colony colour, the production of fruiting bodies and the characteristics of hyphal growth on MEA (Table 1).

Sequence analysis

Both strands of the SSU rDNA of the six representative isolates were sequenced to determine whether these isolates represented *Pesotum* or *Graphium* species. After manual alignment, a total of 1032 base pairs were obtained. Uninformative characters were excluded from the data set, resulting in a total of 908 excluded characters and 124 parsimony informative characters. The heuristic search option generated 20 most parsimonious trees with similar topologies but with different branch lengths. Only one tree was selected for presentation (Fig. 1). The phylogram generated from the SSU sequence data (Fig. 1) indicated that two of the isolates reside in the *G. penicillioides* aggregate, order Microascales. The other four isolates grouped with *Pesotum* (Ophiostomatales). Two of the latter isolates grouped closely with *O. piceae* and *O. ulmi*, while the other two resided in a separate clade.

The ITS rDNA regions of the four *Pesotum* spp. were sequenced in both directions to compare them with *O. quercus* and *O. piceae*. Only sequences for two of the isolates could be unambiguously aligned. The other two isolates were thus excluded from the analysis. The total number of characters after alignment was 579, including 196 bp of ITS1, 158 bp of the 5.8S gene and 225 bp of ITS2. Uninformative characters were excluded from the data analysis leaving a total of 557 characters excluded from the data set. The remaining 22 characters were all parsimony informative. Heuristic searches generated three most parsimonious trees with the same topologies but different branch lengths. One of these trees was selected for presentation (Fig. 2). The ITS tree included sequences of four *O. quercus* isolates, four *O. piceae* isolates and the two *Pesotum* isolates from Ecuador. The two *Pesotum* isolates clearly reside with *O. quercus*. The BLAST search that was done with ITS sequences of the two *Pesotum* isolates that could not be included in the ITS analysis, did not result in a positive identification of these isolates.

Pathogenicity

Inoculations with *G. penicillioides*, *O. quercus* and the *Pesotum* sp. resulted in very small lesions. Control inoculations did not give rise to any lesions. The inoculated fungi could be re-isolated from only slightly coloured wood, up to 10 mm from the lesions. The average lesion length for *G. penicillioides*, *O. quercus,* the *Pesotum* sp. and the control were 12.05 mm, 16.68 mm, 12.9 mm and 12 mm respectively. Lesions associated with *O. quercus* were significantly longer than the others, including the control (P=0.0031). Neither *G. penicillioides* nor the *Pesotum* sp. differed from the control inoculations in terms of lesion length (Fig. 3).

DISCUSSION

In this study SSU and ITS sequence data were used to identify *Graphium*-like isolates collected from diseased *S. parahybum* trees in Ecuador. Three different species, one within the Microascales and two in the Ophiostomatales were isolated from machete wounds. This is in addition to *C. fimbriata* and *C. moniliformis* that were isolated from these wounds in previous studies (Roux *et al*. 2000, Geldenhuis *et al*. 2001, Chapter 2). The *Graphium* sp. identified is most likely related to *G. penicillioides* and the one *Pesotum* sp. is *O. quercus*. The other *Pesotum* sp. appears to represent an undescribed taxon that we have chosen not to name, due to the low number of isolates available. This study represents the first reports of *G. penicillioides* and *O. quercus* from Ecuador and from *S. parahybum*.

Our results have shown that lesions caused by *O. quercus* after inoculation on young *S. parahybum* trees in the greenhouse, differed significantly from the control. These lesions however, did not differ more than 5 mm from the control and although the differences are statistically significant they are not pathogenetically important. *Graphium penicillioides* and the *Pesotum* sp. were also unable to cause any signs of disease. These three fungi were most probably transferred to machete wounds by casual insects and they clearly appear to be saprobic. It was interesting that we were able to re-isolate them from apparently healthy wood in advance of the inoculation wounds. This indicates that they are able to grow inside the tree without causing disease. Although we do not consider them primary pathogens, it is possible that their presence may contribute to sapstain in response to the decline of the tree.

Graphium penicillioides was the most commonly isolated synnematous fungus from the machete wounds on *S. parahybum*. Identification of *Graphium* and *Pesotum* species is difficult because these genera are morphologically very similar. *Graphium penicillioides* isolates were thus distinguished from the other isolates based on cultural characteristics and their identity established using DNA sequence data. Little is known regarding the biology of *G. penicillioides*. The fungus was originally described from *Populus italica* Moench. in Prague (Corda 1837) and there are no reports of it being pathogenic on the trees from which it has been isolated. It clearly has a wide host and distribution range on wounds of deciduous trees.

Six of the 21 *Graphium*-like isolates obtained from *S. parahybum* in this study occur within the genus *Pesotum*. Comparisons of sequence data showed that four of these isolates represent the *Pesotum* anamorph of *O. quercus*. Okada *et al.* (1998) assigned *P. pirinum* (Goid.) Okada & Seifert as the anamorph of *O. quercus*, but Harrington *et al.* (2001) considered *P. pirinum* to be distinct from *O. quercus* and proposed that *G. roboris* Georgescu & Teodoru might be an appropriate anamorph. Resolving this issue did not form part of the scope of this study and until this has been clarified, we will refer to the isolates from Ecuador as *O. quercus*. *Ophiostoma quercus* is a common and widespread fungus, occurring predominantly on hardwoods, but also on conifers (Harrington *et al.* 2001). The fungus was originally described from oak in Yugoslavia (Georgévitch 1926), but for many years, was treated as a synonym of *O. piceae* (Hunt 1956, Upadhyay 1981, Przybyl & de Hoog 1989), with the consequence that many reports of *O. piceae* from hardwoods could have represented *O. quercus*. Its occurrence on native hardwood trees in Ecuador, confirms previous suggestions that it might be native to the southern continents (De Beer *et al.* 2003). Although *O. quercus* has been linked to vascular wilt in Europe (Anonymous 1990), it appears to be only a weak pathogen or a saprobe on *S. parahybum* in Ecuador.

Two isolates from *S. parahybum* could only be identified as representing a *Pesotum* sp. This fungus appears to represent an undescribed species.

This study has extended both the host and geographic range of *O. quercus* and *G. penicillioides*. These fungi, and the unnamed *Pesotum* sp., were also shown not to contribute to the death of *S. parahybum* in Ecuador. Although they are related to plant

pathogenic fungi and were associated with dying trees, we do not believe that they justify inclusion in future pathogenicity tests that are planned for trees in Ecuador plantations.

REFERENCES

Anonymous. (1990) Oak decline and the status of *Ophiostoma* spp. on oak in Europe. *EPPO Bulletin* **20**: 405-423.

Brasier, C.M. (1990) China and the origins of Dutch elm disease: an appraisal. *Plant Pathology* **39**: 5-16.

Corda, A.C.I. (1837) Icones fungorum hucusque cognitorum. Abbildungen der pilze und schwaemme I. Pragae.

Crane, J.L. & Schoknecht, J.D. (1973) Conidiogenesis in *Ceratocystis ulmi*, *Ceratocystis piceae* and *Graphium penicillioides*. *American Journal of Botany* **60**: 346-354.

De Beer, Z.W., Wingfield, B.D. & Wingfield, M.J. (2003) The *Ophiostoma piceae* complex in the Southern Hemisphere: a phylogenetic study. *Mycological Research* **107**: 469-476.

Geldenhuis, M.M., Roux, J., Wingfield, B.D., De Beer, Z.W. & Wingfield, M.J. (2001) Ophiostomatoid fungi associated with diseased *Schizolobium parahybum*. Joint Meeting of the American Phytopathological Society, Mycological Society of America and Society of nematologists. Salt Lake City, Utah, August 25-29.

Georgévitch, P. (1926) *Ceratostomella querci* n. sp. *Comptes Rendus Hebdomadaires des Séances de l'Academie des Sciences* **183**: 759.

Harrington, T.C. (1981) Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. *Mycologia* **73**: 1123-1129.

Harrington, T.C., McNew, D., Steimel, J., Hofstra, D. & Farrell, R. (2001) Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi. *Mycologia* **93**: 111-136.

Hilles, D.M. & Huelsenbeck, J.P. (1992) Signal, noise and reliability in molecular phylogenetic analysis. *Journal of Heredity* **83**: 189-195.

Hughes, S.J. (1958) Revisiones hyphomycetum aliquot cum appendice de nominibus rejiciendis. *Canadian Journal of Botany* **36**: 727-836.

Hunt, J. (1956) Taxonomy of the genus *Ceratocystis*. *Lloydia* **19**: 1-58.

Jacobs, K. & Wingfield, M.J. (2001) Leptographium *species - tree pathogens, insect associates and agents of blue-stain*. American Phytopathological Society Press, St. Paul, Minnesota.

Lacap, D.C., Liew, E.C.Y. & Hyde, K.D. (2003) An evaluation of the fungal 'morphotype' concept based on ribosomal DNA sequences. *Fungal Diversity* **12**: 53-66.

Münch, E. (1907) Die blaufäule des Nadelholzes. *Naturwissenschaftliche Zeitschrift für Forst und Landwirtschaft* **5**: 531-573.

Okada, G., Jacobs, K., Kirisits, T., Louis-Seize, G.W., Seifert, K.A., Sugita, T., Takematsu, A. & Wingfield, M.J. (2000) Epitypification of *Graphium penicillioides* Corda, with comments on the phylogeny and taxonomy of graphium-like synnematous fungi. *Studies in Mycology* **45**: 169-188.

Okada, G., Seifert, K.A., Takematsu, A., Yamaoka, Y., Miyazaki, S. & Tubaki, K. (1998) A molecular phylogenetic reappraisal of the *Graphium* complex based on 18S rDNA sequences. *Canadian Journal of Botany* **76**: 1495-1506.

Okada, G., Takematsu, A. & Takamura, Y. (1997) Phylogenetic relationships of the hyphomycete genera *Chaetopsina* and *Kionochaeta* based on 18S rDNA sequences. *Mycoscience* **38**: 409-420.

Przybyl, K. & de Hoog, G.S. (1989) On the variability of *Ophiostoma piceae*. *Antonie van Leeuwenhoek* **55**: 177-188.

Raeder, U. & Broda, P. (1985) Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* **1**: 17-20.

Rayner, R.W. (1970) *A mycological colour chart*. Commonwealth Mycological Institute and British Mycologica Society, Kew, Surrey, UK.

Roux, J., Wingfield, M.J., Montenegro, F., Geldenhuis, M.M. & Wingfield, B.D. (2000) *Ceratocystis* diseases of *Schizolobium parahybum* in Ecuador. Proceedings of the 38th Congress of the Southern African Society for Plant Pathology, Grahamstown, January 26-28.

Seifert, K.A. & Okada, G. (1993) *Graphium* anamorphs of *Ophiostoma* species and similar anamorphs of other ascomycetes. In Ceratocystis *and* Ophiostoma*: Taxonomy, Ecology and Pathogenicity* (M.J. Wingfield, K.A. Seifert & J.F. Webber, eds): 27-41. APS Press, St. Paul, Minnesota.

Swofford, D.L. (1998) *PAUP: phylogenetic analysis using parsimony*. Version 4.0 Beta version. Sinauer Associates, Sunderland, Massachusetts.

Upadhyay, H.P. (1981) *A monograph of* Ceratocystis *and* Ceratocystiopsis. The University of Georgia Press, Athens, Georgia, USA.

Upadhyay, H.P. & Kendrick, W.B. (1974). A new *Graphium*-like genus (conidial state of *Ceratocystis*). *Mycologia* **66**: 181-183.

Webber, J.F. & Brasier, C.M. (1984) The transmission of Dutch elm disease: a study of the processes involved. In *Vertebrate microbial interactions* (J.M. Anderson, A.D.M. Rayner & D.W.H. Walton, eds.): 271-306. Cambridge University Press.

White, T.J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenies. In *PCR protocols: A Guide to Methods and Amplifications* (M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White, eds.): 315-322. Academic Press Inc., San Diego, California, USA.

Wingfield, M.J., Kendrick, B. & Van Wyk, P.S. (1991) Analysis of conidium ontogeny in anamorphs of *Ophiostoma*: *Pesotum* and *Phialographium* are synonyms of *Graphium*. *Mycological Research* **95**: 1328-1333.

Zhou, X.D., De Beer, Z.W., Wingfield, B.D. & Wingfield, M.J. (2001) Ophiostomatoid fungi associated with three pine-infesting bark beetles in South Africa. *Sydowia* **53**: 290-300.

Zhou, X.D., De Beer, Z.W., Wingfield, B.D. & Wingfield, M.J. (2002) Infection sequence and pathogenicity of *Ophiostoma ips*, *Leptographium serpens* and *L. lundbergii* to pines in South Africa. *Fungal Diversity* **10**: 229-240.

Table 1. The three morphological groups of the *Graphium* and *Pesotum* species isolated from *S. parahybum*. Total number of isolates in each group is presented in parentheses, and numbers of isolates sequenced in this study are printed in bold type.

Morpho-logical group	Isolate numbers[a]	Colony colour		Fruiting structures	Characteristics of hyphal growth on MEA
		Reverse	Top		
Group 1	**CMW5564**; **CMW5573** (2)	white, center olivaceous buff (21'''d)	white, center olivaceous buff (21'''d)	dominant	suppressed growth, colony margin smooth
Group 2	**CMW5565**; CMW5567; **CMW5568**; CMW5575 (4)	buff (19''f)	white, center olivaceous buff (21'''d)	dominant	aerial mycelium present, colony margin smooth
Group 3	CMW5551; **CMW5554**-5556; CMW5558-5563; **CMW5566**; CMW5570-5572; CMW5574 (15)	buff (19''f)	greenish glaucous (33'''f), margin buff	sparse	suppressed growth, colony margin smooth

[a] CMW refers to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Table 2. Fungal isolates used in DNA sequence comparisons. Numbers of isolates sequenced in this study are printed in bold type and those used in pathogenicity trials are underlined.

Species	Culture no.	Host	Origin	GenBank no.	
				ITS	SSU
Ceratocystis fimbriata	[a]C89	-	-	-	U32418
Chaetomium elatum	[b]UCB81-063	-	-	-	M83257
Graphium penicillioides	[c]**CMW5566**	*S. parahybum*	Ecuador	-	AY351895
	CMW5554	*S. parahybum*	Ecuador	-	AY351894
	[d]CBS506.86	*Ulmus procera*	UK	-	AB007652
	CBS320.72	forest soil	Solomon Islands	-	AB007653
	CBS470.71	*Fagus sylvatica*	Germany	-	AB007681
	CBS781.85	*Orthotomicus erosus*	South Africa	-	AB007682
	[e]JCM9301	*Salix* sp.	Netherlands	-	AB007654
G. putredinis	JCM7866	grass	Japan	-	AB007683
Microascus cirrosus	[f]UAMH963	-	-	-	M89994
Neurospora crassa	Unknown	-	-	-	X4971
Ophiostoma ainoae	JCM9356	*Ips typographus*	Japan	-	AB007665
O. bicolor	JCM9358	*I. typographus*	Japan	-	AB007666
O. cucullatum	JCM8815	*I. typographus*	Japan	-	AB007664
O. europhioides	JCM9360	*I. typographus*	Japan	-	AB007667
O. penicillatum	JCM9362	*Picea jezoensis*	Japan	-	AB007668
O. piceae	CMW2468	*P. abies*	France	AF493240	-
	CMW7644	*P. abies*	Austria	AF493245	-
	CMW7646	*P. abies*	Poland	AF493247	-
	CMW7648	*P. sitchensis*	UK	AF493249	-
	JCM6016	*Betula platyphylla*	Japan	-	AB007663
O. quercus	CMW2463	*Fagus sylvatica*	France	AF493239	-
	CMW7645	*Quercus robur*	Austria	AF493246	-
	CMW7647	*Q. robur*	Poland	AF493248	-
	CMW7650	*Quercus* sp.	UK	AF198238	-
	CMW5565	*S. parahybum*	Ecuador	AY351899	AY351901
	CMW5568	*S. parahybum*	Ecuador	AY351900	AY351896
O. stenoceras	UCB57-013	-	-	-	M85054
O. ulmi	[g]MH75	-	-	U23424	-
	[h]ATCC32437	*U. americana*	USA	-	M83261
Pesotum fragrans	CBS 219.83	*P. abies*	Norway	-	AB007656
Pesotum sp.	**CMW5573**	*S. parahybum*	Ecuador	-	AY351897
	CMW5564	*S. parahybum*	Ecuador	-	AY351898
Podospora anserina	Unknown	-	-	-	X54864
Pseudallescheria boydii	Unknown	-	-	-	U43915
Sporothrix schenckii	ATCC14284	human	USA	-	M85053

[a] C = Culture Collection of T.C. Harrington, Department of Plant Pathology, Iowa State University, Iowa, USA. [b] UCB = Culture Collection of University of California Berkeley, CA, USA. [c] CMW = Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. [d] CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands. [e] JCM = Japanese Collection of Microorganisms, RIKEN (The Institute of Physical and Chemical Research), Saitama, Japan. [f] UAMH = University of Alberta Microfungus Collection and Herbarium, Edmonton, Alberta, Canada. [g] MH = Culture Collection of M. Hubbes, Faculty of Forestry, University of Toronto, Ontario, Canada. [h] ATCC = American Type Culture Collection, Manassas, VA, USA.

Figure 1. One of the most parsimonious phylogenetic trees produced by a heuristic search of the SSU sequence data. *Neurospora crassa* was used as outgroup taxon. Bootstrap values were derived from 1000 samples and are indicated above the branches of the tree. Length of the tree = 238 steps, CI = 0.664, RI = 0.891, RC=0.592 and g1= -0.57.

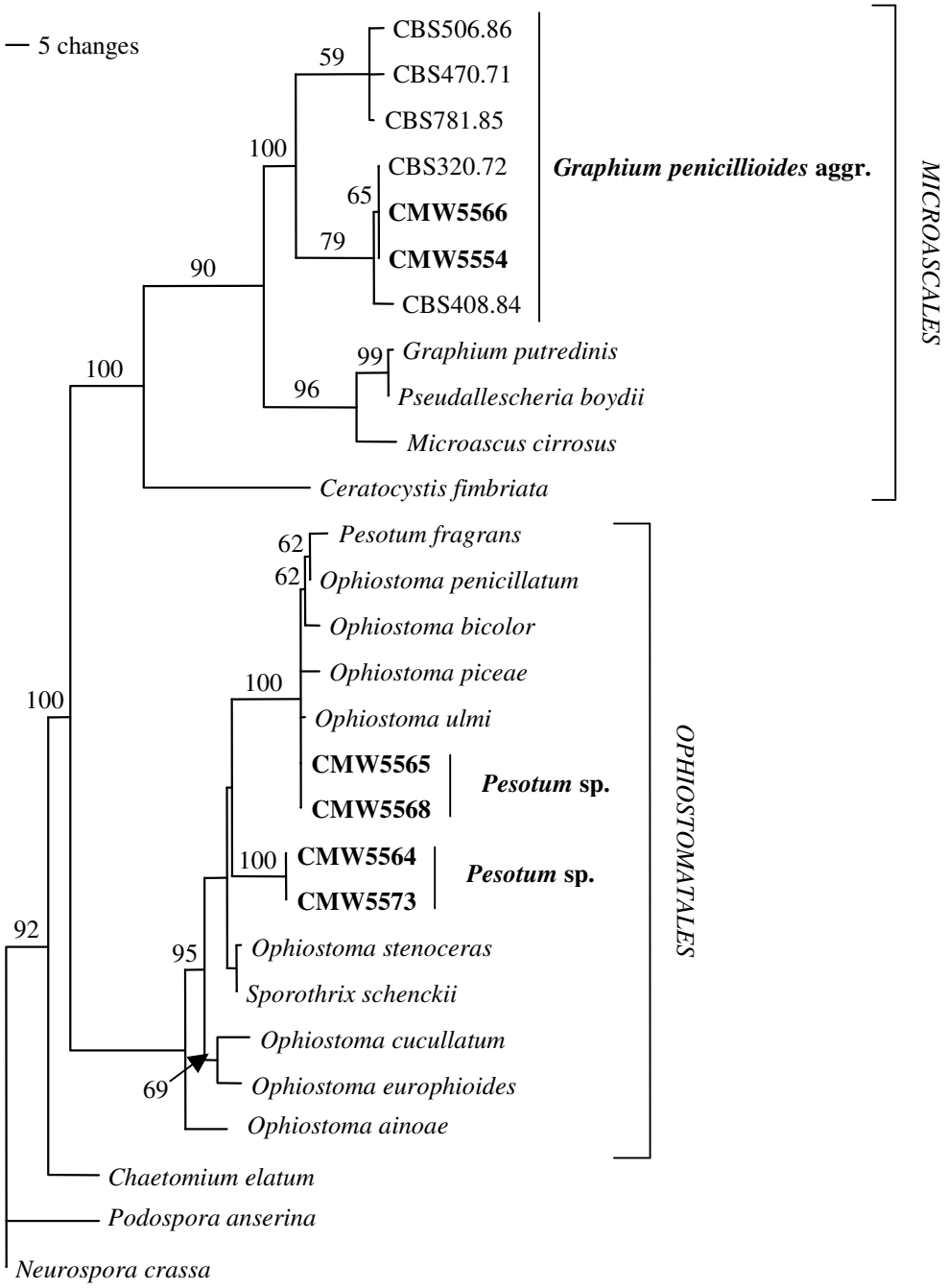


Figure 2. One of the most parsimonious phylogenetic trees constructed from ITS sequence data. A heuristic search was done with *Ophiostoma ulmi* as outgroup taxon. Bootstrap values (1000 replications) are indicated above the tree branches. Length of the tree = 24 steps, CI = 0.958, RI = 0.984, RC=0.943 and g1 = -0.934.

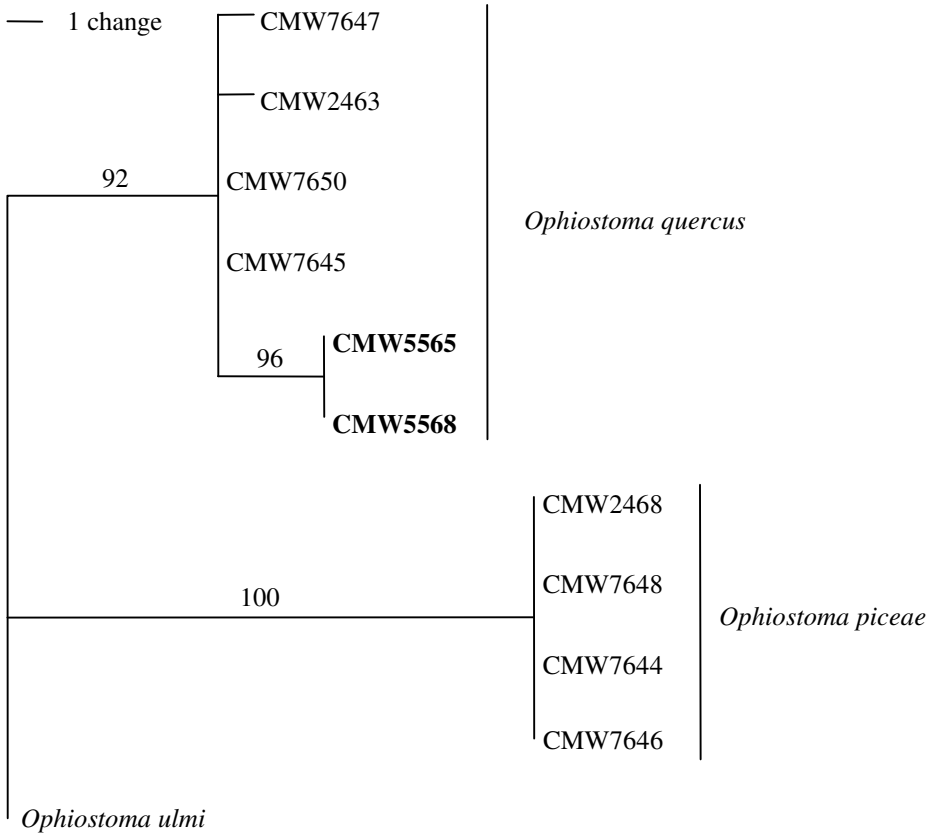
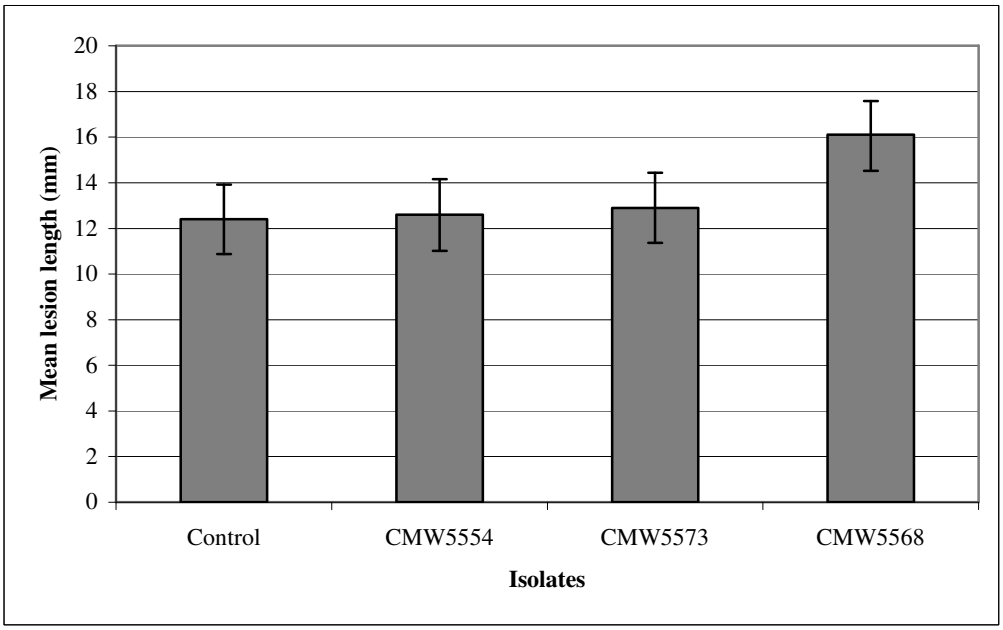

1 change
CMW7647
CMW2463
92
CMW7650
CMW7645
96
CMW5565
CMW5568
Ophiostoma quercus
CMW2468
CMW7648
100
CMW7644
CMW7646
Ophiostoma piceae
Ophiostoma ulmi

Figure 3. Mean lesion length after inoculation of *Schizolobium parahybum* with *Graphium penicillioides* (CMW 5554), the *Pesotum* sp. (CMW 5573) and *Ophiostoma quercus* (CMW 5568). Bars represent mean (Least Squares Means) lesion lengths for each isolate. Lesion length caused by *O. quercus* differed significantly from the control (P=0.0031), while *G. penicillioides* and the *Pesotum* sp. showed no significant differences.


20
18
16
14
12
10
8
6
4
2
0
Mean lesion length (mm)
Control
CMW5554
CMW5573
CMW5568
Isolates

Chapter 4

Development of polymorphic markers for the root pathogen *Thielaviopsis basicola* using ISSR-PCR*

Thielaviopsis basicola is a soil-borne fungal pathogen affecting many important agricultural crops. Little is known regarding the population biology or origin of this pathogen. Polymorphic markers developed for *Ceratocystis fimbriata*, a species complex phylogenetically closely related to *T. basicola*, were tested and found not to be useful for *T. basicola*. In this study 14 primer pairs, seven of which resulted in the amplification of single polymorphic fragments in *T. basicola* were developed. These primers will enable further studies on this economically important pathogen, and will result in an enhanced understanding of its population structure in different parts of the world.

* This chapter was published as: Geldenhuis, M.M., Roux, J., Wingfield, M.J. & Wingfield, B.D. (2004) Development of polymorphic markers for the root pathogen *Thielaviopsis basicola* using ISSR-PCR. *Molecular Ecology Notes* **4**: 547-550.

Thielaviopsis basicola is a soil-borne plant pathogenic fungus that is found in many parts of the world (Nag Raj & Kendrick 1975). It causes serious root diseases on a wide range of economically important crop plants including cotton, beans, carrots and tobacco. In some cases, it is one of the most important constraints to production (Yarwood 1981).

Very little is known regarding the origin or genetic diversity of *T. basicola*. This is partially due to a lack of appropriate tools to assess these characteristics. In this regard, co-dominant molecular markers have proved to produce highly reliable markers, providing information regarding the origin, spread and probable success of pathogen management practices. The aim of this study was to develop appropriate markers that can be used to gain an enhanced understanding of the population biology of *T. basicola*.

Thielaviopsis basicola is phylogenetically closely related to the important canker and wilt pathogen *Ceratocystis fimbriata* (Paulin & Harrington 2000). Microsatellite primers recently developed for *C. fimbriata* (Barnes *et al*. 2001a; Table 2) were tested using DNA extracted from two *T. basicola* isolates (CMW 5463, CMW 4098). These primers were AG 1/2, AG 7/8, AG 15/16, AG 17/18, CF 11/12, CF 15/16, CF 21/22 and CF 23/24. PCR mixtures and reaction conditions were the same as those described by Barnes *et al*. (2001a). In addition to the specific annealing temperature for each primer pair described by Barnes *et al*. (2001a; Table 2), temperatures two degrees below and above the specific annealing temperature were also tested. None of these primers successfully amplified DNA for either of the *T. basicola* isolates.

In order to develop co-dominant polymorphic markers for *T. basicola*, the internal-short sequence repeat (ISSR)-PCR technique (Van der Nest *et al*. 2000, Burgess *et al*. 2001) was used. Two isolates of *T. basicola* from South Africa (CMW 5482, CMW 5528) and two from Ecuador (CMW 4098, CMW 4457) were used for marker development. These isolates and the developed markers were subsequently compared with *T. basicola* isolates from different hosts and parts of the world (Table 1). DNA was extracted from all fungal isolates using the protocol described by Barnes *et al*. (2001b). All isolates used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

ISSR-PCR was performed on a South African *T. basicola* isolate (CMW 5482) using seven primers, namely 5' DDB(CCA)$_5$, 5' DHB(CGA) $_5$, 5' NDB(CA) $_7$C, 5' YHY(GT) $_5$G, 5' DBD(CAC) $_5$, 5' (CAT) $_5$, and 5' NDV(CT)$_5$ following the approach of Barnes *et al*. (2001a) except that an annealing temperature of 49°C was used. The resulting amplicons were cloned, colonies screened for inserts of suitable size and these were then sequenced. Inserts were sequenced with T7 and SP6 using an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq®DNA Polymerase, FS (Perkin-Elmer, Warrington, U.K.) following the manufacturers protocols, on an ABI Prism 377 DNA sequencer. Sequences were screened for tandem repeats (n>2) and primers designed to flank these regions. No perfect tandem repeats of longer than 8 repeats were found.

Fourteen primer pairs were designed to flank microsatellite-like regions. These were tested on the two South African and two Ecuadorian isolates (Table 1). PCR reactions were carried out in a total volume of 50 µl on a HYBAID thermocycler (Teddington, UK). The PCR mix included 2 ng DNA template, Expand HF buffer containing 1.5 mM $MgCl_2$ (supplied with the enzyme), 0.2 µM of each primer, 200 µM of each dNTP and *Taq* Expand™ High Fidelity polymerase mixture (1.75 U) (Roche). Reaction conditions were the same as those described by Burgess *et al*. (2001). Specific annealing temperatures were used for each primer pair (Table 2). The PCR products were separated using PAGE (6% polyacrylamide in 50 mM TBE buffer for 7h at 140V) and visualized by silver staining (Blum *et al*. 1987). Five of the primer pairs produced multiple bands, 2 primer pairs were monomorphic and the remaining 7 primer pairs produced one band that was polymorphic for isolates from South Africa and Ecuador (Table 2).

One primer from each polymorphic primer pair was labeled with the phosphoramidite fluorescent dyes FAM or TET (MWG) (Table 2). The same PCR reactions and conditions described above were used with the labeled primers to amplify all isolates (Table 1). Differences in product size were determined, relative to the internal size standard (TAMARA) by separating the labelled PCR products using PAGE on an ABI Prism 377 DNA sequencer. Analyses were done using GeneScan® 2.1 (Perkin-Elmer Corp.) and Genotyper® (Perkin-Elmer Corp.).

For the 16 *T. basicola* isolates from 8 different countries, 11 genotypes and 36 alleles could be detected across the 7 loci (Table 1). The number of alleles per locus ranged from

3 to 9. Each isolate had a different genotype except for the two South African isolates that had the same genotype. Five of the Ecuador isolates also had the same genotype, resulting in 4 genotypes out of the 8 isolates from carrots. The different genotypes, observed from only a few isolates from a single host, suggest that there is some degree of diversity in Ecuador.

In this study the ISSR-PCR technique was used to successfully develop seven co-dominant polymorphic markers for the important root pathogen *T. basicola*. The results suggest a different genetic composition for different geographical regions. This indicates that the markers will be valuable in assessing diversity and spread of the pathogen within countries and between continents. Knowledge gained from the application of these markers should contribute to the development of improved management strategies to reduce the impact of *T. basicola*.

REFERENCES

Barnes, I., Gaur, A., Burgess, T., Roux, J., Wingfield, B.D. & Wingfield, M.J. (2001a) Microsatellite markers reflect intra-specific relationships between isolates of the vascular wilt pathogen, *Ceratocystis fimbriata*. *Molecular and Plant Pathology* **2**: 319-325.

Barnes, I., Roux, J., Wingfield, M.J., Coetzee, M.P. & Wingfield, B.D. (2001b) Characterisation of *Seiridium* spp. associated with cypress canker based on ß-tubulin and histone sequences. *Plant Disease* **85**: 317-321.

Blum, H., Beier, H. & Gross, H.J. (1987) Improved silverstaining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**: 93-99.

Burgess, T., Wingfield, M.J. & Wingfield, B.D. (2001) Simple sequence repeat (SSR) markers distinguish among morphotypes of *Sphaeropsis sapinea*. *Applied and Environmental Microbiology* **67**: 354-362.

Nag Raj, T.R. & Kendrick, B. (1975) *A monograph of* Chalara *and allied genera*. Wilfred Laurier University Press: Waterloo, Ontario, Canada. 200pp.

Paulin, A.E. & Harrington, T.C. (2000) Phylogenetic placement of anamorphic species of *Chalara* among *Ceratocystis* species and other ascomycetes. *Studies in Mycology* **45**: 209-222.

Van der Nest, M.A., Steenkamp, E.T., Wingfield, B.D. & Wingfield, M.J. (2000) Development of simple sequence repeat (SSR) markers in *Eucalyptus* from amplified inter-simple sequence repeats (ISSR). *Plant Breeding* **119**: 433-436.

Yarwood, C.E. (1981) The occurrence of *Chalara elegans*. *Mycologia* **73**: 524-530.

Table 1: Alleles (base pairs) observed at each of the seven loci for 16 *T. basicola* isolates from eight different countries.

Isolate number[1]	**Country**	**NG 3/4**	**NG 5/6**	**NG 13/14**	**NG 15/16**	**NG 17/18**	**NG 19/20**	**NG 21/22**
CMW 5482, CMW 5528	South Africa	435	451	304	385	341	324	378
CMW 4098, CMW 4100, CMW 4381, CMW 4685, CMW 4689	Ecuador	405	448	303	378	341	341	382
CMW 4684		405	449	304	378	341	341	382
CMW4686		395	433	300	378	346	332	385
CMW 4457		395	434	300	378	347	331	385
CMW 5451	USA	427	452	304	386	341	316	379
CMW 6714	Australia	396	445	301	378	342	341	385
CMW 5896	Uganda	408	445	301	378	341	331	385
CMW 7065	Netherlands	408	446	301	378	341	331	385
CMW 7067	Belgium	405	454	303	377	341	351	385
CMW 7070	Switzerland	435	451	304	385	341	316	378
Number of alleles		6	9	4	4	4	5	4

[1]CMW refers to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Table 2: Primer pairs designed for amplification of *Thielaviopsis basicola* sequence characterised amplified regions.

Primer pair	Primer sequence	Core sequence	Tm[a] (ºC)	Ta[b] (ºC)	GC %	Banding pattern	Genbank Accession no.
NG1	5' GCT GGT GGG CGG AGA ATG 3'	*A_2CTA_5*A4GA2GA8*$(GA_2GA)_2CA_2GA$*	60.5	62	66.7	Monomorphic	AY559430
NG2	5' GGA TGG CCA GGG CCC CTC 3'		65.1		77.8		
NG3[c]	5' GGC CCA GGC CAA AGG CAG 3'	*$(C_2AT)C_2AC(C_2AT)_4(C_2AC_2A_2T)_3C_2AC$*$(C_2T_2)_3$*$A_3CA_5$*	62.8	62	72.2	Polymorphic	AY559433
NG4	5' GCT ATC AAA GGG CAT GGC 3'		58.8		57.9		
NG5[c]	5' CCT TTG ATG TCT CCT CCT GTC 3'	*$CATC(CATA)_4$*$T_3CT_3C_3T_7GT_2(GCT)_3$*	59.8	64	52.4	Polymorphic	AY559434
NG6	5' CCT GAG TCG TCT GCT TGT GG 3'		61.4		60		
NG7	5' CCA GTC CTG ATT GAT CGC C 3'	Sequence rich in C and T repeats	58.8	60	57.9	Monomorphic	AY559440
NG8	5' GAG ATG GTC TAT GGC CGC 3'		58.2		61.1		
NG9	5' CCC ACC TGC CGA ACA ACG '3	Sequence rich in A repeats	60.5	60	66.7	Multiple bands	AY559441
NG10	5' CTG ACT CTG AAG CCC GTC 3'		58.2		61.1		
NG11	5' CTG TGA CGT CTG TAC GTC TC 3'	*$CT_2GT_2GCT(GT_2CT_2)_2GT_2$*	59.4	61	55	Multiple bands	AY559439
NG12	5' GAC GCC CAT GCC GGT GTC 3'		62.8		72.2		
NG13[d]	5' GGG GAC GCG ACT TAG TGC C 3'	*$A_2(GA)_4A_2(GA)_2$*	63.1	64	68.4	Polymorphic	AY559435
NG14	5' GTC CAG AAT CTG CCC TGA CG 3'		61.4		60		
NG15[d]	5' GCG AGT TTG CGG GAG TTT G 3'	*A_5*$A_5CGA_2GA_8$*$(GA)_4$*$(C_2AG_2)_2GAC(C_2AG_2)C_2A_2G_2A_2$	58.8	62	57.9	Polymorphic	AY559437
NG16	5' CGC TAC GCT GAG GGT CCC 3'	*	62.8		72.2		
NG17[c]	5' GGA GAA GCC TCG ATG TGT AG 3'	*$(T_2C)_2C(T_2C_2)T_4G_2(T_2C_2)_2T_2(CAT)_2$*	59.4	62	55	Polymorphic	AY559436
NG18	5' CCG CCA GGA TCA GCC GGG 3'		65.1		77.8		
NG19[d]	5' GGC CAG CAG AGC CCC AAG 3'	*$T_4A(T_2C)_2T_3CT_2C_2T_4$*$(CT)_2CACT(CA)_2$	62.8	62	72.2	Polymorphic	AY559432
NG20	5' CAA GAC TAC CAC GGC ACC G 3'	$(CT)_2$*$(CT)_4CA(CT)_3CACTCA(CT)_2CA$* $(TCTG)_2$*$TC_3)_2T_2CA_2C_3$*	61.0		63.2		
NG21[c]	5' GAA GAG CAA TCT ACA GTG CGC 3'	*$T_8CA_3CA_2GA_6$*$C_2T_8(CT)_2C_2(CT)(CCT)_2(CT_4)_2T_3$*	59.8	62	52.4	Polymorphic	AY559438
NG22	5' GCA GTC GAG GGA GCC TAA G 3'		61.0		63.2		
NG23	5' GAC TGC CCC GCC AAA CTC 3'	*$(CA)_4GA(CA)_3$*	60.5	60	66.7	Multiple bands	AY559442
NG24	5' GGT AGT CTG GGA TCT GGG 3'		58.2		61.1		
NG25	5' GGT GGA CAC GAG TGG CTC 3'	*$T(CT_5)_3T_8$*$(GA)_4$*$GT(CT)_4$*	60.5	62	66.7	Multiple bands	AY559443
NG26	5' GCC TGG CCT GTG CTG GTC 3'		62.8		72.2		
NG27	5' CGT CTA TTT GCT GCG GTA GC 3'	*$(GT)_7CT$*	59.4	62	55	Multiple bands	AY559431
NG28	5' GCT GCG CCA GCT GTG TGA G 3'		63.1		68.4		

[a]Tm = melting temperature
[b]Ta = annealing temperature
[c]primer labelled with FAM
[d]primer labelled with TET
*Variable length of sequence

Chapter 5

Clonality in South African isolates and evidence for European origin of the root pathogen, *Thielaviopsis basicola*

Thielaviopsis basicola is a soil-borne fungal pathogen with a wide host range and a cosmopolitan distribution. It causes disease on many agricultural crops and in South Africa, is the causal agent of black pod rot of groundnuts and black root-rot on chicory. Knowledge of the population diversity of *T. basicola* could provide valuable information regarding management strategies, the possible movement, origin, and reproductive strategies of the fungus. The objective of this study was to determine the population diversity of *T. basicola* isolates from groundnuts and chicory in South Africa using co-dominant polymorphic markers. These markers were also used to compare isolates from South Africa with those from other hosts and geographic regions. Seven loci revealed nine alleles and two genotypes, one on groundnut and one on chicory, differing at only two loci, in South Africa. *Thielaviopsis basicola* isolates from eight different countries and ten different hosts revealed seventeen genotypes across the seven loci, with 39 different alleles. The lack of diversity for the two South African host-related populations of isolates suggests that *T. basicola* was introduced into South Africa. Some evidence is provided for a European origin of the pathogen, possibly linked to trade in root crops.

INTRODUCTION

Thielaviopsis basicola (Berk. & Broome) Ferraris (syn. *Chalara elegans* Nag Raj & Kendrick) is a soil-borne fungus with a world-wide distribution (Nag Raj & Kendrick 1975). It has a wide host range and has been found on more than 137 plant genera (Yarwood 1981). *Thielaviopsis basicola* causes root rot disease on many agricultural crops including chicory (Prinsloo *et al*. 1991), bean (Tabachnik *et al*. 1979), cotton (Mathre *et al*. 1966), groundnut (Tabachnik *et al*. 1979), tobacco (Gayed 1972), and tomato (Koike & Henderson 1998) and is an important post harvest pathogen on fresh market carrots (Punja *et al*. 1992).

No sexual state has been observed for *T. basicola*, although DNA-based phylogenetic studies indicate that it resides with *Ceratocystis* Ellis & Halsted in the Microascales (Paulin-Mahady *et al*. 2002). The fungus reproduces via endoconidia and thick walled chlamydospores enabling it to survive in the soil for long periods of time (Nag Raj & Kendrick 1975). The pathogen adheres to root surfaces and can be transferred to new areas on seed or by insects (Labuschagne & Kotzé 1991, Stanghellini *et al*.1999). *Thielaviopsis basicola* is an important pathogen in South Africa where it causes black root rot of chicory (*Cichorium intybys* L.) and black pod rot of groundnuts (*Arachis hypogaea* L.) (Prinsloo 1980, Prinsloo *et al*. 1991).

Very little is known regarding the origin of *T. basicola* in South Africa, or elsewhere in the world. The objective of this study was to use co-dominant polymorphic markers recently developed for *T. basicola* (Geldenhuis *et al*. 2004) to study the population diversity of *T. basicola* from groundnuts and chicory in South Africa. We also used these markers to compare isolates representing the two South African populations with those available to us from other hosts and geographic origins.

MATERIALS AND METHODS

Fungal cultures and isolations

Thielaviopsis basicola isolates from groundnuts and chicory in South Africa were collected in several areas. Isolates from groundnuts were obtained from 205 groundnut samples randomly taken from a black pod rot field near Jan Kempdorp and an infested field in

Potchefstroom (Fig. 1). One hundred and forty five diseased plant samples were taken from Jan Kempdorp and 60 samples from Potchefstroom. The chicory isolates were from 50 diseased chicory plants from the chicory processing mill at Alexandria in the Eastern Cape Province (Fig. 1).

Isolations from groundnuts and chicory were done using carrots as bait (Moller & De Vay 1968). The diseased tissue was placed tightly between two surface sterilised carrot slices and incubated at 25 ºC in moist chambers. After 5-6 days, the carrots were examined for fungal growth and the groundnut pods were cracked open to find *T. basicola* sporulating on the kernels and hull tissue. Conidial masses were transferred to 2% malt extract agar (MEA) (20g/l malt extract and 20g/l agar, Biolab Diagnostics Ltd, Midrand, South Africa) containing streptomycin sulphate (0.4g/l) (SIGMA).

Single conidial cultures were made for all isolates used in the study. Conidia were scraped from the MEA surface with a needle and placed in 8 ml sterile distilled water. The spore suspension (1 ml) was transferred to and spread on the surface of 2% water agar plates (20g/l agar, Biolab). Excess water was removed and the plates were incubated at 25 ºC for 16h. Single germinating conidia were transferred to 2% MEA, incubated at 25 ºC and resulting cultures were stored at 4 ºC.

Isolates of *T. basicola* from other areas were from a wide variety of hosts, geographic areas and some were specifically from international culture collections (Table 1). All isolates used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa and representative South African isolates have been deposited in the culture collection of the Centraalbureau voor Schimmelcultures, Urecht, Netherlands.

Population genetic analyses

Isolates were transferred to fresh 2% MEA in Petri dishes and incubated at 25 ºC. After ten days DNA was extracted from the isolates using the protocol described by Barnes *et al*. (2001). Seven fluorescently labeled short sequence repeat (SSR) primers designed for *T. basicola* (Geldenhuis *et al*. 2004) were used in PCR amplification of all isolates (Table 1). The same PCR reaction mixtures and conditions were used as described by Geldenhuis *et al*. (2004).

Differences in PCR product sizes were determined by separating the fluorescently labeled PCR products using PAGE on an ABI Prism 377 DNA sequencer. Results were analyzed using the GeneScan® 2.1 program (Perkin-Elmer Corp.) and Genotyper® (Perkin-Elmer Corp.). Samples for GeneScan analysis were prepared as described by Burgess *et al.* (2001a).

The gene diversity of each locus of the South African population as a whole (both groundnut and chicory isolates) was calculated using the equation $H = 1-\Sigma_k x_k^2$ where H is the gene diversity and x_k is the frequency of the k^{th} allele (Nei 1973). The gene diversity of the population was then calculated by adding the gene diversities of each locus and dividing it by the number of loci. The gene diversity for the remaining isolates used in this study could not be determined due to the small sample sizes.

Representative isolates from the different hosts and geographic regions were selected for distance analysis. The total nucleotide length for each allele was used to calculate distances (D_{AD}) using the MICROSAT program (http://human.stanford.edu/microsat). The distance matrix obtained from these calculations was then analyzed with MEGA version 2.1 (Kumar *et al*. 2001), using the neighbour joining option to produce a distance tree (Fig. 2).

RESULTS

Fungal cultures and isolations

A total of 79 *T. basicola* isolates were obtained from diseased groundnuts and 15 isolates were obtained from chicory. Each of these isolates originated from a different plant. Fifty three groundnut isolates were obtained from Jan Kempdorp and 26 from Potchefstroom. All the chicory isolates originated from a random collection from different delivery trucks at the chicory processing factory.

Population genetic analyses

The seven fluorescently labeled polymorphic primers successfully amplified all of the isolates used in the study. Genescan analysis for the South African isolates revealed nine alleles across the seven loci (Table 2). Only two genotypes were observed for this population (Table 1). All the groundnut isolates represented the same genotype, with no

differences between those from the two different areas. The chicory isolates also represented a single genotype, but it was different to that from groundnut (Table1).

The worldwide collection of *T. basicola* isolates had 39 alleles across the seven loci (Table 2), resulting in 17 genotypes (Table 1). The number of alleles per locus ranged from four to ten. There were four genotypes from eight isolates from carrots in Ecuador, while five isolates from the Netherlands all had different genotypes. Only one genotype was observed for the nine Australian isolates and one for the three isolates from the USA. Single isolates from Switzerland and Belgium all represented different genotypes, and these were also different to those in all other countries tested (Table 1). No genotypes were shared between any of the non-South African isolates available for this study.

Genescan analysis revealed no variation within the groundnut and chicory isolates (Table 1). For this reason, no further analyses were performed on these two populations, separately. The gene diversity (H) of the South African *T. basicola* population (groundnut and chicory) was 0.077.

The distance tree compiled for all representative isolates revealed two distinct clades (Fig. 2). The first of these (Clade A) included three sub-clades one of which included all groundnut isolates. A second sub-clade included the chicory isolates together with one isolate from Switzerland and one from the Netherlands. The third sub-clade consisted of three isolates from lettuce in the USA and one isolate from the Netherlands. The second major clade (Clade B) included isolates from Ecuador, Australia, Uganda, the Netherlands, and Belgium. Most of the isolates in this clade originated from carrots.

DISCUSSION

Results of this study using co-dominant polymorphic markers have shown that the important root pathogen *T. basicola* in South Africa is represented by a genetically uniform population. Thus, a relatively large collection of isolates included only two different genotypes. Furthermore, these were relatively closely related and sub-divided based on their host of origin. These results provide strong evidence to suggest that *T. basicola* has been introduced into South Africa and a comparison with isolates from other parts of the world suggests that the fungus originated in Europe.

The gene diversity of the South African *T. basicola* population was very low when compared with that of other fungi (Goodwin *et al*. 1992, Wikler & Gordon 2000). Recently established populations would be expected to have low gene diversity, while a pathogen would typically have the highest gene diversity in its country of origin (Gordon *et al*.1996, McDonald 1997). Multiple introductions of a fungus into a country could also result in a high gene diversity (Burdon & Roelfs 1985, Correll *et al*.1992, Burgess *et al*. 2001b). The low gene diversity of *T. basicola* from South Africa in the present study supports the view that it has been introduced into this country and that there have been few introductions.

Two major clades emerged from distance analysis for the *T. basicola* isolates used in this study. One clade included groundnut and chicory isolates from South Africa residing together with isolates from the Netherlands, Switzerland, and the USA. Chicory isolates were especially close to isolates from the Netherlands and Switzerland. Although additional isolates from these countries would be required to clarify their relatedness to those from South Africa, intercontinental spread, probably originally from Europe seems likely to have occurred. Isolates from Ecuador, Australia, Uganda, the Netherlands, and Belgium resided in the second major clade. Isolates in this clade included those from carrots in Ecuador, Uganda, and Australia and it seems likely that they also originated in Europe.

The number of *T. basicola* isolates from plants other than groundnuts and chicory (South Africa), was low and insufficient to support statistical calculations relating to populations. However, the fact that four genotypes were present in a small collection of isolates from a single host (carrots) in Ecuador suggests a high level of genetic diversity in that country. Similarly, the fact that five isolates from the Netherlands all had different genotypes also indicates high levels of diversity in that country. These results could be misleading due to a small number of isolates, but in comparison to results from South Africa, they indicate clear trends. Furthermore, the fact that distance analysis shows isolates from Europe reside within both major clades together with isolates from countries such as Ecuador, suggest that the fungus is native to Europe and that it has been distributed to other countries. This could easily have occurred through the widespread distribution of bulbs and root crops from Europe to other parts of the world, over long periods of time.

The *T. basicola* isolates from groundnuts all represent the same genotype although they were collected from different areas. Jan Kempdorp and Potchefstroom are separated by a distance of ~300 km. This suggests that the pathogen was introduced to the different areas from the same source, probably from infected seed. Labuschagne & Kotzé (1991) demonstrated that black pod rot could be transmitted through contaminated seed and they found that even seed appearing healthy could be infected.

Our results based on distance analysis suggest that *T. basicola* has been subjected to host specialisation. Isolates residing in one of the two major clades represent those from carrots. In contrast, isolates from chicory, groundnuts, and lettuce all resided in the second major clade. The isolates from the Netherlands originated from different hosts, and they all represented a different genotype. This appears to represent host selection by genotypes. These results are consistent with those of Punja & Sun (1999) who showed, using RAPDs, that isolates from the same host and geographical region tend to group together. Evidence of some degree of host specificity in *T. basicola* has also been reported for isolates that are pathogenic to one host but not to another (Keller & Shanks 1955, Lloyd & Lockwood 1963).

The low diversity of the South African *T. basicola* population suggests that breeding for resistance to this pathogen will be easier than it would have been with a genetically diverse pathogen. Knowledge of the genetic structure of a pathogen provides information regarding the possible success of control measures. A population with a high diversity would more easily adapt to new environments and be more difficult to control (McDonald & McDermott 1993, McDonald 1997). Our results suggest resistance to *T. basicola* in South African groundnut and chicory cultivars is likely to be relatively durable. The markers that we have used in this study should also be helpful in assessing the population diversity of isolates on other crops and from other parts of the world.

REFERENCES

Barnes, I., Roux, J., Wingfield, M.J., Coetzee, M.P. & Wingfield, B.D. (2001) Characterisation of *Seiridium* spp. associated with cypress canker based on ß-tubulin and histone sequences. *Plant Disease* **85**: 317-321.

Burdon, J.J. & Roelfs, A.P. (1985) Isozyme and virulence variation in asexual reproducing populations of *Puccinia graminis* and *P. recondita* on wheat. *Phytopathology* **75**: 907-913.

Burgess, T., Wingfield, M.J. & Wingfield, B.D. (2001a) Simple sequence repeat markers distinguish among morphotypes of *Sphaeropsis sapinea*. *Applied and Environmental Microbiology* **67**: 354-362.

Burgess, T., Wingfield, M.J. & Wingfield, B.D. (2001b) Comparisons of genotypic diversity in native and introduced populations of *Sphaeropsis sapinea* isolated from *Pinus radiata*. *Mycological Research* **105**: 1331-1339.

Correll, J.C., Gordon, T.R. & McCain A.H. (1992) Genetic diversity in California and Florida populations of the pitch canker fungus *Fusarium subglutinans.* f. sp. *pini*. *Phytopathology* **82**: 415-420.

Gayed, S.K. (1972) Host range and persistence of *Thielaviopsis basicola* in tobacco soil. *Canadian Journal of Plant Science* **52**: 869-873.

Geldenhuis, M.M., Roux, J., Wingfield, B.D. & Wingfield. M.J. (2004) Development of polymorphic DNA markers for the root pathogen *Thielaviopsis basicola* using ISSR-PCR. *Molecular Ecology Notes* **4**: 547-550.

Goodwin, S.B., Spielman, L.J., Matuszak, J.M., Bergeron, S.N. & Fry, W.E. (1992) Clonal diversity and genetic differentiation of *Phytophthora infestans* populations in Northern and Central Mexico. *Phytopathology* **82**: 955-961.

Gordon, T.R., Storer, A.J. & Okamoto, D. (1996) Population structure of the pitch canker pathogen, *Fusarium subglutinans* f. sp. *pini*, in California. *Mycological Research* **100**: 850-854.

Keller, J.R. & Shanks, J.B. (1955) Poinsettia root rot. *Phytopathology* **45**: 552-558.

Koike, S.T. & Henderson, D.M. (1998) Black root rot, caused by *Thielaviopsis basicola*, on tomato transplants in California. *Plant Disease* **82**: 447 (Abstr.).

Kumar, S., Tamura, K., Jakobsen, I.B. & Nei, M. (2001) *Molecular evolutionary genetics analysis software*. Arizona State University, Tempe, Arizona, USA.

Labuschagne, N. & Kotzé, J.M. (1991) Incidence of *Chalara elegans* in groundnut seed samples and seed transmission of blackhull. *Plant Pathology* **40**: 639-642.

Lloyd, A.B. & Lockwood, J.L. (1963) Effect of soil temperature, host variety and fungus strain on *Thielaviopsis* root rot of peas. *Phytopathology* **53**: 329-331.

Mathre, D.E., Ravenscroft, A.V. & Garber, R.H. (1966) The role of *Thielaviopsis basicola* as a primary cause of yield reduction in cotton in California. *Phytopathology* **56**: 1213-1216.

McDonald, B.A. & McDermott, J.M. (1993) Population genetics of plant pathogenic fungi. *Bioscience* **43**: 311-319.

McDonald, B.A. (1997) The population genetics of fungi: Tools and techniques. *Phytopathology* **87**: 448-453.

Moller, W.J. & De Vay, J.E. (1968) Carrots as a species-selective isolation media for *Ceratocystis fimbriata*. *Phytopathology* **58**: 123-126.

Nag Raj, T.R. & Kendrick, B. (1975) *A monograph of* Chalara *and allied genera*. Wilfrid Laurier University Press, Waterloo.

Nei, M. (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Science* **70**: 3321-3323.

Paulin-Mahady, A.E., Harrington, T.C. & McNew, D. (2002) Phylogenetic and taxonomic evaluation of *Chalara*, *Chalaropsis*, and *Thielaviopsis* anamorphs associated with *Ceratocystis*. *Mycologia* **94**: 62-72.

Prinsloo, G.C. (1980) *Thielaviopsis basicola* associated with a pod disease of groundnuts in South Africa. *Phytophylactica* **12**: 25- 26.

Prinsloo, G.C., Baard, S.W. & Ferreira, J.F. (1991) Organisms associated with black root rot of chicory in South Africa. *Phytophylactica* **23**: 59-67.

Punja, Z.K., Chittaranjan, S. & Gaye, M. (1992) Development of black root rot caused by *Chalara elegans* of fresh market carrots. *Canadian Journal of Plant Pathology* **14**: 299-309.

Punja, Z.K. & Sun, L-J. (1999) Morphological and molecular characterisation of *Chalara elegans* (*Thielaviopsis basicola*), cause of black root rot on diverse plant species. *Canadian Journal of Botany* **77**: 1801-1812.

Stanghellini, M.E., Rasmussen, S.L. & Kim D.H. (1999) Aerial transmission of *Thielaviopsis basicola*, a pathogen of corn-salad, by adult shore flies. *Phytopathology* **89**: 476-479.

Tabachnik, M., Devay, J.E., Garber, R.H. & Wakeman, R.J. (1979) Influence of soil inoculum concentrations on host range and disease reactions caused by isolates of *Thielaviopsis basicola* and comparison of soil assay methods. *Phytopathology* **69**: 974-977.

Wikler, K. & Gordon T.R. (2000) An initial assessment of genetic relationships among populations of *Fusarium circinatum* in different parts of the world. *Canadian Journal of Botany* **78**: 709-717.

Yarwood, C. E. (1981) The occurrence of *Chalara elegans*. *Mycologia* **73**: 524-530.

Table 1: *Thielaviopsis basicola* isolates used in this study.

Isolate number[1]	**Alternative designation**[2]	**Country**	**Host**	**Number of isolates**	**Genotype profile**
CMW 5463-5541		South Africa	Groundnuts	79	2314671
CMW 7622-7633			Chicory	15	1314681
CMW 4098, CMW 4100, CMW 4381, CMW 4685, CMW 4689		Ecuador	Carrots	5	5213353
CMW 4684				1	5214363
CMW 4686				1	4231114
CMW 4457				1	3241124
CMW 5451-5453		USA	Lettuce	3	1414582
CMW 5896,		Uganda	Carrots	1	3212434
CMW 5916			Carrots	1	3212445
CMW 6714-6716, CMW 6718-6723		Australia	Carrots	9	5222234
CMW 7065	CBS 341.33	Netherlands	*Primula* sp.	1	3212444
CMW 7066	CBS 342.33		*Euphorbia pulcherrima*	1	1314681
CMW 7068	CBS 413.52		*Lathyrus odoratus*	1	1314582
CMW 7069	CBS 414.52		*Primula* sp.	1	3212441
CMW 7071	CBS 430.74		*Betula* sp.	1	32224(10)4
CMW 7067	CBS 487.48	Belgium	*Paphiopedilum* sp.	1	6113394
CMW 7070	CBS 150.67	Switzerland	*Nicotiana tabacum*	1	1314671

[1]CMW refers to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

[2]CBS refers to the culture collection of the Centraalbureau voor Schimmelcultures, Baarn & Delft, The Netherlands.

Table 2: Alleles observed at 7 loci for *Thielaviopsis basicola* isolates from South Africa (SA), Ecuador (ECU), USA, Uganda (UGA), Australia (AUS), the Netherlands (NET), Switzerland (SWI) and Belgium (BEL). Allele sizes are indicated in the number of base pairs.

Locus	Allele	SA	ECU	USA	UGA	AUS	NET	SWI	BEL
NG3/4	395	-	2	-	-	-	-	-	-
	396	-	-	-	-	9	-	-	-
	405	-	6	-	-	-	-	1	-
	408	-	-	-	2	-	3	-	-
	427	-	-	3	-	-	1	-	-
	435	94	-	-	-	-	1	-	1
NG5/6	433	-	1	-	-	-	-	-	-
	434	-	1	-	-	-	-	-	-
	445	-	-	-	1	9	-	-	-
	446	-	-	-	1	-	2	-	-
	448	-	5	-	-	-	-	-	-
	449	-	1	-	-	-	-	-	-
	451	79	-	-	-	-	-	-	1
	452	15	-	3	-	-	2	-	-
	454	-	-	-	-	-	-	1	-
	457	-	-	-	-	-	1	-	-
NG 13/14	300	-	2	-	-	-	-	-	-
	301	-	-	-	2	9	3	-	-
	303	-	5	-	-	-	-	1	-
	304	94	1	3	-	-	2	-	1
NG15/16	377	-	-	-	-	-	-	1	-
	378	-	8	-	2	9	3	-	-
	385	94	-	-	-	-	2	-	1
	386	-	-	3	-	-	-	-	-
NG17/18	341	94	6	3	2	-	4	1	1
	342	-	-	-	-	9	1	-	-
	346	-	1	-	-	-	-	-	-
	347	-	1	-	-	-	-	-	-
NG19/20	316	15	-	3	-		2	-	1
	324	79	-	-	-	-	-	-	-
	331	-	1	-	2	-	3	-	-
	332	-	1	-	-	-	-	-	-
	341	-	6	-	-	9	-	-	-
	351	-	-	-	-	-	-	1	-
NG21/22	378	94	-	-	-	-	2	-	1
	379	-	-	3	-	-	1	-	-
	382	-	6	-	-	-	-	-	-
	385	-	2	-	1	9	2	1	-
	392	-	-	-	1	-	-	-	-
TOTAL ISOLATES		94	8	3	2	9	5	1	1

Figure 1. The main groundnut and chicory producing areas in South Africa.

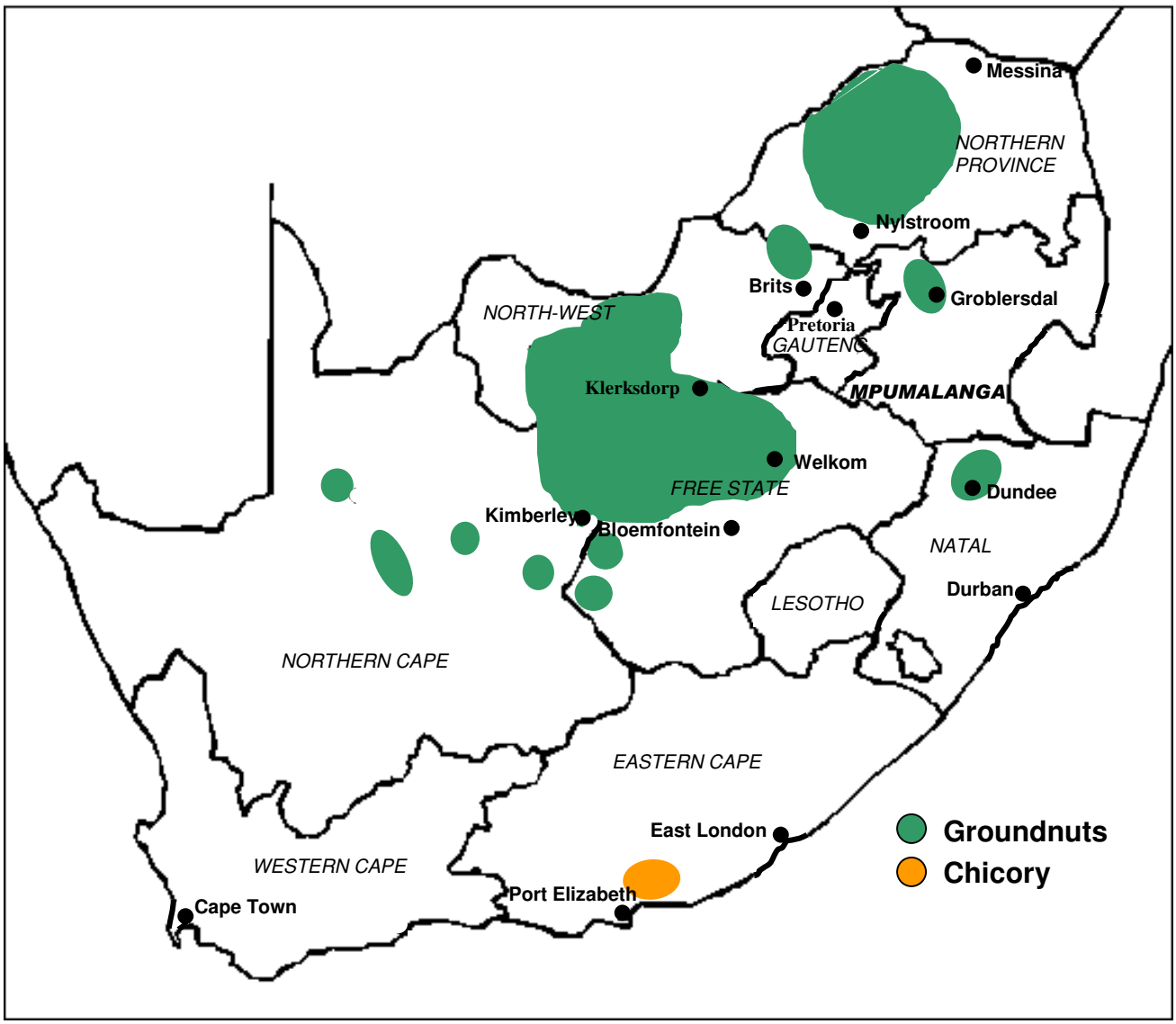
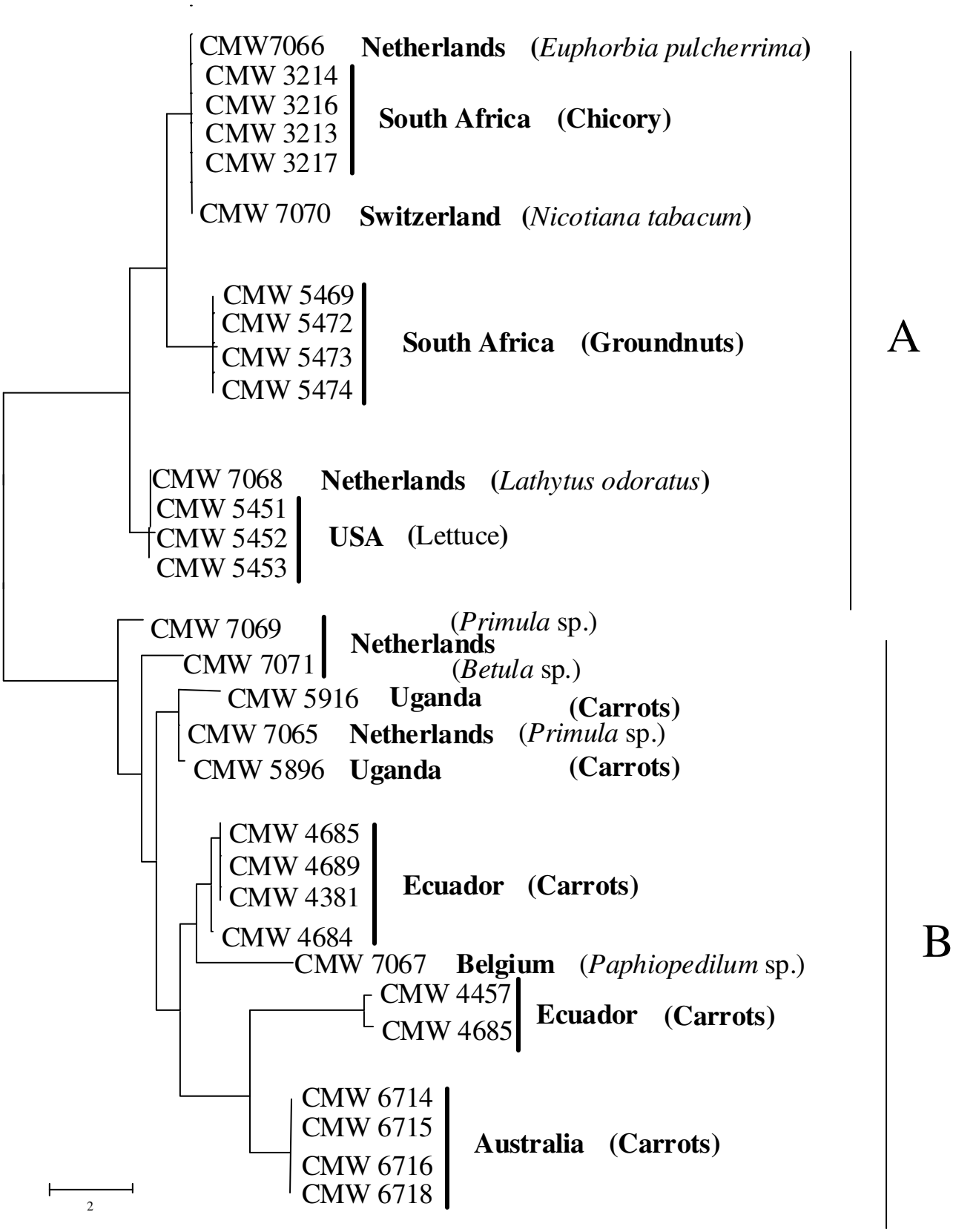

Messina
NORTHERN PROVINCE
Nylstroom
Brits
Groblersdal
NORTH-WEST
Pretoria
GAUTENG
Klerksdorp
MPUMALANGA
Welkom
FREE STATE
Dundee
Kimberley
Bloemfontein
NATAL
Durban
LESOTHO
NORTHERN CAPE
EASTERN CAPE
East London
WESTERN CAPE
Port Elizabeth
Cape Town
Groundnuts
Chicory

Figure 2. Isolates of *Thielaviopsis basicola* representing different hosts, countries and genotypes were used to compile a neighbour joining distance tree. The genetic distances were calculated using D_{AD} on total nucleotide length for each allele at the 7 loci.


CMW7066 Netherlands (Euphorbia pulcherrima)
CMW 3214
CMW 3216
CMW 3213
CMW 3217
South Africa (Chicory)
CMW 7070 Switzerland (Nicotiana tabacum)
CMW 5469
CMW 5472
CMW 5473
CMW 5474
South Africa (Groundnuts)
A
CMW 7068 Netherlands (Lathytus odoratus)
CMW 5451
CMW 5452
CMW 5453
USA (Lettuce)
CMW 7069
Netherlands (Primula sp.)
CMW 7071 (Betula sp.)
CMW 5916 Uganda (Carrots)
CMW 7065 Netherlands (Primula sp.)
CMW 5896 Uganda (Carrots)
CMW 4685
CMW 4689
CMW 4381
CMW 4684
Ecuador (Carrots)
CMW 7067 Belgium (Paphiopedilum sp.)
CMW 4457
CMW 4685
Ecuador (Carrots)
CMW 6714
CMW 6715
CMW 6716
CMW 6718
Australia (Carrots)
B
2

SUMMARY

Schizolobium parahybum is native to Ecuador, South America, where plantations of this tree is being established. Development of these plantations has been unsuccessful due to a serious die-back disease. A survey on the role fungal pathogens might play in disease development were conducted by isolating possible pathogenic fungi from streaks in the xylem and from machete wounds from dying trees. The primary aim of this study was to identify the isolated fungi and to determine their pathogenicity to *S. parahybum* through a series of greenhouse trials.

Ceratocystis fimbriata, *C. moniliformis*, *Thielaviopsis basicola*, *Graphium penicillioides*, *Ophiostoma quercus* and a *Pesotum* species were identified as possible pathogens of *S. parahybum*. Inoculation trials conducted with these fungi revealed that *C. fimbriata* and *C. moniliformis* were able to cause significant lesions on young *S. parahybum* trees under greenhouse conditions. These fungi were, however, not consistently isolated from diseased trees and the lesions in greenhouse trials were not consistently produced. *Graphium penicillioides*, *O. quercus* and the *Pesotum* species were unable to cause any notable lesions.

Thielaviopsis basicola caused lesions that differed significantly from the control inoculations of *S. parahybum*. These results were intriguing, as *T. basicola* is not a known tree pathogen, but is predominantly known to cause disease of agricultural crops such as groundnuts and chicory. The ability of *T. basicola* to cause lesions on *S. parahybum* initiated the second part of the thesis that dealt with a population diversity study of this pathogen. Comparison of *T. basicola* isolates from Ecuador and other parts of the world with isolates from groundnut and chicory in South Africa revealed that the Ecuador isolates did not originate from *S. parahybum* but from the carrots that was used to isolate them. Although *T. basicola* from carrots were able to cause lesions on *S. parahybum*, we did not investigate this phenomenon further as this fell beyond the scope of this thesis.

Seven polymorphic primers were developed for *T. basicola* using the ISSR-PCR technique. These primers were used to determine the population diversity of *T. basicola* from groundnuts and chicory populations in South Africa. Results showed a low diversity

for both populations and suggest that *T. basicola* was introduced to South Africa. The markers were also used to compare isolates from the groundnut and chicory populations with *T. basicola* isolates from other hosts and geographical regions. This indicated that *T. basicola* may be native to Europe from where it possibly spread to other countries.