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BIOLOGY OF *BOTRYOSPHAERIA DOTHIDEA* AND *SPHAEROPSIS SAPINEA* AS ENDOPHYTES OF EUCALYPTS AND PINES IN SOUTH AFRICA

BY

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Dissertation submitted in fulfilment of requirements for the degree

DOCTOR OF PHILOSOPHY

to the Faculty of Science,

Department of Microbiology and Biochemistry,

University of the Free State

Republic of South Africa

September 2001

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CONTENTS

Preface

Botryosphaeria dothidea and *Sphaeropsis sapinea* are two well-known pathogens of eucalypts and pines, respectively, in many parts of the world, including South Africa. Knowledge concerning the biology and host relationships of these two economically important pathogens is relatively extensive. The endophytic nature of these fungi has, however, only been recognised relatively recently. The aim of this study was, therefore, to investigate various aspects of the endophytic infections of these fungi in more detail and to relate this phenomenon to their disease etiology. In addition, taxonomic questions pertaining to these fungi in South Africa have also been considered.

The first chapter of this dissertation presents a literature review of *B. dothidea* and *S. sapinea*, and particularly focuses on similarities and differences between the two fungi. These pathogens are compared on all aspects of their biology. The available literature suggests that they are very similar, both in terms of their taxonomy and biology.

The presence of endophytic infections caused by *B. dothidea* in eucalypt leaves and by *S. sapinea* in pine seed cones is described in chapter two. Different plant parts were studied to determine which tissue types are infected endophytically by these two fungi. This was also the first report of the endophytic infections caused *S. sapinea* in healthy seed cones.

In chapter three, I consider the endophytic infections caused by *B. dothidea* in eucalypt leaves more intensively. A particular focus of this study was to determine the mode of infection, the spatial distribution and the possible origin of endophytic infections within healthy leaves. Knowledge obtained from this study could contribute to current understanding of the role of these endophytic infections in healthy leaves, in pathogenesis.

During the course of this study, I came to recognise that more than one species of *Botryosphaeria* is associated with disease symptoms on eucalypts in South Africa.

The aim of chapter four was to study two sets of isolates more closely. This was done using morphology, pathogenicity and rDNA sequence data.

Sphaeropsis sapinea is believed to be an introduced fungus to South Africa. This is due to the fact that this fungus is restricted to coniferous trees that are exotic in this country. In chapter five I consider the structure of the South African population of *S. sapinea*. This was achieved using a large representative set of isolates and population parameters relevant to the population.

The presence of endophytic infections of *S. sapinea* in symptomless seed cones prompted me to question the role of such infections in hail associated die-back of pines in South Africa. Chapter six addresses the role of these infections in pathogenesis of various age classes of pine trees, following hail damage. This was achieved by sequential dissection and isolation procedures.

Chapter seven represents a case study on the susceptibility to *S. sapinea* die-back, of two provenances of *Pinus greggii*, after hail damage. Modern hybridisation programmes continuously necessitates that the Forestry Industry evaluates new pine species for tolerance to *S. sapinea*. This presented a unique opportunity to evaluate a new species to South Africa, at a very diverse family and seed source level.

During studies of canker diseases of eucalypts caused by *B. dothidea*, and particularly as I studied endophytic infections on these trees, I commonly encountered species of *Valsa* associated with stem cankers. Although this was perhaps slightly outside the main scope of this dissertation, it was of interest to determine the identity and role of these fungi in the stem canker complex of eucalypts. To achieve this goal I made use of morphological and rDNA sequence data.

All chapters in this dissertation deal with some aspects of the endophytic infections caused by *B. dothidea* on eucalypts and *S. sapinea* on pines in South Africa. It is the first time that such a study has been conducted in South Africa and it is my sincere hope that it will contribute to the understanding of the disease etiology of these two important pathogens.

This thesis represents a compilation of manuscripts that were prepared over a period of five years. Each chapter is an individual entity and some redundancy between chapters has been unavoidable.

Acknowledgements

I wish to express my sincere thanks and appreciation to the following:

My Heavenly Father, for this study would not have been possible without His mercy and love.

My wife, Cornette for her love, understanding and continued support.

My supervisors, Prof Mike Wingfield, Prof Teresa Coutinho and Prof Pedro Crous for allowing my own views and guidance extraordinair.

The Department of Microbiology and Biochemistry for the opportunity and facilities to undertake this study.

The National Research Foundation for financial support.

Dr. George Carroll, Dr. Bob Blanchette, Dr. André Drenth, Dr. Michael Milgroom and Dr. Gerry Adams for valued suggestions, revisions of manuscripts and co-authorship of publications in this thesis.

CHAPTER 1

Botryosphaeria dothidea and *Sphaeropsis sapinea*, two important opportunistic pathogens in forest plantations, especially in South Africa.

Botryosphaeria dothidea and _Sphaeropsis sapinea_, two important opportunistic pathogens in forest plantations, especially in South Africa.

In many parts of the world, especially the Southern Hemisphere, plantations of exotic tree species have been established. These include species of *Pinus* and *Eucalyptus*, which are amongst the most widely planted commercial tree species. The forestry industry in South Africa depends almost exclusively on these two genera of trees. Commercial plantations are concentrated in the eastern parts of the country and cover approximately 1.4 million ha (Denison & Kietzka, 1993). Diseases of eucalypts and pines have had a substantial impact on the industry and there are numerous pathogens that are well-established (Wingfield *et al.*, 1991). Two pathogens, *Botryosphaeria dothidea* (Moug. Ex Fr.) Ces et de Not. and *Sphaeropsis sapinea* (Fr:Fr.) Dyko and Sutton cause die-back and canker diseases of eucalypts and pines, respectively. Both have already caused considerable loss to South African Forestry.

Botryosphaeria dothidea was first described by Cesati and de Notaris in 1863 when they established the genus *Botryosphaeria*. Many synonyms exist for this fungus including *Botryosphaeria ribis* (Tode and Fr.) Grossenb. and Duggar, which is the one most commonly used (Witcher & Clayton, 1963). Internationally, *B. dothidea* has a wide distribution (Punithalingam & Holliday, 1973) and is associated with diseases of some 70 plant genera, including *Eucalyptus* and *Pinus* species (Davison & Tay, 1983; Hodges, 1983; Webb, 1983; Barnard *et al.*, 1987; Shearer *et al.*, 1987; Smith *et al.*, 1994).

Sphaeropsis sapinea is a pathogen of conifers, including 48 pine species in 39 countries in both the Northern and Southern Hemisphere. The fungus was first described as *Sphaeria pinea* Desm. by Desmazieres in 1842 as a saprobe on *Pinus sylvestris* L. needles from France. Many synonyms exist, with *Diplodia pinea* (Desm.) Kickx, Petrak and Sydow the most widely used (Punithalingam & Waterston 1970). Sutton (1980), revised the taxonomy of the fungus and has provided a full list of synonyms for it.

Since the discovery of *B. dothidea* and *S. sapinea*, in excess of 60 scientific publications have been devoted to each, recognising them as long standing threats to their hosts, when they are grown in monoculture. Knowledge pertaining to the biology and etiology of diseases associated with these pathogens has increased tremendously, especially during the last 20 years. Although disease symptoms associated with *B. dothidea* on eucalypts, has been the topic of only a limited number of papers, there are similarities between its etiology and ecology in both forest plantations and in orchards of other tree crops. The most significant contributions regarding disease etiology and ecology of *S. sapinea* originate from the United States, South Africa and New Zealand, where the fungus has caused significant damage to exotic and indigenous pines. The aim of this review is to provide a assessment of the literature on the biology and ecology of *B. dothidea* and *S. sapinea* and especially highlight similarities and differences between the two pathogens.

TAXONOMIC HISTORY

Both *B. dothidea* and *S. sapinea* are well known pathogens that were described during the late 1800s. *Botryosphaeria dothidea* has retained its original binomial as established by Cesati & de Notaris in 1863. It has an extensive list of synonyms that include *Botryosphaeria ribis* (Tode and Fr.) Grossenb. and Duggar, *Botryosphaeria mali* (Putterill) and *Botryosphaeria berengeriana* de Not. Pennycook & Samuels (1985) refer to this group as *B. dothidea sensu lato* and I concur with this interpretation. In my view, the morphological variation of species in this genus was not appreciated in early descriptions of new species. A contributing factor towards the uncertainty which arose regarding the taxonomic delimitation of *B. dothidea*, is its wide host range. The description of *B. mali* from apple in South Africa by Putterill (1919), is a good example of the early tendency to describe new species based on small morphological differences and different hosts.

The phylogenetic placement of *Botryosphaeria* itself, is still an unresolved matter. Barr (1972) placed *Botryosphaeria* in the Dothideales, only to later accept it in the Botryosphaeriaceae (Pleosporales) (Barr, 1979). However, it was placed in the

Dothideaceae (Dothideales) by Sivanesan (1984) and later in the Botryosphaeriaceae (Dothideales) by Hawksworth *et al* (1995). Studies using 18S rDNA gene sequences were to date unable to place *Botryosphaeria* with confidence in either the Dothideales or the Pleosporales (Berbee, 1996; Hanlin & Hanlin, 1999). Generally, it seems that the classification of Hawksworth *et al* (1995) is accepted as correct (Denman *et al*, 2000).

The presence of an anamorphic state, linked to an ascomycetous fungus such as *B. dothidea*, could have contributed to the taxonomic uncertainty when attempting to identify these fungi. The teleomorph is not commonly encountered in nature, resulting in the use of anamorphic characters for identification. The anamorph state of *B. dothidea* currently resides in two genera, i.e. *Fusicoccum* Corda and *Dothiorella* Sacc. There appears to be a great deal of confusion as to whether *Fusicoccum* or *Dothiorella* may be considered the correct genus to accommodate the anamorph of *B. dothidea*. According to Sutton (1980), the status of *Fusicoccum* and the type species, *Fusicoccum aesculi* Cda. apud Strum, is uncertain. The fungus was described and illustrated by Saccardo (1880b, 1886, quoted from Sutton, 1980), apparently without taking the original material of Corda into consideration. This description by Saccardo was later assumed to be a misapplication, when Petrak (1922, quoted from Sutton, 1980) placed *F. aesculi* in *Dothiorella*, as *Dothiorella aesculi* Petrak, citing it as the anamorph of *B. berengeriana*. However, both Sutton (1980) and Morgan-Jones & White (1987) were of the opinion that Saccardo did not misapply the name *F. aesculi*, which implies that fungi currently residing in *Dothiorella* may actually be more correctly placed in *Fusicoccum*. Von Arx & Müller (1954) seemingly disregarded the work of Petrak and cited *F. aesculi* as the anamorph of *B. berengeriana*. Later, Pennycook & Samuels (1985) suggested that *Macrophomopsis* Petrak should be synonymized with *Fusicoccum* and thus *Macrophomopsis coronillae* (Deamazieres) Petrak with *F. aesculi*. However, *Fusicoccum aesculi* is the name most frequently used for the anamorph of *B. dothidea* (Sutton, 1980; Pennycook & Samuels, 1985; Morgan-Jones & White, 1987; Sutton & Arauz, 1991). A recent study by Crous & Palm (1999) attempted to clarify the above mentioned uncertainty and confirmed the validity of the genus *Fusicoccum* and its type species *F. aesculi*.

Currently, the most confusing situation regarding the taxonomy of *B. dothidea* concerns the *Dothiorella* anamorph from different hosts. Grossenbacher & Duggar (1911) regarded *Dothiorella ribis* Sacc. as the anamorph of *B. dothidea* (as *B. ribis*). This view was later discarded by Shear *et al.* (1925) who reported the presence of intermixed microconidia with normal conidia and regarded the anamorph described by Grossenbacher & Duggar (1911) as a synonym of *Dothiorella gregaria* Sacc. However, *D. gregaria.* was already considered a synonym of *F. aesculi* and was subsequently also moved to *D. aesculi* by Petrak (1922). The reports by Wiehe (1952) of *D. gregaria*, as the anamorph of *B. dothidea* (as *B. ribis*), causing die-back on the oil tung tree *(Aleurites montana* Forsk.) and Webb (1983) of *Dothiorella eucalypti* (Berk. and Br.) Sacc., as the anamorph of *B. dothidea*, causing seed capsule abortion of *Eucalyptus camaldulensis* Dehnh. amply illustrates the confusion. Later still, Gardner & Hodges (1990) reported a *Dothiorella* sp., possibly *Dothiorella vulgaris* de Trav., causing twig die-back of *Myrica faya* Ait. from Madeira as the anamorph of *B. dothidea* (as *B. ribis*). *Dothiorella dominicana* Petr. and Cif. causing mango decline and stem end rot of mango fruit is regularly referred to as a later synonym of *F. aesculi* (Ploetz *et al.*, 1996). The above mentioned problems have been addressed to some extent by Crous & Palm (1999), when they reassessed three anamorphic genera of *Botryosphaeria*, including *Dothiorella*, and concluded that the fungi in this genus could better be accommodated in *Diplodia*. They subsequently re-described the type species *Dothiorella pyrenophora* Sacc. as *Diplodia pyrenophora* (Sacc.) Crous & M.E. Palm. This study only started to address the identity of isolates previously treated as *Dothiorella*, and an extensive re-assessment of all isolates is needed.

Throughout the literature, the conidial dimensions reported for anamorphs of *B. dothidea*, irrespective of whether they are referred to as *Fusicoccum* sp. or *Dothiorella* sp., overlap considerably (Table 1). I believe that, based on literature, difficulty may be encountered in distinguishing the genera *Fusicoccum* and *Dothiorella* as far as species identification of the anamorph of *B. dothidea* is concerned. Certainly all original material will have to be re-examined and neotypes collected where original material was lost. I also believe that *B. dothidea* is morphologically variable on

different hosts and concur with Sutton (1980) in using *F. aesculi* as the correct anamorph of *B. dothidea*. Currently, the taxonomy of *Botryosphaeria* is being revised, incorporating ITS phylogeny with morphology (Jacobs & Rehner, 1998; Crous & Palm, 1999). Such studies are paving the way towards an exhaustive re-evaluation of *B. dothidea* and ultimately the genus, *Botryosphaeria*.

The situation regarding the taxonomy of *S. sapinea* is much less confusing than that of *B. dothidea*, partly because the fungus occurs on a restricted range of coniferous trees and partly because it has no known teleomorph. The fungus was originally described as *Sphaeria pinea* by Desmazieres in 1842 as a saprobe on *Pinus sylvestris* needles from France. Petrak & Sydow (1927) proposed a new binomial, *Macrophoma pinea* (Desm.) Petrak & Syd., to accommodate what they viewed as the lectotype of *Macrophoma*, *Macrophoma macrosperma* (Karst.) Berl. & Vogl. and the earlier epithet of *S. pinea*. Later Petrak (1961) reassessed the situation and concluded that *M. pinea* was a later homonym of *M. pinea* Pass. (syn. *Dothiorella pinea* (Pass.) Petrak & Sydow) and placed the lectotype under the name *Macrophoma sapinea* (Fr.) Petrak. Punithalingam & Waterston (1970) published a list of synonyms under *Diplodia pinea* (Desm.) Kickx, Petrak and Sydow that included *M. pinea*. The relevance of *Macrophoma* was discussed by Sutton (1980) when he regarded the genus *Macrophoma* to be a later synonym of the conserved genus *Sphaeropsis*. The fungus known as *D. pinea* was thus accommodated in *Sphaeropsis sapinea* (Fr.) Dyko & Sutton, separate from *Diplodia* based on differences in conidial development. *Sphaeropsis sapinea* thus included isolates that can develop a faint septum prior to germination (in the strict *Macrophoma* sense) and those that produce conidia holoblastically with percurrent proliferation (Sutton, 1980; Minter *et al.*, 1982).

The existence of distinct groups of isolates in *S. sapinea* was first reported by Palmer & Stewart (1982) when they recognised that isolates from *P. resinosa* and *P. banksiana* differed in cultural appearance, pathogenicity and conidial dimensions. These variants were initially designated as "red pine type" from *P. resinosa* and "jack pine type" from *P. banksiana*. Palmer (1991) more comprehensively listed the differences between what she termed the A and B types. Isozyme banding showed

that type A and B isolates could be distinguished on the basis of different patterns. Little variation, however, occurred within each type and they were thought to be very closely related (Palmer, 1991). Wang *et al.* (1985) found that confusion could arise due to variation in conidial dimensions as well as cultural differences, making it difficult to distinguished the morphotypes. By making use of SEM, it was possible to distinguish mature conidia of type A from type B conidia as the former had smooth walls, whereas the latter had pitted walls. Intermediate cultures and young type B conidia were found to have smooth conidial walls. Isolates obtained from non-wounded *P. resinosa* shoots yielded type A isolates with smooth walls, whereas isolates from wounded twigs yielded type B isolates with pitted walls. The authors argued that since the pitted walls in type B isolates were found to be a constant characteristic, this characteristic could be used to greater effectiveness to distinguish between the two morphotypes. Ultrastructural studies on the conidial walls of Type A and B isolates revealed that both morphotypes possess a single cell wall layer that is separated into a outer electron dense layer and an inner hyaline layer. In type A conidia, the electron dense layer is continuous whereas with type B conidia, some inconsistencies in this layer corresponded with the location of pits (Wang *et al.*, 1986). In an evaluation of the conidial morphology of 50 isolates of *S. sapinea* from various parts of the world, Swart *et al.* (1993) concluded that pitted cell walls of mature conidia was extremely variable, with smooth walls being the norm. These authors suggested that wall pitting is a poor characteristic to distinguish conidia of the A and B types.

Smith & Stanosz (1995) found that storage and subculturing had an effect on the morphological criteria for differentiating between the A and B morphotypes of *S. sapinea*. Using RAPD markers isolates from the north central USA could be divided into two very distinct groups with Type A isolates more similar to one another (>85% similarity) than Type B isolates (<59% similarity). Morphotype A was found to be the more aggressive pathogen (Palmer *et al.*, 1987; Blodgett & Stanosz, 1997), to lack host specialization (Stanosz *et al.*, 1996), to have a wide host range (Stanosz *et al.*, 1996) and it has the widest distribution (Wang *et al.*, 1985). In contrast, isolates of the B morphotype are less pathogenic (Palmer *et al.*, 1987; Blodgett & Stanosz, 1997),

thought to be restricted to *P. resinosa* and *P. banksiana* (Stanosz *et al.*, 1996) in north central United States (Wang *et al.*, 1985; Stanosz *et al.*, 1996), but now also found on more species and distributed wider (Stanosz *et al.*, 1999). In recent studies that included *S. sapinea* isolates from Indonesia and Mexico (de Wet *et al.*, 2000) and Canada (Hausner *et al.*, 1999), the existence of a third morphotype, C (de Wet *et al.*, 2000) and forth morphotype, I (Hausner *et al.*, 1999) were reported. Type C isolates could be distinguished from both type A and B on the basis of differing RAPD banding patterns and conidial dimensions, whereas, type I isolates was different on the basis of RFLP ribotypes, conidial dimensions and wall pitting.

A canker disease of Italian cypress (*Cupressus sempervirens* L.) was described by Solel *et al.* (1987) and attributed to a fungus closely resembling *S. sapinea*. The fungus was consequently designated as a *forma specialis* of *S. sapinea* restricted to cypress and named *Sphaeropsis sapinea* f. sp. *cupressi*. The validity of this *forma specialis* was, however, challenged by Swart *et al.* (1993) who showed, by using conidial morphology and allozyme analyses, that these two fungi were not as closely related as reported by Solel *et al.* (1987). These authors thus refrained from using the species name "*sapinea*" when referring to the *Sphaeropsis* sp. from cypress.

A teleomorph has never been associated with *S. sapinea*. The formation of spermatia in some cultures (Wingfield & Knox-Davies, 1980) and a single unconfirmed report of sexual structures (Laughton, 1937) are the only indications of the possibility that the teleomorph exists. It is also possible that the teleomorph was lost as a result of speciation of this fungus on coniferous hosts, diverging from the teleomorph. *Sphaeropsis sapinea*, despite the absence of a teleomorph, is closely related to fungi in the genus *Botryosphaeria*. A recent study by Jacobs & Rehner (1998) based on ITS phylogeny, showed that *S. sapinea* is closely related to *Botryosphaeria obtusa* (Schw.) Shoemaker (anamorph *Sphaeropsis* spp. possibly *S. malorum* Peck).

HISTORICAL BACKGROUND IN SOUTH AFRICA

In forestry, *Botryosphaeria dothidea* was first reported in South Africa, associated with leaf lesions and tip blight of *Eucalyptus* species in the western Cape (Crous *et al.*, 1989). Later, Smith *et al.* (1994) reported that it is responsible for widespread die-back and canker symptoms on a range of *Eucalyptus* spp. (*Eucalyptus grandis* Hill: Maid., *Eucalyptus nitens* Deane et Maid. Maid., *Eucalyptus macarthurii* Deane et Maid. and *Eucalyptus smithii* R.T. Bak.). It would appear that *B. dothidea* was unknown, as a eucalypt pathogen, in South Africa until the late 1980's, as it was never mentioned in a comprehensive list of fungi associated with eucalypts in South Africa from as early as 1910 (Lundquist & Baxter, 1985). I, however, do not attribute its recent appearance in the literature to an introduction, but rather to the fact that very little work was done on eucalypt diseases until recently. Thus, this fungus was probably overlooked. Currently, *B. dothidea* is considered to be one of the more important and widespread problems relating to *Eucalyptus* production in South Africa.

Sphaeropsis sapinea was first shown to be pathogenic to various *Pinus* spp. cultivated in South Africa by Fisher (1912). This followed the first report of the fungus in 1909 from the Fort Cunynghame State Forest in the eastern Cape Province (Waterman, 1943). Prior to 1930, *P. radiata* was the major pine species affected by this fungus in South Africa (Lundquist, 1987). Losses due to post-hail associated die-back caused by *S. sapinea* were so serious that planting of *P. radiata* was discontinued in the summer rainfall areas by 1925 and replaced with *Pinus patula* Schl. and Cham.

During the early 1930's it was believed that *P. patula* was resistant to *S. sapinea*. Die-back of *P. patula* occurred sporadically at first but steadily increased and by 1940 *P. patula* was no longer considered to be resistant to infection by *S. sapinea* (Lundquist, 1987). During the late 1930's, *S. sapinea* was considered to be the most important fungal pathogen of *P. radiata* in South Africa, especially after hail damage (Laughton, 1937). By then, the host range in South Africa included *Pinus pinaster* Ait., *Pinus taeda* L., *Pinus muricata* D. Don., *Pinus caribaea* Morelet, *Pinus canariensis* C. Sm. and *Pinus halepensis* Mill. By the early 1960's, *S. sapinea* was

believed to be the most important forestry pathogen in South Africa (Lückhoff, 1964), with the most serious losses due to hail associated die-back occurring on *P. patula* in summer rainfall areas.

In a countrywide survey of the occurrence of *S. sapinea*, Swart *et al.* (1985) reported 39 cases of disease development associated with *S. sapinea* over a two year period. In 70 % of these cases, hail damage and drought stress were found to be primary factors contributing to *S. sapinea* symptom development, clearly indicating that the potential danger posed by this fungus was not declining. It is estimated that between 1923 and 1983 there were 11 outbreaks of *S. sapinea* induced die-back of *P. pinaster* and 25 of *P. radiata* in the southern Cape province alone (Zwolinski *et al.*, 1990b). *Sphaeropsis sapinea* continues to be regarded as the most economically important pine pathogen in South Africa.

DISEASE ETIOLOGY

The evidence for the relatedness of *B. dothidea* and *S. sapinea* is supported by their very similar disease etiology. The following section presents a comparison of the etiology of *B. dothidea* and *S. sapinea* and focuses primarily on similarities between these pathogens.

Mode of infection--The long standing view of the mode of infection and subsequent disease development by both *B. dothidea* and *S. sapinea* is one of wound infection (Wiehe, 1952; Witcher & Clayton, 1963; Schreiber, 1964; Foster & Marks, 1968; Marks & Minko, 1969; Wright & Marks 1970; Milholland, 1972; Punithalingam & Holliday, 1973; Weaver 1974; Von Broembsen, 1986; Smith *et al.*, 1994) leading to symptom development in the presence of environmental stress (Wene & Schoeneweiss, 1980; Hodges, 1983; Herbert & Grech, 1985; Shearer *et al.*, 1987; Pusey, 1989; Cline, 1994; Smith *et al.*, 1994). The ability of these fungi to also infect unwounded tissue was first recognized in the early 1970's. Both fungi were found to be able to infect stems and leaves through direct penetration of lenticels and stomata (Brookhauser & Peterson, 1971; Milholland, 1972; Weaver, 1974; Chou, 1976a, b;

Walla & Peterson, 1976; Chou, 1978; Brown & Hendrix, 1981; Michailides, 1991; Smith, 1995). Both fungi are also known to cause die-back and canker symptoms on *Eucalyptus* and *Pinus* spp. (Haddow & Newman, 1942; Marks & Minko, 1969; Chou, 1976a; Bega *et al.*, 1978; Davison & Tay, 1983; Hodges, 1983; Webb, 1983; Chou, 1984; Palmer & Nicholls, 1985; Shearer *et al.*, 1987; Palmer, 1991; Smith *et al.*, 1994).

Investigations regarding the infection of lenticels and stomata and the role of these infections in disease development, began during the early 1990's. Various researchers have reported that both *B. dothidea* and *S. sapinea* are able to become established as latent endophytic infections in leaves and stems of various hosts including eucalypts (Fisher *et al.*, 1993; Smith, 1995; Smith *et al.*, 1996a, b) and pines (Stanosz *et al.*, 1997; Smith *et al.*, 1996b). The infection of stomata of *E. grandis* leaves by germ tubes of *B. dothidea* was demonstrated by Smith (1995), who also showed that many individual infections may occur in single a leave. No evidence is available to suggest that these leaf infections play any role in shoot die-back and branch cankers. *Sphaeropsis sapinea*, however, was found to infect needles through stomata and that these needles were subsequently killed (James *et al.*, 1991). Such infections would lead to rapid colonisation and subsequent death of the cambium of shoots and branches (James *et al.*, 1991). The actual host tissue in which these infections reside and the course of development from initial endophytic infection to colonisation of tissue and subsequent symptom development is unknown and needs further study.

Disease symptoms and associated losses--Reports of extensive losses due to *B. dothidea* in eucalypt plantations is not common, with the few reports available, mainly dealing with isolated case studies of seed capsule abortion (Webb, 1983), death of *Eucalyptus radiata* Sieb. in species selection trials (Shearer *et al.*, 1987), twig and branch cankers of natural growing *Eucalyptus marginata* Donn. ex Sm. (Davison & Tay, 1983), coppice failiure of *E. grandis* (Barnard *et al.*, 1987) and a case of root diseases of *P. taeda* and *P. elliottii* (Hodges, 1983). Smith *et al.*, (1994) reported a more widespread occurrence of stem cankers and shoot die-back from South Africa, affecting many species, clones and commercial hybrids of eucalypts.

There are many reports on disease symptom expression and associated losses caused by *S. sapinea*, and these have contributed to a better understanding of the pathogen. Disease symptoms on plantation pines, caused by *S. sapinea*, can be classified as shoot die-back (Haddow & Newman, 1942; Marks & Minko, 1969; Chou, 1976a; Bega *et al.*, 1978; Chou, 1984; Palmer & Nicholls, 1985) and crown wilt (Haddow & Newman, 1942; Chou 1984; Chou 1987; Palmer, 1991). These symptoms are similar to those caused by *B. dothidea* on eucalypts.

Shoot die-back is usually restricted to current year shoots of eucalypts (Smith *et al.*, 1994) and pines (Buchanan, 1967; Chou, 1984). These shoots often die rapidly and characteristically may form curled tips (Haddow & Newman, 1942; Smith *et al.*, 1994). Both fungi can infect the pith tissue of these shoots and cause necrosis and desiccation of pith cells (Marks & Minko, 1969; Smith *et al.*, 1994). Shoot die-back can in successive years, cause tree death (Peterson, 1981b), but these symptoms generally lead to damage rather than death.

Crown wilt manifests itself as the same symptom when caused either by *B. dothidea* on eucalypts or by *S. sapinea* on pines. This symptom is the result of lesions on lateral branches reaching and girdling the main stem (Shearer *et al.*, 1987), causing all tree parts above this lesion to die. Both fungi are also able to infect the cortex and pith-tissue surrounding cankers on the main stem, causing discoloration of the wood (Chou 1984, 1987; Smith *et al.*, 1994).

Both *B. dothidea* and *S. sapinea* have been reported to cause root disease of pines (Crandall 1938; Hodges 1983; Wingfield & Knox-Davies, 1980). This symptom has never been reported in eucalypts. *Botryosphaeria dothidea* has never been reported to be a serious nursery pathogen, whereas *S. sapinea* is commonly found to cause collar rot of *P. resinosa* seedlings (Palmer & Nicholls, 1985).

Wounding and stress--Botryosphaeria dothidea is capable of infecting its host through wounds (Wiehe, 1952; Witcher & Clayton, 1963; Schreiber, 1964;

Milholland, 1972; Punithalingam & Holliday, 1973; Weaver, 1974; Brown & Britton, 1986; Von Broembsen, 1986; Smith *at al.*, 1994), however, few reports deal with this subject in any depth. The most frequent reports of the infection of wounds by *S. sapinea* are those linked to hail damage (Van der Westhuizen, 1968; Brown *et al.*, 1981; Swart *et al.*, 1987; Zwolinski *et al.*, 1990a) and pruning wounds (Gilmour, 1964; Chou, 1984; Chou & MacKenzie, 1988). The elevated severity of disease symptoms when wounding is accompanied by environmental stresses is well documented (Brown *et al.*, 1981; Swart *et al.*, 1987; Nicholls & Ostry, 1990). Hail damage is important in South African and has been associated with 51% of cases where shoot blight and dead top, as a result of *S. sapinea* infection following hail damage, had occurred (Swart *et al.*, 1987). It is also well known that where hail damage occurs together with drought, the disease severity is highly elevated (Swart *et al.*, 1987). The above mentioned two factors are related to such an extent that only 7% of reported *S. sapinea* die-back occurrences were due to hail damage in the absence of drought (Swart *et al.*, 1987). Brown *et al.*, (1981) also found that *P. elliottii*, normally very tolerant to infection by *S. sapinea*, succumbed to extensive shoot blight and top die-back after being damaged by hail.

A case study from South Africa illustrates the importance of hail damage and its impact together with *S. sapinea* infections of pines more clearly. Zwolinski *et al.* (1990b) calculated the loss of wood and potential volume after a hail storm in the south-eastern Cape Province, and found that a loss of merchantable timber in prematurely harvested sites was 28% of the volume, while the loss in potential production was as high as 55%. On sites where timber was not prematurely harvested the loss in predicted volume was 11.4%. Based on this case study, the authors predicted a possible annual loss of R 9.5 million per year (1986 values) for the South African Forestry Industry, due to *S. sapinea*.

Drought stress (Wright & Marks, 1970; Bega *at al.*, 1978; Herbert & Grech, 1985; Swart *et al.*, 1985; Chou, 1987; Pusey, 1989; Palmer, 1991), overstocking (Wright & Marks, 1970; Bega *at al.*, 1978; Wingfield & Knox-Davies, 1980), excessive fertilization (De Kam *et al.*, 1991), frost (Wene & Schoeneweiss, 1980; Palmer, 1991;

Cline, 1994; Smith *et al.*, 1994), hail damage (Brown *et al.*, 1981; Swart *et al.*, 1987; Zwolinski *et al.*, 1990a; Palmer, 1991), defoliation (Old *et al.*, 1990) heavy snow and insect damage (Nicholls & Ostry, 1990) may predispose trees to such an extent that they may be infected by *B. dothidea* and *S. sapinea*. *Sphaeropsis sapinea* colonization and hyphal growth was found to be more pronounced in the stems of artificially inoculated *P. radiata* seedlings subjected to drought stress (Chou, 1987). This phenomenon was also evident with infections in drought stressed stems of *Pinus nigra* Arnold, *Pinus sylvestris* L. and *Pinus thunbergiana* Franco, where fungal growth became more pronounced with a increasingly negative water potential from -0.1 to -1.2 MPa (Bachi & Peterson, 1985). McPartland & Schoeneweiss (1984), reported the same phenomenon for *B. dothidea*. Hyphae of *B. dothidea* in unstressed, artificially inoculated, *Betula alba* L. stems were thin, contorted and restricted to the vicinity of the inoculation point. This was in contrast to hyphae in drought stressed stems which were thick, branched and spread out extensively through xylem vessels.

Sporulation and dispersal--*Botryosphaeria dothidea* can sporulate on dead shoots (Haddow & Newman, 1942; Drake, 1971), slash or prunings (Sutton, 1981), bark of older wood (Michailides, 1991;) and bark associated with stem cankers (Michailides, 1991). *Sphaeropsis sapinea* can sporulate on dead shoots (Chou, 1976a; Laing & Chi, 1980), slash or prunings (Chou, 1984), attached or mummified seed cones (Haddow & Newman, 1942; Slagg & Wright, 1943; Laing & Chi, 1980; Peterson, 1981b; Chou, 1984; Johnson *et al.*, 1985; James *et al.*, 1991), needles or needle fascicles (*S. sapinea* - Haddow & Newman, 1942; Laing & Chi, 1980; Peterson, 1981b), bark of older wood (Haddow & Newman, 1942; Peterson, 1981b) and bark associated with stem cankers (Palmer, 1991). In most of above mentioned situations, these fungi may often be alone in sporulating on infected tissue.

Retained and infected plant parts play an integral role in the redistribution of inoculum within the tree canopy and within orchards or plantations (Pusey 1989; Peterson 1981a; Johnson *et al.*, 1985). This is especially relevant in pine plantations where *S. sapinea* sporulates on retained seed cones. This may occur on otherwise healthy trees and is an important source of inoculum build-up within the canopy, which will in turn

infect young shoots (Peterson, 1981a). Johnson *et al.* (1985) illustrated this point by reporting that trees with more retained seed cones, on which *S. sapinea* was sporulating, were more heavily diseased. No pycnidia were evident on cones in areas where shoot die-back did not occur. Seed cones tend to be infected early in the growing season during the second year when the cones expand rapidly. These cones become infected on trees in the absence of die-back symptoms (Peterson, 1977). These findings were supported by Smith *et al.* (1996a) when they described *S. sapinea* to be present as a symptomless latent endophyte in various cone parts of healthy two-year-old cones of *P. patula* and *P. radiata*.

Botryosphaeria dothidea is dispersed through both conidia and ascospores, although conidia have been shown to be the primary source of inoculum (Michailides, 1991; Sutton, 1981). Both ascospores and conidia require a specific temperature range and sufficient moisture to germinate (Sutton & Arauz, 1991). The production and exudation of conidia of *S. sapinea* is highly dependent on high humidity and periods of wetness (Chou 1976b). The production of conidia was found to coincide with high rainfall (Brookhauser & Peterson, 1971; Palmer *et al.*, 1988), but is more dependant on suitable temperatures following rainfall (Swart *et al.*, 1987).

Conidia of *S. sapinea* are associated with various insects including the pine spittle bug (*Aphrophora parallela* Say.) (Haddow & Newman, 1942), the pitch nodule moth (*Petrova sabiniana*) (Hunt, 1969) and the ovipositor holes of *Pissodes nemorensis* (Swart *et al.*, 1987). Zwolinski *et al.* (1995) found that the activity of two cambiophagous insects, *P. nemorensis* and *Orthotomicus erosus* were different regarding their association with *S. sapinea*. These authors found that *P. nemorensis* activity in hail damaged trees already infected by *S. sapinea*, have the effect of enhancing symptom development associated with *S. sapinea*. In addition it was found that *S. sapinea* infections could be secondary to *P. nemorensis* activity, in healthy tissue. Activity of *O. erosus* was found to be restricted to tissue previously infected by *S. sapinea*. There is no evidence to suggest a relationship between insect activity on eucalypts and *B. dothidea*.

CONCLUSIONS

- *Botryosphaeria dothidea* and *Sphaeropsis sapinea* are closely related fungi probably residing in the same genus, but generally infecting either hardwoods (*B. dothidea*) or conifers (*S. sapinea*). They are opportunistic pathogens that occur as symptomless endophytes and they tend to cause serious disease only when trees are stressed.
- Diseases caused by *B. dothidea* on eucalypts in South Africa are relatively well known and include shoot die-back as well as branch and main stem cankers. The role of latent endophytic leaf infections in disease development is an important issue to resolve.
- The taxonomy of *B. dothidea* is currently being revised. In South Africa, isolates from different disease symptoms indicate a degree of morphological variation. The question whether this variation may be attributed to natural variability within *B. dothidea* or by the presence of more than one species remains to be answered.
- *Sphaeropsis sapinea* is a well-known threat to the pine industry of South Africa and its etiology has been studied extensively. The ability of this pathogen to cause latent endophytic infections in seemingly healthy pines is cause for concern and the role of such infections in disease development needs to be investigated.
- *Sphaeropsis sapinea* is well established in South Africa but nothing is known of its population diversity. Diversity within the population is assumed to be relatively low, because of the general view that *S. sapinea* is an introduced as opposed to an indigenous fungus. Also because no teleomorph is known for it. Knowledge of the population structure of *S. sapinea* may prove valuable in the management of this disease.
- South Africa has relied on relatively few species of pine to sustain our Forestry Industry. In recent years, various factors have forced the Industry to look towards expanding to incorporate new species and to engage hybridisation and cloning programs. Although knowledge exists regarding susceptibility of tried and tested pine species, this is not the case for new species, especially under South African conditions.

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Table 1. Conidial dimensions reported for the anamorphic states of *B. dothidea*.

	Conidium dimensions			
Species name	Length (μm)	Breadth (μm)	Conidial shape	Source
Dothiorella sp.	12.5 - 33.7	3.8 - 7.7	Ellipsoid - fusoid	English *et al.*, 1975
B. dothidea	17.8 - 31.7	4.0 - 7.9	-	"
Dothiorella - like	18 - 31	4.5 - 8	-	Grossenbacher & Duggar, 1911
Fusicoccum sp.	14 - 23	3 - 4.5	Fusoid to clavate	Morgan Jones & White, 1987
F. aesculi	15 - 32	4 - 9	Fusoid	Pennycook & Samuels, 1985
B. mali	32.4	4.8	-	Putterill 1919
B. ribis	10 - 29	4 - 9	-	Shear *et al.*, 1925
Dothiorella sp.	17 - 25	5 - 7	Fusoid	Sivanesan, 1984
B. dothidea	14 - 32	4 - 9	Fusoid	Smith *et al.*, 1994
B. dothidea	10 - 23	3 - 7	Fusoid	Spiers, 1977
B. ribis	16 - 27	4 - 7	-	Stevens & Shear, 1929
F. aesculi	18 - 25	4 - 4.5	Fusoid	Sutton, 1980
M. coronillae	24 - 28	6.5 - 7.5	Fusoid	"
Dothiorella sp.	12 - 30	4 - 8	Both ends tapered	Von Arx & Müller, 1954
D. eucalypti	17 - 24	2 - 5	-	Webb, 1983
Dothiorella sp.	13 - 25	6 - 10	-	Whiehe, 1952

CHAPTER 2

Sphaeropsis sapinea and *Botryosphaeria dothidea* endophytic in *Pinus* spp. and *Eucalyptus* spp. in South Africa

Published as: SMITH, H., WINGFIELD, M.J., CROUS, P.W. and COUTINHO, T.A. (1996). *Sphaeropsis sapinea* and *Botryosphaeria dothidea* endophytic in *Pinus* spp. and *Eucalyptus* spp. in South Africa. *South African Journal of Botany* **62,** 186-88.

Sphaeropsis sapinea and *Botryosphaeria dothidea* endophytic in *Pinus* spp. and *Eucalyptus* spp. in South Africa

Sphaeropsis sapinea (Fr.: Fr.) Dyko & B. Sutton and the anamorph of *Botryosphaeria dothidea* (Moug.) Ces. et De Not. are morphologically and ecologically similar fungi that cause serious canker and die-back diseases of *Pinus* and *Eucalyptus* spp. respectively, in South Africa. In this paper the presence of both these fungi as symptomless endophytes in healthy, pine and eucalypt tissue was demonstrated. *Sphaeropsis sapinea* was present in 50% of young, green, *P. patula* Schl. et Cham., and 90% of *P. radiata* D. Don cones. In contrast, it was virtually absent from the cones of *P. elliottii* Engalm. et Vasey and *P. taeda* L. *B. dothidea*, on the other hand, was found to be common in all the *Eucalyptus* spp. tested, occurring in 93% of *E. smithii* R. T. Bak., 77% of *E. camaldulensis* Dehnh., 63% of *E. grandis* Hill ex Maid. and 57% of *E. nitens* (Deane et Maid.) Maid. leaves tested. The enigma of the rapid ingress of both these fungi in stressed or damaged trees might thus be explained by their endophytic habit.

The forestry industry in South Africa is economically dynamic and rapidly expanding. Currently, approximately 1 400 000 ha is planted to exotic *Eucalyptus* and *Pinus* species. As the estimated production of wood and fiber is expected to double by the year 2005 and the land area suitable for establishing plantations is limited to less than 2 000 000 ha, the industry is challenged to optimization (Denison & Kietzka, 1993). Plantations are concentrated in the eastern parts of the country and include various climatic areas. Invariably, some plantations are established in marginal areas where the impact of stress-related pathogens is accentuated. The impact of fungal diseases on the industry has been ignored in the past but is rapidly gaining recognition (Wingfield, 1987; Wingfield *et al*, 1991).

Many fungal pathogens are well established and cause diseases of *Pinus* and *Eucalyptus* spp. in South Africa (Wingfield, 1987; Wingfield *et al*, 1991). These can account for millions of Rands of losses due to reduced wood quality, loss of volume and tree mortality (Zwolinski *et al*, 1995). Two important pathogens that are very similar both in

ecology and morphology are *Sphaeropsis sapinea* (Fr.:Fr.) Dyko & B. Sutton and the anamorph of *Botryosphaeria dothidea* (Moug.) Ces. et De Not. *Sphaeropsis sapinea* is generally considered to be an opportunistic wound and stress-related die-back and canker pathogen of *Pinus* (Swart & Wingfield, 1991), whereas *B. dothidea* has a similar ecology on *Eucalyptus* (Smith *et al*, 1994).

Sphaeropsis sapinea is one of the most common fungi occurring on *Pinus* spp. and was first described under the name *Sphaeria pinea* Desm. in 1842 (Sutton, 1980). Many synonyms exist for this fungus with *Diplodia pinea* (Desm.) J. Kickx f. probably the most widely used (Punithalingam & Waterston, 1970). Although this pathogen has been reported from many countries, it is most notorious in South Africa where it causes extensive infection and mortality of *Pinus radiata* D. Don and *Pinus patula* Schl. *et* Cham. after hail damage (Laughton, 1937; Swart *et al*, 1987). Many disease symptoms are associated with infections by *S. sapinea*, but shoot blight and top die-back are most common (Swart & Wingfield, 1991). Frequent hailstorms and drought contribute largely to the extensive nature of die-back caused by this fungus in South Africa. Management of losses due to this pathogen in plantations is difficult and largely restricted to selection of *Pinus* spp. for disease tolerance (Swart & Wingfield, 1991).

Like *S. sapinea, B. dothidea* has been known on woody plants for many years and has had a confused taxonomic history. The cosmopolitan nature and wide host range of this pathogen has been recognised for many years (Smith, 1934). On *Eucalyptus*, *B. dothidea* causes a wide range of symptoms including leaf spots, shoot die-back as well as branch and stem cankers (Barnard *et al*, 1987; Crous *et al*, 1989; Davison & Tay, 1983; Shearer *et al*, 1987; Smith *et al*, 1994; Webb, 1983). Infection and subsequent symptom development associated with this fungus is aided by the presence of wounds (Witcher & Clayton, 1963) and environmental stress (Crist & Schoeneweiss, 1975).

In South Africa, *B. dothidea* is associated with many important disease symptoms on *Eucalyptus* spp. In most cases, symptom development is associated with trees under stress (Smith *et al*, 1994). There is, however, good evidence for variation in susceptibility of various *Eucalyptus* spp. to this pathogen.

Recently, *B. dothidea* has been found to occur as a symptomless endophyte in *Eucalyptus nitens* (Deane *et* Maid.) Maid. in England (Fisher *et al*, 1993). The latter study prompted us to consider whether this pathogen might also occur in healthy *Eucalyptus* trees in this country. Given the ecological and taxonomic similarities of *B. dothidea* and *S. sapinea*, we also questioned whether the latter fungus might similarly exist in healthy pine tissue as a symptomless endophyte. The aim of this preliminary study was thus to consider the possible existence of these important pine and eucalypt pathogens as symptomless endophytes. Implications to the South African forestry industry are also considered.

MATERIALS AND METHODS

Pine tissue--Healthy, green, mature, but unopened cones (Gifford & Foster, 1988) of four *Pinus* spp. were collected from commercial stands during March 1995. These included *Pinus elliottii* Engalm. *et* Vasey (Kwambomanbi area, Northern Natal), *P. patula* (Kwambonambi area, Northern Natal), *P. radiata* (Humansdorp area, Eastern Cape) and *Pinus taeda* L (Kwambonambi area, Northern Natal). Ten cones from each pine species (2 trees with 5 cones per tree) were surface sterilised by an immersion sequence in 96 % ethanol for 1 min.; undiluted bleach (3.5 - 5 % available chlorine) for 5 min.; 96 % ethanol for 30 sec and rinsed in sterile water. All cones were opened under sterile conditions and eight seeds, eight seed wings, eight tissue segments from ovuliferous scales and eight segments of pith tissue from each cone were placed in Petri dishes containing MEA (2 % Biolab, malt extract agar), supplemented with 200 mg/l chloramphenicol to suppress bacterial growth. Plates were incubated at 20 °C in the dark for 10 days. Dark, fast growing fungi were transferred to agar plates containing water agar (WA) on which sterile pine needles had been placed, and allowed to form pycnidia. Isolates of *S. sapinea* were identified based on their characteristic conidia using light microscopy.

Eucalypt tissue--Healthy leaves of four *Eucalyptus* spp. were collected from commercial stands during February 1995. These included *Eucalyptus camaldulensis* Dehnh. (Kwambonambi area, Northern Natal), *E. grandis* Hill ex Maid. (Kwambonambi area,

Northern Natal), *E. nitens* (Piet Retief area, Southeastern Transvaal) and *Eucalyptus smithii* R. T. Bak (Piet Retief area, Southeastern Transvaal). Thirty leaves from each of the four species (2 trees with 15 leaves per tree) were surface sterilised as previously described. Following surface sterilisation, each leaf was divided into five pieces and these were placed in Petri dishes containing MEA. Plates were incubated at 20°C in the dark for 10 days. Dark, fast growing fungi were transferred to 2 % MEA and incubated under continuous cool fluorescent light to induce sporulation. Isolates of *B. dothidea* were identified on the basis of their characteristic conidia using light microscopy.

RESULTS AND DISCUSSION

Sphaeropsis sapinea was a common inhabitant of various parts of healthy, asymptomatic cones of *P. radiata* and *P. patula* but was virtually absent in similar tissue from *P. elliottii* and *P. taeda* (Table 1). The relative abundance of this important pathogen in the four *Pinus* spp. tested, closely matches their relative susceptibility to this pathogen (Swart & Wingfield, 1991). Thus, amongst *Pinus* spp. commonly propagated in South Africa, *P. radiata* and *P. patula* are known to be the most susceptible and *P elliottii* and *P. taeda* most resistant to infection by *S. sapinea*.

Sphaeropsis sapinea has been found to be able to survive as edophytic infections in the wood of *Pinus sylvestris* (Petrini & Fisher, 1988), but the fact that *S. sapinea* can live as a symptomless endophyte in healthy pine cone tissue, has not previously been recognised. Rather, the fungus is generally thought to primarily infect wounded and stressed tissue and its occurrence on dying or dead tissue has been considered an indication of a primarily saprotrophic habit. Results of this study might imply that the fungus preferentially infects healthy cone tissue remaining latent until the onset of stress. Proliferation in pine tissue, other than cones, would then originate from existing infections rather than from new infections of wounded or stressed tissue.

Sphaeropsis sapinea is one of the most important pathogens of pines in South Africa, causing extensive die-back in plantations of susceptible species after hail (Swart *et al*, 1987). Trees are rapidly colonised by the fungus and become blue-stained and die within

a few weeks. The speed at which this colonisation takes place has always been enigmatic. The fact that this fungus is most likely extensively present within living tissues of trees suggests that it is stress due to hail, rather than infection of hail wounds alone, that leads to rapid colonisation and tree death. This intriguing aspect of the biology of *S. sapinea* is currently being investigated.

Botryosphaeria dothidea was commonly isolated from leaves of all four *Eucalyptus* spp. considered in this study (Table 2). In some species such as *E. smithii*, virtually every leaf examined was infected with this fungus. *B. dothidea* is evidently a common inhabitant of asymptomatic *Eucalyptus* leaves in South Africa, thus supporting the results of Fisher *et al.* (1993) who considered *E. nitens* in England. Based on these results, it seems likely that the occurrence of *B. dothidea* on leaf spots and on dead and dying *Eucalyptus* leaves might well be derived from earlier latent endophytic infections of healthy leaves. This would be in contrast to the dogma suggesting that the fungus is a saprophyte that colonises dead tissue.

Like *S. sapinea*, infections of *Eucalyptus* spp. associated with *B. dothidea* in South Africa develop rapidly after the onset of environmental stress such as frost, hot winds or drought. The rapid development of disease symptoms, and particularly under conditions not likely to be conducive to spore germination, has been a source of confusion. The presence of latent endophytic infections of the fungus in healthy tissue would provide a logical explanation for symptom development, as it is observed under field conditions.

Endophytic fungi are those with the ability to colonise healthy plant tissue without exhibiting virulence or symptom expression (Carroll, 1990; Rollinger & Langenheim, 1993), thus not causing obvious damage (McCutcheon *et al*, 1993). These fungi occur entirely within the host tissue (Todd 1988) and include latent pathogens and fungi with mycorrhizal associations (Petrini, 1991). The nature of endophytic relationships is variable, with latent pathogens also sharing an endophytic relationship with their hosts. Pathogens can thus remain latent for long periods of time, causing symptoms only when the physiological or ecological conditions favour virulence (Carroll, 1986; Bettucci & Saravay, 1993; Kulik, 1984; McOnie, 1967; Nataniels & Taylor, 1983; Tokunaga & Ohira, 1973). Based on the results of this study we suggest that *S. sapinea* and *B. dothidea* be considered as latent pathogens capable of endophytic infections. This is in

contrast to the view that they are primarily opportunists that preferentially colonise wounded and stressed tissue.

The fact that *S. sapinea* and *B. dothidea* exist endophytically in healthy pine and eucalypt trees, respectively, has important implications for the South African forestry industry. For example, the presence and relative abundance of these fungi in healthy tissue might provide a reflection of the inherent susceptibility of species to these pathogens. This seems to be the case with *S. sapinea* which was primarily present in species known to be most susceptible to it (Swart & Wingfield, 1991). It may also be possible to eliminate endophytic infections in high value trees such as those in valuable seed orchards through chemical treatments, consequently reducing losses in these situations. Clearly, the endophytic nature of *S. sapinea* and *B. dothidea* in pines and eucalypts is deserving of further intensive investigation.

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Table 1. Number of cones or cone parts with endophytic infections of *S. sapinea* in four species of *Pinus*

	% *S. sapinea* isolates[1]				
Pinus spp.	% cones infected	% seed infected	% seed blades infected	% pith tissue infected	% ovuliferous scales infected
P. elliottii	0	0	0	0	0
P. patula	50	19	20	30	18
P. radiata	90	81	85	89	74
P. taeda	10	0	0	1	0

[1]10 cones, 80 seeds (8 / cone), 80 seed blades (8 / cone), 80 pith tissues (8 / cone) and 80 ovuliferous scales (8 / cone) were sampled for each *Pinus* sp.

Table 2. Number of leaves and leaf segments with *B. dothidea* endophytic infections in four species of *Eucalyptus*

Eucalyptus spp.	No. of leaves tested	% of leaves infected[1]	% of leaf segments infected[2]
E. camaldulensis	30	77	60
E. grandis	30	63	58
E. nitens	30	57	47
E. smithii	30	93	77

[1]Percentage of leaves infected with *B. dothidea* in each species of *Eucalyptus*.

[2]Percentage of leaf segments that yielded isolates of *B. dothidea*. Each leaf sampled was cut into five pieces, yielding a total of 150 segments per species.

CHAPTER 3

Infection of healthy *Eucalyptus* leaves by *Botryosphaeria dothidea.*

Submitted as: Smith, H., Coutinho, T.A. & Wingfield, M.J. (2001). Infection of healthy *Eucalyptus* leaves by *Botryosphaeria dothidea. South African Journal of Botany.*

Infection of healthy *Eucalyptus* leaves by *Botryosphaeria dothidea*.

Botryosphaeria dothidea is associated with serious diseases of *Eucalyptus* in South Africa. Despite this, very little is known regarding the time and manner in which it infects these trees. The fungus is known to occur endophytically in leaves of various *Eucalyptus* species in England and South Africa. In this study we consider the ability of *B. dothidea* to infect apparently healthy *Eucalyptus* leaves. Scanning electron microscopy revealed that pycnidiospores of *B. dothidea* were able to infect healthy leaves directly through stomata. In addition, isolations from segments of apparently healthy leaves showed that the presence of *B. dothidea* is the result of multiple infection events, which apparently fail to result in symptom development.

The forestry industry in South Africa uses 1.2 % of the country's total land area, amounting to 1 400 000 ha for exotic plantations (Denison & Kietzka, 1993). Pathogens, especially those that are well established in the country, can cause significant losses in these plantations (Wingfield *et al.*, 1991). One of these pathogens, *Botryosphaeria dothidea* (Moug.) Ces. de Not is of particular importance in stressed *Eucalyptus* trees, where it is commonly associated with die-back and cankers (Smith *et al.*, 1994).

Botryosphaeria dothidea is the most important species in the genus *Botryosphaeria* and is responsible for various diseases of economically important crops (Brown & Hendrix, 1981; English *et al.*, 1975; Gerlach *et al.*, 1974; Herbert & Grech, 1985; Maas & Uecker, 1984; Michailides & Ogawa, 1986; Pusey *et al.*, 1986; Rumbos, 1987). This pathogen causes seed capsule abortion (Webb, 1983), twig die-back, branch and stem cankers (Davison & Tay, 1983; Shearer *et al.*, 1987; Smith *et al.*, 1994) as well as coppice failure (Barnard *et al.*, 1987) of *Eucalyptus* spp. and *Pinus* spp. (Hodges, 1983). Historically, it has been regarded mainly as a wound and stress-related pathogen (Wiehe, 1952; Witcher & Clayton, 1963).

Botryosphaeria dothidea has been isolated as a symptomless endophyte from *Eucalyptus nitens* Deane et Maid. Maid. in England (Fisher *et al.*, 1993), *Eucalyptus camaldulensis* Dehnh., *Eucalyptus grandis* Hill ex Maid., *E. nitens* and *Eucalyptus smithii* R. T. Bak in South Africa (Smith *et al.*, 1996a; Smith *et al.*, 1996b) as well as from *Quercus petraea* (Matt.) Lieb. in Austria (Halmschlager *et al.*, 1993). Thus the necessity for, and relevance of wounds for successful infection and subsequent symptom development has been questioned. The fungus can establish itself in elm stems without apparent wounds (Luttrell, 1950), mature and immature apple fruit (Parker & Sutton, 1993) and can also infect *Pistacia vera* L. through stomata and lenticels (Michailides, 1991). A closely related species, *Botryosphaeria eucalyptorum* Crous, H. Smith et M.J. Wingf. sp.nov. also occurs as a die-back and canker pathogen of eucalypts in the South Africa (Smith *et al.*, 2000). This species was, however, not linked to endophytic infections of leaves.

Botryosphaeria dothidea is able to survive on dead, woody material (Michailides 1991; Weaver, 1974), where it reproduces both sexually and asexually (Michailides, 1991; Smith *et al.*, 1994; Sutton, 1981). The teleomorph of this fungus is found on dead twigs and branches in *Eucalyptus* plantations, but is difficult to obtain under laboratory conditions on artificial medium (Smith *et al.*, 1994). Pycnidia of the anamorph (*Fusicoccum aesculi* Cda apud Strum), however, are relatively common in culture when the fungus is grown on artificial media, and subjected to continuous cool florescent light (Jeffers, 1991; Smith *et al.*, 1994; Sutton & Arauz, 1991).

This study represents part of a series of investigations of the biology of *B. dothidea* on *Eucalyptus* species in South Africa (Smith *et al.*, 1994; Smith *et al.*, 1996a,b). *Botryosphaeria dothidea* commonly occurs in apparently healthy leaves of *Eucalyptus* in South Africa, and one objective of this study was to determine how infection occur and which leaf tissues are infected. Furthermore, it was of interest to determine whether the presence of this fungus in apparently healthy leaves is as a result of single or multiple infections and whether these infections remain localised or extensive colonisation of the leaf tissue occurs.

MATERIALS & METHODS

Mode of infection--Pycnidiospores of *B. dothidea* were used in infection studies. Pycnidiospores were obtained by growing an isolate of *B. dothidea* (BOT 0007) on 2% MEA (Malt Extract Agar, Biolab) for 2-3 weeks under continuous cool florescent light at 25°C. Pycnidia formed randomly on the agar, especially around the perimeter of dishes, after approximately two weeks. Unicellular, hyaline, fusiform pycnidiospores with truncate bases (17-25 x 5-7 μm) were collected from cirrhi that exuded from pycnidia. Pycnidiospores were suspended in sterile distilled water (~1400 spores / ml) and sprayed onto the upper surface of two month-old leaves of preconditioned, 15 month-old *E. grandis* seedlings. The branches bearing inoculated leaves were enclosed in plastic bags for three days to maintain high humidity. Inoculated seedlings were kept in a glasshouse with an average daily temperature of approximately 25°C. Trees were preconditioned to ensure the absence of as much as possible of the natural endophytic community within these leaves. Trees were kept in a greenhouse at 25°C for three months prior to inoculation. Leaves were tested for the presence of endophytic fungi during the week prior to artificial inoculations.

Scanning electron microscopy--In order to visualise the germination and infection processes on the surface of artificially infected leaves by pycnidiospores of *B. dothidea*, leaf segments were prepared for electron microscopy, three days after inoculation. Leaves were cut into small segments (5-mm squares), fixed in 3% glutaraldehyde and 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated in a graded acetone series, critical-point dried and coated with gold palladium. Specimens were examined using a JSM 6400 scanning electron microscope.

Light microscopy--Light microscope examination of the internal infections in artificially as well as naturally infected *Eucalyptus* leaves were performed on cleared leaf samples. Leaf samples were cleared in 2% KOH at 50°C for four days. The 2% KOH were replaced each day with fresh solution. After four days leaf samples were cleared of chlorophyll and were straw coloured. The KOH was washed from leaf tissue with three changes of water. The cleared leaf samples were suspended in 3%

H_2O_2 for 30 minutes, after which they were washed in water. The leaf pieces were stained with 0.05% Trypan Blue in lactophenol at 50°C overnight. They were then dehydrated in an ethanol series (50%, 70%, 80%, 95% & 100%), followed by ethanol / xylene (1:1) and xylene. Each of the above steps lasted 30 minutes (Dr. Jeffrey Stone, personal communications). Preparations were viewed with a light microscope for the presence of fungal thalli in leaf tissue. Cleared leaf samples of naturally infected *E. grandis* leaves were examined for the spatial distribution and relative infection density by the complete endophytic assemblages found in these leaves (Smith *et al.*, 1996b). Artificially infected leaves as described in the first section were also microscopically examined to establish the tissues in which these infections reside after initial infection.

Tissues infected--In order to determine whether *B. dothidea* infections inside healthy *Eucalyptus* leaves resided in the epidermal layer or deeper within the mesophyll cells a randomly collected sample of naturally infected *E. grandis* leaves from two plantations the in Mpumalanga Province were used. Naturally infected leaves from this sample were surface sterilised (Smith *et al.*, 1996 a,b) and confirmation that they were infected by *B. dothidea* was obtained following incubation of leaf pieces on 2% MEA, supplemented with 200mg/l chloramphenicol to suppress bacterial growth. Ten leaves from this sample were selected and from each leaf five, 2-cm squares were cut and surface sterilised. These squares were placed in 20-ml sterile water containing 0.5-g dematacеous earth. The leaf pieces were rotated for up to six hours until the scouring effect had removed the cuticle and epidermal layers. The absence of the epidermal cells was verified by light microscopy. The leaf pieces were then placed on 2% MEA in Petri dishes and incubated at 25°C for up to five days. The identity of *B. dothidea* isolates was confirmed after sporulation.

Macro dissections--Macro dissections of four apparently healthy *E. grandis* leaves were initially performed to determine the number of infections per leaf. In this case the leaves were surface sterilised as previously mentioned and each individual leaf was divided into five segments. These segments were cultured as described previously. The point of origin of all *B. dothidea* isolates from individual leaves was

carefully noted. Isolates of *B. dothidea* obtained from these leaves were tested for their genotypic diversity within individual leaves. Genotypic diversity was determined using vegetative compatibility tests. All determinations of vegetative compatibility were done on Oat Meal Agar (OMA). Oat meal (60 g/l) was steamed in a water bath at 70 ^{o}C for 2 hours with periodic stirring. It was separated from the liquid by filtration through a double layer of cheesecloth resulting in approximately 600 ml of oat meal suspension. Agar (20 g) was melted in 400 ml distilled water and then added to oat meal suspension and autoclaved. The medium was thoroughly mixed prior to dispensing into plastic Petri dishes. Isolates of *B. dothidea* from each leaf were paired in all possible combinations. Six isolates were placed approximately 1 cm apart on each OMA plate in such a way as to pair all isolates with themselves and with all other isolates. Dishes were incubated at 20 ^{o}C in the dark for 2 weeks. The occurrence of different VCG's was determined on the basis of barrage formation between isolates (Anagnostakis, 1983).

Micro dissections--The macro dissection technique proved to be inadequate to determine the extent of localisation of endophytic infections caused by *B. dothidea*. A micro dissection technique was thus used to determine the degree to which *B. dothidea* colonises healthy leaf tissue after infection. Four naturally infected leaves of *E. grandis* were selected for micro dissection. From each of the ten leaves, two 1-cm squares were cut from approximately the same leaf parts, and surface sterilised as previously mentioned. The surface sterilised squares were aseptically dissected into 2-mm squares and these were placed in sequence onto 2% MEA in Petri dishes. Squares yielding isolates of *B. dothidea* were noted after positive identification and infection maps for this fungus were constructed.

Statistical analysis--Data obtained from micro dissections were tested for the independent nature of individual infections according to the Poisson distribution and for significance by χ^2 ($P = 0.05$).

RESULTS

Mode of infection--None of the leaves tested yielded isolates of *B. dothidea* and few other endophytes were present in these leaves. Artificial infection with conidia of *B. dothidea* proved to be successful. Scanning micrographs revealed that conidia of *B. dothidea* readily germinated on the surfaces of artificially infected leaves. Germ tubes from conidia grew towards and directly penetrated stomata on the upper surfaces of *E. grandis* leaves (Fig. 1a). Direct penetration of the epidermis was never observed. Leaves removed from trees after three days cleared well and light microscopy revealed few conidia of *B. dothidea* on the leaf surface. The sub-stomatal hyphae were, however, visible inside the sub-stomatal chamber. No thalli were evident within epidermal cells of artificially infected leaves but were present in the apoplast between mesophyll cells (Fig. 1b). Inspection of naturally infected *E. grandis* leaves revealed an extremely high frequency of infection. Thalli of the natural endophytic assemblage of these leaves were visible amongst mesophyll cells and were particularly concentrated near the leaf veins.

Tissues infected--Light microscopy of cleared naturally and artificially infected *E. grandis* leaves revealed that endophyte thalli is predominantly found in the apoplast amongst mesophyll cells. The scouring procedure effectively removed the cuticle and epidermis. Naturally infected leaf pieces with the epidermis removed, however, still yielded isolates of *B. dothidea* following culturing on 2% MEA. Infections caused by this fungus is thus not of the epidermis but of deeper lying mesophyll cells.

Macro dissections--*Botryosphaeria dothidea* was recovered from all four leaves (leaf 1 = 10 isolates, leaf 2 = 11 isolates, leaf 3 = 13 isolates and leaf 4 = 12 isolates). Isolates obtained from macro dissections were restricted to those infections which had occurred close to the cut edges of the leaf pieces, thus not accounting for infections in the middle of individual segments. All isolates obtained from the four leaves were tested for their genotypic diversity within individual leaves. All isolates grew well on OMA and colonies made contact with each other within 3-4 days. The fungus became dark grey to black after 1 week. Incompatibility reactions resulted in dark mycelial

barrage lines and were most obvious approximately 2 weeks after inoculation, after which mycelial growth became very dense and the reactions were no longer obvious. Compatible isolates merged without the formation of any barrage lines (Fig. 1c). Without exception it was found that each isolate from individual leaves accounted for a distinct genotype or vegetative compatibility group (VCG), indicating that the presence of *B. dothidea* inside healthy leaves is as a result of multiple infection events.

Micro dissections--Micro dissections of 1-cm squares of naturally infected *E. grandis* leaves were found to solve the problem of discrimination against infections that had occurred away from cut edges of cultured leaf pieces, and could thus identify individual infection sites. Results obtained after culturing of micro dissection leaf pieces indicated that they were only sparsely infected by *B. dothidea*, with the highest infection frequency being five infected segments from a possible 25 (Table 1). *Botryosphaeria dothidea* was rarely isolated from two 2-mm segments adjacent to one another. These results clearly indicate that endophytic infections caused by *B. dothidea* results in minimal colonisation of leaf tissue when these are healthy.

Statistical analyses--Analyses of micro dissection data clearly indicated that natural infection events by *B. dothidea* in *E. grandis* leaves are random and non-related, thus fitting the Poisson distribution model ($\lambda = 2.4$, $\chi^2 = 3.3$, $P = 0.05$) (Mead & Curnow, 1983).

DISCUSSION

In this study, we were able to show that germ tubes from conidia of *B. dothidea* infect *E. grandis* leaves directly through stomata. Although these infections were observed to occur after artificial inoculations of the leaves, we believe that conidia of *B. dothidea* are able to infect leaves in a similar manner in plantations during periods of high humidity and temperature. These results support the findings by Michailides (1991) working with *Pistacia*. Penetration of the stomata presumably allows *B. dothidea* to establish itself undetected within the leaf tissue as a latent infection, as no

necrosis or symptom development was observed on leaves from which *B. dothidea* was isolated as an endophyte. This is consistent with the behaviour of similar fungi such as those causing postharvest rot of cranberries (*Gordronia cassadrae* Peck, *Botryosphaeria vaccinii* (Shear) Barr., *Phyllosticta elongata* G.J. Weidemann and *Physalospora vaccinii* (Shear) Arx & Müller), which were found in immature asymptomatic fruit (Jeffers, 1991).

The clearing and staining of artificially infected leaves revealed that germtubes from conidia of *B. dothidea*, penetrates the stomata, enters the sub-stomatal chamber and thereafter resides in the apoplast amongst mesophyll cells. The combination of light microscopy and scouring with dematacеous earth conclusively showed that *B. dothidea* is confined to the apoplast and that this fungus does not become established within the epidermis. Clearing and staining of leaves has also been successfully used to locate epidermal infection by *Rhabdocline parkeri* in Douglas fir needles (Stone, 1987).

The wide range of endophytic fungi reported to occur in healthy *Eucalyptus* leaves (Fisher *et al.*, 1993; Smith *et al.*, 1996b) was visualised in naturally infected leaves of *E. grandis* in this study. These leaves were found to be heavily infected with endophytic fungi, of which *B. dothidea* is one. The determination of the contribution to the total endophytic assemblage, by *B. dothidea* could in future be a challenging research aspect.

Many isolates of *B. dothidea* were found in leaves of *E. grandis* in this study. These tended to be of distinct VCG's and thus were distinct genetic entities. The generation of large numbers of genetic entities (VCG's) is achieved through sexual reproduction (Leslie, 1993). *Botryosphaeria dothidea* is able to reproduce sexually in association with many of its hosts (Shear *et al.*, 1925; Spiers, 1977; Stevens & Shear, 1929; Wiehe, 1952), including *Eucalyptus* species in South Africa (Smith *et al.*, 1994). The sexual structures are normally formed on dead twigs, branches and bark of infected trees (Smith *et al.*, 1994; Weaver, 1974). The occurrence of different VCG's among isolates of *B. dothidea* indicates that this fungus is well established in South Africa. It

is also apparent that the population is not the result of clonal multiplication, but rather of extensive sexual reproduction.

The number of *B. dothidea* isolates obtained from individual whole leaves was relatively small in comparison to those found by other authors. For example, Stone (1987) reported between 15 and 20 infections per mm^2 on Douglas-fir (*Pseudotsuga menziesii* (Mirb) Franco) needles infected by *Rhabdocline parkeri* Shrew. In this study, leaf pieces used for isolations were relatively large and smaller pieces may have resulted in a larger number of isolates. The results are, however, sufficient to conclude that *Eucalyptus* leaves are subjected to multiple infections in nature, and that these remain asymptomatic, at least as long as the leaf is healthy and not stressed.

All leaves used in this study were of approximately the same age. The age of tissue sampled is likely to play an important role in the number of infections, with more infections occurring in older tissue (Bernstein & Carroll, 1977; Petrini & Carroll, 1981). At this time, it is not known what the influence of leaf age is on the infection frequency of *B. dothidea* on *Eucalyptus* in South Africa.

Infections that account for the presence of *B. dothidea* in asymptomatic leaves should be termed latent endophytic infections by a latent pathogen (Petrini, 1991). Notwithstanding its pathogenic abilities on *Eucalyptus* (Barnard *et al.*, 1987; Davison & Tay, 1983; Shearer *et al.*, 1987; Smith *et al.*, 1994; Webb, 1983), this fungus is included in the broad definition of an endophyte (Petrini, 1991). The extent of the latent period and factors that result in the fungus becoming a virulent pathogen deserve further study.

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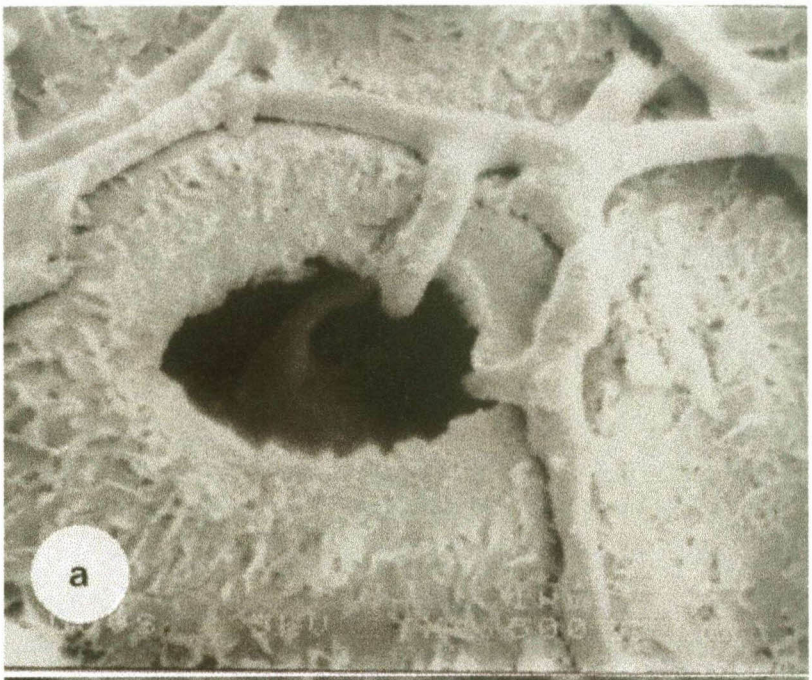
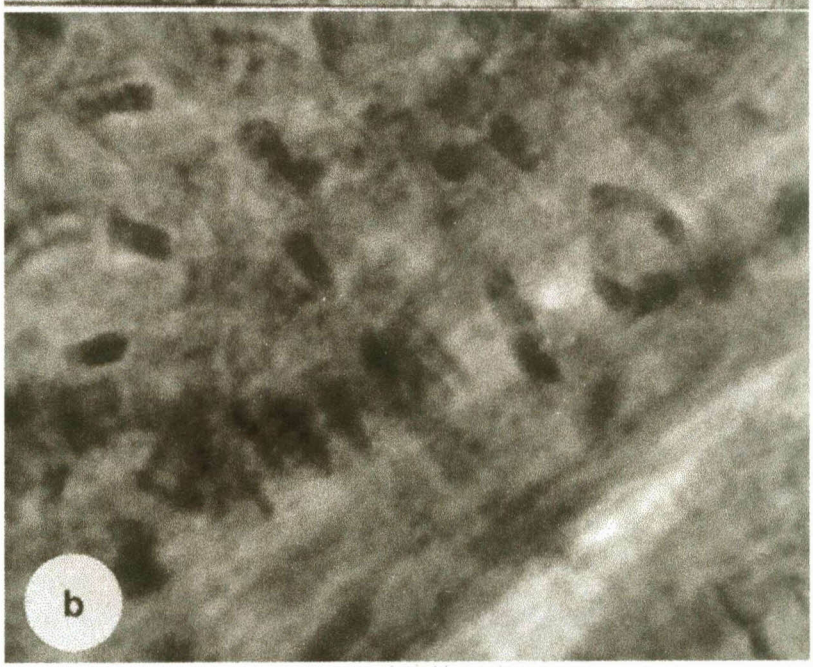
Table 1. Frequency of leaf segments infected with *B. dothidea* resulting from the micro dissection technique.

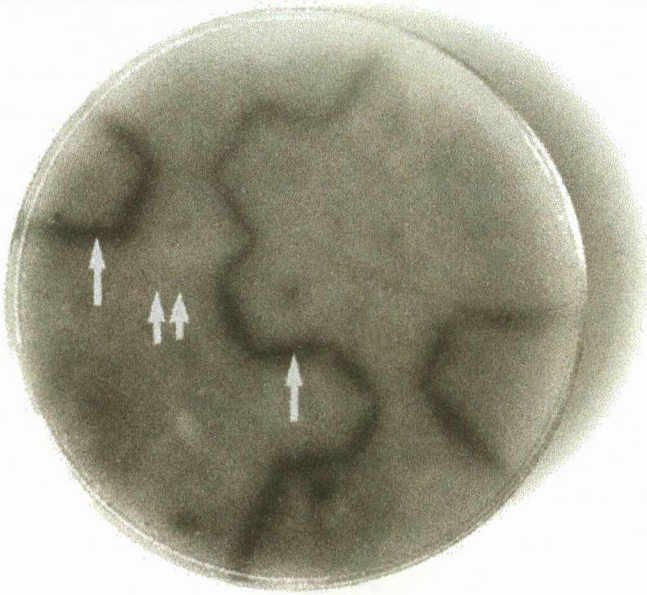
Count of infected segments	Observed frequency
0	4 [1/]
1	3
2	2
3	5
4	4
5	2

[1/] On four occasions original 2 cm square leaf pieces yielded no micro-dissected 2 mm squares infected with *B. dothidea.*

Figure 1. (a) Germ tube originating from pycnidiospore of *B. dothidea* penetrating stomate on the upper surface of an *E. grandis* leaf. (bar = 1 μm) (b) Thalli amongst mesophyll cells. (c) Mycelial barrage lines (single arrow) between incompatible isolates and the absence of barrage lines (double arrow) between compatible isolates of *B. dothidea.*







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CHAPTER 4

Botryosphaeria eucalyptorum sp. nov., a new species in the *B. dothidea*-complex on *Eucalyptus* in South Africa

Published as: SMITH, H., CROUS, P.W., WINGFIELD, M.J., and COUTINHO, T.A. (2000). *Botryosphaeria eucalyptorum* sp. nov., a new species in the *B. dothidea*-complex on *Eucalyptus* in South Africa. *Mycologia* **93,** 277-285.

Botryosphaeria eucalyptorum sp. nov., a new species in the *B. dothidea*-complex on *Eucalyptus* in South Africa

Two morphologically similar fungi are associated with canker and die-back of eucalyptus in South Africa, one of which was identified as part of the *Botryosphaeria dothidea*-complex. In this study, the identity of the other fungus was determined by comparing morphology, pathogenicity and DNA sequence analysis of isolates of both taxa. Based on these results, a new species, *Botryosphaeria eucalyptorum*, and its anamorph *Fusicoccum eucalyptorum*, are described. Although the teleomorph is morphologically similar to other taxa in the *B. dothidea*-complex, conidial characteristics of the anamorph are distinct, as well as the sequences of the nrDNA internal transcribed spacers ITS1 and ITS2. Like *B. dothidea*, the fungus is pathogenic to *Eucalyptus*, although there do not appear to be clear differences in pathogenicity between these two species.

Botryosphaeria dothidea (Moug.: Fr.) Ces. & De Not. was first described by Cesati and de Notaris in 1863 when they established the genus *Botryosphaeria*. The fungus has a cosmopolitan distribution and is associated with diseases of at some 70 plant genera (Smith, 1934; Punithalingam & Holliday, 1973), including *Eucalyptus* and *Pinus* species (Davison & Tay, 1983; Hodges, 1983; Webb, 1983; Barnard *et al*, 1987; Shearer *et al*, 1987; Smith *et al*, 1994). *Botryosphaeria dothidea* causes die-back and canker symptoms on various *Eucalyptus* species in South Africa (Smith *et al*, 1994) and is thus of concern to commercial forestry in this country.

Considerable controversy exists surrounding the taxonomic status of *B. dothidea* and *Botryosphaeria ribis* (Tode & Fr.) Grossenb. & Duggar. Some authors regard the species as synonyms (Arx & Müller, 1954; Witcher & Clayton, 1963; English *et al*, 1975; Spiers, 1977; Brown & Hendrix, 1981; Maas & Uecker, 1984; Pusey, 1989), while others have treated them as separate taxa (Smith, 1934; Punithalingam & Holliday, 1973; Rumbos, 1987; Rayachhetry *et al*, 1996). Pennycook & Samuels (1985) recognized the diversity within *B. dothidea*, and consequently referred to it as *B. dothidea* sensu lato. If the proposed synonymy of *B. dothidea* and *B. ribis* is upheld (Arx & Müller, 1954), the name *B. dothidea* (1863) would

have priority, as it predates *B. ribis* (1911) (Witcher & Clayton, 1963). However, recent molecular data (Jacobs & Rehner, 1998) suggest that separate species exist in this complex. Whether these species can be attributed to *B. dothidea* or *B. ribis*, however, remains to be determined.

The teleomorphs of *Botryosphaeria* are rarely encountered in nature, and much confusion has surrounded the taxonomic classification of the anamorphs. The general similarity based on the morphological descriptions of the anamorphs, and difficulties encountered in inducing strains to sporulate in culture, has resulted in confusion relating to the delimitation of species. This is aptly illustrated by the fact that different species of *Fusicoccum* Corda or *Dothiorella* Sacc. have in the past been linked to either *B. dothidea* or *B. ribis* (Arx & Müller, 1954; Grossenbacher & Duggar, 1911; Webb, 1983; Gardner & Hodges, 1990).

Fusicoccum aesculi Corda, the accepted anamorph sate of *B. dothidea* (Arx & Müller, 1954; Sutton, 1980; Pennycook & Sameuls, 1985), has been reported as a common endophyte from asymptomatic leaves of various *Eucalyptus* spp. in South Africa (Smith *et al*, 1996a, b). Recently, a species of *Botryosphaeria* resembling species in the *B. dothidea*-complex, but with an anamorph morphologically distinct from *F aesculi*, consistently has been isolated from stem cankers on *Eucalyptus* spp. in South Africa. Thus, two species of *Botryosphaeria* appear to occur within the same niche on *Eucalyptus* in this country.

The internal transcribed spacers (ITS1 and ITS2) of the nrDNA operon has been successfully employed to analyze intra- and interspecific relationships in various fungi (Berbee & Taylor, 1993). In some cases these studies have allowed species delimitation where morphological characters have not been useful (Anderson & Stasovski, 1992; Rehner & Uecker, 1994; Witthuhn *et al*, 1998). The aim of the present study was to determine whether there were one or two species of *Botryosphaeria* on *Eucalyptus* in South Africa, and if two, whether they were equally pathogenic to *Eucalyptus*.

MATERIALS AND METHODS

Iisolates.—Most isolates included in this study were collected in 1995 during a survey of eucalypt diseases in the Mpumalanga Province of South Africa. Three isolates were collected from indigenous Myrtaceae growing in the KwaZulu-Natal Province (Table 1). Isolations were made from twigs with die-back symptoms as well as from branch and main stem cankers.

Morphology and cultural characteristics.—Isolates were placed on 2% malt extract agar (MEA) (20 g/L malt extract and 12 g/L agar; Biolab, Midrand, Johannesburg) under continuous fluorescent light for up to 3 wk at 25 C to promote sporulation. Growth rate was determined by placing three single conidial isolates on MEA plates in the dark, at temperatures ranging from 5--35 C at 5° intervals. Three replicate plates were used per isolate for each temperature. Two perpendicular measurements were obtained after 4 d for each colony, and averages determined. Colony colors (upper surface and reverse) were determined using the color charts of Rayner (1970). Wherever possible, thirty measurements were made of mature structures mounted in lactophenol, the 95% confidence intervals determined, and the extremes given in parentheses. Type specimens are lodged at the National Collection of Fungi, Pretoria (PREM), and ex-type cultures maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA isolation.—Isolates (Table I) were grown on MEA in Petri dishes for 4 d at 25 C in the dark. Template DNA was obtained by an extraction method modified from Raeder & Broda (1985). Mycelium was scraped off the surface of 4-d-old cultures with a scalpel and transferred to sterile Eppendorf tubes (1.5 mL) and 100 µL of an extraction buffer (200 mM Tris-HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added to each tube. Tubes were immersed in liquid nitrogen and mycelium was ground with a pestle until a homogenous solution was obtained. At this stage, a further 400 µL of extraction buffer were added to each tube. Extraction of template DNA was achieved by repeated addition of phenol (350 µL) and chloroform (150 µL) with centrifugation (13 000 rpm, 1 h). Template DNA was precipitated overnight at -20 C with isopropanol (0.54 volume) and 3 M NaAc (0.1 volume). The DNA was pelleted at 13 000 rpm for 10 min at 4 C. The resulting pellets were washed with cold

70% ethanol (100 µL) and dried. The dried pellets of template DNA were re-suspended in sterile water (100 µL).

PCR.--PCR amplifications were performed with the primers ITS1 and ITS4 (White *et al*, 1990). The amplified fragments included the 3' end of the small subunit (SSU) rRNA gene, the 5.8S gene, part of the large subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) regions 1 and 2. The PCR reaction mixture contained 2.5 units of *Taq* polymerase (Boehringer Mannheim, Mannheim, Germany), the buffer supplied with the enzyme, 250 µM dNTPs, 6.25 mM $MgCl_2$ and 0.5 µM of each primer. Initial denaturation was performed at 93 C for 3 min, followed by 35 cycles of primer annealing at 58 C for 45 s, chain elongation at 72 C for 90 s and denaturation at 92 C for 30 s. Final chain elongation took place at 72 C for 15 min. The PCR products were separated on 1.5% agarose gels, stained with ethidium bromide and visualized under UV light.

The PCR fragments amplified were approx. 560 bp in size. These were purified using the QIAquick PCR purification kit. Both strands of the PCR products were sequenced using the Thermo Sequenase Dye Terminator Cycle Sequencing Pre-mix Kit (Amersham Life Science). Samples were run on an ABI Prism 377 DNA sequencer and the sequence analyzed using Sequence Navigator (Perkin-Elmer). The primers ITS1, ITS4, CS2 and CS3 (Wingfield *et al*, 1996) were used in sequencing reactions. The nucleotide sequences were aligned using CLUSTAL W (Thompson *et al*., 1994), improved manually where necessary, and analyzed using PAUP* version 4.0b2a (Swofford, 1999). The alignment used in this study is available upon request from the authors. Maximum parsimony trees were generated using the heuristic search option with 1000 random addition replicates with the tree bisection reconnection (TBR) algorithm. Support for the clades was assessed by 1000 bootstrap replicates (Felsenstein, 1985). *Mycosphaerella africana* Crous & MJ Wingf. was used as outgroup because *Botryosphaeria* and *Mycosphaerella* are both placed in the Dothideales (Crous, 1998). Sequence data of isolates were deposited in GenBank and TreeBASE (AF283675-283690; S539).

Pathogenicity.—Isolates BOT 7, 19, 21, 25, 30 (*B. dothidea*) and BOT 2, 11, 16, 24 and 32 (*Botryosphaeria* sp.) were evaluated for their pathogenicity in inoculation trials. Inoculations

were conducted on a susceptible *E. grandis* clone (ZG 14) in the Kwambonambi area, KwaZulu-Natal province in early spring (Sep 1997). The 6-mo-old trees had stem diameters of between 4 and 6 cm. Trees were actively growing during the time that the trials were conducted and no environmental stresses were apparent. Bark disks (5-mm diam) were removed with a cork borer (10 trees per isolate, 1 isolate per tree) and replaced with an agar disk colonized with mycelium from 4-d-old isolates growing on MEA. Controls were inoculated with sterile MEA disks. Wounds were sealed with masking tape to reduce desiccation. Lesion lengths (mm) were recorded 1 mo after inoculation. The entire trial was repeated once on trees in the same plantation.

RESULTS

Morphology and cultural characteristics.—All isolates of the unnamed *Botryosphaeria* sp. produced *Fusicoccum* anamorphs as defined by Crous & Palm (1999). The unnamed *Botryosphaeria* sp. had fusoid ascospores (20--)23--26(--28) x (7--)8--9(--11) µm, overlapping somewhat with the more ovoid to ellipsoid ascospores of *B. ribis* (17--23 x 7--10 µm; Punithalingam & Holliday, 1973) and *B. dothidea* (13--)19--27(--35) x (6--)8--11(--14) µm; Pennycook & Samuels, 1985). The *Fusicoccum* conidia of the unnamed *Botryosphaeria* sp. [(20--)22--25(--28) x (6--)7--8 µm *in vivo*, (18--)20--23(--25) x 7--8(--12) µm *in vitro*] were similar in length, but slightly wider than those of *F. aesculi* [18--25(--30) x 4--4.5(--5) µm (immature type *in vivo*; Crous & Palm, 1999); (15--)20--26(--32) x (4--)5--6(--9) µm *in vitro*; Pennycook & Samuels, 1985)], and the *Fusicoccum* anamorph of *B. ribis* (16--31 x 4.5--8 µm *in vivo*; Grossenbacher & Duggar, 1911; 14--23 x 3--4.5 µm *in vitro*; Morgan-Jones & White, 1987). In addition, conidia of *B. dothidea* and *B. ribis* vary from being fusiform to narrowly ellipsoidal (widest just above the middle), while conidia of the unnamed *Botryosphaeria* sp. are ovoid to clavate, generally having their widest point closer to the apex.

ITS sequence data and phylogeny.—The PCR of all isolates consistently produced amplification products of approx. 560 bp. The DNA sequences of all the *Botryosphaeria* isolates were found to be similar. It was possible to align the data manually by inserting gaps in the sequence data. With *M. africana* as the outgroup, 152 most parsimonious trees of 302 steps were produced from the aligned 529 bp sequence data, using the heuristic search option

of PAUP* (Fig. 1) (CI = 0.904, HI = 0.096, RI = 0.854). Gaps were treated as a fifth character (gapmode = newstate). The trees differed only in the position of isolates within terminal groupings and not amongst clades. The overall topology of all 152 most parsimonious trees was, therefore, identical. Four principal clades were formed, designated as clades I to IV. All the branch points of the clades had bootstrap values (Hillis & Bull, 1993) greater than 70%.

Clade I had a bootstrap support value of 97% and included asexual *Fusicoccum* isolates that matched the description of *F. aesculi*. Clade II had 100% bootstrap support and consisted of two *Fusicoccum* isolates that also are part of the *B. dothidea*-complex (Jacobs & Rehner, 1998, AF027750 and AF027746). Clade III had 99% bootstrap support and included 5 isolates of the unnamed *Botryosphaeria* sp. from *Eucalyptus*. Clade IV consisted of a single isolate of *F. luteum* (Jacobs & Rehner, 1998, AF027745).

Pathogenicity.—All isolates screened in this study produced lesions in the secondary phloem 1 mo after inoculation (Table 2). The most virulent isolate was BOT 7, while BOT 25 was least virulent. Both of these isolates belonged to the *F. aesculi* (= *B. dothidea*) group (clade I) (Fig. 9). Isolates representing the unnamed *Botryosphaeria* sp. (clade III) did not differ significantly from one another based on lesion length ($P = 0.05$). The five *F. aesculi* (= *B. dothidea*) isolates tested (clade I) caused significantly longer lesions (species mean = 34.3 mm) than the five isolates of the unnamed *Botryosphaeria* sp. (clade III) (species mean = 26.9 mm; contrast testing $P = 0.05$).

Based on the morphological differences observed (primarily in conidium taper), as well as differences in pathogenicity and DNA phylogeny, we describe the unnamed species from *Eucalyptus* as new.

Botryosphaeria eucalyptorum Crous, H. Smith et M. J. Wingf. sp. nov. Figs.1--8

Anamorph. **Fusicoccum eucalyptorum** Crous, H. Smith et M. J. Wingf. sp. nov.

Ascostromata in contextu hospitis inclusa, usque ad 300 µm diametro, erumpescentia, solitaria, botryosa, stromatiformia, atrobrunnea vel nigra, cum ostiolis centralibus nigris.

Asci clavati, inter paraphyses filiformes interspersi, 70--140 x 15--21 µm, octosporati, bitunicati cum loculo apicali bene evoluto. Ascosporae irregulariter biseriatae, hyalinae, unicellulares, granulares, cum aetate pallide brunnescens, (20--)23--26(--28) x (7--)8--9(--11) µm, juventute valde inaequilaterae, maturitate minus ita, fusoides, medio latissimae, fundis obtusis, apicibus obtusis vel subobtusis. Pycnidia in contextu hospitis inclusa, solitaria vel botryosa, stromatiformia, globosa, usque ad 450 µm diametro; paries pycnidii e stratis 6--8 formata, e textura angulari brunnea composita, ad intima hyalinescens. Cellulae conidiogenae holoblasticae, hyalinae, subcylindricae, 10--25 x 3.5--6 µm, percurrenter cum 1--2 proliferationibus prolificentes, vel in plano eodem periclinaliter minuter incrassatae. Conidia hyalina, granularia, ovoidea vel subclavata, apicibus obtusis, in fundo subtruncato vel obtuse rotundato angustata, interdum cum fimbria marginali minuta, in conidiis junioribus manifesta, (18--)23--25(--28) x (6--)7--8 µm.

Ascostromata embedded in host tissue, up to 300 µm diam., becoming erumpent, solitary, botryose, stromatic, dark brown to black, with central, black ostioles. *Asci* clavate, interspersed amongst filiform paraphyses, 70--140 x 15--21 µm, 8-spored, bitunicate with a well-developed apical chamber. *Ascospores* irregularly biseriate, hyaline, unicellular, granular, becoming light brown with age, (20--)23--26(--28) x (7--)8--9(--11) µm, prominently inequilateral when young, less so when mature, fusoid, widest in the middle, base obtuse, apex obtuse to subobtuse. *Pycnidia* embedded in host tissue, solitary or botryose, stromatic, globose, up to 450 µm diam.; pycnidial wall, 6--8 cell layers thick, composed of brown *textura angularis*, becoming hyaline towards the inner region. *Conidiogenous cells* holoblastic, hyaline, subcylindrical, 10--25 x 3.5--6 µm, proliferating percurrently with 1--3 proliferations, or proliferating at the same level with minute periclinal thickening. *Conidia* hyaline, granular, ovoid to slightly clavate, apex obtuse, tapering towards a subtruncate or bluntly rounded base, sometimes with a minute marginal frill visible on younger conidia, (20--)22--25(--28) x (6--)7--8(--9) µm *in vivo*, (18--)20--23(--25) x 7--8(--12) µm *in vitro*. *Cultures.* Colonies iron gray (25""'k) (underneath), and olivaceous gray (25""'i) (surface), with extensive gray aerial mycelium, and smooth colony margins. Colonies obtaining a radius of 21--24 mm diam on MEA after 4 d in the dark at 25 C. Cardinal temperatures for growth were min above 5 C, max below 35 C, opt 25 C.

Anamorph. Fusicoccum eucalyptorum Crous, H. Smith et M.J. Wingf. Sp. Nov.

Etymology. In reference to its host, *Eucalyptus*.

Hosts. *Eucalyptus grandis* Hill: Maid. and *E. nitens* (Deane et Maid.) Maid.

Distribution. Mpumalanga (White River, Sabie), South Africa.

Specimens examined. SOUTH AFRICA. MPUMALANGA: Sabie, *Eucalyptus grandis*, 1995, *H. Smith* (HOLOTYPE of *B. eucalyptorum* , PREM 56603), (HOLOTYPE of *F. eucalyptorum*, PREM 56604).

DISCUSSION

We conclude that two species of *Botryosphaeria*, namely *B. dothidea*, and *B. eucalyptorum* occur on *Eucalyptus* in South Africa, based on morphological and cultural characteristics, pathogenicity and partial nrDNA sequence data.

Botryosphaeria eucalyptorum closely resembles *B. ribis* (Punithalingam & Holliday, 1973; Sivanesan, 1984) and *B. dothidea* (Pennycook & Samuels, 1985), but the ascospores of *B. eucalyptorum* are more fusoid and slightly longer. Conidia of *F. eucalyptorum*, the anamorph of *B. eucalyptorum*, are consistently wider, and have a more distinct taper, compared to the smaller, more fusoid conidia of anamorphs (especially *F. aesculi*) in the *B. dothidea*-complex.

The validity of *B. ribis* as a separate species from *B. dothidea* has been a matter of debate for many years (Arx & Müller, 1954; Rumbos, 1987; Rayachhetry *et al*, 1996). Sequence data of Jacobs & Rehner (1998), supports observations of the above mentioned authors, that isolates described as *B. dothidea* and *B. ribis* respectively, group together in strongly supported clades. This provides further proof that more than one species occurs in the *B. dothidea*-complex. Whether any of these taxa can now be attributed to *B. ribis* remains to be shown. At present no ex-type cultures of either species are available for comparison with isolates thought to represent *B. dothidea* or *B. ribis*. What is necessary is an epitypification of these species and a collection of representative isolates for molecular study. Until such time, we suggest that the synonymy proposed by Arx & Müller (1954) is followed, as no conclusive evidence has yet been presented that *B. ribis* and *B. dothidea* are separate taxa.

Isolates of *B. eucalyptorum* used in this study were found to have limited variability in their pathogenicity. Lesions produced after inoculations did not differ significantly amongst isolates. In contrast, isolates representing the fungus we know as *B. dothidea*, produced lesions that differed significantly between isolates. The fact that these data are based on only five isolates of each species could explain the lack of variability amongst the *B. eucalyptorum* isolates. Based on the available data, isolates of *B. eucalyptorum* were less virulent than that of *B. dothidea*. It is, however, clear that *B. eucalyptorum* is pathogenic to eucalypts.

DNA sequence data was used successfully in this study to separate isolates of *B. eucalyptorum* from others in the *B. dothidea*-complex. The variability amongst isolates representing *F. aesculi* (= *B. dothidea*) as reported by Jacobs & Rehner (1998) was reaffirmed in this study. Isolates of *F. aesculi* were found to be present in two distinct, but closely related terminal clades (clades I and II, Fig. 9). Isolates of *F. aesculi* present in these clades originate from different hosts and continents, indicating that, although there appears to be much variability within the *B. dothidea*-complex, these fungi are closely related. Isolates of *F. aesculi* could be distinguished as distinct from those of *B. eucalyptorum* (= *F. eucalyptorum*). The overall phylogeny indicates, however, that all *Fusicoccum* species represented in this study are closely related.

Botryosphaeria eucalyptorum has thus far been associated only with cankers on the main stems of *E. grandis* and *E. nitens*. Although this fungus appears to be less pathogenic than *B. dothidea,* the fact that it is pathogenic should be considered by the Forestry Industry as being significant. *Botryosphaeria dothidea* is a common endophyte of eucalypt leaves in South Africa (Smith *et al*, 1996a), and continuous monitoring could in future reveal that *B. eucalyptorum* also occurs as an endophyte. This study contributes to the current understanding of *Botryosphaeria* and more specifically to species in the *B. dothidea*-complex. Much work is, however, still needed to fully understand and reclassify this variable group of fungi.

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Table 1. Identity and origin of isolates studied

Isolate No.	GenBank No.	Teleomorph	Anamorph	Host	Location	Collector
BOT 275	AF238689		*Sphaeropsis sapinea*	*Pinus patula* Schl. & Cham.	Indonesia	M.J. Wingfield
CMW 3141	AF238675	*Botryosphaeria dothidea*	*Fusicoccum aesculi*	*Cercis canadensis* L.	Columbia, U.S.A.	K.A. Jacobs
BOT 682	AF283680	"	"	*Syzygium guinesee* (Willd.) DC.	KwaZulu-Natal, South Africa	H. Smith
BOT 681	AF283676	"	"	*Heteropyxis natalensis* Harvey	"	"
BOT 683	AF283677	"	"	*Syzygium cordatum* Hochst.	"	"
BOT 7	AF283678	"	"	*Eucalyptus grandis* Hill: Maid.	Mpumalanga, South Africa	"
BOT 21	AF283681	"	"	"	"	"
BOT 30	AF283682	"	"	"	"	"
BOT 19	AF283683	"	"	*Eucalyptus smithii* R.T. Bak.	"	"
BOT 25	AF283679	"	"	*E. grandis*	Swaziland	"
BOT 11	AF283684	*Botryosphaeria* sp.	*Fusicoccum* sp.	"	Mpumalanga, South Africa	"
BOT 16	AF283687	"	"	"	"	"
BOT 2	AF283688	"	"	*Eucalyptus nitens* (Deane et Maid.) Maid.	"	"

Table 1. continued

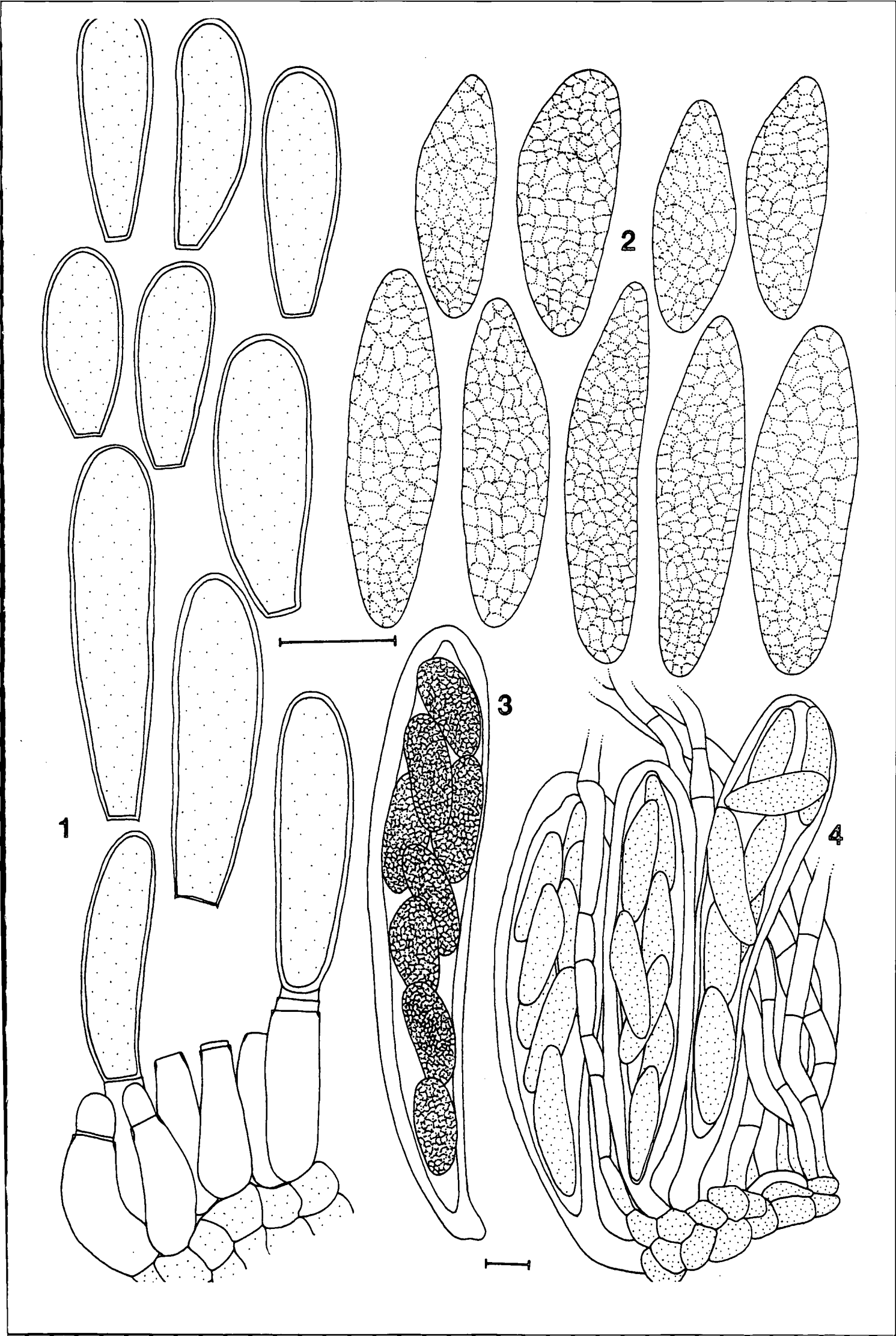
BOT 24	AF283686	"	"	*E. grandis*	"	"
BOT 32	AF283685	"	"	"	"	"
CMW 3025	AF283690	*Mycosphaerella africana*	-	*Eucalyptus viminales* Labill.	Stellenbosch, South Africa	P.W. Crous

Table 2. Lesion lengths produced by *Botryosphaeria dothidea* and *B. eucalyptorum* after inoculation of the *Eucalyptus grandis* clone (ZG 14)

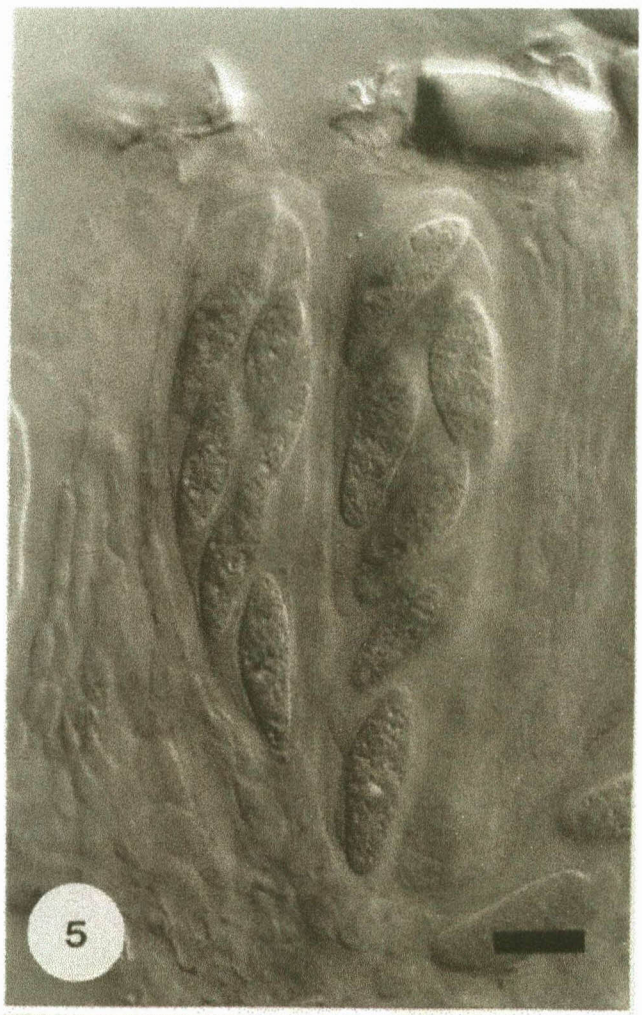
Isolate no.	Identification	Lesion length (mm)[a]
BOT 7	*B. dothidea* (ITS clade I)	109 a
BOT 19	"	98 a
BOT 21	"	90 a
BOT 2	*B. eucalyptorum* (ITS clade III)	66 b
BOT 32	"	57 b
BOT 16	"	55 b
BOT 24	"	46 b
BOT 11	"	45 b
BOT 30	*B. dothidea* (ITS clade I)	23 c
BOT 25	"	22 c

[a] Lesion lengths represent the mean of two replications of ten trees each. Numbers follwed by a different letter are significantly different (P=0.05).

Figures. 1--4. *Botryosphaeria eucalyptorum* and its anamorph *Fusicoccum eucalyptorum*. 1. Conidia and conidiogenous cells. 2. Ascospores. 3. Mature ascus. 4. Developing asci and pseudoparaphyses. Bars = 10 μm.


1
2
3
4

Figures. 5–8. Differential interference micrographs of *Botryosphaeria eucalyptorum* and its anamorph *Fusicoccum eucalyptorum*. 5. Developing asci with pseudoparaphyses. 6. Young ascus. 7, 8. Conidia. Bar = 10 μm.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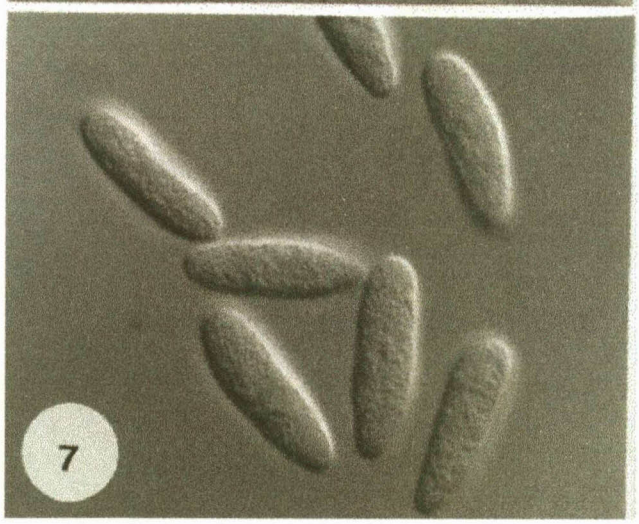
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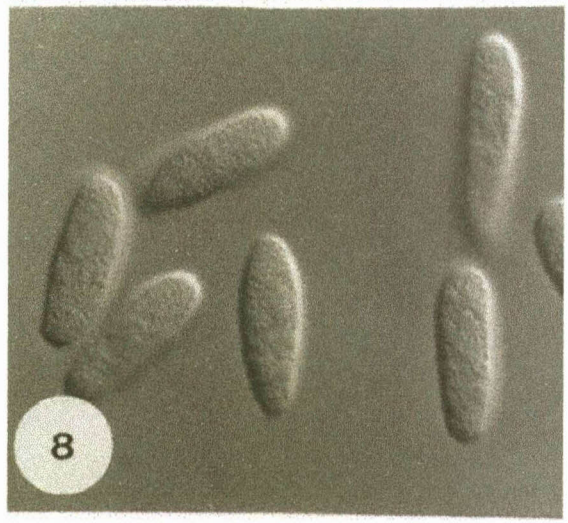
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Figure 9. Phylogenetic relationships amongst *Botryosphaeria dothidea*, *B. eucalyptorum* and related taxa based on parsimony analyses of the ITS1 and ITS2 rRNA operon DNA sequence data. The phylogram is rooted to *Mycosphaerella africana*. Bootstrap frequencies of higher than 55% are indicated (1000 replications) below internodes, and branch lengths, proportional to the number of steps, are indicated above internodes. Roman numerals represent groupings as used in the text and tables.

CHAPTER 5

Genotypic diversity of *Sphaeropsis sapinea* from South Africa and Northern Sumatra

Published as: Smith, H, Wingfield, M.J., de Wet, J. & Coutinho, T.A. (2000). Genotypic diversity of *Sphaeropsis sapinea* from South Africa and Northern Sumatra. *Plant Disease* **84**: 139-142.

Genotypic diversity of *Sphaeropsis sapinea* from South Africa and Northern Sumatra

Sphaeropsis sapinea is the most important pathogen of *Pinus* spp. in South Africa. The fungus, which reproduces only asexually, occurs on exotic *Pinus* spp. In this study, the diversity of the *S. sapinea* population in South Africa was compared with a population from Northern Sumatra. The populations for both countries were obtained from exotic *P. patula* plantations. The phenotypic diversity of these populations was assessed using vegetative compatibility tests. The percentage maximum genotypic diversity, based on Stoddard and Taylor's index, for the South African population was 30.5% compared to 1.5% for the Northern Sumatran population. Based on the number of phenotypes, the South African *S. sapinea* population was significantly more diverse (P = 0.05) than that of the Northern Sumatran population. The results indicate that the population of *S. sapinea* in South Africa has, in all likelihood, arisen as a result of introductions of the fungus on pine seeds, imported from various parts of the world, during the last century.

Sphaeropsis sapinea (Fr:Fr.) Dyko & Sutton (= *Diplodia pinea* (Desm.) Kickx, Petrak & Sydow) is the most important pine pathogen in South Africa, causing serious annual losses, due to die-back after hail (Swart *et al*, 1987a; Swart *et al*, 1987b; Zwolinski *et al*, 1990a,b). *S. sapinea* ocurs on pines, which are exotic in the country, suggesting that it may be an introduced pathogen. The pathogen has been present in South Africa since the turn of the century and was first reported in 1909 from the eastern Cape Province (Lundquist, 1987). Since that time, numerous outbreaks of die-back after hail, have been reported mostly from the summer rainfall areas of the Mpumalanga Province, where *Pinus patula* Schl. & Cham is grown extensively. This fungus is also reported to cause diseases of pines elsewhere in the world (Punithalingam & Waterston, 1970), although the extent of the disease appears to be less severe than it is in South Africa (Swart *et al*, 1987a).

Recently studies have shown that *S. sapinea* is present as latent infections in third-year, mature seed cones of *P. patula* and *Pinus radiata* D. Don (Smith *et al*, 1996). Latent infections have also been reported from the stems of pine seedlings in the United States (Stanosz *et al*, 1997). In South Africa, *S. sapinea* colonizes cones extensively and has been recovered from the cone pith tissue, seeds, seed wings and ovuliferous scales. It is the most abundant in the cone pith tissue (Smith *et al*, 1996).

The fungus is widespread in South Africa and occurs wherever pines are cultivated, including Mpumalanga Province, Northern Province, KwaZulu Natal, Eastern Cape Province and Western Cape Province. *S. sapinea* has recently been recorded from Northern Sumatra and Indonesia, where it causes tip die-back on various *Pinus* spp., including the exotic *P. patula* and native *Pinus merkusii* Jungh & de Vriese (Wingfield, unpublished). Plantations of *P. patula* are commonly established adjacent to natural stands of *P. merkusii* in Northern Sumatra.

No teleomorph has been associated with *S. sapinea*. All indications are that only asexual reproduction occurs, and this would lead to clonal lineages within a population (McDonald, 1997). By measuring the level of genotypic diversity within a population, it is possible to gain an indication of whether sexual or asexual reproduction occurs (McDonald, 1997). Levels of genotypic diversity can be obtained as multilocus genotypes derived from molecular markers or by determining vegetative compatibility groups (VCGs) (Leslie, 1993). The use of VCGs to determine the variability within fungal populations has been widely and successfully used in recent years (Adams *et al*, 1990; Leslie, 1993; Meijer *et al*, 1994; Proffer & Hart, 1988).

The objective of this study was to determine the diversity within the South African *S. sapinea* population on introduced pines and to compare this with the diversity of a population of the fungus collected from trees in Northern Sumatra, where young plantations of *P. patula* occurred in close proximity to a native population of pines. It was hypothesized that the population in South Africa would be relatively uniform, typical of an introduced pathogen, and that isolates from Northern Sumatra where native pines occur, would be more diverse.

MATERIALS AND METHODS

Sampling in South Africa--*Sphaeropsis sapinea* isolates were obtained only from *P. patula* in the Sabie region of Mpumalanga Province, South Africa.. A three level hierarchical sampling strategy was followed. The sampling commenced in a commercial plantation of 15-year-old *P. patula* trees on the farm Klipkraal. An individual tree, approximately in the middle of the plantation, was selected and 10 third year mature seed cones were sampled from it. The second level of sampling comprised 50 randomly selected trees from the same plantation. From each of these 50 trees, one, third year mature seed cone was sampled. The third level of sampling comprised two plantations, both within a 50-km radius from the first sampling site (Klipkraal). On each of these plantations, Hebron and Renosterhoek, 50 third year seed cones were collected from 50, approximately 15-year-old, *P. patula* trees.

Sampling in Northern Sumatra--*Sphaeropsis sapinea* isolates were obtained only from *P. patula* in the Habinsaran region of Northern Sumatra. Shoots with die-back were collected from three plantations of approximately 5-year-old *P. patula* trees. These plantations were in the same region where native stands of *P. merkusii* occurred. All three plantations were within 20 km from one another. A single dead shoot was collected from each of 12 trees in the Habinsaran plantation #1, seven dead shoots from each of five trees in the Habinsaran plantation #2 and eight dead shoots from each of seven trees in the Habinsaran plantation #3.

Isolations--The mature third year seed cones were cut into halves under sterile conditions in the laboratory. Cone pith tissue pieces were placed on 2% MEA (malt extract agar, Biolab) in Petri dishes, supplemented with 200-mg/l chloramphenicol to suppress bacterial growth, and incubated at 25° C. Fast growing, darkly pigmented colonies were transferred to water agar plates with sterile pine needles on which *S. sapinea* isolates formed pycnidia for confirmation of their identity. Shoots with die-back were placed on moist filter paper in Petri dishes and incubated at 25° C. *Sphaeropsis sapinea* pycnidia formed on the surface of pine tissue from which single

conidial isolates were obtained. Isolates, positively identified as *S. sapinea*, were transferred to MEA slants in culture vials and stored at 4° C. The hierarchical sampling of *S. sapinea* isolates from South Africa was comprised of 107 isolates from three sampling levels. A total of 83 isolates were obtained from die-back shoots in Northern Sumatran plantations.

Vegetative compatibility tests--Vegetative compatibility tests with the South African isolates were first done within each sampling level of the hierarchy. Isolates within each sampling level were paired in all possible combinations on OMA (oatmeal agar). Oatmeal (60 g/l) was steamed in a water bath at 70° C for 2 hours with periodic stirring. It was then separated from the liquid by filtration through a double layer of cheesecloth resulting in approximately 600 ml of oatmeal suspension. Agar (20 g, Biolab) was melted in 400 ml distilled water and then added to oatmeal suspension and autoclaved. The medium was thoroughly mixed prior to dispensing into plastic assay dishes (20-cm square, Nunc Industries).

Representative VCGs from each sampling level were then paired in all possible combinations. The same approach was used for the Northern Sumatran isolates of *S. sapinea*, where isolates were first paired within plantations and then as representative VCGs between plantations. Isolates were placed approximately 1 cm apart on the OMA plates in such a way as to pair all isolates with themselves and with all other isolates. Assay plates were incubated at 20° C in the dark for 4-6 days. The occurrence of different VCGs was determined on the basis of barrage line formation between incompatible isolates (Anagnostakis, 1983). *Sphaeropsis sapinea* isolates grew actively on OMA and colonies made contact with each other within 2-4 days. Incompatible reactions resulted in dark mycelial barrage lines (Fig. 1) and were most obvious approximately 5 days after inoculation. After this time mycelial growth became very dense and the reactions were no longer visible. Compatible isolates (Fig. 1) merged without the formation of any barrage lines. All isolates are available in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Geographic differentiation--Isolates within each population of *S. sapinea*, belonging to the same VCG, were treated as individual phenotypes since the genetic background was unknown. Stoddart and Taylor's (Stoddart & Taylor, 1988) index of genotypic diversity was used for this haploid fungus to measure and statistically compare the diversity of the different phenotypes in the different populations. Thus, the null hypothesis that no significant difference in genotypic diversity exists between the South African and Northern Sumatran populations of *S. sapinea*, was tested. The genotypic diversity ($G^{\wedge}$) of the sampled population was calculated for each population according to the method of Stoddart & Taylor (1988). The percentage of maximum diversity was calculated by dividing genotypic diversity ($G^{\wedge}$) of the observed population by the sample size (N) (McDonald *et al*, 1994). This facilitates the direct comparison the South African and Northern Sumatran populations, despite their different sample sizes. Genotypic diversity for the two populations was tested for significant differences using the Students t-test (P = 0.05) (Stoddart & Taylor, 1988).

RESULTS

South African population--A total of 62 VCGs were found among the 107 isolates from the three plantations (Table 1). The 44 isolates of *S. sapinea* obtained from the Klipkraal plantation were represented by 16 VCGs (largest observed VCG = 11 isolates). The 31 isolates obtained from the Hebron plantation accounted for 23 VCGs (largest observed VCG = 3 isolates) and the 32 isolates from the Renosterhoek plantation also for 23 VCGs (largest observed VCG = 4 isolates). Although these three plantations were within 50 km of one another, no common VCGs were found to occur between plantations.

Northern Sumatran population--Four VCGs were found amongst the 83 isolates from three plantations (Table 1). The 11 isolates obtained from Habinsaran plantation #1 were represented by two VCGs (largest observed VCG = 6 isolates). The 31 isolates from Habinsaran plantation #2 accounted for two VCGs (largest observed VCG = 30 isolates) and the 41 isolates obtained for Habinsaran plantation #3 also for two VCGs (largest observed VCG = 39 isolates). In the Habinsaran plantations #2 and

#3, where several isolates originated from the same tree, it was found that isolates from the same tree belonged to the same VCG, with the exception of one isolate in plantation #2 and two isolates in plantation #3. A large common VCG which included 75 isolates was found to occur amongst the three plantations. In contrast, the South African population of *S. sapinea* was found to be very diverse with 62 VCGs, compared to the low diversity of the Northern Sumatran population that consisted of only four VCGs.

Geographic differentiation--The percentage of maximum genotypic diversity for the South African sampled population of *S. sapinea* was 30.5% compared to the 1.5% of the Northern Sumatran sampled population (Table 1). The South African population was significantly more diverse ($P = 0.05$) than the Northern Sumatran one (South Africa: $G\hat{} = 32.6$, STD = 18.6 and Northern Sumatra: $G\hat{} = 1.2$, STD = 26.5). The null hypothesis, that no significant difference in diversity exists between the South African and Northern Sumatran populations of *S. sapinea*, was consequently rejected. A high degree of clonality was evident in the Northern Sumatran population, where the dominant VCG comprised 90% of the total sample and was present in all three plantations sampled. In contrast, the dominant South African VCG comprised only 10% of the total sample and was present in only one plantation.

DISCUSSION

This is the first study to consider diversity and geographic differentiation in the important pine pathogen *S. sapinea*. Contrary to expectation, the results showed that the introduced population of *S. sapinea* in South Africa is very diverse, while the opposite is true for the population from Northern Sumatra. The results from this study clearly indicate that the pathogen has a broad diversity base in South Africa.

By determining the occurrence and frequency of distinct VCGs in populations of *S. sapinea* from South Africa and Northern Sumatra, and assuming that each VCG represented a particular genotype, we were able to compare the genotypic diversity of these populations in a statistically meaningful way. Although the inferences that can

be made from determining genotypic as opposed to gene diversity is limited, we were able to make various assumptions concerning the population structures of the South African and Northern Sumatran populations of *S. sapinea*.

Genotypic diversity is based on the number and frequency of genotypes occurring in a population (Stoddart & Taylor, 1988). The genotypic diversity of an observed sample ($G^{\wedge}$) is a robust method of testing genotypic diversity in asexually reproducing fungi (Stoddart & Taylor, 1988). This method has low type I errors but lacks the ability to distinguish highly repeated genotypes (large clones) from genotypes that occur rarely, within a population, as is the case in the Northern Sumatran population. As a result, highly repeated genotypes greatly reduce the genotypic diversity of the observed sample and cause high type II errors (Stoddart & Taylor, 1988). In this case, however, large type II errors are more the result of the clonal population structure (Northern Sumatran population) than the lack of power of the test (Stoddart & Taylor, 1988).

The discrepancies in the expected VCG diversity within the sampled populations of *S. sapinea* from South Africa and Northern Sumatra is interesting and we have put forward a number of hypotheses which need further investigation. The diverse population of *S. sapinea* present in South Africa is, in our opinion, largely due to the fact that this fungus is an endophytic latent colonizer of mature seed cone tissue, including seed, of *P. patula* (Smith *et al*, 1996). The diverse nature of the fungus from South African *P. patula* is consistent with the hypothesized occurrence of multiple introductions into the country, over a long period of time. This would most likely have occurred because the South African Forestry Industry has imported pine seed for many decades from many parts of the world where pines are grown and found naturally. The broad genetic base existing in South Africa also suggests that this fungus may have an undiscovered teleomorph or that the sexual stage was lost fairly recently. Laughton (Laughton, 1937) mentioned the presence of rarely formed perithecia, unfortunately the anamorph connection was never made, rendering the report as being of dubious value.

There are various possible hypotheses to explain the low diversity within the *S. sapinea* population from Northern Sumatran *P. patula*. Native *P. merkusii* occurs in Northern Sumatra, with the exotic *P. patula* only established fairly recently. The low diversity of *S. sapinea* occurring on this exotic pine may thus be as a result of a limited number of introduced VCGs on seed from a common origin. Alternatively the *S. sapinea* population occurring on the native *P. merkusii* may not yet have moved onto *P. patula*. In light of the fact that *S. sapinea* causes tip die-back of both *P. merkusii* and *P. patula* it can be argued that, from the indigenous population of *S. sapinea* occurring on *P. merkusii*, only one or more selectively fit VCGs may have moved onto the recently introduced *P. patula*. The selective pressure in such a situation may be pathogenicity, enabling a dominant VCG or VCGs to predominate in the population sampled from *P. patula*. In order to distinguish between the above mentioned two hypotheses, a detailed sampling and population study is needed from native *P. merkusii* in Northern Sumatra.

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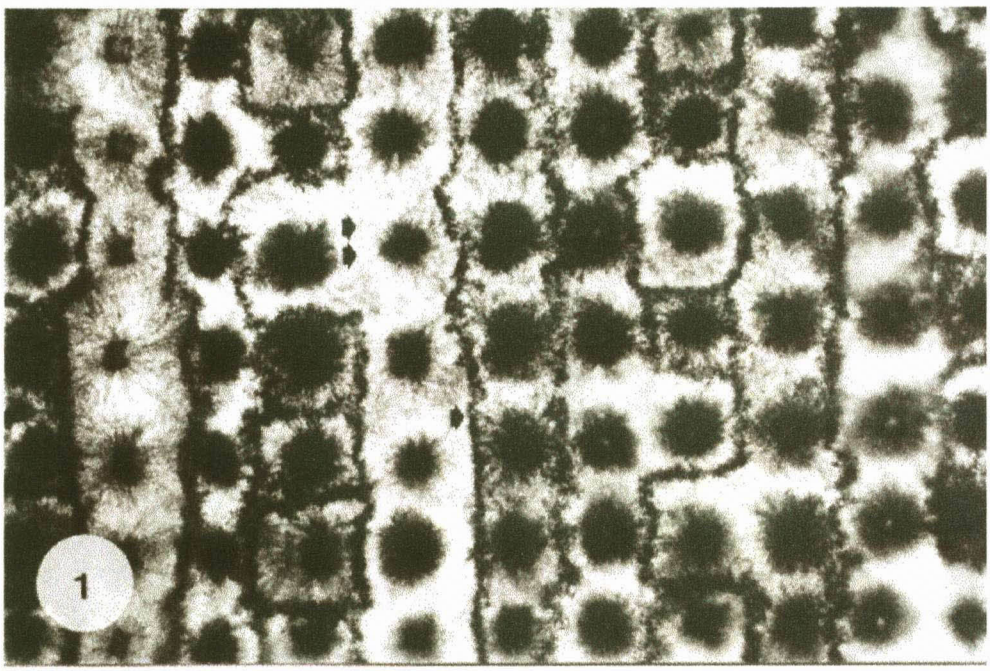
Table 1. *Sphaeropsis sapinea* vegetative compatibility groups (VCGs) from *P. patula* seed cones from three plantations in South African and shoots with die-back from three plantations in North Sumatra.

Location	Sample size	No. VCGs	No. isolates
Klipkraal (South Africa)	44	1	11
		1	7
		3	4
		1	3
		1	2
		9	1
Hebron (South Africa)	31	2	3
		4	2
		17	1
Renosterhoek (South Africa)	32	1	4
		2	3
		2	2
		18	1
Total	107	62	
Genotypic diversity (G^)	32.6		
Standard deviation (STD)	18.6		
Maximum percentage of genotypic diversity (G^/N%)	30.5%		

Location	Sample size	No. VCGs	No. isolates
Habinsaran #1 (North Sumatra)	11	1	6
		1	5
Habinsaran #2 (North Sumatra)	31	1	30
		1	1
Habinsaran #3 (North Sumatra)	41	1	39
		1	2
Total	83	4	
Genotypic diversity (G^)	1.2		
Standard deviation (STD)	26.5		
Maximum percentage of genotypic diversity (G^/N%)	1.5%		

Figure 1. Dark barrage lines (arrow) formed between incompatible isolates of *S. sapinea* belonging to different VCGs and confluent mycelium (double arrows) between compatible isolates belonging to the same VCG.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CHAPTER 6

The role of latent *Sphaeropsis sapinea* infections in post-hail associated die-back of *Pinus patula*

Submitted for publication as: Smith, H., Wingfield, M.J. & Coutinho, T.A. (2001). The role of latent *Sphaeropsis sapinea* infections in post-hail associated die-back of *Pinus patula*. *Forest Ecology and Management*. (Accepted).

The role of latent *Sphaeropsis sapinea* infections in post-hail associated die-back of *Pinus patula*

Sphaeropsis sapinea is an economically important pathogen of pines in South Africa. The most serious disease caused by this fungus is die-back of *Pinus patula* and *Pinus radiata* after hail. In this study we investigate the role of latent *S. sapinea* infections in seed cones of *P. patula* in post-hail associated die-back. *Pinus patula* seed cones were found to be infected during the second year of development. Extensive colonization of all cone tissues occurred in the third year when cones mature, prior to seed discharge. Vegetative compatibility tests showed that *S. sapinea* is present as a single vegetatively compatible group in third year seed cones. The presence of *S. sapinea* in colonized third year seed cones apparently result from a single successful infection in each cone. The probable role of latent infections by *S. sapinea* was considered in a case study of hail damaged *P. patula* trees of three age classes. Mortality was most severe in a stand of 25-year-old trees (92%) followed by a stand of 14-year-old trees (32%) and a 5-year-old stand (9%). Isolations from diseased trees showed that *S. sapinea* could be recovered from darkly stained branch pith, which was continuous with infected pith in third year seed cones. In contrast, the fungus was absent in the non-discolored pith of branches on healthy trees of the same age. Our results indicate that *S. sapinea* infects branch pith tissue directly from previously infected, attached seed cones to cause rapid die-back and mortality of hail damaged trees.

In South Africa, *Sphaeropsis sapinea* (Fr:Fr.) Dyko & Sutton was first reported in 1909 from the Fort Cunynghame State Forest in the eastern Cape Province (Lundquist 1987a). Prior to 1930, *Pinus radiata* D. Don was the most commonly planted pine species and it was seriously damaged by *S. sapinea* (Lundquist 1987b). Losses due to die-back caused by *S. sapinea* after hail damage was so serious that planting of *P. radiata* was discontinued in the summer rainfall and thus hail prone areas (Swart & Wingfield, 1991). By 1925, *Pinus patula* Schl. & Cham. had replaced most *P. radiata* in these areas and is now the most widely planted *Pinus* species in South Africa.

During the early 1930's it was believed that *P. patula* was tolerant to infection by *S. sapinea.* It was this fact that motivated its deployment in hail prone areas. Occurrences of die-back on *P. patula* occurred sporadically at first but steadily increased and by 1940, *P. patula* was no longer considered to be tolerant to invasion by the pathogen (Lundquist 1987b).

By the early 1960's *S. sapinea* was considered to be the most important pathogen of plantation grown forest trees in South Africa (Lückhoff, 1964; Swart *et al.*, 1985). The most serious losses associated with the fungus occurred after hail on *P. patula* in summer rainfall areas. It is estimated that between 1923 and 1983 there had been 11 outbreaks of *S. sapinea* induced die-back of *P. pinaster* and 25 of *P. radiata* in the southern Cape Province alone (Zwolinski *et al.*, 1990). *Sphaeropsis sapinea* remains the most economically important pine pathogen in South Africa, despite the fact considerable effort is made to reduce its impact (Swart *et al.*, 1985).

Sphaeropsis sapinea is well-known for its capacity to infect wounds (Wright & Marks, 1970; Palmer 1991). These arise naturally or through forestry practices (Marks & Minko, 1969; Gilmour, 1964; Chou, 1984; Swart *et al.*, 1985; Chou & MacKenzie, 1988) and insect damage (Swart *et al.*, 1987). In South Africa the pathogen is most frequently reported to infect pines through wounds caused by hail (Van der Westhuizen, 1968; Brown *et al.*, 1981; Swart *et al.*, 1987; Zwolinski *et al.*, 1990).

Sphaeropsis sapinea has recently been shown to occur as a latent pathogen in various healthy pine tissues (Stanosz *et al.*, 1997; Smith *et al.*, 1996). Stanosz *et al.* (1997) reported that *S. sapinea* is able to cause latent infections in stem portions of nursery grown *Pinus resinosa* Ait. seedlings. Furthermore, Smith *et al.* (1996) showed that such latent infections are common in mature un-opened seed cones of *P. patula* and *P. radiata* in South Africa. In the latter study, the fungus was recovered from seed cone pith tissue, seeds, seed wings and ovuliferous scales. The pith tissue of cones was found to be the

most frequently infected. Both these authors speculated that this phenomenon might explain the rapid disease development under stress.

In this paper, we consider the probable role of latent infections caused by *S. sapinea* in *P. patula* seed cones, in post-hail associated die-back. Particular attention is given to the stage of cone development when infections occur and the number of infections that may occur in individual cones. The result of latent infections and the influence of tree age on mortality following hail damage is assessed using a case study. Finally, we consider the possibility that *S. sapinea* may be present in the form of latent infections in the bark of *P. patula* trees, and that these infections may contribute the development of stem and branch cankers associated with hail wounds.

MATERIALS AND METHODS

Laboratory Studies

Stage of seed cone development

Collection of cones--Seed cones in three age classes, as well as current year pollen cones, were collected from a 14-year-old stand of *P. patula* in the Sabie area, Mpumalanga Province, South Africa. Seed cones represented current year, second year actively expanding and third year mature un-opened cones. Thirty seed cones of each age class, as well as 30 pollen cones, were collected randomly in the stand (10 seed and pollen cones from each of three trees). All sampled cones were transported to the laboratory and stored at 4°C. Isolations from these cones were done within 48 h of collection.

Isolation and culture methods--All cones were separated according to age, briefly submerged in 96% ethanol and flamed. In all cases the cones were cut in half using sterilized pruning shears. Isolations from cone pith tissue, seeds, seed wings and ovuliferous scales were made by placing tissue pieces into Petri dishes containing 2% MEA (Malt Extract Agar, Biolab) supplemented with 200 mg/L chloramphenicol to suppress bacterial growth. From each third year cone, eight pieces of pith tissue, eight

seeds, eight seed wings and eight pieces of ovuliferous scale were sampled. Seed wings were not sampled for the second and current year seed cones. The current year seed and pollen cones were cut in half and placed, with the cut surface facing the medium, without dissection. All plates were incubated at 23°C for up to 2 weeks. Fast growing, darkly pigmented colonies were transferred to 2% MEA in culture vials and stored at 4°C. These isolates were plated onto water agar, with sterile pine needles on the surface, to induce sporulation. Isolates were identified as *S. sapinea* based on pycnidial and conidial morphology.

Number of S. sapinea infections in cones.

Selection of isolates--Ten, third year mature seed cones with high frequencies of *S. sapinea* recovery were selected for further study. Sixteen *S. sapinea* isolates were selected from each of these cones (four each from pith tissue, seeds, seed wings and ovuliferous scales) to represent a sampling of the entire cone.

Somatic compatibility tests--The 16 *S. sapinea* isolates originating from each of the 10 seed cones were evaluated for their diversity based on somatic compatibility groups (Anagnostakis, 1983). Isolates within each seed cone were paired in all possible combinations on oatmeal agar (OMA) plates. Representative somatic compatibility groups from each cone were further paired with isolates of all other cones.

Oatmeal (60 g/L) was steamed in a water bath at 70 °C for 2 hours with periodic stirring. The solid particles were separated from the liquid by filtration through a double layer of cheesecloth resulting in approximately 600 ml of oatmeal broth. Agar (Biolab) (20 g) was melted in 400 ml distilled water added to the oatmeal broth and the final mixture was autoclaved. The medium was thoroughly mixed prior to dispensing into plastic assay dishes (25 cm square, Nunc). Isolates were placed approximately 1 cm apart on the OMA plates such that all isolates were paired with themselves and with all other isolates. Dishes were incubated at 20 °C in the dark for 4-6 days. Different somatic compatibility

groups were identified based on the presence of barrage lines between incompatible isolates.

Field study

Behavior of latent S. sapinea infections following hail damage.

Field site--During December 1995, a severe hailstorm occurred in the Sabie area, Mpumalanga Province, South Africa. The damage caused by the hail was widespread and uniformly damaged *P. patula* stands were in close proximity to each other. These stands included 5, 14 and 25-year-old trees. Mature seed cones were present in large numbers on the 14 (50-100 cones per tree) and 25-year-old (> 100 cones per tree) trees, but were absent from the 5-year-old trees. In the months following the hailstorm up to March 1996, severe die-back and mortality occurred on trees. Undamaged 13 and 5-year-old stands of *P. patula* in the proximity were used as controls in isolation studies.

Isolations--During March 1996, diseased tissue including dead shoots as well as cankered branches and stems were collected from the three stands of *P. patula*. Isolations were made by placing small pieces of discolored cortex, pith and wood tissues from symptomatic branches and shoots onto the surface of 2% MEA, in Petri dishes. Isolates were identified as representing *S. sapinea* after sporulation.

Tissue colonization--In order to consider the role of latent infections, in seed cones, seven branches with attached cones were collected from hail damaged 14-year-old *P. patula* trees. These branches and attached cones were split and isolations were made from the pith tissues of the seed cone, the cone stipe, the pith tissue continuous with the cone and the branch and the pith tissue of the branch. This isolation procedure was also used on seven branches and attached seed cones of undamaged 13-year-old *P. patula* trees that were collected outside the hail damaged area. In addition, isolations were made from the pith tissue of seven branches of the damaged 5-year-old *P. patula* trees, that did not bear mature seed cones. Only 14-year-old trees were considered due to the fact that this was the only cone bearing age class with comparable undamaged trees available.

Assessment of mortality--Mortality varied greatly among the three age classes of *P. patula* trees damaged by the hail. The trees of all three age classes were in separate plantings in close proximity to one another and thus presented an opportunity to compare the outcome of die-back caused by *S. sapinea* after hail, directly amongst these different age classes. An estimation of the mortality in each age class of trees was done by scoring a block of 100 trees. Trees were scored only as dead or alive during September 1996.

Latent infections in the bark.

Collection of bark samples--During March 1997, healthy intact bark was collected from 5-year-old *P. patula* trees damaged by hail as well as from trees of approximately the same age that were outside the hail area. Fifty bark discs were removed with a sterile cork borer from 10 healthy and 10 hail damaged trees (five per tree). The five bark disk from each tree were sampled randomly from the main stem and branches. These bark discs were placed, with the inner bark facing the medium, on 2% MEA in Petri dishes. The fungal isolates growing from the bark pieces were identified as representing *S. sapinea* using the technique described earlier.

RESULTS

Laboratory Studies

Stage of seed cone development--*Sphaeropsis sapinea* was not recovered from the 40 current year seed or pollen cones sampled. The fungus was recovered from only 20% of the rapidly expanding second year seed cones and from 60% of the mature unopened third year seed cones (Table 1). The presence of *S. sapinea* in second year seed cones was restricted to the outer ovuliferous scales. In contrast, extensive colonization of the entire cone including the seeds, seed blades and cone pith tissue, of the third year seed cones was recorded (Table 1).

Number of S. sapinea infections in cones--*Sphaeropsis sapinea* isolates grew well on OMA and colonies made contact with each other within 2-4 days. Incompatible reactions resulted in dark mycelial barrage lines and were most obvious after approximately 5 days of incubation. After this time mycelial growth became very dense and the reactions were no longer clear. Compatible isolates merged without the formation of any barrage zones. *Sphaeropsis sapinea* was found, without exception, to be present in all 10 sampled seed cones as a single somatic compatibility group. Isolates from each of the 10 seed cones, however, accounted for a different somatic compatibility group.

Field Study

Isolation of the pathogen--*Sphaeropsis sapinea* was consistently isolated from diseased and dead shoots as well as cankers on branches and stems, representing all three *P. patula* age classes. The fungus was consistently associated with die-back symptoms and tree mortality.

Tissue colonization--The pith tissue of seed cones, cone stipes and branches was darkly discolored in the case of the 14-year-old *P. patula* trees damaged by hail (Fig. 1a). *Sphaeropsis sapinea* was consistently isolated from discolored pith tissues of the seed cones, the cone stipes, the pith tissues connecting the cones with the branches and the pith tissue of the branches. In contrast, discoloration of the branch pith in healthy undamaged 13-year-old trees that were outside the hail-damaged area, was absent (Fig. 1b). In this case, *S. sapinea* was present only as latent infections in the seed cone and cone stipe pith tissues. Branches sampled from the hail damaged 5-year-old *P. patula* trees did show canker development caused by *S. sapinea* surrounding hail wounds (Fig. 1c). The pith of these branches was, however, healthy, not discolored (Fig. 1d) and yielded no isolates of the pathogen.

Assessment of mortality--Mortality of hail damaged *P. patula* trees varied greatly among different age classes. The 25-year-old stand had the highest percentage of dead trees (92%), followed by the 14-year-old stand (34%) and the 5-year-old stand (9%). We

believe that the trees scored in each age class were representative of the whole stand and although the sample number was quite small, the differences was very clear.

Latent infections in the bark--The bark discs sampled from both hail-damaged and undamaged 5-year-old *P. patula* trees were rarely infected with *S. sapinea*. From the 100 bark discs sampled, only 16 were infected with the fungus, nine from hail damaged trees and seven from undamaged trees. Hail-damaged trees were thus not more heavily infected than undamaged trees.

DISCUSSION

Results of this study have provided strong evidence that latent infections in mature *P. patula* seed cones, caused by *S. sapinea*, play an important role in post-hail associated die-back, and more specifically in tree mortality. *Sphaeropsis sapinea* appears to infect seed cones during the second year of development when they are actively and rapidly expanding. Our results support those by Peterson (1977), who also found that seed cones tend to be infected early in the growing season during the second year, when cones expand rapidly. Infections during this time are localized in the outer ovuliferous scales. Extensive colonization occurs only during the third year, prior to seed discharge, when cones become mature and inactive.

The fact that *S. sapinea* could not be recovered from current year seed and pollen cones suggests that these organs are not infected in the first year of growth. The reason for this situation is unclear. The sampling date may have had an influence on the time period that the current year cones were on trees, coupled with environmental conditions conducive to conidial discharge and successful infection. Thus, conidia of *S. sapinea* may have been present on the cone surface and destroyed with the flaming procedures followed, prior to isolation. Pollen cones may not be infected because they are short-lived. Second year seed cones are actively expanding and metabolically active, which possibly prevents *S. sapinea* from colonizing the cones extensively. The active defense mechanisms in such

actively growing tissue would presumably inhibit infections, so that they remain discrete and localized.

The presence of *S. sapinea* in mature cones as a single somatic compatibility group per cone has three possible explanations. It could be argued that seed cones of *P. patula* were infected repeatedly during active expansion, but that these infections have a low rate of successful establishment. Alternatively, it could be that a single seed cone is successfully infected by a number of conidia belonging to the same vegetative compatibility group or that a unique infection event by only one conidium per cone takes place. The 10 seed cones sampled for the somatic compatibility group studies were, however, distributed throughout a stand of trees and *S. sapinea* isolates from each seed cone belonged to a different somatic compatibility group. It was also recently found that although *S. sapinea* is an introduced fungus in South Africa, it has a broad genetic base with many somatic compatibility groups in the Sabie area population (Smith *et al*, 2000). We, therefore, believe that repeated infections with a low rate of success are responsible for the presence of latent infections caused by *S. sapinea* in seed cones.

The colonization of the third year seed cones by *S. sapinea* is restricted to the different cone parts and the pith tissue of the cone stipe. The branch pith tissue of healthy undamaged 13-year-old *P. patula* trees was found not to be infected or colonized by *S. sapinea*. The pith tissues of third year mature seed cones as well as the pith tissues of seed cone stipes are metabolically inactive and brown (Fig. 1b). In contrast, the pith tissue of healthy branches is metabolically active (Esau, 1953) and green. The latent infection and colonization by *S. sapinea*, of third year mature unopened seed cones of *P. patula*, is thus confined to the inactive pith tissue of the third year mature seed cones, when trees are healthy and not stressed.

The colonization of branch pith tissue by *S. sapinea* changes after trees are stressed by environmental factors such as hail. The discoloration of the branch pith of hail-damaged 14-year-old trees was found to be extensive. Hail invariably stressed the affected trees to

such an extent that the latent *S. sapinea* infections inside the pith tissue of third year seed cones were activated to spread into the pith tissue of the branches. The fungus thus appeared to overcome the defense barriers in healthy branch pith tissues and to colonize these tissues rapidly and extensively. At this point we can only speculate that environmental stress may have a profound impact on the efficiency of defense mechanisms (Woodward, 1992). In the case of environmental stress such as severe hail, *S. sapinea* is apparently able to colonize branch and stem pith tissues both proximal and distal to the points of infection. Here, these points of infection are primarily previously infected third year seed cones.

Branch pith colonization by *S. sapinea* appears to occur rapidly (Haddow & Newman, 1942; Marks & Minko, 1969). This would be facilitated considerably by the fact that the fungus is already prevalent in the pith tissue of the seed cones and the seed cone stipes. The pith tissue is largely comprised of parenchyma cells that are virtually devoid of chloroplasts in mature tissue. In older branches the cells are mostly empty and may act as accumulation sites for crystals and tannins. (Esau, 1953). The presence of large intracellular spaces in this tissue (Esau, 1953) may explain the rapid rate at which it becomes colonized by *S. sapinea.*

Observations from this study suggest strongly that tree susceptibility due to *S. sapinea* infections and colonization after hail, increases as trees age. We believe that the extremely high mortality seen in the 25-year-old stand of *P. patula* is the direct result of extensive branch pith colonization by *S. sapinea*, which originates from the numerous third year seed cones on these older trees. The mortality of *P. patula* trees appears to be related to the large number of third year seed cones on older trees. This is in contrast to lower levels of mortality on young trees that do not carry mature seed cones.

Sphaeropsis sapinea was consistently associated with die-back symptoms as well as with branch and stem cankers of three age classes of *P. patula* damaged by hail. This was also true for 5-year-old *P. patula* trees that showed extensive die-back, often with more than

half of the main stem that had died. In younger trees the mortality was, however, very low. The symptoms observed during this study are consistent with those reported for crown wilt (Chou, 1984; Haddow & Newman, 1942; Palmer, 1991) as well as branch and stem cankers (Palmer, 1991) caused by *S. sapinea* wound infections. We believe that infections of hail wounds on young trees that do not carry mature seed cones leads to crown wilt, with tree parts above the infection site dying off, but that these infections rarely result in tree death.

Colonization of the pith by *S. sapinea* moves down from a point of infection and subsequent canker development (Chou, 1984; 1987). This observation was confirmed in our study. Isolations from the pith tissue of branch segments from hail damaged 5-year-old *P. patula* trees, revealed that this tissue is not colonized by *S. sapinea*, while healthy. These branch segments sampled were from the living lower parts of branches that had died back. Hail wounds on the bark of these branch segments had developed into cankers infected with *S. sapinea*, but the pith tissue was unaffected at the time of sampling. Hail wounds on dead and living branch segments were, however, of the same age and may have been infected by *S. sapinea* simultaneously. The fact that resistance to *S. sapinea* infection and disease development increases with wood and bark age (Chou, 1987), lower on stems and branches, may explain why these trees did not die back indefinitely. Die-back was halted at a specific stage and trees started to recover and produced new growth as early as August, during the spring of 1996.

Despite the fact that *S. sapinea* is known to infect hail wounds (Van der Westhuizen, 1968; Brown *et al.*, 1981; Swart *et al.*, 1987; Zwolinski *et al.*, 1990), we have found that the fungus also persists in asymptomatic bark of *P. patula* trees. The accepted dogma that hail wounds are infected by conidia of *S. sapinea*, and that this leads to rapid tree death is in our view, an oversimplification of the actual course of events. Latent *S. sapinea* infections in the bark probably become active after the onset of environmental stress factors such as hail. This would enable the fungus to colonize the damaged tissue surrounding such wounds. *Sphaeropsis sapinea* was found to occasionally cause lesions

around control inoculation wounds in artificial inoculation trials conducted on *P. radiata* and *P. patula* (FW Wolfaardt, Department of Microbiology and Biochemistry, University of the Free State, P.O. Box 339, Bloemfontein 9300, South Africa, personal communication). These lesions may have been the result of latent infections of *S. sapinea* present in the bark of these trees.

Sphaeropsis sapinea sporulates prolifically on cones, after they have opened and discharged seeds (Haddow & Newman, 1942; Slagg & Wright, 1943; Laing & Chi, 1980; Peterson, 1981; Chou, 1984; Johnson *et al.*, 1985; James *et al.*, 1991). The inoculum produced on these retained cones plays an important role in the survival of the fungus. The conidia produced on these cones may infect current year shoots (Johnson *et al.*, 1985) and also the second year seed cones. Seed cones thus appear to play an important role in the persistence and survival of *S. sapinea* on pine trees. It is our view that breeding programs aimed at selection for female sterility may lead to a significant reduction in damage caused by *S. sapinea*.

Results of this study suggest strongly that post-hail associated die-back of *P. patula* may result from two distinct forms of infection. Extensive die-back and high levels of mortality found in older trees bearing large numbers of mature third year seed cones. Alternatively, low mortality and recovery of young trees is found where cones are absent or in low numbers.

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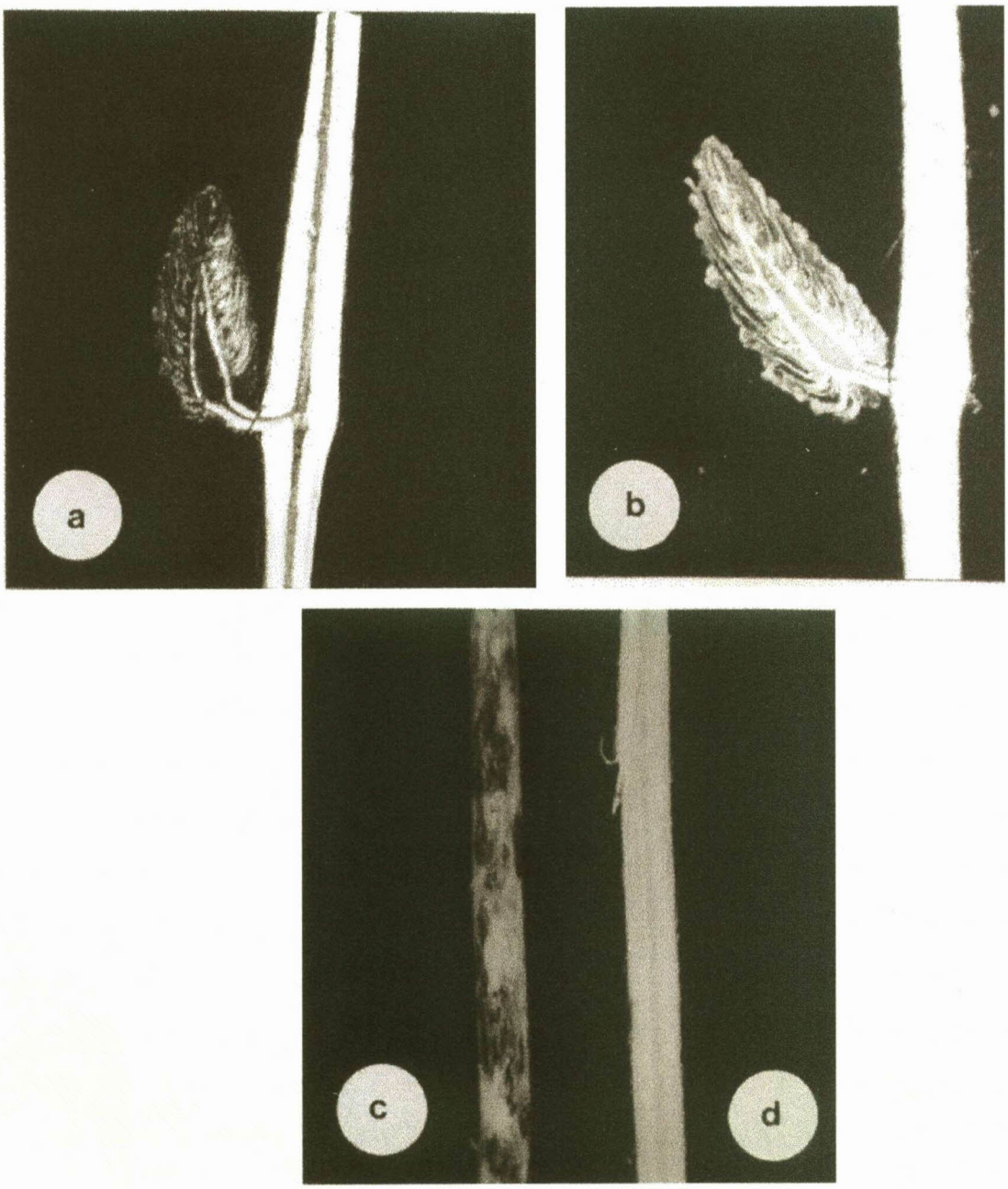
Table 1. Presence of *S. sapinea* in different tissues of three age classes of *P. patula* seed cones and current year pollen cones.

Cone age	% cones infected	% Cone tissue infected with *S. sapinea* [1/]			
		seed	seed blades	pith tissue	ovuliferous scales
Current year (pollen)	0	-	-	-	-
Current year (seed)	0	0	0	0	0
Second year (seed)	20	0	0	0	2.5
Third year (seed)	60	23	22	58	19

[1/] 30 cones, 240 seeds (8 / cone), 240 seed blades (8 / cone), 240 pith tissues (8 / cone) and 240 ovuliferous scales (8 / cone) were sampled.

Figure 1. (a) Darkly discolored branch and seed cone pith from a hail-damaged 14-year-old *P. patula* tree. (b) Absence of branch pith discoloration from a healthy 13-year-old *P. patula* tree. (c) Canker caused by *S. sapinea*, surrounding a hail wound. (d) Healthy non-discolored branch pith from a hail-damaged 5-year-old *P. patula* tree.


a
b
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d

CHAPTER 7

Relative susceptibility of northern and southern provenances of *Pinus greggii* to infection by *Sphaeropsis sapinea*

Submitted for publication as: Smith, H., Wingfield, M.J., Wolfaardt, F. W. & Coutinho, T.A. (2001). Relative susceptibility of northern and southern provenances of *Pinus greggii* to infection by *Sphaeropsis sapinea*. *Forest Ecology and Management*. (Accepted).

Relative susceptibility of northern and southern provenances of *Pinus greggii* to infection by *Sphaeropsis sapinea*

Sphaeropsis sapinea is a well-known pathogen of *Pinus* spp. that causes severe die-back, in South Africa. In this study, 65 families representing both the northern and southern populations of *P. greggii* were evaluated for their tolerance to infection and subsequent die-back caused by *S. sapinea*. Families were evaluated for tolerance following natural infection after hail damage, as well as through inoculation. Variation in tolerance of trees after natural infections, occurred amongst families of both the northern and southern provenances, but highly significant differences in tolerance were observed between the two provenances. *Pinus greggii* trees of the southern provenances were significantly more susceptible to natural infection after hail damage. Artificial infection was not suitable for prediction of susceptibility to natural infections after hail damage. These observations have significant practical implications for plantation establishment in South Africa.

The search for *Pinus* species that are able to grow well on marginal sites and thus to sustain the development of commercial plantation forestry (Dvorak *et al.*, 1996), has recently focused on species such as *Pinus greggii* Engelm. *Pinus greggii* is a member of the closed cone pine group and occurs in two distinctly separate locations in central and northern Mexico between latitudes 20° to 26° N (Dvorak *et al.*, 1996). The central or southern distribution of *P. greggii* is variable and includes environments ranging from those with high rainfall (1600 mm annually) and good soils to sites with rocky shallow soils and low rainfall (800 mm annually). These areas overlap with the distribution range of the closely related *Pinus patula* Schl. & Cham. In contrast, the northern distribution of *P. greggii* is much less variable and generally drier (650 mm annual precipitation) and colder (Dvorak *et al.*, 1996). *Pinus* species growing at high altitude (1200 – 2800 m) and in areas with low annual precipitation, are most desirable for commercial planting in marginal areas with elevated sites in the tropics and subtropics. This has consequently led to the establishment of *P. greggii* provenance trials in South Africa, Colombia and Brazil by the Central American and Mexico Coniferous Resources Cooperative (CAMCORE) (Dvorak *et al.*, 1996).

Provenance trials of *P. greggii* established by CAMCORE have incorporated seed collections from the most complete distribution of this pine species in natural stands. These include a wide spectrum of genetic variability within the species. Results from these trials have indicated significant differences in growth rates (Dvorak *et al.*, 1996), morphology (Donahue & Lopez-Upton, 1996) and terpene composition (Donahue *et al.*, 1997). The hypothesis that the northern and southern provenances of *P. greggii* might represent different species has, however, not been fully tested and further genetic studies are required to consider this question completely.

A *P. greggii* provenance trial was established on the farm In de Diepte, Mpumalanga Province, South Africa (25° S, 1200 mm annual precipitation, 2150 m altitude) in 1992. During December 1995, trees in this trial were severely damaged by hail. The hail damaged the trees represented in the trial uniformly and extensive die-back was evident as early as March 1996. *Sphaeropsis sapinea* (Fr.:Fr.) Dyko & Sutton was found to be the causal agent of die-back symptoms observed on all pine species present in the trial.

Sphaeropsis sapinea is the most important pine pathogen in South Africa and causes extensive die-back of *P. patula* and *Pinus radiata* D. Don (Swart *et al.*, 1985, 1987 a,b; Zwolinski *et al.*, 1990 a,b) after hail damage. *Pinus elliottii* and *P. taeda* are relatively tolerant to infection and subsequent die-back caused by *S. sapinea* (Slagg & Wright, 1943; Bega *et al.*, 1978; Swart *et al.*, 1988). Dvorak *et al.*, (1996) noted that *P. greggii* appeared to be more tolerant to post-hail associated die-back, caused by *S. sapinea*, than was *P. patula* but did not elaborate on this hypothesis.

The aim of this study was to assess differences in relative susceptibility of the southern and northern provenances of *P. greggii* to post-hail associated die-back caused by *S. sapinea.* Comparisons were made with *P. patula*, which is known to be highly susceptible and *P. elliottii* and *P. taeda* that are resistant to infection by *S. sapinea.* Branch inoculations were also used as a measure of predicting susceptibility or tolerance of the pine species to infection and thus post-hail associated die-back caused by *S. sapinea.*

MATERIAL & METHODS

Experimental layout--The trial was planted in a compact family design with open-pollinated families from each provenance grouped together within each of four replications. Six-tree plots in rows represented each family in every replication. Spacing between trees was 3.0 m x 3.0 m. The trial included 13 families from the southern and 51 families from the northern provenances of *P. greggii* and controls of *P. patula* (3 seed lots), *Pinus elliottii* Engelm. (1 seed lot) and *Pinus taeda* L. (2 seed lots). The southern provenances originated from Laguna Seca (6 families) and Laguna Atezca (1 family) in Hidalgo and El Madroño (6 families) in Queretaro. The northern provenances originated from La Tapona (18 families), Ojo de Agua (16 families), Las Placetas (2 families) and Cerro El Potosi (2 families) in Nuewo Leon and Jamé (7 families) and Cañon Los Lirios (6 families) in Coahuila (Table 1).

Natural die-back--The extent of natural die-back of all pine species represented in the trial was measured, three months after the hail damage, during March 1996. At this time, trees started to regenerate, thus assuming the start of re-growth and the end of active die-back. Die-back was measured as the difference between tree height and the height of the first living branch. Measurements were taken of all trees in the trial and to the nearest 0.01 m.

Isolations were made by dissecting tissue pieces from lesion margins of branches and stems that showed die-back symptoms (50 randomly selected trees from trial site). These tissue pieces were placed on 2% malt extract agar (MEA, Biolab) in Petri dishes and incubated at 25 °C for up to two weeks. Dark gray to black isolates were transferred to water agar with sterile pine needles (WA, Biolab). Pycnidia of *Sphaeropsis sapinea* formed on needles and the fungus was recovered from more than 90% of tissue pieces sampled in this way.

Inoculations--Replication one of the provenance trial was used in inoculation studies. Six trees, from each of the families of *P. greggii* as well as controls on *P. patula*, *P. elliottii* and *P. taeda*, were inoculated with an isolate of *S. sapinea* (CMW 1184, PREM 48859) that had previously been shown to be virulent in pathogenicity tests. The isolate was grown on 2% MEA in Petri dishes for 5 days prior to inoculation. Inoculations were made during March 1996 (autumn) and repeated in September 1996 (spring). Inoculations were done on the

undamaged bark of branches (approximately 3 cm diameter) minimally damaged by the hail. One branch was inoculated per tree. A cork borer (8 mm diameter) was used to remove bark discs. Bark discs were replaced by agar discs of the same size on which *S. sapinea* was growing. The wounds were sealed with masking tape to restrict desiccation. The lengths of the lesions that had formed in the cambium were measured after four weeks.

Statistical analysis--Analyses of variance was computed for the height of natural die-back and lesion length. Differences amongst families and within provenances were tested for significance using Tukey's procedure for the comparison of means ($p \leq 0.05$). The mean susceptibility of provenances of *P. greggii* was compared by contrast testing with *F*-distribution ($p \leq 0.05$) (Mead & Curnow, 1983). The effectiveness of using inoculations to predict susceptibility in pines to post-hail associated die-back was tested by using Spearman's coefficient of rank correlation (Ostle & Malone, 1994).

RESULTS

Natural die-back--The three southern provenances of *P. greggii* differed significantly from one another ($p \leq 0.05$) (Table 2), with trees from Laguna Atezca showing the largest degree of die-back (mean = 202.6 cm, $s^2 = 0.00$), followed by those of El Madroño (mean = 193.1 cm, $s^2 = 7.25$) and Laguna Seca (mean = 140.9 cm, $s^2 = 11.32$). There were no significant differences in natural die-back, caused by *S. sapinea*, amongst *P. greggii* families from the southern provenances of Laguna Atezca in Hidalgo and El Madroño in Queretaro (Table 2). Family 24 of the El Madroño provenance showed the most severe die-back of all families of the southern provenances (family mean = 222.1 cm, $s^2 = 32.45$) and family 102 of the Laguna Seca provenance was the least severely damaged (family mean = 108.9 cm, $s^2 = 20.04$). Significant differences occurred amongst families of the Laguna Seca provenance, with family 108 showing significantly more die-back than families 102 and 103 (Table 2). All southern provenances had significantly ($p \leq 0.05$) more die-back than the *P. taeda* (mean = 79.6 cm, $s^2 = 6.33$) and P. elliottii (mean = 12.7 cm, $s^2 = 0.00$) seed sources, included as positive controls for tolerance to *S. sapinea* infections. No significant difference ($p \leq 0.05$), however, existed between southern provenances and the *P. patula* seed sources (mean =

148.8, s^2 = 6.33), that were included as positive controls for susceptibility to S. sapinea infection.

The nine northern provenances of *P. greggii*, did not differ significantly from one another in terms of die-back ($p \leq 0.05$) (Table 2). The provenance Cañon Los Lirios showed the highest degree of die-back (mean = 30.9 cm, s^2 = 1.03) followed by Jamé (mean = 29.3 cm, s^2 = 6.03), Las Placetas (mean = 28.9 cm, s^2 = 2.59), Ojo de Agua (mean = 24.4 cm, s^2 = 5.19), La Tapona (mean = 23.3 cm, s^2 = 7.13) and Cerro El Potosi (mean = 18.0 cm, s^2 = 3.06). There were no significant differences in die-back amongst families belonging to the provenances of Las Placetas, Cerro El Potosi and Ojo de Agua from Nuevo Leon as well as Cañon Los Lirios from Coahuila. The northern provenance family that showed the most severe die back was family 151 from the La Tapona provenance (family mean = 66.2 cm, s^2 = 16.90) whereas family 135 from the Ojo de Agua provenance (family mean = 6.4 cm, s^2 = 4.53) showed the least die-back. The families with the most severe die-back were from the Jamé provenance (families 95 & 92) and the La Tapona provenance (families 151 & 163). Trees in these families did not exhibit significant differences from *P. taeda* seed sources, but showed significantly less die-back than the *P. patula* seed sources (Table 2). Die-back of trees of northern provenances of *P. greggii* did not differ significantly ($p \leq 0.05$) from those of the *P. elliottii* seed source, but showed significantly ($p \leq 0.05$) less die-back than both the *P. taeda* and *P. patula* seed sources. Trees from northern provenances of *P. greggii* (overall mean = 21.9 cm, s^2 = 11.28) had significantly less die-back than trees of southern provenances (overall mean = 169.7 cm, s^2 = 21.44) after hail and subsequent *S. sapinea* infection.

Inoculations--No significant differences ($p \leq 0.05$) in lesion development were evident amongst families from the southern as well as families from the northern provenances of *P. greggii* and controls at either inoculation date (Table 3). The only exception was trees from the southern provenance Laguna Atezca that developed significantly larger lesions than all other provenances. This provenance, however, is comprised of only one family, that may explain the discrepancy. Northern provenances showed a tendency to produce smaller lesions, following inoculation with *S. sapinea*.

The low values of Spearman's ranking correlation coefficients (March inoculation $r_s = 0.318$; September inoculation $r_s = 0.252$) indicates that a weak relationship exists between the natural *S. sapinea* induced die-back following hail damage and lesion development following inoculation of branches. The fact that 90% (March inoculation) and 94% (September inoculation) of the differences observed between natural die-back and inoculations are unexplained (March inoculation $r_s^2 = 0.101$; September inoculation $r_s^2 = 0.064$) indicate that inoculation of branches is not a good parameter in the prediction of the tolerance of pine species, provenances or families to infection by *S. sapinea* following hail damage. This point may be best illustrated by the ranking discrepancies of the *P. elliottii* trees that were included for comparison due to their known tolerance to *S. sapinea* infection. Amongst the 70 families of *P. greggii*, *P. elliottii*, *P. taeda* and *P. patula* included in this trial, *P. elliottii* was ranked 61st on a scale of tolerance to natural die-back (1st = most susceptible). In contrast, it was ranked 7th (March inoculation) and 11th (September inoculation) respectively on a scale of lesion development in inoculation trials (1st = largest lesion developed).

DISCUSSION

Trees of *P. greggii* representing the provenances from central (southern) and northern Mexico could easily be distinguished from one another based on their susceptibility to *S. sapinea* infection after hail. Trees of the southern provenances were significantly more susceptible to infection by the pathogen and subsequently displayed more dramatic die-back than those of the northern provenances. The differences in *S. sapinea* associated die-back observed between the southern and northern provenances of *P. greggii* are perhaps not surprising given the fact that these trees differ notably in morphology (Donahue & Lopez Upton, 1996), growth (Dvorak *et al.*, 1996) and terpene chemistry (Donahue *et al.*, 1997).

The susceptibility of southern as opposed to northern provenances of *P. greggii* to *S. sapinea* infection after hail is an important characteristic, when selecting provenances with potential for commercial planting or hybridization programmes in South Africa. Summer rainfall areas frequently have hail-storms that cause devastating losses due to die-back caused by S. sapinea (Swart *et al.*, 1987 a,b, Zwolinski *et al.*, 1990 a,b). *Sphaeropsis sapinea* is the most important pathogen of pines in South Africa. It is thus an important discovery that some

forms of *P. greggii* are tolerant to this pathogen, and this will have important implications for the Forestry Industry.

Pinus greggii is highly desirable due to its tolerance to altitude and drought. It is thus a major advantage that this species can hybridize with *P. patula*, the most important Pinus sp. planted in South Africa. There is thus an opportunity to breed for drought tolerance and high altitude adaptation in *P. patula*, through hybridization with *P. greggii*. The problem, however, is that *P. patula* is very susceptible to *S. sapinea* infection. In our opinion there is tremendous potential for hybridization of *P. patula* and the northern provenances of *P. greggii* to obtain *S. sapinea* tolerance.

Using inoculation studies, we were able to determine the relative susceptibility of northern and southern provenances of *P. greggii* to *S. sapinea*. Northern provenances were not significantly more tolerant to inoculations with *S. sapinea* but showed a tendency to form smaller lesions, indicating similarities with die-back after hail. This technique could, however, not distinguish the tolerance of different families within provenance. The failure of this technique to predict response of different families under natural conditions is amply illustrated by the low Spearman's ranking coefficients obtained. This insensitivity may be reduced by increasing the period from inoculation to lesion measurement to more than 4 weeks. Pinus elliottii was extremely tolerant to natural post-hail associated die-back but showed significant lesion development after artificial inoculation. *Pinus elliottii* is widely regarded to be tolerant to infection by *S. sapinea* (Slagg & Wright, 1943; Bega *et al.*, 1978; Swart *et al.*, 1988). The reason why this species displayed susceptibility in these trees is unclear and deserves further study.

There exists a strong possibility that the use of the northern provenances of *P. greggii* in hybridization programs with *P. patula*, will lead to increases in tolerance to S. sapinea infection. This, however, will be a slow process and the material produced will need to be thoroughly tested under field conditions before definitive predictions can be made. The results obtained in this study may, however, vary as a result of intraspecific variation within *S. sapinea* (Swart *et al.*, 1987b) populations from different parts of the world.

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Table 1. Location of provenances of *Pinus greggii* in Mexico used in this study.

Provenances	Latitude – Longitude[a/]	Elevation (m)[a/]
SOUTHERN REGION:		
Laguna Seca, Hidalgo	21°02' N – 99°10' W	1670 - 1830
El Madroño, Queretaro	21°16' N – 99°10' W	1650 - 1730
Laguna Atezca, Hidalgo	20°49' N – 98°46' W	1250 – 1420
NORTHERN REGION:		
Jamé, Coahuila	25°21' N – 100°37' W	2500 - 2590
Las Placetas, Nuevo Leon	24°55' N – 100°11' W	2370 - 2520
Cañon Los Lirios, Coahuila	25°22' N – 100°29' W	2260 - 2460
Cerro El Potosi, Nuevo Leon	24°54' N – 100°12' W	2430 - 2500
Ojo de Agua, Nuevo Leon	24°53' N – 100°13' W	2115 - 2400
La Tapona, Nuevo Leon	24°43' N – 100°10' W	2090 - 2350

[a/](Dvorak *et al.*, 1996)

Table 2. Mean die-back of families of *Pinus greggii* and controls, caused by *Sphaeropsis sapinea* after hail.

Family	Provenance / Selection	Average die-back (cm)[1/]
24	El Madroño	222.1 a
35	Laguna Atezca	202.6 ab
9	El Madroño	198.2 ab
11	El Madroño	189.7 abc
7	El Madroño	186.4 abcd
16	El Madroño	185.7 abcd
108	Laguna Seca	180.8 abcde
12	El Madroño	176.3 abcdef
999	*P. patula* 2nd generation orchard	166.2 bcdef
105	Laguna Seca	156.7 bcdefg
997	*P. patula*	141.1 cdefgh
998	*P. patula* 1st generation orchard	139.0 defgh
106	Laguna Seca	135.4 efgh
100	Laguna Seca	134.5 efgh
103	Laguna Seca	129.3 fgh
102	Laguna Seca	108.9 ghi
995	*P. taeda* 2nd generation mix	92.2 hij
996	*P. taeda* Texas origin	66.9 ijk
151	La Tapona	66.1 ijkl
95	Jamé	50.0 jklm
92	Jamé	43.3 jklm
131	Ojo de Agua	43.3 jklm
163	La Tapona	41.4 klm
138	Ojo de Agua	40.8 klm
136	Ojo de Agua	39.4 klm
153	La Tapona	37.1 klm
147	La Tapona	34.3 klm
89	Cañon Los Lirios	34.2 klm
66	Las Placetas	34.0 klm
85	Cañon Los Lirios	33.0 klm
145	Ojo de Agua	31.9 klm
82	Cañon Los Lirios	31.0 klm
166	La Tapona	30.8 klm
93	Jamé	30.2 klm
79	Cañon Los Lirios	29.8 klm
81	Cañon Los Lirios	29.4 klm
148	La Tapona	28.9 klm
77	Cañon Los Lirios	28.2 klm
157	La Tapona	28.2 klm
119	Ojo de Agua	28.0 klm
143	Ojo de Agua	28.0 klm
126	Ojo de Agua	27.4 klm

Table 2. continue

Family	Provenance / Selection	Average die-back (cm)
128	Ojo de Agua	26.8 klm
98	Jamé	25.7 klm
48	Cerro El Potosi	24.2 klm
97	Jamé	24.0 klm
65	Las Placetas	23.7 klm
144	Ojo de Agua	20.3 klm
167	La Tapona	20.2 klm
150	La Tapona	20.2 klm
146	La Tapona	19.5 klm
121	Ojo de Agua	19.5 klm
127	Ojo de Agua	18.3 klm
123	Ojo de Agua	18.3 klm
155	La Tapona	18.2 klm
125	Ojo de Agua	16.9 lm
90	Jamé	16.3 m
154	La Tapona	16.0 m
91	Jamé	15.8 m
118	Ojo de Agua	15.0 m
169	La Tapona	13.8 m
152	La Tapona	13.2 m
994	*P. elliottii* 2nd generation orchard	12.7 m
156	La Tapona	12.0 m
47	Cerro El Potosi	11.9 m
164	La Tapona	10.8 m
142	Ojo de Agua	10.6 m
149	La Tapona	10.1 m
135	Ojo de Agua	6.4 m
159	La Tapona	5.6 m

[1/]Means in a column followed by the same letters are not significantly different from each other ($p \leq 0.05$).

Table 3. Means of natural die-back (cm) and lesion length (mm) observed in *Pinus greggii* provenances from the southern and northern regions as well as control species.

Provenance	Natural die-back[1/]	Inoculation[1/]	
		March	September
SOUTHERN REGION:			
Laguna Atezca	202.6 a	21.7 a	15.5 a
El Madroño	193.1 b	17.1 b	15.0 a
Laguna Seca	140.6 c	14.2 b	23.5 a
NORTHERN REGION:			
Cañon Los Lirios	30.9 e	10.1 b	16.8 a
Jamé	29.3 e	7.2 b	14.8 a
Las Placetas	28.9 e	8.2 b	17.5 a
Ojo de Agua	24.4 e	6.1 b	13.2 a
La Tapona	23.7 e	6.4 b	13.7 a
Cerro El Potosi	18.0 e	13.0 b	17.9 a
CONTROL SPECIES			
Pinus patula	148.8 c	17.5 b	21.7 a
Pinus taeda	79.6 d	12.4 b	18.4 a
Pinus elliottii	12.7 e	14.2 b	19.5 a

[1/]Means in columns followed by the same letter do not differ significantly from each other (contrast testing $p \leq 0.05$). Contrast testing were used to compare provenances with one another despite the different number of families contributing to each provenance.

CHAPTER 8

Cytospora and *Cytospora*-like fungi from *Eucalyptus* species and their phylogenetic relationships based on DNA sequence homologies

Submitted for publication as: Adams, G., Smith, H. Roux, J. & Wingfield, M.J. (2001). *Cytospora* and *Cytospora*-like fungi from *Eucalyptus* species and their phylogenetic relationships based on DNA sequence homologies. *Plant Pathology* (Accepted).

Cytospora and _Cytospora_-like fungi from _Eucalyptus_ species and their phylogenetic relationships based on DNA sequence homologies

Cultures of *Cytospora* isolated from *Eucalyptus* trees in South Africa, Congo, Thailand, Venezuela, Mexico, Uganda and Australia, as well as *Cytospora*-like isolates from Indonesia were compared based on the homology of the internal transcribed spacer regions and the 5.8S ribosomal DNA of the nuclear ribosomal DNA repeat unit. Phylogenetic analysis clustered isolates of *Cytospora* from *Eucalyptus* into at least three unrelated groupings. A fourth grouping included isolates that morphologically resembled *Cytospora* in culture except for the structure of the walls of the pycnidia. These *Cytospora*-like cultures were identified as *Phomopsis* species based on sequence homology. The cultures formed alpha conidia that were significantly shorter than *Phomopsis* species previously described from *Eucalyptus*. Genetic variation in DNA sequence was high among and within the three groupings of *Cytospora* from *Eucalyptus*. One grouping included isolates of the *Valsa ceratosperma* teleomorph from Uganda and isolates others have described as *V. ceratosperma* from Australia. The grouping was not related to isolates of *V. ceratosperma* from the Congo. Sequences for *V. ceratosperma* on hosts other than *Eucalyptus* were not homologous to any of the isolates from *Eucalyptus*. Results of this study show that the current description of *V. ceratosperma* encompasses several distinctly different species.

Cytospora eucalypticola van der Westhuizen was first described as a pathogen causing cankers and death of *Eucalyptus saligna* Sm. in Tzaneen, Northern province, South Africa (van der Westhuizen, 1965a, b). The disease continues to be reported in this area, particularly on seedlings of *E. grandis* A.W. Hill ex Maiden under closed canopies. In South Africa, *Eucalyptus* species, hybrids and clones occasionally suffer *Cytospora* bark lesions, branch dieback and stem cankers following stresses. For example, plantations of *E. dunnii* Maiden can collapse from *Cytospora* stem canker following combined drought and frost injury. *Cytospora* occurs on stems of young hybrid *E. grandis* clones suffering from bacterial wilt or root rot diseases, termite damage or machete wounds. The

teleomorph is seldom present but has been found abundant on bark lesions at the bases of *E. grandis* X *E. tereticornis* Sm. hybrids in the Congo following fire injury (Roux *et al*, 2000) and on *E. grandis* in Ugandan wetlands. Cytospora canker is also an occasional disease wherever *Eucalyptus* is grown in commercial plantations in the southern hemisphere, including Australia (Fraser & Davison, 1986; Davison & Tay, 1983), Africa, Indonesia, Thailand, South and Central America (MJW unpublished) and rarely in the northern hemisphere in California (Cooke & Harkness, 1881), Florida (Alfieri *et al*, 1994), Japan (Old *et al*, 1991), and the former Soviet Union (Gvritishvili, 1982). Additionally, *C. eucalypticola* is an endophyte in xylem in branches and leaves of *Eucalyptus* (Smith *et al*, 1999; Bettucci & Saravay, 1993; Fisher *et al*, 1993).

Old *et al* (1991) considered that the *Cytospora* species on *Eucalyptus* in Australia agreed with the description of *C. eucalypticola* while the teleomorphs in Australia and Japan resembled *Valsa ceratosperma* (Tode:Fr.) Maire [anamorph = *C. sacculus* (Schwein.) Gvrit.]. He suggested retaining the anamorph name *C. eucalypticola* rather than *C. sacculus* because uncertainty remained as to whether the *Cytospora* and *Valsa* from *Eucalyptus* in Australia were the same as those from *Eucalyptus* in Japan and elsewhere in the Northern Hemisphere. The *C. eucalypticola* from South Africa and Australia had small narrow conidia (3-4 X 0.7-1.0 μm), unbranched conidiophores and locules of variable size and irregular arrangement in the pycnidium (van der Westhuizen, 1965a; Old *et al*, 1991). The *Cytospora* on *Eucalyptus* in Japan had small narrow conidia but locules were uniform in size and arranged radially. Additionally, they differed consistently and distinctly in culture characteristics (light brown with sparse fruiting) from the Australian and South African isolates (dark olivaceous with abundant fruiting) (Old *et al*, 1991). *Cytospora australiae* Spregazzini (synonym *C. eucalyptina* Spregazzini) also described from *Eucalyptus* had longer and wider conidia (4-6 X 2-3 μm) and regular radially arranged locules (van der Westhuizen, 1965a). *Valsa* fruiting bodies from *Eucalyptus* in Australia and Japan were similar to *V. ceratoperma* from other hosts (Old *et al*, 1991; Kobayashi, 1970). The teleomorphs from *Eucalyptus* in the U.S.S.R. were also described as *V. ceratosperma* by Gvritishvili (1982) but those in

California and Florida were described as *Valsa eucalypti* M.C. Cooke & Harkness (Cooke & Harkness, 1881; Alfieri *et al*, 1994). The latter may be a *Leucostoma* species (Spielman, 1985). *Valsa ceratosperma*, in the studies of Old *et al* (1991), varied considerably in ascospore size on different continents. Australian specimens from *Eucalyptus* had spores that ranged from 7-8 X 1.5-1.8 μm, compared to Japanese specimens at 5.6-7 X 1.8-2.0 μm. Spores of *V. ceratosperma* from other hosts in Japan ranged from 5.5-9.0 X 1.0-2.0 μm whereas those from England had a mean of 10.5 X 2.5 μm. The breadth of the size range seems too liberal to precisely circumscribe a species of *Valsa*.

Phomopsis species may resemble *Cytospora* species in culture and sometimes in nature because of the common absence of beta conidia (ß-conidia), particularly if alpha conidia (α-conidia) are allantoid. *Phomopsis eucalypticola* K.M. Old & Z. Q. Yuan (teleomorph = *Diaporthe eucalypticola* K.M. Old & Z. Q. Yuan) was described as an opportunistic colonizer of stem wounds of *Eucalyptus* spp. in Australia (Yuan *et al*, 1995). The anamorph formed long necked pycnidia with ellipsoid hyaline one-celled two-guttulate α-conidia, 5-7.5 X 2-3.0 μm; ß-conidia generally were not present in culture. *Diaporthe eucalypti* Harkness (= *D. medusaea* Nitschke, anamorph *Phomopsis rudis* (Fr.) Höhnel) has been described from *Eucalyptus* leaves in California (Harkness, 1884) with α-conidia, 7.3 X 2.4 μm (Kobayashi, 1970). The two species differ primarily in ascospore shape, ascospore appendages, size of ß-conidia and the erumpent nature of the necks (Yuan *et al*, 1995). A different species with α-conidia of similar size but larger ß-conidia, *Phomopsis eucalypti* Zerova, has been reported to cause a severe leaf disease of seedlings on several species of *Eucalyptus* (Mohanan & Sharma, 1987).

Testing isolates of *Cytospora* and *Phomopsis* from *Eucalyptus* spp. for pathogenicity and host range has not been accomplished because disease is not readily induced (Old *et al*, 1986; Old & Kobayashi, 1988; Shearer *et al*, 1987; Yuan *et al*, 1995). It is not unusual to experience difficulty in inducing disease with pathogenic endophytes like *Cytospora* and *Phomopsis* species (Schoeneweiss, 1983). For example, to initiate *Cytospora* canker

formation on *Populus* spp., the moisture content of the bark and xylem (whole cutting) must be reduced to approximately 30 % of saturation in the living branch (Bloomberg, 1962). Following initiation of disease, canker growth can then cease when the water potential changes to levels more favorable to plant growth.

Characters other than morphology and pathogenicity are needed to delineate *Cytospora* and *Cytospora*-like pathogens of *Eucalyptus* into species. Host specificity is not a verifiable characteristic for this genus and the anamorphs vary distinctly in certain collections, while those formed in nature differ considerably from those formed in culture. Teleomorphs are rare, and the morphology of the teleomorphs often is not distinguishing at the species level. DNA sequence can provide a large number of characters to use to assess the relationships among isolates and species of *Cytospora* and *Cytospora*-like pathogens. The large number of characters also permits the statistical testing of hypothesized phylogenetic relationships among these organisms.

Cytospora spp. are commonly isolated from disease symptoms on *Eucalyptus*. Where material is stored, this is generally in the form of cultures and voucher specimens with fruiting structures are seldom available for study. Our objective in this study was, therefore, to compare isolates of *Cytospora* and *Cytospora*-like fungi from *Eucalyptus* spp. collected in many parts of the world. Furthermore, we determined their relationships based on homology of DNA sequence of the internal transcribed spacers and the 5.8S gene of the nuclear ribosomal DNA operon (ITS-rDNA). We also attempted to determine the phylogenetic relationships of these isolates to other species of *Cytospora* and their teleomorphs.

MATERIALS AND METHODS

Material examined--Information on the geographic origins of the relevant isolates used in this study is given in Table I. At the time of the study, few herbarium specimens

accompanied these cultures. Teleomorphs matching the morphology of *V. ceratosperma* were collected on *Eucalyptus* in Uganda and Congo (Table I).

For morphological studies each isolate was inoculated onto 2 % malt extract agar (50 ml) in a sterile chamber. Twigs of *E. grandis* approximately 0.5 cm diameter and 7 cm long were surface disinfected for 1 sec in 70 % ethanol, 5 min in 0.525 % sodium hypochlorite and rinsed in sterile water. Following 5 days of fungal growth at room temperature, the disinfected twigs were placed upright (5 twigs/isolate/chamber) with 1 cm of their basal end in the agar culture (Chang *et al*, 1989). Isolates were also inoculated onto autoclaved leaves of *E. grandis* on 2 % malt extract agar. Once hyphae colonized the leaf tissue, the leaves were placed on water agar and incubated in the above conditions until pycnidia developed to maturity. Following 20 days incubation at 20 C in 12 hr cool white fluorescent light and 12 hr dark, the pycnidia that formed on leaves and bark were examined by phase contrast microscopy. Specimens were examined for shape and size of conidia, conidiophores, wall, neck and locule structure. Specimens were stained with lactophenol cotton blue and mounted permanently in polyvinyl alcohol lactoglycerol that were solidified by heating 1 hr at 60 C (Koske & Tessier, 1983).

DNA preparation, enzymatic amplification and sequencing--Total genomic DNA was extracted from all isolates (Table I). Isolates were grown in 25 mL of 2 % malt extract broth at room temperature for 5 to 10 days. Mycelia were then harvested by vacuum filtration through miracloth (Calbiochem-Novobiochem Corp., La Jolla, California), lyophilized, and stored at -20 C. DNA was extracted from lyophilized hyphae by one of two methods of extraction, a standard sodium dodecyl sulfate (SDS) phenol method (Lee *et al*, 1988) and a cetyltrimethyl-ammonium bromide (CTAB) method (Raeder & Broda, 1985). Hyphae were ground to a fine powder in liquid nitrogen with a pestle. One to 2 mL of the extraction buffer was added and mixed. The extract was then centrifuged to remove solids, purified with phenol : chloroform : isoamyl alcohol (24:24:1) extractions and precipitated with isopropanol and centrifugation. The precipitate was air dried under vacuum and then dissolved in 50 µL TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Approximately 2.5 ng of the total genomic DNA was used per 100 uL reaction mixture

for polymerase chain reaction amplification (PCR) (White *et al*, 1990). Various brands of prepackaged buffers and polymerases were used for PCR amplification during this research. Reaction mixtures were approximately those of White *et al* (1990). Primers used in the amplifications included ITS1, ITS2, ITS3, and ITS4 for the ITS-rDNA (White *et al*, 1990).

The cycling reactions were performed in a DNA Thermal Cycler (Perkin-Elmer Corp., Norwalk, CT) or similar machine. Thermal cycling was programmed for a 2 min hot start of 94 C followed by 35 cycles of 1 min 94 C, 1 min 50 C, 45 s at 72 C. The 45 s at 72 C was extended each cycle by 4 s. The amplification ended with an additional 7 min extension of 72 C. PCR amplification products were separated by agarose gel electrophoresis, stained with ethidium bromide and examined under ultraviolet light. Two hundred uL of each product (not exposed to ultraviolet light) were purified by using the DNA binding resin and protocol of Wizard PCR Preps DNA purification system (Promega Corp., Madison, Wisconsin).

Sequencing was performed using a *Taq* DyeDeoxi Terminator™ cycle system, the ABI Catalyst 800, and the ABI Prism 373A or 377 fluorescence sequencer (PE Applied Biosystems, Foster City, California). Sequencing reactions were carried out using the Big Dye fluorescent labeling sequencing kit (PE Applied Biosystems). Amplified double-stranded PCR products were sequenced independently along both strands with the primers listed above. Sequences were merged and aligned using ESEE version 1.09e (Cabot & Beckenbach, 1989), visually edited and aligned, then a composite consensus sequence was proofread. Sequences have been deposited in GenBank (AF192314-21, AF260263-6, Table I). An alignment of the sequences has been submitted to TreeBASE as SN471.

Initially, the ITS-rDNA sequences of *Cytospora* and *Cytospora*-like isolates from *Eucalyptus* were compared in distance analysis with a total of 80 taxa in the Valsaceae that we have sequenced (GCA unpublished). The initial analysis was computed using

PHYLIP version 3.5 (Felsenstein, 1997) with two Kimura distances (DNADIST) and the neighbor joining algorithm (Saitou & Nei, 1987). A reduced set of data containing 38 taxa was then analyzed more thoroughly. The pruned taxa were additional members of groups already well represented in the subset, or entire groups distant from the relevant groups near to the groupings containing *Cytospora* and *Cytospora*-like isolates from *Eucalyptus*. The presence or absence of the pruned taxa did not affect placement of the *Cytospora* sequences in the final analysis. The phylogeny of the 38 taxa data set was computed with PAUP version 4.0 beta (Swofford, 1999) using maximum parsimony (Swofford & Maddison, 1987). A tree from the most parsimonious trees was displayed using TreeView (Page 1996), Fig. 1. To develop a consensus tree, 2000 heuristic searches (Hedges, 1992) were performed by bootstrapping (Felsenstein, 1985) with the tree bisection-reconnection branch swapping algorithm (TBR), MAXTREES unrestricted, and saving no more than 100 shortest trees. Confidence intervals for branches on the consensus tree were inserted into the most parsimonious tree (Fig. 1). *Diatrype stigma* (Hoffm.:Fr.) Fr. (anamorph = *Libertella betulina* Desmaz.) and *Diatrypella frostii* Peck. (anamorph = *Cytosporina* sp.) were chosen to serve as an outgroup because the teleomorphs of Diatrypales are similar in morphology to the Diaporthales and the anamorphs are somewhat similar in morphology to *Cytospora* Ehrenb.:Fr. The Diatrypales was representative of a strongly bootstrap-supported clade distant to all the included taxa.

RESULTS

DNA sequences of *Cytospora* and *Cytospora*-like fungi from *Eucalyptus* have been deposited and accessioned in the National Center for Biotechnology Information, GenBank (Table I). The length of DNA sequence for each isolate, inclusive of introduced gaps to permit alignment of the entire set, was 571 nucleotides.

The initial neighbor joining analysis of 80 *Cytospora* taxa yielded a phylogeny that places the *Cytospora*-like isolates from *Eucalyptus* within the genus *Diaporthe*. *Leucostoma*

sequoiae Bonar is the species with a true *Cytospora* anamorph closest to the Diaporthales. *Leucostoma sequoiae* forms an anamorph with morphological features of the *Torsellia* type (GCA unpublished; Spielman, 1985). In this study, *L. sequoiae* represents the root of the monophyletic clade that includes *Cytospora* anamorphs of *Valsa, Leucostoma, Valsella,* and *Valseutypella* (data on *Valseutypella* unpublished) when the phylogeny is rooted to the outgroups *Diatrype*, *Diaporthe* or other genera in the Diaporthales that we have tested.

Parsimony analysis of the data set of *Eucalyptus* isolates and 24 other taxa (total 38 taxa) is represented in a cladogram (Fig. 1). The names in italics in Fig. 1 represent unique DNA sequences that correspond to isolate codes given in Table I. The host, *Eucalyptus*, and the country of origin are included in an abbreviated form in each italicized name. Heuristic analysis gave 9 most parsimonious trees of 709 steps and consistency index of 0.574, retention index of 0.690, and a re-scaled consistency index of 0.396. The well-known species of *Valsa, Leucostoma* and *Valsella* (Barr, 1978; Kobayashi, 1970; Spielman, 1985) are remote from the *Cytospora* isolates from *Eucalyptus* including *V. ceratosperma* from other hosts (Fig. 1). The *Cytospora*-like isolates, all from Indonesia, cluster within the genus *Diaporthe* (Clade P) with an 82 % bootstrap confidence level (BCL), therefore we consider them putative *Phomopsis* species. The *Cytospora* isolates cluster in several clades (Clade 1, BCL 88 %; Clade 2, BCL 100 %; Clade 3, BCL 100 %) within the monophyletic group of *Cytospora* anamorphs of *Leucostoma, Valsa* and *Valsella* species (BCL 85 %).

Pycnidia formed primarily on the surface rather than embedded in the bark of inoculated twigs and leaves. Pycnidia and their locules are similar in morphology to those formed in agar culture rather than those from nature. The conidia of the *Eucalyptus* isolates of *Cytospora* (Clades 1, 2, 3) and *Cytospora*-like fungi (Clade P) are not readily distinguishable. They are small relative to a microscope micrometer scale and measurements are not precise without further magnification of the X1500 microscope image. Conidia of each isolate of EUCAINDO1 (CMW4309, 4314) (n=50) stain darkly

with lactophenol cotton blue and they are straight, 2.8 X 0.9 μm, with 1-2 guttules. Conidia of EUCAINDO2 and EUCAINDO3 stain lightly, are allantoid to straight, do not contain guttules, and are 2.9 X 0.9 μm and 3.1 X 0.9 μm, respectively. Conidiogenous cells are phialides and conidiophores (6.6-11.6 X 0.7-1.0 μm) are branched in the *Cytospora*-like fungi. Conidia of the *Cytospora* isolates are allantoid and vary slightly: In Clade 1, conidia of EUCACONGO (n=50) are 4.0 X 1.0 μm; EUCAMEXI, 3.4 X 0.9 μm; EUCASAFR1 and EUCASAFR2, 4.1 X 0.8 μm; EUCATHIA, 3.4 X 0.9 μm; EUCAVENZ, 3.4 X 0.9 μm. In Clade 2, conidia of EUCASAFR3 are 4.1 X 0.9 μm; EUCASAFR4, 4.5 X 1.0 μm. In Clade 3, conidia of EUCAAUST are 3.9 X 0.9 μm; EUCASAFR5, 3.8 X 0.8 μm; EUCAUGAND, 3.9 X 0.9 μm. Conidia of all isolates are shorter and significantly narrower than those of *C. australiae* and the conidiophores are unbranched (9.0-17.0 X 0.9-1.0 μm). Pycnidia are unilocular on inoculated leaves, twigs and agar medium. Measurements of the morphological characteristics of each of the isolates of *Cytospora* from *Eucalyptus* fall within the species description of *C. eucalypticola.*

Distinct differences between the *Cytospora* and *Cytospora*-like fungi are evident in the wall of the pycnidium, particularly when examining the neck (beak) area. The *Cytospora*-like fungi have elongated cells in the plectenchymatous wall (Fig. 2) that approaches a textura porrecta. The *Cytospora* isolates form a typical parenchymatous wall of textura prismatica. In either group of fungi, the wall may become a crust-like sclerenchyma on the globe of the pycnidium that can confuse diagnosis. On the surface of the neck of the pycnidium, the *Cytospora*-like fungi of EUCAINDO2 show a surface cell protruding from the tissue distributed at regular intervals like short blunt hairs (Fig. 3). Similar cells are illustrated on the neck of *P. eucalypticola* by Yuan *et al* (1995).

In agar culture, *Cytospora* (Clades 1, 2, 3) and *Cytospora*-like (Clade P) fungi occasionally form distinct necks, however, only the *Cytospora*-like fungi form long necks (1-4 times as long as the diameter of the pycnidium globe) on inoculated *Eucalyptus* leaves on the surface of water agar (Fig. 5). The pycnidium of the *Cytospora*-like fungi is

macroscopically distinct on *Eucalyptus* leaves in culture. The globe of the pycnidium (but not the neck) has a thick woolly coating of hyphae on the surface that extends into the air radially (Fig. 6). Although hyphae may form a mat over some pycnidia of the *Cytospora* isolates, the hyphae do not extend into the air radially like those of the *Cytospora*-like fungi. Most *Cytospora* isolates form pycnidia that are glabrous. Sutton (1980) described the conidiomata of *Cytospora* and *Phomopsis* as stromatal (phialostromatineae) rather than pycnidial (phialopycnidiineae). In agar and on inoculated leaves and twigs the morphology of *Cytospora* and the *Cytospora*-like fungi (putative *Phomopsis* species) form a distinctly flask-shaped pycnidium.

DISCUSSION

The results of our phylogenetic study support the conclusion that the isolates of *Cytospora* and *Cytospora*-like fungi from *Eucalyptus* in the Southern Hemisphere and Mexico represent at least four distinct groups that are only distantly related to one another. The *Cytospora* species on *Eucalyptus* spp. display a genetic diversity that is as great as that measurable within the genus *Valsa*, inclusive of *Leucostoma* and *Valsella*. The genetic diversity shown in this study is displayed as changes in nucleotide substitutions and insertions or deletions within the internal transcribed spacers I and II and the 5.8S rDNA sequence.

The *Cytospora* isolates forming Clade 1 have a geographical distribution over three continents (Thailand, Mexico, Venezuela, Congo and South Africa) and a high level of genetic variation that seems to parallel this distribution. Based on the genetic diversity present in the sequences in Clade 1 compared to that present in the other *Valsa* and *Leucostoma* species in the phylogeny, Clade 1 may include at least three separate species. One of the species includes isolates from Congo that form a teleomorph with morphological characteristics similar to those of *V. ceratosperma*. Clade 2 and Clade 3 each may contain one species. Clade 3 includes isolates from Uganda that also form a teleomorph with the morphological characteristics of *V. ceratosperma*. Isolates from

Eucalyptus are not close to isolates of *V. ceratosperma* from other hosts included in this phylogenetic study. We believe that the teleomorphs formed on *Eucalyptus* will need re-interpretation and diagnosis.

Despite the geographical origin of the isolates used in this study, we hypothesize that they most likely evolved in Australia and Tasmania, probably on different species of *Eucalyptus* and in different regions of the continent. Historical and modern transport of seed and propagation material to other continents and forces of natural selection are most likely responsible for the predominance of a particular population of a species among our samples. Alternatively, some of the three species could have evolved on other genera of plants in the plant family Myrtaceae and moved onto an introduced *Eucalyptus* species. Such an event could then be magnified as favored *Eucalyptus* selections were shipped between continents by the international forestry industry.

The *Cytospora*-like fungi from Indonesia (Clade P) may be *Phomopsis* isolates that fail to form ß-conidia in culture. Clade P, therefore, may represent two *Diaporthe* species. The isolates in Clade P form conidia that are about half the length of those reported for *P. eucalypticola* (Yuan *et al*, 1995). Isolates in EUCAINDO1 form straight conidia with two guttules, branched conidiophores, and elongated cells in the pycnidium wall similar to *P. eucalypticola,* but conidia are significantly shorter. The conidia of isolates in EUCAINDO1, EUCAINDO2 and EUCAINDO3 also are significantly shorter than those of *P. rudis* and *P. eucalypti.* Herbarium material corresponding directly to these isolates is not available. However, the isolates of Clade P may represent two undescribed species of *Diaporthe.*

The Australian isolates of *Cytospora* were considered by Old *et al* (1991) to *be C. eucalypticola* based on morphology similar to this South African species. South African isolate CMW940 (EUCASAFR5) and our Ugandan isolates are related to the Australian species, however, the majority of *Cytospora* isolates from *Eucalyptus* spp. in South Africa are not related to the Australian isolates. We are uncertain which clade (Clade 1, 2 or 3) might correspond to *C. eucalypticola* sensu van der Westhuizen. Unfortunately,

Cytospora and *Phomopsis* species can not be identified from fruiting bodies produced in culture (Uecker, 1988) and further work linking field collected specimens directly to DNA sequences will be needed to resolve this question.

Recognition of distinct populations and species among the *Cytospora* and *Cytospora*-like fungi causing cankers on *Eucalyptus* might have value in the testing of disease resistance in *Eucalyptus* species, hybrids, or clones. We do not know whether differences in relative virulence on *Eucalyptus* among the four species are significant in particular environments but it might be important to guard against introduction of new strains into plantations. Considering the endophytic nature of *Cytospora* and *Phomopsis* in trees, restricting importation of the pathogens on *Eucalyptus* spp. would be difficult and beyond the capabilities of most nationally administered plant health inspection services. In fact the future movement of these pathogens to new continents may be the inevitable consequence of the rapidly emerging use and importance of intensive forestry in hybrid and clonal *Eucalyptus* (Denison & Kietzka, 1993). International companies developing hybrids and clones generally evaluate the performance of the new plant material in different regions.

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Table 1. Taxa studied in phylogenetic analysis of the internal transcribed spacer of the nuclear rDNA, their geographic and host origins, voucher specimen numbers, strain numbers, cladogram codes and GenBank accession numbers.

Taxon	Geographic origin.	Host E.=Eucalyptus	Specimen No.	Culture No.	Cladogram code	GenBank No.
Cytospora sp.	Australia	*E. marginata.*	-	ATCC 56123	EUCAAUST	AF192314
Cytospora sp.	NSW Australia	*E.* sp.	-	CMW1157 [a]	EUCAAUST	AF192314
Cytospora sp.	Batlow, Australia	*E. delegatensis*	-	CMW1158	EUCAAUST	AF192314
Cytospora sp.	Batlow, Australia	*E. delegatensis*	-	CMW1159	EUCAAUST	AF192314
Cytospora sp.	Pretoria, S. Africa (SA)	*E. camaldulensis*	-	PPRI 5297 [b]	EUCASAFR3	AF260264
Cytospora sp.	Seven Oaks, SA	*E. grandis*	-	CMW940	EUCASAFR5	AF260265
Cytospora sp.	Tzaneen, SA	*E. grandis*	-	CMW627	EUCASAFR1	AF192318
Cytospora sp.	Matubatuba, SA	*E. camaldulensis*	-	CMW628	EUCASAFR2	AF192319
Cytospora sp.	KwaMbonambi, SA	*E. grandis*	-	CMW1237	EUCASAFR4	AF260263
Cytospora sp.	KwaMbonambi, SA	*E. grandis*	-	CMW1238	EUCASAFR4	AF260263
Cytospora sp.	KwaMbonambi, SA	*E. grandis* (*E. g*)	-	CMW1240	EUCASAFR4	AF260263
Valsa sp.	Tchittanga, Congo	*E. g* X *tereticornis*	MSC368317 [c]	CMW5260	EUCACONGO	AF192315
Valsa sp.	Tchittanga, Congo	*E. g* X *tereticornis*	MSC368318	CMW5261	EUCACONGO	AF192315
Valsa sp.	Tororo, Uganda	*E. grandis*	MSC368319	CMW5262	EUCAUGAND	AF260266

Table 1 continued

Valsa sp.	Entebbe, Uganda	*E. grandis*	MSC368320	CMW5263	EUCAUGAND	AF260266
Valsa sp.	Itojo, Uganda	*E. grandis*	MSC368321	CMW5264	EUCAUGAND	AF260266
Cytospora sp.	Mexico	*E. grandis*	-	CMW516	EUCAMEXI	AF192317
Cytospora sp.	Mexico	*E. grandis*	-	CMW517	EUCAMEXI	AF192317
Cytospora sp.	Acarigua, Venezuela	*E. camaldulensis*	-	CMW3393	EUCAVENZ	AF192321
Cytospora sp.	Acarigua, Venezuela	*E. camaldulensis*	-	CMW3394	EUCAVENZ	AF192321
Cytospora sp.	Thailand	*E. camaldulensis*	-	CMW464	EUCATHIA	AF192320
Cytospora-like	Indonesia	*E. urophylla*	-	CMW461	EUCAINDO3	AF192316
Cytospora-like	Indonesia	*E. urophylla*	-	CMW462	EUCAINDO3	AF192316
Cytospora-like	Indonesia	*E. urophylla*	-	CMW460	EUCAINDO2	AF192313
Cytospora-like	Sumatra, Indonesia	*E. grandis*	-	CMW4047	EUCAINDO2	AF192313
Cytospora-like	Sumatra, Indonesia	*E. grandis*	-	CMW4309	EUCAINDO1	AF192312
Cytospora-like	Sumatra, Indonesia	*E. grandis*	-	CMW4310	EUCAINDO1	AF192312
Cytospora sp.	South Africa	*Mangifera indica*	-	PPRI6767	MANGO	AF260267
Valsa ceratosperma	Michigan, USA.	*Rhus typhus*	MSC368322	GCAsumac1[d]	VCERATO2	AF192324
Valsa ceratosperma	Japan	Malus pumila	-	ATCC 56632	VCERATO1	AF192326
Cytospora cedri	-	-	-	CBS196.50	CCEDRI	AF192311

Table 1 continued

Diaporthe vaccinii	Michigan, USA	*Vaccinium corymbosum*	-	GCADvaccP	DIAPORTHEV	AF191166
Diatrypella frostii	Illinois, USA	Acer *sp.*	-	ATCC52484	DFROSTII	AF192322
Diatrype stigma	Sweden	*Rhamnus frangula*	-	ATCC64170	DIATRYPEST	AF192323

[a] Accession number of the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agriculture Biotechnology Institute (FABI), University of Pretoria

[b] Accession number of the Plant Protection Research Institute, Agriculture Research Center, Pretoria

[c] Accession number of the Beal-Darlington herbarium at Michigan State University (MSC)

[d] Accession number in the collection of Gerard C. Adams (GCA)

Figure. 1. Cladogram of the analysis of DNA sequence of the internal transcribed spacers and the 5.8S rDNA gene of the ribosomal DNA operon of 38 isolates of *Cytospora* (C) and their *Valsa* (V), *Leucostoma* (L), and *Valsella* teleomorphs, and *Cytospora*-like fungi and their *Diaporthe* teleomorphs. Taxa are designated by a prefix for the genus followed by an abbreviated species epithet and a number representative of a population with a distinct sequence (ie., *VCERATOSP2* = *Valsa ceratosperma* from the U.S.A.; *VCERATOSP1* = from Japan). Isolates from *Eucalyptus* spp. are in italics and are designated by the prefix *EUCA* followed by an abbreviated country code (ie., *EUCAINDO1* = isolate from *Eucalyptus* in Indonesia). Another designation *MANGO* represents a *Cytospora* sp. on mango fruit from South Africa known only from cultures. *DIATRYPEST* = *Diatrype stigma*, *DFROSTII* = *Diatrypella frostii*, *DIAPORTHEV* = *Diaporthe vaccinii*. The tree shown is one of the most parsimonious trees determined by the Heuristic algorithm. Branch lengths are calculated by maximum parsimony and correspond to genetic distance (expected nucleotide substitutions per site). The scale bar is of a branch length equal to 10 nucleotide substitutions. Numbers at nodes are bootstrap indices of support (2000 replications), in percentage.

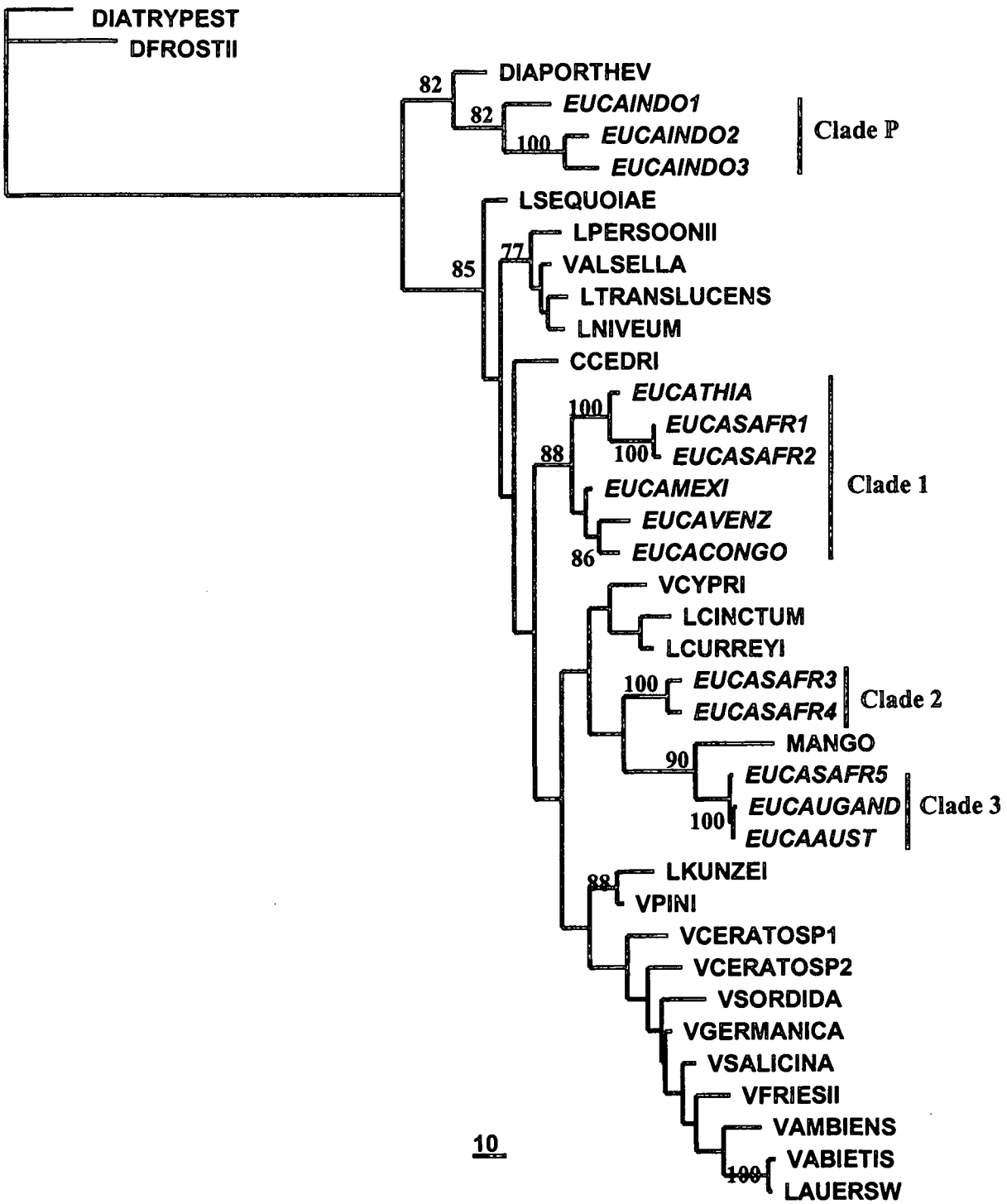
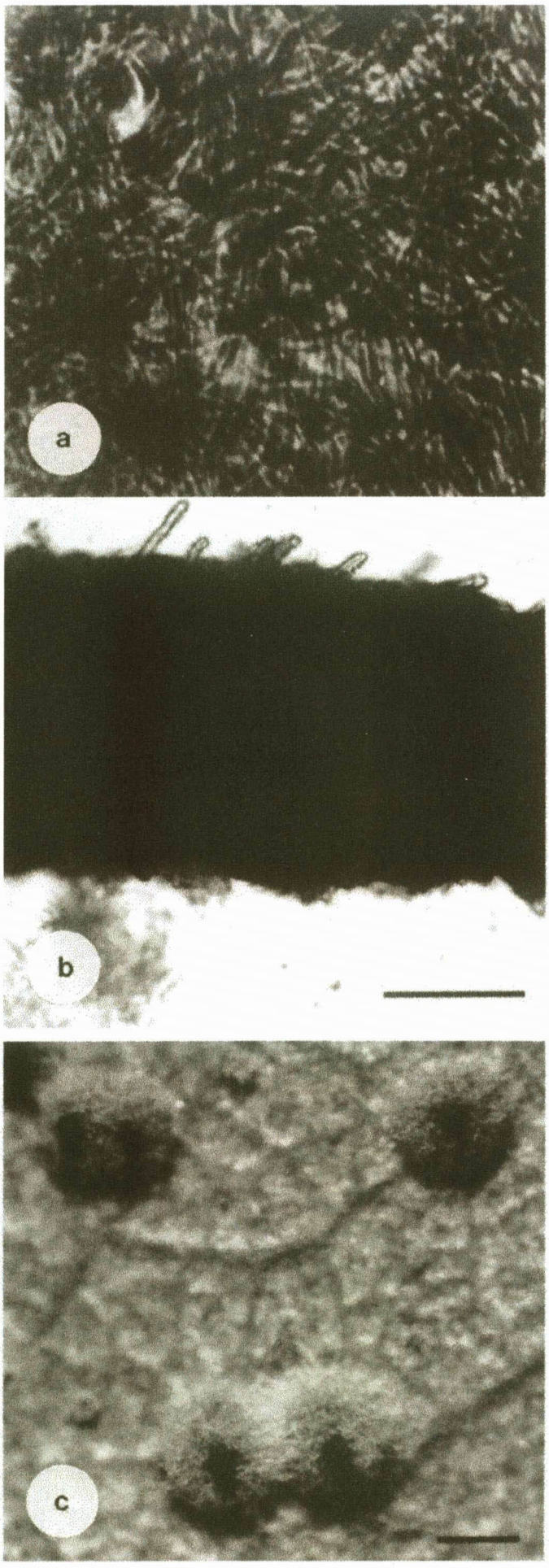

DIATRYPEST
DFROSTII
82
DIAPORTHEV
82
EUCAINDO1
100
EUCAINDO2
EUCAINDO3
Clade P
LSEQUOIAE
77
LPERSOONII
85
VALSELLA
LTRANSLUCENS
LNIVEUM
CCEDRI
100
EUCATHIA
EUCASAFR1
88
100
EUCASAFR2
EUCAMEXI
EUCAVENZ
86
EUCACONGO
Clade 1
VCYPRI
LCINCTUM
LCURREYI
100
EUCASAFR3
EUCASAFR4
Clade 2
MANGO
90
EUCASAFR5
EUCAUGAND
100
EUCAAUST
Clade 3
LKUNZEI
88
VPINI
VCERATOSP1
VCERATOSP2
VSORDIDA
VGERMANICA
VSALICINA
VFRIESII
VAMBIENS
VABIETIS
100
LAUERSW
10

Figure. 2. (a) The cellular structure of the wall of the neck (beak) of the pycnidium of a species of *Phomopsis* from *Eucalyptus.* In culture the *Phomopsis* resembles *Cytospora* species except in the morphology of the wall. Photograph was taken at X1000 with phase contrast microscopy, (b) the neck of the pycnidium of a species of *Phomopsis* from *Eucalyptus* having short blunt cells regularly distributed along the surface. Photograph was taken at X100 with bright field microscopy. Bar is 10 μm in length and (c) hyphae extending from the walls of pycnidia of a species of *Phomopsis* from *Eucalyptus* grown on leaves on agar. *Cytospora* species from *Eucalyptus* do not form hyphae extending from the pycnidium wall. Bar is 1 mm in length.


a
b
c

Keywords: *Botryosphaeria dothidea*, *Sphaeropsis sapinea*, *Eucalyptus*, *Pinus*, endophyte, population genetics, vegetative compatibility, sequencing

Summary

Botryosphaeria dothidea and *Sphaeropsis sapinea* both very important pathogens in the South African forestry context. These fungi are well established in the country and contribute substantially to annual losses incurred. Currently very little can be done to control the fungi and the damage they cause. The understanding of their respective disease etiologies is thus of great importance to develop relevant counter measures. The overall aim of this dissertation was to investigate various poorly understood aspects of these two fungi and to try and relate the results to practical contributions towards controlling the impact the two pathogens have. Studies have been conducted during the course of five years and each study represents an independent research investigation.

The introductory chapter presents a review of the literature pertaining to all aspects of biology, history and taxonomy of *B. dothidea* and *S. sapinea*. The two fungi are clearly very similar in all these aspects and perhaps the only clear difference is that *S. sapinea* is restricted to pines in South Africa. Many other similarities and some differences between these two important pathogens are highlighted and many of these have provided the background for further investigations.

In chapter two the presence of *B. dothidea* and *S. sapinea* is demonstrated as symptomless endophytes in healthy, pine and eucalypt tissue. *Botryosphaeria dothidea* was found to be common in all the *Eucalyptus* spp. tested, occurring at high percentages in symptomless leaves of *Eucalptus smithii*, *E. camaldulensis*, *E. grandis* and *E. nitens*. *Sphaeropsis sapinea* was, in contrast, only present in young, green *Pinus patula* and *P. radiata* cones, but virtually absent from the cones of *P. elliottii* and *P. taeda*.

Botryosphaeria dothidea is associated with die-back and canker diseases of eucalypts in South Africa. Despite this fact, little is known about the infection process. The fungus is known to occur endophytically in leaves of various *Eucalyptus* species in South Africa. In chapter three I consider the ability of *B. dothidea* to infect apparently healthy *Eucalyptus* leaves and the subsequent location and structure of these

infections once inside leaf tissue. Scanning electron microscopy revealed that conidia of *B. dothidea* can infect healthy leaves through stomata. These infections ultimately reside amongst mesophyll cells and constitute a number of individual infections per leaf.

Two morphologically similar fungi are associated with die-back and canker of eucalypts in South Africa. The one was identified as part of the *Botryosphaeria dothidea*-complex. In chapter four, the identity of the second fungus was determined by comparing morphology, pathogenicity and DNA sequence analysis of isolates of both taxa. Based on results obtained, *Botryosphaeria eucalyptorum*, and its anamorph *Fusicoccum eucalyptorum*, are described as a new species. I found that the teleomorph is morphologically similar to other taxa in the *B. dothidea*-complex, but conidial characteristics of the anamorph are distinct, as well as the sequences of the nrDNA internal transcribed spacers ITS1 and ITS2. As is the case with *B. dothidea*, the fungus is pathogenic to *Eucalyptus*, there do not, however, appear to be differences in pathogenicity between the two.

Sphaeropsis sapinea is the most important pathogen of pines in South Africa. The fungus, which reproduces only asexually, occurs only on exotic pines. In chapter five, I investigated the diversity of the *S. sapinea* population in South Africa and compared it with a population from Northern Sumatra. Both populations were obtained from exotic *P. patula* plantations. The phenotypic diversity of these populations was assessed using vegetative compatibility tests. The percentage maximum genotypic diversity, based on Stoddard and Taylor's index, for the South African population was much higher than the Northern Sumatran population, thus indicating that the South African *S. sapinea* population was more diverse than the Northern Sumatran population. These results support the hypothesis that the population of *S. sapinea* in South Africa has been introduced from various parts of the world, during the last century.

In chapter six, I investigated the role that latent *S. sapinea* infections in seed cones of *P. patula*, play in post-hail associated die-back. *Pinus patula* seed cones were found to be infected during the second year of development, with extensive colonisation

only occurring in the third year when cones mature, prior to seed discharge. Vegetative compatibility tests revealed that the presence of *S. sapinea* in individual third year seed cones is confined to a single genetic entity. *Sphaeropsis sapinea* colonisation of third year seed cones thus, apparently results from a single successful infection per cone. The probable role of latent infections by *S. sapinea* indicated that tree age and by implication, increased numbers of attached seed cones, contributes to more severe die-back after hail damage.

The control of damage caused by *S. sapinea* is highly dependant on a dynamic hybridisation programme. Alternative species of pines is thus constantly evaluated for potential. In chapter seven, 65 families representing both the northern and southern populations of *P. greggii* were evaluated for their tolerance to infection and subsequent die-back caused by *S. sapinea*. Families were evaluated following natural infection after hail damage, as well as by artificial inoculation. Variation in tolerance occurred and was highly significant between the two provenances, with the northern provenance proving to be very tolerant. *Pinus greggii* trees of the southern provenances were comparable with P. patula. The potential of the families from northern origins has to be investigated further.

Cultures of *Cytospora* isolated from *Eucalyptus* trees in South Africa, Congo, Thailand, Venezuela, Mexico, Uganda and Australia, as well as *Cytospora*-like isolates from Indonesia were compared in chapter eight. Comparisons were based on the homology of the internal transcribed spacer regions and the 5.8S ribosomal DNA of the nuclear ribosomal DNA repeat unit. Isolates clustered into at least three unrelated groupings, with a fourth grouping that included isolates that morphologically resembled *Cytospora*. Results from this chapter indicated that the current description of *Valsa ceratosperma* encompasses several distinctly different species and needs to be further refined.

Botryosphaeria dothidea and *S. sapinea* are two of the most important pathogens of eucalypts and pines in South Africa. The fact that they exist as symptomless endophytes in trees has added a fascinating aspect to our understanding of their role in tree diseases. In the past, they have generally been considered to be wound infecting

opportunistic fungi. Results of these studies have shown that this is not so and that they are clearly able to infect healthy trees. They are unlikely to be able to infect dead or moribund tissue. The investigations presented in this dissertation have added considerable knowledge to our understanding of *B. dothidea* and *S. sapinea* and will also promote efforts to reduce disease caused by them. However, there are many questions that remain to be answered pertaining to them and it is my hope that this study will provide a foundation and stimulus for further work.

Opsomming

Botryosphaeria dothidea en *Sphaeropsis sapinea* is albei baie belangrike patogene vir die Suid Afrikaanse bosbou industrie. Hierdie fungi is lank gevestig in die land en dra in 'n groot mate by tot jaarlikse verliese vir die bosbou industrie. Huidiglik is daar baie min wat gedoen kan word om hierdie patogene of die skade wat hulle aanrig te beheer of te beperk. Dit is dus uiters belangrik dat ons hul siekte ontwikkeling verstaan sodat relevante beheer of bestuurs praktyke daar gestel kan word. Die oorkoepelende doel van hierdie studie was dus om aandag te gee aan verskeie aspekte van hierdie twee patogene, om sodoende 'n bydrae te maak tot die praktiese bekamping van skade deur hulle aangerig. Studies was oor 'n vyf jaar tydperk en elke hoofstuk verteenwoordig 'n onafhanklike navorsings projek.

Die inleidende hoofstuk is 'n oorsig van die relevante literatuur met betrekking tot die biologie, geskiedenis en taksonomie van *B. dothidea* en *S. sapinea*. Die twee fungi is baie naby vervant en kom ooreen in baie aspekte, met dalk die enigste duidelike verskil, die feit dat *S. sapinea* alleenlik op denne in Suid Afrika voorkom. Baie ander ooreenkomste en verskille tussen die twee belangrike patogene word dus uitgelig en skep dus ook die basis vir verdere navorsing.

In hoofstuk twee demonstreer ek die voorkoms van *B. dothidea* en *S. sapinea* as simptoomlose endofiete in gesonde bloekom en denne weefsel. *Botryosphaeria dothidea* het algemeen voorgekom in die gesonde blare van al die *Eucalyptus* spp. wat getoets was, naamlik *Eucalptus smithii*, *E. camaldulensis*, *E. grandis* en *E. nitens*. *Sphaeropsis sapinea* was, daarenteen, slegs gevind in jong, groen këels van *Pinus patula* en *P. radiata* maar het so te sê ontbreek in die këels van *P. elliottii* en *P. taeda*.

Botryosphaeria dothidea word geassosieer met terugsterwing en kanker formasie van bloekoms in Suid Afrika. Afgesien van hierdie feit is daar baie min bekend oor die meganisme van infeksie. Die fungus is bekend dat dit voorkom as endofiet in die blare van verskeie *Eucalyptus* spesies in Suid Afrika. In hoofstuk drie, kyk ek dus na die vermoë van *B. dothidea* om gesonde blare te infekteer en ook na die lokasie van die tallie in blaar weefsel na infeksie. Skandeer electron mikroskopie is gebruik om

vas te stel dat konidia van *B. dothidea* wel gesonde blare deur huidmondjies kan infekteer. Hierdie infeksies oorleef dan tussen mesofiel selle en die teenwoordigheid van die fungus in blare was die gevolg van veelvuldige infeksies in elke blaar.

Twee mofhologies naverwante fungi word geassosieer met terugsterwing en kanker formasie van bloekoms in Suid Afrika. Die een is geidentifiseer as lid van die *Botryosphaeria dothidea*-kompleks. In hoofstuk vier, word die identiteit van die ander fungus bepaal met die hulp van vergelykings in morfologie, patogenisiteit en DNA opeenvolging analise met isolate van beide taksa. Gebaseer op die resultate word *Botryosphaeria eucalyptorum* en die anamorf *Fusicoccum eucalyptorum* as 'n nuwe spesie beskryf. Die teleomorf was morfologies baie naby verwant aan ander taksa in die *B. dothidea*-kompleks, met die verskille sover dit konidium karakter trekke, die unieke anamorf en die veskille in basispaar opeenvolging van die nrDNA interne getranskibeerde spasies ITS1 en ITS2. Soos met *B. dothidea*, is die nuwe fungus ook patogenies op *Eucalyptus* maar dit blyk dat daar wel verskille in patogenisiteit bestaan.

Sphaeropsis sapinea is die ekonomies mees belangrike patogeen van denne in Suid Afrika. Die fungus kom slegs op eksotiese denne voor en reproduseer slegs ongeslagtelik. In hoofstuk vyf onsersoek ek die populasie diversiteit van *S. sapinea* in Suid Afrika en vergelyk dit met 'n populasie van Noordelike Sumatra. Beide populasies is versamel in eksotiese *P. patula* plantasies. Die fenotiepiese diversiteit van hierdie populasies is bepaal deur van vegetatiewe vereenigbaarheids studies gebruik te maak. Die persentasie maksimum genotiepeise deversiteit, gebaseer op Stoddard en Taylor se indeks, het getoon dat die Suid Afrikaanse populasie baie meer divers is as die Noord Sumatra populasie. Hierdie resultate ondersteun die hipotese dat die populasie van *S. sapinea* in Suid Afrika die land binne gebring was vanuit verskeie ander dele van die wêreld gedurende die afgelope eeu.

In hoofstuk ses, bestudeer ek die rol wat latente infeksies in këels, deur *S. sapinea,* speel in die siekte ontwikkeling na hael skade. *Pinus patula* këels word in die tweede jaar geinfekteer, maar word eers in die derde jaar gekoloniseer voordat saad vrystelling plaasvind. Vegetatiewe vereenigbaarheids toetse het bewys dat *S. sapinea*

in individuele këels voorkaom as 'n enkele genetiese entitieit. Dus lyk dit asof die kolonisasie van drie jaar oue këels deur *S. sapinea* onstaan as 'n enkele infeksie per këel.

Die beheer van skade veroorsaak deur *S. sapinea* is afhanklik van 'n dinamiese teelprogram. Alternatiewe spesies van denne word voordurend geevalueer vir potensiaal. In hoofstuk sewe word 65 famielies van die noordelike en suidelike populasies van *P. greggii* uit Meksiko geevalueer vir moontlike weerstand teen *S. sapinea*. Famielies was geevalueer deur van natuurlike infeksies na hael en van kunsmatige inokulasies gebruik te maak. Famieleis van die noordelike populasie was baie meer weerstandbiedend en dus word hul potensiaal verder ondersoek.

Botryosphaeria dothidea en *S. sapinea* is twee baie belangrike patogene vir die bosbou bedryf in Suid Afrika. Die feit dat beide kan voortbestaan as simptoomlose endofiete het 'n baie interressante faset van hul biologie bloot gelê. In die verlede was beide hoofsaaklik geken as patogene wat slegs wonde kan infekteer. Resulate van hierdie studies het egter bewys dat hulle ook in staat is om gesonde weefsels te infekteer. Dit lyk egter ook asof beide nie in staat sou wees om dooie material te infekteer nie. Die resulate wat hier voorgehou word het baie bygedra tot ons kennis rondom *B. dothidea* en *S. sapinea,* en sal dien om verdere navorsing te stimuleer.