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PREFACE

The genus *Ceratocystis* includes numerous plant pathogens, including devastating pathogens of hardwood tree species and wood staining agents. The pathogenic species are known to cause cankers, wilting and mortality of their hosts. The species causing vascular staining do not affect the host or wood structure, but reduces the value of the products produced. It is well documented that fungi in this genus require wounds for infection and are disseminated to these wounds by insects. Both the role that wounds as well as insects play in the biology and ecology of these fungi have been studied to some extent, however, no studies of this nature have been performed on these fungi in Africa.

Understanding the difficulties and short-comings in the taxonomy of the genus *Ceratocystis* is important for the accurate identification of previously undescribed species. Chapter one of this dissertation reviews the past problems experienced with the taxonomy of the genus *Ceratocystis*. This chapter further reviews the biology and ecology of the genus in general and focuses on the impact specific species within the genus has on the hosts they infect. An emphasis of this chapter is the association of species within the genus with insects. This section of the chapter reviews various groups of insects associated with *Ceratocystis* spp., adaptations of the fungi for the purpose of dispersal and the levels of dependence between the fungi and their insect associates.

Research relating to fungal pathogens of forestry crops in Africa has been neglected in the past. Chapter two of this thesis investigates the diversity of *Ceratocystis* spp. infecting wounds of hardwood species used in commercial forestry in Southern and Eastern African countries including South Africa, Tanzania, Kenya and Malawi. This chapter reports two known *Ceratocystis* spp., five previously undescribed *Ceratocystis* spp. and one undescribed *Thielaviopsis* sp. and investigates the pathogenicity of these fungi on their respective hosts.

Ceratocystis albifundus, the causal agent of wilt and canker of non-native *Acacia mearnsii*, has been reported to be one of the most serious pathogens of this tree in Africa. It has been hypothesised to be native to the African continent. This is based on the high genetic diversity of this fungus on non-native *A. mearnsii* in South Africa and Uganda and the fact that *C. albifundus* has only been reported from the African continent. This fungus also occurs on a number of native African tree species and does not seem to lead to disease on these hosts. Chapter three of this thesis investigates the population structure of this fungus from native as well as non-native hosts using microsatellite markers. It is the most extensive population study of this pathogen to date and provides interesting information on the possible origin of the fungus in Africa.

It has been well documented that *Ceratocystis* spp. are associated with insects such as nitidulid beetles and flies. However, no studies have been performed on the possible insects associated with *Ceratocystis* spp. in Africa. Knowledge of the insects and their biology could contribute to the formulation of management strategies to reduce the impact of disease in plantations. Chapter four of this dissertation reports on the identity of nitidulid beetles associated with *C. albifundus* in both a native ecosystem and non-native *A. mearnsii* plantation. This study also provides information regarding the effect climate has on the number of insects as well as the fluctuation of the number of fungal isolates obtained.

As *Ceratocystis* spp. require wounds for infection, numerous studies have been performed on factors relating to wounds that affect the infection success of *Ceratocystis* spp. However, most of these studies have been performed in the Northern Hemisphere. To date, no information is available regarding the influence of wound age and size on the infection success of *C. albifundus*. Chapter five investigates these factors on the infection success of *C. albifundus* on *A. mearnsii* in the Gauteng Province of South Africa. We also investigated the possible decrease of infection success of *C. albifundus* due to pre-inoculation of the wounds by the saprophytic fungus *Ophiostoma quercus* under field conditions.

Studies in this dissertation show the need to increase our knowledge pertaining to the fungal biodiversity within non-native forestry plantations as well as the native flora of

Africa. It indicates the importance of understanding the biology and ecology of these fungi and the need for research in this respect. It finally highlights the importance of understanding the interaction of native fungi on non-native flora and *visa versa*.

Chapter 1

Biology and ecology of Ceratocystis species with an emphasis on their insect associations

1.1. INTRODUCTION

The genus *Ceratocystis* Ell. & Halst. includes many pathogens of mostly woody and some herbaceous plant species, globally. These fungi result in a variety of disease symptoms, including branch and stem cankers, vascular staining, wilting and root disease, often leading to mortality of infected plants (Kile 1993). Some of the well known tree pathogens include *C. fagacearum* (Bretz) Hunt, the causal agent of oak wilt (Bretz 1952, Sinclair *et al*. 1987), *C. fimbriata sensu stricto* (*s.s*.) Ell. & Halst. that causes black rot of sweet potato (Halstead 1890, Engelbrecht & Harrington 2005), *C. albifundus* M. J. Wingf., De Beer & M. J. Morris the cause of Ceratocystis wilt of Australian wattle trees (Morris *et al*. 1993, Wingfield *et al.* 1996), *C. laricicola* Redfern & Minter infecting larch (Redfern *et al*., 1987), *C. polonica* (Siem) Moreau that results in blue stain of Norway spruce (Solheim 1986) and *C. platani* (Walter) Engelbrecht & Harrington which is the cause of an important canker stain disease of plane trees (Mook 1940, Walter 1946, Ferrari & Pichenot 1975, Tsopelas & Angelopoulos 2004, Engelbrecht & Harington 2005).

Ceratocystis spp. pose and increasing threat to plantation forestry based on hardwood species in the tropics and Southern Hemisphere. Whereas before the 1980's there were no reports of hardwood tree species being affected by *Ceratocystis* spp., a number of reports of such diseases have emerged in the last 20 years particularly form Africa (Wingfield 1990, Morris *et al*. 1993, Roux *et al.* 2000, Roux *et al.* 2001a,b, Roux *et al.* 2004b, Roux *et al.* 2005) and South America (Ribeiro *et al*. 1985, Barnes *et al*. 2003a, Rodas *et al*. 2008). These reports have included the description of previously unknown *Ceratocystis* spp., thus also illustrating the limited information currently available regarding the species diversity of this genus.

Information pertaining to *Ceratocystis* spp. in Africa is limited. Apart from the few records from plantations there are reports of these fungi from agricultural crops and indigenous plants (Kihurani *et al*. 2000, Crous *et al*. 2000). Studies in the last five years have resulted in a number of reports of *Ceratocystis* spp. from native trees in southern and eastern Africa, including the description of previously unknown species (Roux *et al.* 2005, 2007, Kamgan *et al*. 2008). This highlights the lack of information

regarding these fungi on the continent and emphasising the fact that many important pathogens still await description and study.

The taxonomic history of the genus *Ceratocystis* is complex and has been debated extensively in the past (De Hoog & Scheffer 1984, Upadyay 1993, Samuels 1993, Wingfield 1993). Numerous species concepts have been applied since the establishment of the genus and only with the use of the phylogenetic species concept have the most crucial issues been resolved. However, problems still persist for emerging groups now recognised to reside in the genus, as neither the morphological, nor the phylogenetic species concepts are sufficiently robust to delineate species with certainty (Engelbrecht & Harrington 2005, Wingfield *et al*. 2006). Currently, a combination of the morphological, biological and phylogenetic species concepts is necessary to delineate species (Engelbrecht & Harrington 2005, Wingfield *et al*. 2006).

Ceratocystis spp. have evolved several characteristics to ensure successful dispersal and infection of plants. These specifically include characteristics making them suitable for insect dispersal (Leach *et al*. 1934, Ingold 1961, Griffin 1968, Lanza & Palmer 1977). The insects either create wounds, or visit fresh wounds on plants, thus disseminating *Ceratocystis* spp. Species in the genus can have casual vectors such as nitidulid beetles (Coleoptera: Nitidulidae) (Jewell 1956, Moller & DeVay 1968, Harrington 1987) and flies (Griswold 1953, Moller & DeVay 1968, Hinds 1972) or mutualistic vectors such as bark beetles (Coleoptera: Scolytidae) (Kirschner 2001). Both the fungal and insect associates have a number of chemical and physical adaptations to facilitate the association between them (Leach *et al*. 1934, Ingold 1961, Lanza & Palmer 1977).

The aim of this review is to briefly summarise the taxonomic history of the genus *Ceratocystis*, to provide a review of knowledge pertaining to the biology of these fungi and most importantly to consider their symbiotic relationships with insects. Other important issues, such as the economic importance of these fungi and their requirement for wounds as infection sites are also highlighted using key examples. The intention here is to provide a foundation for the studies presented in the thesis that follows the review. The latter product focuses specifically on expanding the

knowledge base regarding species of *Ceratocystis* on non-native plantation tree species in Africa.

1.2. TAXONOMIC HISTORY OF THE GENUS *CERATOCYSTIS*

The taxonomic history of the genus *Ceratocystis* is complex and has encompassed many changes during the course of the past 120 years (Figure 1). The genus was first established by Ellis & Halsted in 1890 for the species *Ceratocystis fimbriata*, after it was found associated with black rot of sweet potato in the United States of America (USA) (Halsted 1890). During subsequent years, *C. fimbriata* was treated in many different genera, including *Ophiostoma* Sydow & Sydow, *Sphaeronema* Sacc., *Endoconidiophora* Münch, *Rostrella* Zimm. and *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr. (Upadhyay 1981).

Much of the confusion surrounding the taxonomy of *Ceratocystis* and similar genera was due to the similarity of morphological structures of these fungi. Fungi in the above mentioned genera typically all have long, beaked ascomata with spores produced at their apices in sticky masses. Ascospores are generally produced in evanescent asci (Elliot 1923). It is thus not surprising that today, morphology is not used as the only technique for species identification and new descriptions in these genera.

Numerous techniques and criteria have been applied to clarify the taxonomy of the genus *Ceratocystis*. One of the first techniques to be used was the separation of genera based on their associated anamorphs (Melin & Nannfeldt 1934, Bakshi 1951, Kendrick 1971, Nag Raj & Kendrick 1975, Upadhyay 1981). Another technique commonly applied was to distinguish between genera based on ascospore morphology (Bakshi 1951, Olchowecki & Reid 1974, Upadhyay & Kendrick 1975). Biochemical characteristics, such as the composition of the cell walls and sensitivity and tolerance to antibiotics, were also distinguishing factors for genera (Fergus 1956, Smith *et al.* 1967, Jewell 1974, Weijman & De Hoog 1975, Harrington 1981). Ultrastructural differences, for example, the development of teleomorph structures, types of cells present and the construction of the cell walls were also used to distinguish between genera in *Ceratocystis sensu lato* (Benny & Kimbrough 1980, Van Wyk *et al.* 1991,

Van Wyk *et al*. 1993). More recently, DNA sequence data have been utilized to distinguish between genera and species (Hausner *et al*. 1992, 1993a, b, c, Spatafora & Blackwell 1993, 1994, Wingfield *et al*. 1994, 1996, Witthuhn *et al*. 1998, 1999, Barnes *et al.* 2003a, Johnson *et al.* 2005, Van Wyk *et al.* 2004a, b, 2006). The latter approach that has applied the phylogenetic species complex has contributed substantially to resolving confusion regarding the taxonomic position of *Ceratocystis*.

The difficulty with the morphological description of *Ceratocystis* and the species accommodated in the genus is well illustrated from the first description of *Ceratocystis*. Halsted & Fairchild (1891), incorrectly identified the ascocarps of *C. fimbriata* as pycnidia and the ascospores as conidia. This was perhaps understandable given the fact that the asci delequese very early in the development of the ascomata. Saccardo (1892), unaware of the oversight of Ellis & Halsted, transferred *C. fimbriata* to *Sphaeronema* as *S. fimbriatum*. In 1918, *S. fimbriatum* was transferred to *Linostoma* (Fr.) Hohn., a genus split from *Ceratostomella* Sacc., based on its dark coloured perithecia with long necks and ovoid asci containing spores arranged in several rows (Von Höhnel 1918). However, the name *Linostoma* had previously been assigned to a genus of flowering plants in the *Thymeleacaeae* Juss., forcing Sydow and Sydow (1919) to establish the genus *Ophiostoma.* They relegated all the species previously in *Linostoma* to this newly established genus, separate from *Ceratostomella*.

Approximately three decades after *Ceratocystis* was first described, Elliott (1923), not accepting the changes made by Von Hönel (1918), found that the incorrectly identified pycnidia of *C. fimbriata* were in fact perithecia with ascospores emerging from deliquescent asci. He transferred *O. fimbriatum* to *Ceratostomella* (Elliot 1923). Later, Melin & Nannfeldt (1934), reduced the genus *Endoconidiophora*, which was established for species that formed conidia endogenously, to synonymy with *Ceratostomella*. They also transferred *Ceratostomella fimbriata*, *Ce. paradoxa* (Dade) Moreau and *Ce. adiposa* Butl. back to *Ophiostoma*, due to the fact that they had *Chalara* (Corda) Rabenh. conidial states (Melin & Nannfeldt 1934). Melin & Nannfeldt (1934), stated that the oldest genus name, *Endoconidiophora,* should be used, but this name would be confusing and thus they suggested the genus name *Ophiostoma,* until a more suitable name could be found.

Bakshi (1951), not accepting the taxonomic placement of *Ceratocystis* and other genera at that time, reduced *Ceratostomella, Linostoma*, *Ophiostoma* ,*Grosmannia, Rostrella* Zimmermann, *Ceratocystis* and *Endoconidiophora* to three, namely *Ceratostomella* (including only *Ceratostomella*), *Ophiostoma* (including *Linostoma*, *Ophiostoma* and *Grosmannia*) and *Ceratocystis* (including of *Rostrella*, *Ceratocystis* and *Endoconidiophora*). The type species, *O. fimbriatum,* was also transferred to *Ceratocystis* as *C. fimbriata* (Bakshi 1951). This caused considerable debate amongst mycologists working with these fungi with some authors not accepting this classification (Moreau 1952, Von Arx 1952, Von Arx & Műller 1954). Upadhyay (1981), chose to synonymise all the above genera and treated them as one genus, namely *Ceratocystis*. However, together with this genus he established *Ceratocystiopsis* based on the differences in the morphology between these fungi and *Ceratocystis* (Upadhyay & Kendrick 1975).

Species of *Ceratocystis* have been noted to have distinct differences based on morphology, physiology and molecular data when compared to *Ceratocystiopsis* and *Ophiostoma* (Smith *et al*. 1967, Weijman & De Hoog 1975, Harrington 1981, De Hoog & Scheffer 1984, Hausner *et al*. 1993a), while common characteristics are often shared between *Ceratocystiopsis* and *Ophiostoma* (De Hoog & Scheffer 1984, Hausner *et al*. 1993a, Wingfield 1993). Wingfield (1993), therefore synonymised the genus *Ophiostoma* with *Ceratocystiopsis*. The taxonomic debate was muted when Hausner *et al*. (1993a, b, c) utilized DNA sequence data for distinction between *Ceratocystis* and *Ophiostoma*.

The emergence of DNA-based techniques has had a significant impact on the taxonomic status of *Ceratocystis* and other fungi. These techniques, and particularly DNA sequence-based phylogenies, have shown clearly that *Ceratocystis* represents a very distinct genus and resides in a different order (Microascales) from the morphologically similar genera with which it had been confused in the past (Hausner *et al*. 1993a, b, c, Spatafora & Blackwell 1994). Several studies using DNA sequence data of the large sub-unit (LSU), ribosomal RNA (rRNA) (Hausner *et al*., 1993a,b, Wingfield *et al*. 1994) and the small sub-unit (SS) rRNA (SSrRNA) (Hausner *et al*.

1992, 1993a, b) have confirmed the uniqueness of the genus *Ceratocystis*. Recently, this was supported by Blackwell and co-authors (2006).

An important result of using DNA sequence data to elucidate the taxonomy of the genus *Ceratocystis* was the discovery by Witthuhn *et al*. (1998) that two distinct phylogenetic groups could be recognised within the genus. These two groups were referred to as the Fimbriata group and the Coerulescens group (Witthuhn *et al*. 1998). However, due to the similarity of some of the species at a single gene, multiple gene geneologies have become a necessity in studying *Ceratocystis* spp. (Van Wyk *et al.* 2004a, b, Engelbrecht & Harrington 2005, Johnson *et al*. 2005, Van Wyk *et al*. 2006, 2007a, b, Kamgan *et al*. 2008). Commonly used genes in multi gene phylogenies include, the Transcription Elongation Factor 1-α (EF1-α), the Internal Transcribed Spacer Region including the 5.8S rRNA operon (ITS), the Beta-tubulin gene regions and Mating type genes (Barnes *et al.* 2003a, Marin *et al*. 2003, Johnson *et al.* 2005, Van Wyk *et al.* 2004a, b, 2006, 2007a, b). Using multi gene phylogenies, it has been found that *Ceratocystis* in fact represent at least three distinct genera (Wingfield *et al.* 2006). However, this distinction has not yet been formalised and currently, *Ceratocystis* is best thought of as including three phylogenetic groups, namely the Fimbriata, Coerulescens and Moniliformis groups (Wingfield *et al*. 2006).

Within some of the groups in the genus *Ceratocystis*, the phylogenetic species concept is not sufficient to delineate between species. Due to this insufficiency, Engelbrecht & Harrington (2005), applied a combination of the morphological and biological species concepts to separate species within the *C. fimbriata s.l.* species complex. In the study, the authors separated *C. fimbriata s.s.* from other taxa in what has now become known as the *C. fimbriata s. l.* species complex. The complexity of the *C. fimbriata s.l.* group, however, requires considerable additional work and new species are being described within this complex annually. In future studies, a combination of the morphological, biological and phylogenetic species concepts will prove necessary to clarify the taxonomic status of species complexes in the genus *Ceratocystis*.

1.3. IMPORTANCE OF *CERATOCYSTIS* SPP.

The genus *Ceratocystis* includes both saprophytes and important primary plant pathogens of angiosperms and gymnosperms. The saprophytic fungi are usually known for their sap staining ability (Von Shrenck 1903, Kile 1993). Although these fungi do not have a detrimental effect on their hosts, they decrease the value of the timber products produced from them (Davidson 1935). The pathogenic species include fungi causing diseases of agronomic crops, fruit trees and timber trees including conifers and hardwood species (Kile 1993, Roux & Wingfield 2007). *Ceratocystis* spp. infect roots, stems and fruit, causing a range of symptoms from rot and canker to stains, wilt and death of their hosts. Species in the genus includes some of the best known tree pathogens including *C. fagacearum*, the cause of oak wilt in the USA (Bretz 1952, Sinclair *et al*. 1987), *C. cacaofunesta* Engelbrecht & Harrington the cause of canker disease of *Theobroma cacao* L. (cacao) (Desrosiers 1958, Malaguti 1952, Idrobo 1958, Goberdhan 1959, Havord 1962, Schieber 1969, Bezerra 1997, Delgado & Suarez 2003) and *C. platani*, the cause of canker of plane trees (Mook 1940, Walter 1946, Walter *et al*. 1952, McCracken & Burkhardt 1977, Panconesi 1981, Matasci & Gessler 1997, Panconesi 1999, Tsopelas & Angelopoulos 2004).

In the following section a number of the most important examples of *Ceratocystis* spp. causing diseases of agricultural crops and trees are highlighted. As the focus of this review is on *Ceratocystis* spp. and their role in forest species, we only present a short overview of some of the most important *Ceratocystis* spp. on agricultural plants and forestry species, with emphasis on examples of *Ceratocystis* spp. causing diseases of forest trees, to present some background for the rest of the chapters presented in this dissertation. For more information on diseases of agricultural crops reference can be made to publications by Harrington (2004). An extensive list of *Ceratocystis* spp. that are proven tree pathogens (Table 2) and those which are considered saprophytes and staining agents or for which no clear role as pathogens have been established (Table 3) in forests and plantations are presented at the end of this review.

1.3.1. *Ceratocystis* spp. infecting agricultural crops

Ceratocystis cacaofunesta

Ceratocystis cacaofunesta, previously part of the *C. fimbriata s.l* species complex,

was first reported infecting cacao (*Theobroma cacao*), as *C. fimbriata,* in 1918 from South America (Desrosiers 1958, Delgado & Suarez 2003) and has since then been recorded killing trees in numerous countries (Malaguti 1952, Idrobo 1958, Goberdhan 1959, Havord 1962, Schieber 1969, Bezerra 1997). Although *C. cacaofunesta* has had an impact on cacao production, the disease currently has little effect as it is managed by the use of resistant planting stock (Simmonds 1994).

Ceratocystis fimbriata s.s.

Ceratocystis fimbriata s.s. was the first *Ceratocystis* sp. reported to infect an agricultural crop. It was first reported causing black rot of sweet potatoes (*Ipomoea batatas* L.) in the USA in 1890 (Halsted 1890). One year after its first discovery, almost all sweet potato growers in the main sweet potato production area in the USA were affected by black rot (Halsted & Fairchild 1891). Although black rot of sweet potato had a destructive impact on the production of the crop, it is today relatively uncommon as a result of the implementation of integrated disease control (Kihurani *et al.* 2000).

Ceratocystis paradoxa

Ceratocystis paradoxa was first reported from pine-apple (*Ananas comosus* (L.) Merr.) in France in 1886 (De Seynes 1886). Since then, the fungus has been reported from coconut palm (*Cocos nucifera* L.) (Dade 1928), oil palm (*Elaeis guineensis*), date palm (*Arecu cathecu* L.) (Kile 1993) and sugar cane (*Saccharum officinarum* L.) (Lewton-Brain 1907, McMartin 1937, Chang & Jensen 1974). The disease occurs in the tropics (Ploetz *et al*. 2003) and has been reported from approximately 40 countries (Wismer 1961).

A number of modes of transmission have been reported for *C. paradoxa*. Numerous authors feel the most common mode of transmission is by means of insects. These include *Carpophilus hemipterus* L., *Urophorus humeralis* F., *Haptoncus ocularis* Fairmaire (Chang & Jensen 1974) and flies (Chi 1949). It has also been reported that the fungus can be dispersed by means of infected soil and cuttings, and via air

dispersal (Wismer 1961). Similar to a number of *Ceratocystis* spp., *C. paradoxa* requires wounds for infection. These wounds could be created by insects, rats and mechanical damage (Fawcett 1931, Wismer 1961, Chan & Jensen 1974) or silvicultural practices such as the preparation of cuttings (Wismer 1961).

Symptoms associated with pineapple disease include butt rot and leaf spot of pineapples and rot of pineapple fruits (De Seynes 1886, Ploetz *et al*. 2003), germination failure sugar cane seeds, rot of sugar cane cuttings and stems and death of pineapple stalks and wilting of leaves (Wismer 1961). The disease affects all sugar cane growing areas (Wismer 1961) and has been reported to lead to serious crop failure (McMartin 1944, Chi 1949, Antoine 1956).

Crop losses of between 20 and 80% have been reported (Dickson *et al*. 1931). To date, a number of control strategies have been developed for pineapple disease which include cultural techniques such as selection of cutting size and planting stock and seed treatment (Wismer 1961), selecting the correct planting time as well as the use of fungicides (Wakker & Went 1898, Ploetz *et al.* 2003).

1.3.2. *Ceratocystis* spp. infecting fruit-tree crops

Ceratocystis fimbriata s.l.

Numerous fungi that resembles *C. fimbriata* s.s. in morphology have been lumped together as *C. fimbriata s.l.* These fungi have been reported from a multitude of hosts and from most continents. Their impact on agricultural crops is significant, causing diseases such as canker of stone fruit (*Prunus* spp. L.) (DeVay *et al*. 1963), canker wilt and death of coffee (*Coffea* L.) (Obregon 1936, Pontis 1951, Szkolnik 1951, Echandi & Segall 1956) and citrus (*Citrus* L.) (Contreras & Marmelioz 1984, Instituto Colombiano Agropecuario 1993). The fungus has been reported to be the most serious pathogen of coffee and has affected approximately 800 000 hectares of coffee cultivating areas in South America (Castro 1998). *Ceratocystis fimbriata s.l.* infection of citrus has had a significant affect on the citrus industry. Since its report in 1981 to 1994, the disease had lead to the death of 10% of lemon trees in Colombia

(Mourichon 1994).

Ceratocystis manginecans

Ceratocystis manginecans M. van Wyk, A. Al Adawi & M. J. Wingf. was first reported as *C. fimbriata* in 2005, infecting mango trees in Oman (Van Wyk *et al*. 2005, Al Adawi *et al*. 2006). In 2007, the fungus infecting mango was re-described as a distinct species namely *C. manginecans* (Van Wyk *et al*. 2007a). This pathogen leads to gummosis as well as vascular discolouration, cankers and wilting of leaves (Van Wyk *et al*. 2005, Al Adawi *et al*. 2006). Tree mortality was observed to occur six months after the appearance of symptoms (Al Adawi *et al*. 2006).

1.3.3. *Ceratocystis* spp. as pathogens of forest and plantation trees

Ceratocystis albifundus

Ceratocystis albifundus, the causal agent of Ceratocystis wilt of Australian *A. mearnsii* in Africa, is considered one of the most important pathogens of non-native *A. mearnsii* trees on the continent (Morris *et al*. 1993, Roux & Wingfield 1997, Roux *et al*. 1999, Roux *et al*. 2005). Infection by *C. albifundus* leads to wilting and finally mortality of trees (Roux *et al*. 1999). This pathogen affects trees of all ages and is able to kill trees ao all ages under field conditions. The fungus has also been shown to lead to wilting and mortality of mature trees within six weeks after artificial inoculation (Roux *et al*. 1999). The fungus has also been reported on eight native host genera from the African continent (Roux *et al*. 2007). Although *C. albifundus* produces lesions after artificial inoculation on native tree species, mortality has not been observed in the native vegetation (Roux *et al*. 2007).

Ceratocystis fagacearum

Ceratocystis fagacearum causes oak wilt (Bretz 1952, Sinclair *et al*. 1987) of *Quercus* spp. L. in the USA. Other known hosts of this fungus are *Castanea mollissima* Blume, *C. sativa* Mill., *Lithocarpus densiflorus* (Hook. & Arn.) Rehd., *Castanopsis sempervirens* (Kellogg) Hjelmqvist and most genera in the Fagaceae (Bretz 1952). In 1944, a survey of oak trees in Wisconsin revealed that more than half the trees in a localised area of 40.4 ha had been killed by *C. fagacearum* (Rexrode & Brown 1983).

A second study investigating eight counties in Wisconsin reported that approximately 11% of the annual growth increase of oak forests was offset by mortality of the trees caused by oak wilt (Rexrode & Brown 1983).

Ceratocystis fagacearum seems to have a loose association with a number of insect species that are not known to be primary pests and are not able to create wounds on the host trees (Gibbs 1980, Juzwik & French 1983). These insects include *Carpophilus dimidiatus, Ca. sayi, Euporaea labilis* and *E. peltoides*. Transmission of *C. fagacearum* by nitidulid beetles is significant in overland spread of the fungus and the establishment of new infection centres (Cease & Juzwik 2001). The beetles are attracted to sporulating mats on recently killed oak trees, and after feeding on these mats, they are covered in fungal propagules which they subsequently spread to other trees (Juzwik & French 1983). These insects only disseminate the fungus to fresh wounds and do not seem to benefit from the association.

Ceratocystis fimbriata s.l.

Species in the *C. fimbriata s.l.* species complex have been reported to cause death of several plantation grown tree species. Hosts include rubber trees (*Hevea brasiliense* Muell.) (Olson & Martin 1949), *Eucalyptus* spp. (Roux *et al*. 2000) and *Acacia* spp. (Ribeiro *et al.* 1985). This fungus was first reported to cause disease and mortality of non-native hardwood tree species in the late 1980's when it was reported from *A. decurrens* Willd. in South America (Ribeiro *et al.* 1985). It was later reported from *Eucalyptus* spp. growing in the Republic of Congo (Roux *et al*. 2000), where infection leads to rapid wilting, discoloration of the xylem and mortality of trees ranging from six months to four years (Roux *et al*. 2000). Shortly thereafter, the fungus was also reported as the cause of disease of *Eucalyptus* spp. in Uganda and Uruguay (Roux *et al*. 2001a, Barnes *et al*. 2003b).

In 2004, *C. fimbriata s.l.* was reported to infect wounds of *Eucalyptus* spp. in South Africa (Roux *et al*. 2004b). It is, however, interesting to note that to date, *C. fimbriata s.l.* has not been reported to be associated with disease of *Eucalyptus* spp. in South Africa. This could be due to the influence of climate as the areas where *C. fimbriata s.l.* has been reported to cause disease of *Eucalyptus* spp., is more tropical than the areas where *C. fimbriata s.l.* has not been reported to cause disease. Another

explanation for the difference in the pathology of the fungus on these hosts in the different areas could be due to the presence of resistant plant material in the areas where the fungus does not lead to severe disease problems.

Ceratocystis fujiensis

Ceratocystis fujiensis M. J. Wingf., Yamaoka & Marin is a pathogen of *Larix kaempferi* (Lamb.) Carrière that has recently been described from Japan (Marin *et al*. 2005). This species was reported during a study of two closely related species, *C. polonica* and *C. laricicola* in Europe and Japan (Marin *et al*. 2005). *Ceratocystis fujiensis* is morphologically indistinguishable from *C. laricicola* but has been shown to be a distinct species based on DNA sequence comparison and ecological aspects. The ecological aspects include the insect associates of these two species, as *C. laricicola* is associated with *Ips cembrae* Heer, and *C. fujiensis* is associated with *I. subelongatus* Motsch. (Marin *et al*. 2005). *Ceratocystis fujiensis* is currently restricted to Asia and in association with its insect vector, is able to kill *Larix* spp., thus posing a substantial quarantine threat to forestry in the Northern Hemisphere (Marin *et al*. 2005) as neither *C. fujiensis* nor its associated insects occur in these regions.

Ceratocystis laricicola

Ceratocystis laricicola was first reported to infect European larch (*Larix decidua* Miller) in 1972 and is vectored by the larch bark beetle *I. cembrae* (Redfern *et al*. 1987). It is generally accepted that beetle attacks alone could in some cases lead to tree mortality, however, inoculation trials have shown that *C. laricicola* is an aggressive pathogen of larch and most probably an important component of tree death (Redfern *et al*. 1987). To date, the fungus has been reported from numerous countries including Europe (Pfeffer 1995), Scotland, Denmark (Crooke & Bevan 1957, Redfern *et al*. 1987, Stauffer *et al*. 2001) and Germany (Crooke & Bevan 1957, Redfern *et al*. 1987).

Ceratocystis pirilliformis

Ceratocystis pirilliformis was first reported colonising wounds on *Eucalyptus* spp. in Australia (Barnes *et al*. 2003a). Shortly after the report of this fungus from Australia, a study was performed to investigate *Ceratocystis* spp. infecting wounds of *Eucalyptus* spp. in South Africa. Similar to the study in Australia, *C. pirilliformis* was frequently isolated from wounds (Roux *et al*. 2004b, Kamgan *et al*. 2009). Although *C. pirilliformis* has not been associated with disease of *Eucalyptus* spp. under natural conditions, it was shown to be able to produce lesions after being inoculated onto *Eucalyptus* seedlings under greenhouse conditions and ~10cm diameter trees under field conditions (Roux *et al*. 2004b). In that study, it was also found that *C. pirilliformis* displayed the same levels of pathogenicity as *C. fimbriata* on *Eucalyptus* spp. (Roux *et al*. 2004b).

Ceratocystis polonica

Ceratocystis polonica, first reported from Poland in the 1930's, is an important insect-associated pathogen of *Picea* Dietrich spp. (Siemaszko 1939). It has been reported to cause severe damage to these trees in Norway (Christiansen & Bakke 1988) and destructive outbreaks during which millions of *Picea* trees were killed have been documented from North and Central Europe (Postner 1974, Christiansen & Bakke 1988). From 1985 to 1994, *C. polonica* and its insect associate, *Ips typographus* L., have been responsible for the loss of approximately 14.1 million cubic meters of *Picea* wood in Austria, Switzerland and Germany (Führer 1996). The most recent outbreak of *I. typographus* in central and Western Europe started around 1992 and has been reported to be triggered by adverse climatic conditions (Führer 1996, Kirisits 2001a).

It has been suggested that *I. typographus* is the primary cause of mortality of *P. abies* trees (Christiansen & Bakke 1988) and that after attack by the beetles, *C. polonica* establishes in the galleries of these insects. In order to overcome the biochemical and structural defence mechanisms of the trees, these beetles will mass attack the living trees, thereby causing mortality (Raffa & Klepzig 1992). Evidence has been provided to suggest that *I. typographus* is in fact not the primary cause of *P. abies* mortality, but that mortality is caused by *C. polonica*. Mass inoculation trials with *C. polonica* simulating attacks by *I. typographus* resulted in tree death (Horntveldt *et al*. 1983,

Christiansen 1985, Christiansen *et al*. 1987, Solheim 1988, Croise *et al*. 1998, Kirisits 1998, Krokene & Solheim 1998, Yamaoka *et al.* 2000, Kirisits & Offenthaler 2002).

1.3.4. Staining and/or saprophytic *Ceratocystis* spp. on forest trees

Saprophytic and sap stain fungi do not lead to tree mortality or negatively effect the wood structure, but does lead to the decrease in timber value. In the past, the term staining fungi was in some cases used to refer to fungi that are also pathogenic in nature and could lead to tree mortality (Gibbs 1993). Staining fungi has also been described as generalists (Seifert 1993) with more that one species occurring on a single piece of timber (Davidson 1935, Campbell 1960, Olchowecki & Reid 1974, Seifert 1993).

The discoloration caused by *Ceratocystis* spp. varies from blue, grey to black (Seifert 1993) and is due to the darkly pigmented hyphae that colonise the wood (Hartig 1878, Hedgcock 1906, Munch 1907, Croan & Highley 1995). Hyphae that cause the discolouration are concentrated in the parenchyma and resin ducts of colonised wood and often occur in the tracheids, but are in general unable to enter wood cell walls (Seifert 1993, Croan & Highley 1995). These *Ceratocystis* spp. do not lead to mortality or to structural damage of the timber (Boyce 1961, Chow 1983, Blanchette *et al*. 1992, Seifert 1993). Some of the effects staining fungi in the genus *Ceratocystis* have on wood include minor dry weight loss (Eslyn & Davidson 1976), minor strength loss (Seifert 1993) and toughness loss (Findlay & Pettifor 1937, Chapman & Scheffer 1940). The wood production quality damage caused by staining fungi have in the past been controlled by chemical treatment and the use of antagonistic fungi exhibiting antagonism targeted metabolites (Benko & Henningson 1986, Croan & Highley 1991, 1994, Hiratsuka *et al*. 1994).

Staining fungi in the genus of *Ceratocystis* are dispersed by numerous means. They are generally associated with insects and rely on them for dispersal (Leach *et al*. 1934, Leach 1940, Mathiesen 1950, Mathiesen-Käärik 1953). These associations have been noted to be either a close or a loose association (Gibbs 1993). Mathiesen-Käärik (1953), also stated that air dispersal is possible in species such as *C. coerulescens*. Dowding (1969) showed that air dispersal is possible as long as the air conditions were not to dry.

Ceratocystis coerulescens is common on *Pinus* spp. and *Piceae* spp. in Europe (Munch 1907, Bakshi 1950, Griffen 1968, Olchowecki & Reid 1974, Upadhyay 1981). It has been reported to be associated with a number of insects including *Ips schmutzenhofferi*, *Orthotomicus proximus* and *Pityogenus chacographus* (Mathiesen 1950, Mathiesen-Käärik 1953). *Ceratocystis coerulescens* commonly infects recently cut logs and broken roots (Seifert 1993) and has been referred to as a saprophyte or wound coloniser (Wingfield *et al*. 1997) and has not been reported to be a pathogen of *Pinus* or *Picea* spp. Interestingly, *C. coerulescens* has however been reported as a severe pathogen on a single host genus namely *Acer* spp. (Kile 1993).

1.4. WOUNDS AS INFECTION SITES FOR *CERATOCYSTIS* SPP.

It is documented that *Ceratocystis* spp. require wounds for infection (DeVay *et al*. 1963, Kile 1993). These wounds can originate from various sources including wind and hail damage, growth cracks, insect feeding, animals and human activities such as grafting, pruning and harvesting practices. Fresh wounds attract sap-feeding insects that may carry *Ceratocystis* spp. to these substrates and disperse ascospores from diseased to healthy hosts (Moller & DeVay 1968). Spores can also be spread to wounds in wind-borne frass (Iton 1960, Kile 1993).

A species in the *Ceratocystis fimbriata s.l.* species complex commonly infects peach trees wounded during harvesting of the fruit as the fruit is broken from the stem (DeVay *et al*. 1963). A species in this complex has also been reported to infect coffee plants in Colombia through stem wounds made by workers who support themselves against the stems of the plants to prevent slipping on the steep hills on which the coffee plants are grown (Marin *et al*. 2003). Pruning wounds are also common entry points for members of the *C. fimbriata s.l* species complex, and the fungus can be carried on machetes or other pruning tools (Walter 1946, Teviotdale & Harper 1991).

It has been reported that *C. platani* can infect trees via underground root grafts in perennial plants (Walter 1946, Kile 1993). This occurrence is common in areas where trees of the same species grow in close proximity and where root systems graft in

native ecosystems (Walter 1946, Kile 1993). This form of dispersal has also been reported for *C. fagacearum* in natural and urban forests (Accordi 1986, Kile 1993).

Natural forces commonly cause wounds that can be infected by *Ceratocystis* spp. Strong winds and hail are common sources of wounds. *Ceratocystis albifundus*, for example, has been shown to infect several native tree species after strong winds damaged stems and branches (Roux *et al*. 2007). Similarly, *C. albifundus* has also been isolated from hail and insect damage on *A. mearnsii* in South Africa (Roux & Wingfield 1997).

Insects such as bark beetles (Coleoptera: Scolytinae) make wounds that facilitate the infection of host trees by *Ceratocystis* spp. Beetles bore through the bark to excavate egg galleries in the underlying phloem. During this process, the wound is directly inoculated with fungi (Bramble & Holst 1940, Leach 1940). Examples of fungi that are specifically vectored by bark beetles include *C. laricicola* associated with *I. cembrae* (Redfern *et al*. 1987), *C. polonica* associated with the bark beetle *I. typographus* (Horntvedt 1988, Solheim 1986, Krokene & Solheim 1996), *C. fujiensis* associated with *I. subelongatus* (Yamaoka *et al*. 1998), *C. fagacearum* associated with *Pseudopityophthorus minutissimus* Zim. (Ambourn *et al*., 2005) and *C. rufipennis* associated with *Dendroctonus rufipennis* Kirby (Harrington & Wingfield 1998).

Recently *C. atrox* M. van Wyk & M.J. Wingf. was reported from tunnels of the wood-boring insect *Phoracantha acanthocera* Macleay (Cerambicydae: Coleoptera) (Macleay) (Van Wyk *et al*. 2007b). These, insects, although not necessarily associated with specific *Ceratocystis* spp., greatly assist these fungi in infection of suitable hosts through the wounds that they create. Another example of this is *C. polychroma* associated with *Hexamitodora semivelutina* Hell. (Coleoptera: Cerambycidae) on *Syzygium aromaticum* L. Merr. & Perry (Van Wyk *et al*. 2004a).

It has been well documented that *Ceratocystis* spp. infect artificially made wounds on trees. In this regard, two species, *C. eucalypti* and *C. pirilliformis* were first discovered in trials where *Eucalyptus* spp. in Australia had been intentionally wounded (Kile 1996, Barnes *et al*. 2003). Similarly, *C. fimbriata s.l.* and *C. moniliformis* are well-known from wounds on *Eucalyptus* spp. in South Africa (Roux

et al. 2004b). The use of artificial wounds has in recent years been used widely to collect *Ceratocystis* spp. from trees on which they do not necessarily cause disease. Two studies investigating *Ceratocystis* spp. infecting medicinal bark harvesting wounds on native tree species in Africa, isolated *C. albifundus* (Roux *et al*., 2004a), and two previously undescribed species, *C. savannae* Kamgan & Jol. Roux and *C. tsitsikammensis* Kamgan & Jol. Roux (Kamgan *et al*. 2008). Likewise, a study in Colombia yielded a previously undescribed species, *C. neglecta* M. van Wyk, Jol. Roux & C. Rodas from artificially made wounds on *Eucalyptus* spp. (Rodas *et al*. 2008). A number of other wounding studies are underway in various parts of the world and these will most likely yield numerous other undescribed species from hosts and countries not previously surveyed.

Successful infection of wounds by *Ceratocystis* spp. is dependant on a number of physical and environmental factors. Species in the *C. fimbriata s.l.* species complex, for example, are able to infect their hosts when viable fungal propagules are deposited onto relatively superficial bark wounds (DeVay *et al.* 1968). In contrast, *C. fagacearum* can only infect the host tree if viable fungal propagules come into contact with freshly exposed wood (xylem) of the host (Kuntz & Drake 1957).

Temporal factors have been shown to affect the success of infection by *Ceratocystis* spp. (Bostock & Middleton 1987, Biggs 1989, Teviotdale & Harper 1991). Numerous studies have shown that *C. fagacearum* could not cause infection when wounds were older than 24 hours (Morris *et al*. 1955, Kuntz & Drake 1957, Gibbs 1980). It has been reported that this loss of susceptibility of wounds over time could be attributed to the loss of the thin film of moisture present on fresh wounds, along with the formation of a periderm after wounding. Biggs (1989), attributed the increase in resistance of wounds to infection over time to the accumulation of suberin. Wound infection by other microorganisms has been shown to influence infection success of pathogens. It has been reported that the colonization of wounds by the saprophytic fungus, *Ophiostoma piceae* (Munch) H. Sydow & Sydow, prior to the artificial inoculation of the wound with *C. fagacearum*, prevented colonization and infection by the pathogen (Gibbs 1980). A similar influence has been observed for bacteria with the inhibition of *Botrytis cinerea* (De Bary) Whetzel by a bacterial species in the genus *Pseudomonas* Migula (Barka *et al*. 2002).

Climatic factors such as temperature and relative humidity also influence the germination of spores and infection of *Ceratocystis* spp. (Cole & Fergus 1956). In this study, the authors reported that fungi could survive at extremely low temperatures for a period of time under laboratory conditions, as spores withstood freezing at -10°C after 83 days (Cole & Fergus 1956). The authors also observed differences in the response of conidia compared to that of ascospores. The thermal death point of the ascospores (42°C - 44°C) were higher than that of the conidia (40°C - 42°C). They also found that relative humidity and temperature had a significant influence on the germination and survival of fungi.

In forestry operations, wounding of trees is common. In operations such as pruning, a fresh, open wound is created when the branches are cut or sawn off, or where double stems are reduced. Accidental wounds are also often created when timber is removed in thinning operations. Furthermore, timber is often infected by *Ceratocystis* spp. after harvesting, resulting in blue stain of the harvested product. This is especially common where timber is not debarked immediately, allowing insects and fungi to survive under the bark. Management operations that reduce the occurrence of wounds and that involve the speedy removal of bark could, therefore, reduce infection of timber by *Ceratocystis* spp.

1.5. INSECT ASSOCIATIONS WITH *CERATOCYSTIS* SPP.

It is a well established fact that *Ceratocystis* spp. are dispersed by arthropods (Sinclair *et al*. 1987). Hartig (1878), first recognised the interrelationship between insect damage, discolouration of wood and fungi during his study of blue-stain in the sapwood of conifers. Münch (1907, 1908), also observed that blue-stain in living trees and lumber is associated with attack by bark beetles. Since these reports, a number of studies have been compiled regarding various aspects of the association of fungi with bark beetles and numerous reports of such associations have been made for *Ceratocystis* spp. (Table 1).

Ceratocystis spp. have associations with three broad categories of insects. These categories are (1) Bark beetles (phloeophagous insects), (2) nitidulid beetles and (3)

more general insects (Harrington 1987, Kirisits 2004). These associations can be very specific, such as those with bark beetles, or general. In the following section, the various groups of insects associated with *Ceratocystis* spp. will be discussed and the different types of associations between *Ceratocystis* spp. and insects as well as the various adaptations by *Ceratocystis* spp. to facilitate these relationships will be considered.

1.5.1. Interdependence of insects and their associated *Ceratocystis* spp.

There is considerable debate regarding the interdependence of *Ceratocystis* spp. and their associated insects. A point of much debate is whether the fungus or the insect is the primary cause of tree death, or whether it is a combination of the two. Examples where the interdependence has been studied include *C. fagacearum*, *C. polonica* (Christiansen 1985, Solheim 1988, Kirisits 1998, Krokene & Solheim 1998, Yamaoka *et al.* 2000, Kirisits & Offenthaler 2002) and *C. laricicola* (Crooke & Bevan 1957, Redfern *et al*. 1987, Yamaoka *et al.* 1998, Stauffer *et al*. 2001, Kirisits 2001a, b).

The symbiosis involving *Ips typographus* and *C. polonica* aptly illustrates the differing opinions regarding the nature of the interaction between bark beetles and their fungal associates. It has been suggested that *I. typographus* is the primary cause of mortality of *P. abies* trees (Christiansen & Bakke 1988) and that after attack by the beetles, *C. polonica* establishes in the galleries of these insects. In order to overcome the biochemical and structural defence mechanisms of the trees, these beetles will mass attack the living trees, thereby causing mortality (Raffa & Klepzig 1992). On the other hand, evidence has been provided to suggest that *I. typographus* insects are in fact not the primary cause of *P. abies* mortality, but that mortality is caused by *C. polonica*. Experiments in which trees have been mass inoculated with *C. polonica*, to simulate the situation when trees are attacked by *I. typographus*, has resulted in considerable blue stain and also tree death (Horntveldt *et al*. 1983, Christiansen 1985, Christiansen *et al*. 1987, Solheim 1988, Croise *et al*. 1998, Kirisits 1998, Krokene & Solheim 1998, Yamaoka *et al.* 2000, Kirisits & Offenthaler 2002). It is thus hypothesised that *C. polonica* increases the effect of *I. typographus* infestation and makes the host more suitable for attack and reproduction of its insect vector (Whitney 1982, Harrington 1993, Paine *et al.* 1997).

Tree infesting insects and *Ceratocystis* spp. may exist in symbiotic relationships. *Ceratocystis* spp. are dependent on insects for dissemination, while the insects in some cases rely on these *Ceratocystis* spp. to lower the defences of the trees (Raffa & Klepzig 1992, Krokene 1996, Paine *et al*. 1997). An important pathogen, *C. laricicola* seems to have such an association with its insect symbiont, *I. cembrae* (Redfern *et al*. 1987, Yamaoka *et al.* 1998, Stauffer *et al*. 2001, Kirisits 2001b). *Ips cembrae* is regarded as a secondary pest of larch, and has played a significant role in the establishment of *C. laricicola* in Scotland, after it was introduced in the 1950's (Crooke & Bevan 1957, Redfern *et al.* 1987). The insect involved in the symbiosis benefits from the association in the sense that the fungus lowers the defence mechanisms of the host tree, thus allowing the beetles to colonise the trees (Redfern *et al*. 1987).

Insects have been reported to assist their associated fungi with "transport" as in the case of *C. fagacearum*, the causal agent of oak wilt (Bretz 1952, Sinclair *et al*. 1987). *Ceratocystis fagacearum* seems to have a loose association with a number of insect species (*Carpophilus dimidiatus, Ca. sayi, Euporaea* spp.) that are not known to be primary pests and are not able to create wounds on the host trees (Juzwik & French 1983). Therefore, the insects only transport/disseminate the fungus to fresh wounds, that act as suitable sites for infection by the fungus, and they do not seem to benefit from the association.

1.5.2. Adaptations of *Ceratocystis* spp. for the purpose of insect dispersal

Insect associated *Ceratocystis* spp. possess a number of adaptations to ensure the success of the symbiosis. One of these adaptations is the production of fruity odours that attract insects (Lanza & Palmer 1977). Not all *Ceratocystis* spp., however, produce fruity aromas. Species of *Ceratocystis* spp. that produce fruity odors include species in the *C. fimbriata s.l* species complex, *C. pirilliformis*, *C. fagacearum* and *C. moniliformis* Hedgcock (Mathiesen-Kräkik 1953, Kirschner 2001, Barnes *et al*., 2003a). Numerous studies have identified a number of monoterpenes to play a role in the production of these fruity odors. These are citronellol, geraniol, nerol, linalool, alphaterpiniol and neral (Lanza *et al.* 1976, Lanza & Palmer 1977).

The ascospores of *Ceratocystis* spp. are presented in slimy (mucilaginous) masses at the apices of long ascomatal necks (Ingold 1961). These long necks lift the spores of *Ceratocystis* spp. above competing fungi (Leach *et al*. 1934, Griffin 1968, Malloch & Blackwell 1993). Some species, however, do not produce long necks and have adapted by producing their spore masses in thread-like tendrils (Wingfield 1993). The slimy spore masses produced at the apices of long ascomatal necks increases the possibility for the spores to adhere to the bodies of insects due to an adhesive layer around the spores (Leach *et al.* 1934, Griffin 1968, Malloch & Blackwell 1993). Ascospore shape has also been reported to have an influence on the adhesion of these spores to the insects (Malloch & Blackwell 1993). It has been hypothesised that the concave shape of the spores produced by *Ceratocystis* spp., facilitates the spores coming into contact with the insects at more than one point, and that the numerous expanded contact areas could assist in the spores not being dislodged from the insect body during transmission (Malloch & Blackwell 1993). Spores also poses protective sheaths than enable them to be ingested by insects and pass unharmed through their intestinal tracts (Leach 1940, Moller & DeVay 1968).

1.5.3 Categories of insects associated with *Ceratocystis* spp.

1.5.3.1 Bark Beetles

Bark beetles (Coleoptera: Scolytidae) create wounds that facilitate infection by *Ceratocystis* spp. and assist in the dissemination of these fungi from one host to another. Beetles bore through the bark to excavate egg galleries in the underlying phloem. During this process, the wounds are directly inoculated with fungi carried on the exoskeleton of the beetles, as well as through spores in the digestive tracts of some beetles (Bramble & Holst 1940, Leach 1940).

Tree infecting *Ceratocystis* spp. associated with bark beetles include *C. laricicola, C. polonica, C. fujiensis* and *C. rufipenni* M. J. Wingf., T. C. Harr. & H. Solheim. *Ceratocystis laricicola* is specifically associated with *I. cembrae* in Europe (Redfern *et al*. 1987). Research has shown that *C. laricicola* is highly pathogenic to larch trees, lowering the defence mechanisms of the host tree and thus allowing the beetle to colonise the trees (Redfern *et al.* 1987). This fungus/beetle association has been well studied and is known from Europe (Pfeffer 1995), Scotland, Denmark (Crooke &

Bevan 1957, Redfern *et al*. 1987, Stauffer *et al*. 2001) and Germany (Crooke & Bevan 1957, Redfern *et al*. 1987).

Ceratocystis polonica is known to infect *Picea abies* (Bruegger) P. Schmidt and is associated with the bark beetle *I. typographus* (Horntvedt 1988, Solheim 1986, Krokene & Solheim 1996). Since the 1970's, it has lead to significant damage in Europe (Christiansen & Bakke 1988, Fűhrer 1996). Furniss *et al*. (1990), reported that the spores of *C. polonica* are disseminated on the exoskeleton, or in the guts of the insects. The fungus will infect the phloem of the tree after the beetles attack healthy trees (Krokene & Solheim 2001). Mass attack by the beetles and subsequent infection by the fungus ultimately leads to mortality of the host trees (Christiansen & Bakke 1988). However, the pathogenicity of *C. polonica* without the interaction of the beetles has been established during mass inoculation tests, where the fungus alone was inoculated into small wounds on the tree and proved to be pathogenic (Horntvedt *et al*. 1983, Krokene & Solheim 1998, Harrington *et al*. 2002).

Ceratocystis fujiensis is associated with *I. subelongatus* in Japan (Yamaoka *et al*. 1998, Stauffer *et al*. 2001). The beetle infests larch trees (*Larix kaempferi*) (Koizumi 1990, Yamaoka *et al*. 1998), inoculating *C. fujiensis* into the trees and resulting in tree death (Yamaoka *et al*. 1998, Stauffer *et al*. 2001). *Ceratocystis fujiensis* has been proven to be highly pathogenic, resulting in tree mortality within 100 days after mass inoculation of 30 year-old Japanese larch (Yamaoka *et al*. 1998).

Ceratocystis rufipenni is associated with *Dendroctonus rufipennis* Kirby (Harrington & Wingfield 1998). This beetle has been described as a weak to moderately aggressive pest of *Picea* spp. in Canada and the U.S.A. (Harrington & Wingfield 1998, Six & Klepzig 2004). A number of species associated with *D. rufipennis* has been reported to cause symptoms on *Picea* spp. during inoculation trials, however, *C. rufipenni* seems to be the most aggressive (Horntvedt *et al*. 1983, Solheim 1988, Solheim & Safranyik 1997). Once the fungus has infected its host, it colonises the sapwood and phloem (Solheim 1995). The fungus colonises new hosts rapidly and is often found at the leading edge of fungal growth spreading towards the sapwood of *D. rufipennis* infested trees (Solheim 1995).

1.5.3.2. Nitidulid beetles

Nitidulid beetles, or sap-feeding beetles (Coleoptera: Nitidulidae), have been reported as vectors of a number of *Ceratocystis* spp., including *C. fagacearum*, *C. moniliformis*, *C. fimbriata* (Collins & Kalnins 1965, Moller & DeVay 1968, Juzwik *et al*. 1998) and *C. paradoxa* (Dade) Moreau (Chan & Jensen 1974). Some authors view Nitidulids as the most important insect vector group of *Ceratocystis* spp. in the nort and central states of the USA (Juzwik 2001). Nitidulid beetles do not make wounds but visit wounds made by other factors such as insect or animal feeding as well as, natural forces or mechanical damage (Connell 1956, Juzwik *et al*. 1999). The adults of these beetles are attracted to, and live in fermenting plant sap, decaying fruit, or fungi (Downie & Arnett 1996). These beetles and their larvae actually feed off the sap of the host trees as well as on the fungal mats (Moller & DeVay 1968, Cease & Juzwik 2001). As these beetles feed on the fungal mats, viable pathogen propagules attach to their bodies and are spread with them (Juzwik & French 1983, Apple *et al*. 1990). In this manner, they assist in the establishment of new oak wilt infection centres, either within the same stands or in adjacent or more distant stands (Juzwik 2001).

1.5.3.3. Generalist organisms

A number of generalist organisms, including insects other than bark beetles and Nitidulid beetles, transmit *Ceratocystis* spp. These include phoretic mites (Himelick & Curl 1958, Moller & DeVay 1968, Moser *et al*. 1985, 1997), nematodes (Vovlas *et al*. 1994) and flies (Diptera) (Himelick & Curl 1958, Moller & DeVay 1968, Bridges & Moser 1983, Moser 1997). As early as the late 1950's the mite, *Garmania bulbicola* Oudemans was identified to be able to transmit *C. fagacearum* to artificial wounds (Hemlick & Curl 1958). Similarly, a species of the mite *Tarsonemus* has been reported in studies of a member of the *C. fimbriata s.l.* species complex (Moller & DeVay 1968) and more recently *C. fujiensis* (Moser *et al*. 1997).

Flies (Diptera) have been reported to be associated with *Ceratocystis* spp. (Collins & Kalnins 1965, Moller & DeVay 1968). Already in the mid 1960's *Drosophila* spp. were reported to be an important associate of the well known *C. fagacearum* (Collins & Kalnins 1965). Similarly, in a study by Moller & DeVay (1968), the authors also reported two fly species to be associated with *C. fimbriata* including, *Drosophila*

melanogaster Meigen and *Chymomyza procnemoides* Wheeler. The authors, however, felt the association of these two species with *C. fimbriata* was a casual one.

A limited number of studies have identified more general organisms as associates of *Ceratocystis* spp. In a study of banana diseases and pests, *C. paradoxa* was reported to be associated with nematodes (Vovlas *et al*. 1994). In this study, *C. paradoxa* was found to be transmitted by the nematode *Helicotylenchus multicinctus* Cobb associated with root systems of declining bananas. There are also single reports of *Ceratocystis* species associated with organisms such as ants (Greiff & Currah 2007) and mice (Goto *et al*. 1954).

1.5.4. Association levels between insects and *Ceratocystis* spp.

There are two distinct categories of associations between *Ceratocystis* spp. and insects. Some species, such as those in the *C. fimbriata s.l* complex and *C. moniliformis* clades, have a casual relationship with insects. These are the species that tend to produce fruity odours which attract many species of flies (Diptera) and sap beetles (Nitidulidae) (Himelick & Curl 1958). Another group of *Ceratocystis* spp., mostly those in the *C. coerulescens* group tend not to produce fruity odours. These species rely on very close relationships with specific insects, particularly bark beetles, for their dispersal (Harrington & Wingfield 1998).

Some *Ceratocystis* spp. have been associated with a number of different insect species, forming no specific relationships with particular insect species. These *Ceratocystis* spp. are all characterized by the production of fruity volatiles that attract generalist insects (Moller & DeVay 1968, Kirisits 2004). Species of *Ceratocystis* that have this loose form of relationship with insects include *C. fimbriata s.l.*, *C. fagacearum* and *C. moniliformis* (Mathiesen-Kräärik 1953, Kirschner 2001). *Ceratocystis fagacearum*, for example, has been reported from nitidulid beetles (Bretz 1952, Collins & Kalnins 1965, Juzwik *et al*. 1998) and flies (Hemelick & Curl 1958). Similarly, a species in the *C. fimbriata s.l* species complex has been reported from nitidulid beetles, flies and mites (Moller & DeVay 1968).

A number of *Ceratocystis* spp. have associations with specific insect species. In these cases, one insect species is associated with only one fungus. If these relationships did

not exist or failed, the fungi involved would not be able to disseminate effectively. These *Ceratocystis* spp. do not produce the same fruity volatiles as those produced by *Ceratocystis* spp. with loose associations with insects. Examples here are *C. laricicola,* associated with the bark beetle *I. cembrae* (Redfern *et al.* 1987), *C. fujiensis* associated with *I. subelongatus* (Yamaoka *et al.* 1998), *C. rufipenni* associated with *D. rufipennis* (Solheim & Safranyik 1997, Wingfield *et al.* 1997) and *C. polonica* which is associated with *I. typographus* (Solheim 1986, Christiansen & Solheim 1990, Krokene & Solheim 1996, Harrington & Wingfield 1998, Kirisits *et al.* 2000).

Ceratocystis laricicola is specifically associated with *I. cembrae* in Europe (Redfern *et al.* 1987). This fungus/beetle association has been well studied and is known from various countries (Crooke & Bevan 1957, Redfern *et al.* 1987, Pfeffer 1995, Stauffer *et al.* 2001). *Ceratocystis laricicola* has not been reported being associated with any other insects and is involved in a symbiotic relationship with *I. cembrae*.

The close association between *C. polonica* and the bark beetle *I. typographus* (Horntvedt 1988, Solheim 1986, Krokene & Solheim 1996) has led to significant losses of *P. abies* trees in Europe (Christiansen & Bakke 1988, Fűhrer 1996). In this association, spores of *C. polonica* are disseminated on the exoskeleton, or in the intestinal tract, of the beetle (Furniss *et al.* 1990) and infects the *Picea* trees after mass attack by the beetles, ultimately leading to trees death (Christiansen & Bakke 1988).

Ceratocystis fujiensis, associated with *I. subelongatus*, is inoculated into its host tree when the beetle infests larch trees (Koizumi 1990, Yamaoka *et al.* 1998). Similar to *C. polonica*, *C. fujiensis* has been proven to be highly pathogenic to its host, but is not able to infect the host tree without the assistance of its insect associate (Yamaoka *et al.* 1998, Stauffer *et al.* 2001).

Ceratocystis rufipenni, associated with *D. rufipennis,* is an example of a close association of a fungus with an insect, but not the reciprocal situation (Harrington & Wingfield 1998, Six & Klepzig 2004). *Ceratocystis rufipenni* has only been found associated with *D. rufipennis* (Six & Klepzig 2004). *Ceratocystis rufipenni* has been shown to be the most aggressive fungus associated with *D. rufipennis* (Horntvedt *et*

al. 1983, Solheim 1988, Solheim & Safranyik 1997) and is usually found at the leading edge of fungal growth (Solheim 1995). This indicates that *C. rufipenni* is directly dependent on *D. rufipennis* for dissemination and could suggest that *C. rufipenni* assists *D. rufipennis* to overcome the tree's defence mechanisms.

Not all associations between insects and *Ceratocystis* spp. are easily defined. An example of this is the association between *C. rufipenni* and *D. rufipennis*. Although a close relationship between *C. rufipenni* and *D. rufipennis* was reported by Wingfield *et al*. (1997), the fungus was not isolated from these beetles in later studies (Six & Bentz 2003, Six & Klepzig 2004). These contradictions in results could be due to difficulties in isolating the fungus or the incubation temperature (Solheim 1995). It could also be due to fungal succession. *Ceratocystis rufipenni* is usually only isolated from the leading edge of the lesions on freshly infected wood (Solheim 1995, Six & Bentz 2003). It could be that when the insects emerge from the wood (1-2 years after attack), saprophytic fungi had colonised the wood where the beetles developed and that *C. rufipenni* was not present at the time of emergence (Solheim 1995). It could also be that the main mode of dissemination of *C. rufipenni* by *D. rufipennis* is in the intestinal tract of the insect (Harrington *et al*. 1996), thereby also explaining the limited success in isolating the fungus from the insect. Although the biology of *C. rufipenni* is very similar to that of *C. polonica* (Christiansen 1985, Solheim & Safranyik 1997), their association with their respective insects differs significantly. Both these fungi are highly pathogenic on their respective hosts and are commonly isolated from the leading edges of the infections. *Ceratocystis polonica* is, however, frequently isolated from its associated beetle *I. typographus*, whereas *C. rufipenni* is not frequently isolated from *D. rufipennis*.

The fact that bark beetles are important forest pests, and that many of their fungal associates cause destructive tree diseases, emphasizes the need for further and detailed investigation into these associations. Although a vast number of studies have been reported on the relationships between insects and *Ceratocystis* spp., the limited knowledge of these in Africa creates a void in research in this field. Understanding the interactions between these organisms will inevitably help combat these forest pests and pathogens. For example, the wilt pathogen *C. albifundus,* one of the most threatening pathogens of plantations of non-native *A. mearnsii*, is hypothesised to be

native to the African continent (Roux *et al.* 2001c, Barnes *et al.* 2005) and has not been studied regarding its possible insect vectors. Knowledge pertaining to the biology and ecology of the insect vectors of these pathogens could assist in the control of the insect as well as the pathogens.

1.6. CONCLUSIONS

Ceratocystis spp. include many economically important plant pathogens including a group that cause diseases of trees and sap-stain of timber worldwide. Losses incurred by these pathogens include tree mortality, growth reduction and the decrease in value of timber. Wounds and the insects that form part of a symbiotic relationship with these fungi are important factors for the dispersal and infection of these pathogens. However, very little information is available on these fungi in Africa. As the forestry industry in a number of African countries have entered a dramatic growth and development stage, the recent reports of a number of wilt pathogens in the genus *Ceratocystis* in some African countries emphasize the need for further studies on this group of fungi on the continent.

Effective management of tree diseases and pests rely on a number of factors. These include comprehensive information of the threatening organism's biology, ecology and origin. Once the origin of a pathogen is known, risk assessment could be improved and centres can be identified for possible research into control measures (Linde *et al*. 2002). At a deeper level, knowledge of pathogenic fungi's genetic structure is also important role. With globalisation and the increasing movement around the world of people and commodities, the possibility of new introductions of plant diseases and pests increase. Although a pathogen or pest is present in a country, the introduction of new genotypes could pose a greater threat than the one that currently exists. Therefore, to reduce the threat of pests and pathogens, it is necessary to have a comprehensive understanding about their genetic diversity and movement.

Answering the many questions pertaining to the origin and dispersal of *Ceratocystis* spp. between countries and continents could assist in the restriction of further spread of these pathogens and the threat they pose to the forestry industry and natural ecosystems. In similar fashion, knowledge pertaining to their symbiotic relationships

with insects could also assist in the formulation of management strategies. Studies in the thesis that follow this review, focus on *Ceratocystis* spp. infecting wounds on non-native plantation hardwood tree species in southern and eastern Africa. These studies will focus mainly on the morphology, phylogeny and pathogenicity of these fungi. Furthermore, the insect vectors associated with these fungi and the role they play in the dissemination and biology of the fungi isolated are considered. Use is made of population diversity studies with polymorphic DNA markers to obtain knowledge pertaining to the possible origin of the most important fungi obtained during the studies. This knowledge should be valuable in the development of management and quarantine strategies against these pathogens.

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TABLE 1. List of reported associations of *Ceratocystis* spp. and their associated insects.

***Ceratocystis* spp.**	**Insect spp.**	**Host**	**Country**	**Reference**
C. atrox	*Phorocantha acanthocera*	*Eucalyptus* spp.	Australia	VanWyk *et al*. 2007b
C. bhutanensis	*Ips schmutzenhofferi*	*P. spinulosa*	Bhutan	Van Wyk *et al.* 2004a
C. coerulescens	*I. acuminatus*	*Pinus sp*		Mathiesen 1950, Mathiesen-Käärik 1953
	Orthotomicus proximus	*P. sylvestris*		Mathiesen 1950, Mathiesen-Käärik 1953
	Pityogenus chalcographus	*P. sylvestris*		Mathiesen 1950, Mathiesen-Käärik 1953
C. fagacearum	*Carpophilus brachypterus*	Unknown		Henry 1944, Bretz 1952, Juzwik & French 1983
	Ca. dimidiatus	Unknown		Henry 1944, Bretz 1952, Juzwik & French 1983
	Ca. sayi	Unknown		Cease & Juzwik 2001, Juzwik *et al*., 2004
	Epuraea labilis	Unknown		Henry 1944, Bretz 1952, Juzwik & French 1983
	E. peltoides	Unknown		Henry 1944, Bretz 1952, Juzwik & French 1983
C. fimbriata	*Ca. freemani*	Unknown		Moller & DeVay 1968
	Chymomyza procnemoides	Unknown		Moller & DeVay 1968
	Euporea spp.	Aspen		Hinds 1972
C. fujiensis	*I. subelongatus*	*Larix kaempferi*	Japan	Yamaoka *et al.* 1998, Marin *et al*. 2005
C. laricicola	*I. cembrae*	*L. deciduas*	Europe	Redfern *et al*. 1987, Kirisits *et al*. 2000, Stauffer *et al.* 2001
C. manginecans	*Hypocryphalus mangiferae*	*Mangifera indica*	Oman	Al-Adawi *et al*. 2006
C. moniliformopsis	*Eucryphia lucia*	*Eucalyptus oblique*	Australia	Yuan & Mohammed 2002
	Nothofagus cunninghamii	*E. oblique*	Australia	Yuan & Mohammed 2002
C. paradoxa	*Urophorus humeralis*	Sugar cane	USA	Chang & Jensen 1974
	Carpophilus hemipterus	Sugar cane	USA	Chang & Jensen 1974

	Haptoncus ocularis	Sugar cane	USA	Chang & Jensen 1974
***Ceratocystis* spp.**	**Insect spp.**	**Host**	**Country**	**Reference**
C. polonica	*I typographus*	*P. abies*	Europe	Siemaszko 1939, Mathiesen 1950, Mathiesen 1951, Mathiesen Käärik 1953, Harding 1985, 1989, 1995, Solheim 1986, Furniss *et al.* 1990, Solheim 1992a, b, 1993a, b, Virri & Von Weisenberg 1995, Krokene & Solheim 1996, Viiri 1997, Grubelnik 1998, Harrington & Wingfield 1998, Kirschner 1998, Kirisits *et al*. 2000, Kirschenr 2001, Salle *et al.* 2003, Jankowiak 2005, Viiri & Lieutier 2004
	I. amitinus	*Pi. Cembra*		Kirisits *et al*. 2000
	I. duplicates			Valkama 1995, Krokene & Solheim 1996
	H. palliates	*P. abies*		Krokene & Solheim 1996
	H. palliates	*Pi. Sylvestri*		Krokene & Solheim 1996
	H. palliates	*L. kaempveri*		Krokene & Solheim 1996
	Pityogenus chalcographus	*P. abies*		Kirisits 1996, Krokene & Solheim 1996, Kirisits *et al*. 2000
	Polygraphus polygraphus	*P. abies*		Krokene & Solheim 1996
C. polychroma	*Hexamitodera semivelutina*	*Syzygium aromaticum*	Sulawesi	Van Wyk *et al*. 2004
C. rufipenni	*D. rufipennis*	*P. engelmannii*	Canada	Harrington & Wingfield 1998

TABLE 2. List of *Ceratocystis* spp. reported as the causal agents of forest and plantation tree diseases under field and/or greenhouse conditions.

***Ceratocystis* spp.**	**Host**	**Country**	**Reference**
C. albifundus	*Acacia mearnsii*	South Africa	Morris *et al*. 1993, Roux *et al*. 1997, 199b
	A. mearnsii	Kenya	Roux *et al*. 2005
	A. mearnsii	Tanzania	Roux *et al*. 2005
	A. mearnsii	Uganda	Roux *et al.* 2001
C. coerulescens	*Acer* spp.	USA	Kile 1993
C. fagacearum	*Quercus* spp.	USA	Henry 1943, Bretz 1952, Hunt 1956, Upadhyay 1981
C. fimbriata s.l.	*A. decurrens*	Brazil	Ribeiro *et al*. 1985
	Eucalyptus spp.	Brazil	Laila *et al*. 1999
	Eucalyptus spp.	Republic of Congo	Roux *et al*. 2000
	Eucalyptus spp.	Uganda	Roux *et al*. 2001b
	Eucalyptus spp.	Uruguay	Barnes *et al.* 2003
	Eucalyptus spp.	South Africa	Roux *et al*. 2004
C. fujiensis	*L. kaempferi*	Japan	Yamaoka *et al.* 1998
	Piceae spp.	Japan	Marin *et al*. 2005
C. laricicola	*L. deciduas*	Europe	Redfern *et al*. 1987, Kirisits *et al*. 2000, Stauffer *et al.* 2001
	Larix spp.	Scotland	Harrington & Wingfield 1998
C. pirilliformis	*Eucalyptus* spp.	South Africa	Roux *et al*. 2004
C. platani	*Platanus* spp.	USA	Mook 1940, Walter 1946,
	Platanus spp.	France	Ferrari & Pichenot 1975
	Platanus spp.	Southern Europe	Panconesi 1999
	Platanus spp.	Greece	Tsopelas & Angelopoulos 2004
	Platanus spp.	Italy	Panconesi 1981
C. polonica	*P. abies*	Europe	Mathiesen 1950, Mathiesen-Käärik 1953, Postener 1974, Harding 1985, Christiansen & Bakke 1988, Harding 1995, Solheim 1986, Furniss *et al.* 1990, Krokene & Solheim 1996, Grubelnik 1998, Harrington & Wingfield 1998, Salle *et al.* 2003, Jankowiak 2005
	P. abies	Austria	Führer 1996
	P. abies	Switzerland	Führer 1996
	P. abies	Germany	Führer 1996
	Piceae spp.	Poland	Siemaszko 1939, Mathiesen 1951, Hunt 1956
	Piceae spp.	Sweden	Siemaszko 1939, Mathiesen 1951, Hunt 1956
	Pi. Cembra	Norway	Harrington & Wingfield 1998
	Pi. Sylvestri		Kirisits *et al*. 2000
C. polonica	*L. kaempveri*		Valkama 1995, Krokene & Solheim 1996
C. populicola	*Populus tremuloides*	Canada & USA	Johnson *et al*., 2005

***Ceratocystis* spp.**	**Host**	**Country**	**Reference**
C. populicola	*Populus tremuloides*	Poland	Gremmen & De Kam 1977, Przybyl 1984, Johnson *et al*. 2005
	Populus tremuloides	Quebec	Vujanovic 1999, Johnson *et al*. 2005
C. rufipenni	*P. engelmannii*	Canada	Harrington & Wingfield 1998
C. smalleyi	*Carya cordiformis*	USA	Johnson *et al*. 2005

TABLE 3. List of *Ceratocystis* spp. reported from forest and plantation tree species but with no clear association with disease or mortality of these trees.

Ceratocystis spp.	**Host**	**Country**	**Reference**
C. albifundus	*Acacia cafra*	South Africa	Roux *et al*. 2007
	Burkea africana	South Africa	Roux *et al*. 2007
	Combretum molle	South Africa	Roux *et al*. 2007
	Co. zeyheri	South Africa	Roux *et al*. 2007
	Faurea saligna	South Africa	Roux *et al*. 2007
	Ocna pulcra	South Africa	Roux *et al*. 2007
	Ozoroa paniculosa	South Africa	Roux *et al*. 2007
	Terminalia sericia	South Africa	Roux *et al*. 2007
	Brachystegia speciformis	Zambia	Roux *et al*. 2004
	Tulbergia nitidula	Zambia	Roux *et al*. 2004
	Parinari curatelifolia	Zambia	Roux *et al*. 2004
	Julbinardia spp.	Zambia	Roux *et al*. 2004
	Brachystegia busei	Malawi	Roux *et al*. 2004
C. atrox	*Eucalyptus* spp.	Australia	VanWyk *et al*. 2007b
C. bhutanensis	*P. spinulosa*	Bhutan	Van Wyk *et al.* 2004b
C. caryae	*Carya* spp.	USA	Johnson *et al*. 2005
	Ulmus spp.	USA	Johnson *et al.* 2005
	Ostrya virginiana	USA	Johnson *et al.* 2005
C. coerulescens	*Acer* spp.	Canada	Griffin 1968
	Pinus spp.	Scotland	Bakshi 1950
	Pinus spp.	Sweden	Lagerberg *et al.* 1927
	Piceae spp.	USA	Upadhyay 1981, Olchowechi & Reid 1974
	Piceae spp.	Germany	Munch 1907, Hunt 1956
	Pseudotsuga spp.	Germany	Upadhyay 1981, Davidson 1935
	Quercus spp.	Germany	Upadhyay 1981
	Fagus spp.	-	Upadhyay 1981
C. douglasii	*Pseudotsuga* spp.	USA	Wingfield *et al*. 1997
C. eucalypti	*Eucalyptus* spp.	Australia	Kile *et al*. 1996
C. moniliformis	*Liquidamber* spp.	USA	Hedgcock 1906
	Quercus spp.	Scotland	Bakshi 1951
	Quercus spp.	Japan	
	Pycnanthus komba	Cameroon	Luc 1952
	Calamus maximus	Philippines	Roldan 1962
	Endospermum peltatum	Philippines	Roldan 1962
	Parkea javanica	Philippines	Roldan 1962
	Fagus grenata	Japan	Kitajima 1936
	Eucalyptus spp.	South Africa	Roux *et al.* 2004
C. moniliformopsis	*E. oblique*	Australia	Yuan & Mohammed 2002
	E. oblique	Australia	

Ceratocystis spp.	**Host**	**Country**	**Reference**
C. pinicola	*Pinus* spp.	England	Harrington & Wingfield 1998
C. pirilliformis	*Eucalyptus* spp.	Australia	Barnes *et al*. 2003a
C. resinifera	*Piceae* spp.	Norway	Harrington & Wingfield 1998
C. resinifera	*Piceae* spp.	Scandinavia	Lagerberg *et al*. 1927, Roll-Hansen & Roll-Hansen 1980, Harrington &Wingfield 1998
C. savannae	*Acxacia nigrescens*	South Africa	Kamgan *et al*. 2008
	Combretum zeyheri	South Africa	Kamgan *et al*. 2008
	Terminalia sericea	South Africa	Kamgan *et al*. 2008
	Sclerocarya birrea	South Africa	Kamgan *et al*. 2008
	Burkea Africana	South Africa	Kamgan *et al*. 2008
C. tsitsikammensis	*Rapanea melanophloeos*	South Africa	Kamgan *et al*. 2008
	Ocotea bulata	South Africa	Kamgan *et al*. 2008
C. tribiliformis	*P. merkusii*	Indonesia	Van Wyk *et al*. 2006
C. variospora	*Quercus* spp.	USA	Davidson 1944
	Betula platyphylla	Japan	Johnson *et al*. 2005
C. virescens	*Liquidambar* spp.	USA	Davidson 1944
	Lirodendron spp.	USA	Davidson 1944
	Nassa spp.	USA	Davidson 1944
	Fagus spp.	USA	Davidson 1944
	Magnolia spp.	USA	Davidson 1944
	Quercus spp.	USA	Davidson 1944

Appendix 1

Chapter 2

Ceratocystis species on *Acacia mearnsii* and *Eucalyptus* spp. in eastern and southern Africa including six new species

(Heath *et al.* 2009, Fungal Diversity 34: 41-68)

ABSTRACT

Species of *Ceratocystis* include well-known plant pathogens causing cankers, vascular wilt and root diseases, as well as many species that are agents of sap stain of lumber. A number of *Ceratocystis* spp. have been reported from Africa, but the continent is generally poorly sampled in terms of these fungi. The aim of this study was to consider the presence of *Ceratocystis* spp. infecting wounds on plantation-grown, non-native, *Acacia mearnsii* and *Eucalyptus* spp. in Kenya, Malawi, Tanzania and South Africa. Isolates were collected from cut stumps and artificially induced wounds on the stems of trees. They were subsequently identified based on morphological characteristics and DNA sequence comparisons for the ribosomal RNA Internal Transcribed Spacer region, including the 5.8S operon as well as partial sequences of the β-tubulin and the Transcription Elongation Factor 1α genes. Analyses showed that isolates represent six previously undescribed species from Malawi, South Africa and Tanzania, while *C. moniliformis* was found for the first time in Tanzania. The undescribed *Ceratocystis* spp. are provided with the names *C. zombamontana prov. nom., C. polyconidia prov. nom., C. tanganyicensis prov. nom., C. obpyriformis prov. nom., C. oblonga prov. nom.* and *T. ceramica prov. nom.* The wilt pathogen *C. albifundus* was also commonly found on *A. mearnsii* in Tanzania and in Kenya. All the new species described in this study were pathogenic on the hosts from which they were originally isolated.

2.1. INTRODUCTION

The genus *Ceratocystis* includes some of the best-known plant pathogens in the world, responsible for a wide range of disease symptoms including stem cankers, vascular wilts and root diseases (Kile 1993). Most of these important pathogens are related to *C. fimbriata* Ell. & Halst. *sensu lato* (*s.l.*). Some have recently been provided with new names while others are recognized as unique based on phylogenetic inference (Wingfield *et al.* 1996, Barnes *et al.* 2003, Engelbrecht & Harrington 2005, Johnson *et al.* 2005, Van Wyk *et al.* 2007, Rodas *et al.* 2008). Well known tree diseases caused by *Ceratocystis* spp. include oak wilt caused by *C. fagacearum* (Bretz) Hunt (Bretz 1952, Sinclair *et al.* 1987), canker stain disease of plane trees (*Platanus* spp.) caused by *C. platani* Engelbrecht et Harrington (Engelbrecht & Harrington 2005) and wattle wilt of *Acacia mearnsii* De Wild. caused by *C. albifundus* M. J. Wingf., De Beer and M. J. Morris (Morris *et al.* 1993, Wingfield *et al.* 1996). Many species, particularly those in the *C. coerulescens* (Münch) Bakshi species complex are agents of sap stain of lumber (Münch 1907) and various species, especially in the *C. moniliformis* Hedgcock *s.l.* species complex, appear to be saprophytes (Davidson 1935, Van Wyk *et al.* 2006b).

Ceratocystis spp. residing in the *C. fimbriata s.l.* species complex require wounds for infection (DeVay *et al.* 1963, Kile 1993). These wounds can emerge from wind and hail damage, growth cracks, insect and other animal damage as well as human activities such as grafting and pruning. Insects carry the *Ceratocystis* spp., which are ecologically adapted to this mode of dissemination, to the wounds, where infection can take place (DeVay *et al.* 1963, Kile 1993). Stumps remaining from freshly harvested trees are also commonly infected by species of *Ceratocystis* (Roux *et al.* 2004). Recent studies have shown that artificially induced wounds are also commonly infected by *Ceratocystis* spp. and provide an opportunity to trap species infecting wounds from the environment (Barnes *et al.* 2003, Roux *et al.* 2004, Rodas *et al.* 2008).

Relatively little is known regarding *Ceratocystis* spp. occurring in Africa (Roux *et al.* 2005). During the course of the last two decades, there have been numerous studies

investigating these fungi on trees in the region. These have largely emerged from the discovery of a serious wilt disease of *Acacia* spp. now known to be caused by *C. albifundus* (Morris *et al*. 1993, Wingfield *et al*. 1996, Roux *et al*. 2001a,b, 2005). More recently, *C. fimbriata s.l.* was reported to result in rapid wilting and death of *Eucalyptus* spp. in the Republic of Congo (Roux *et al*. 2000) and Uganda (Roux *et al*. 2001a). This fungus has also been isolated from wounds on *Eucalyptus* trees in South Africa (Roux *et al.* 2004) but although it was pathogenic in inoculation tests, it has not been associated with disease under natural conditions. Most recently, two other species of *Ceratocystis*, *C. pirilliformis* Barnes and M. J. Wingf. and *C. moniliformis* have been recorded from *Eucalyptus* spp. in South Africa (Roux *et al*. 2004).

Population growth and globalisation is placing increasing pressure on native forests in Africa. For this reason, extensive forestry programmes, largely based on non-native species have been established. Diseases have already emerged as presenting serious constraints to the long term sustainability of forest plantations in Africa (Gibson 1964, Roux *et al*. 2005) and this is likely to be an increasing trend in the future. Concern regarding diseases has prompted surveys for important groups of tree pathogens including species of *Ceratocystis*. The aim of this study was thus to expand current knowledge relating to *Ceratocystis* spp. in Africa, particularly those occurring on wounds on non-native *A. mearnsii* and *Eucalyptus* spp. in eastern and southern Africa.

2.2. MATERIALS AND METHODS

2.2.1. Collection of isolates

Isolates were collected from *A. mearnsii* and *Eucalyptus* spp. at four localities (Piet Retief, Tzaneen, Pietermaritzburg and Lothair) in South Africa and one each in Malawi (Zomba Mountain), Tanzania (Njombe) and Kenya (Thika). Collections were made from the stumps of freshly felled *Eucalyptus* spp. and *A. mearnsii* as well as from artificially induced wounds on the stems of *Eucalyptus* trees. In the case of the stumps, samples were collected between four days and four weeks after felling, by removing pieces of wood displaying stained vascular tissue and/or the presence of fungal growth.

Stem wounds were made on *Eucalyptus* trees using the technique previously described by Barnes *et al*. (2003). Twenty trees were selected randomly at each study site and wounds were made on the stems, approximately 1.5 meters from the ground. Approximately 100 cm^2 of bark was removed from the stems to expose the cambium. A horizontal slit was made into the xylem of the wound, approximately five mm deep. Samples were collected after six weeks by removing a piece of wood and bark from the top and bottom corners of the wound site and transferred to the laboratory in brown paper bags for further study.

Wood sections were examined for the presence of fruiting structures of *Ceratocystis* spp. In addition, wood pieces displaying vascular discoloration were baited for *Ceratocystis* spp. by placing these between two carrot slices (five mm thick) and incubating them at 25°C for 7-10 days (Moller & DeVay 1968). Pieces of wood were also incubated in containers with moist tissue paper at 25°C for seven days to induce the formation of fruiting structures.

Once ascomata of *Ceratocystis* spp. were found, spore masses were lifted from their apices and transferred to 2% (w/v) malt extract agar (MEA) (Biolab, Midrand, South Africa) supplemented with streptomycin sulphate (0.001 g vol^{-1}, SIGMA, Steinheim, Germany). Plates were then incubated at approximately 25ºC under natural day/night conditions. Isolates were purified on 2% MEA and are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative isolates were also deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. In order to prepare herbarium specimens, cultures bearing fruiting structures were dried on 30% glycerol and deposited with the National Fungal Herbarium of South Africa (PREM), Pretoria.

2.2.2. Morphology and growth in culture

All isolates collected in this study were grouped based on their culture morphology on 2% MEA after five days and were then studied microscopically for further

differentiation. Representative isolates of each group were selected for further identification using DNA sequence comparisons.

For identification based on morphology, pure cultures were maintained on 2% MEA until fruiting structures formed. Fungal structures were mounted on glass slides in lactic acid and examined under a Zeiss Axioskop microscope. Images were captured using a HRc Axiocam digital camera and Axiovision 3.1 software (Carl Zeiss Ltd., Germany). Fifty measurements were made for each taxonomically relevant structure and averages and standard deviations (st. dev) were determined for each of these structures. Measurements are presented in this study as (minimum -) mean minus st. dev. - mean plus st. dev. (- maximum). Colours of cultures were defined based on the mycological colour charts of Rayner (1970).

Two test isolates of each species were selected to study growth in culture. These included one chosen to represent the holotype specimen and one of the paratypes. Growth rates of known species were not determined. Studies of growth in culture were performed by placing an agar disk (four mm diameter) overgrown with mycelium (mycelial side down) of selected five-day old isolates at the centres of 90mm Petri dishes containing 2% MEA. Petri dishes were incubated in the dark at temperatures ranging from 5°C to 35°C at 5°C intervals. Colony diameters were measured after seven days. Two measurements, perpendicular to each other, were made for each culture. Five replicates of each test strain were used at each temperature and averages of the ten measurements taken for each isolate were computed. The entire experiment was repeated once.

2.2.3. DNA isolation, PCR reactions and sequence analyses

DNA of representative *Ceratocystis* isolates (Table 1) was extracted using the method described by Van Wyk *et al*. (2006a). Three gene regions were amplified for sequencing and phylogenetic analyses. The ribosomal RNA Internal Transcribed Spacer regions (ITS) 1 and 2, and the 5.8S operon, were amplified using the primers

ITS1 and ITS4 (White *et al*. 1990). Part of the beta-tubulin (*β*-tubulin) gene was amplified with primers Bt1a and Bt1b (Glass & Donaldson 1995) and part of the Transcription Elongation Factor-1α (EF-1α) gene was amplified using the primers EF1F and EF1R (Jacobs *et al*. 2004).

Polymerase chain reaction (PCR) mixtures, for all three gene regions, consisted of 1 x Expand HF Buffer containing 1.5 mM $MgCl_2$ (Roche Diagnostics, Mannheim, Germany, supplied with the enzyme), 200 μM of each dNTP, FastStart *Taq* enzyme (2 U) (Roche Diagnostics, Mannheim, Germany), 200 ηM of the forward and reverse primers, and 2-10 ng DNA. Reactions were adjusted to a total volume of 25 μL with sterile water. The PCR programme was set for 4 min at 95°C for initial denaturation of the DNA. This was followed by 10 cycles consisting of a denaturation step at 95°C for 40s, an annealing step for 40s at 55°C and an elongation step for 45s at 70°C. Subsequently, 30 cycles consisting of 94ºC for 20s, 55ºC for 40s with a 5s extension step after each cycle, and 70ºC for 45s were performed. A final step of 10 min at 72ºC completed the programme. Amplification of the DNA for the three gene regions was confirmed under ultraviolet (UV) illumination using gel electrophoresis with 2% agarose in the presence of ethidium bromide. Amplicons were purified using 6% Sephadex G-50 columns following the manufacturer's instructions (Steinheim, Germany).

PCR amplicons were sequenced in both directions using the ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, California, USA), with the same primers as those used for DNA amplification. Sequencing reactions were run on an ABI PRISM™ 3100 Autosequencer (Applied BioSystems, Foster City, California, U.S.A) and sequences were analysed using Sequence Navigator version 1.0.1 (Applied BioSystems, Foster City, California, USA). Sequences were compared with those of closely related *Ceratocystis* and *Thielaviopsis* spp. obtained from GenBank (http://www.ncbi.nlm.nih.gov), resulting in three datasets. The first set comprised three gene regions for isolates representing the *C. fimbriata s.l.* species complex, the second of the ITS gene region of *Thielaviopsis* spp. together with a

small number of *Ceratocystis* spp., and the third set was made up of three gene regions for species in the *C. moniliformis s.l.* species complex. Sequences were aligned using the web interface (http://align.bmr.kyushu-u.ac.jp/mafft/software/) of MAFFT (Katoh *et al.* 2002) and confirmed manually.

Analyses were performed using Phylogenetic Analysis Using Parsimony (PAUP) version 4.0b10* (Swofford 2002). To determine whether the sequences for the multiple gene regions could be combined into single datasets, partition homogeneity tests (Swofford 2002) were conducted. Gaps were treated as a fifth character and trees were obtained via stepwise addition of 1 000 replicates with the Mulpar option in effect. The heuristic search option based on parsimony with stepwise addition was used to obtain the phylograms. Confidence intervals using 1000 bootstrap replicates were calculated. *Ceratocystis virescens* (Davidson) Moreau was designated as the monophyletic sister out-group taxon. All sequences derived from this study were deposited in GenBank (Table 1).

Phylogenetic trees based on Bayesian probabilities using a Markov Chain Monte Carlo (MCMC) algorithm were generated using MrBayes version 3.1.1 (Ronquist & Huelsenbeck 2003). For each gene, a model of nucleotide substitution was determined using Mr Modeltest (Nylander 2004) and these were included for each gene partition in MrBayes. One million random trees were generated using the MCMC procedure with four chains and sampled every 100th generation. To avoid including trees that were sampled before convergence, tree likelihood scores were assessed to determine the number of trees that were formed before the stabilization. Trees outside the point of convergence were discarded by means of the burnin procedure in MrBayes.

2.2.4. Pathogenicity tests

The relative pathogenicity of the *Ceratocystis* spp. isolated from the various hosts was determined in inoculations performed in a greenhouse. An *E. grandis* W. Hill ex Maiden clone and *A. mearnsii* seedlings were maintained under greenhouse conditions

for two weeks to acclimatise them to these conditions, prior to inoculation. The greenhouses were subjected to natural day/night conditions (~13 hours daylight/ ~11 hours darkness) and a temperature of approximately 25ºC was maintained. Tree diameters varied from 10 to 15 mm. The two fastest growing isolates for each of the six *Ceratocystis* spp. (CMW23809, CMW23818, CMW23807, CMW23808, CMW23802, CMW23803, CMW15992, CMW15999, CMW15235, CMW15236, CMW15242, CMW15248) were selected for the inoculation tests. Twenty trees were inoculated with each test strain. In addition, ten trees were inoculated with sterile MEA plugs, to serve as controls.

Wounds were made on the tree stems using a cork borer (5 mm diam) in such a way that a disc of bark was removed to expose the cambium. Mycelial plugs of a similar size were taken from the edges of seven-day-old actively growing cultures and placed in the wounds with the mycelium facing the cambium. Wounds were sealed with laboratory film (Parafilm "M", American National Can™ Chicago, Illinois, USA) to protect the inoculated fungus and the cambium from desiccation. Lesion lengths were measured six weeks after the trees were inoculated. The experiment was repeated once and the data were pooled for analyses. The differences between variables were log10 transformed to obtain a normalized distribution. The data were then subjected to a univariate procedure using SAS (SAS Version 8.2, 2001). To determine whether the inoculated fungi were responsible for the lesion development, re-isolations were made from the lesions and the fungi identified based on morphological characteristics.

2.3. RESULTS

2.3.1. Isolates

Isolations from ascomata and mycelium on wounds or carrot baits yielded cultures that, based on morphology, clearly represented a number of different *Ceratocystis* spp. A total of 136 isolates were obtained in this study. Of these, 106 were isolated from *A. mearnsii*, 23 cultures from *Eucalyptus* spp. and seven isolates were of a *Thielaviopsis* sp. from *Eucalyptus* spp. for which no sexual fruiting bodies were found (Table 1).

Symptoms associated with fungal infection of the wounds and stumps were a discoloration of the vascular tissue in a streaked pattern, developing above and below the wounds. Vascular streaking of the wood associated with the cut stumps spread downwards into the roots.

2.3.2. Morphology and growth in culture

The *Ceratocystis* spp. obtained in this study could be separated into eight distinct groups based on culture morphology. Selected isolates representing these groups (designated Group A to Group H) were chosen for further study and others have been preserved (Table 1). Group A isolates were from *A. mearnsii* in Tanzania (CMW15773, CMW15781), South Africa (CMW23825, CMW23838) and Kenya (CMW 24685, CMW 24686) and produced light-coloured mycelium bearing ascomata with light coloured bases, similar to those of *C. albifundus*. Group B isolates (CMW15235, CMW15236, CMW15251) from *E. grandis* in Malawi, produced light brown to greyish-white colonies (17""i) and perithecia with black ascomatal necks. Group C isolates (CMW23806, CMW23807, CMW23808) were obtained from *A. mearnsii* from South Africa and had greyish-white colonies (21"k) and perithecia with black ascomatal necks similar to those of *C. pirilliformis.* Group D isolates (CMW15991, CMW15992, CMW15999) were from *A. mearnsii* in Tanzania and produced dark grey to greenish colonies (25"m) similar to those of *C. fimbriata s.l.* This fungus produced abundant chlamydospores not typically found in *C. fimbriata s.s.* Isolates (CMW23809, CMW23818, CMW23819) residing in Group E were from *A. mearnsii* in South Africa and produced dark brown to greenish-brown colonies (19"i) and perithecia with black ascomatal necks and had an extremely strong alkaloid odour. Group F isolates (CMW23802, CMW23803, CMW23804) were from *A. mearnsii* in South Africa and produced white-coloured colonies that turned light brown with age and were similar to those of *C. savannae* Kamgan Nkuekam and Jol. Roux. These isolates, however, produced nodules on the hyphae, a characteristic not found in *C. savannae* (Kamgan *et al*. 2008). Group G isolates were from *Eucalyptus* spp. in Tanzania (CMW22284, CMW22289) and South Africa (CMW17587, CMW17960). These isolates were characterized by hyaline to grey to black mycelium, similar to that found in *C.*

moniliformis. Group H isolates (CMW15245, CMW15246, CMW15248) represented a *Thielaviopsis* sp., from a *Eucalyptus* sp. in Malawi without any production of sexual structures and were characterized by light to dark brown mycelium (21"b).

No growth rate studies were performed for Groups A and G as these represent known species. The optimal growth for isolates (CMW15236, CMW15235) representing Group B was 20-25°C. No growth was observed at 5°C and 35°C. At 20 and 25°C an average of 41mm of growth was observed after 14 days. The optimal growth for Group C (CMW23808, CMW23807) isolates was 25°C and they did not grow at 5°C or at 35°C. At 25°C, an average of 52mm growth was observed after seven days. Group D isolates (CMW15992, CMW15999) grew optimally at 20°C but did not grow at 5°C or at 35°C and reached an average of 27mm of growth after seven days. Group E isolates (CMW23809, CMW23818) grew optimally at 20-25°C with minimal growth at 5°C and 35°C and produced an average growth of 81mm in seven days. Isolates in Group E produced an average growth of 72mm at 20°C and 81mm at 25°C after seven days. Group F isolates (CMW23803, CMW23802) grew optimally at 25°C. Growth was also observed at 5°C and at 35°C. Cultures reached an average of 83mm of growth after seven days. Group H isolates (CMW15245, CMW 15248) produced optimal growth at 25°C reaching an average of 90mm of growth after four days. No growth was observed at 5°C, 10°C or at 35°C.

2.3.3. Phylogenetic analyses

DNA sequencing yielded amplicons of ~500bp for both the ITS and *β*-tubulin gene regions and amplicons of ~750bp for the EF1-α gene region. Partition homogeneity tests showed that data from the three gene regions could be combined for both the *C. fimbriata s.l.* and *C. moniliformis s.l.* datasets. The *C. fimbriata s.l.* data set had a P value of 0.001 and the *C. moniliformis s.l.* data set a P value of 0.16 for the partition homogeneity tests.

One most parsimonious tree was obtained for the combined data set of the *C. fimbriata s.l.* group (Figure 1). This tree had a length of 1597 base pairs. There were 1915 characters, with 1135 of these characters being constant, 37 characters being parsimony-uninformative and 743 characters being parsimony-informative, with Consistency Index (CI) = 0.7276, Retention Index (RI) = 0.9003 and Rescaled Consistency (RC) = 0.6551. In the phylogenetic tree, *C. fimbriata s.s.*, *C. platani* Engelbrecht and Harrington, *C. cacaofunesta* (Walter) Engelbrecht and Harrington, *C. pirilliformis, C. polychroma* M. van Wyk and M.J. Wingf., *C. albifundus*, *C. caryae* J.A. Johnson and Harrington, *C. smalleyi* J.A. Johnson and Harrington, *C. variospora* (Davids.) C. Moreau, *C. populicola* J.A. Johnson and Harrington, *C. tsitsikammensis* Kamgan Nkuekam and Jol. Roux and *C. atrox* M. van Wyk and M.J. Wingf. all represented distinct clades, supported by high bootstrap values. The isolates collected in this study formed four separate and distinct clades. Groups emerging from the phylogenetic analyses were consistent with the groups defined based on their morphology.

The isolates obtained from *A. mearnsii* from Tanzania (Group A, Group D) grouped in two clades. Group D isolates (CMW15991, CMW15992, CMW15999) resided in a distinct clade (Clade 1), close to *C. tsitsikammensis* with strong bootstrap support (100%). Group A isolates (CMW24806, CMW24861) resided in the *C. albifundus* clade (Clade 2), with 100% bootstrap support. Isolates (CMW24685, CMW24686) from *A. mearnsii* from Kenya, treated as Group A (CMW24685, CMW24686), also grouped in the *C. albifundus* clade (Clade 2). Isolates from *A. mearnsii* in South Africa resided in three clades (Group A, Group C, Group E). The first group of isolates, Group A (CMW23825, CMW23838) grouped within the *C. albifundus* clade (Clade 2). The other two groups of isolates (CMW23806, CMW23807, CMW23808) designated Group C (Clade 3) and Group E (CMW23809, CMW23818, CMW23819) (Clade 4) resided in two distinct clades with strong (100%) bootstrap support, close to the *C. pirilliformis* clade. Isolates (CMW15235, CMW15236, CMW15251) obtained from *E. grandis* from Malawi, designated Group B, also formed a distinct clade, separate from any other isolates and with strong (100%) bootstrap support, close to *C. pirilliformis* (Clade 5). Posterior probability values calculated for the branch nodes supported the bootstrap

values for all these clades and suggested that four previously undescribed species in the *C. fimbriata s.l.* group were collected during this study (Figure 1).

Three most parsimonious trees were obtained for the data set including the *Thielaviopsis* spp. (Figure 2). In the phylogenetic tree, *C. bhutanensis* M. van Wyk, M.J. Wingf. and T. Kirisits, *C. omanensis* Al-Subhi, M.J. Wingf, M. van Wyk and Deadman, *T. ovoidea* (Nag Raj et Kend.) Paulin, Harrington et McNew, *T. populi* (Veldeman ex Kiffer et Delan) Paulin, Harrington et McNew, *T. basicola* (Baker et Br.) Ferr., *C. polonica* (Siem.) C. Moreau and *C. resenifera* Harrington and M.J. Wingf. all represented distinct clades, supported by high bootstrap values. *Thielaviopsis* isolates collected from *Eucalyptus* in Tanzania (Group H) formed a separate, well supported clade (Figure 2). This tree had a length of 265 steps, the total number of characters were 468, with 303 of these being constant and 165 parsimony-informative, with CI = 0.8868, RI = 0.9690 and RC = 0.8593. In the phylogenetic tree, the *Thielaviopsis* isolates obtained in this study grouped most closely to *C. bhutanensis* and *C. omanensis*, separate from other known *Thielaviopsis* spp. and with strong bootstrap support (Figure 2). These isolates were then further compared to all *Ceratocystis* spp. in the *C. moniliformis s.l.* group using all three gene regions, confirming their unique nature, as well as their affinity to *C. bhutanensis* and *C. omanensis* (Figure 3).

Two most parsimonious trees were obtained for the combined data set of the *C. moniliformis s.l.* group, of which one is presented (Figure 3). This tree had a length of 578 steps, the total number of characters were 1273, with 844 constant, three characters parsimony-uninformative and 426 parsimony-informative characters, with CI = 0.9135, RI = 0.9595 and RC = 0.8765. In the phylogenetic tree, *C. savannae*, *C. omanensis*, *C. bhutanensis, C. moniliformis*, *C. tribiliformis* M. van Wyk and M.J. Wingf. and *C. moniliformopsis* Z.Q. Yuan and C. Mohammed all resided in distinct clades, supported by high bootstrap values. Clades emerging from the phylogenetic analyses were consistent with those (Group G, Group F, Group H) that emerged based on morphology for the African isolates. Isolates (CMW23802, CMW23803, CMW23804) obtained from *A. mearnsii* from South Africa (Group G) grouped in a distinct clade (Clade 6)

with strong (100%) bootstrap support, closest to *C. savannae*. Isolates in Group F obtained from *Eucalyptus* in Malawi (CMW15242, CMW15245, CMW15248) grouped in a distinct clade (Clade 7) with strong (100%) bootstrap support, most closely related to the isolates obtained from *A. mearnsii* from South Africa (Clade 6) and *C. savannae*. Isolates in Group H obtained from *Eucalyptus* spp. in Tanzania (CMW22284, CMW22289) and South Africa (CMW17587, CMW17960) grouped together within the *C. moniliformis* clade (Clade 8). Posterior probability values calculated for the branch nodes supported the bootstrap values (Figure 3).

Taxonomy

Comparison of DNA sequence data for the African isolates resulted in distinct phylogenetic lineages, consistent with the groups that emerged from morphological comparisons. These thus showed that five previously undescribed *Ceratocystis* spp. and one new *Thielaviopsis* sp. were amongst the isolates collected in this study (Table 2, Table 3). These fungi are consequently described as new taxa.

Ceratocystis zombamontana **R.N. Heath & Jol. Roux. sp. nov.** **(Fig. 4)**

(MB511245)

Etymology: The name refers to the Zomba mountain in Malawi where this fungus was first isolated.

Coloniae badiae. *Colla ascomatum* atrobrunnea vel nigra, apicem versus pallescentia (264-) 315 – 442 (-535) µm longa, apicibus (13-) 156 – 21 (-23) µm latis. *Ascosporae* lateraliter visae cucullatae vel pileiformes (4-) 5 – 6 (-6) X (3-) 3 – 4 (-5) µm, e supra visae doliiformes (5-) 6 – 7 (-8) X (5-) 6 – 7 (-8) µm. *Anamorpha Thielaviopsis* cum conidiophoris phialidicis in mycelio singulis, hyalinis. Conidiophorae primariae ampulliformes. Conidia primaria cylindrica baciliformia; secondaria doliiformia. *Chlamydosporae* absunt.

Colonies hair brown (17""i), reverse hair brown (17""i). *Mycelium* mostly superficial and smooth, sparse tawny olive (19"i) aerial mycelium. *Optimal temperature* for growth 20-25°C, minimal growth at 5°C and no growth at 35°C. Slow growing, reaching

41mm in 14 days at 20 and at 25°C. *Ascomatal bases* dark brown to black, subglobose to ovoid, (130-) 152 – 196 (-222) µm long, (106-) 132 – 181 (-208) µm wide. Ornamentation absent. *Ascomatal necks* dark brown to black at base becoming lighter brown to hyaline towards apices, (264-) 315 – 443 (-535) µm long, (24-) 30 – 38 (-42) µm wide at bases of necks, (13-) 16 – 21 (-23) µm wide at tips of necks. *Ostiolar hyphae* divergent, hyaline, (16-) 20 – 25 (-27) µm long. *Asci* not observed. *Ascospores* accumulating in round, white to yellow (yellow-buff 19d) masses at the apices of the ascomatal necks, embedded in sheath tissue, aseptate, hyaline, cucullate (hat-shaped) in side view, (4-) 5 – 6 (-6) µm long, (3-) 3 – 4 (-5) µm wide without sheath, doliiform in top view, (5-) 6 – 7 (-8) µm long, (5-) 6 – 7 (-8) µm wide including sheath.

Thielaviopsis anamorph: *Conidiophores* occurring singly on mycelium, conidiophores phialidic, hyaline, primary conidiophores flask shaped (55-) 69 – 101 (-119) µm long, (4-) 5 – 7 (-8) µm wide at bases, (3-) 3 – 5 (-5) µm wide at tips. Secondary conidiophores flaring, (72-) 74 – 117 (-128) µm long, (4-) 4 – 6 (-7) µm wide at bases, (4-) 4 – 4 (-6) µm wide at tips. Phialidic *conidium* development through ring wall building, *conidia* of two types, formed singly or in chains, primary conidia cylindrical, baciliform, (15-) 16 – 21 (-26) µm long, (3-) 3 – 4 (-6) µm wide, secondary conidia barrel-shaped, (5-) 6 – 7 (-9) µm long, (3-) 3 – 4 (-4) µm wide. *Chlamydospores* absent. *Specimens examined*: Malawi, Zomba Mountain (S 15° 21.269 E 035° 18.163). Isolated from wounds on *Eucalyptus grandis*. Collected: R.N. Heath and J. Roux, 2004. **Holotype**, PREM59804, **culture ex-type** CMW15236 = CBS122296.

Additional specimens: Malawi, Zomba Mountain (S 15° 21.269 E 035° 18.163). Isolated from wounds on *Eucalyptus grandis*. Colected: R.N. Heath and J. Roux, 2004. **Paratype**, PREM59805, **culture ex-type** CMW15235 = CBS122297; CMW15251 = CBS122298 = PREM59806; CMW15242 = PREM59807.

***Ceratocystis polyconidia* R.N. Heath & Jol. Roux. sp. nov. (Fig. 5)**

(MB511246)

Etymology: The name refers to the abundance of primary conidia produced.

Coloniae isabellinae. *Colla ascomatum* atrobrunnea vel nigra, apicem versus pallescentia vel hyalina (326-) 589 – 429 (-694) µm longa, apicibus (11-) 13 – 19 (-23) µm latis. *Ascosporae* lateraliter visae cucullatae vel pileiformes (3-) 3.1 – 4.1 (-5) X (3.5-) 4.3 – 5.3 (-5.8) µm, e supra visae doliiformes. *Anamorpha Thielaviopsis* cum conidiophoris phialidicis in mycelio singulis, hyalinis. Conidiophorae primariae ampulliformes. Conidiophorae secondariae expansae. Conidia primaria cylindrica baciliformia; secondaria doliiformia. *Chlamydosporae* terminales, singulae, parietibus crassis, subglobosae, brunneae.

Colonies isabella (19"i), reverse isabella (19"i). *Mycelium* mostly superficial, smooth, segmented, sparse hazel (17"i) aerial mycelium. *Optimal temperature* for growth 20-25°C. Minimal growth at 5°C and 35°C. Fast growing, reaching 81mm in 7 days at 25°C. *Ascomatal bases* dark brown to black, globose, (121-) 153 – 221 (-269) µm long, (133-) 153 – 223 (-277) µm wide. No ornamentation. *Ascomatal necks* dark brown to black at bases becoming lighter brown to hyaline towards apices, (326-) 389 – 429 (-694) µm long, (20-) 26 – 34 (-39) µm wide at bases of necks, (11-) 13 – 19 (-23) µm wide at tips of necks. *Ostiolar hyphae* divergent, hyaline, (36-) 39 – 47 (-57) µm long. *Asci* not observed. *Ascospores* accumulating in round, white to yellow (yellow-buff 19d) masses at the apices of the ascomatal necks, cucullate in side view, (3-) 3 – 4 (-5) µm long, (4-) 4 – 5 (-6) µm wide without sheath and (4-) 4 – 5 (-6) µm wide with sheath, doliiform in top view.

Thielaviopsis anamorph: Primary and secondary *conidiophores* occurring singly on mycelium, conidiophores phialidic, hyaline, primary conidiophores flask shaped, (72-) 74 – 117 (-128) µm long, (4-) 4 – 6 (-7) µm wide at bases, (6-) 6 – 8 (-8) at the widest point, (4-) 4 – 4 (-6) µm wide at tips. Secondary conidiophores flaring at apices, (38-) 52 – 87 (-105) µm long, (4-) 5 – 7 (-7) µm wide at bases, (5-) 6 – 8 (-9) µm wide at tips. Phialidic *conidium* development through ring wall building, *conidia* of two types, formed singly or in chains, primary conidia cylindrical, baciliform, (14-) 18 – 25 (-28) µm long, (4-) 4 – 6 (-6) µm wide, secondary conidia barrel-shaped, (8-) 9 – 11 (-13) µm long, (5-) 6 – 8 (-8) µm wide. *Chlamydospores* terminal, single, thick walled, sub-globose, argus brown (13m), (9-) 9 – 11 (-13) µm long, (8) 11 – 14 (-16) µm wide.

Specimens examined: South Africa, Piet Retief (S 26° 58' 68.5", E 030° 54' 28.3"). Isolated from cut stumps of *Acacia mearnsii*. Collected: R.N. Heath, 2006. **Holotype**, PREM59788, **culture ex-type** CMW23809 = CBS122289.

Additional specimens: South Africa, Piet Retief (S 26° 58' 68.5", E 030° 54' 28.3"). Isolated from cut stumps of *Acacia mearnsii*. Collected: R.N. Heath, 2006. **Paratype**, PREM59789, **culture ex-type** CMW23818 = CBS122290; CMW23819 = CBS122821 = PREM59790; CMW23817 = PREM59791, CMW23810 = PREM59863.

***Ceratocystis tanganyicensis* R.N. Heath & Jol. Roux sp. nov. (Fig. 6)**

(MB511247)

Etymology: The name refers to the famous lake Tanganyika in Tanzania, not far from where this fungus was first isolated.

Coloniae eburneo-atrovirides. *Colla ascomatum* atrobrunnea vel nigra, apicem versus pallescentia vel hyalina (302-) 366 – 484 (-558) µm longa, apicibus (13-) 14 – 18 (-21) µm latis. *Ascosporae* lateraliter visae cucullatae vel pileiformes (3-) 3 –5 (-6) X (4-) 4 – 6 (-8) µm vagina exclusa, e supra visae doliiformes. *Anamorpha Thielaviopsis* cum conidiophoris phialidicis. Conidiophorae primariae ampulliformes. Conidiophorae secondariae expansae. Conidia primaria cylindrica baciliformia; secondaria doliiformia. *Chlamydosporae* in hyphis singulae, globosae.

Colonies dark ivory green (25"m), reverse ivory green (25"m). *Mycelium* mostly superficial and smooth, sparse tawny olive (19"i) aerial mycelium. *Optimal temperature* for growth 20°C. No growth at 5°C or at 35°C. Slow growing, reaching 27mm in 7 days at 20°C. *Ascomatal bases* dark brown to black, subglobose, (127-) 149 – 190 (-216) µm long, (119-) 138 – 177 (-205) µm wide. No ornamentation. *Ascomatal necks* dark brown to black at base becoming lighter brown to hyaline towards apices, (302-) 366 – 484 (-558) µm long, (19-) 24 – 32 (-37) µm wide at bases of necks, (13-) 14 – 18 (-21) µm wide at tips of necks. *Ostiolar hyphae* divergent, hyaline, (17-) 39 – 47 (-47) µm long. *Asci* not observed. *Ascospores* accumulating in round, white to yellow (yellow-buff 19d) masses at the apices of the ascomatal necks, cucullate in side

view, (3-) 3 – 5 (-6) µm long, (4-) 4 – 6 (-8) µm wide without sheath and (5-) 6 – 7 (-8) µm wide with sheath, doliiform in top view.

Thielaviopsis anamorph: Primary and secondary *conidiophores* occurring singly on mycelium, conidiophores phialidic, hyaline, primary conidiophores flask shaped, (49-) 60 – 116 (-179) µm long, (4-) 5 –7 (-8) µm wide at bases, (4-) 6 – 8 (-9) at the widest point, (3-) 4 – 5 (-8) µm wide at tips. Secondary conidiophores flaring at the apices, (43-) 55 – 85 (-98) µm long, (4-) 5 – 7 (-8) µm wide at bases, (5-) 6 – 8 (-9) µm wide at tips. Phialidic *conidium* development through ring wall building, *conidia* of two types, formed singly or in chains, primary conidia cylindrical, bacilliform, (12-) 14 – 19 (-24) µm long, (3-) 4 – 5 (-5) µm wide, secondary conidia barrel-shaped, (3-) 3 – 9 (-13) µm long, (6-) 7 –10 (-12) µm wide. *Chlamydospores* developing singly on hyphae, argus brown (13m), globose, (10-) 10 – 13 (-14) µm long, (3-) 10 – 12 (-13) µm wide.

Specimens examined: Tanzania, Njombe area (S 09° 16.366 E 034° 38.765). Isolated from cut-stumps of *Acacia mearnsii*. Collected R.N. Heath and J. Roux, 2004. **Holotype**, PREM59800, **culture ex-type** CMW15992 = CBS122293.

Additional specimens: Tanzania, Njombe area (S 09° 16.366 E 034° 38.765). Isolated from cut-stumps of *Acacia mearnsii*. Collected R.N. Heath and J. Roux, 2004. **Paratype**, PREM59801, **culture ex-type** CMW15999 = CBS122294; CMW15991 = CBS122295 = PREM59802; CMW15993 = PREM59803, CMW15988 = PREM59864.

***Ceratocystis obpyriformis* R.N. Heath & Jol. Roux sp. nov.** **(Fig. 7)**

(MB511248)

Etymology: The name refers to the distinctly obpyriform shape of the ascomatal bases.

Coloniae olivaceae. *Colla ascomatum* atrobrunnea vel nigra, apicem versus pallescentia (477-) 569 – 675 (-708) µm longa, apicibus (13-) 16 – 21 (-26) µm latis. *Ascosporae* lateraliter visae cucullatae vel pileiformes (3-) 3 – 4 (-6) µm longa, apicibus (3-) 4 – 5 (-7) µm latis vagina exclusa, e supra visae doliiformes. *Anamorpha Thielaviopsis* cum conidiophoris phialidicis in mycelio singulis, hyalinis. Conidiophorae primariae ampulliformes. Conidiophorae secondariae expansae. Conidia primaria cylindrica baciliformia; secondaria doliiformia. *Chlamydosporae* absunt.

Colonies olivaceous (21"k), reverse olivaceous (21"k). *Mycelium* mostly superficial and smooth, sparse white aerial mycelium. *Optimal temperature* for growth at 25°C. No growth at 5°C or at 35°C. Fast growing, reaching 52mm in 7 days at 25°C. *Ascomatal bases* dark brown to black, obpyriform, (152-) 177 - 217 (-233) µm long, (149-) 166 – 206 (-228) µm wide. No ornamentations. *Ascomatal necks* dark brown to black at base becoming lighter brown to hyaline towards apices, (477-) 569 – 675 (-708) µm long , (26-) 28 – 36 (-45) µm wide at bases of necks, (13-) 16 – 21 (-26) µm wide at tips of necks. *Ostiolar hyphae* divergent, hyaline, (34-) 37 – 47 (-54) µm long. *Asci* not observed. *Ascospores* accumulating in round, white to yellow (yellow-buff 19d) masses at the apices of the ascomatal necks, embedded in sheath, aseptate, hyaline, cucullate in side view, (3-) 3 – 4 (-6) µm long, (3-) 4 – 5 (-7) µm wide without sheath and (4-) 5 – 8 (-8) µm wide with sheath, doliiform in top view.

Thielaviopsis anamorph: Primary and secondary *conidiophores* occurring singly on mycelium, conidiophores phialidic, hyaline, primary conidiophores flask shaped, (57-) 75 – 124 (-157) µm long, (4-) 5 – 6 (-7) µm wide at bases, (3-) 6 – 8 (-8) µm at the widest point, (3-) 4 – 5 (-5) µm wide at tips. Secondary conidiophores flaring at apices, (54-) 56 – 74 (-83) µm long, (4-) 4 – 6 (-7) µm wide at bases, (5-) 6 –8 (-9) µm wide at tips. Phialidic *conidium* development through ring wall building, *conidia* of two types, formed singly or in chains, primary conidia cylindrical, bacilliform, (14-) 15 – 20 (-24) µm long, (3-) 3 – 5 (-6) µm wide, secondary conidia barrel-shaped, (8-) 10 – 12 (-13) µm long, (5-) 6 – 8 (-9) µm wide. *Chlamydospores* absent.

Specimens examined: South Africa, Piet Retief (S 26° 58' 68.5", E 030° 54' 28.3"). Isolated from cut stumps of *Acacia mearnsii*. Collected: R.N. Heath, 2006. **Holotype**, PREM59796, **culture ex-type** CMW23808 = CBS122511.

Additional specimens: South Africa, Piet Retief (S 26° 58' 68.5", E 030° 54' 28.3"). Isolated from cut stumps of *Acacia mearnsii*. Collected: R.N. Heath, 2006. **Paratype**, PREM59797, **culture ex-type** CMW23807 = CBS122608; CMW23806 = CBS12609 = PREM59798; CMW27862 = PREM59799.

***Ceratocystis oblonga* R.N. Heath & Jol. Roux sp. nov.** **(Fig. 8)**

(MB511249)

Etymology: The name refers to the oblong shape of the secondary conidia.

Coloniae iuvenes albae, fuscantes. *Colla ascomatum* atrobrunnea vel nigra, apicem versus pallescentia (405-) 5025 – 721 (-881) µm longa, apicibus (12-) 13 – 18 (-23) µm latis. *Ascosporae* lateraliter visae cucullatae vel pileiformes (3-) 3 – 4 (-4) X (4-) 5 – 6 (-6) µm vagina exclusa, e supra visae doliiformes. *Anamorpha Thielaviopsis* cum conidiophoris phialidicis in mycelio singulis, hyalinis. Conidiophorae secondariae expansae. Conidia primaria cylindrica baciliformia; secondaria oblonga, apicibus truncatis. *Chlamydosporae* absunt.

Colonies white when young, becoming deep colonial buff (21"b), reverse grayish sepia (15""i). *Mycelium* superficial producing aerial mycelia. *Hyphae* granular. *Optimal temperature* for growth at 20-25°C. Minimal growth at 5°C and 35°C. Fast growing, reaching 83mm in 7 days at 25°C. *Ascomatal bases* dark brown to black, obpyriform, (149-) 206 – 329 (-372) µm long, (130-) 180 – 254 (-315) µm wide with conical spines, (8-) 11 – 16 (-19) µm long. *Ascomatal necks* dark brown to black at base becoming lighter brown to hyaline towards apices, (405-) 502 – 721 (-881) µm long, (30-) 46 – 69 (-76) µm wide at bases of necks, (12-) 13 – 18 (-23) µm wide at tips of necks with disciform bases. *Ostiolar hyphae* divergent, hyaline, (22-) 22 – 27 (-31) µm long. *Asci* not observed. *Ascospores* accumulating in round, white to yellow (yellow-buff 19d) masses at the apices of the ascomatal necks, embedded in sheath, aseptate, hyaline, cucullate (hat-shaped) in side view, (3-) 3 – 4 (-4) µm long, (4-) 5 – 6 (-6) µm wide without sheath and (6-) 7 – 8 (-8) µm with sheath, doliiform in top view.

Thielaviopsis anamorph: Primary and secondary *conidiophores* occurring singly on mycelium, conidiophores phialidic, hyaline, primary conidiophores flask shaped, (19-) 21 – 35 (-41) µm long, (2-) 3 – 4 (-4) µm wide at bases, (2-) 3 – 4 (-5) µm at the widest point, (2-) 2 – 3 (-3) µm wide at tips. Secondary conidiophores flaring at apices, (23-) 29 – 50 (-59) µm long, (3-) 3 – 4 (-5) µm wide at bases, (4-) 4 – 6 (-6) µm wide at tips. Phialidic *conidium* development through ring wall building, *conidia* of two types, formed singly or in chains, primary conidia cylindrical, bacilliform, (12-) 14 – 19 (-23) µm long, (3-) 3 – 5 (-5) µm wide, secondary conidia oblong, apices truncate, (5-) 6 – 7 (-9) µm long, (3-) 4 – 5 (-6) µm wide. *Chlamydospores* absent.

Specimens examined: South Africa, Piet Retief (S 26° 58' 68.5", E 030° 54' 28.3"). Isolated from cut stump of *Acacia mearnsii*. Collected: R.N. Heath, 2006. **Holotype**, PREM59792, culture **ex-type** CMW23803 = CBS122291.

Additional specimens: South Africa, Piet Retief (S 26° 58' 68.5", E 030° 54' 28.3"). Isolated from cut stump of *Acacia mearnsii*. Collected: R.N. Heath, 2006. **Paratype**, PREM59793, **culture ex-type** CMW23802 = CBS122820; CMW23804 = CBS122292 = PREM59794; CMW23805 = PREM59795.

***Thielaviopsis ceramica* R.N. Heath & Jol. Roux. sp. nov. (Fig. 9)**

(MB511250)

Etymology: The name originates from the historic name of the geographical region (Zomba, Malawi) where the fungus was found. Historically, the name Zomba originated from the fact that early settlers used the area to produce clay pots (called Zoomba omba), where after the region was called Zomba. "*ceramica*" means clay in Greek.

Coloniae iuvenes albae, cum aetate atrobubalinae. Mycelium granulare. Conidiophorae phialidicae in mycelio singulae, hyalinae. *Conidiophorae* primariae ampulliformes (20-) 22 – 30 (-36) µm longae, basi (2-) 3 – 4 (-5) µm, apicibus (2-) 2 – 2 (-3) µm latae. *Conidiophorae* secondariae expansae (23-) 33 – 46 (-53) µm longae, basi (1-) 2 –3 (-3) µm, apicibus (2-) 2 – 3 (-3) µm. *Conidia* primaria cylindrica baciliformia; secondaria doliiformia. *Chlamydosporae* absunt.

Colonies white when young, becoming deep colonial buff (21"b). *Mycelium* superficial producing aerial mycelia, granular. *Optimal temperature* for growth at 25°C. No growth at 5, 10 or at 35°C. Fast growing, reaching 90mm in four days at 25°C. *Conidiophores* occurring singly on mycelium, conidiophores phialidic, hyaline, primary conidiophores flask shaped, (20-) 22 – 30 (-36) µm long, (2-) 3 – 4 (-5) µm wide at bases, (3-) 3 – 4 (-5) µm wide at middle, (2-) 2 – 2 (-3) µm wide at tips. Secondary conidiophores flaring at apices, (25-) 35 – 46 (-53) µm long, (1-) 2 – 3 (-3) µm wide at bases, (2-) 2 – 3 (-3) µm wide at tips. Phialidic *conidium* development through ring wall building, *conidia* of two types, formed singly or in chains, primary conidia cylindrical,

bacilliform, (4-) 6 – 8 (-10) µm long, (1-) 2 (-3) µm wide, secondary conidia barrel-shaped, (2-) 2 – 3 (-4) µm long, (3-) 4 – 6 (-8) µm wide. *Chlamydospores* absent.
Specimens examined: Malawi, Zomba Mountain (S 15° 21.269 E 035° 18.163). Isolated from wounds on *Eucalyptus grandis*. Collected: R.N. Heath and J. Roux, 2004. **Holotpe**, PREM59808, **culture ex-type** CMW15245 = CBS122299, CMW15251

Additional specimens: Malawi, Zomba (S 15° 21.269 E 035° 18.163). Isolated from wounds on *Eucalyptus grandis*. Colected: R.N. Heath and J. Roux, 2004. **Paratype**: PREM59809, **culture ex-type** CMW15248 = CBS122300; CMW15246 = CBS122624 = PREM59810; CMW15238 = PREM59811, CMW15249 = PREM59865.

2.3.4. Pathogenicity tests

Greenhouse inoculations on *A. mearnsii* trees with *Ceratocystis* isolates collected in this study resulted in distinct lesions, whereas the control inoculations produced no lesions (Figure 10). Lesions associated with all isolates differed significantly from the controls ($P<0.0001$). Although *C. polyconidia* (CMW23809, CMW23818) produced the longest lesions, and *C. obpyriformis* (CMW23807, CMW23808) produced the shortest lesions, there were no statistical differences between the lesion lengths produced by the test isolates. After six weeks, the wounds of the control inoculations had begun to recover and to produce callus tissue. All test organisms were consistently re-isolated from the lesions after six weeks. Both replicates of the experiments produced similar results.

Greenhouse inoculations on the *E. grandis* (ZG14) trees with all *Ceratocystis* and *Thielaviopsis* isolates resulted in distinct lesions, whereas the control inoculations produced no lesions (Figure 11). *Ceratocystis zombamontana* (CMW15235, CMW15236) produced significantly larger lesions than *T. ceramica* (CMW15248, CMW 15245) or the control ($P<0.0001$). However, *T. ceramica* also produced significantly larger lesions than the control inoculations ($P<0.0001$). Both the test organisms were consistently isolated from the lesions. Both replicates of the experiments produced the same results.

2.4. DISCUSSION

This study focused on identifying *Ceratocystis* spp. from two non-native plantation tree species in southern and eastern Africa, led to the discovery of six previously undescribed *Ceratocystis* spp. In addition, *C. albifundus* and *C. moniliformis s.s.* were commonly encountered. Identification of the species arose from a combination of morphological characteristics and comparisons of DNA sequence data, the latter of which were important in recognizing new species for fungi that are morphologically similar.

Two of the previously undescribed species encountered in this study are related to *C. moniliformis* and reside in a group that we refer to as the *C. moniliformis s.l.* species complex. Fungi in the *C. moniliformis s.l.* group can easily be distinguished from other *Ceratocystis* spp. based on the presence of conical spines on their ascomatal bases (Hedgcock 1906, Hunt 1956, Upadhyay 1981). This group also produces disc-like structures at the bases of the ascomatal necks (Bakshi 1951, Hunt 1956). The two species described in this study have been provided with the names *C. oblonga* and *T. ceramica.*

Ceratocystis oblonga grouped close to *C. savannae* within the *C. moniliformis s.l.* species complex, but in a discrete clade with strong bootstrap support. It can also be distinguished from *C. savannae* based on colony colour. In this regard, *C. oblonga* produces white colonies when young, turning colonial buff with age, whereas *C. savannae* produces smokey gray cultures (Kamgan *et al.* 2008). *Ceratocystis oblonga* also has hyphae that have a granular appearance, whereas *C. savannae* has smooth-walled hyphae. Furthermore, *C. savannae* does not produce secondary phialides, but these structures are common in *C. oblonga*. *Ceratocystis oblonga* produces significantly smaller primary and secondary conidiophores than those of *C. savannae* and it produces secondary conidia that are oblong with truncate apices, which separates it from all other *Ceratocystis* spp. Another clear distinction between *C. oblonga* and *C. savannae* is found in the shapes of their ascomatal bases. *Ceratocystis oblonga*

produces obpyriform ascomatal bases in contrast to the globose ascomatal bases produced by *C. savannae*. *Ceratocystis oblonga* also differs from *C. savannae* and most other species in the *C. moniliformis s.l.* group, other than *C. bhutanensis* (Van Wyk *et al*. 2004, Kamgan *et al*. 2008), in the fact that it is able to grow at 5°C, producing colonies of up to 5mm diameter after four days of growth. Together with *C. bhutanensis*, it is one of two species in this group adapted to growth at low temperatures.

Thielaviopsis ceramica is phylogenetically most closely related to *C. bhutanensis*. These species are, however, fungi with vastly different ecologies with one occurring on wounds on *Eucalyptus* spp. in Africa and the other associated with conifer infesting bark beetles in Bhutan (Van Wyk *et al*. 2004). The two species can further be distinguished based on various morphological characteristics. *Thielaviopsis ceramica* produces colonial buff-coloured colonies whereas those of *C. bhutanensis* are cream-buff to dark olive to black in colour. *Thielaviopsis ceramica* also produces slightly longer (4-6 µm) barrel shaped conidia than that of *C. bhutanensis* (3-5 µm). As no teleomorph structures could be induced for *T. ceramica* no other morphological comparisons were possible.

Ceratocystis moniliformis was isolated from *Eucalyptus* stumps in South Africa and Tanzania. This fungus has previously been reported from artificially induced wounds on *Eucalyptus* spp. in South Africa (Roux *et al.* 2004) but this is the first confirmed report of its occurrence elsewhere in Africa. Isolation of *C. moniliformis* was not surprising as it is a fungus with a broad global distribution and host range (Davidson 1935, Bakshi 1951, Hunt 1956, Van Wyk *et al*. 2006b). It is not known to be a pathogen of *Eucalyptus* spp. or other tree species.

Five of the species collected in this study reside in the *C. fimbriata s.l.* species complex. Of these, four were of previously undescribed species which we have provided with the names *C. tanganyicensis*, *C. zombamontana*, *C. polyconidia* and *C. obpyriformis*. Fungi in the *C. fimbriata s.l.* species complex are generally known as virulent pathogens and they include species such as *C. fimbriata s.s.*, *C. platani, C. cacaofunesta* and *C.*

albifundus. Although species in this group are aggressive pathogens, they are not as fast growing as species in the *C. moniliformis s.l.* species complex and in this respect, they can generally be distinguished from the later group based on culture morphology.

Ceratocystis zombamontana is most closely related to *C. pirilliformis* within the *C. fimbriata s.l.* species complex, but it resides in a distinct phylogenetic clade. This species produces hair brown cultures in contrast to the pale olivaceous grey cultures found in *C. pirilliformis. Ceratocystis zombamontana* has shorter ascomatal necks that are wider at the tip compared to that of *C. pirilliformis* and it has significantly larger ascospores than those of the former species. Furthermore, *C. zombamontana* produces flask-shaped primary phialides compared to the cylindrical to lageniform primary phialides of *C. pirilliformis*. Another distinct morphological difference between these two phylogeneticaly closely related species is that *C. pirilliformis* produces clamydospores (Barnes *et al*. 2003), whereas these structures have not been found in *C. zombamontana*.

Ceratocystis tanganyicensis, isolated from *A. mearnsii,* resides in a large clade, with *C. tsitsikammensis,* but forming a distinct clade with strong bootstrap support. Although this species is phylogenetically closest to *C. tsitsikammensis*, it can clearly be distinguished from that species based on culture morphology. In this regard, *C. tanganyicensis* produces ivory green cultures, whereas those of *C. tsitsikammensis* are a greenish olivaceous colour. *Ceratocystis tanganyicensis* also grows optimally at 20°C while *C. tsitsikammensis* grows optimally at 25°C. *Ceratocystis tanganyicensis* can be distinguished from *C. tsitsikammensis* based on the sub-globose ascomatal bases in *C. tanganyicensis* compared to the globose ascomatal bases of *C. tsitsikammensis*. Furthermore, *C. tanganyicensis* produces longer (39-47µm) ostiolar hyphae than *C. tsitsikammensis* (23-38 µm) (Kamgan *et al*. 2008).

Ceratocystis polyconidia and *C. obpyriformis*, from *A. mearnsii* in South Africa reside in distinct clades, however, both grouped closely with *C. pirilliformis* and *C. tanganyicensis* in phylogenetic analyses. *Ceratocystis polyconidia* could be

distinguished from *C. pirilliformis* based on culture morphology as it produces dark ivory grey cultures compared to those of *C. pirilliformis* which are pale olivaceous grey. *Ceratocystis polyconidia* can be distinguished from *C. obpyriformis* and *C. pirilliformis* by its globose ascomatal bases, compared to the pyriform ascomatal bases in the latter species and the obpyriform ascomatal bases in *C. obpyriformis*. *Ceratocystis obpyriformis* can further be distinguished from *C. pirilliformis* and *C. polyconidia* as these species produce chlamydospores (Barnes *et al*. 2003) whereas these structures are absent in *C. obpyriformis*.

Ceratocystis albifundus was isolated from *A. mearnsii* stumps in three of the countries in which collections were made. Identification of this species can easily be achieved using morphology, as it is the only species in the group with light coloured ascomatal bases. Isolation of *C. albifundus* from *A. mearnsii* from Tanzania, Kenya and South Africa was expected as this species has previously been reported from these countries causing wilting and death of trees (Roux & Wingfield 1997, Roux *et al.* 2005). In South Africa it is considered the most important pathogen of *A. mearnsii* and an important factor in plantation health (Roux & Wingfield 1997).

Pathogenicity tests showed that all the species described in this study can give rise to lesions on the host plants from which they were isolated. Under field conditions all the species were isolated from wounds and although inoculation trials gave rise to lesions, it is unknown whether they are able to cause disease in nature. As a number of species in the *C. fimbriata s.l.* group are known to be pathogens, it is possible to speculate that isolates obtained in this study, and residing in the *C. fimbriata s.l.* species complex, could be important pathogens. In contrast, based on knowledge of the isolates residing in the *C. moniliformis s.l.* complex, these are probably not pathogenic in nature, but predominantly saprophytic.

This study presents the most comprehensive consideration of wound-infecting *Ceratocystis* spp. on non-native plantation grown tree species in Southern and Eastern Africa. Recently it has been estimated that there are approximately 171 500 fungal

species in South Africa (Crous *et al.* 2006). The fact that six new fungal species were found in a limited study in three countries emphasizes the distinct lack of knowledge regarding microfungi in Africa. This is even more so if one considers that this study reports three previously undescribed species for South Africa, a country in which research on the health of plantation forestry trees has a significant history.

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Table 1. *Ceratocystis* isolates obtained and used in this study.

Species	Culture nr.	Host	Geographical origin	Collector(s)	GenBank Accession no. ITS. BT, EF1α
C. albifundus	[b] CMW4068	*Acacia mearnsii*	South Africa	J. Roux	DQ520638, EF070429, EF070400
"	[b] CMW5329	"	Uganda	J. Roux	AF 388947, DQ371649, EF070401
"	[a] CMW23823, CMW23824	"	South Africa	R.N. Heath	
"	[a b] CMW23825	"	"	"	EU245010, EU244982, EU244942
"	[a] CMW23826-CMW23837	"	"	"	
"	[a b] CMW23838	"	"	"	EU245009, EU244981, EU244941
"	[a b] CMW24860	"	Tanzania	R.N. Heath & J. Roux	EU245011, EU244983, EU244943
"	[a b] CMW24861	"	"	"	EU245012, EU244984, EU244944
	[a] CMW24862-CMW24887	"	"	"	
"	[a b] CMW24685	"	Kenya	"	EU245013, EU244985, EU244945
"	[a b] CMW24686	"	"	"	EU245014, EU244986, EU244946
"	[a] CMW27876, CMW27877	"	"	"	
C. atrox	[b] CMW19383	*Eucalyptus grandis*	Australia	M.J. Wingfield	EF070414, EF070430, EF070402
"	[b] CMW19385	"	"	"	EF070415, EF070431, EF070403
C. bhutanensis	[b] CMW8217, CBS114289	*Ips schmutzenhoferi*	Bhutan	M.J. Wingfield, T. Kirisits, D.B. Chhetri	AY528957, AY528962, AY528952
C. bhutanensis	[b] CMW8242, CBS112907	"	Bhutan	"	AY528956, AY528961, AY528951
C. cacaofunesta	[b] CMW15051, CBS152.62	*Theobroma cacao*	Costa Rica	A.J. Hansen	DQ520636, EF070427, EF070398
C. cacaofunesta	[b] CMW14809, CBS115169	"	Ecuador	C. Suarez	DQ520637, EF070428, EF070399
C. caraye	[b] CMW14793, CBS114716	*Carya cordiformis*	USA	J. Johnson	EF070424, EF070439, EF070412
C. caraye	[b] CMW14808, CBS115168	*C. ovata*	"	"	EF070423, EF070440, EF070411
C. fimbriata	[b] CMW15049, CBS141.37	*Ipomaea batatas*	"	C.F. Andrus	DQ520629, EF070442, EF070394
C. fimbriata	[b] CMW1547	*I. batatas*	Papua New Guinea	E.C.H. McKenzie	AF264904, EF070443, EF070395
C. moniliformis	[a b] CMW22284	*E. grandis*	Tanzania	J. Roux & R.N. Heath	EU245015, EU244987, EU244947

Species	Culture nr.	Host	Geographical origin	Collector(s)	GenBank Accession no. ITS. BT, EF1α
C. moniliformis	[a] [b] CMW22289	*E. grandis*	Tanzania	J. Roux & R.N. Heath	EU245016, EU244988, EU244948
"	[a] CMW27864 – CMW27875	"	"	"	
"	[a] CMW17584	*Eucalyptus* sp.	South Africa	G. Kamgan Nkuekam	
"	[a] [b] CMW17587	"	"	"	EU245017, EU244989, EU244949
"	[a] [b] CMW17690	"	"	"	EU245018, EU244990, EU244950
"	[a] CMW27863	"	"	"	
"	[b] CMW9590	"	"	J. Roux	AY528985, AY528996, AY529006
"	[b] CMW4114	"	Ecuador	M. J. Wingfield	AY528986, AY528997, AY529007
C. moniliformopsis	[b] CMW10215, CBS115793	*E. oblique*	Australia	Z.Q. Yuan	AY528999, AY528988, AY529009
"	[b] CMW9986, CBS109441	"	"	"	AY528987, AY528998, AY529008
C. oblonga	[a] [b] CMW23802	*A. mearnsii*	South Africa	R.N. Heath	EU245020, EU244992, EU244952
"	[a] [b] CMW23803	"	"	"	EU245019, EU244991, EU244951
"	[a] [b] CMW23804	"	"	"	EU245021, EU244993, EU244953
"	[a] CMW23805	"	"	"	
C. obpyriformis	[a] [b] CMW23806	*A. mearnsii*	South Africa	"	EU245005, EU244977, EU244937
"	[a] [b] CMW23807	"	"	"	EU245004, EU244976, EU244936
"	[a] [b] CMW23808	"	"	"	EU245003, EU244975, EU244935
"	[a] CMW27862	"	"	"	
C. omanensis	[b] CMW11046, CBS118112	*Mangifera indica*	Oman	A. O. Al-Adawi	DQ074739, DQ074729, DQ074734
"	[b] CMW11048, CBS115780	"	"	"	DQ074742, DQ074732, DQ074737
C. pirilliformis	[b] CMW6569	*Eucalyptus nitens*	Australia	M.J. Wingfield	AF427104, DQ371652, AY528982
"	[b] CMW6579	"	"	"	AF427105, DQ371653, AY528983
C. platani	[b] CMW14802, CBS115162	*Platanus occidentalis*	USA	T.C. Harrington	DQ520630, EF070425, EF070396
C. platani	[b] CMW23918	"	Greece	M.J. Wingfield	EU426554, EU426555, EU426556
C. polonica	[b] CMW5026	n/a	n/a	n/a	AY233907

Species	Culture nr.	Host	Geographical origin	Collector(s)	GenBank Accession no. ITS. BT, EF1α
C. polonica	[b] CMW1165	n/a	n/a	n/a	AY233906
C. polychroma	[b] CMW11424, CBS115778	*Syzygium aromaticum*	Indonesia	M.J. Wingfield	AY528970, AY528966, AY528978
"	[b] CMW11436, CBS115777	"	"	"	AY528971, AY528967, AY528979
C. polyconidia	[a b] CMW23809	*A. mearnsii*	South Africa	R.N. Heath	EU245006, EU244978, EU244938
"	[a] CMW23810-CMW23817	"	"	"	
"	[a b] CMW23818	"	"	"	EU245007, EU244979, EU244939
"	[a b] CMW23819	"	"	"	EU245008, EU244980, EU244940
"	[a] CMW23820-CMW23822	"	"	"	
C. populicola	[b] CMW14789, CBS119.78	*Populus* sp.	Poland	J. Gremmen	EF070418, EF070434, EF070406
"	[b] CMW14819, CBS114725	"	USA	T. Hinds	EF070419, EF070435, EF070407
C. resinifera	[b] CMW20931, CBS100202	*Picea* sp.	Norway	H. Solheim	U75616
"	[b] CMW26371, CBS100204	"	USA	T. Hinds	U75618
C. savannae	[b] CMW17300	*Acacia nigrescens*	South Africa	G. Kamgan Nkuekam	EF408551, EF408565, EF408572
"	[b] CMW17279	*Combretum zeyheri*	"	"	EF408552, EF408566, EF408573
C. smalleyi	[b] CMW14800, CBS114724	*Carya cordiformis*	USA	G. Smalley	EF070420, EF070436, EF070408
C. tanganyicensis	[a] CMW15988-CMW15990	*A. mearnsii*	Tanzania	R.N. Heath & J. Roux	
"	[a b] CMW15991	"	"	"	EU244997, EU244969, EU244929
"	[a b] CMW15992	"	"	"	EU244999, EU244971, EU244931
"	[a] CMW15993-CMW15998	"	"	"	
"	[a b] CMW15999	"	"	"	EU244998, EU244970, EU244939
C. tanganyicensis	[a] CMW16000-CMW16007	*A. mearnsii*	Tanzania	R.N. Heath & J. Roux	
"	[a] CMW27878-CMW27892	"	"	"	
C. tribiliformis	[b] CMW13013	*Pinus merkusii*	Indonesia	M.J. Wingfield	AY528993, AY529003, AY529014
"	[b] CMW13015	"	"	"	AY528994, AY529004, AY529015
C. tsitsikammensis	[b] CMW14276	*Rapanea melanophloeos*	South Africa	G. Kamgan Nkuekam	EF408555, EF408569, EF408576

Species	Culture nr.	Host	Geographical origin	Collector(s)	GenBank Accession no. ITS. BT, EF1α
C. tsitsikammensis	[b] CMW14278	*Rapanea melanophloeos*	South Africa	G. Kamgan Nkuekam	EF408556, EF408570, EF408577
C. variospora	[b] CMW20935, CBS 114715	*Quercus alba*	USA	J. Johnson	EF070421, EF070437, EF070409
"	[b] CMW20936, CBS 114714	*Q. robur*	"	"	EF070422, EF070438, EF070410
C. virescens	[b] CMW11164	*Fagus americanum*	"	D. Houston	DQ520639, EF070441, EF070413
"	[b] CMW3276	*Quercus* sp.	USA	T. Hinds	AY528984, AY528990, AY5289991
C. zombamontana	[a b] CMW15251	*Eucalyptus* spp.	Malawi	R.N. Heath & J. Roux	EU245001, EU244973, EU244933
"	[a b] CMW15235	"	"	R.N. Heath & J. Roux	EU245002, EU244974, EU244934
"	[a b] CMW15236	"	"	R.N. Heath & J. Roux	EU245000, EU244972, EU244932
"	[a] CMW15242	"	"	R.N. Heath & J. Roux	
T. basicola	[b] C1602	n/a	n/a	n/a	AF275490
"	[b] C1373	n/a	n/a	n/a	AF275482
T. ceramica	[a b] CMW15245	*Eucalyptus* sp.	Malawi	R.N. Heath & J. Roux	EU245022, EU244994, EU244926
	[a] CMW15237	"	"	"	
"	[a] CMW15238	"	"	"	
	[a] CMW15240, CMW15241	"	"	"	
"	[a b] CMW15246	"	"	"	EU245023, EU244995, EU244927
"	[a b] CMW15248	"	"	"	EU2450024, EU244996, EU244928
	[a] CMW15249	"	"	"	
T. ceramica	[a] CMW17139-CMW17141	*Eucalyptus* sp.	Malawi	R.N. Heath & J. Roux	
"	[a] CMW24169-CMW24172	"	"	"	
T. ovoidea	[b] C1375	n/a	n/a	n/a	AF275483
"	[b] C1376	n/a	n/a	n/a	AF275484
T. populi	[b] CBS484.71	*Populus* sp.	n/a	n/a	AF275479
"	[b] CBS486.71	"	n/a	n/a	AF275480

[a] Isolates obtained in this study
[b] Isolates used in phylogenetic analysis in this study

Table 2. Morphological differences between species described in this study and closely related species in the *C. fimbriata sensu lato* group.

Character	*C. pirilliformis*	*C. zombamontana*	*C. polyconidia*	*C. tanganyicensis*	*C. obpyriformis*	*C. tsitsikammensis*
ASCOMATA						
Base						
Shape	Pyriform to Globose	Obpyriform	Globose	Subglobose	Obpyriform	Globose
Colour	Black	Dark brown to black	Dark brown to black	Dark brown to black	Dark brown to black	Black
Diameter	115-187(-206) µm	(106-)132-181(-208) µm	(133-)153-223(-277) µm	(119-)138-177(-205) µm	(149-)166-206(-228) µm	(124-)143-175(-186) µm
Ornamentation	None	None	None	None	None	None
Neck						
Colour	Black	Dark brown to black	Dark brown to black	Dark brown to black	Dark brown to black	Black
Collar	None	None	None	None	Collar	None
Length	372-683(-778)µm	(264-)315-443(-535) µm	(326-)429-589(694) µm	(302-)365-484(-558) µm	(477)569-675(-708) µm	(217-)321-425(-465) µm
Width (tip)	12-21 (-25) µm	(13-)16-21(-23) µm	(11-)13-19(-23) µm	(13-)14-18(-21) µm	(13-)16-21(-26) µm	n/a
Width (base)	19-33(-40)µm	(24-)30-38(-42) µm	(20-)26-34(-39) µm	(19-)24-32(-37) µm	(26-)28-36(-45) µm	(31-)32-47(62) µm
Ostiolar hyphae						
Shape	Convergent	Divergent	Divergent	Divergent	Divergent	Divergent
Length	n/a	(16-)20-25(-27) µm	(36-)39-47(-57) µm	(17-)39-47(-47) µm	(34.-)39-47(-54) µm	(23-)28-38(-42) µm
Ascospores						
Colour	Hyaline	Hyaline	Hyaline	Hyaline	Hyaline	Hyaline
Shape (side view)	Hat-shaped	Hat-shaped	Hat-shaped	Hat-shaped	Hat-shaped	Hat-shaped
Length	3-5(-7)µm	(4-)5-6(-6) µm	(3-)3-4(-5) µm	(3-)3-5(-6) µm	(3-)3-4(-6) µm	(4-)5-(-7) µm
Width	2-4(-5)µm	(3-)3-4(-5) µm	(4-)4-5(-6) µm	(4-)4-6(-8) µm	(3-)4-5 (-7) µm	(2-)3-4(-5) µm
Aggregation	n/a	Yellow-buff	Yellow-buff	Yellow-buff	Yellow-buff	n/a

Table 3. Morphological differences between species described in this study and closely related species in the *C. moniliformis sensu lato* group.

Character	*C. oblonga*	*C. savannae*	*C. moniliformis*[a]
ASCOMATA			
Base			
Shape	Obpyriform	Globose	Globose
Colour	Dark brown to black	Dark brown	Brown / black
Diameter	(13-)180-254(-315) µm	(155-)178-217(-248) µm	90-180
Ornamentation	(8-)11-16(-19) µm	(1-)3-8(-13) µm	12-16 x 6 µm
Neck			
Colour	Dark brown to black	Dark brown	Na
Collar	Disc shaped	Disc shaped	Na
Length	(405-)502-721(-881) µm	(359-)455-703(-775) µm	Na
Width (tip)	(12-)13-18(-23) µm	(13-)12-21(-24) µm	Na
Width (base)	(30-)46-69(-76) µm	(37-)48-59(-62) µm	Na
Ostiolar hyphae			
Shape	Divergent	Divergent	Divergent
Length	(22-)23-27(31) µm	(17-)25-40(-46) µm	12-18 µm
Ascospores			
Colour	Hyaline	Hyaline	Hyaline
Shape (side view)	Hat-shaped	Hat-shaped	Oval, one side flat
Length	(3)-3-4(-4) µm	(5-)5-5 (-6) µm	4-5 µm
Width	(6-)7-8(-8) µm	(2-)3-3(-4) µm	3-4 µm
Aggregation	Yellow-buff	Straw yellow	Na

[a]Hedgcock 1906

Figure 1. Phylogenetic tree based on the combined ITS, *β*-tubulin and EF1-α gene regions of the *Ceratocystis fimbriata sensu lato* group of isolates. The phylogram was obtained using the heuristic search option based on parsimony. Bootstrap values are indicated above the branches with posterior probability values in brackets. *Ceratocystis virescens* represents the out-group taxon. Clades 1-5 (Bold) indicate isolates identified in this study.

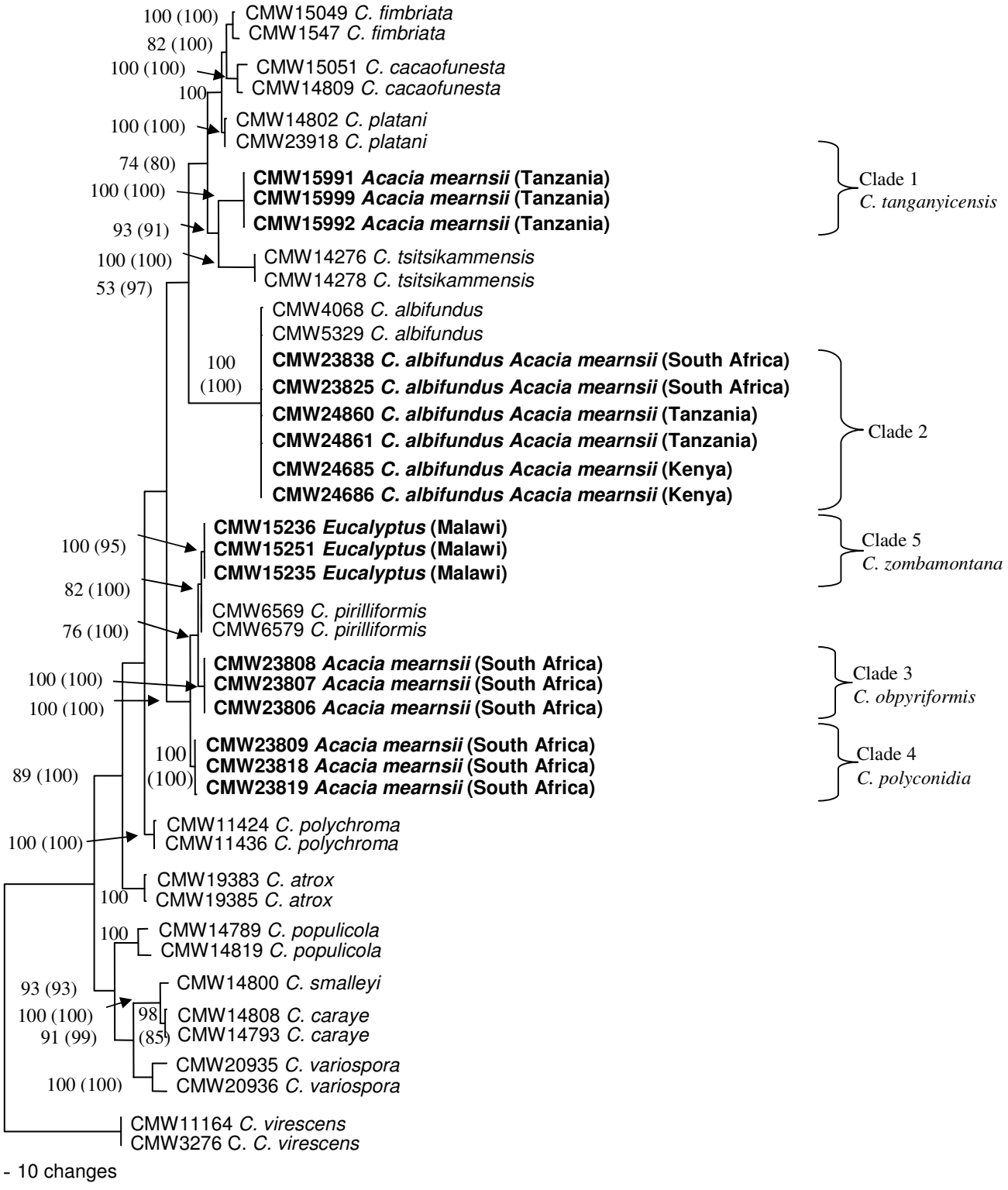

100 (100)
CMW15049 C. fimbriata
82 (100)
CMW1547 C. fimbriata
100 (100)
CMW15051 C. cacaofunesta
100
CMW14809 C. cacaofunesta
100 (100)
CMW14802 C. platani
CMW23918 C. platani
74 (80)
100 (100)
CMW15991 Acacia mearnsii (Tanzania)
CMW15999 Acacia mearnsii (Tanzania)
93 (91)
CMW15992 Acacia mearnsii (Tanzania)
100 (100)
CMW14276 C. tsitsikammensis
53 (97)
CMW14278 C. tsitsikammensis
CMW4068 C. albifundus
CMW5329 C. albifundus
100
(100)
CMW23838 C. albifundus Acacia mearnsii (South Africa)
CMW23825 C. albifundus Acacia mearnsii (South Africa)
CMW24860 C. albifundus Acacia mearnsii (Tanzania)
CMW24861 C. albifundus Acacia mearnsii (Tanzania)
CMW24685 C. albifundus Acacia mearnsii (Kenya)
CMW24686 C. albifundus Acacia mearnsii (Kenya)
100 (95)
CMW15236 Eucalyptus (Malawi)
CMW15251 Eucalyptus (Malawi)
CMW15235 Eucalyptus (Malawi)
82 (100)
CMW6569 C. pirilliformis
76 (100)
CMW6579 C. pirilliformis
100 (100)
CMW23808 Acacia mearnsii (South Africa)
CMW23807 Acacia mearnsii (South Africa)
100 (100)
CMW23806 Acacia mearnsii (South Africa)
100
(100)
CMW23809 Acacia mearnsii (South Africa)
CMW23818 Acacia mearnsii (South Africa)
CMW23819 Acacia mearnsii (South Africa)
89 (100)
100 (100)
CMW11424 C. polychroma
CMW11436 C. polychroma
CMW19383 C. atrox
100
CMW19385 C. atrox
100
CMW14789 C. populicola
CMW14819 C. populicola
93 (93)
CMW14800 C. smalleyi
100 (100)
98
CMW14808 C. caraye
91 (99)
(85)
CMW14793 C. caraye
CMW20935 C. variospora
100 (100)
CMW20936 C. variospora
CMW11164 C. virescens
CMW3276 C. C. virescens
- 10 changes
Clade 1
C. tanganyicensis
Clade 2
Clade 5
C. zombamontana
Clade 3
C. obpyriformis
Clade 4
C. polyconidia

Figure 2. Phylogenetic tree based on the ITS gene region of *Thielaviopsis* spp. and *Ceratocystis* spp. The phylogram was obtained using the heuristic search option based on parsimony. Bootstrap values are indicated above the branches with posterior probability values in brackets. *Ceratocystis virescens* represents the out-group taxon.

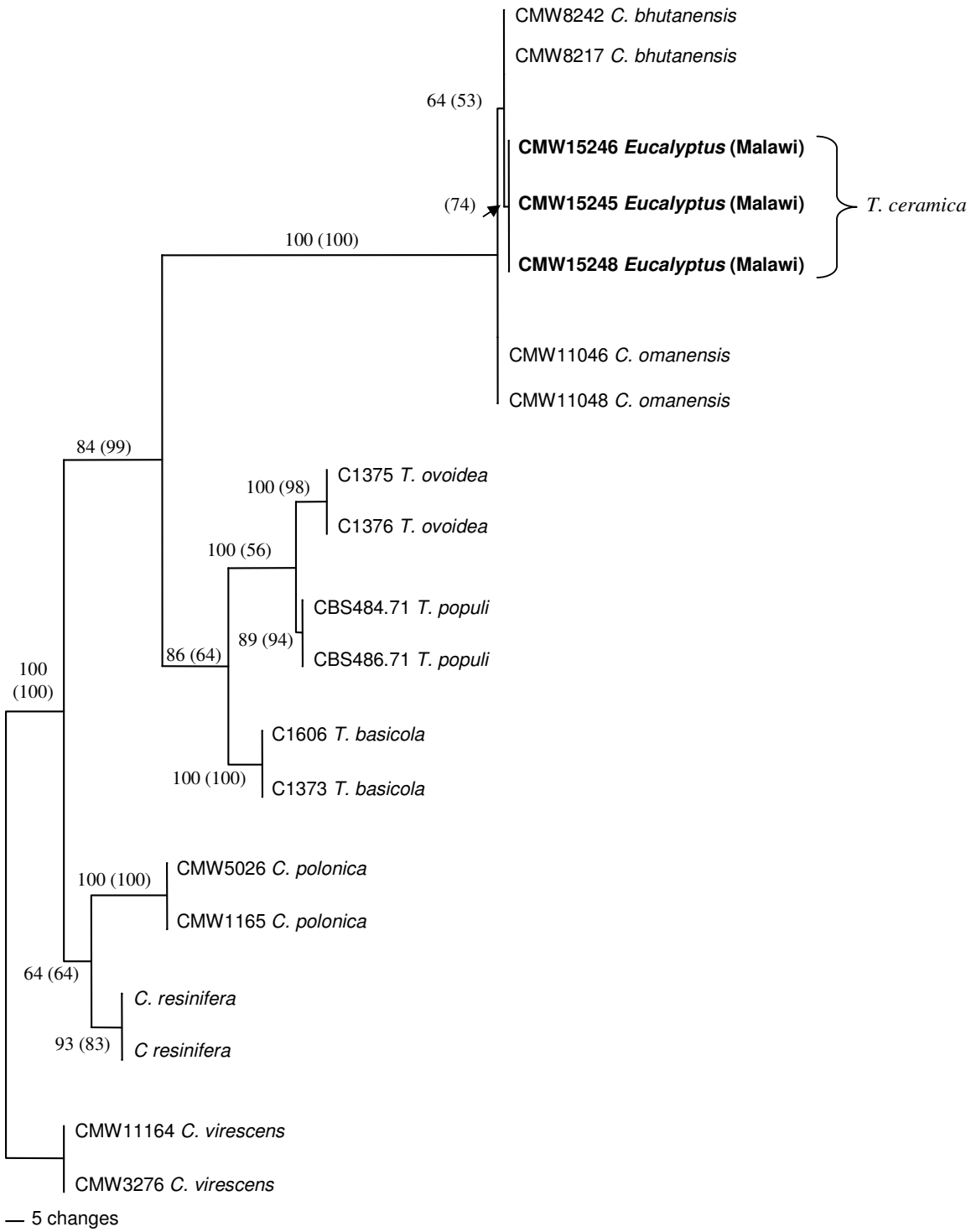

CMW8242 C. bhutanensis
CMW8217 C. bhutanensis
64 (53)
CMW15246 Eucalyptus (Malawi)
(74)
CMW15245 Eucalyptus (Malawi)
T. ceramica
100 (100)
CMW15248 Eucalyptus (Malawi)
CMW11046 C. omanensis
CMW11048 C. omanensis
84 (99)
100 (98)
C1375 T. ovoidea
C1376 T. ovoidea
100 (56)
CBS484.71 T. populi
89 (94)
86 (64)
CBS486.71 T. populi
100
(100)
C1606 T. basicola
100 (100)
C1373 T. basicola
100 (100)
CMW5026 C. polonica
CMW1165 C. polonica
64 (64)
C. resinifera
93 (83)
C resinifera
CMW11164 C. virescens
CMW3276 C. virescens
5 changes

Figure 3. Phylogenetic tree based on the combined ITS, *β*-tubulin and EF1-α gene regions of the *Ceratocystis moniliformis sensu lato* group. The phylogram was obtained using the heuristic search option based on parsimony. Bootstrap values are indicated above the branches with posterior probability values in brackets. *Ceratocystis virescens* represents the out-group taxon. Clades 6-8 (Bold) indicate isolates identified in this study.

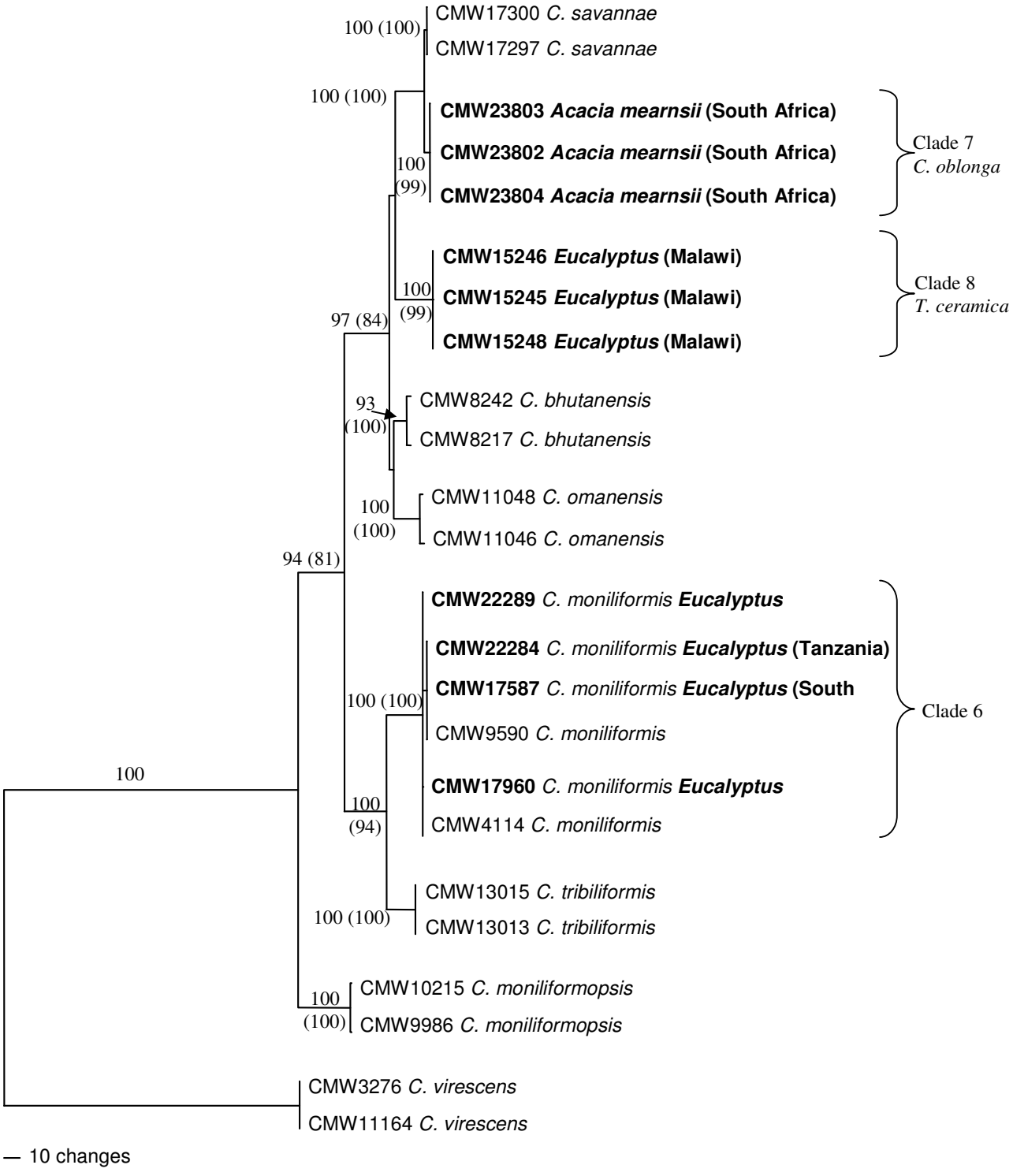

CMW17300 C. savannae
CMW17297 C. savannae
100 (100)
CMW23803 Acacia mearnsii (South Africa)
CMW23802 Acacia mearnsii (South Africa)
CMW23804 Acacia mearnsii (South Africa)
100 (100)
100 (99)
Clade 7
C. oblonga
CMW15246 Eucalyptus (Malawi)
CMW15245 Eucalyptus (Malawi)
CMW15248 Eucalyptus (Malawi)
100 (99)
97 (84)
Clade 8
T. ceramica
CMW8242 C. bhutanensis
CMW8217 C. bhutanensis
93 (100)
CMW11048 C. omanensis
CMW11046 C. omanensis
100 (100)
94 (81)
CMW22289 C. moniliformis Eucalyptus
CMW22284 C. moniliformis Eucalyptus (Tanzania)
CMW17587 C. moniliformis Eucalyptus (South
CMW9590 C. moniliformis
100 (100)
CMW17960 C. moniliformis Eucalyptus
CMW4114 C. moniliformis
100 (94)
Clade 6
100
CMW13015 C. tribiliformis
CMW13013 C. tribiliformis
100 (100)
CMW10215 C. moniliformopsis
CMW9986 C. moniliformopsis
100 (100)
CMW3276 C. virescens
CMW11164 C. virescens
— 10 changes

Figure 4. Morphological characteristics of ***Ceratocystis zombamontana***: A. Obpyriform ascomata. Scale bar = 25µm B. Divergent ostiolar hyphae. Scale bar = 5µm C. Hat-shaped ascospores. Scale bar = 2.5µm D. Flask shaped primary phialide Scale bar = 10µm E. Secondary phialides producing barrel-shaped conidia. Scale bar = 10µm F. Primary conidia, cylindrical (above) and secondary conidium, barrel-shaped (below) Scale bar = 10µm.

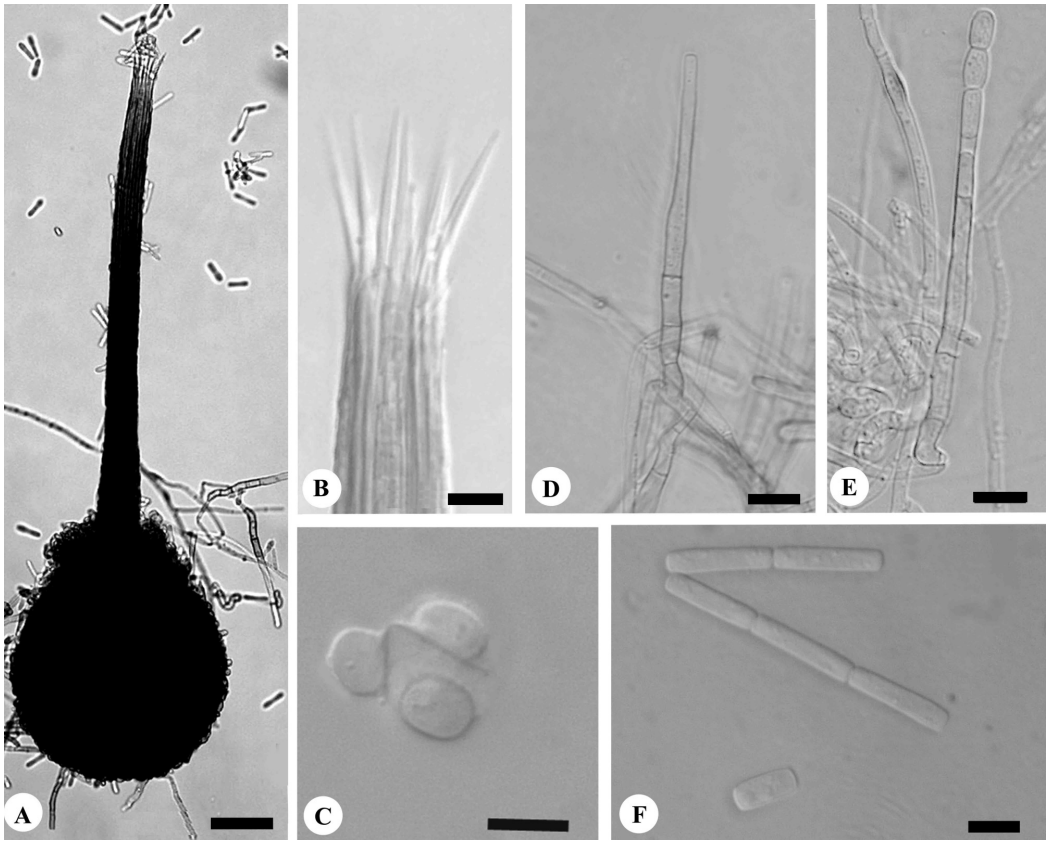

A
B
C
D
E
F

Figure 5. Morphological characteristics of ***Ceratocystis polyconidia***: A. Globose ascomatal base. Scale bar = 100µm B. Divergent ostiolar hyphae with emerging hat-shaped ascospores. Scale bar = 10µm. C. Hat-shaped ascospore. Scale bar = 2.5µm. D. Segmented hyphae. Scale bar = 10µm. E. Flask-shaped primary phialide with emerging cylindrical conidia. Scale bar = 20µm. F. Subglobose chlamydospores. Scale bar = 10µm. G. Secondary phialide producing barrel-shaped conidia in chains. Scale bar = 20µm. H. Barrel shaped conidia in chains. Scale bar = 10µm. I. Cylindrical conidia in chains. Scale bar = 5µm.

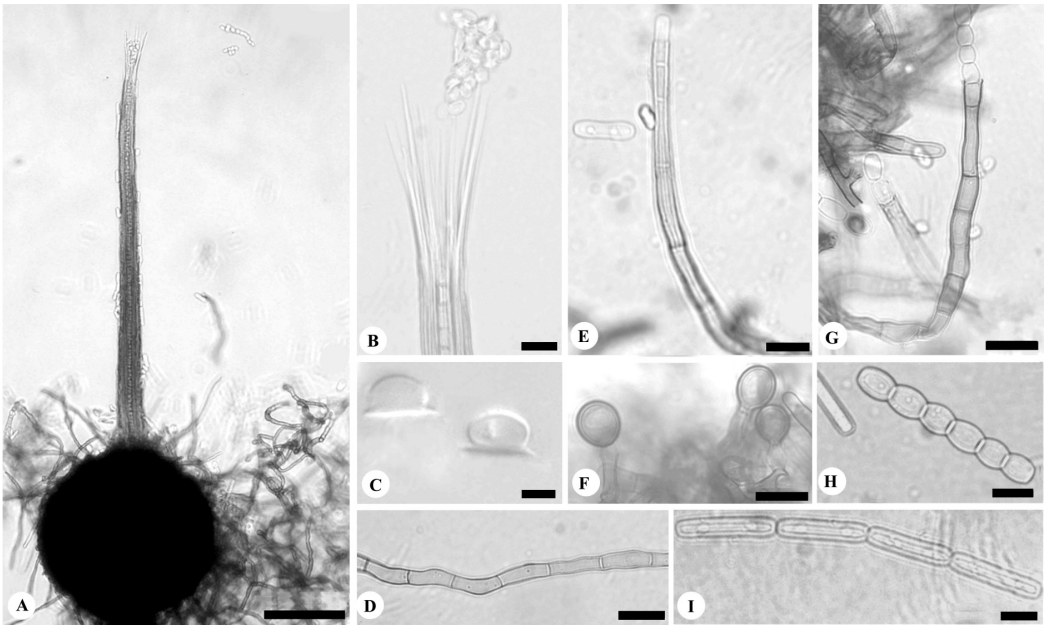

A
B
C
D
E
F
G
H
I

Figure 6. Morphological characteristics of ***Ceratocystis tanganyicensis***: A. Subglobose ascomatal base. Scale bar = 100µm. B. Divergent ostiolar hyphae. Scale bar = 10 µm. C. Hat-shaped ascospores. Scale bar = 5µm. D. Flask shaped primary phialide producing cylindrical conidia. Scale bar = 20µm. E. Cylindrical conidia. Scale bar = 10µm. F. Secondary phialide producing barrel shaped conidia. Scale bar = 5µm. G. Barrel shaped conidia. Scale bar = 5µm. H. Globose chlamydospores produced singly or in chains. Scale bar = 10µm.

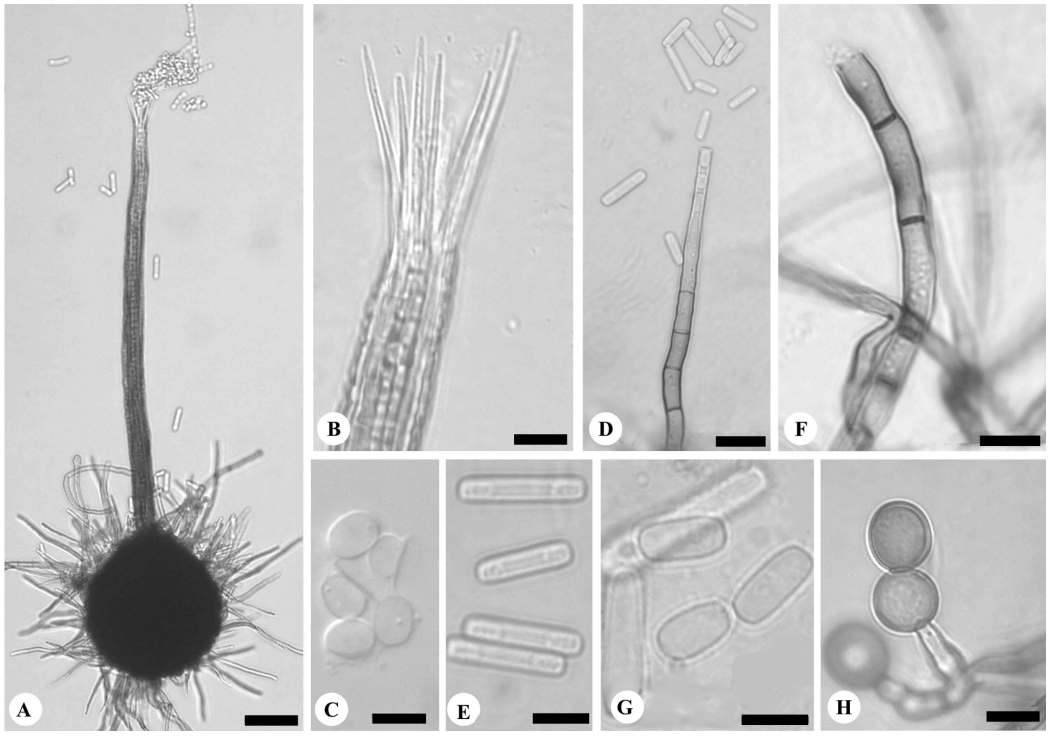

A
B
C
D
E
F
G
H

Figure 7. Morphological characteristics of ***Ceratocystis obpyriformis*** A. Obpyriform ascomatal base. Scale bar = 100µm. B. Divergent ostiolar hyphae with emerging hat-shaped ascospores. Scale bar = 20µm. C. Hat-shaped ascospores. Scale bar = 5µm. D. Primary, flasked shaped phialide producing cylindrical conidia. Scale bar = 50µm. E. Cylindrical conidia. Scale bar = 5µm. F. Secondary phialides. Scale bar = 20µm. G. Barrel shaped conidia in chains. Scale bar = 20µm.

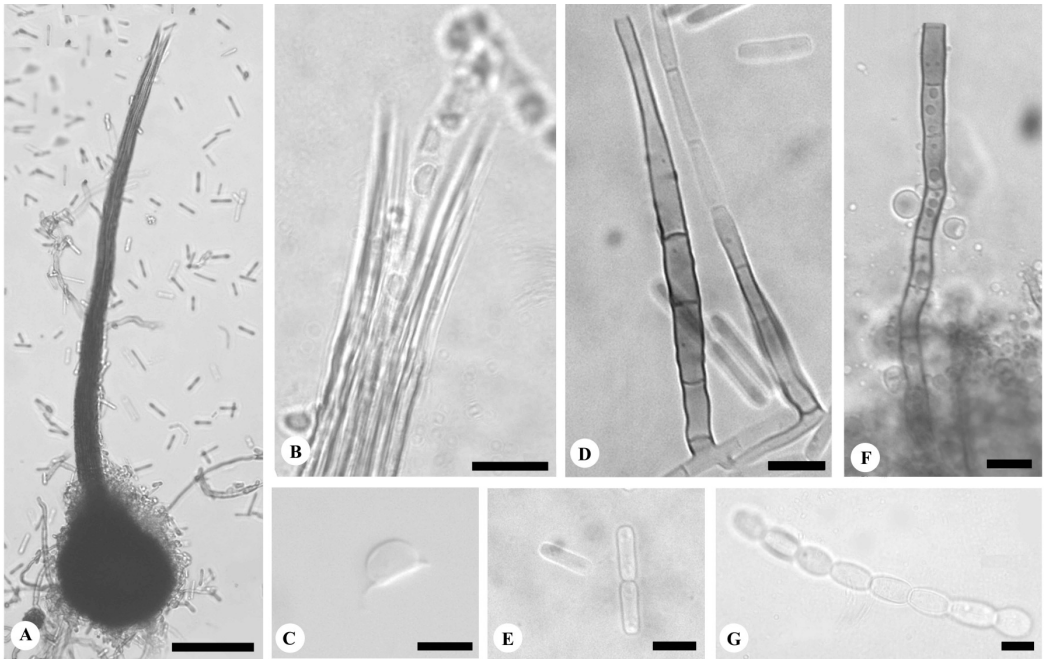

A
B
C
D
E
F
G

Figure 8. Morphological characteristics of ***Ceratocystis oblonga***: A. Obpyriform ascomatal base. Scale bar = 100µm. B. Divergent ostiolar hyphae. Scale bar = 20µm. C. Ascomatal base with short conical spines. Scale bar = 5µm. D. Hat-shaped ascospores. Scale bar = 5µm. E. Flask shaped primary phialide producing cylindrical conidia. Scale bar = 10µm. F. Hyphae with rough walls. Scale bar = 5µm. G. Cylindrical conidia. Scale bar = 10µm. H. Secondary phialide producing barrel-shaped conidia. Scale bar = 10µm. I. Oblong-shaped conidia. Scale bar = 5µm.

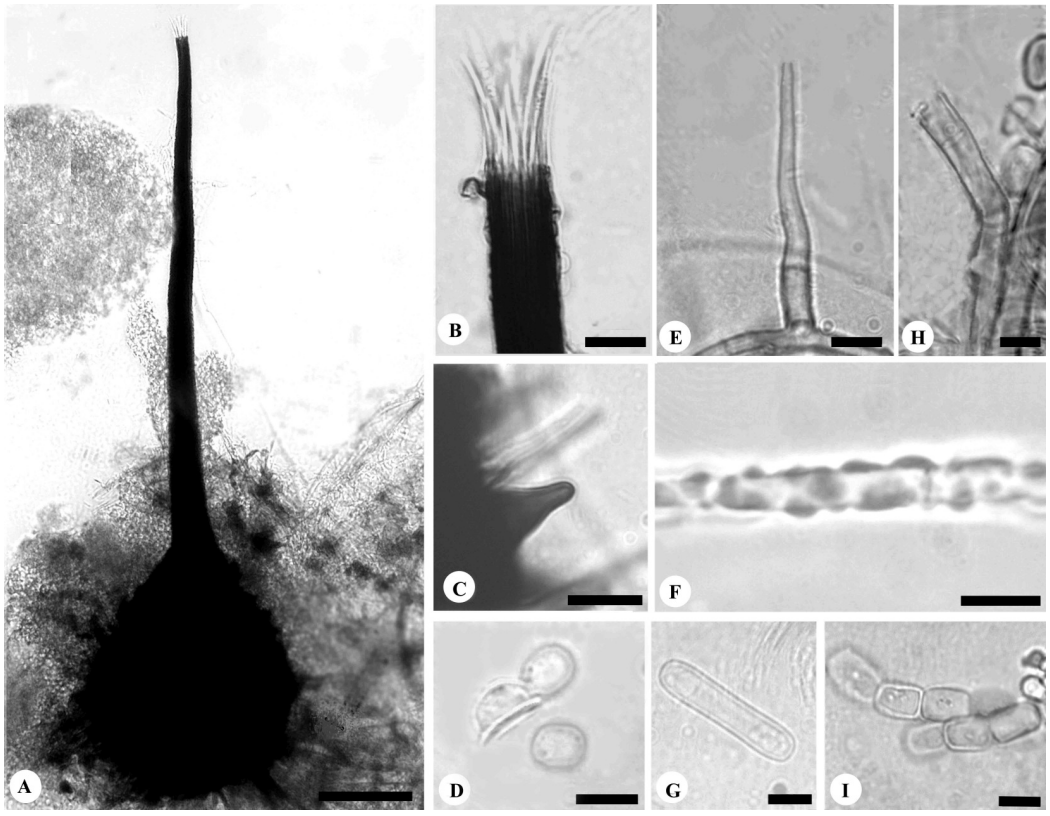

A
B
C
D
E
F
G
H
I

Figure 9. Morphological characteristics of ***Thielaviopsis ceramica***: A. Primary phialide Scale bar = 5µm. B. Flask shaped secondary phialide. Scale bar = 5µm. C. Primary cylindrical conidia. Scale bar = 5µm. D. Secondary barrel-shaped conidia. Scale bar = 2.5µm. E. Hypha with granulated wall. Scale bar = 5µm.

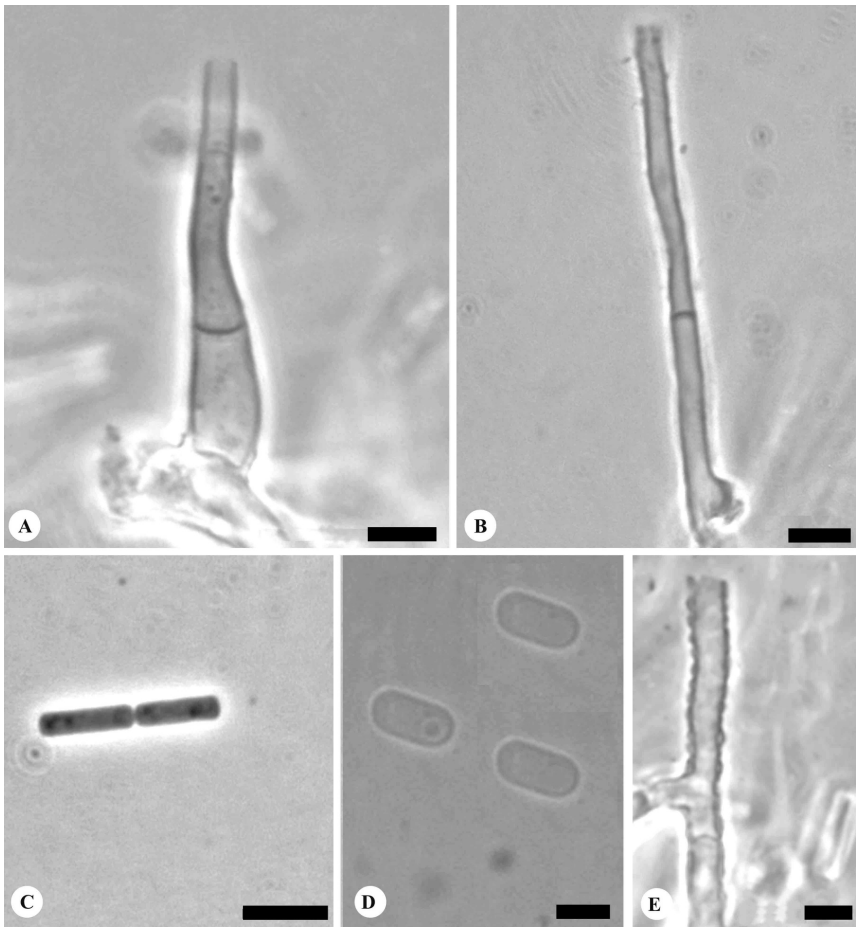

A
B
C
D
E

Figure 10. Bar chart indicating the average lesion length (in millimetres) resulting from inoculation trials with *Ceratocystis oblonga* (CMW23802, CMW23803), *C. obpyriformis* (CMW23807, CMW23808), *Ceratocystis polyconidia* (CMW23809, CMW23818), and *C. tanganyicensis* (CMW15992, CMW15999) onto *Acacia mearnsii.* Different letters above the bars indicate treatments that are statistically different based on a 95 % confidence limit.

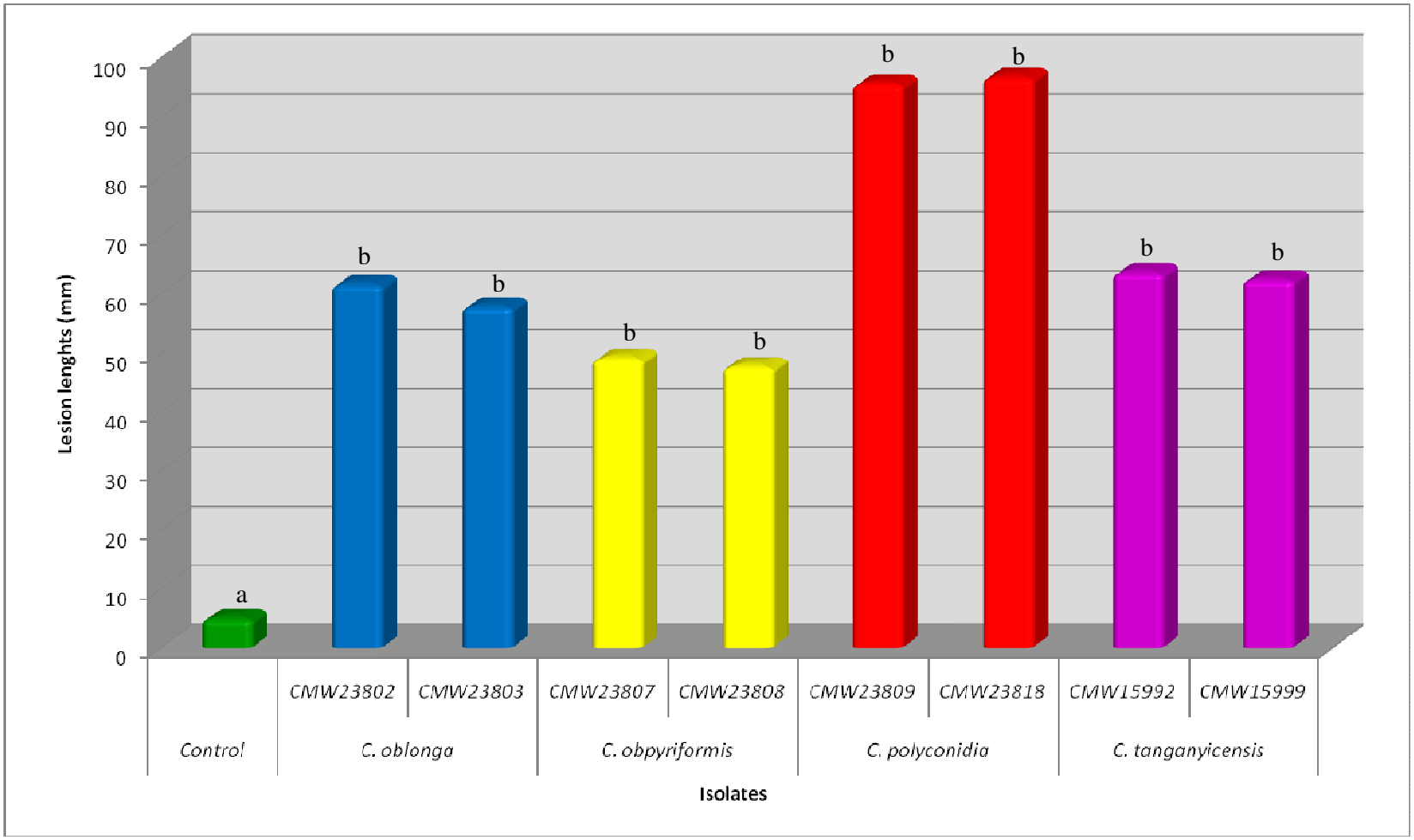
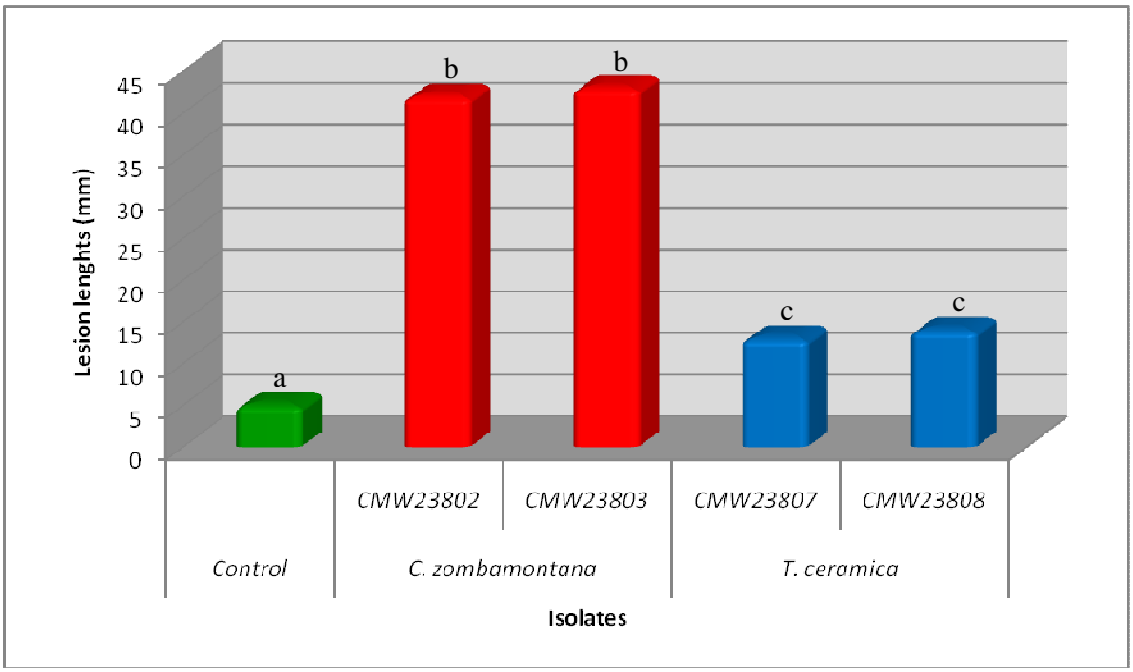

Lesion lenghts (mm)
100
90
80
70
60
50
40
30
20
10
0
a
b
b
b
b
b
b
b
b
CMW23802
CMW23803
CMW23807
CMW23808
CMW23809
CMW23818
CMW15992
CMW15999
Control
C. oblonga
C. obpyriformis
C. polyconidia
C. tanganyicensis
Isolates

Figure 11. Bar chart indicating the average lesion length (in millimetres) resulting from inoculation trials with *C. zombamontana* (CMW15235, CMW15236) and *T. ceramica* (CMW15245, CMW15248) onto *Eucalyptus grandis* (ZG 14). Different letters above the bars indicate treatments that are statistically different based on a 95 % confidence limit.


Lesion lenghts (mm)
45
40
35
30
25
20
15
10
5
0
a
b
b
c
c
CMW23802
CMW23803
CMW23807
CMW23808
Control
C. zombamontana
T. ceramica
Isolates

CHAPTER 3

POPULATION ANALYSES OF CERATOCYSTIS ALBIFUNDUS IN SOUTHERN AND EASTERN AFRICA SUGGEST A PROGRESSIVELY SOUTHERLY HOST COLONISATION ROUTE

ABSTRACT

Ceratocystis albifundus causes wilt and death of non-native *Acacia mearnsii* trees in Southern and Eastern Africa, resulting in considerable economic losses to forestry industries. The fungus is believed to be native to the African continent based on its host and geographic ranges, as well as high level of genotypic diversity on *A. mearnsii* in Uganda and South Africa. Surveys conducted between 2005 and 2007, yielded several populations of *C. albifundus* from various hosts and geographic areas in Southern and Eastern Africa. These isolates provided a first opportunity to consider the diversity of the pathogen from native African trees and to gain further insight into the possible origin and movement of *C. albifundus*. For this purpose polymorphic markers, previously applied to populations of the pathogen, were used. The presence or absence of alleles in populations was analysed using standard population genetic methods. The highest percentage of unique alleles (52%) was observed in the populations from native hosts and those from the northern range of the samples collected (48%). Maximum percentage of genotypic diversity ranged from 30.7% to 100%. The highest percentage of genotypic diversity exhibited by a population was for the northern range of isolates collected. The results suggest that *C. albifundus* is native to regions north of South Africa and that it has more recently become established in the country. These results further support the hypothesis that *C. albifundus* has undergone a host jump from native African trees to plantation grown *A. mearnsii*.

3.1. INTRODUCTION

Ceratocystis albifundus is the causal agent of Ceratocystis wilt of non-native *A. mearnsii* trees in South Africa (Morris *et al*. 1993) and Eastern Africa (Roux *et al*. 2001, 2005). The disease is characterized by the formation of cankers and lesions on the bark of infected trees that also exude copious amounts of gum. Internally, the pathogen causes extensive discolouration of the sapwood and in the final stages of disease development, wilting, die-back and mortality of trees are observed (Roux *et al*. 1999). The wilt disease caused by *C. albifundus* has resulted in significant losses in Southern and Eastern African *A. mearnsii* plantations (Roux *et al*. 2001a, Roux *et al*. 2005) and it poses a significant risk to the future sustainability of forestry operations that depend on these trees.

In recent years, *C. albifundus* has been reported from a number of native tree genera in South Africa (Roux *et al*. 2007), Zambia and Malawi (Roux *et al*. 2004). The first report of *C. albifundus* from a native host was when it was recorded as *C. fimbriata* on *Protea gigantea* (Gorter 1977, Wingfield *et al*. 1996). Later the fungus was reported from eight other native host tree genera in South Africa (Roux *et al*. 2007). On these native hosts, the fungus was found on wounds resulting from strong winds and there was no associated disease or tree death found (Roux *et al*. 2007).

Ceratocystis albifundus has been found only on the African continent and is thought to be native to Africa. Population biology studies on isolates from *A. mearnsii* in South Africa and Uganda have shown a high genetic diversity for the fungus in these two regions (Roux *et al*. 2001b, Barnes *et al.* 2005). Roux *et al*. (2001b) determined the nuclear and mitochondrial gene diversity of this pathogen using a microsatellite marker and mitochondrial restriction enzyme profiles. In that study, gene diversity for *C. albifundus* was high in comparison to other native *Ceratocystis* spp. (Roux *et al*. 2001b). In a study by Barnes *et al*. (2005), isolates of *C. albifundus* from *A. mearnsii* in South Africa and Uganda were compared using polymorphic microsatellite DNA markers to determine the genetic diversity, population structure and gene flow between the two populations. The gene and genotypic diversity was

high in both populations, but they were genetically isolated and gene flow was low. It was, therefore, suggested that the ancestral population had yet to be discovered (Barnes *et al.* 2005).

The report of *C. albifundus* from several native host genera in South Africa (Roux *et al*. 2007), Zambia and Malawi (Roux *et al*. 2004) provided a first opportunity to compare populations from native hosts to those on non-native *A. mearnsii* trees. These populations, together with recently collected populations from non-native *A. mearnsii* trees in Tanzania, makes available the most extensive collection of *C. albifundus* isolates to date for Southern and Eastern Africa. The aim of this study was to consider the population diversity of *C. albifundus* isolates from native and non-native hosts from southern and eastern Africa, using polymorphic microsatellite DNA markers. Previously collected populations of the pathogen from Uganda, Zambia and South Africa were included and additional isolates were collected from native hosts, non-native *A. mearnsii*, as well as from insects in South Africa and non-native *A. mearnsii* in Tanzania.

3.2. Materials and Methods

3.2.1. Isolates

Isolates of *C. albifundus* included in this study originated from previous collections housed in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), and from collections made specifically for this investigation. Isolates sourced from previous studies (Barnes *et al*. 2005, Roux *et al*. 2004, Roux *et al*. 2007) originated from six geographical areas (Figure 1). These included three regions of South Africa (Gauteng Province, Eastern Cape Province, Mpumalanga Province, KwaZulu-Natal), one from Uganda (Kabale region) and Zambia (Kitwe Province). Samples included those from native African hosts (South Africa, Zambia) and those from non-native *A. mearnsii* trees (South Africa, Uganda). Samples from native hosts were obtained from wounds resulting from bark harvesting operations (Roux *et al*. 2004, Roux *et al*. 2007). Those collected from *A. mearnsii*

were obtained from stumps of recently felled trees or from diseased and dying trees (Roux *et al*. 2001).

To increase the population size of *C. albifundus,* collections were made from native and non-native hosts in South Africa and from non-native hosts in Tanzania (Njombe) (Figure 1). Collections were made from cut stumps of non-native *A. mearnsii*, between four days and four weeks after felling, by removing pieces of wood and bark displaying stained vascular tissue or the presence of fungal growth. Isolates from native tree species were collected in South Africa at the Leeuwfontein Collaborative Nature Reserve (Gauteng Province) and southern parts of the Kruger National Park (Limpopo Province). Isolates collected in the Kruger National Park were from natural wounds created by elephants, while isolates from Leeuwfontein Collaborative Nature Reserve were collected from natural wounds, artificially induced wounds and from insects that visit wounds.

Wood and bark sections collected from *A. mearnsii* as well as native tree species were examined for the presence of fruiting structures of *Ceratocystis* spp. Where present, isolations were made from these fruiting structures directly onto 2% (w/v) malt extract agar (MEA) (Biolab, Midrand, South Africa) supplemented with streptomycin sulphate (0.001g/litre, SIGMA, Steinheim, Germany). In addition, wood pieces displaying vascular discoloration were also baited for *Ceratocystis* spp. by placing these between two slices of carrots (5mm thick) and incubating them at 25°C for 7-10 days following the method described by Moller & DeVay (1968). Pieces of wood were also incubated in moist containers at 25°C for seven days to induce the formation of fruiting structures. Once ascomata of *Ceratocystis* spp. were found, spore masses were lifted from their apices and transferred to 2% (w/v) MEA supplemented with streptomycin sulphate (0.001 g/litre). Isolates were identified as *C. albifundus* based on their light coloured ascomatal bases, as this is the only *Ceratocystis* sp. that has this distinguishing characteristic (Wingfield *et al*. 1996). Plates were then incubated at approximately 25ºC under natural day/night conditions. Isolates were purified on 2% (w/v) MEA and are maintained in the culture collection

(CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

3.2.2. DNA extraction, PCR amplification and allele size determination

Genomic DNA of the *C. albifundus* isolates was extracted using the method described by Van Wyk *et al*. (2006). Polymerase chain reactions (PCR) were performed using an Eppendorf Mastercycler PCR machine (Eppendorf AG, Germany). Twenty four fluorescently labelled primer pairs developed for *C. fimbriata* Ell. & Hast. were used for PCR reactions (Barnes *et al*. 2001, Steimel *et al*. 2004). Of these, seven primer pairs that successfully produced PCR products were used for the study. The PCR reaction mixture and conditions used were those described previously (Barnes *et al*. 2001, Steimel *et al.* 2004). Amplified DNA was visualised on 2% (w/v) agarose gels stained with ethidium bromide and viewed under ultra violet light.

Allele sizes of amplified PCR products were determined by electrophoresis on an ABI PRISM^{TM} 3100 Automated DNA sequencer (Applied Biosystems, Foster City, USA) and compared against a GENESCAN–500 LIZ (Applied Biosystems, Warrington, UK) internal size standard. Allele sizes were analysed with GENESCAN and GENEMAPPER software (Applied Biosystems, Foster City, USA). Allele size data from GENESCAN and GENEMAPPER were summarised in spreadsheets and the number of alleles per locus and the total number of haplotypes across all the populations were determined.

3.2.3. Genotypic diversity

The frequency of alleles at each locus was calculated for each geographical region by dividing the number of times each allele occurred by the total number of isolates. Each isolate was assigned a haplotype code according to the observed allele sizes across the microsatellite loci. Genotypic diversity (G_{ST}) was calculated using the equation of Stoddard & Taylor (1988). To compare genotypic diversities between collections of different numbers of isolates, the maximum percentage of genotypic diversity ($\hat{G}$) was calculated (Chen *et al.* 1994). Statistical confirmation that

sufficient numbers of isolates and markers were available for each collection was obtained by plotting genotypic diversity against number of loci sampled with 1000 re-samplings using the computer software program Multilocus v1.3b (Agapow & Burt 2001).

3.3. RESULTS

3.3.1. Isolates

A total of 190 isolates were included in this study (Table 1). Each isolate used for the analyses originated from a different tree or insect. For the analyses, populations were defined based on host and geographical area. The first two populations were defined based on whether the isolates had been obtained from native tree species and insects in native areas (hereafter referred to as the "Native" population) versus isolates from *A. mearnsii* trees (hereafter referred to as the "Non-native" population). The "Native" population consisted of 102 isolates whereas the "Non-native" population consisted of 88 isolates. Furthermore, populations were defined based on geographical origin with isolates obtained from Zambia, Uganda and Tanzania grouped into a "Northern" population (78 isolates) versus isolates obtained from South Africa grouped into a "Southern" population (112 isolates). These "Northern" and "Southern" populations were further subdivided into isolates obtained from native hosts or non-native hosts. A total of eight populations designated as described above were then subjected to analyses (Table 2).

3.3.2. Determination of allele size

A total of 89 alleles were observed across the eight loci detected by the seven markers (one marker produced two loci) used for all the populations, collectively resulting in an average of 10 alleles per locus. The "Native" population exhibited the greatest number of alleles, namely 69, within which 36 (52%) unique alleles were identified (Table 2). This was the population with the highest number of unique alleles. The lowest number of alleles was obtained for the Southern population of non-native trees (33), with only four unique alleles (Table 2).

3.3.3. Genotypic diversity

A total of 447 different haplotypes were observed across all the populations (Table 2). The number of haplotypes per population ranged between 17 and 81. The populations from non-native hosts exhibited the highest number of haplotypes (81). Of these, 444 (99%) haplotypes were unique to the population. The populations obtained from native hosts from the "Northern" population exhibited the lowest number of haplotypes (17) with all (100%) being unique to the population.

The maximum percentage of genotypic diversity ($\hat{G}$) between the various populations ranged between 30.7% and 100% (Table 2). The population from native hosts from the "Southern" population had the lowest $\hat{G}$ of 30.7%, while the population from native hosts from the "Northern" population exhibited the highest $\hat{G}$ value of 100%. The genotypic diversity plotted against the number of loci provided a saturated matrix thereby indicating that all of the populations defined in the study had been sufficiently sampled.

3.4. DISCUSSION

This study presents the first analyses of the population structure of the wilt pathogen *C. albifundus,* collected from non-native as well as native hosts from Southern and Eastern Africa. Previous studies were performed only on isolates collected from *A. mearnsii* from South Africa and Uganda (Barnes *et al*. 2005, Roux *et al*. 2001) and concluded that *C. albifundus* is native to Africa. The limited number of populations, as well as the unavailability of isolates from native hosts, made it difficult for the authors to identify a founder population and resulted in many unanswered questions.

If *C. albifundus* were to have originated on native hosts in Africa, it would be expected that the genotypic diversity would be highest in populations obtained from these hosts (Tsutsui *et al*. 2000). Our results do not fully support this expectation. The population obtained from native African species (southern and northern combined) had a low $\hat{G}$ value (34.9%) compared to the population from non-native

hosts (73.3%). However, when the populations are evaluated individually, a different picture emerges. The population obtained from native hosts from the "Northern" population, exhibited the maximum $\hat{G}$ value (100%), compared to that of the "Southern" native hosts with only 30.7%. Furthermore, the "Northern" population showed nearly double the amount of genotypic diversity (72.3%) compared to the "Southern" population (36.8%). This suggests that the fungus most likely originated from hosts north of South Africa and that it has spread in a southerly direction.

The difference in $\hat{G}$ value between populations obtained from native hosts could be due to the different sampling strategy used to collect the "Northern" and "Southern" populations. The population from native hosts from the "Northern" population was collected from numerous tree species from two sites situated close to each other (~50ha, ~50km), in contrast to the population obtained from native hosts from the "Southern" population. The last-mentioned population was collected in two relatively small geographical areas situated a long distance from each other, with one area situated in the Kruger National park (~100 ha) (Figure 1D) and the other obtained from three collection sites in the Leeuwfontein Collaborative Nature Reserve (~50ha) (Figure 1E). However, the statistical analysis that is used to confirm that sufficient numbers of isolates and markers have been used to sample a population (plotting genotypic diversity against number of loci sampled) reached matrix saturation. This suggests that both populations were fully sampled and that the different sampling strategies cannot be used as an explanation of the differences between the northern and southern populations.

An alternative explanation for the differences between the "Southern" and "Northern" populations isolated from trees native to each region is that *C. albifundus* originated in an area north of South Africa and that it was introduced into the country only recently. This notion also explains the difference in diversity between the *C. albifundus* isolates from non-native tree species in the northern collections, compared to the South African collections, where the "Southern" collections are less diverse than the "Northern" collections. The isolates obtained from non-native species in the north are also less diverse than the isolates obtained from native species in the north. This would be expected if the pathogen had moved from native species to plantation species. In support of this hypothesis, the isolates from the non-native species in the

north have fewer unique alleles than those observed in the corresponding native population.

Our results show that it is not possible to simply compare isolates of *C. albifundus* from native hosts with isolates from non-native hosts throughout the geographic regions from which they were collected. Each region should rather be evaluated on its own for the current set of populations. The data suggest that *C. albifundus* originated in a region to the north of South Africa and that it has progressively followed a southerly route of host colonisation. One explanation for the movement of *C. albifundus* in a southerly direction could be due to climatic changes that have occurred in a southerly direction (Barrett *et al*. 1992). The end of the previous ice age and aridification brought about by the closure of the Indonesian seaway 3-4 million years ago (Cane & Molnar 2001) and this resulted in a gradual increase in temperatures over the southern hemisphere. This warming of the continent in a southerly direction has resulted in the expansion of the habitat of possible hosts of the fungus. To the best of our knowledge, this is the first study to demonstrate the possible correlation between the habitat expansion of plants on the African continent and the spread of a fungus. The data also suggest that *C. albifundus* has made a number of host jumps in the recent past. The first (based on fairly high diversity values) was probably onto non-native species north of southern Africa. These host jumps commonly occur in tree pathogens, although they are poorly understood (Slippers *et al*. 2005). It appears that the pathogen has subsequently been moved into Southern Africa. It is not possible to determine whether this has occurred as the result of movement of non-native or native tree species into this region, but both options are equally likely.

The number of unique alleles exhibited by each population of *C. albifundus* corresponded with the genetic diversity obtained for that population. The "Northern" population exhibited more unique alleles (48%) than the "Southern" populations (41%). Within the "Northern" population, isolates from native hosts (44%) exhibited a much higher percentage of unique alleles than those from non-native hosts (14%). A similar trend was observed within the "Southern" population with the isolates from native trees exhibiting a higher percentage of unique alleles (36%) than those from non-native hosts (12%). These results again indicate that *C. albifundus* most probably

originated north of southern Africa and subsequently followed a progressively southerly host colonisation route.

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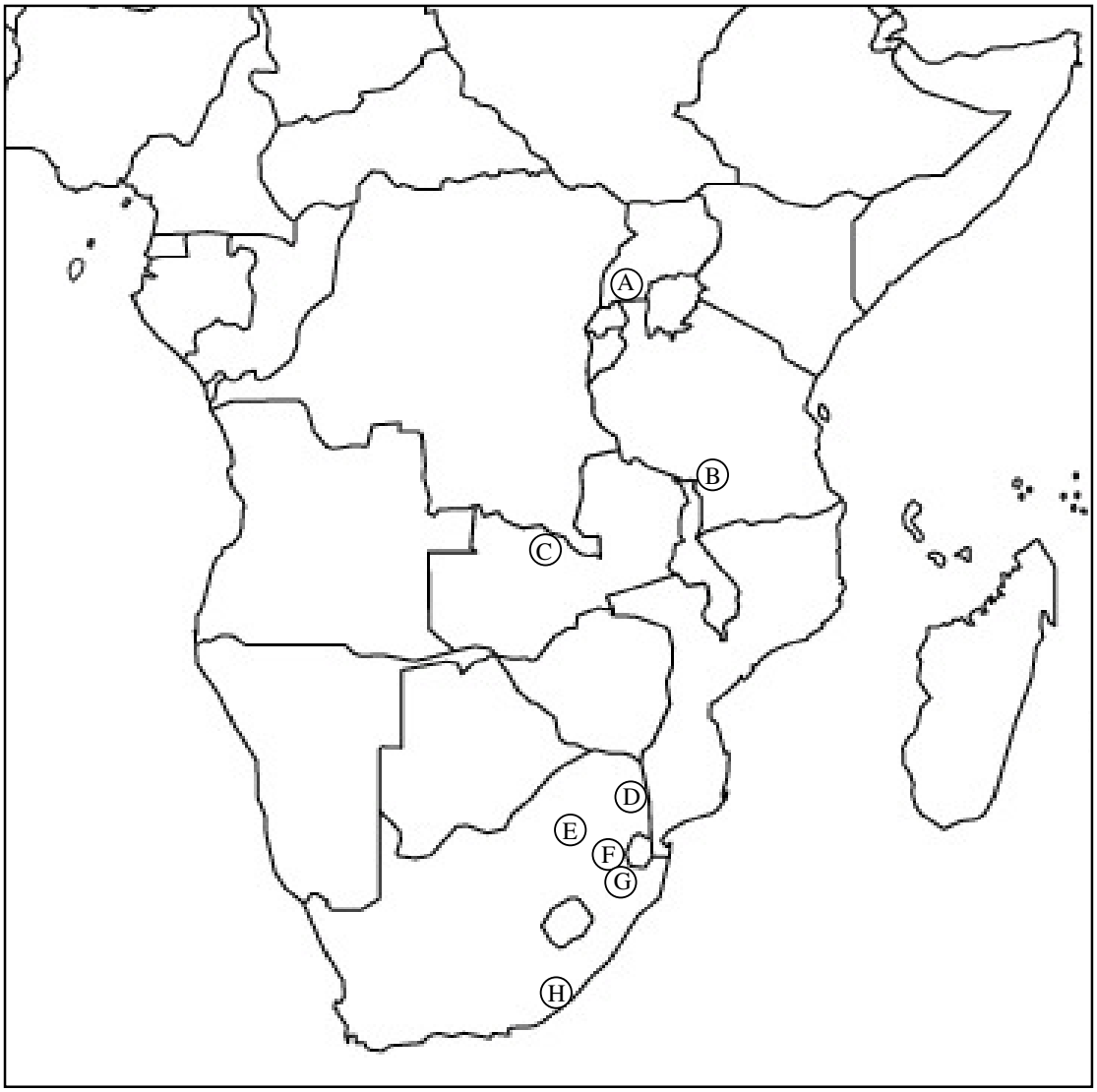
Table 1. List of isolates used in this study.

Culture number	Host	Geographical origin	Collector (s)
CMW17620-CMW17623	Native	Kruger National Park, RSA	R.N. Heath & J. Roux
CMW21146-CMW21154	"	"	"
CMW23840-CMW23848	"	"	"
CMW14157-CMW14163	"	Leeuwfontein Nature Reserve, RSA	"
CMW14165, 14166	"	"	"
CMW14168-CMW14173	"	"	"
CMW14605	"	"	"
CMW17270-CMW17288	"	"	"
CMW17307-CMW17312	"	"	"
CMW17628-CMW17631	"	"	"
CMW17633-CMW17639	"	"	"
CMW17773-CMW17775	"	"	"
CMW21469-CMW21475	"	"	"
CMW21661-CMW21695	"	"	"
CMW22292, 22293	"	"	"
CMW22300-CMW22302	"	"	"
CMW13979,13988	"	Kitwe Province, Zambia	J. Roux
CMW14607-CMW14610	"	"	"
CMW14612-CMW14617	"	"	"
CMW14619, 14620	"	"	"
CMW4076, 4079, 4084	Non-native	KwaZulu-Natal Province, RSA	"
CMW4097	"	Eastern Cape Province, RSA	T.C. Harrington
CMW23823- CMW23839	"	Mpumalanga Province, RSA	R.N. Heath
CMW23849-CMW23853	"	"	"
CMW4074, 4998, 5329	"	Kabale region, Uganda	J. Roux & G. Nakabonge
CMW7111-CMW7114	"	"	"
CMW7153-CMW7156	"	"	"
CMW7160-CMW7162	"	"	"
CMW7208	"	"	"
CMW9173-CMW9175	"	"	"
CMW9177-CMW9180	"	"	"
CMW9182, 9184	"	"	"
CMW9375-CMW9277	"	"	"
CMW15997-CMW15999	"	Njombe, Tanzania	R.N. Heath & J. Roux
CMW16003-CMW16006	"	"	"
CMW24860-CMW24887	"	"	"

Table 2. Summary of values obtained during statistical analyses of polymorphic DNA markers.

Population	No. of isolates	No. of alleles	Unique alleles	No. of haplotypes	Unique haplotypes	$\hat{G}$ -value
Native	102	69	36	77	77	34.9%
Non-native	88	46	13	81	80	73.3%
Northern	78	60	29	71	70	72.3%
Southern	112	53	22	75	74	36.8%
Northern native	17	43	19	17	17	100%
Northern non-native	60	37	5	52	52	66.7%
Southern native	84	38	14	48	48	30.7%
Southern non-native	28	33	4	26	26	93.3%

Figure 1. Map indicating the geographical areas from which *C. albifundus* isolates were obtained for this study. (A) Uganda (Kabale region), (B) Tanzania (Njombe), (C) Zambia (Kitwe, Copperbelt Province), (D) Kruger National Park (South Africa), (E) Leeuwfontein Collaborative Nature Reserve (South Africa), (F) Mpumalanga Province (South Africa), (G) KwaZulu-Natal Province (South Africa), (H) Eastern Cape Province (South Africa).


A
B
C
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CHAPTER 4

INSECT ASSOCIATES OF CERATOCYSTIS ALBIFUNDUS AND PATTERNS OF ASSOCIATION IN A NATIVE SAVANNA ECOSYSTEM IN SOUTH AFRICA

(Heath *et al*. 2008, Environmental Entomology 38:356-384)

ABSTRACT

Species of *Ceratocystis sensu lato* (*s.l.*) include important plant pathogens such as *C. albifundus*, that causes a serious wilt disease of non-native, plantation-grown *Acacia mearnsii* trees in Africa. The aim of this study was to identify the insects associated with *C. albifundus* in South Africa and thus to consider the means by which the pathogen spreads. Insects were collected weekly for 77 weeks in a native ecosystem using modified pitfall traps. Trapped insects were identified and fungi were isolated using carrot baiting and by plating them onto malt extract agar. Fungi were identified using morphological characteristics and DNA sequence comparisons. Three different nitidulid beetles (Coleoptera: Nitidulidae), *Brachypeplus depressus*, *Carpophilus bisignatus* and *Ca. hemipterus* were collected, of which the most common were the *Carpophilus* spp. Two *Ceratocystis* spp., namely *C. albifundus* and *C. oblonga* were isolated from all three insect species. Insect numbers and fungal isolates decreased significantly in the colder months of the year. Of the two *Ceratocystis* spp., *C. oblonga* was most abundant occurring on 0.5% of the *Carpophilus* spp. *C. albifundus* was isolated from 1.1% of the *Brachypeplus* individuals and from 0.01% of the *Carpophilus* individuals. This study presents the first record of insects associated with *C. albifundus* and *C. oblonga* and it provides an indication of environmental influences on fungal and insect populations, which could contribute to future disease management.

4.1. INTRODUCTION

Species of *Ceratocystis sensu lato* (*s.l.*) includes many plant pathogens, the majority of which infect trees (Kile 1993). This group of fungi has been known for more than a century with the type species, *Ceratocystis fimbriata* Ellis & Halstead, described in 1890 as the causal agent of black rot of sweet potato (*Ipomoea batatas* L.) (Halsted 1890). *Ceratocystis fimbriata* is widely recognised as representing a complex of cryptic species, some of which have been described recently. Examples of important pathogens in the *C. fimbriata s.l.* species complex are *C. platani* (J.M. Walter) Engelbrecht & T.C. Harrington (Engelbrecht & Harrington 2005), *C. cacaofunesta* Engelbrecht & T.C. Harrington (Engelbrecht & Harrington 2005) and *C. albifundus* Morris, De Beer & M. J. Wingfield (Wingfield *et al*. 1996). Other well-known pathogens accommodated in *Ceratocystis s.l.*, not related to *C. fimbriata s.l.,* include the oak wilt pathogen *C. fagacearum* Bretz (Bretz 1952) and *C. paradoxa* (Dade) Moreau, which causes disease of numerous crops (Kile 1993). There are also numerous species that are best known as agents of sap stain in lumber, or that have not been shown to be pathogenic (Kile 1993).

Ceratocystis spp. are well adapted to being vectored by insects. In this regard, there are two discrete groups in the genus. Those that produce fruity aromas, have casual vectors such as nitidulid beetles (Coleoptera: Nitidulidae) and flies (Diptera) (Moller & DeVay 1968a, Kirisits 2004). A second suite of species, such as *C. polonica* (Siemazko) Moreau and *C. laricicola* Redfern & Minter live in a mutualism with conifer-infesting bark beetles (Coleoptera: Scolytidae) and do not produce fruity

aromas (Harrington & Wingfield 1998). Other than the production of fruity aromas, *Ceratocystis* spp. are well adapted for dispersal by insects, having long ascomatal necks that give rise to sticky masses of spores that stick easily to insect bodies. It is thought that these long necks not only reduce the competition of surrounding fungi by bearing their spore drops above the competing fungi, but that they could also influence the type of insects that vector them (Malloch & Blackwell 1993). An interesting related adaptation is that some species compensate for short perithecial necks by the production of their spore masses in thread-like tendrils (Wingfield 1993).

Aside from the bark beetle associated species, the species of *Ceratocystis* that has been considered most closely in terms of its insect vectors is the oak wilt pathogen *C. fagacearum*. Numerous studies have shown that nitidulid beetles are the primary vectors of this fungus (Gibbs 1980, Gibbs & French 1980, Juzwik & French 1983). Transmission of *C. fagacearum* by nitidulid beetles is significant in overland spread of the fungus and the establishment of new infection centers (Cease & Juzwik 2001). The beetles are attracted to sporulating mats on recently killed oak trees, and after feeding on these mats, they are covered in fungal propagules which they subsequently spread to other trees (Juzwik & French 1983).

Various insects that are associated with *Ceratocystis* spp. either feed on sap or on the fungi themselves. After the insects have been attracted to the fungi by the fruity odors that they produce (Lanza & Palmer 1977), the spores are ingested or they adhere to their bodies. These insects are attracted to the sweet sap associated with fresh wounds on plants and the fungi are thus transferred to a new substrate as in the case of nitidulid beetles transmitting *C. fimbriata* (Moller & DeVay 1968a). There is also

some evidence that the insects play a role in the over wintering of the fungi, that do not persist on wounds for very long (Moller & DeVay 1968a). Insects are further believed to play a significant role in the spermatization of the fungi with which they are associated, as has been shown in the case of *C. fagacearum* (Thompson *et al.* 1955).

Ceratocystis albifundus resides in the *C. fimbriata s.l.* species complex and it causes a serious wilt disease of non-native *A. mearnsii* de Wild in eastern and southern Africa (Morris *et al.* 1993, Roux *et al.* 2005). The fungus was first discovered in South Africa in the 1980's and was initially treated as *C. fimbriata* (Morris *et al.* 1993) until DNA sequence comparisons became available and it was recognized as a discrete taxon (Wingfield *et al.* 1996). Population biology studies, its occurrence on several native African tree families, as well as reports only from Africa suggest that *C. albifundus* is most likely an African fungus (Roux *et al.* 2001, Barnes *et al.* 2005, Roux *et al.* 2007).

Very little is known regarding the biology of *C. albifundus*, despite the fact that it is an important pathogen. Because of its fruity aroma, it has been assumed that it is vectored by insects similar to those associated with other *Ceratocystis* spp. that produce attractive aromas. The aim of this study was to identify possible insect associates of *C. albifundus* in South Africa. Furthermore, we considered the seasonal occurrence of these vectors in a typical native savanna ecosystem.

4.2. MATERIALS AND METHODS

4.2.1. Study areas

Two study areas were selected for this investigation. One was located on the Leeuwfontein Collaborative Nature Reserve (Leeuwfontein) approximately 60km north-east of Pretoria, Gauteng Province, South Africa. This study area included three sites (S 25° 23' 38.3", E 028° 37' 19.5"; S 25° 22' 39.1", E 028° 37' 23" & S 25° 22' 37.2", E 028° 37' 38.2") and is situated in native savannah vegetation. The area was selected because *C. albifundus* has previously been isolated from several native tree species in this reserve (Roux *et al*. 2007) and due to its close proximity to the laboratory.

The second study area, selected for comparison of insect species, was situated ~40 km south-west of Piet Retief, Mpumalanga Province, South Africa (S 26° 58' 68.5", E 030° 54' 28.3"). This study area, consisting of a single site, is within a plantation of non-native *A. mearnsii* trees, between two compartments. The one compartment was six-years-old and the second compartment had recently been clear felled. The two aforementioned study areas are referred to as the "native" and "non-native" study areas.

4.2.2. Traps and bait

Before commencement of the main trials, several different trap and bait types were tested in pilot trials. The different baits tested included fermenting dough, bananas, pineapples and a mixture of all three baits. The traps that were tested included funnel traps, panel traps and a modified pitfall trap (Southwood & Henderson 2000). Based on results of the pilot trials, freshly cut pineapple (1cm^2 blocks) was selected as the

ideal bait and modified pitfall traps were used for the main experiments. These traps (Figure 1) consisted of a removable cup-shaped bottom section (115mm diameter) in which the bait was placed beneath a sieve to prevent the insects from coming into direct contact with the bait. The top section of the trap consisted of a tube (with 3mm diameter holes) fitting into the cup, which allowed insects to enter the tube. The tube was sealed with a lid (Figure 1) to prevent rainwater from entering the trap or the insects from escaping. The traps were fastened to trees at a height of approximately 1.5m with adjustable straps.

4.2.3. Collection of insects from the "Native" study area

Trapping and collection of insects on Leeuwfontein commenced in mid winter, 2005 (30 June), and proceeded over a period of 77 weeks. The bait was replaced and insects were collected weekly throughout the study period. Insects were removed from the traps with an aspirator and transported to the laboratory in individual glass vials in a cool box at ~5°C. Insect specimens were grouped based on morphological characteristics, counted, and representative samples were mounted or preserved in 70% alcohol for identification.

4.2.4. Collecting of insects from "Non-native" study area

Trapping of insects in the plantation of non-native *A. mearnsii* trees near Piet Retief was undertaken to consider the possible insect associations of *C. albifundus* in commercial plantations of non-native trees, specifically to compare to insects obtained in the native ecosystem. Sampling was performed for only a week during summer 2006 (23-26 April). For this portion of the study, the same trap design, bait and trapping protocol as that used for the native study area, was used. The bait was

replaced and insects collected daily for four days. Insect numbers were not calculated for this portion of the study, as the aim here was not to monitor insect numbers or environmental conditions but merely to compare insect species to those in the native habitat.

Insects were also collected from under bark flaps on the stumps of recently felled *A. mearnsii* trees. This made it possible to compare the incidence of fungus/insect associations for insects collected from traps and insects collected from the stumps. This was also done to confirm that the fungal isolation techniques used were effective. Sampling for this part of the study was done during January (summer) 2008.

4.2.5. Presence of fungal propagules on insect bodies

Insect exoskeletons were inspected for the presence of fungal propagules using scanning electron microscopy (SEM). A total of 25 specimens of each of the morphological groups of insects collected from the "native" area were examined. Specimens were dried in self-indicating silica gel for five days. Once the critical drying point (CDP) was reached, specimens were mounted on stubs using double-sided carbon tape. Half of the specimens were mounted on their dorsal sides and the remaining specimens were mounted on their ventral sides. Specimens were coated with gold using a Polaron E5200C sputter coater (Watford, England) and examined using a JSM-840 scanning electron microscope (JEOL, Tokyo, Japan).

4.2.6. Isolation of fungi

Three methods were used to isolate *Ceratocystis* spp. from the collected insects. Equal numbers of insects were used for each of the three isolation methods. For one

third of the insects, the exoskeletons were surface disinfested by submerging the insects in 96% ethanol for 1 min, in undiluted bleach for 1 min and in 70% ethanol for 1 min and thereafter rinsing them in sterile water. Insects were then macerated onto 2% Malt Extract Agar (MEA) (Biolab, Merck, Midrand, South Africa) amended with 100mg/liter (100ppm) Streptomycin sulphate (Sigma, Steinheim, Germany) to inhibit bacterial growth and incubated at 25°C for two to seven days. Plates were then examined using a dissection microscope.

Another third of the insects were killed with forceps and placed directly onto the surface of 2% MEA amended with Streptomycin sulphate with either the dorsal or ventral sides facing the agar surface. Samples were then incubated for two to four days at 25°C after which plates were examined using a dissection microscope. Pure cultures were obtained by making hyphal tip transfers.

With a third of the insects, five to 10 individuals were placed between two carrot discs, approximately 10 mm thick (Moller & DeVay 1968b). The carrot discs were prepared by soaking in distilled water amended with 300mg/liter (300ppm) Streptomycin sulphate for 10min. The carrot discs, containing the insects, were incubated at 25°C for four to seven days. Once ascomata had formed on the carrot surfaces, single spore drops were transferred to 2% MEA amended with 100mg/litre (100ppm) Streptomycin sulphate in order to obtain pure cultures. Only one isolate of the various fungal species were isolated from each individual insect. All isolates obtained in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

4.2.7. Identification of isolates

Ceratocystis isolates obtained from the insects were grouped based on culture morphology after seven days of growth at room temperature on 2% MEA. Isolates were identified based on structural morphology following published descriptions for *Ceratocystis* spp. Fungal structures were mounted on glass slides in lactophenol and examined under a Zeiss Axioskope (Carl Zeiss Ltd., Germany) microscope and images were captured using a HRc Axiocam digital camera and Axiovision 3.1 software (Carl Zeiss Ltd., Germany).

Identifications of the *Ceratocystis* isolates were confirmed by comparing DNA sequences for two isolates of each morphospecies, with those previously published. Three gene regions were used for the DNA sequence comparisons. These were the Internal Transcribed Spacer regions (ITS1, ITS2), including the 5.8S rDNA region, amplified using primers ITS1 and ITS4 (White *et al*. 1990), part of the Beta-tubulin (*β*-tubulin) gene region using primers Bt1a and Bt1b (Glass & Donaldson 1995) and the Transcription Elongation Factor 1α (EF-1α) region using the primers EF1F and EF2R (Jacobs *et al*. 2004). Polymerase chain reaction (PCR), sequencing, purifying, data processing and data analyses protocols were the same as those used by Van Wyk *et al*. (2006).

4.2.8. Statistical analyses of data

Data collected in this study included the number of insects collected per week on Leeuwfontein, the number of fungal isolates obtained from these insects, as well as various climate variables. After insects had been collected and divided into groups based on morphological characteristics, the total number of insects residing in each

morphological group was enumerated. Once fungal isolates had been obtained from the insects and identified, the total number of each species was noted.

Climate data were obtained from the Range and Forage Department, Roodeplaat research station of the Agricultural Research Council (ARC) (S 25° 60' 41", E 028° 35' 42"), approximately 30km from Leeuwfontein. The weather data collected included rainfall (mm/day), daily maximum temperature (°C), daily minimum temperature (°C), wind speed (M/s), maximum daily relative humidity (%) and minimum daily relative humidity (%).

For the purpose of statistical analyses, the data were divided into two sets. One dataset was used to determine which variables influenced the number of fungal isolates obtained during the study period. Data for the number of fungal isolates were transformed into binary values where values below zero were converted to zero and those greater than zero to one. Data were subjected to the stepwise logistic procedure determining the predictive variables using SAS (SAS Version 8.2, 2001).

The second data set was used to determine which climatic variables had an influence on the number of insects collected during the study period. Data were subjected to a stepwise regression on the predictive variables using SAS (SAS Version 8.2, 2001). The significance level for entry of variables into the model was set at 0.05 and the significance level for variables remaining in the model was set at 0.1.

The fungus / insect association was calculated by deviding the total number of fungal isolates (per individual fungul species) by the total number of insects (per individual insect species) collected. This value was converted to a percentage value by multiplying the association level calculated by 100.

4.3. RESULTS

4.3.1. Collection of insects from the "Native" study area

Insects were found in the traps at Leeuwfontein throughout the study period, with 38,262 insects collected over the 77 week study period. A clear seasonal trend was observed in the number of insects collected, with the lowest numbers of insects (4,752) collected in the winter and spring (June 2005 – October 2005 and 22 June 2006 – 26 October 2006) seasons respectively (Figure 2).

At the start of the 2005/2006 summer (mid October), the number of insects increased gradually and these fluctuated over the summer months, gradually decreasing from early May 2006 to stabilize at relatively low numbers in winter 2006 (June – September). A similar trend was observed for the 2006 summer, with the number of insects increasing from the end of October. A total of 33, 510 insects were collected during the summer and autumn months during the study period. In contrast to the summer of 2005/2006, the number of insects collected in the summer of 2006 increased more rapidly at the beginning (October) of the 2006 summer season (Figure 2).

Three main groups of insects were identified based on morphological characteristics. These represented two *Carpophilus* spp. (28,252 insects) and one *Brachypeplus* sp.

(2,041 insects). The two *Carpophilus* spp. were identified as *Ca. bisignatus* Boheman, and *Ca. hemipterus* L. (Figure 3). The *Brachypeplus* sp. was identified as *B. depressus* Erichson (Figure 3). Other insects collected in the traps included Coleoptera, Diptera & Hymenoptera (7969). The numbers of insects collected for each of the two *Carpophilus* spp. differed significantly with *Ca. bisignatus* being more abundant (21,188) than *Ca. hemipterus* (7,064).

4.3.2. Collection of insects from "Non-native" study area

The same three species of nitidulid beetles (*Ca. bisignatus*, *Ca. hemipterus*, and *B. depressus*), collected in the native study area, were found in the *A. mearnsii* plantations. Other insects collected in the traps included Coleoptera and Diptera. Mainly *Ca. hemipterus* (6) and *B. depressus* (23) were collected in 2008 from under bark flaps from the stumps of recently felled trees.

4.3.3. Presence of fungal propagules on insect bodies

Scanning electron microscopy revealed hat shaped ascospores on the dorsal sides of seven insects (Figure 4). These spores had similar morphology to those produced by *Ceratocystis* spp. isolated from the insects, varying from 4µm–6µm in size. Ascospores were observed on two of the 25 *B. depressus* beetles scanned. Of the 50 *Carpophilus* beetles examined, ascospores were observed on only two *Ca. hemipterus* and three *Ca. bisignatus* individuals. The spores occurred singly on the insects and were not abundant. No spores were observed on the ventral sides of the beetles.

4.3.4. Identification of isolates

The *Ceratocystis* spp. obtained in this study represented two species. Some isolates were easily identified as *C. albifundus* based on morphology because this species is the only *Ceratocystis* sp. known to produce ascomata with light-coloured bases. This is in contrast to the second species obtained that had white, fast growing colonies that turned brown with time, with dark coloured ascomatal bases and granular hyphae. It was not possible to accurately distinguish these isolates from closely related species such as *C. savannae* Kamgan-Nkuekam & Jol. Roux (Kamgan *et al*. 2008), necessitating DNA sequencing to confirm their identity.

Analyses of DNA sequences showed that isolates with light coloured ascomatal bases grouped within the *C. albifundus* clade (Bootstrap support of 100%). Isolates that could not be identified based on morphology alone grouped in a clade (Bootstrap support, 98%) with the recently described species, *C. oblonga* R. N. Heath & Jol. Roux (Heath *et al*. 2009), distinct from all other species in the *C. moniliformis s.l.* Hedgcock group.

4.3.5. Association of fungi with insects

Of the three methods used to isolate *Ceratocystis* spp. from the insects, only two yielded positive results. When the insects were surface disinfested, they yielded only bacteria and, therefore, these insect numbers were excluded from the levels of association calculations. *C. oblonga* was isolated from the insects that were placed directly on agar as well as those placed between the carrots, while *C. albifundus* was obtained only using the carrot baiting technique. For the calculation of association levels for *C. albifundus*, only the number of insects placed between carrots was included.

In total, 22 of the 680 (3.2%) *B. depressus* individuals used in the carrot baiting yielded isolates of *C. albifundus*. Two isolates were obtained from 2,354 *Ca. hemipterus* (0.08%) and one isolate was obtained from 7,062 (0.02%) *Ca. bisignatus* individuals. In contrast, 137 of the 18,832 (0.73%) *Carpophilus* spp. yielded *C. oblonga* and only three (0.44%) of the *B. depressus* individuals yielded this fungus. Of the *C. oblonga* isolates obtained from *Carpophilus* spp., 29 isolates were from *Ca. hemipterus* and 108 isolates were from *Ca. bisignatus* (Table 1).

Both the insect numbers and success of fungal isolation showed considerable seasonal fluctuation (Figure 2). *Ceratocystis* spp. were obtained from the insects only during the summer months, with the first isolates obtained in the 22nd wk (24-30 November 2005) of sampling. Successful isolation of *Ceratocystis* spp. ceased in week 48 (1-8 June 2006) of sampling. *Ceratocystis* spp. began to reappear in isolations at the beginning of summer in week 75 (30 November 2006) of the sampling period (Figure 2). A total of 165 *Ceratocystis* isolates were obtained from 30,293 insects during the entire study period. However, as one third of the insects (10098) had been surface sterilized and no fungal isolates were expected from them the effective number of insects from which *Ceratocystis* isolates could be expected was 20,195.

Isolation of *Ceratocystis* spp. from the insects collected from traps in the non-native study area gave similar results to those obtained from the native study site. In this area, *C. oblonga* (11 isolates) was isolated only from *Carpophilus* spp. and all but one of the *C. albifundus* isolates (7) originated from the *Brachypeplus* sp. with one isolate obtained from *Ca. bisignatus*.

The six *Ca. hemipterus* and 23 *B. depressus* collected from under bark flaps of stumps from recently felled *A. mearnsii* trees in 2008 yielded a total of 38 *Ceratocystis* isolates. Six of the *Ca. hemipterus* individuals yielded six *C. oblonga* isolates and three of the same insects gave rise to three *C. albifundus* isolates. Twenty-one *C. oblonga* isolates and eight *C. albifundus* isolates were collected from the 23 *B. depressus* insects collected from under the bark flaps. No *Ca. bisignatus* were obtained from the stumps (Figure 3).

4.3.6. Statistical analyses of data

Analyses of one climate data set, to determine which variables influenced the number of fungal isolates obtained, indicated that there were linear relationships present. Analyses indicated that the number of *C. oblonga* isolates obtained was influenced by minimum daily relative humidity (Chi-Square=10.81, P=0.001, Degrees of freedom=1). The association of predicted probabilities and observed responses for this interaction produced a concordance percentage of 78.4%. Analyses further indicated that the number of *C. albifundus* isolates obtained was influenced by an interaction between wind speed and the number of *B. depressus* collected (Chi-Square=9.31, P=0.0023, Degrees of freedom=1). The association of predicted probabilities and observed responses for this interaction produced a concordance percentage of 95.2%.

Analyses of the second data set, to determine which climatic variables influenced the number of insects collected, showed the presence of linear relationships. With regard to the number of *B. depressus* collected, a number of variables had a linear relation

(F-value=13.64, R-square=0.5426). These included daily maximum temperature, maximum daily relative humidity, daily minimum temperature, minimum daily relative humidity, the interaction between daily maximum temperature and maximum daily relative humidity and the interaction between daily minimum temperature and minimum daily relative humidity. However, only daily maximum temperature (t-value=2.81, P=0.0064, Df=1) and maximum daily relative humidity had significant probability values (t-value=2.73, P=0.0080, Df=1).

With regard to the number of *Carpophilus* spp. collected, only two of the tested variables had a linear relationship (F-value=13.43, R-square=0.4896). These were daily maximum temperature and maximum daily relative humidity. The linear relation between the climatic variable, daily maximum temperature, and the number of insects collected was significant (t-value=4.55, P>0.0001, Df=1). The linear relationship with maximum daily relative humidity identified was, however, not supported by the probability value (t-value=-0.29, P=0.7762, Df=1).

4.4. DISCUSSION

Prior to this study, nothing was known regarding the mode of spread of the wilt pathogen *C. albifundus*. We have thus confirmed that, similar to other *Ceratocystis* spp., *C. albifundus* has associations with nitidulid beetles. Virtually nothing is known of the biology of nitidulid beetles in South Africa and this study was, therefore, also the first to consider the influence of climate on the population numbers of these insects in the country and to identify some of the fungi associated with them. Results

also provide valuable information that might be used to limit spread of the disease of *A. mearnsii* caused by *C. albifundus* in South Africa.

Three insect species were identified as associates of *C. albifundus* and *C. oblonga* collected in this study. These were *B. depressus, Ca. hemipterus* and *Ca. bisignatus*. Very little is known regarding these insects in Africa and none has been reported to be associated with fungi in South Africa. *Brachypeplus depressus* has, however, been reported to be the most common *Brachypeplus* sp. in Africa (Kirejtshuk & Barcley 2007).

Carpophilus spp. have previously been reported as vectors of *Ceratocystis* spp. (Moller & DeVay 1968a, Juzwik & French 1983). *Carpophilus hemipterus* in particular, is a known agricultural pest and has been reported as an associate of *C. fimbriata* (Moller & DeVay 1968a). No evidence could, however, be found of prior associations between fungi and *Ca. bisignatus* and *B. depressus*.

It was interesting that only two species of *Ceratocystis* were isolated from insects in this study. Various other *Ceratocystis* spp. are known to occur on wounds of trees in the areas studied. These include *C. savannae* in the native study area (Kamgan *et al*. 2008), *C. obpyriformis* R.N. Heath & Jol. Roux and *C. polyconidia* R.N. Heath & Jol. Roux in the non-native study area (Heath *et al*. 2009). All of these fungi occur on wounds and are presumably vectored by insects similar to those encountered in this study. The results may suggest that there could be some host specialisation in terms of the species of *Ceratocystis* vectored, or that some level of competition exists between these fungi. Alternatively, these *Ceratocystis* spp. may occur at much lower

levels than *C. albifundus* and *C. oblonga*. Our data could also have been skewed by the low number of *Ceratocystis* spp. recovered from the insects collected in traps.

Ceratocystis oblonga was more commonly isolated from nitidulid beetles than was *C. albifundus*. *Ceratocystis oblonga* is a species recently described from cut stumps of non-native *A. mearnsii* in South Africa (Heath *et al.* 2009). The relatively large numbers of isolates of *C. oblonga* compared to those of *C. albifundus* could be due to differences in the odours produced by the two fungi, with *C. oblonga* being more attractive to the insects than *C. albifundus*. Alternatively, competition between the fungi on the substrate or isolation technique could have influenced these results. *Ceratocystis oblonga* grows fast (83mm in 7d) (Heath *et al.* 2009) compared to the relatively slow growing *C. albifundus* (20mm in 8d) (Wingfield *et al.* 1996) on 2% MEA. This could account for the fact that *C. albifundus* was more frequently isolated from insects subjected to carrot baiting than from insects placed on 2% MEA plates. This study represents the first report of *C. oblonga* from an other organism than *A. mearnsii* and it expands the geographic range of this fungus. It was predominantly isolated from the two *Carpophilus* spp. The study performed in the *A. mearnsii* plantations showed that *C. oblonga* has a percentage association with *Carpophilus* spp. of 100%. A large number of isolates of *C. oblonga* (21) also originated from *B. depressus* (23) collected from *A. mearnsii.*

Differences in isolation success were observed between insects collected in traps compared with those collected from stumps of recently felled *A. mearnsii* trees. Isolations from insects collected from bark flaps resulted in an association of 100% for *C. oblonga* with *Ca. hemipterus* and 91% with *B. depressus*. *Ceratocystis*

albifundus had a 50% association with *Ca. hemipterus* and 35% association with *B. depressus*. These levels of association were noticeably higher than those obtained for *C. albifundus* with *B. depressus* (3.2%) and *Carpophilus* spp. (0.03%) collected in traps in the native study sites. Similar results have been reported in previous studies with *C. fagacearum* where low associations (0.7%) were found between *C. fagacearum* and *Ca. truncatus* Muttay collected from traps in contrast to high association levels (79.8%) obtained from insects collected from fungal mats (Norris 1956, Gibbs 1980, Juzwik & French 1983). The lower recovery rates of *Ceratocystis* spp. from insects collected in traps could be due to the fact that the insects and spores are exposed to harsh environmental conditions during flight possibly reducing the viability and number of spores. In contrast, insects collected from fungal mats would not have been exposed to the same severe environmental conditions. The fact that we isolated the *Ceratocystis* spp. from free flying insects collected in traps, and from insects obtained from fungal mats, clearly shows that these insects play a role in the spread of these fungi.

Climatic factors had a significant influence on the population fluctuation of the nitidulid beetles. Not surprisingly, these insects were much more abundant in the spring and summer months (87%) than in the colder winter months (12%). These observations are similar to those for studies on the transmission of *C. fagacearum* by free-flying and fungus-mat-inhabiting nitidulids in Minnesota (Yount *et al*. 1955, Juzwik & French 1983). In those studies, it was shown that nitidulid beetle numbers are strongly influenced by temperature, with the insects over wintering under the bark of trees, in debris or in rotting fruit on the soil surface during the winter (James *et al*. 1995, Hossain & Williams 2003). Although we did not consider where these insects

might occur during the winter, they most likely occupy similar niches in South Africa to those of the better-studied Northern Hemisphere species.

Climatic factors had an influence of the number of fungal isolates obtained. For *C. oblonga*, minimum relative humidity was the only climatic variable with a significant influence on isolation. Interestingly, the number of *C. albifundus* isolates obtained was influenced by wind speed. This result was unusual as the only instance where winds have been reported to play a role is in the dissemination of fungal spores in the frass of insects (Iton 1960) and it might be incidental and an aberration.

Ceratocystis albifundus infects wounds on trees and results of this study suggest strongly that nitidulid beetles are the primary vectors that move the pathogen from one tree to another. *Acacia mearnsii* trees require corrective pruning to improve growth form after establishment and to correct damage caused by animal grazing (Dunlop & Goodricke 2000). We assume that these wounds are visited by nitidulid beetles that then transmit the pathogen. Given that the insects appear not to be active in the winter months, pruning should be undertaken only during this period to restrict the spread and infection by the fungus.

In this study we showed that *C. albifundus* and *C. oblonga* are involved in a relationship with three nitidulid beetles that most likely act as their vectors. It seems that these relationships are not specific as both fungal species were isolated from more than one insect species. This is not surprising as both the fungi produce fruity odours and are expected to have a loose association with the insect vectors (Himelick & Curl 1958). Vectorship of these insect however needs to be confirmed by further

studying the frequency of contaminated beetles visiting commonly known infection courts, the number of contaminated beetles emerging from diseases material and experimental caging studies. There are at least 11 *Ceratocystis* spp. known from Africa, however, no research regarding the possible insect associations of these species or their role in the biology and possible control strategies of the fungal pathogens have been performed. Further research is required into the possible insect associations of these species on the continent as this could provide valuable information to facilitate management and control strategies.

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Table 1. Association percentages for *Ceratocystis* spp. isolated from three nitidulid species collected from baited pitfall traps in native study area and from under bark flaps on non-native *A. mearnsii* stumps in South Africa.

	Ceratocystis albifundus				*Ceratocystis oblonga*			
	Traps	Nr. of insects assayed	Bark flaps	Nr. of insects assayed	Traps	Nr. of insects assayed	Bark flaps	Nr. of insects assayed
B. depressus	3.2%	680	35%	23	0.44%	680	91%	23
Ca. hemipterus	0.08%	2 354	50%	6	1.2%	2 354	100%	6
Ca. bisignatus	0.02%	7 067	-		1.5%	7 067	-	

Figure 1. Illustration of a section through the trap used in this study.

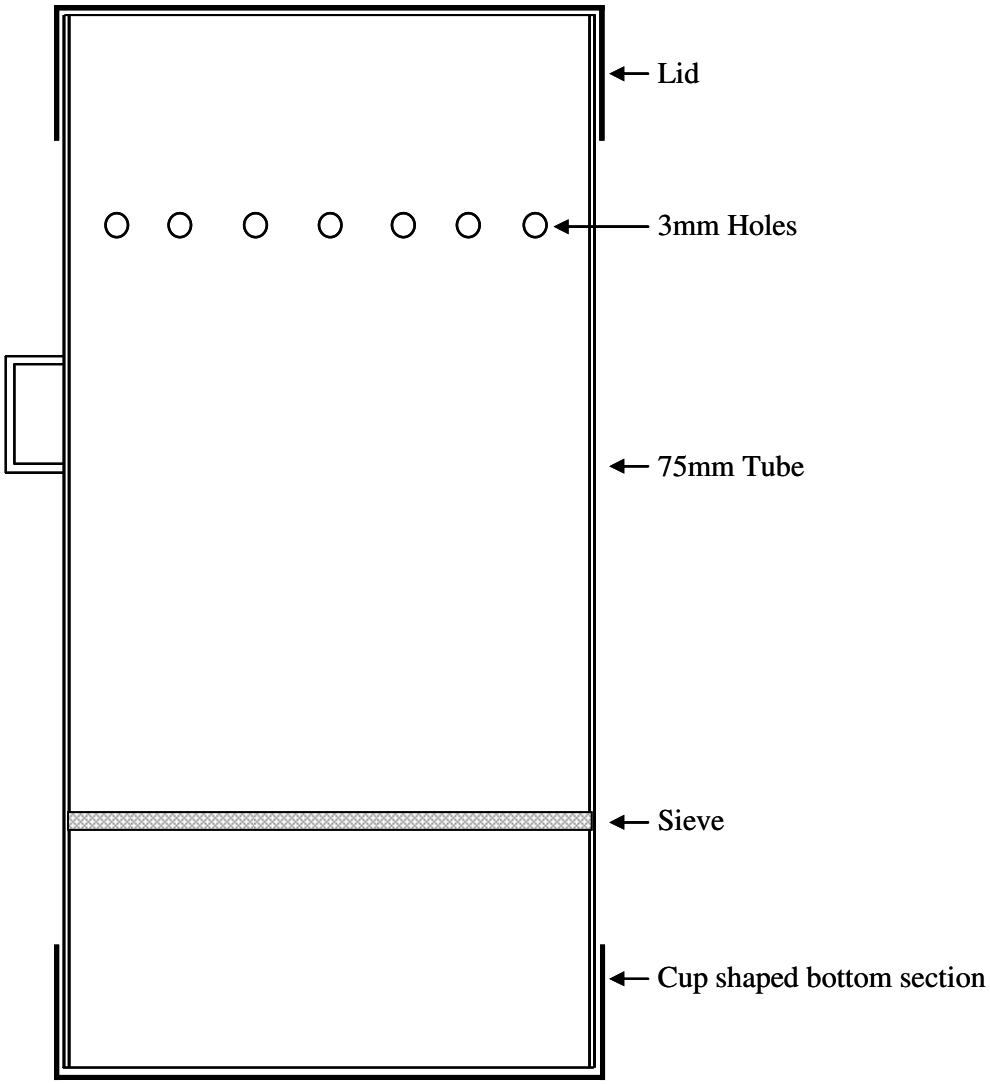

Lid
3mm Holes
75mm Tube
Sieve
Cup shaped bottom section

Figure 2. Graph illustrating the fluctuation of the total numbers of individual insects species and number of *Ceratocystis* isolates collected for the duration of the study.

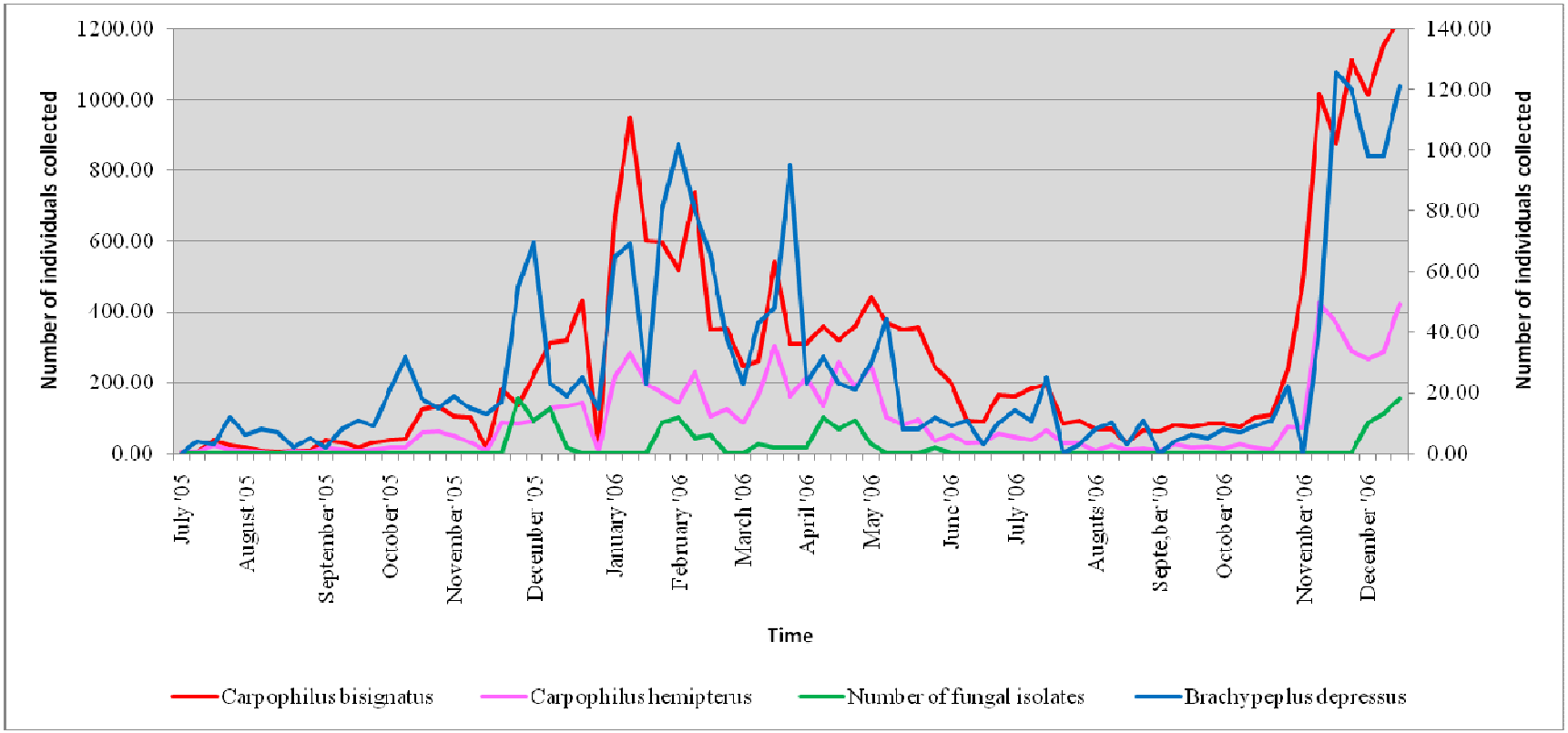

Number of individuals collected
1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Number of individuals collected
July '05
August '05
September '05
October '05
November '05
December '05
January '06
February '06
March '06
April '06
May '06
June '06
July '06
Augusts '06
Septe,ber '06
October '06
November '06
December '06
Time
Carpophilus bisignatus
Carpophilus hemipterus
Number of fungal isolates
Brachypeplus depressus

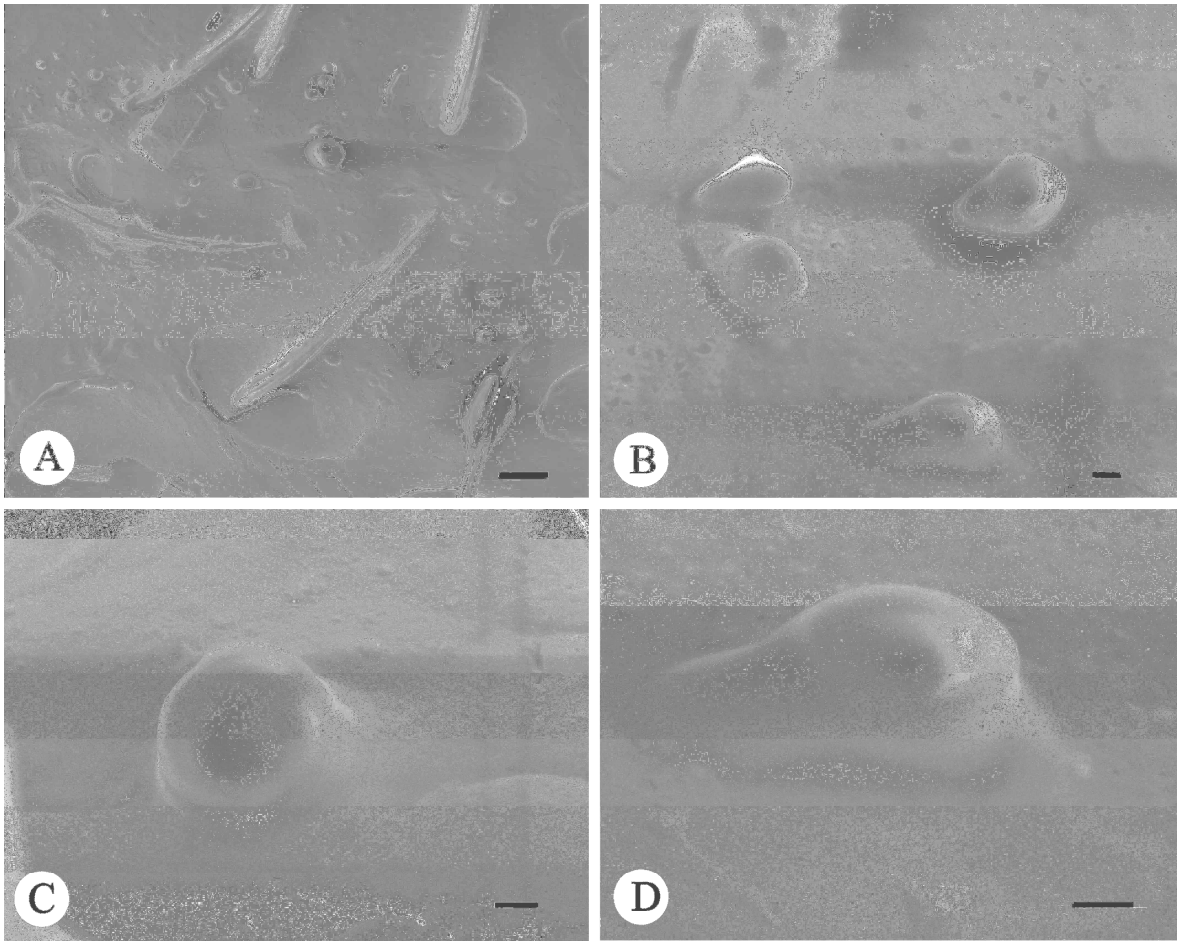
Figure 3. Insects collected from the non-native study site (A) *B. depressus*, (B) *C. hemipterus*, (C) *C. bisignatus*. Scale bar = 1000µm.

A

B

C

Figure 4. Scanning Electron Microscope images illustrating (A) fungal propagules on the insect body, (B) hat-shaped ascospores characteristic of *Ceratocystis* spp. observed on *B. depressus*, (C) hat-shaped ascospores characteristic of *Ceratocystis* spp. observed on *Ca. bisignatus*, (D) hat-shaped ascospores characteristic of *Ceratocystis* spp. observed on *Ca. hemipterus.*


A
B
C
D

Chapter 5

FACTORS INFLUENCING INFECTION OF ACACIA MEARNSII BY THE WILT PATHOGEN CERATOCYSTIS ALBIFUNDUS IN SOUTH AFRICA

ABSTRACT

It is well known that species of *Ceratocystis*, which cause canker and wilt diseases on trees require wounds for infection. In this regard, a number of physical and environmental factors influence the success of wound colonization by these fungi. The aim of this study was to consider the influence of wound age, stem diameter and colonization of wounds by *Ophiostoma quercus* on the success of infection by *Ceratocystis albifundus,* an important wilt pathogen of *Acacia mearnsii* in southern and eastern Africa, under field conditions. This was done by performing controlled inoculations on *A. mearnsii* trees, of different diameters, with *C. albifundus* at different time intervals after wounding and after pre-inoculation of wounds with *O. quercus* at the time of wounding. The success of infection by *C. albifundus* decreased significantly from eight hours after wounding and it was higher on stems of greater diameter. Pre-infection of wounds by *O. quercus* reduced the infection success of *C. albifundus*. The overall results showed that managing wounds created during forestry operations and treating wounds with naturally occurring, non-virulent fungi, such as *O. quercus,* could reduce the prevalence of infections by *C. albifundus*.

5.1. INTRODUCTION

Ceratocystis albifundus De Beer, M.J. Wingf. & Morris (Ascomycetes, Microascales) is the causal agent of Ceratocystis wilt and die-back of non-native plantation-grown *Acacia mearnsii* De Wild. trees in southern and eastern Africa and is considered the most important pathogen of *A. mearnsii* trees in Africa (Morris *et al*. 1993, Wingfield *et al*. 1996 Roux *et al*. 2005). The disease was first reported on *A. mearnsii* in South Africa (Morris *et al.* 1993), but it has subsequently also been recorded from Uganda (Roux & Wingfield 2001), Kenya and Tanzania (Roux *et al.* 2005). Symptoms of infection include gum exudation, wood-discoloration, stem cankers, rapid wilting and tree death (Morris *et al.* 1993, Roux *et al.* 1999). The fungus has been reported from native tree species in Africa, but has not been reported to cause disease on its native hosts (Roux *et al*., 2007).

Ceratocystis spp. require wounds for infection (Norris 1953, Bretz 1952, DeVay *et al*. 1963, Kile 1993). Such wounds can arise from wind (Roux *et al*. 2007), hail damage (Roux *et al*., 1995), growth cracks (Teviotdale & Harper 1991), insect and animal feeding (Walter 1946, Bretz 1952, DeVay *et al*. 1963), harvesting (Teviotdale & Harper 1991, Marin *et al*. 2003) and silvicultural practices such as grafting and pruning (Walter 1946, Teviotdale & Harper 1991, Dunlop & Goodricke 2000). Infection is dependant on a number of physical and environmental factors. For example, species in the *C. fimbriata sensu lato* (*s.l.*) species complex are able to infect their hosts when viable fungal propagules are deposited onto bark wounds (DeVay *et al.* 1968). Other *Ceratocystis* spp., such as *C. fagacearum* (Bretz) Hunt, can only infect if viable fungal propagules are deposited onto freshly exposed wood of the host (Kuntz & Drake 1957). Temporal factors also affect the success of infection by *Ceratocystis* spp. For example, Kuntz & Drake (1957) showed that *C. fagacearum* could not cause infection when wounds were o$lder than 24 hours. Climatic factors such as temperature and relative humidity have also been shown to influence germination of spores and infection by *Ceratocystis* spp. (Cole & Fergus 1956). Gibbs (1980), showed that colonization of wounds by the saprophytic fungus,

Ophiostoma piceae (Munch) H. Sydow & Sydow, prior to infection by *C. fagacearum*, prevented colonization by the pathogen.

Ceratocystis spp. have evolved several strategies to reach wounds and subsequently infect trees. The most common of these is through associations with insects including bark beetles (Coleoptera: Scolytidae) (Webber & Gibbs 1989, Kirisits 2004), nitidulid beetles (Coleoptera: Nitidulidae) (Himelick & Curl 1958, Moller & DeVay 1968, Harrington 1987) and flies (Moller & DeVay 1968, Hinds 1972). These fungi are morphologically adapted to insect dispersal having their ascospores in sticky drops at the apices of long-necked sporocarps (Dowding 1984). Many species of *Ceratocystis*, particularly those not associated with bark beetles (Webber & Gibbs 1989, Kirisits 2004), also produce fruity aromas that attract insects to fungal sporulating structures that subsequently visit fresh wounds on trees. In this regard, *C. albifundus* has recently been reported to be associated with nitidulid beetles (*Carpophilus* spp. and *Brachypeplus* spp.) that visit wounds on native and non-native trees in South Africa (Heath *et al*. 2009).

Infection of *A. mearnsii* by *C. albifundus* has been associated with hail wounds, as well as with pruning wounds on these trees (Roux *et al.* 1995). *Acacia mearnsii* trees typically require corrective pruning to improve growth form after establishment and to correct damage caused by animal grazing (Dunlop & Goodricke 2000). Wounds arising from these operations provide ideal infection sites for *C. albifundus*.

Although selection and breeding for resistance against the disease caused by *C. albifundus* has been promoted, the fungus remains one of the most important pathogens of *A. mearnsii* in South Africa. Therefore, an understanding of the factors involved in the successful infection of wounds by *C. albifundus* could contribute to improved disease management options in plantations. In this study we aimed, 1) to consider the influence of wound age on the success of infection by *C. albifundus* under field conditions, 2) investigated the possible influence of the colonization of wounds by the commonly occurring wound-infecting *Ophiostoma quercus* (Georgév.) Nannf. (De Beer *et al*. 2003) on the success of infection by *C. albifundus* under field

conditions. The third aim of this study was to consider whether stem diameter affects the infection success of *C. albifundus*.

5.2. MATERIALS AND METHODS

5.2.1. Preparation of inoculum

An isolate (CMW4095) of *C. albifundus* collected in 1998 from diseased *A. mearnsii* in South Africa, was selected for artificial inoculations. Only one isolate was used for the study as low variance in virulence between isolates of *C. albifundus* was proven by De Beeer (1994). An *O. quercus* isolate (CMW24164), collected from a four-week-old wound on an *A. mearnsii* stump in 2006, also in South Africa, was selected for tests to consider the effect of pre-infection by this fungus on the infection success of *C. albifundus*. Both isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Pure cultures of the test isolates were grown on 2% (w/v) malt extract agar (MEA) (Biolab, Midrand, South Africa) supplemented with Streptomycin Sulphate (0.001 g/vol, SIGMA, Steinheim, Germany) and incubated at 25ºC under natural day/night conditions for seven days prior to inoculation. Spore suspensions were made by washing spores from the surface of seven-day old cultures, with sterile water. Concentrations of spores were adjusted to 1 x 10^6 spores per ml with sterile water using a Neubauer 1/100 mm haemocytometer (BEOCO, Germany). Suspensions were prepared no longer than ~60 minutes before inoculation and were maintained at ~5ºC prior to application. Viability of the spores in the suspensions was confirmed by plating 1ml of spore suspension onto 2% (w/v) MEA at the time of inoculation and confirming growth after five days.

5.2.2. Wounding and inoculation of trees

The study was conducted in a two-year-old stand of *A. mearnsii* situated 35 km east of Pretoria, South Africa (S 25° 23' 38.3", E 028° 37' 19.5"). The inoculation study was performed using a completely randomised design with regard to treatments as well as the diameter of tree stems. Trees ranging from 10 mm to 32 mm in diameter were

wounded by cutting the stems approximately 50 cm above the soil surface with pruning shears, thereby simulating stem reduction practices applied in silvicultural regimes for *A. mearnsii* cultivation in South Africa (Figure 1a). This method produced a stub with a flush cut exposing the xylem cells of the stems. Inoculations commenced in March 2007 (late summer) and the study was completed in May 2007 (autumn), thus also consistent with the time at which corrective pruning often occurs.

A total of 400 trees were pruned for the experiment. Twenty different treatments were applied, with each treatment applied to 20 trees. The 20 treatments included a water control (negative control), a positive control with *C. albifundus*, a positive control with *O. quercus* and a combination of *C. albifundus* and *O. quercus* inoculated at wounding. Eight treatments included those where *C. albifundus* was inoculated onto wounds two, six, eight, 24, 48 (2 days), 124 (5 days), 196 (8 days) and 346 hours (14 days) after wounding. The remaining eight treatments were fresh wounds which were inoculated with *O. quercus* at the time of wounding, followed by inoculation with *C. albifundus* at two, six, eight, 24, 48 (2 days), 124 (5 days), 196 (8 days) and 346 hours (14 days) after wounding. Inoculations were made by dispensing 2 ml spore suspensions directly onto the wounds using disposable syringes. The entire experiment was repeated once consecutively.

5.2.3. Assessment of infection

The experiments were terminated six weeks after each treatment commenced as this time period has been shown to be sufficient for disease development on trees of similar size under field conditions in South Africa (Roux *et al*. 1999). Inoculated stubs were collected by cutting the stems at the soil surface and transporting the samples in separate plastic bags to the laboratory. In the laboratory, the diameter of each stem was measured and the bark removed. Every inoculated wound was assessed visually for the presence or absence of vascular discoloration. Re-isolations were performed to correlate with visual assessment of *C. albifundus* infection.

5.2.4. Statistical analyses

Statistical analyses were performed using SAS (SAS Version 8.2, 2001). The infection data were converted to binary format with the presence of infection represented by a “1” and the absence of infection represented by “0”. Because this

response variable was binary, a logistic model could be applied to reproduce the logit as a linear model of the predictor variables. The predictor variables were Treatment (a categorical variable with 20 levels), Replicate (a categorical variable with two levels) and Tree Diameter (a continuous variable). The logistic analysis models the variable lin{P/(1-P)}, the logit, as a linear function of the predictor variables, where P is the probability of a specific combination of predictor values to yield an infection.

The significance of each predictor to predict the logit was calculated. The concordance percentage was calculated to serve as a measure of the "goodness of fit" of the applied model. Confidence intervals for the true model parameters were calculated using the estimated parameters and their standard errors. Furthermore, to provide for specific chosen combinations of predictor values (Treatment, Replicates and Tree Diameter), not present in the data, such combinations were added to the dataset while the response variable was absent. This was done to exclude the "spiked" observations when the model was fitted, and to include them when estimated P - values were calculated for the specific added combinations. Finally, 95 % confidence intervals were calculated for the P-values for the "spiked" observations.

5.3. RESULTS

5.3.1. Assessment of infection

The only exterior symptoms observed on inoculated stems, six weeks after inoculation were death of the tips of the stems (Figure 1b) and yellowing of the bark bark and staining of the wood. *Ceratocystis* infection was distinguished from infection by *O. quercus* based on the fact that *C. albifundus* causes light to dark brown streaking (Figure 1d) whereas *O. quercus* resulted in stain, when present, having a distinct blue colour (Figure 1e). The majority (95%) of the 40 negative water controls (Figure 1c) showed no symptoms of *C. albifundus* or *O. quercus* infection. Those control treatments that did show infection (5%) were considered to be due to natural infection since *C. albifundus* naturally occur in the Gauteng Province (Roux *et al.*, 2007). Re-isolation of *C. albifundus* from infected stems followed a similar trend to infection assessed visually (Table 1). The Chi-square probability value showed significant treatment differences within the total dataset ($P < 0.0001$). The predictor variable, replicate, had a significant effect on the analyses ($P = 0.0008$). This could be due to

the trees used in the repeat trial entering the dormancy period due to the change in season (Robinson *et al*., 2004). However, no interaction existed between the predictor variables treatment and replicates (P = 0.4745), therefore, treatment means could be calculated across replicates.

5.3.2.1. Time after wounding

The time after wounding had a significant influence on the infection success of *C. albifundus*. The greatest number of stems with symptoms of *C. albifundus* infection was observed in the 6hr treatment where *C. albifundus* was inoculated alone, with 31 of the 40 trees (77.5%) showing infection. Infection of stems inoculated with *C. albifundus* at the time of wounding, two, six and eight hours after wounding was significantly higher than that of all other treatments ($P \leq 0.0344$). The levels of infection of stems that were inoculated with *C. albifundus* at 124, 196 and 346 hours after wounding were not significantly different from the control treatments for this experiment, nor did they differ significantly from each other (Figure 2).

5.3.2.2. Pre-inoculation with O. quercus

Pre-inoculation of stems with *O. quercus* had a significant influence on the infection success of *C. albifundus*. Stems inoculated with *O. quercus* and *C. albifundus* concurrently at the time of wounding exhibited the highest levels of infection and differed significantly from all other treatments. All other treatments inoculated with *O. quercus* at the time of wounding, and *C. albifundus* at different time intervals thereafter, were significantly different from the control treatment (Figure 3). Pre-inoculation with *O. quercus* at time of wounding and with *C. albifundus* at 196 hours was not significantly different from the control treatments. All other treatments differed significantly from the control treatments.

5.3.2.3. Influence of stem diameter on infection

The diameter of the stems, and thus surface area of the wound exposed to infection, had a significant influence on the infection success of *C. albifundus* ($P = 0.0002$). The analysis of maximum likelihood estimates produced an estimate value of 0.0617 for tree diameter as a predictor variable with one degree of freedom (Figure 4). This indicated that with every 10 mm increase in stem diameter, the probability of infection by *C. albifundus* increased by 6.2%.

5.4. DISCUSSION

Results of this study showed clearly that infection of wounds on *A. mearnsii* by *C. albifundus* is strongly influenced by the condition of the wounds. Wounds became less susceptible to infection over time and after 24 hours they had a very low level of susceptibility. Likewise, pre-inoculation of wounds with *O. quercus* significantly decreased the ability of *C. albifundus* to infect wounds. Potential of infection also increased as the size of the stems increased.

Loss of susceptibility to infection by *C. albifundus* with increasing wound age was not surprising. Similar results have been found in many studies of pathogens, including *C. fimbriata, Leucostoma cincta* and *L. persoonii*, which infect woody plants (Bostock & Middleton 1987, Biggs 1989, Teviotdale & Harper 1991). Specifically in the case of *Ceratocystis*, the results of this study are similar to those on *C. fagacearum* where wounds on oak trees are typically found not to be susceptible to infection after 24 hours (Morris *et al*., 1955, Kuntz & Drake 1957, Gibbs 1980).

The present study did not investigate the possible reasons why wound susceptibility decreases with time. Previous studies attributed loss of susceptibility of wounds to *C. fimbriata* on Almond trees to the fact that the thin film of moisture required on the wound for the germination of spores disappears with time. Other studies have attributed the increase in resistance of wounds to infection with time to the accumulation of an impervious lingo-suberized layer (Biggs 1984, 1985 a, b, 1989, Oven *et al*., 1999, Stobbe *et al*., 2002, Robinson *et al*., 2004). Numerous authors have stated that this layer is responsible for preventing moisture los from the wound as well as preventing fungal infection (Biggs 1992, Woodward 1992). The formation of a necrophylactic periderm has also been reported as a wound response that could prevent fungal infection (Biggs 1985a). It is, however, unlikely that the formation of the necrophylactic periderm is the primary defence against fungal infection as this layer only forms after the impervious lingo-suberized layer has formed (Woodward 1992, Oven & Terelli 1994, Oven *et al*., 1999).

Pre-inoculation of wounds with *O. quercus* had a significant effect on infection with *C. albifundus*. Thus, colonisation of fresh wounds by *O. quercus* approximately two hours prior to infection by *C. albifundus* could reduce the chance of infection by the pathogen significantly. In a similar study, Gibbs (1980) showed that where *O. piceae* was inoculated onto fresh wounds approximately 24 hours prior to *C. fagacearum*, the chance of infection by the pathogen was greatly reduced. *Ophiostoma quercus* was selected for the current study because it is commonly found on wounds on *A. mearnsii* trees (unpublished data) and has not been reported to decrease the value or structural integrity of the timber. Damage caused by sapatain fungi such as *O. quercus* have been reported to be cosmetic (Seifert 1993). Another consideration was that *Ophiostoma* spp. have a biology similar to that of *Ceratocystis* spp. and are also carried to wounds by insects (Brasier 1990, Kirisits 2004). Gibbs (1980), suggested that colonisation of wounds by *O. piceae* may be common in nature, reducing the natural occurrence of oak wilt. The same situation seems to be possible in South Africa in the case of *C. albifundus* infection and the colonisation of wounds with *O. quercus*.

The diameter of the stems inoculated in this study had a significant effect on the infection success of *C. albifundus.* As the stem diameter increased, and thus the size of the exposed wound (horizontal cut) surface area, the probability of infection increased. This could be due to a more rapid drying of the smaller stems compared to the larger stems. The fact that stem diameter has an influence on the infection success of *C. albifundus*, makes the results of this study applicable to the prescribed pruning protocols for *A. mearnsii* trees in South Africa. Current silvicultural prescriptions for the management of *A. mearnsii* suggest that pruning should be undertaken on stems two to four meters tall (Dunlop & Goodricke 2000). If the age at which pruning conducted is reduced, the diameter of the stems would be smaller and infection be less likely.

Successful management of plantation forest diseases relies on a combination of selection and breeding and sound silvicultural practices. The results obtained in this study could assist forestry companies in southern and eastern Africa in formulating silvicultural regimes that could reduce the impact of *C. albifundus* infection and mortality after pruning. The results obtained in this study could also be applied to

better management of numerous tree growing industries. Furthermore, this study also raises the possibility of using *O. quercus* to reduce the infection of recently cut stems by *C. albifundus*. Although this study supports findings obtained in previous studies using other *Ceratocystis* spp., this is the first study of its kind on the African continent and the first to provide possible management prescriptions for the fully regulated forestry management system followed in numerous countries world-wide.

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Table 1. Comparison of *C. albifundus* isolated from vascular streaking to visual infection assessed

Treatment	**Number of visual symptoms observed**	**Number of *C. albifundus* isolates obtained**
Water control	2	0
Ceratocystis albifundus, 0 hrs	30	10
Ophiostoma quercus, 0 hrs	15	3
C. albifundus & *O. quercus*, 0 hrs	28	5
C. albifundus, 2 hrs	28	9
O. quercus 0 hrs & *C. albifundus* 2 hrs	15	0
C. albifundus, 6 hrs	31	9
O. quercus 0 hrs & *C. albifundus* 6 hrs	16	1
C. albifundus, 8 hrs	29	5
O. quercus 0 hrs & *C. albifundus* 8 hrs	11	0
C. albifundus, 24 hrs	18	2
O. quercus 0 hrs & *C. albifundus* 24 hrs	9	0
C. albifundus, 48 hrs	9	0
O. quercus 0 hrs & *C. albifundus* 48 hrs	8	0
C. albifundus, 124 hrs	5	0
O. quercus 0 hrs & *C. albifundus* 124 hrs	10	0
C. albifundus, 196 hrs	6	0
O. quercus 0 hrs & *C. albifundus* 196 hrs	5	0
C. albifundus, 346 hrs	3	0
O. quercus 0 hrs & *C. albifundus* 346 hrs	7	0

Figure 1. Wound morphology and symptoms of infection on *A. mearnsii.* (a) typical wound made for the inoculation, (b) external symptoms including dying of the tips of the stems, (c) water control showing no signs of infection, (d) vascular streaking caused by *C. albifundus* (indicated by arrow), (e) vascular streaking caused by *O. quercus* (indicated by arrow) on positive treatment with *O. quercus*..

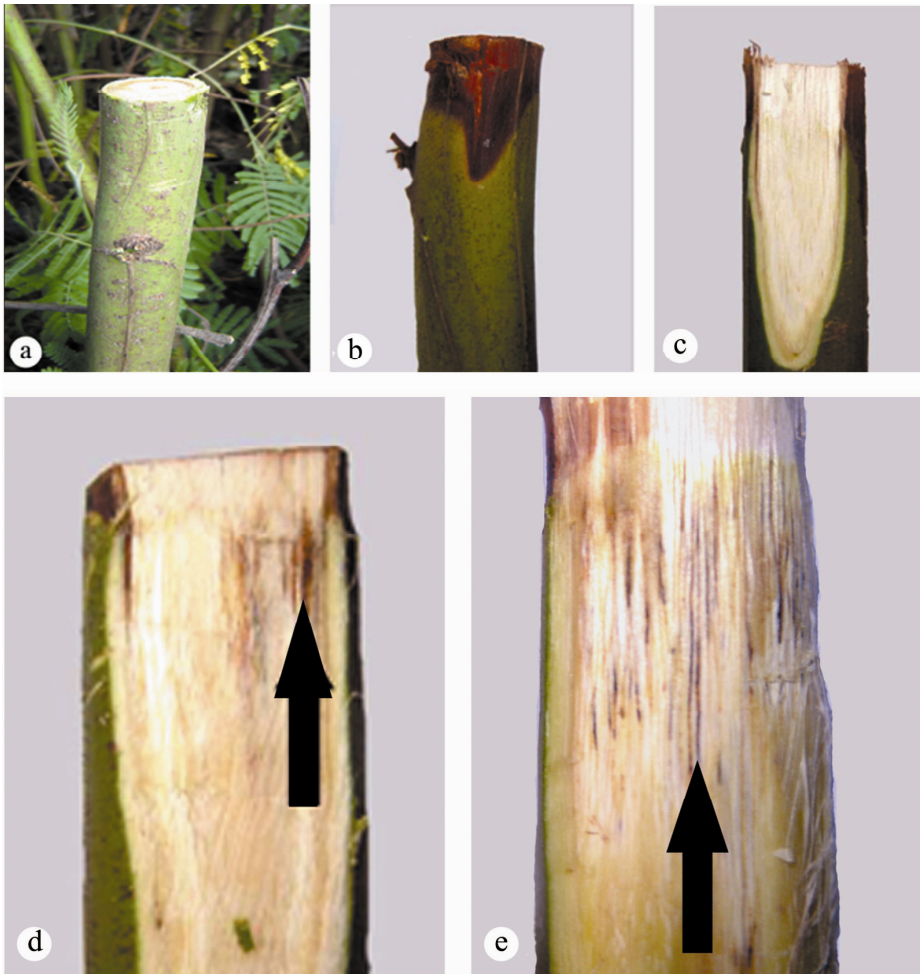

a
b
c
d
e

Figure 2. Number of horizontal stem cuts (wounds) infected with *C. albifundus* assessed visually for each treatment. Different letters above bars indicate treatment results significantly different from each other at a 5 % significance level. Figure shows combined data for both experiments (2x20 trees).

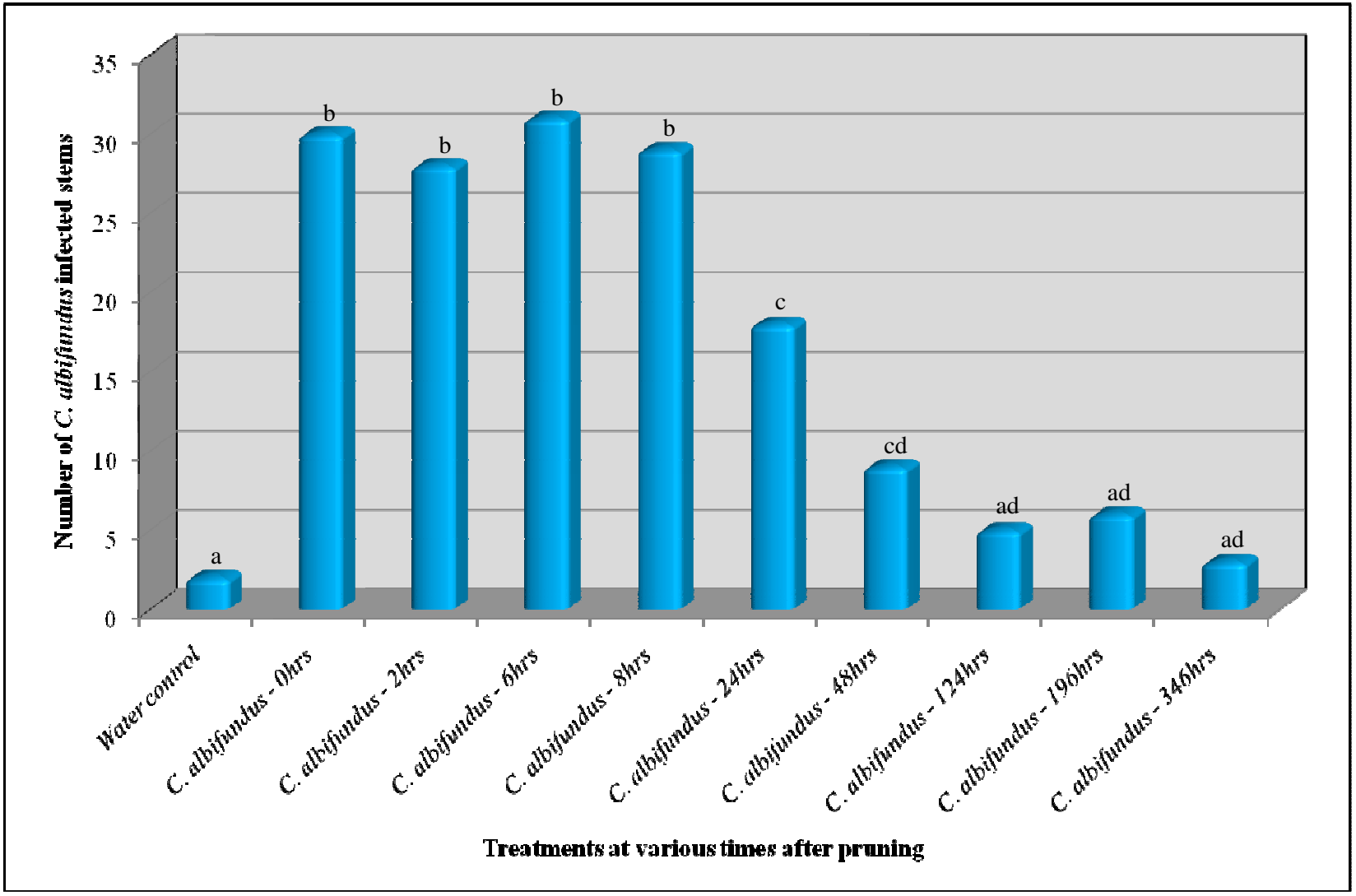

Number of C. albifundus infected stems
35
30
25
20
15
10
5
0
a
b
b
b
b
c
cd
ad
ad
ad
Water control
C. albifundus - 0hrs
C. albifundus - 2hrs
C. albifundus - 6hrs
C. albifundus - 8hrs
C. albifundus - 24hrs
C. albifundus - 48hrs
C. albifundus - 124hrs
C. albifundus - 196hrs
C. albifundus - 346hrs
Treatments at various times after pruning

Figure 3. Number of horizontal stem cuts (wounds) infected with *C. albifundus* assessed visually for each treatment. Different letters above bars indicate treatment results significantly different from each other at a 5 % significance level. Figure shows combined data for both experiments (2x20 trees).

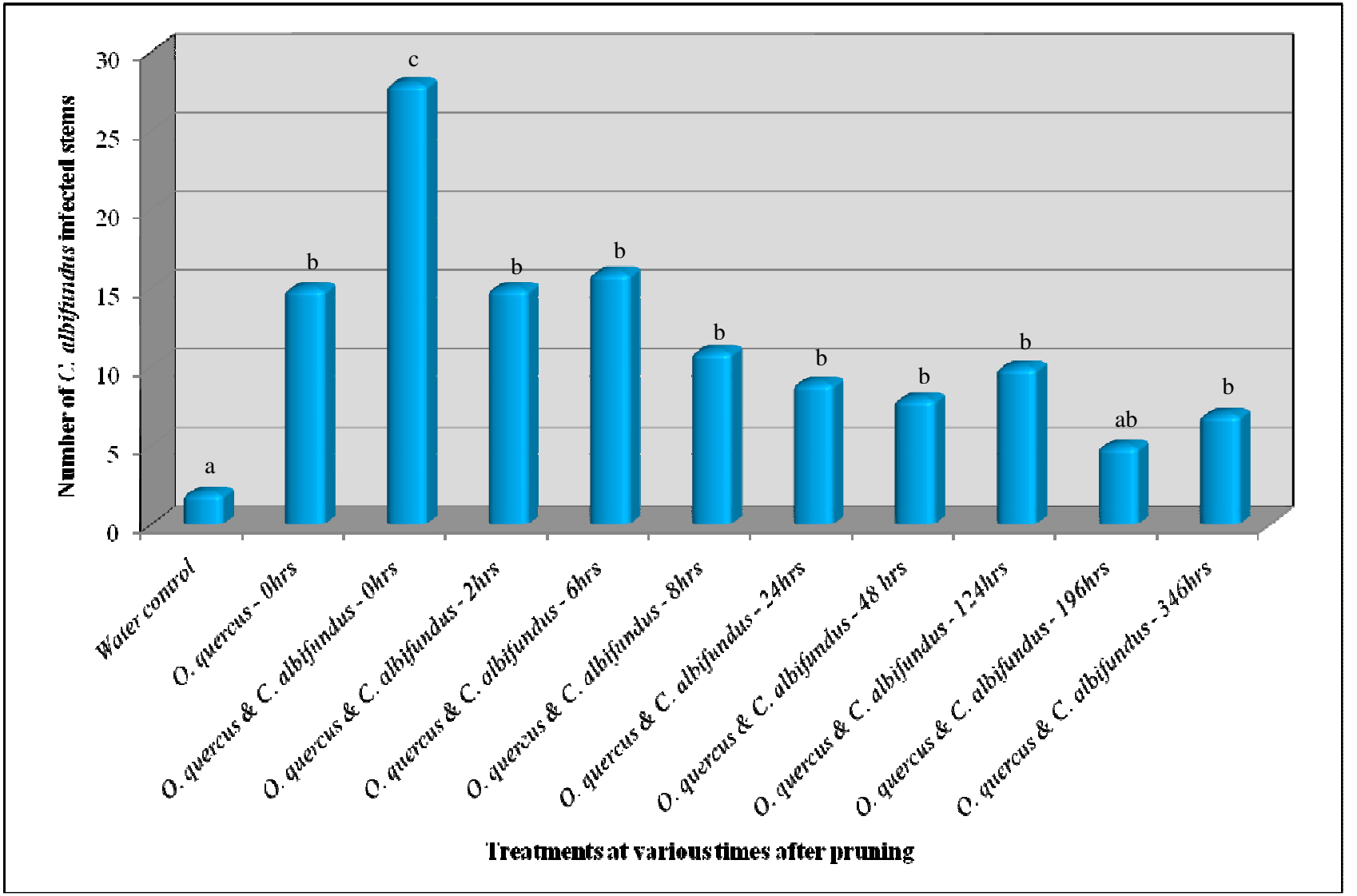
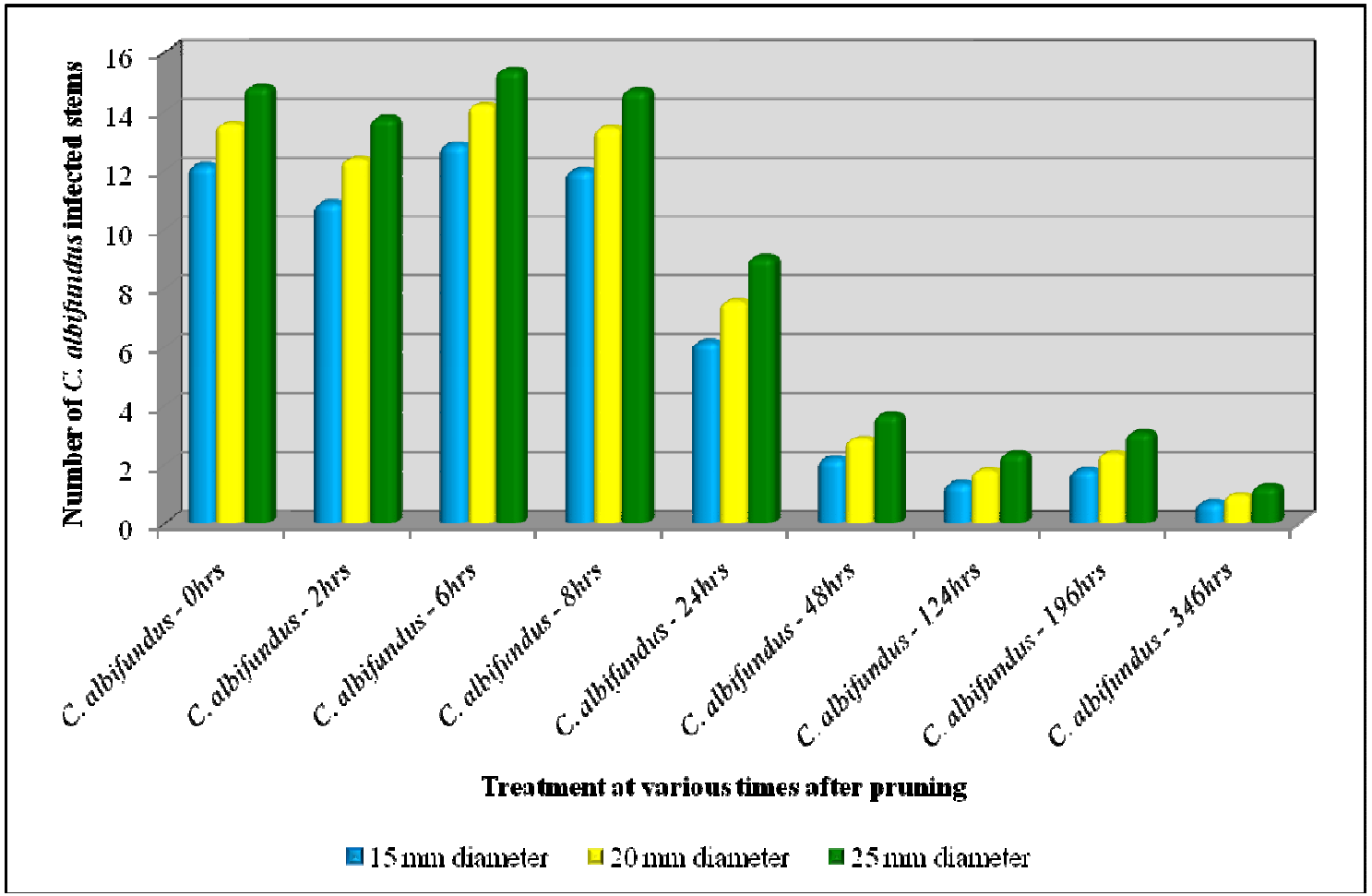

Number of C. albifundus infected stems
30
25
20
15
10
5
0
a
b
c
b
b
b
b
b
b
b
ab
b
Water control
O. quercus - 0hrs
O. quercus & C. albifundus - 0hrs
O. quercus & C. albifundus - 2hrs
O. quercus & C. albifundus - 6hrs
O. quercus & C. albifundus - 8hrs
O. quercus & C. albifundus - 24hrs
O. quercus & C. albifundus - 48 hrs
O. quercus & C. albifundus - 124hrs
O. quercus & C. albifundus - 196hrs
O. quercus & C. albifundus - 346hrs
Treatments at various times after pruning

Figure 4. The effect of diameter on infection success of *C. albifundus* on horizontal stem cuts (wounds) obtained from analysis for specific chosen combinations of the predictor values (Treatment, Replicates and Tree Diameter) at a 5 % significance level obtaining a percentage concordance of 80.5% and Wald Chi-square value of 13.67 and *P* – 0.0002. Figure shows combined data for both experiments (2x20 trees).


Number of C. albifundus infected stems
16
14
12
10
8
6
4
2
0
C. albifundus - 0hrs
C. albifundus - 2hrs
C. albifundus - 6hrs
C. albifundus - 8hrs
C. albifundus - 24hrs
C. albifundus - 48hrs
C. albifundus - 124hrs
C. albifundus - 196hrs
C. albifundus - 346hrs
Treatment at various times after pruning
15 mm diameter
20 mm diameter
25 mm diameter

SUMMARY

The genus *Ceratocystis* includes many important tree pathogens. Some of the well known tree diseases caused by *Ceratocystis* spp. include oak wilt caused by *C. fagacearum* in the USA, Ceratocystis wilt of wattle caused by *C. albifundus* in Africa and canker stain disease of plane trees in Europe caused by *C. platani*. Apart from *C. albifundus,* and the disease it causes on Australian *Acacia* spp. in southern and eastern Africa, relatively little is known regarding the *Ceratocystis* spp. on the African continent. Recent studies have lead to the discovery of several previously undescribed species, as well as a number of new distribution and host reports for *Ceratocystis* spp., especially in South Africa. Many areas of research pertaining to *Ceratocystis* spp., however, still deserve attention in Africa. Studies conducted for this dissertation investigated several aspects pertaining to the *Ceratocystis* spp. occurring on commercially important plantation grown tree species, including the taxonomy, biology and insect associations of the species infecting wounds on *A. mearnsii* and *Eucalyptus* spp.

The first chapter of this dissertation provided a review of the literature pertaining to the biology, ecology and taxonomic history of *Ceratocystis* spp. A strong focus of this chapter was the association of *Ceratocystis* spp. with insects. In this section, examples of known insects associations were mentioned while the levels of association and interaction or interdependence of these associations were discussed. This review highlights the need for research in this field in Africa. To date, only six *Ceratocystis* spp. have been reported from tree species in Africa and no literature is available pertaining to the possible insects associations of these species.

Human population growth is placing increasing pressure on native forests in Africa. To address this problem, extensive plantation forestry programmes, based on non-native tree species, have been established. Pests and diseases, however, are threatening the long term sustainability of these plantations. The threat of host jumps, and the ease with which pathogens move across the globe exasperates this problem. Concern regarding diseases caused by *Ceratocystis* spp. has initiated surveys for this important group of

tree pathogens. Chapter two in this thesis described five previously unknown *Ceratocystis* spp. and one *Thielaviopsis* sp. from four African countries. These species were *C. oblonga*, *C. polyconidia*, *C. obpyriformis*, *C. tanganyicensis*, *C. zombamontana* and *T. ceramica*. As all of these species were able to cause lesions following artificial inoculations on the tree species they were isolated from, they could be pathogenic and pose a threat to these trees in the areas where they are grown. This, however, needs to be tested under field conditions for an extended period of time to determine whether these species are pathogenic or only staining agents. The high number of species described from this single study highlights the need for further research in forestry in Africa.

One of the most serious pathogens threatening non-native *A. mearnsii* trees grown in commercial plantations in Africa is the wilt pathogen, *C. albifundus*. Ceratocystis wilt of wattle commonly affects trees after hail damage, but the fungus is also able to infect through other wounds such as pruning wounds. It has been hypothesised that the fungus is native to the African continent. The discovery of *C. albifundus* on native hosts in Africa enabled us to perform a population study on this fungus using microsatellite markers and for the first time incorporate populations from both native and non-native hosts on the African continent. The results of this chapter support the hypothesis that the fungus has performed a host jump from native hosts to non-native hosts and not the reciprocal. Although results from this study provided some insight into the possible origin of the fungus, it could not provide conclusive proof. Based on the data, it appears that *C. albifundus* originated from a range of native hosts north of South Africa with which it co-evolved. It then established itself on the non-native trees via multiple introductions in a southerly direction.

Ceratocystis spp. are well-known for their insect associations. However, no information regarding this aspect of the biology of *Ceratocystis* spp. was known for Africa. Research conducted for this dissertation identified three insect species in association with *C. albifundus* and the newly described fungus, *C. oblonga*. These were *Brachypeplus depressus*, *Carpophilus hemipterus* and *Ca. bisignatus*. Results show a possible closer association between *C. albifundus* and *B. depressus* and between *C.*

oblonga and *Ca. hemipterus* and *Ca. bisignatus*. However, these fungi form part of a group that are known to have non-specific associations with insects. Both *C. albifundus* and *C. oblonga* could thus be associated with the insects identified in this study. We also provided evidence that climatic factors have an influence on the population size and abundance of the insects associated with the *Ceratocystis* spp. This study not only provided ecological and biological information pertaining to the fungus and the insects, but also provides information that could be applied in the formulation of control strategies to reduce the impact of *C. albifundus* and other *Ceratocystis* spp. in South Africa.

To date, limited control strategies are available for the management of Ceratocystis wilt of *A. mearnsii*. Currently, the only management strategy that is followed is the use of disease tolerant plant families and recommendations to avoid tree wounding as far as possible. Many of the recommendations are based on information from Northern Hemisphere countries and not necessarily the most effective or accurate for Africa. In Chapter five, we investigated the effect wound age and size has on the infection success of *C. albifundus*. We also investigated the influence pre-inoculation of wounds with *Ophiostoma quercus* has on the infection success of *C. albifundus*. Results obtained indicated that both these factors have a significant influence on infection success. The results of this study indicated that infection by *C. albifundus* decreases significantly eight hours after wounding. The study results also indicated that infection of *C. albifundus* increases significantly as the size of the wound increases and that pre-inoculation of wounds with *O. quercus* decreased the infection success of *C. albifundus*.

Research conducted for this dissertation has highlighted the importance of studies focusing on fungal diseases occurring in the commercial plantations of southern and eastern Africa. With a total of six previously undescribed fungal species being reported in this thesis, it makes one question as to what number of fungal species still awaits to be discovered. It also raises the question of how many species on the native flora are able to infect and threaten economically important non-native crops. It has also reiterated the importance of understanding the biology of these fungi and the role this information could play in the formulation of disease control strategies. For the future,

knowledge pertaining to these fungi and their biology in an aim to manage diseases could be achieved by expansive and continued surveys of the commercial plantations. This can only be achieved effectively by the establishment of collaborations between research and forestry institutions and between countries.