

STUDIES ON THE DIVERSITY AND BIOLOGY OF THE PINE PATHOGEN *SPHAEROPSIS SAPINEA*

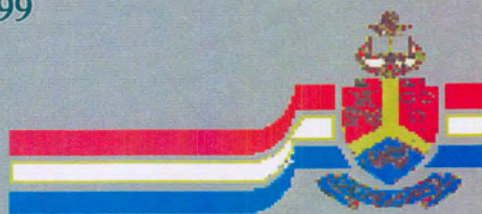
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A thesis submitted in partial fulfillment of the requirements for the degree of
MAGISTER SCIENTIAE

In the Faculty of Biological and Agricultural Sciences, Department of
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DECLARATION

I, the undersigned, hereby declare that the thesis submitted herewith for the degree Magister Scientiae to the University of Pretoria, contain my own independent work and has hitherto not been submitted for any degree at any other University.

J de Wet

Juanita de Wet

January 2000

***I have nothing to offer but blood, toil, tears
and sweat***

Winston Churchill



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The least of things with a meaning is worth more in life than the greatest of things without it.

Carl Jung



Mondi
Ltd.



sappi
forests



TREE PATHOLOGY CO-OPERATIVE
PROGRAMME



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PREFACE

Sphaeropsis sapinea is an opportunistic pathogen causing a variety of disease symptoms on conifers world-wide. In South Africa, annual losses due to post-hail associated *S. sapinea*-induced die-back of *Pinus radiata* are estimated at 3.8 million US dollars (1986 values). Management of *S. sapinea* is based on the planting of resistant pine species in areas less affected by adverse environmental conditions that could potentially stress the trees and lead to infection. The discovery of dsRNA fragments in the cytoplasm of this pathogen, and the subsequent description of two viruses (SsRV1 and SsRV2) provides a new exiting option of biological control. The implementation of dsRNA as a biocontrol strategy for management of *S. sapinea* diseases will, however, require an extensive re-evaluation of the biology and genetics of this well-known and important pathogen. This thesis represents a series of studies conducted over a two-year period to investigate various aspects of the biology and diversity of this fungus as well as its association with dsRNA fragments.

Chapter One represents a literature review on the taxonomy, ecology, host range, epidemiology and disease symptoms associated with *S. sapinea*. Information pertaining to recent advances on the genetic structure of populations of this fungus and dsRNA fragments associated with it, are also included. These factors are likely to be key elements in the more effective management of *S. sapinea* diseases and in the implementation of quarantine practices that will prevent future spread of the pathogen.

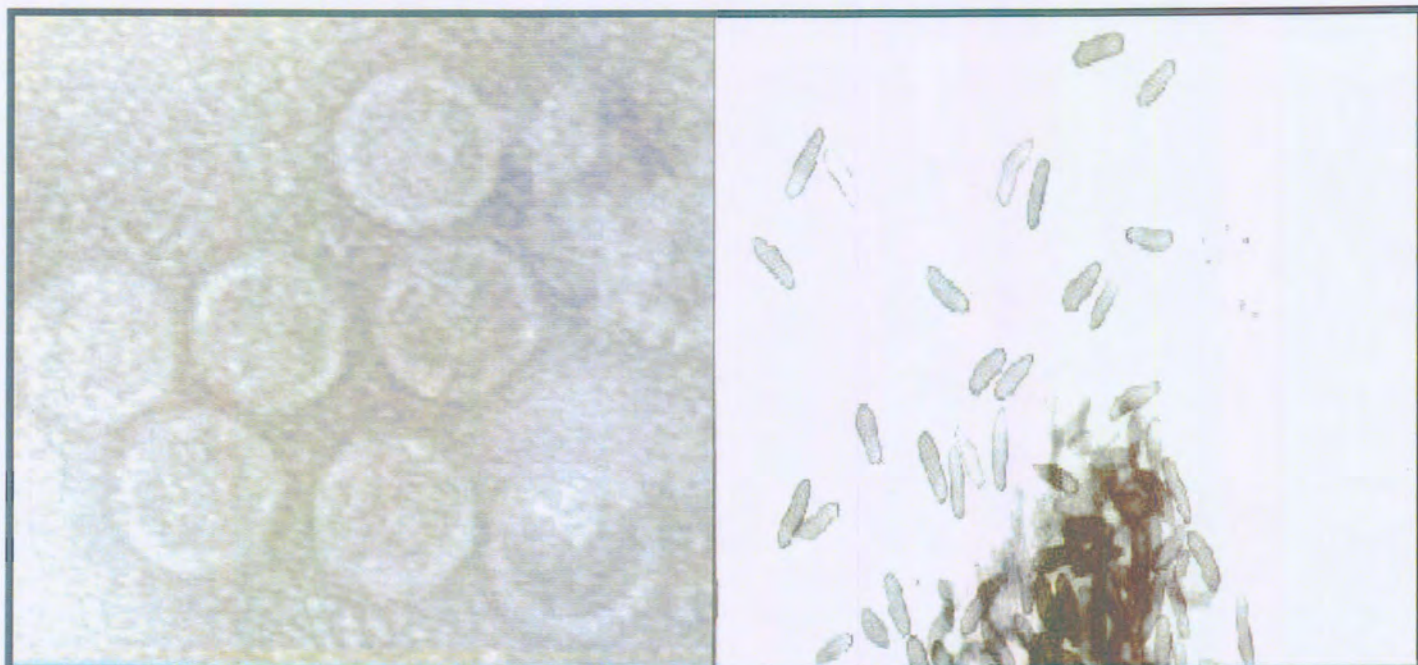
The objective of Chapter Two was to characterise of *S. sapinea* isolates collected from South Africa, Indonesia and Mexico. The A and B morphotypes of the fungus are distinguishable based on cultural and conidial morphology, growth rate and pathogenicity. Considerable variation was, however, observed in phenotypes of *S. sapinea* isolates collected from the various geographical regions under consideration. Alternative techniques such as RAPDs, known from previous reports to being able to distinguish between the A and B morphotypes, and DNA sequences were used to characterise this set of *S. sapinea* isolates.

A third group of *S. sapinea* isolates from Indonesia and Mexico, distinct from the previously described A and B morphotype groups, emerged from RAPD analysis. In Chapter Three isolates belonging to what I have designated as the C morphotype of *S. sapinea* were characterised based on morphology and pathogenicity. Isolates representing all three morphotypes were also compared based on cultural and conidial characteristics, *in vitro* growth rate and virulence.

Chapter Four represents a comparison of the range of diversity for five geographically distant *S. sapinea* populations. These five populations represent both presumed native and introduced populations of the fungus. Assessment of diversity was based on phenotypic characteristics (VCGs). The genetic structure of the fungal population could impact on management strategies by impairing the breeding for resistance and the effective spread of biocontrol agents. This knowledge could also give an indication as to where the fungus might have originated.

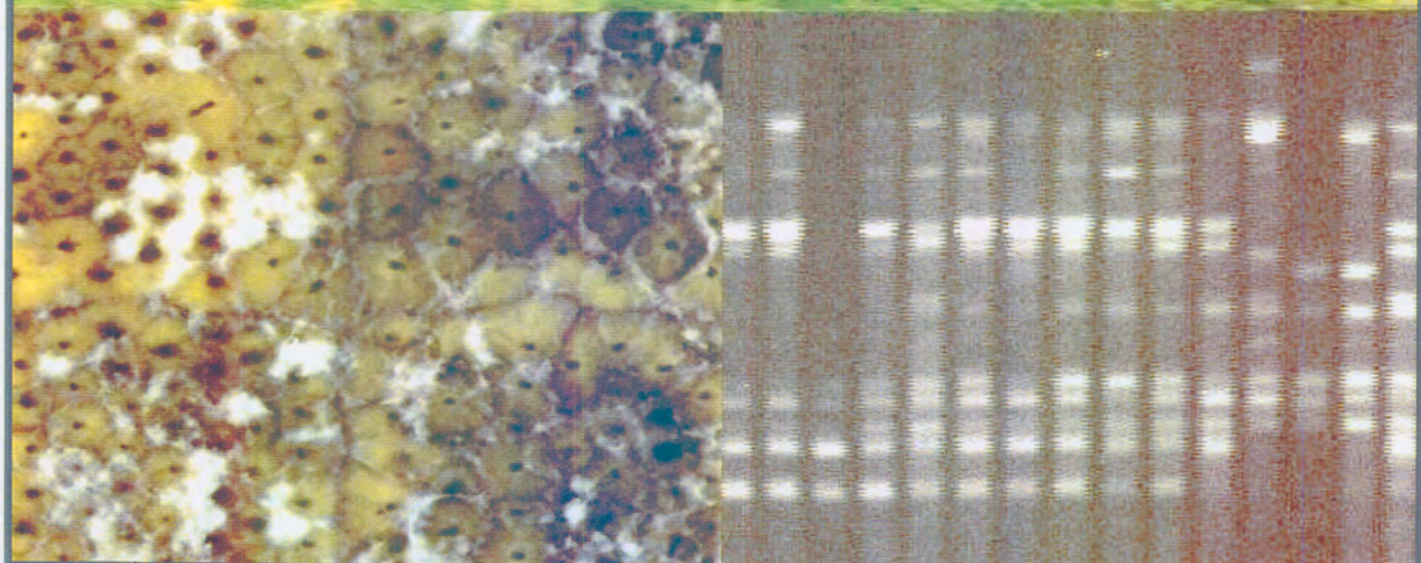
Sphaeropsis sapinea is well-known for its association with dsRNA fragments of which two have already been characterised at the DNA sequence level (SsRV1 and SsRV2). Chapter Five deals with the variation observed in size and number of different dsRNA fragments associated with *S. sapinea* isolates, from a wide range of regions. Cultural characteristics, *in vitro* growth rate and pathogenicity of the isolates were, furthermore, used to characterise the different phenotypes of the fungus that might be correlated to the presence of dsRNA.

In this thesis, I have attempted to increase the available knowledge on *S. sapinea*, using isolates from a wide variety of sources. It is my wish that data emerging from these studies will contribute to more effective management of diseases caused by this fungus. Ultimately, effective disease control will depend on an integrated management strategy that will require a comprehensive knowledge of the biology of *S. sapinea*.



CHAPTER 1

SPHAEROPSIS SAPINEA: AN IMPORTANT PATHOGEN OF CONIFERS



INTRODUCTION

Sphaeropsis sapinea (Fr.:Fr.) Dyko & Sutton is a well-known opportunistic pathogen that has been reported from virtually every country in the world where *Pinus* spp. are grown (Eldridge, 1961; Buchanan, 1967; Punithalingam & Waterston, 1970; Gibson, 1979; Swart, Knox-Davies & Wingfield, 1985). It is best known by its synonym *Diplodia pinea* (Desm.) Kickx., which is the causal agent of the disease commonly known as Diplodia die-back of pine (Waterman, 1943; Punithalingam & Waterston, 1970; Sutton, 1980). The fungus manifests itself, either as a dormant endophyte causing latent infections in its host (Smith *et al.*, 1996; Stanosz *et al.*, 1997), or as a saprophyte resulting in blue stain of timber (Birch, 1937; Laughton, 1937; Waterman, 1943; Da Costa, 1955; Millikan & Anderson, 1957; Eldridge, 1961; Buchanan, 1967; Marks & Minko, 1969; Peterson, 1977; Brown, Bevege & Stevens, 1981; Swart *et al.*, 1985; Swart & Wingfield, 1991b).

Two morphotypes of *S. sapinea* have been described based on morphological differences between isolates from the north central United States (Wang *et al.*, 1985; Wang, Blanchette & Palmer, 1986; Palmer, Stewart & Wingfield, 1987; Palmer, 1991). The criteria for the differentiation of the A and B morphotypes include colour and appearance of mycelium, dimensions of conidia, conidial-wall morphology and virulence. Other forms of the fungus may also exist in centres of diversity of *Pinus*.

Sphaeropsis sapinea causes extensive economic loss to commercial forestry where *Pinus* spp. are grown as exotics (Da Costa, 1955; Lückhoff, 1964; Buchanan, 1967; Punithalingam & Waterston, 1970; Currie & Toes, 1978; Gibson, 1979; Swart & Wingfield, 1991b; Zwolinski, Swart & Wingfield, 1990a&b). Currently, the exploitation of resistant pine species, together with optimal management, such as pruning at times when infection does not occur, are used to reduce the potential of pine plantations being infected by *S. sapinea* (Gibson, 1979; Burdon, Currie & Chou, 1980; Swart & Wingfield, 1991a). New possibilities for control have also recently been considered when dsRNA virus particles (VLPs) were found to occur naturally within some *S. sapinea* isolates (Wu, Palmer & Adams, 1989; Preisig, Wingfield & Wingfield, 1998; Steenkamp *et al.*, 1998). Biocontrol based on hypovirulence is, however, a new

and relatively unexplored field of study and much research remains to be done before VLP's can be exploited as mediators of hypovirulence.

Extensive reviews of the biology and taxonomy of *S. sapinea* have been published in the past (Waterman, 1943; Lückhoff, 1964; Chou, 1984; Swart *et al.*, 1985; Swart & Wingfield, 1991b). Since then, a considerable amount of new information regarding biocontrol through mycoviruses, genetic structure of geographically separated populations and the taxonomic status of the fungus have been added to the knowledge-base of this well-established pine pathogen. The aim of this review is to summarise all knowledge on *S. sapinea* and to provide a foundation to formulate an integrated disease management strategy for the associated disease.

TAXONOMY

Sphaeria pinea Desm. was first reported as pathogen of conifers in 1842 from *P. sylvestris* L. trees in France (Eldridge, 1961; Buchanan, 1967). Subsequent to this original description many synonyms have been used for this well-known pathogen, of which *Diplodia pinea* (Desm.) Kickx. is the most common (Waterman, 1943; Punithalingam & Waterston, 1970; Sutton, 1980). The name *D. pinea* was reviewed by Sutton (1980) and transferred to *Sphaeropsis* based on differences in conidium ontogeny between *Diplodia* and *Sphaeropsis*. At that time the genus *Diplodia* was characterised by the monoblastic formation of conidia, while *S. sapinea* forms its conidia through holoblastic, annelidic proliferation (Sutton, 1980; Minter, Kirk & Sutton, 1982).

Sphaeropsis sapinea lacks a teleomorph in its life-cycle and is, thus, classified under the Phylum Deuteromycotina, Class Coelomycetes (Sutton, 1980). Spermatia and spermatophores have, however, been observed on *P. elliotii* Engelm. et Vasey and *P. taeda* L. in South Africa, which may indicate the existence of a reduced sexual phase (Wingfield & Knox-Davies, 1980a). The spermatia differ from the conidia or asexual spores in that they are smaller (2.4-6.0 x 0.9-1.8µm), hyaline, cylindrical and have flattened ends where they attach to the spermatophores (Wingfield & Knox-Davies, 1980a).

Sphaeropsis sapinea produces conidia (30-45 x 10-16µm) in small black pycnidia (up to 250µm) that can occur separately or in groups on stems, needles or foliar parts of the host (Fig. 1) (Haddow & Newman, 1942; Buchanan, 1967; Punithalingam & Waterston 1970; Sutton, 1980; Swart, Wingfield & Grant, 1993). Each pycnidium produces 400-500 thick-walled, usually non-septate, yellow to brown-coloured conidia that darken with age (Haddow & Newman, 1942). The conidia are oblong to clavate with blunt basal ends and rounded at the apex (Barker, 1979).

The A and B morphotypes of *S. sapinea* can be distinguished based on cultural characteristics, conidial morphology and virulence among isolates originating from the north Central United States (Wang *et al.*, 1985; Wang *et al.*, 1986; Palmer *et al.*, 1987; Palmer, 1991). The A morphotype isolates have fluffy white to grey-green mycelium, while the B morphotype isolates have white to black mycelium that is appressed to the agar surface (Wang *et al.*, 1985; Smith & Stanosz, 1995). The conidia of A morphotype isolates are slightly larger (33.5-34.3 x 12.6-12.8µm), with smooth walls compared to conidia of B morphotype isolates that are smaller (33.5-34.3 x 11.6-12.1µm) and have pitted walls (Wang *et al.*, 1985; Smith & Stanosz, 1995). Sporulation of A morphotype cultures is light-dependant, while light is not necessary for sporulation of B morphotype cultures (Palmer *et al.*, 1987; Smith & Stanosz, 1995). While isolates representing the A morphotype have been collected from various pine species, world-wide, isolates representing the B morphotype display host specificity towards *P. banksiana* Lamb. and *P. resinosa* Aiton. from the north central United States (Wang *et al.*, 1985; Smith & Stanosz, 1995). Furthermore, the A morphotype is more virulent than the B morphotype (Wang *et al.*, 1985 & 1986; Palmer *et al.*, 1987; Palmer, 1991; Smith & Stanosz, 1995). The difference in virulence between the two morphotypes of *S. sapinea* could potentially impact on the choice of a suitable management strategy (Swart & Wingfield, 1991b; Stanosz, Smith & Guthmiller, 1996).

Isozyme profiles were initially used to distinguish between the A and B morphotypes of *S. sapinea* (Palmer *et al.*, 1987; Swart *et al.*, 1991). Isozymes, or isofunctional (iso = constant) enzymes, are different forms of the same enzyme that catalyse different reactions, they are consistently found in all organisms and are highly polymorphic between organisms (Glynn & Reid, 1969). Palmer *et al.* (1987) distinguished between

authentic A and B morphotype isolates from the north central United States based on two polymorphic isozymes, alcohol dehydrogenase and sorbitol dehydrogenase, from a total of six isozymes tested. Swart *et al.* (1991) compared the isozyme profiles of South African *S. sapinea* isolates with those of isolates representing the A and B morphotypes from the USA. A general conclusion was that all South African isolates probably represent the A morphotype of *S. sapinea* and that conidial wall morphology is a poor characteristic on which to differentiate between the two morphotypes of the fungus.

The existence of two distinct “types” of *S. sapinea* has been confirmed using random amplified polymorphic DNA (RAPDs), coupled with cluster and parsimony analysis (Smith & Stanosz, 1995; Stanosz *et al.*, 1996). Smith & Stanosz (1995) produced polymorphic fragments, using eight RAPD primers, that serve as specific fingerprints to distinguish between the A and B morphotypes, and to determine the degree of similarity between them. They also showed that north central United States isolates belonging to the A morphotype are more similar to each other (>85% similarity) than to isolates in the B morphotype (<59% similarity) (Smith & Stanosz, 1995).

ECOLOGY, GEOGRAPHICAL DISTRIBUTION AND HOST RANGE

In early reports of *S. sapinea* (as *D. pinea*), the fungus was mostly considered to be saprophytic on pine timber and one of the main agents of blue stain (Birch, 1937; Laughton, 1937; Waterman, 1943; Da Costa, 1955; Millikan & Anderson, 1957; Eldridge, 1961). In 1912 it was shown to be pathogenic on various *Pinus* spp. in South Africa (Fisher, 1912). Subsequent to these early reports, both the A and B morphotypes of *S. sapinea* have been shown to be pathogenic to wide range of *Pinus* spp. world-wide (Wang *et al.*, 1985 & 1986; Palmer *et al.*, 1987; Palmer, 1991).

Sphaeropsis sapinea is generally considered to be an opportunistic pathogen that initiates infection if the defence mechanisms of the host are weakened and conditions are favourable for infection (Waterman, 1943; Buchanan, 1967; Marks & Minko, 1969; Peterson, 1977; Brown *et al.*, 1981; Swart *et al.*, 1985; Swart & Wingfield, 1991b).

Recently, *S. sapinea* was also shown to be able to exist as a symptomless endophyte in pine cones and stems of healthy pine seedlings and mature trees (Smith *et al.*, 1996; Stanosz *et al.*, 1997). As an endophyte, the fungus causes latent infections in healthy pine tissue and disease symptoms develop only when trees are stressed (Smith *et al.*, 1996; Stanosz *et al.*, 1997).

Sphaeropsis sapinea is a ubiquitous fungus that has been reported from various conifers in 25 countries between latitudes of 50° north and 25° south (Eldridge, 1961; Buchanan, 1967; Punithalingam & Waterston, 1970; Gibson, 1979; Swart *et al.*, 1985). It is well-known for its association with members of the genus *Pinus*, but it has also been reported on other members of the Coniferophyta such as *Abies*, *Larix*, *Picea*, *Thuja* and *Pseudotsuga* (Eldridge, 1961; Buchanan, 1967; Punithalingam & Waterston, 1970; Gibson, 1979; Swart, Wingfield & Knox-Davies, 1988). Young, exotic *P. radiata* and *P. patula* plantations are especially susceptible to infection, making it a matter of great concern and importance in South Africa, New Zealand and Australia where these two species dominate the forestry industry (Lückhoff, 1964; Buchanan, 1967; Punithalingam & Waterston, 1970; Currie & Toes, 1978; Gibson, 1979; Zwolinski *et al.*, 1990a&b).

The 30 *Pinus* spp. susceptible to *S. sapinea* infection show variation in their degree of tolerance to this fungus (Gibson, 1979; Rees & Webber, 1988). *Pinus radiata*, *P. pinaster* Ait. and *P. patula* are most susceptible species, while *P. elliotii* Engelm. et Cham., and *P. taeda* L. are more resistant to *S. sapinea* infection (Laughton, 1937; Waterman, 1943; Lückhoff, 1964; Van der Westhuizen, 1968; Punithalingam & Waterston, 1970; Gibson, 1979; Brown *et al.*, 1981; Swart *et al.*, 1988). Susceptibility is, however, highly variable and influenced by external factors such as temperature, moisture availability and the turgor pressure of the tissue (Stahl, 1968).

EPIDEMIOLOGY

Sphaeropsis sapinea is a weak or opportunistic pathogen that requires suitable conditions to initiate infection (Birch, 1937; Laughton, 1937; Waterman, 1943; Buchanan, 1967; Marks & Minko, 1969; Brown *et al.*, 1981). Environmental

conditions, physical wounding and poor management are the three major factors that can predispose conifers to infection. Environmental conditions favouring infection include drought, hail, extreme temperatures and long photoperiods (Laughton, 1937; Waterman, 1943; Millikan & Anderson, 1957; Stahl, 1968; Wright & Marks, 1970; Nicholls, 1977; Bega *et al.*, 1978; Wingfield & Knox-Davies, 1980a&b; Brown *et al.*, 1981; Swart *et al.*, 1985; Chou, 1987; Nicholls & Ostry, 1990; Zwolinski *et al.*, 1990a&b; Palmer, 1991; Blodgett & Stanosz, 1993). Physical wounding also plays an important role and is mainly caused by hail, cambiphagous insects and pruning (Laughton, 1937; Haddow & Newman, 1942; Gilmour, 1964; Bega *et al.*, 1978; Barker, 1979; Nicholls, 1977; Brown *et al.*, 1981; Palmer, 1991). Poor management can lead to over-stocking and nutrient deficiencies as a result of competition and thus infection (Lückhoff, 1964; Barker, 1979; Wingfield & Knox-Davies, 1980a; Brown *et al.*, 1981).

Agents of predisposition, such as drought, initiate *S. sapinea* infection and enhance fungal growth in the host tissue by means of reducing host defence mechanisms, making them less fit to cope with, otherwise, latent infections of the fungus (Schoeneweiss, 1975). The reduction in host defences is postulated to be the result of a reduction in the monoterpene content, that is toxic to the fungus, in pine oleoresin (Smith, 1964; Chou & Zabkiewicz, 1976; Brown *et al.*, 1981; Chou, 1982). Enhancement in fungal growth can, also, be the result of an increase in the availability of the carbohydrate pool to the fungus, in drought-stressed tissue (Bachi & Peterson, 1985). Wounds caused by hail, insect feeding and pruning not only reduce the defence mechanisms, but also provide entry sites for water- or wind-dispersed conidia (Birch, 1937; Laughton, 1937; Waterman, 1943; Buchanan, 1967; Marks & Minko, 1969; Brown *et al.*, 1981).

The principal means of dispersal of *S. sapinea* is through conidia-bearing raindrops blown by wind to susceptible hosts (Laughton, 1937; Eldridge, 1961; Buchanan, 1967; Brookhouser & Peterson, 1971; Peterson, 1977; Swart *et al.*, 1985; Swart & Wingfield, 1991a&b). Spore dispersal takes place only after conidial maturation, when the conidia ooze, in mucilaginous masses, from the ostioles of pycnidia, onto the surface of needles (Haddow & Newman, 1942; Eldridge, 1961; Brookhouser & Peterson, 1971). As the conidia dry, they stick firmly to one another and to the host tissue, from where infection can be initiated (Eldridge, 1961; Brookhouser & Peterson, 1971).

Infection of non-wounded pine tissue by *S. sapinea* occurs through stomatal pits and not directly through the epidermis (Brookhouser & Peterson, 1971). Conidia reach maturity in the Spring or early Summer and germinate in the summer when the temperatures and relative humidities are high (Buchanan, 1967; Van der Westhuizen, 1968; Brookhouser & Peterson, 1971; Peterson, 1977; Chou, 1982). The germ tubes enter the host via open stomata, and the fungus grows into the mesophyll from where the colonisation spreads to the phloem and cortical tissue (Laughton, 1937). It is assumed that the fungus produces toxins or antiauxins that disrupt the normal cambial function leading to bark and cambial necrosis, discoloration of the needles (Marks & Minko, 1969; Brookhouser & Peterson, 1971) and finally girdling of the shoots and loss of needles (Laughton, 1937; Brown *et al.*, 1981).

Insects do not play a direct role in the dispersal of *S. sapinea* conidia, but facilitate the further colonisation of healthy cambial tissue and enhance the general severity and impact of the disease (Haddow & Newman, 1942; Wingfield & Palmer, 1983; Zwolinski, Swart & Wingfield, 1994). In the north central United States, the eastern pine shoot borer (*Eucosma gloriola* Heinr.), pitch nodule maker (*Petrova albicapitana* Busk.), red pine shoot moth (*Dioryctria resinosella* Mat.) and the pine root weevil (*Hylobius rhizophagus* Mil., Benj., & Warn.) are always associated with *S. sapinea* on *P. banksiana* Lamb. (jack pine) and *P. resinosa* Aiton (red pine) (Wingfield & Palmer, 1983). In South Africa, only two cambiphagous insects, *Pissodes nemorensis* and *Orthotomicus erosus*, have consistently been associated with *S. sapinea* (Zwolinski *et al.*, 1994).

Mortality due to *S. sapinea* infection is high, especially in plantations of susceptible species, but complete recovery of infected trees can occur (Marks & Minko, 1969; Nicholls & Ostry, 1990). The mechanism of recovery is unknown, but as soon as stress is eliminated and the conditions become favourable, tree growth resumes and fungal development ceases (Laughton, 1937; Palmer, 1991). Generally, the host responds to fungal infection by forming callus that impairs or arrests further formation of cankers and by forming lateral branches to replace dead leaders in trees with die-back (Laughton, 1937; Palmer, 1991).

DISEASE SYMPTOMS

The most common symptoms associated with *S. sapinea* infection are post-hail associated die-back or dead-top of pines (Millikan & Anderson, 1957) and blue stain of pine timber (Da Costa, 1955; Eldridge, 1961) (Fig. 2). There are, however, reports of various other, less conspicuous, disease symptoms such as twig blight (Buchanan, 1967), shoot blight (Purnell, 1957), whorl cankers (Gilmour, 1964) and recurved stems (Marks & Minko, 1969). Disease symptoms appear four to ten days after initiation of infection, providing environmental conditions are optimal for germination of the conidia and growth of the fungus (Brookhouser & Peterson, 1971; Peterson, 1977; Chou, 1982; Swart, Wingfield & Knox-Davies, 1987a&b).

- **Die-back and shoot blight**

Die-back and shoot blight, caused by *S. sapinea*, leads to a loss in the normal growth of terminal shoots (Fisher, 1912; Nicholls, 1977; Peterson, 1977). Wright and Marks (1970) reported a reduction of up to 40% in growth of dead-topped trees in comparison with unaffected trees. Die-back is characterised by shrivelled terminal shoots that are brittle and bend over, as well as needles that become brown and fall (Fisher, 1912; Eldridge, 1961; Nicholls, 1977; Peterson, 1977). Resin is exuded on these shriveled shoots and the remaining needles (Fisher, 1912; Peterson, 1977). On the inside, the infected woody tissue will turn to a purple-brown colour and after a few weeks, pycnidia are formed (Swart *et al.*, 1985).

Post-hail associated *S. sapinea*-induced die-back is common in stands of the more susceptible *P. radiata* and *P. patula* species (Zwolinski, *et al.*, 1990a&b). Devastation and large scale timber losses of *P. radiata* following hailstorms, for example, have been recorded from the southern Cape Province of South Africa (Zwolinski, *et al.*, 1990a&b). The disease is characterised by drastic reduction in the total and green foliage six weeks after a hail storm. The highest degree of leader mortality occurs four months after a hail storm and lasts up to one year, after which regeneration of the foliage occurs (Zwolinski, *et al.*, 1990a&b).

- **Whorl cankers and crown wilt**

Sphaeropsis sapinea-induced cankers on *Pinus* spp. reduce normal growth, whilst in severe cases, where cankers girdle trees, mortality is observed (Nicholls, 1977). Severe whorl cankers can lead to crown wilt where a portion of the crown is killed (Chou, 1984). Cankers are elongated, depressed areas at the whorl of the tree originating from infected cambium and cortical tissue (Gilmour, 1964; Marks & Minko, 1969; Swart *et al.*, 1985). Resin is exuded from the dead areas of cambium and the tissue becomes necrotic (Wright & Marks, 1970; Palmer, 1991).

- **Collar rot and a root disease**

Sphaeropsis sapinea is known to cause collar rot in seedlings (Punthalingham & Waterson, 1970) and to kill plants after infection in the root crown area (Waterman, 1943). Collar rot is characterised by the discoloration of the root collar area followed by foliage chlorosis and exudation of resin (Swart *et al.*, 1985). In South Africa and Swaziland *S. sapinea* was reported to cause a root disease in *P. elliottii* and *P. taeda* (Wingfield & Knox-Davies, 1980a; Swart *et al.*, 1985 & 1987a&b). This disease is characterized by dark blue, resinous lesions on radial parts of lateral roots that can extent to a height of two meters up the tree trunk (Wingfield & Knox-Davies, 1980a).

- **Blue stain**

Blue stain is the result of the saprophytic nature of *S. sapinea* and is characterised by the blue to black discoloration of the pine timber that leads to its devaluation (Laughton, 1937; Da Costa, 1955; Eldridge, 1961). The discoloration is the result of fungal mycelium that grows in the ray parenchyma after penetrating the sapwood through the inner bark and cambium (Da Costa, 1955; Eldridge, 1961). However, the strength of the wood is not affected, as the lignified walls of the infected pine logs remain intact and free from mycelial colonisation (Da Costa, 1955).

POPULATION GENETICS

Genetic diversity in a fungal population refers to the degree of genetic variation among a group of individuals that constitute that population (Burdon & Silk, 1997). In sexual-reproducing populations, a high degree of diversity is normally observed as a result of meiosis and consequent nuclear exchange between individuals (Perkins, 1991). In contrast, asexually reproducing populations are expected to have a lower degree of diversity due to the absence of meiosis (Perkins, 1991). In such populations recombinants are, however, generated through heterokaryosis, whereby heterokaryons (multinucleate cells with variable chromosome numbers) are formed (Burdon & Silk, 1997), parasexual cycles of somatic diploidisation, mitotic cross-over and haploidisation (Fincham & Day, 1965; Leung & Williams, 1986; Leslie, 1993; Burdon & Silk, 1997), and mutagenesis in certain loci (Burdon & Roelfs, 1985). These alternative recombinant-generating strategies, however, occur rarely, since individuals in asexual populations have the advantage of gaining immortality in a certain niche (Perkins, 1991). Individuals in populations with a low population diversity is, however, faced with extinction in changing environmental conditions, as they do not have the ability to pool their genes to adapt to changes (Perkins, 1991).

Sphaeropsis sapinea lacks the ability to generate heterogeneity through meiosis and is thus assumed to have a highly uniform genetic structure (Coates, 1988). For the same reason, it is assumed that an introduced population of this fungus will have less diversity than the native population where the fungus originated and has more time to become differentiated. This assumption was, however, recently contradicted when the degree of diversity in a South African and Indonesian *S. sapinea* population was determined through the use of vegetative compatibility groups (VCGs) (Smith *et al.*, 2000). A higher population diversity was found in the introduced South African population in comparison to one from Indonesia where native *Pinus* spp. are known to occur (Smith *et al.*, 2000). The larger number of VCGs in South Africa must be attributed to multiple introductions of the pathogen with pine seed imports into South Africa from many parts of the world (Smith *et al.*, 2000).

DISEASE MANAGEMENT

The most efficient and practical disease management strategy to date is through planting of pine species that are more resistant to *S. sapinea* infections (Lückhoff, 1964; Wright and Marks, 1970; Gibson, 1979; Burdon *et al.*, 1980; Swart *et al.*, 1985; Swart & Wingfield, 1991a). Pruning, scheduled for times of the year when infection by the pathogen is not likely to occur, is another management strategy that reduces the possible initiation of host predisposition through stress (Swart & Wingfield, 1991a). Careful site selection is, furthermore, needed to minimise mechanical damage and to compensate for the influence of environmental factors (Swart & Wingfield, 1991a). In the summer rainfall areas of South Africa (south-western and southern Cape) where hail storms frequently occur the more susceptible *P. radiata* and *P. patula* species are, for example, substituted by the more resistant *P. elliottii* (Swart *et al.*, 1987a&b; Swart & Wingfield, 1991a). Despite these current integrated management strategies to control *S. sapinea*, enormous economic losses are still suffered annually (Zwolinski *et al.*, 1990a).

A reduction in virulence mediated through virus-like particles (VLPs) is known as hypovirulence, one of the most recent advances towards successful biocontrol of destructive fungal plant pathogen populations (Day *et al.*, 1977; Elliston, 1981; Buck, 1986; Boland, 1992). The best studied example of hypovirulence was successfully implemented as biocontrol strategy is in *Cryphonectria parasitica* (Murrill) M.E. Barr., causal agent of the notorious disease known as chestnut blight (Day *et al.*, 1977; Anagnostakis, 1982; Fulbright *et al.*, 1983). The natural occurrence of dsRNA virus-like particles in the cytoplasm of this pathogen was linked to the observation of reduced virulence and altered phenotypes in virus-infected strains (Day *et al.*, 1977). These dsRNA virus-like particles are being exploited as biological control agents to prevent the elimination of the American chestnuts (Anagnostakis, 1982; Nuss, 1992).

Recently, dsRNA was isolated from isolates representing both morphotypes of *S. sapinea* (Wu *et al.*, 1989; Steenkamp *et al.*, 1998; Preisig *et al.*, 1998). Subsequently, the dsRNA genomes of two viral fragments co-infecting South African *S. sapinea* isolates were characterised and designated SsRV1 and SsRV2 (Preisig *et al.*, 1998).

Their presence has, however, not been linked to any phenotypic changes in the pathogen or to a reduction in virulence (Wu *et al.*, 1989; Preisig *et al.*, 1998; Steenkamp *et al.*, 1998). The slower growth observed in the USA B morphotype of *S. sapinea* could also not be linked to the presence of these dsRNA virus particles (Wu *et al.*, 1989).

The SsRVs in *S. sapinea* forms part of the family Totiviridae, and not the Hypoviridae such as the hypovirulence-related dsRNA viruses of *C. parasitica* (Preisig *et al.*, 1998). The SsRVs, however, have the potential of being implemented as vectors for genetic manipulation of *S. sapinea* in order to reduce its impact on commercial forestry in exotic pine plantations (Preisig *et al.*, 1998; Steenkamp *et al.*, 1998). Hypovirulence-conferring genes isolated from the dsRNA viruses of *C. parasitica* could, for example, be incorporated into the viral genomes of the SsRVs. These genetically altered SsRVs might then be introduced back in *S. sapinea* populations in order to induce hypovirulence. Effective transmission of such dsRNA-containing biocontrol agents, however, requires a relative low degree of genetic diversity in a population of the pathogen (Caten, 1972). The high diversity in the South African population of *S. sapinea* (Smith *et al.*, 2000) would, therefore, reduce the efficacy of such a control strategy (Caten, 1972; Anagnostakis, Hau & Kranz, 1986). Mechanical spread via fungal protoplast systems could, however, be used as an alternative method of spreading biocontrol agents in highly diverse populations (Lhoas, 1971; Ghabrial, 1980).

CONCLUSIONS

Sphaeropsis sapinea is an opportunistic, cosmopolitan pathogen of conifers, and is relatively well controlled. In South Africa, *S. sapinea* is, however, considered to be one of the most important pathogens of *P. patula* and *P. radiata* after the onset of stress through hail (Swart *et al.*, 1987a&b; Zwolinski *et al.*, 1990a). Timber losses due to post-hail associated die-back have been estimated at more than half the potential value of wood produced and about a quarter of the potential volume (Swart *et al.*, 1987a; Zwolinski *et al.*, 1990a). Tremendous economical losses suffered in the summer rainfall areas of South Africa, where these susceptible pine species are widely planted,

requires a re-examination of the current status of the fungus and the formulation of an integrated disease management strategy. Of all the recent advances in this regard, the most notable are as follows:

- Two types of *S. sapinea* have been described based on morphological criteria. The possibility of the existence of additional types cannot be excluded, simply because morphological criteria are not significantly robust to detect them. The development of molecular techniques provides a tool for studying and re-evaluating the current taxonomy of *S. sapinea*, which will improve strategies to exclude new genotypes from entering new environments.
- The discovery of virus-like particles (VLPs), as natural inhabitants of the cytoplasm of *S. sapinea*, presents a novel means to reduce the impact of this destructive pine pathogen. Although, these dsRNA particles have not been linked to hypovirulence in *S. sapinea*, their presence alone provides a tool for genetic manipulation.
- Information on the genetic structure of the fungal population is of great importance to determine the long-term durability of control strategies. The *S. sapinea* population in South Africa, for example, has a very diverse genetic structure, which increases the ability of the fungus to overcome resistance in *Pinus* spp. Biocontrol via dsRNA is furthermore, hampered in the South African population of the fungus, by the large number of VCGs that exist in that population.

Successful management of *S. sapinea* in exotic forestry plantations has not, as yet, been fully achieved. This is probably due to a lack of knowledge of the current taxonomy of the fungus, the genetic status of *S. sapinea* populations and the association with dsRNA viral fragments that could be exploited for biocontrol. This review is the first step towards the ultimate goal of formulating an integrated management strategy for *S. sapinea*, especially in South Africa and other regions dependent on exotic pines for commercial forestry.

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Figure 1. Morphology of *Sphaeropsis sapinea* (A) Dark-coloured, thick-walled conidia.
(B) Asexual fruiting body (pycnidium) with excised conidia.

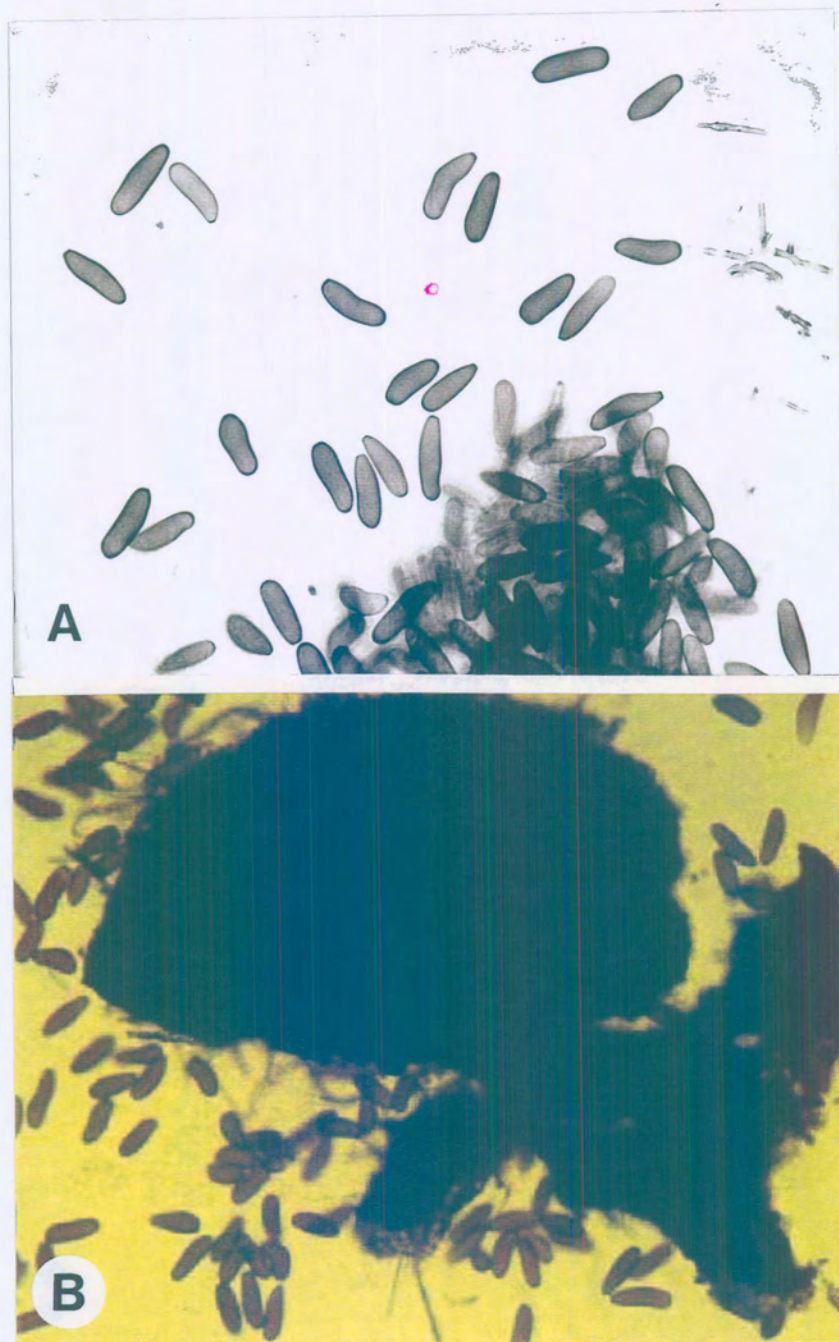
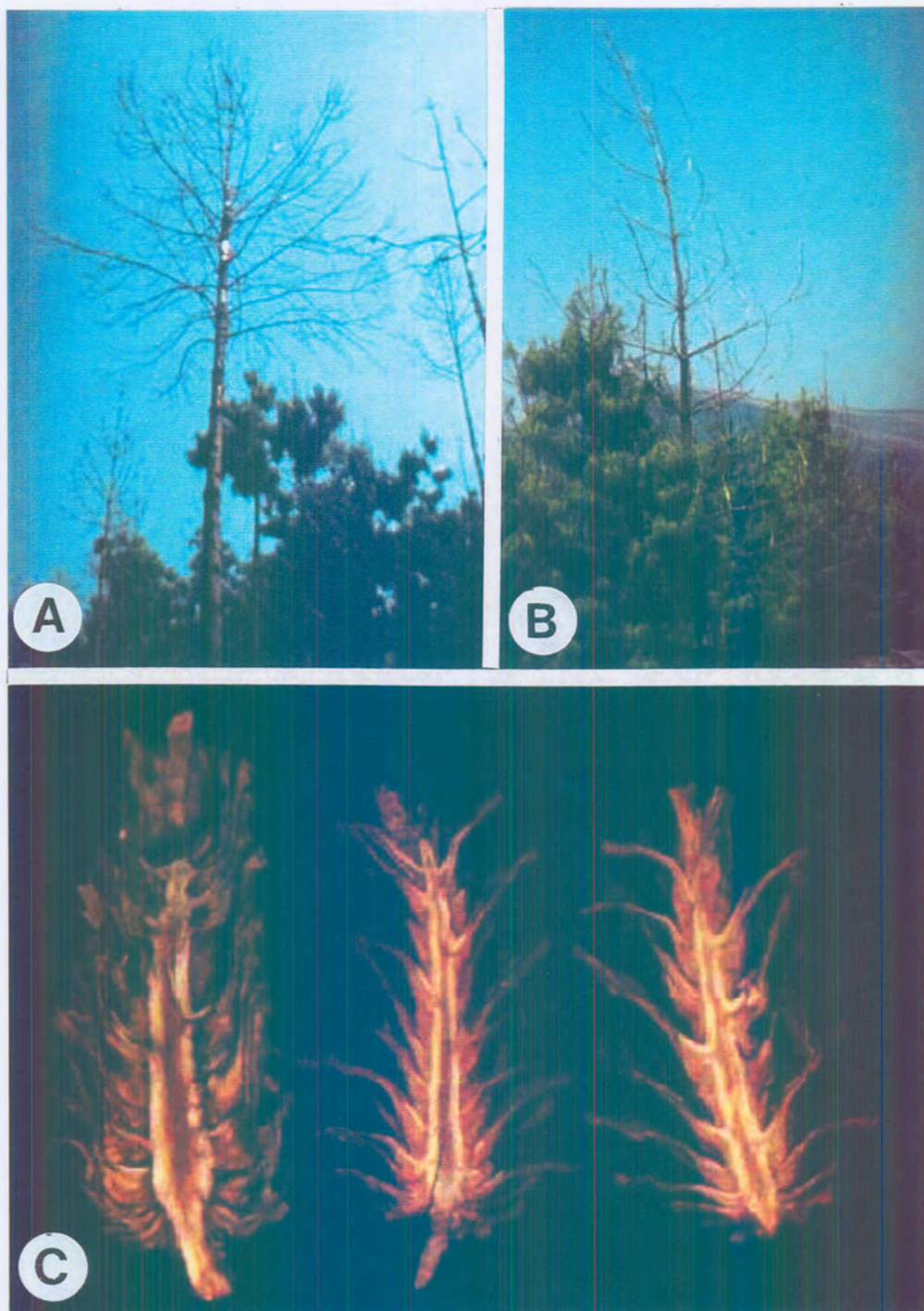
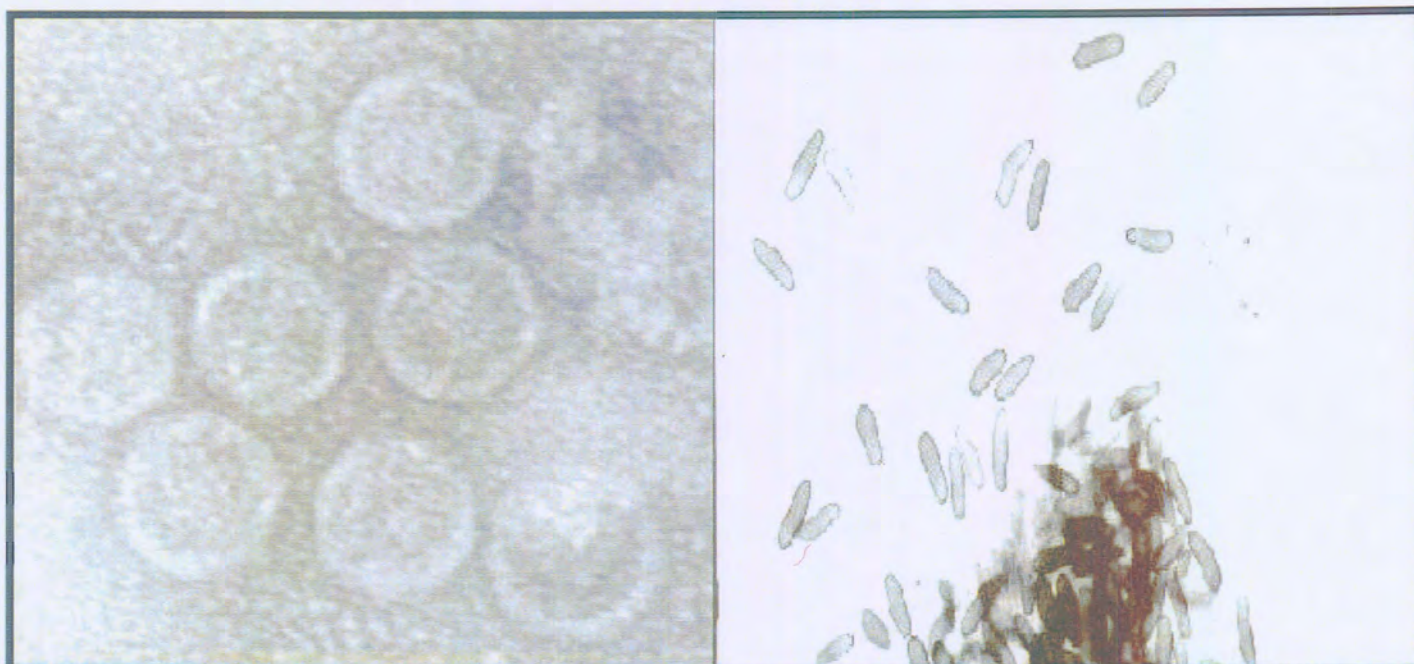


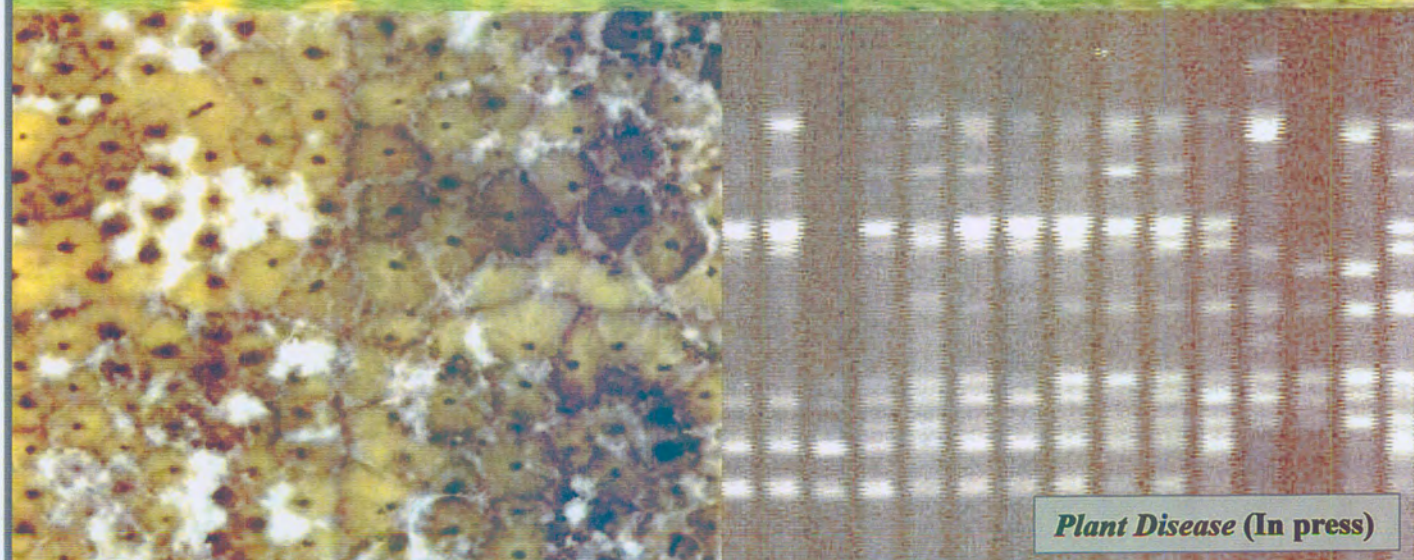
Figure 2. Disease symptoms associated with *Sphaeropsis sapinea*. (A) & (B) Die-back of the leader shoots of pines, usually associated with severe hail damage. (C) Discoloration of the pith tissue of pine cones. This discoloration is know as blue stain in pine timber.





CHAPTER 2

CHARACTERISATION OF *SPHAEROPSIS* *SAPINEA* ISOLATES FROM SOUTH AFRICA, MEXICO AND INDONESIA



Plant Disease (In press)



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ABSTRACT

De Wet, J., Wingfield, M.J., Coutinho, T.A., and Wingfield, B.D. (2000). Characterisation of *Sphaeropsis sapinea* isolates from South Africa, Mexico and Indonesia. *Plant Disease* (In press).

Post-hail associated die-back of *P. patula* and *P. radiata* trees, induced by *Sphaeropsis sapinea* (= *Diplodia pinea*), is a common and important disease in commercial pine plantations. Two morphotypes, A and B, have been described for this fungus based on differences in cultural characteristics, conidial morphology and virulence among isolates from the north central United States. The existence of the two described morphotypes was later verified through the use of random amplified polymorphic DNA (RAPD) analyses and designated as the A and B RAPD marker groups. The objective of this study was to characterise a set of *S. sapinea* isolates from South Africa, Indonesia and Mexico using RAPD analysis and DNA sequencing of the internal transcribed spacer (ITS) region of the ribosomal DNA operon. Sizes of conidia and culture morphology were, furthermore, used to compare the three groups of *S. sapinea* isolates that emerged from the RAPD analysis. Two of the RAPD groups included isolates from the United States, representing the A and B morphotypes, while the third RAPD group accommodated seven Indonesian and one Mexican isolate. ITS sequences of all the *S. sapinea* isolates were highly homologous and resolved only the A and B RAPD groups. The ITS sequences of the isolates in the third RAPD group were the same as those of the A RAPD group. Conidia of isolates representing the A and B morphotypes were approximately the same size but those of the third RAPD group were significantly longer. RAPD analysis had enabled us to identify a third group of *S. sapinea* that is different to the well-recognised A and B groups. Isolates in this third RAPD group also have a distinct morphological characteristic (length of conidia) and thus represent a third discrete morphological group, which we refer to as the C morphotype of *S. sapinea*.

INTRODUCTION

Sphaeropsis sapinea (Fr.:Fr) Dyko & Sutton (= *Diplodia pinea* (Desmaz) J. Kickx. Fl.) is an opportunistic pathogen of conifers (Birch, 1937; Buchanan, 1967; Marks & Minko, 1969; Brown, Bevege & Stevens, 1981). It has a world-wide distribution and is well-known for the damage that it causes to exotic pine plantations (Purnell, 1957; Eldridge, 1961; Gibson, 1980; Swart, Knox-Davies & Wingfield, 1985). In South Africa, the fungus is an important die-back pathogen of various *Pinus* spp. (Swart, Wingfield & Knox-Davies, 1987; Swart & Wingfield, 1991). Predisposition of the host plays an essential role in the initiation of *S. sapinea* infections by reducing the tree resistance or by providing infection sites (Waterman, 1943; Lückhoff, 1964; Marks & Minko, 1969). Predisposition factors include extreme environmental conditions or physical wounding through hail and pruning (Bega *et al.*, 1978; Nicholls & Ostry, 1990; Swart & Wingfield, 1991). In South Africa, the common occurrence of hailstorms enhances the susceptibility of *Pinus radiata* D. Don. and *P. patula* Schl. & Cham. trees to infection by *S. sapinea*, resulting in substantial timber losses each year (Lückhoff, 1964; Gibson, 1980; Zwolinski, Swart & Wingfield, 1990).

Infection by *S. sapinea* results in various disease symptoms, of which die-back is the most common (Millikan & Anderson, 1957). Other symptoms frequently associated with *S. sapinea* infection include whorl cankers (Marks & Minko, 1969; Wright & Marks, 1970), root disease (Wingfield & Knox-Davies, 1980) and crown wilt (Chou, 1982). The fungus was also found as a saprophytic inhabitant of sapwood, resulting in blue stain (Laughton, 1937). In cone and stem tissue of healthy pine trees, *S. sapinea* was found to exist as an endophyte (Smith *et al.*, 1996; Stanosz *et al.*, 1997). As an endophyte, the fungus is in a latent state, and only results in disease symptoms if the host becomes stressed.

Two morphotypes of *S. sapinea* have been described in the United States (Wang *et al.*, 1985; Palmer, Stewart & Wingfield, 1987). These are characterised based on differences in conidial dimension, cell wall morphology, mycelial appearance, growth rate in culture, virulence and isozymes (Wang *et al.*, 1985; Palmer *et al.*, 1987; Swart *et al.*, 1991). The A morphotype has fluffy white to grey-green mycelium and grows

faster than the B morphotype. The B morphotype has white to black mycelium appressed to the agar and grows more slowly than the A morphotype. The A morphotype was also reported to be more virulent than the B morphotype. The categorization of *S. sapinea* isolates from the United States into two groups based on morphology is furthermore supported through RAPD analysis (A and B RAPD marker groups) (Smith & Stanosz, 1995; Stanosz, Smith & Guthmiller, 1996; Blodgett & Stanosz, 1999).

While a substantial effort has been made to characterise isolates of *S. sapinea* in the United States by categorising them into groups based on morphology, virulence and RAPDs, very little work has been conducted on differentiation within the species from other parts of the world. In South Africa, some isolates have the same characteristics as A morphotype isolates from the United States based on isozyme profiles, but no South African isolates were found to have characteristics typical of B morphotype isolates (Swart *et al.*, 1991). The objective of this study was to characterise a set of *S. sapinea* isolates from South Africa, Mexico and Indonesia, based on comparisons with standard isolates from the United States representing the A and B RAPD marker groups using RAPD analysis, as previously described (Smith & Stanosz, 1995), ITS sequence comparisons, as well as, culture and conidial morphology.

MATERIALS AND METHODS

Fungal isolates

A set of 20 *S. sapinea* isolates obtained from various sources was used in this study (Table 1). The South African and Mexican isolates were obtained by direct isolation from the pith tissue of *P. radiata* and *P. greggii* Engelm. cones, respectively, while the Indonesian isolates were obtained from pycnidia on *P. patula* shoots with die-back symptoms. *Sphaeropsis sapinea* isolates from the United States representing the A and B RAPD groups were supplied by Dr. M. Palmer and Dr. G. Stanosz, and were used as comparative standards in all tests (Table 1). All the isolates are maintained in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Isolates were transferred to 2% water agar (WA) (Biolab), supplemented with sterile pine needles, and incubated at 25°C in constant light, to induce sporulation. After positive identification based on conidial and pycnidial morphology (Sutton, 1980), single conidial isolates were generated, and these were grown on 2% malt extract agar (MEA) (Biolab) at 25°C in the dark. All *S. sapinea* isolates were transferred to 2% MEA slants and stored at 4°C.

DNA extractions

DNA was extracted from the freeze-dried mycelium of 20 single conidial isolates of *S. sapinea*, from various parts of the world (Table 1), using the technique described by Raeder and Broda (1985). The isolates were grown on 2% MEA Petri dishes (9 cm) in the dark at 25°C, one week prior to the DNA extraction. The DNA concentrations of the samples were determined using a spectrofluorometer and diluted to 5 ng/μl for use in RAPD analysis.

RAPD analyses

Primers DS2 (5'-CTGCGACTGAATCTTGCAG) and DS3 (5'-GCGAAATGTGTCCTTTGATG) (Smith & Stanosz, 1995) were used to differentiate between the A and B morphotypes of *S. sapinea*. The PCR conditions and primers used were those described by Smith & Stanosz (1995) (first amplification: 3.75 mM MgCl₂, second amplification: 1.5 mM MgCl₂). To ensure that the RAPD banding profiles were reproducible, amplification reactions were repeated several times, with separately extracted DNA. DNA fragments were separated on an 0.8% agarose (w/v) gel (Promega) containing ethidium bromide (10 μg/ml). From each sample, 15 μl was electrophoresed in Tris-boric acid-EDTA (TBE) (pH 8.0) electrophoresis buffer at 100 Volts for 2-3 hours, and the separated fragments were visualised under UV light.

The RAPD banding profiles for each primer were scored for the presence or absence of bands. The banding patterns of *S. sapinea* isolates from South Africa, Indonesia and Mexico were compared with those known to be of the A group (CMW190, CMW4329, CMW4332) and the B group (CMW189, CMW4333, CMW4334). The data set was

analysed using the phylogenetic package PHYLIP version 3.5. The distance matrix was calculated through DNADIST using Kimura-2 as parameter, and an unrooted tree was generated using the Neighbor-Joining method.

Sequencing

The internally transcribed spacer (ITS) region of 20 *S. sapinea* isolates (Table 1), was amplified using primers ITS1 (5'-TCCGTAGGTGAACCTGCGGG-3') and ITS4 (5'-GCTGCGTTCTTCATCGATGC-3') (White *et al.*, 1990). The following temperature profile was used: 3 minutes initial denaturation at 93°C, followed by 35 cycles of 45 seconds at 58°C, 1½ minute at 72°C and 30 seconds at 92°C. The final cycle consisted of a 15 minute primer extension-step at 72°C. A 50 µl reaction mixture was used, containing: 5 Units *Taq* polymerase, 10xPCR buffer (10 mM Tris-HCl; 1,5 mM MgCl₂; 50 mM KCl; pH 8,3), 25 mM MgCl₂, 2,5 mM each dNTP (all purchased from Boehringer Mannheim), 15 mM of each primer (ITS1 and ITS4), 1 µl template DNA (variable concentrations) and sterile water.

Both DNA strands were sequenced using primers ITS1 and ITS 4 as well as the internal primers CS2 (5'-CAATGTGCGTTCAAAGATTCG-3') and CS3 (5'-CCAATCTTTGAACGCACATTG-3') (Visser *et al.*, 1995). Sequencing reactions were carried out using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) and an ABI Prism™ 377 DNA sequencer (Perkin Elmer). All the reactions were done using protocols recommended by the manufacturers.

The sequence data for *S. sapinea* isolates as well as outgroup sequences of *Botryosphaeria dothidea* (Moug.) Ces de Not and *Lasiodiplodia theobromae* (Pat) Griffon & Maubl. were processed using Sequence Navigator version 1.0.1 (Perkin Elmer). These DNA sequences were aligned with each other, as well as, with ITS sequences of three *S. sapinea* isolates (94.07 - AF027758; 94.05 - AF027757; 93.31 - AF027756), previously sequenced by Jacobs & Rehner (1998). Analysis of sequences was achieved using Phylogenetic Analysis Using Parsimony (PAUP) version 3.1.1 (Smithsonian Institution, 1993).

Culture and conidial characteristics

Colour and morphology of fungal mycelium on 2% MEA (Biolab) was noted after 15 days incubation at 25°C in the dark. Conidial morphology and dimensions were also examined after sporulation was successfully induced on 2% WA (Biolab) supplemented with sterile pine needles and incubation at 25°C in constant light. The lengths and widths of 50 conidia per isolate were measured using bright field microscopy, MicroVideo Capture System and Auto-Montage (Syncroscopy, Synoptics, LTD. Cambridge, UK). The colour of the conidia and the presence of septa were, furthermore, recorded using bright field microscopy.

RESULTS

RAPD analyses

RAPD profiles, consisting of 7-10 amplified DNA fragments ranging in size from 300-1500bp, were produced for each of the primers DS2 and DS3 (Fig. 1). Using these primers, it was possible to distinguish between the United States isolates, representing the A and B RAPD groups, which were used as standards. The dendrogram (Fig. 2), generated from the analyses showed that the *S. sapinea* isolate from South Africa (CMW1185) grouped together with standard isolates of the A RAPD group (CMW190, CMW4329, CMW4332). Three of the Mexican isolates (CMW4896, CMW4897, CMW4898) and two of the Indonesian isolates (CMW4885, CMW4886) grouped with standard isolates of the B RAPD group (CMW189, CMW4333, CMW4334). One Mexican isolate (CMW4899) and the remaining seven Indonesian isolates (CMW4876, CMW4877, CMW4878, CMW4879, CMW4880, CMW4881, CMW4883) grouped separately from both the standard A and B RAPD group isolates.

Sequence analyses

Sequences of approximately 540 bp were obtained from the ITS region of the rDNA operon and aligned for *S. sapinea* isolates from Indonesia, Mexico, South Africa as well as for standard isolates of the A and B RAPD groups (Fig. 3). The amplified DNA

products from one isolate of *B. dothidea* and two isolates of *L. theobromae*, used as outgroups, were similar in size. Sequences of all the *S. sapinea* isolates used in this study had a high degree of similarity. Two single-base pair differences were observed in the ITS1 region of the rDNA operon in all the B RAPD group isolates when compared with the A RAPD group isolates. An adenine base was inserted at position 125 and a thymine at position 191 in all the B RAPD group isolates.

The South African *S. sapinea* isolate (CMW1185) and all the Indonesian isolates had ITS sequences that were very similar to the ITS sequences of the A RAPD group isolates (Fig. 3). One Mexican isolate (CMW4900) had a ITS sequence similar to the A RAPD group isolates and the rest (CMW4896, CMW4897, CMW4898) to the B RAPD group isolates. The isolates of the third RAPD group were indistinguishable from the A RAPD group isolates based on their ITS sequences.

Culture and conidial characteristics

Isolates of the A RAPD group (CMW190 and CMW1885) had fluffy black-white mycelium characteristic of the described A morphotype and isolates of the B RAPD group (CMW189 and CMW4898) displayed black suppressed mycelium characteristic of the described B morphotype. Isolates of the third RAPD group had either fluffy mycelium or suppressed mycelium and were thus, indistinguishable from the A and B morphotypes. The Indonesian isolates in this group (CMW4876, CMW4877, CMW4878, CMW4879, CMW4880, CMW4881, CMW4883) were fluffy, while the Mexican isolate (CMW4899) had mycelium that grew close to the agar surface.

The size of conidia represented the only distinguishable characteristic between the three RAPD groups of *S. sapinea* (Fig. 4). The conidia of isolates in the third RAPD group were significantly longer than those of conidia of either the A or B RAPD groups ($df = 2$; $F = 15.259$; $p = 0.001$). The widths of conidia were not significantly different among the three RAPD groups ($df = 2$; $F = 0.296$; $p = 0.750$). Length/width ratios were not useful in distinguishing between isolates of the three RAPD groups ($df = 2$; $F = 3.570$; $p = 0.064$).

DISCUSSION

In this study we were able to characterise a set of *S. sapinea* isolates from South Africa, Mexico and Indonesia, and to compare them with the A and B RAPD marker groups, using a previously described technique (Smith & Stanosz, 1995; Stanosz *et al.*, 1996). Furthermore, we were able to show that a set of isolates from Indonesia and Mexico did not group with standard isolates of the A and B RAPD marker groups. We were also able to correlate molecular differences with differences in morphology for the three RAPD groups. This is the first report of *S. sapinea* isolates grouping outside the two defined RAPD marker groups. Prior to this study, the categorisation of *S. sapinea* isolates, into A and B RAPD marker groups, was exclusively done on isolates collected in the United States. This is the first report where the RAPD technique (Smith & Stanosz, 1995) has been implemented on *S. sapinea* isolates from other countries.

Isolates from outside the United States used in this study included those from South Africa, Mexico and Indonesia. Mexico represents a centre of diversity for the genus *Pinus*, and it was perhaps not surprising to find, together with the B RAPD group, a unique sub-group of *S. sapinea* in this area. This is the first time that the B RAPD group of the fungus has been found outside the United States. Although less richly endowed with *Pinus* spp., *Pinus merkusii* is native in Indonesia and the third RAPD group of *S. sapinea* was also found in isolates from this area. The fact that the newly defined sub-group of *S. sapinea* occurred in two geographically distant countries (Mexico and Indonesia) suggests that it is probably quite wide spread across the natural range of *Pinus*. That it has not been found previously is probably linked to the fact that relatively small collections have been studied in the past.

The origin of *S. sapinea* in South Africa is not presently known. In the previous study of Swart *et al.* (1991), based on isozyme comparisons, isolates grouped with the A morphotype of the United States. Although based on a very small sample, our study confirmed this result. The apparent occurrence of a single group of *S. sapinea* in South Africa is perhaps unusual given the fact that recent findings indicate that the fungus has probably been repeatedly introduced into the country from a wide range of sources (Smith *et al.*, 2000).

In this study, ITS sequence data were used in conjunction with the RAPD analysis to compare the *S. sapinea* isolates. Differences in the sequence data were sufficient to separate *S. sapinea* isolates into two groups representing the A and B morphotypes (=A and B RAPD groups). Using these data it was also possible to distinguish *S. sapinea* from two other well-defined *Botryosphaeria* spp., *B. dothidea* and *B. rhodina* (Anamorph=*L. theobromae*). Although, only minor differences in ITS sequence occurred between standard isolates of the A and B groups, they were distinguishable from each other based on two single base-pair differences in the ITS1 region of the rRNA operon. These two differences occurred consistently in all the B group isolates and were never present in isolates of the A group. ITS sequences of the isolates in the third RAPD group were, however, indistinguishable from ITS sequence data from isolates in the A group. The inability to distinguish between the ITS sequences of isolates in the A and third RAPD group indicates that isolates in the A and B groups are well-defined, while those in the newly emerging third RAPD group are probably in the process of differentiation from isolates in the A group.

Morphological distinction between *S. sapinea* isolates belonging to the A and B morphotypes is based on culture characteristics and conidial wall texture (Palmer *et al.*, 1987; Wang *et al.*, 1985). Although, these morphological characteristics are variable and not particularly robust (Swart *et al.*, 1991), isolates belonging to the third RAPD group were clearly distinguishable from the A and B morphotype groups based on conidial size. A study of the morphology of these isolates has thus, confirmed the existence of the third RAPD group as a distinct morphological group, which we now refer to as the C morphotype of *S. sapinea*.

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We are grateful to Dr. G. Stanosz and Dr. M. Palmer for supplying standard *S. sapinea* isolates, representing the A and B RAPD marker groups, from the United States. Financial support for this research was provided by the National Research Foundation (NRF) and the members of the Tree Pathology Co-operative Programme (TPCP).

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Table 1. *Sphaeropsis sapinea* isolates used in this study.

Isolates ^{a/}	Morphotype	Host	Origin	Collector
CMW 190*	A	<i>P. banksiana</i>	United States	M.A. Palmer
CMW 4329*	A	<i>P. ponderosa</i>	"	G. A. Stanosz
CMW 4332	A	<i>P. nigra</i>	"	"
CMW 189*	B	<i>P. resinosa</i>	"	M. A. Palmer
CMW4333*	B	"	"	G. A. Stanosz
CMW4334	B	"	"	"
CMW4876*	Unknown	<i>P. patula</i>	Indonesia	M. J. Wingfield
CMW4877*	Unknown	"	"	"
CMW4878*	Unknown	"	"	"
CMW4879*	Unknown	"	"	"
CMW4880*	Unknown	"	"	"
CMW4886	Unknown	"	"	"
CMW4881*	Unknown	"	"	"
CMW4883*	Unknown	"	"	"
CMW4885	Unknown	"	"	"
CMW1185*	Unknown	<i>P. radiata</i>	South Africa	"
CMW4896	Unknown	<i>P. greggii</i>	Mexico	"
CMW4897	Unknown	"	"	"
CMW4898*	Unknown	"	"	"
CMW4899*	Unknown	"	"	"

^{a/} Isolates CMW4329, CMW4332, CMW4333 and CMW4334 are also known as 94-35, 95-69, 215 and 474, respectively, in the collection of Dr. G. Stanosz. Isolates marked with an asterisk (*) were included in comparisons of culture and conidial characteristics.

Figure 1. RAPD gel profiles for various *S. sapinea* isolates. (A) Primer DS2 (B) Primer DS3. Lane M, molecular weight marker (λ DNA cut with *HindIII*&*EcoRI*); lane C, water control; lane A, United States RAPD group A (CMW190, CMW4329, CMW4332); lane B, United States RAPD group B (CMW189, CMW4334, CMW4333); lanes 1 to 10, Indonesia (CMW4876, CMW4877, CMW4878, CMW4879, CMW4880, CMW4886, CMW4887, CMW4881, CMW4883, CMW4885); lane 11, South Africa (CMW1185); lanes 12 to 15, Mexico (CMW4896, CMW4897, CMW4898, CMW4899).

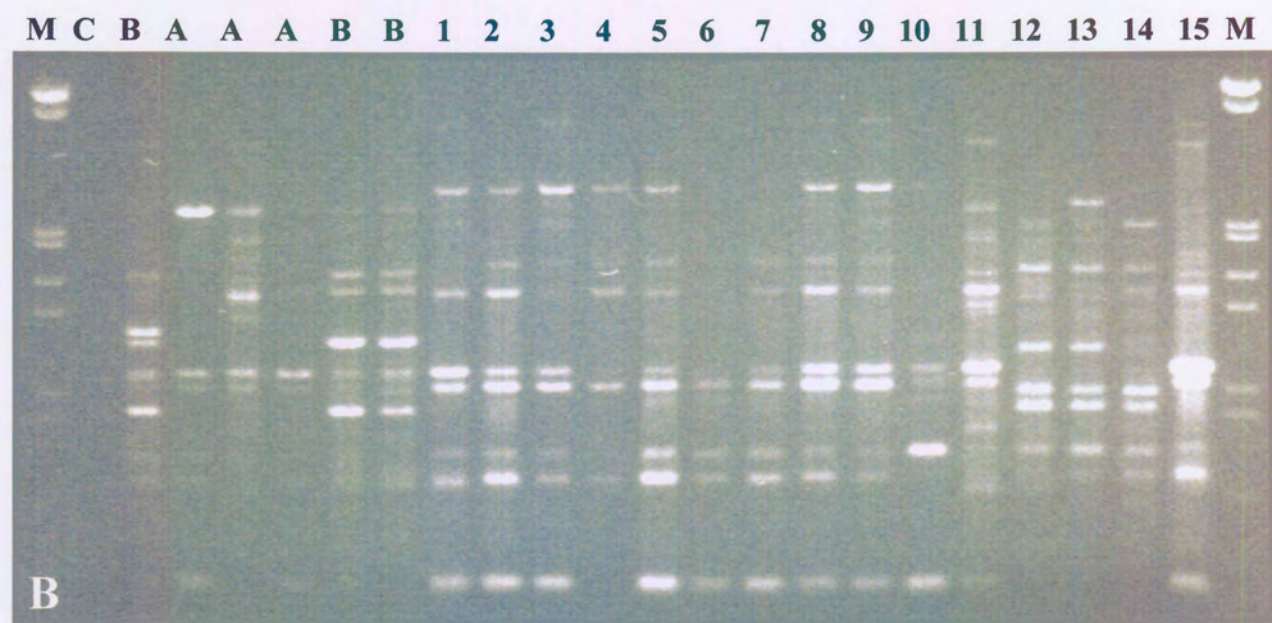
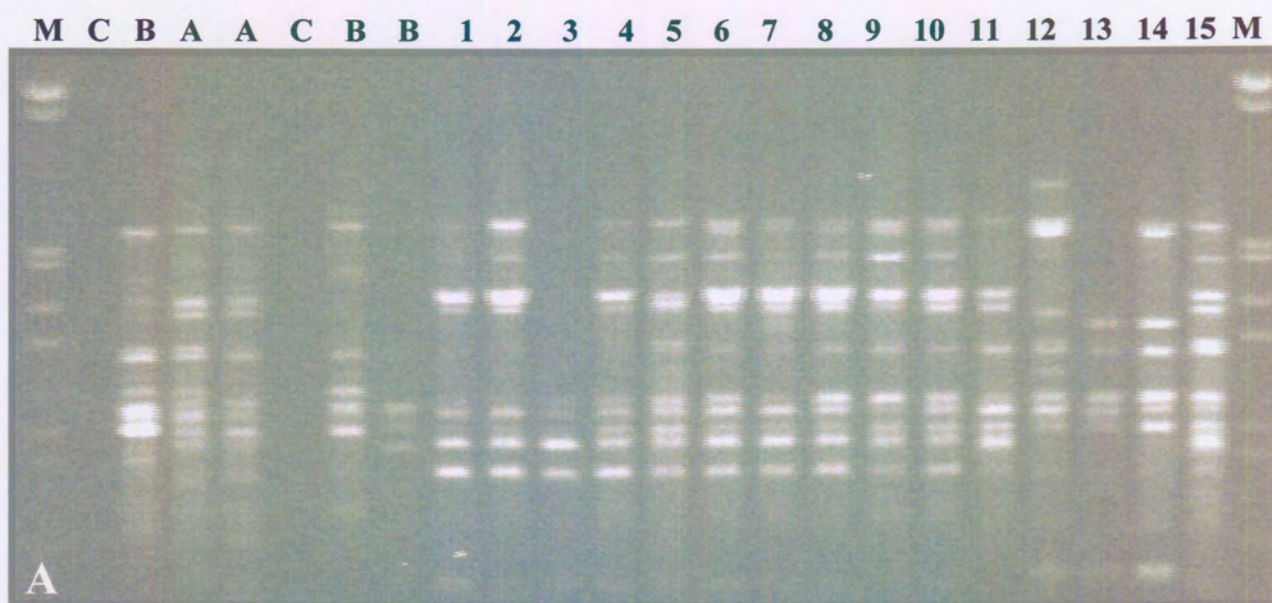


Figure 2. Dendrogram, generated from RAPD data, for *Sphaeropsis sapinea* isolates from Indonesia, Mexico and South Africa, as well as United States isolates representing the A and B RAPD marker groups (CMW190, CMW4329, CMW4332, CMW189, CMW4334, CMW4333). The data set was distance analysed using PHYLIP (version 3.5), with the distance matrix calculated through DNADIST (Kimura-2 parameter) and an unrooted tree was generated using the Neighbor-Joining method.

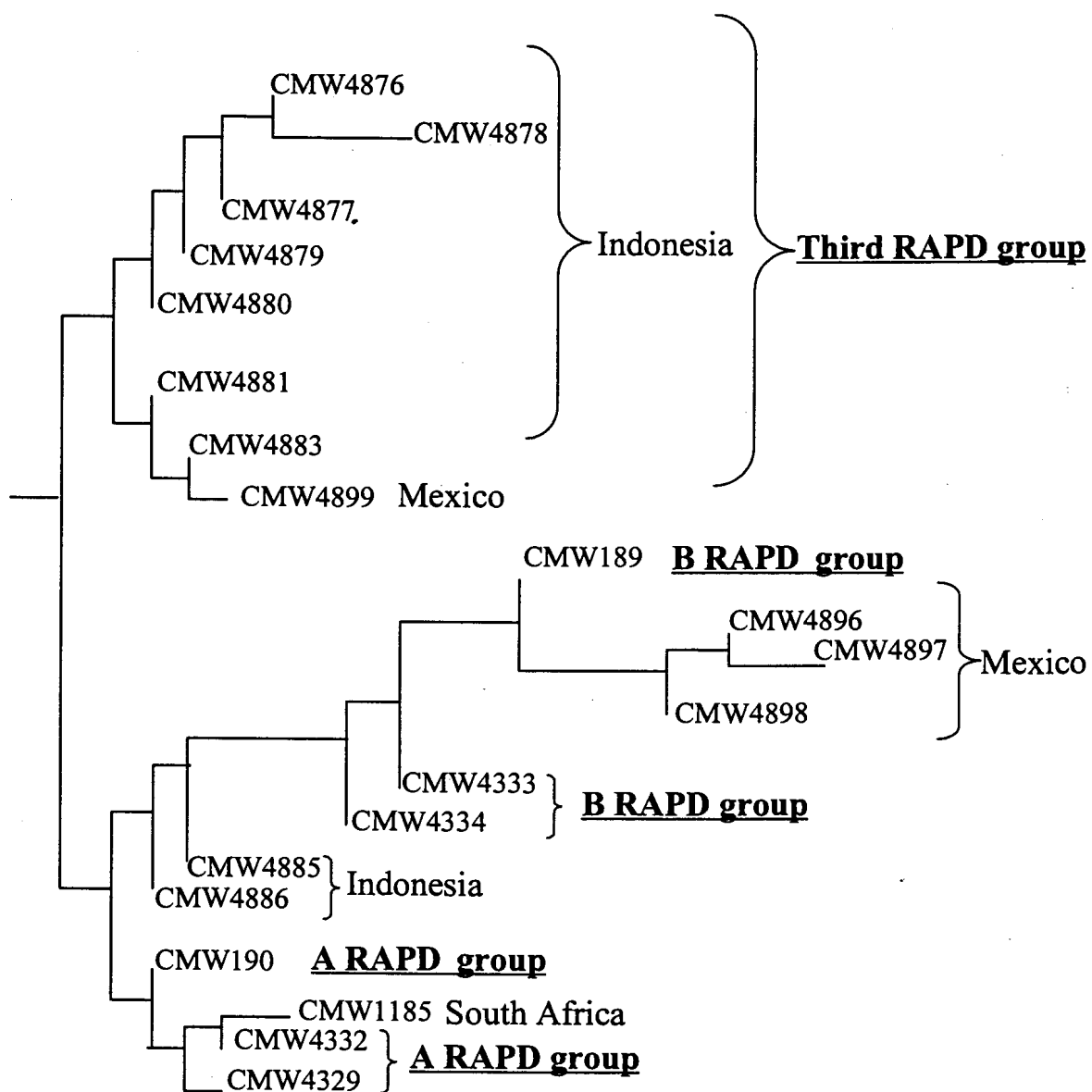


Figure 3. Dendrogram, with bootstrap values (100 replicates), generated from DNA sequence data of the ITS region of the rRNA operon for *Sphaeropsis sapinea* isolates from Indonesia, Mexico and South Africa, as well as USA isolates representing the A and B RAPD marker groups (CMW190, CMW4329, CMW4332, CMW189, CMW4334, CMW4333) using Phylogenetic Analysis Using Parsimony (PAUP, version 3.1.1). Outgroup sequences of *Botryosphaeria dothidea* and *Lasiodiplodia theobromae*, as well as, GenBank sequences of three *S. sapinea* isolates (94.07 - AF027758; 94.05 - AF027757; 93.31 - AF027756), sequenced by Jacobs & Rehner (1998), were included.

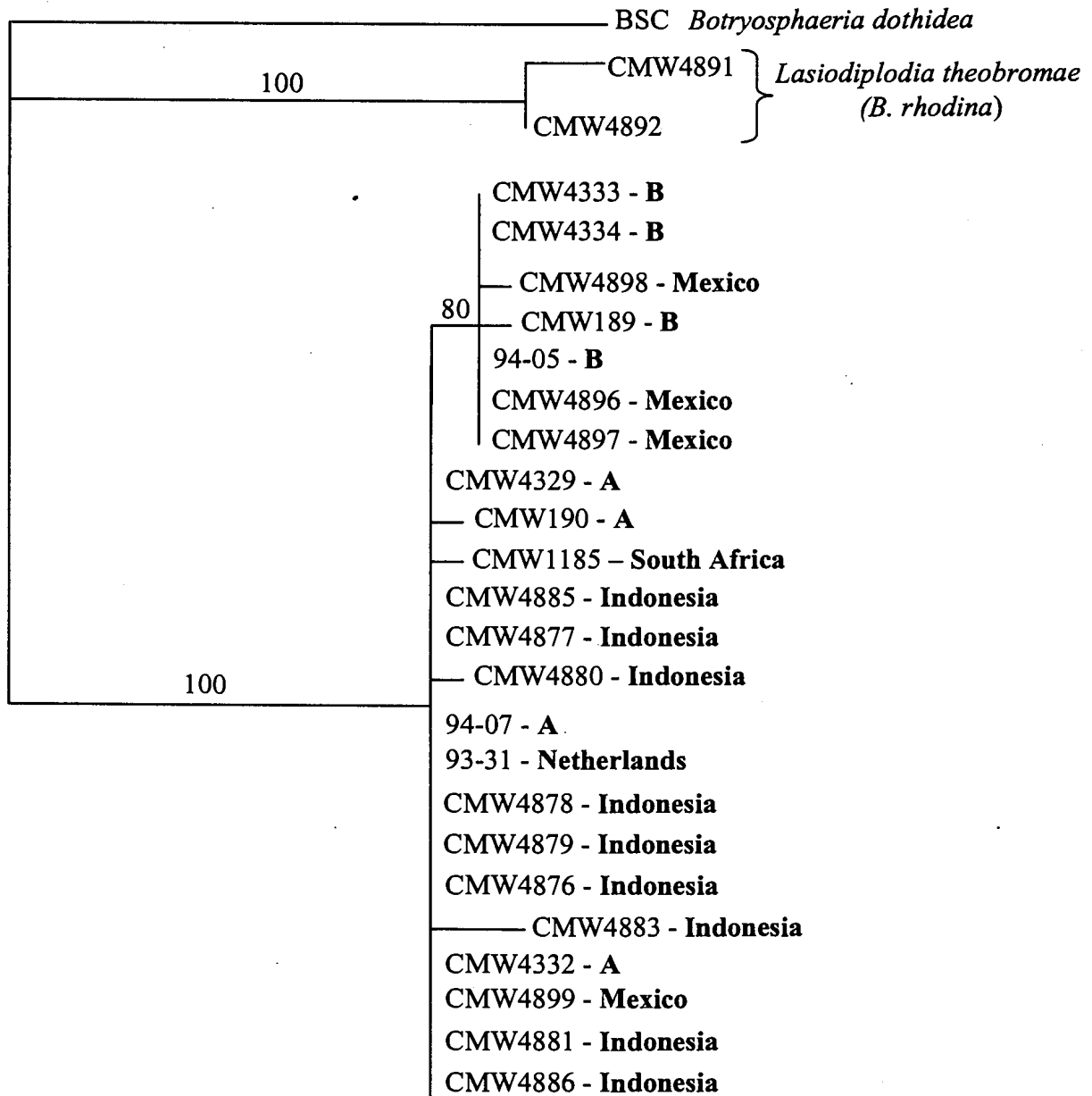
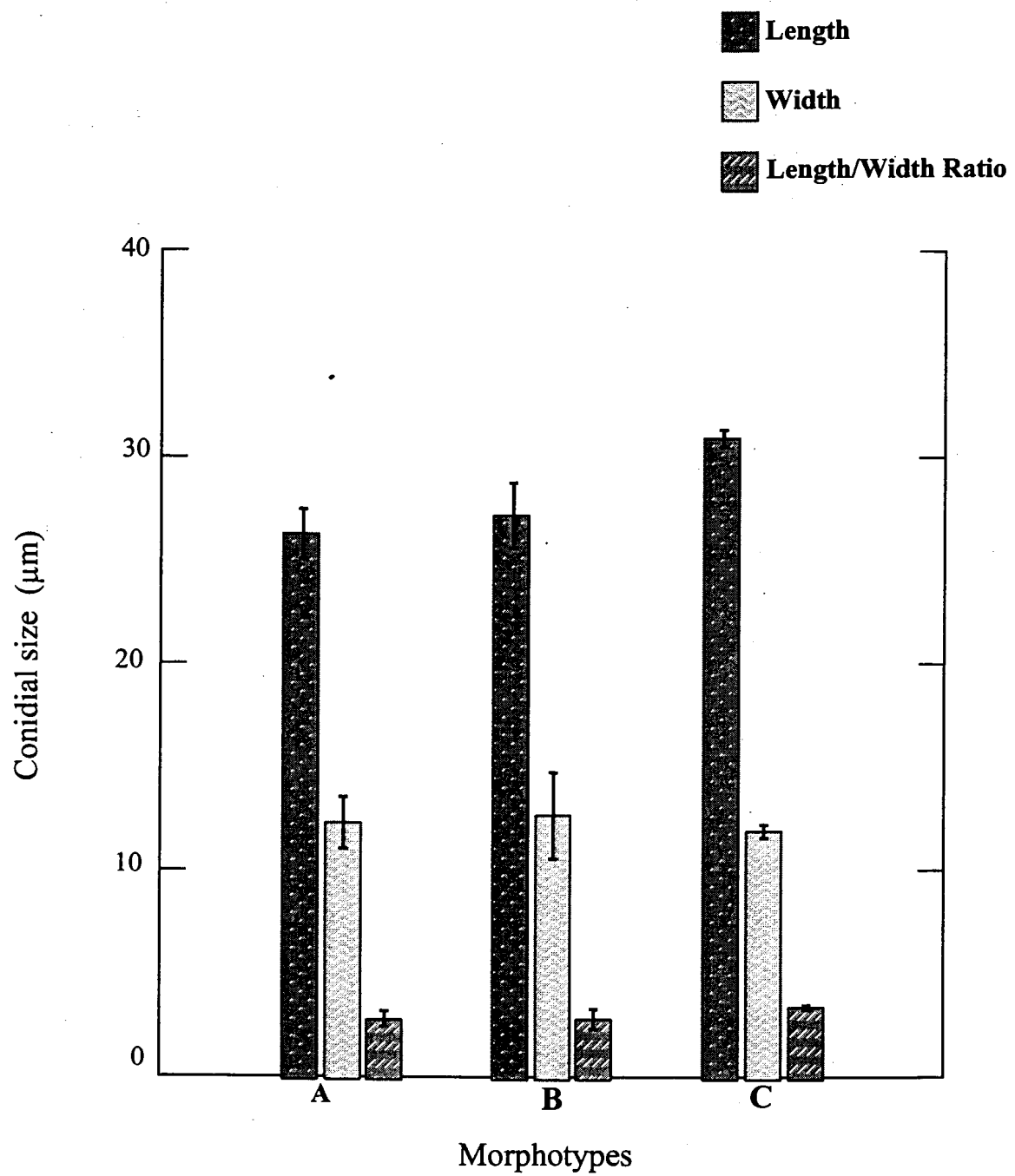
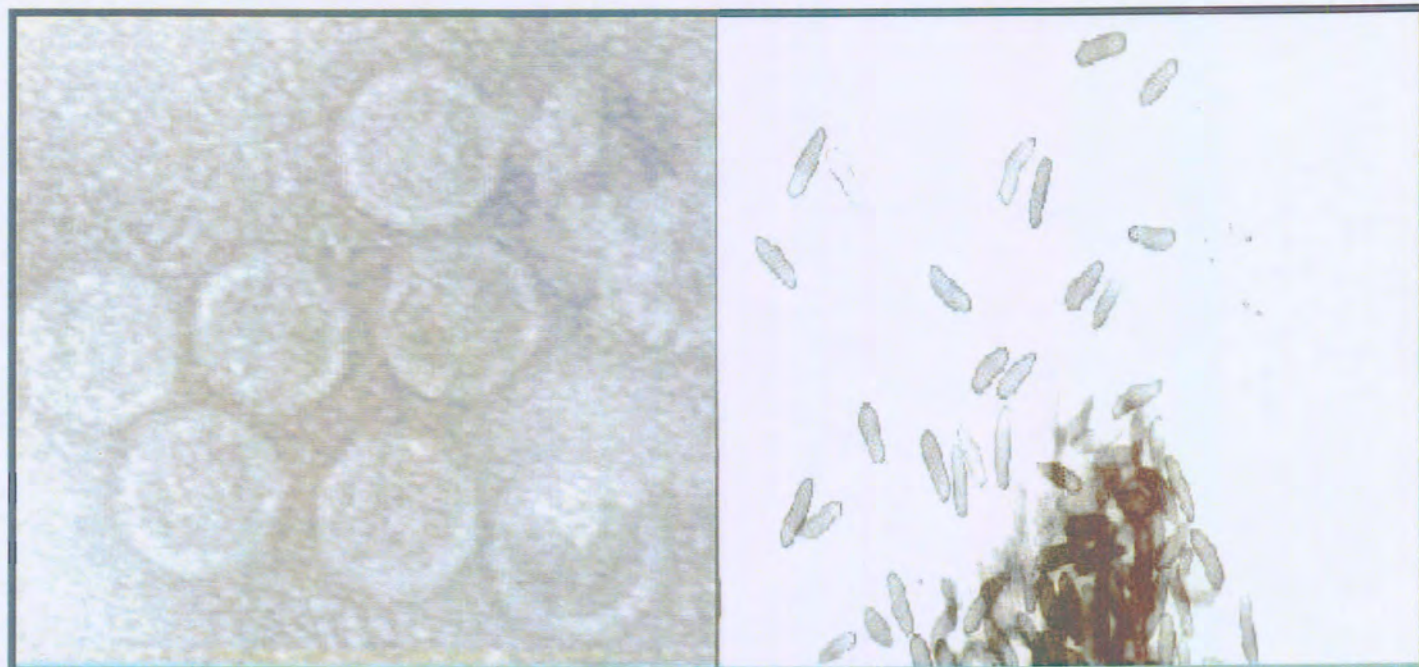


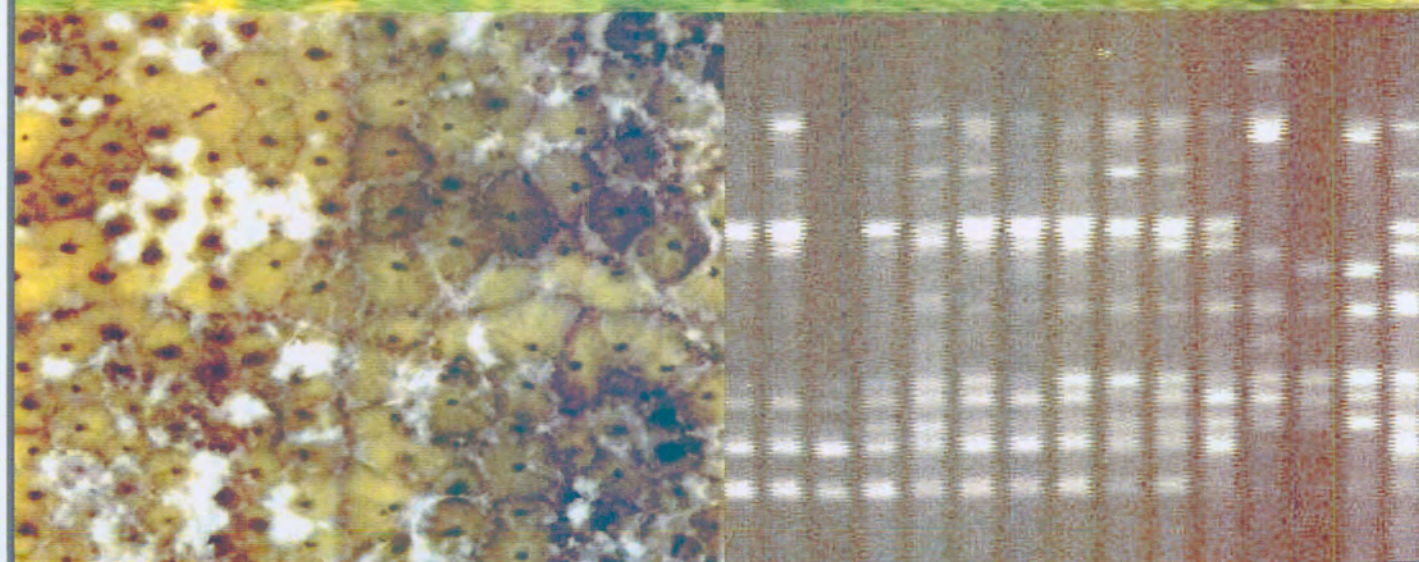
Figure 4. Mean lengths, widths and length/width ratios ($\pm$ S.E.M.) of *Sphaeropsis sapinea* conidia for A RAPD group isolates (CMW190, CMW4329 and CMW1185), B RAPD group isolates (CMW189, CMW4333 and CMW4898) and eight unique RAPD group isolates (CMW4899, CMW4876, CMW4877, CMW4878, CMW4879, CMW4880, CMW4881, CMW4883). Lengths and widths of 50 conidia per isolate were measured using bright field microscopy, MicroVideo Capture System and Auto-Montage computer programmes. Analysis of Variance (ANOVA) based on the least squares means were used to analyse data (SYSTAT version 7.0.1).





CHAPTER 3

CHARACTERISATION OF THE C MORPHOTYPE OF *SPHAEROPSIS SAPINEA* BASED ON MORPHOLOGY AND PATHOGENICITY



ABSTRACT

Sphaeropsis sapinea is an opportunistic pathogen of *Pinus* spp. and other conifers. In healthy, unstressed trees the fungus occurs as an endophyte, causing latent symptomless infections. Infection occurs when host trees are predisposed by environmental or mechanical factors. The A and B morphotypes of *S. sapinea* represent two distinct groups of the fungus initially distinguished from each other based on morphological characteristics. The existence of these two morphotypes has furthermore been confirmed through the use of RAPDs and DNA sequencing of the ITS region of the rDNA operon. A third RAPD group based on RAPD analysis for a group of *S. sapinea* isolates from Indonesia and Mexico has recently been reported and designated morphotype C. The objective of this study was to characterise isolates of the C morphotype of *S. sapinea* based on morphology, growth characteristics and pathogenicity. Based on culture and conidial characteristics, isolates of the three morphotypes displayed minor differences but were distinguishable based on conidial size. No consistent difference in the texture of conidial walls or growth rate was found between the A and C morphotype isolates. C morphotype isolates were, however, considerably more virulent than both the A and B morphotype isolates. The discovery of the much more virulent C morphotype of *S. sapinea* has serious implications for management of this pathogen, as well as, quarantine practises in countries where pine seeds are imported.

INTRODUCTION

Sphaeropsis sapinea (Fr.:Fr.) Dyko & Sutton (= *Diplodia pinea* (Desm.) Kickx.) is an opportunistic pathogen associated with a number of diseases of conifers (Eldridge, 1961; Buchanan, 1967; Punithalingam & Waterston, 1970; Gibson, 1979; Swart, Knox-Davies & Wingfield, 1985). It has a cosmopolitan distribution and extensive host range of more than 30 *Pinus* spp. as well as other conifers. Disease symptoms associated with *S. sapinea* include post-hail associated die-back, cankers, shoot blight, a root disease, collar rot and blue stain (Da Costa, 1955; Millikan & Anderson, 1957; Purnell,

1957; Eldridge, 1961; Gilmour, 1964; Buchanan, 1967; Marks & Minko, 1969; Wingfield & Knox-Davies, 1980). As opportunist, the fungus requires host stress in order to initiate a pathogenic response (Laughton, 1937; Waterman, 1943; Buchanan, 1967; Marks & Minko, 1969; Brown, Bevege & Stevens, 1981). Predisposing factors include conducive environmental conditions, mechanical damage and insects that reduce the resistance to host defence (Haddow & Newman, 1942; Millikan & Anderson, 1957; Gilmour, 1964; Lückhoff, 1964; Wright & Marks, 1970; Nicholls, 1977; Bega *et al.*, 1978).

Two morphotypes, A and B, have been described for *S. sapinea* isolates from the north central United States (Wang *et al.*, 1985; Palmer, Stewart & Wingfield, 1987). Differentiation of the two morphotypes is based on cultural and conidial characteristics, growth rates and growth requirements in culture, as well as pathogenicity and host range (Wang *et al.*, 1985; Wang, Blanchette & Palmer, 1986; Palmer *et al.*, 1987; Palmer, 1991; Smith & Stanosz, 1995). In culture, A morphotype isolates have white to grey-green aerial mycelium and isolates of the B morphotype have black to white mycelium appressed to the surface of the agar. The smooth-walled conidia of the A morphotype are, furthermore slightly longer and wider than the pitted conidia of the B morphotype (Wang *et al.*, 1985; Wang *et al.*, 1986; Palmer *et al.*, 1987). Isolates of the A morphotype generally do not require light for sporulation and also grow more rapidly than the B morphotype isolates. The A morphotype is reported to be more virulent on a wider host range than the B morphotype, that was until recently, restricted to *P. banksiana* Lamb. and *P. resinosa* Aiton. from the north central United States (Wang *et al.*, 1985; Smith & Stanosz, 1995). B morphotype isolates do, however, appear to occur in other parts of the world and on hosts other than *P. banksiana* and *P. resinosa* (Stanosz, Swart & Smith, 1999).

Isolates of *Sphaeropsis sapinea* are highly variable in culture and more phenotypes, in addition to the two already described, are commonly observed. Swart *et al.* (1991) described this pathogen as representing a “morphological continuum without definite types and strains” based on isozyme studies conducted on a collection of South African isolates. The existence of the A and B morphotypes has more recently been confirmed through the use of a random amplified polymorphic DNA (RAPD) technique and the two groups were identified as the A and B RAPD marker groups (Smith & Stanosz,

1995; Stanosz, Smith & Guthmiller, 1996). In a recent study, a third RAPD group was identified from a collection of Indonesian and Mexican isolates (De Wet *et al.*, 2000). ITS sequences were, however, unable to distinguish them from the A RAPD group isolates.

The third RAPD group of *S. sapinea* was designated as the C morphotype based on RAPD banding profiles and conidial size (De Wet *et al.*, 2000). The conidia of the C morphotype are significantly longer than those of both the A and B morphotype. No significant differences in the widths of the conidia between the three morphotypes were observed, although the A morphotype has previously been distinguished from the B morphotype based on its slightly longer and wider conidia (Wang *et al.*, 1985; Palmer *et al.*, 1987).

The objective of this study was to characterise isolates of the C morphotype of *S. sapinea* based on cultural and conidial characteristics. *In vitro* growth studies and pathogenicity tests were also used to compare the three morphotypes. Characterisation of newly emerging types of *S. sapinea* is considered as a first step towards re-evaluation of the current management strategies of this pathogen.

MATERIALS AND METHODS

Fungal isolates

A set of 14 *S. sapinea* isolates from the United States, South Africa, Mexico and Indonesia was selected for morphological characterisation based on traits traditionally used to define the A and B morphotypes. These isolates included six A and B morphotype isolates and eight C morphotype isolates (Table 1). Assignment to a morphotype was based on RAPD banding profiles, ITS sequences and conidial size (De Wet *et al.*, 2000).

Sphaeropsis sapinea isolates from South Africa and Mexico were obtained by direct isolation from the pith tissue of *P. radiata* and *P. greggii* Engelm. cones, respectively, while the Indonesian isolates were obtained from pycnidia on *P. patula* shoots with die-back symptoms. Dark-coloured fungal colonies, obtained after primary isolations, were

transferred to 2% Water Agar (WA) (2% m/v Biolab agar) supplemented with sterile pine needles to induce sporulation. Identification of *S. sapinea* isolates was based on conidial and pycnidial morphology (Sutton, 1980).

Single conidial cultures were generated by spreading conidia, released from excised pycnidia, onto the surface of 2% WA in Petri dishes. The plates were then incubated at 25°C in a mixture of white and near-ultraviolet light, set to provide 12 hour light/dark cycles, for at least 16 hours to allow for germination to take place. Four germinating conidia for each isolate were transferred to 2% Malt Extract Agar (MEA) (2% m/v Biolab malt extract; 2% m/v Biolab agar) in Petri dishes and incubated at 25°C. All the single conidial cultures were transferred to 2% MEA McCartney bottle-slants and stored at 4°C. All isolates are maintained in the Culture Collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Culture and conidial characteristics

Colony colour and morphology of all *S. sapinea* isolates (Table 1) were noted on 2% MEA in Petri dishes (9 cm). After 15 days of incubation at 25°C in the dark, colony colour was assessed using the mycological colour chart of Rayner (1970). Conidial morphology was examined using bright field microscopy after sporulation was induced on 2% WA supplemented with sterile pine needles and incubation at 25°C in a mixture of white and near-ultraviolet light, set to provide 12 hour light/dark cycles. The texture of conidial walls was also examined using a JEOL 840 Scanning Electron microscope at 5 kV and sample preparation methods similar to those used by Wang *et al.*, 1985. Excised pycnidia on sterile pine needles were fixed with 2.5% glutaraldehyde in a 0.075M phosphate buffer (pH 7.4-7.6), followed by osmium fixation using 1% osmium tetroxide (OsO₄). The material was dehydrated in 70% ethanol, followed by three steps in 100% ethanol. Material was then critical-point dried using a Bio-Rad E3000 critical point dryer and CO₂, mounted on aluminium stubs and plated with a mixture of gold-palladium.

Growth study

An *in vitro* growth study was conducted on the 14 *S. sapinea* isolates representing the previously described A and B morphotypes, as well as the recently described C morphotype (De Wet *et al.*, 2000) (Table 1). Mycelial plugs (10 mm diam.) from the margins of one week-old, single conidial cultures were transferred to Petri dishes (9 cm) containing 2% (MEA). Four Petri dishes for each isolate were incubated at each of the four temperatures (10°, 15°, 25° and 30°C) in the dark. The growth rate of these isolates was determined by measuring colony diameters at the four temperatures after 2, 4, 8 and 15 days. Growth rates (mm/day) were statistically analysed using mean separation procedures (SYSTAT, version 7.0.1). Differences in growth rate among *S. sapinea* isolates, representing the three morphotypes, were analysed using the Analysis of Variance and the Tukey pairwise comparisons test (SYSTAT, version 7.0.1). The null hypothesis that no significant differences in growth rate exist among the three morphotypes of *S. sapinea*, was also tested.

Pathogenicity tests

Sphaeropsis sapinea isolates (Table 1) were used for the artificial inoculation of both Granny Smith apples and one-year-old *P. patula* seedlings. Inoculations of apples have, in the past, been effectively used to predict the virulence of isolates of *Cryphonectria parasitica* (Murr.) Barr (Elliston 1985), as well as for isolates of *S. sapinea* (Steenkamp, 1996). The inoculum was prepared by growing single conidial isolates on 2% MEA at 25°C a week prior to inoculation. Sterile 2% MEA plugs were inoculated as controls. A completely randomised block design was used and all tests were repeated once.

Tests on Granny Smith apples. Prior to inoculation, Granny Smith apples were surface sterilised using 70% ethanol and 1% NaClO. A wound (10 mm wide and 5 mm deep) was made in the surface of the apple using a cork borer. Mycelial plugs of the same size were taken from the margins of one-week-old, actively growing *S. sapinea* cultures and placed mycelium-surface down, in the wounds made in the apples. Four apples were inoculated for each isolate. The wounds were sealed with masking tape to

prevent desiccation and incubated at room temperature. After two weeks lesion lengths, widths and surface areas were measured and means compiled.

Tests on *P. patula* seedlings. One hundred and fifty one-year-old *P. patula* seedlings (main stem *ca.* 10 mm diam.) were inoculated with the same *S. sapinea* isolates used in tests on apples (Table 1). A superficial wound (*ca.* 3 mm diam.) to the cambium was made on the stem of the seedlings using a scalpel. Mycelial plugs of the same size were taken from the margins of one-week-old, actively growing *S. sapinea* cultures and placed mycelium-surface down, in the wounds made on the stems. Ten seedlings were inoculated for each isolate. The wounds were sealed with masking tape to prevent desiccation. The tests were carried out in a controlled environment greenhouse with an average daytime temperature of 25°C. Lesion lengths were measured after two weeks.

Results for both the tests, on apples and pine seedlings, were statistically analysed using mean separation procedures (SYSTAT, version 7.0.1). Differences in virulence among *S. sapinea* isolates, representing the three different morphotypes, were analysed using the Analysis of Variance and the Tukey pairwise comparisons test (SYSTAT, version 7.0.1). The null hypothesis that no significant differences virulence exist among the three morphotypes of *S. sapinea*, was also tested.

RESULTS

Culture and conidial characteristics

The A morphotype isolates had fluffy, dark mouse grey to mouse grey mycelium and the B morphotype isolates displayed mouse grey to pale mouse grey mycelium appressed to the surface of the medium. These cultural characteristics are typical of the two morphotypes (Wang *et al.*, 1985, Palmer *et al.*, 1987). Isolates of the C morphotype had cultural characteristics that included those of both the A and B morphotypes (Table 2). It was thus impossible to distinguish the C morphotype isolates from those representing the A and B morphotypes (Fig. 1).

Conidia of all 14 *S. sapinea* isolates included in this study were dark brown with thick walls. Conidia of the A and C morphotype isolates rarely had septa and never had more than one septum per conidium. At least two and frequently more septa were, however, observed in the conidia of the B morphotype isolates.

The A morphotype isolates had conidial walls that were generally smooth and B morphotype isolates had pitted conidial walls (Table 2). Conidia of isolates belonging to the C morphotype all had smooth walls and this made them indistinguishable from those of the A morphotype (Table 2). The conidial walls of three of the C morphotype isolates (CMW4876, CMW4878 and CMW4880), although smooth, had a rougher texture than those of the other C morphotype isolates (CMW4877, CMW4879, CMW4881, CMW4883 and CMW4899) (Fig. 2).

Growth rate

All *S. sapinea* isolates included in this study had optimal growth at 25°C and slower growth at 10°, 15° and 30°C (Table 3). After 8 days, all the isolates incubated at 25°C had completely covered the surface of the medium in Petri dishes. No significant differences were observed in mean growth rates (mm/day) for isolates of the three morphotypes of *S. sapinea* at 10° and 15° (Table 4). At 25°C and 30°C, the mean growth rate for isolates belonging to the A and C morphotypes were, however, significantly greater than those of isolates belonging to the B morphotype (Table 4).

Pathogenicity

Tests on Granny Smith apples. Inoculation of *S. sapinea* isolates into Granny Smith apples all resulted in lesions whereas control inoculations with sterile MEA plugs showed no lesion development (Fig. 3). The surface areas of lesions produced on apples by the A and B morphotype isolates were small and generally did not differ significantly from each other (Table 5). However, the South African A morphotype isolate (CMW1185) and the Mexican B morphotype isolate (CMW4898) produced larger areas of infection than other isolates of those particular morphotypes. Generally, the C morphotype isolates produced significantly larger areas of infection than either the A and B morphotype isolates. One exception was the Mexican C morphotype

isolate (CMW4899), that resulted in a smaller area of infection, similar to those of the B morphotype (Table 5). Based on mean surface area of infection, isolates of the C morphotype were significantly different to isolates of either the A and B morphotype (Table 6).

Tests on *P. patula* seedlings. Inoculation of pine seedlings with *S. sapinea* isolates resulted in lesions, whereas control inoculations with sterile MEA plugs resulted in no lesion development (Fig. 3). Lesions produced by A and B morphotype isolates were generally very small, with those of the B morphotype significantly smaller (Table 5). Isolates of the C morphotype however, produced significantly longer lesions those of either the A and B morphotypes (Table 7). Similar results were obtained for tests conducted on both Granny Smith apples and *P. patula* seedlings.

DISCUSSION

In this study, isolates of the three morphotypes of *S. sapinea* were found to be very similar, based on cultural and conidial characteristics. It was, however, possible to distinguish between the A and B morphotypes based on previously described cultural and conidial characteristics (Wang *et al.*, 1985; Palmer *et al.*, 1987). These features did not enabled us to distinguish between isolates of the C morphotype and those of the previously described A and B morphotypes. The A morphotype isolates have fluffy, aerial mycelium while the B morphotype has mycelium appressed to the agar surface. The C morphotype isolates, however, have cultural characteristics in common with both the A and B morphotype isolates. Some of the C morphotype isolates thus had fluffy mycelium, while others had appressed mycelium. The colony colour of the C morphotype isolates furthermore, ranged between the typical black-white for A morphotype isolates to black-grey for B morphotype isolates. There thus appears to be a continuum of colony morphology and colour for isolates representing the three morphotypes of *S. sapinea*.

Apart from the previously reported differences in size (De Wet *et al.*, 2000), conidial characteristics considered in this study were insufficient to distinguish between isolates of the three morphotypes of *S. sapinea*. The conidial walls of the A morphotype

isolates were found to be generally smooth and those of the B morphotype isolates were pitted, which is consistent with previous observations (Wang *et al.*, 1985; Palmer *et al.*, 1987). The conidia of most C morphotype isolates had smooth walls making them indistinguishable from those of the A morphotype. The conidia of some C morphotype isolates, however, had a rougher texture but no definite pits were observed as for those of the B morphotype. The septation observed in the A and C morphotype conidia was, also, the same but different to that of the B morphotype conidia. Thus, both the A and C morphotype conidia usually have no or only one septum, while the B morphotype conidia can be multi-septate. The fact that C morphotype conidia are longer than those of both the A and B morphotypes (De Wet *et al.*, 2000), is thus the only useful morphological characteristic that can be used to distinguish between the three morphotypes of *S. sapinea*.

Morphological characteristics are highly variable and dependant on many factors such as the genetic variation within a population, as well as external factors such as environmental conditions, the nutrient composition of the growth medium and sample preparation methods for microscopy (Wang *et al.*, 1985, Wang *et al.*, 1986, Swart & Wingfield, 1991; Swart *et al.*, 1991). Reports of isolates of *S. sapinea* that are neither typical of the A or B morphotypes have been made (Wang *et al.*, 1985; Swart *et al.*, 1991; Stanosz *et al.*, 1999). Results of this study suggest that the C morphotype isolates have morphological characteristics that are most like those of A morphotype isolates. This is consistent with recently published sequence data that also show C morphotype isolates as being more similar to the A than to the B morphotype isolates (De Wet *et al.*, 2000). The C morphotype was first recognised based on RAPDs and this approach allows reliable recognition of the three forms of *S. sapinea*.

No differences were detected in *in vitro* growth rate of isolates representing the A and C morphotypes of *S. sapinea* but they grow more rapidly than isolates belonging to the B morphotype. However, isolates belonging to the C morphotype of the fungus were obviously more pathogenic than those isolates of the A and B morphotypes. Lesions produced by the A morphotype were larger than those of the B morphotype and this is consistent with previously published results (Wang *et al.*, 1985 & 1986; Palmer *et al.*, 1987; Palmer, 1991; Smith & Stanosz, 1995). The B morphotype isolates produced small or even no lesions when inoculated into *P. patula* seedlings. This could be due to

host specificity, as this form of *S. sapinea* was until recently only known on *P. resinosa* Aiton. and *P. banksiana* Lamb. (Wang *et al.*, 1985; Smith & Stanosz, 1995; Stanosz *et al.*, 1999). Results of pathogenicity tests on both Granny Smith apples and *P. patula* seedlings were similar and it might be possible to use apples for rapid virulence screening, as is done for this pathogen (Steenkamp, 1996).

The discovery of a third form of *S. sapinea*, now known as the C morphotype of the fungus, has important quarantine implications. Prior to its discovery, the A morphotype of the fungus was recognised as considerably more pathogenic than the B morphotype (Wang *et al.*, 1985 & 1986; Palmer *et al.*, 1987; Palmer, 1991; Smith & Stanosz, 1995). The A morphotype is also the form of the fungus that has assumed a wide distribution in countries of the world such as South Africa, Australia and New Zealand where pines are grown as exotics (Lückhoff, 1964; Marks & Minko, 1969; Currie & Toes, 1978; Swart *et al.*, 1991). This wide distribution might be attributed to the higher level of pathogenicity of this form of the fungus. The fact that isolates of the C morphotype of *S. sapinea* are considerably more virulent than those of the A morphotype, is important. *Sphaeropsis sapinea* is well recognised to be seed-borne and it has apparently been widely distributed in countries that grow pines as exotics, together with seed (Currie & Toes, 1978; Rees & Webber, 1988; Smith *et al.*, 1996; Smith *et al.*, 2000). The high level of pathogenicity of C morphotype isolates suggests that great care should be taken when importing seed from Indonesia and Mexico. The fact that the fungal species is already in a country should not be used to imply reduced risk, and the danger of introducing additional and more virulent genotypes of *S. sapinea* should be carefully considered.

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Table 1. *Sphaeropsis sapinea* isolates used in this study.

Isolates	Morphotype ^{a/}	Host	Origin	Collector
CMW190	A	<i>P. banksiana</i>	United States	M.A. Palmer
CMW1185	A	<i>P. radiata</i>	South Africa	W.J. Swart
CMW4329	A	<i>P. ponderosa</i>	"	G. A. Stanosz
CMW189	B	<i>P. resinosa</i>	United States	M. A. Palmer
CMW4898	B	<i>P. greggii</i>	Mexico	M.J. Wingfield
CMW4333	B	<i>P. resinosa</i>	"	G. A. Stanosz
CMW4876	C	<i>P. patula</i>	Indonesia	M. J. Wingfield
CMW4877	C	"	"	"
CMW4878	C	"	"	"
CMW4879	C	"	"	"
CMW4880	C	"	"	"
CMW4881	C	"	"	"
CMW4883	C	"	"	"
CMW4899	C	<i>P. greggii</i>	Mexico	"

^{a/} A, B and C refers to the described A, B and C morphotypes of *S. sapinea* (Wang *et al.*, 1985 & 1987; Palmer *et al.*, 1987; De Wet *et al.*, 2000).

Table 2. Culture characteristics, conidial dimensions and morphology of isolates representing the three morphotypes of *Sphaeropsis sapinea*.

Isolates	Morphotype	Colony morphology ^{a/}	Number of septa	Texture of conidial walls ^{b/}
CMW190	A	Fluffy, dark mouse grey-mouse grey	0-1	Smooth
CMW1185	A	"	"	"
CMW4329	A	"	"	"
CMW189	B	Suppressed, mouse grey-pale mouse grey	0-3	Pitted
CMW4333	B	"	"	"
CMW4898	B	Suppressed, mouse grey	0-1	"
CMW4876	C	Fluffy, fuscous black-pale mouse grey	0-1	Rough surface
CMW4877	C	"	"	Smooth
CMW4878	C	Fluffy, dark mouse grey-pale mouse grey	"	Rough surface
CMW4879	C	Fluffy, fuscous black-pale mouse grey	"	Smooth
CMW4880	C	"	"	Rough surface
CMW4881	C	"	"	Smooth
CMW4883	C	"	"	"
CMW4899	C	Suppressed, mouse grey-pale mouse grey	"	"

^{a/} Cultural characteristics are based on the morphology and colour of mycelium on 2% MEA after 15 days of incubation at 25°C in the dark. Colony colour was assessed according to the mycological colour chart of Rayner (1970). "Fluffy" indicates to aerial mycelia with a cotton wool appearance while "suppressed" refers to mycelium growing close to the surface or within the growth medium.

^{b/} Texture of the conidial walls as observed from using a JEOL Scanning electron microscope.

Table 3. Mean growth rates of *Sphaeropsis sapinea* isolates, representing the three morphotypes, at four different temperatures (10°, 15°, 25° and 30°C).

Isolates	Morphotype	Mean growth rate (mm/day) ^{a/}			
		10°C	15°C	25°C	30°C
CMW190	A	3.5 ab	5.5 ab	20.0 ab	9.9 ab
CMW1185	A	4.1 ab	6.3 ab	18.6 ab	8.7 ab
CMW189	B	2.6 a	3.7 a	13.7 c	8.3 ab
CMW4898	B	3.7 ab	5.0 ab	13.8 c	6.0 a
CMW4876	C	4.6 b	7.0 b	21.8 ab	12.2 b
CMW4877	C	3.7 ab	5.7 ab	15.0 c	8.9 ab
CMW4878	C	3.7 ab	6.1 ab	20.6 ab	11.1 b
CMW4879	C	3.2 ab	5.1 ab	17.0 ab	8.0 ab
CMW4880	C	4.8 b	6.5 ab	23.5 a	12.7 b
CMW4881	C	3.2 ab	4.8 ab	17.8 ab	10.0 ab
CMW4883	C	3.6 ab	5.5 ab	18.4 ab	10.6 ab
CMW4899	C	5.0 b	7.2 b	20.0 ab	12.1 b

^{a/} The growth rates of isolates incubated at 25°C were calculated after 8 days since the mycelia had covered the surface area of the MEA in the Petri dish. The growth rates of isolates incubated at 10°, 15° and 30°C were only calculated after 15 days. Values within a column with the same letter do not differ significantly with the Tukey pairwise comparisons test ($p < 0.05$).

Table 4. Analysis of variance for the growth rate for isolates representing the three morphotypes *Sphaeropsis sapinea* at four different temperatures (10°, 15°, 25° and 30°C).

Source of variation ^{a/}	Sum of squares (SS)	Degrees of freedom (df)	Mean of squares (MS)	F-ratio	Probability (p)
Temp – 10°C	15.9	11	1.4	0.7	0.73
Error	25.1	12	2.1		
Temp – 15°C	43.5	11	4.0	0.4	0.91
Error	110.1	12	9.2		
Temp – 25°C	247.2	11	22.5	4.4	0.01
Error	62.0	12	5.2		
Temp – 30°C	209.9	11	19.1	3.1	0.03
Error	73.4	12	6.1		
Morphotypes*	102.2	2	51.1	5.9	0.01
Error	181.1	21	8.6		

Least squares			
Source of variation ^{b/}	means (LSM) ^{c/}	Standard error (SE)	Population size (N)
A	19.3 a	1.5	3
B	13.7 b	1.5	3
C	19.3 a	0.7	8

^{a/} Growth rate for *S. sapinea* isolates at four different temperatures (Table 3). The asterisk (*) refers to the mean growth rate for the three morphotypes of *S. sapinea* as source of variation.

^{b/} Mean growth rate for the three *S. sapinea* morphotypes at 25°C.

^{c/} Values within a column followed by the same letter do not differ significantly with the Tukey pairwise comparisons test ($p < 0.05$).

Table 5. Lesion lengths, widths and areas on Granny Smith apples, as well as lesion lengths on *Pinus patula* seedlings after inoculation with isolates representing the three morphotypes of *Sphaeropsis sapinea*.

Isolates	Morphotype	Granny Smith apples ^{a/}			<i>P. patula</i> seedlings ^{b/}
		Mean lesion length (mm)	Mean lesion width (mm)	Area of lesion (mm ²)	Mean lesion length (mm)
CMW190	A	11.5 a	10.5 a	24.6 a	29.1 b
CMW4332	A	11.2 a	10.6 a	24.8 a	23.3 b
CMW1185	A	49.5 d	49.8 c	553.8 c	43.2 c
CMW189	B	10.8 a	10.2 a	21.5 a	9.0 a
CMW4334	B	17.8 b	13.6 a	42.0 a	14.9 a
CMW4898	B	25.1 c	23.1 b	116.0 b	22.3 b
CMW4876	C	31.6 c	30.1 b	228.3 b	68.3 c
CMW4877	C	47.2 d	45.1 c	491.3 c	91.1 c
CMW4878	C	25.9 c	23.9 b	114.8 b	24.1 b
CMW4879	C	31.5 c	29.4 b	193.8 b	68.0 c
CMW4880	C	36.1 c	31.1 b	268.8 b	64.0 c
CMW4881	C	49.6 d	46.4 c	577.5 c	40.8 c
CMW4883	C	53.7 d	49.1 c	655.5 c	74.2 c
CMW4899	C	11.4 a	10.5 a	24.1 a	78.0 c
CONTROL	-	0	0	0	0

^{a/} Mean lesion lengths, widths and areas were determined after two independent inoculation trials in which four Granny Smith apples per isolate were inoculated. The area of the lesion was determined by calculating the number of 1 mm x 1 mm squares represented by the infected area. Values within a column followed by the same letter do not differ significantly with the Tukey pairwise comparisons test ($p < 0.05$).

^{b/} Mean lesion lengths were determined after two independent inoculation trails in which ten *P. patula* seedlings per isolate were inoculated. Values within a column followed by the same letter do not differ significantly with the Tukey pairwise comparisons test ($p < 0.05$).

Table 6. Analysis of variance for the pathogenicity of isolates representing the three morphotypes *Sphaeropsis sapinea* after inoculation of Granny Smith apples.

Source of variation ^{a/}	Sum of squares (SS)	Degrees of freedom (df)	Mean of squares (MS)	F-ratio	Probability (p)
Length	3.4	10	0.4	46.6	0.000
Error	0.3	37	0.007		
Width	3.6	10	0.4	45.8	0.000
Error	0.3	37	0.008		
Area	15.1	10	1.5	39.8	0.000
Error	1.4	37	0.038		
Morphotypes*	12.8	2	6.4	77.1	0.000
Error	3.7	45	0.083		

Least squares			
Source of variation ^{b/}	means (LSM) ^{c/}	Standard error (SE)	Population size (N)
A	1.4 a	0.087	3
B	1.4 a	0.096	3
C	2.4 b	0.055	8

^{a/} Lengths, widths and surface area of infection for 11 *S. sapinea* isolates (Table 1 except CMW1185, CMW4898 and CMW4899). The asterisk (*) refers to the mean surface area for the three morphotypes of *S. sapinea* as source of variation.

^{b/} Mean surface area for the three *S. sapinea* morphotypes.

^{c/} Values within a column followed by the same letter do not differ significantly with the Tukey pairwise comparisons test ($p < 0.05$).

Table 7. Analysis of variance for the pathogenicity of isolates representing the three morphotypes *Sphaeropsis sapinea* after inoculation of *Pinus patula* seedlings.

Source of variation ^{a/}	Sum of squares (SS)	Degrees of freedom (df)	Mean of squares (MS)	F-ratio	Probability (p)
Lesion length	1.9	10	0.2	1.6	0.24
Error	1.3	11	0.1		
Morphotypes*	1.3	2	0.6	6.5	0.01
Error	1.9	19	0.1		

Least squares			
Source of variation ^{b/}	means (LSM) ^{c/}	Standard error (SE)	Population size (N)
A	1.4 a	0.2	3
B	1.0 b	0.2	3
C	1.7 c	0.1	8

^{a/} Lesion lengths for 11 *S. sapinea* isolates (Table 1 except CMW1185, CMW4898 and CMW4899). The asterisk (*) refers to the mean lesion length for the three morphotypes of *S. sapinea* as source of variation.

^{b/} Mean lesion length for the three *S. sapinea* morphotypes.

^{c/} Values within a column followed by the same letter do not differ significantly with the Tukey pairwise comparisons test ($p < 0.05$).

Figure 1. Cultural characteristics of *Sphaeropsis sapinea* isolates on 2% MEA after 15 days incubation at 25°C in the dark. (A) A morphotype culture (CMW190); (B) B morphotype culture (CMW189); (C) and (D) C morphotype culture (CMW4899, CMW4876).

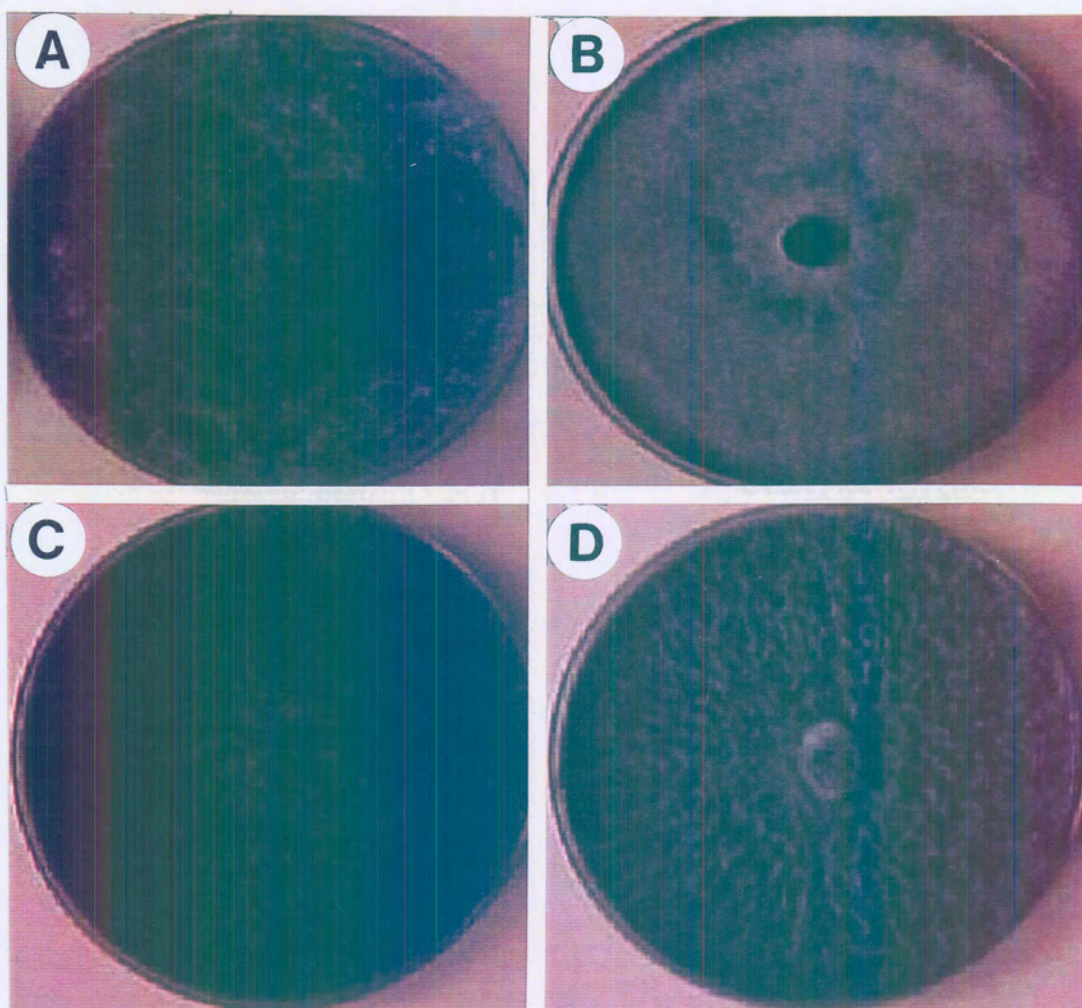


Figure 2. Conidial wall morphology of isolates representing the C morphotype of *Sphaeropsis sapinea*. (A) CMW4876; (B) CMW4880; (C) CMW4883. Scale bar = 10 μm .

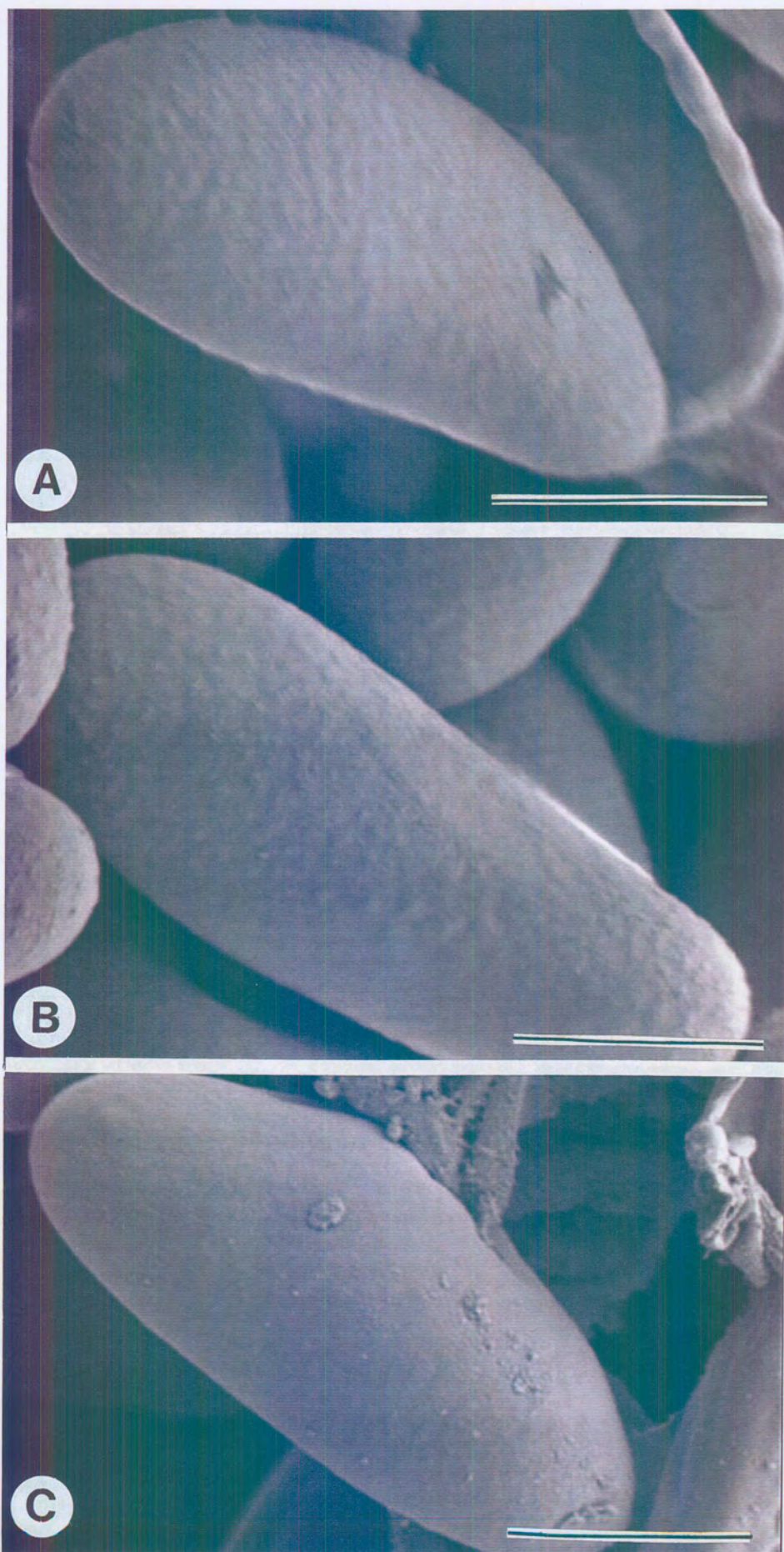
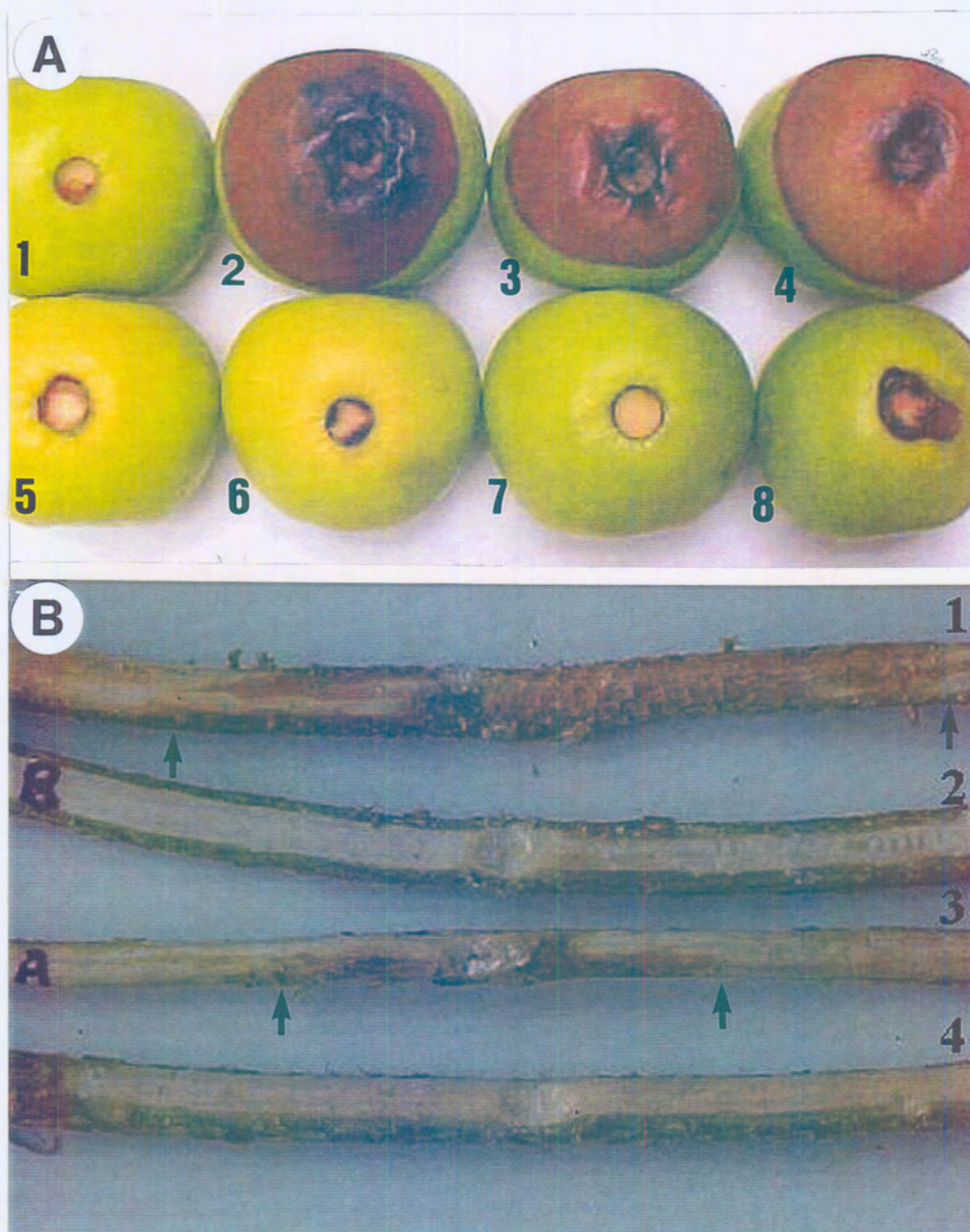
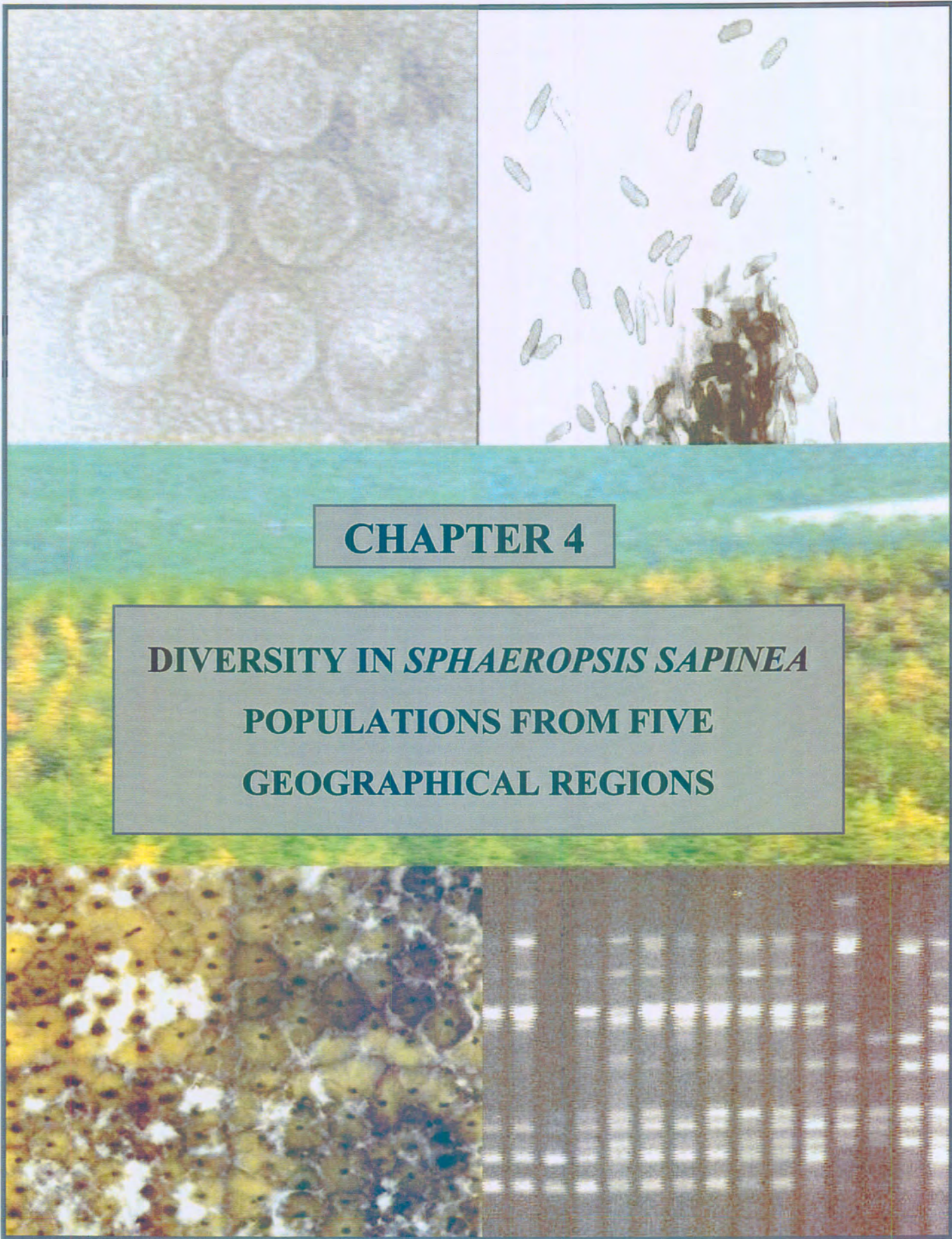


Figure 3. Lesions formed after inoculation with isolates representing the three morphotypes of *Sphaeropsis sapinea*. **(A)** Granny Smith apples: 1 = sterile control, 2–4 = isolates representing the C morphotype (CMW4877, CMW4880, CMW4883), 5–6 = isolates representing the A morphotype (CMW4332 and CMW190) and 7–8 = isolates representing the B morphotype (CMW189 and CMW4334). **(B)** *Pinus patula* seedlings: 1 = isolate representing the C morphotype (CMW4876), 2 = isolate representing the B morphotype (CMW189), 3 = isolate representing the A morphotype (CMW190) and 4 = sterile control. The arrows indicate the length of the lesions, one week after inoculation.



The background of the page is a composite image. The top half is split into two panels: the left panel shows several circular, light-colored spores with a textured surface, and the right panel shows a cluster of elongated, spindle-shaped spores. The bottom half of the page features a landscape with green grass and yellow wildflowers. Overlaid on this landscape are two text boxes. The top box contains the chapter title, and the bottom box contains the main title of the study.

CHAPTER 4

DIVERSITY IN *SPHAEROPSIS SAPINEA* POPULATIONS FROM FIVE GEOGRAPHICAL REGIONS



ABSTRACT

Sphaeropsis sapinea is an opportunistic pathogen associated with many disease symptoms commonly found on *Pinus* spp. and other conifers world-wide. The fungus causes extensive damage in forestry plantations where *Pinus* spp. are planted as exotics. In this study, the genetic diversity in five *S. sapinea* populations from Mexico, California, Switzerland, Madagascar and Colombia was assessed. These five collections represented presumed introduced, as well as native populations of the fungus. The number of vegetative compatibility groups (VCGs) in each population was assessed by pairing single conidial *S. sapinea* isolates on Oatmeal agar and identifying VCGs on the basis of dark barrage lines between isolates of different origin. The number and frequency of VCGs was then used to calculate four parameters, number of VCGs (S) to sample size (N) ratio, Shannon diversity index (Hs), genotypic diversity (G) and maximum % of genotypic diversity, which reflected the degree of diversity in each of the populations. The highest degree of diversity (52%) was found in the presumed introduced Malagasy population and the lowest degree of diversity (9%) in the presumed native Mexican population. The other three populations (California, Switzerland, Colombia) had diversity levels that ranged from 9% to 52%. Although, no significant overall difference in diversity was found between the five *S. sapinea* populations, a significant difference in diversity exists between the presumed introduced Malagasy population and the presumed native populations from Mexico, California and Switzerland. The assessment of the degree of diversity in the five *S. sapinea* populations, included in this study, enabled us to predict the influence of the population genetic structure on the management of Diplodia die-back in different regions of the world.

INTRODUCTION

Sphaeropsis sapinea (Fr.:Fr) Dyko & Sutton (= *Diplodia pinea* (Desmaz) J. Kickx. Fl.) is the causal agent of many disease symptoms commonly observed on *Pinus* spp. (Eldridge, 1961; Buchanan, 1967; Punithalingam & Waterston, 1970; Gibson, 1979;

Swart, Knox-Davies & Wingfield, 1985). These include top die-back (Millikan & Anderson, 1957; Purnell, 1957; Buchanan, 1967), cankers and other stem malformation (Gilmour, 1964; Marks & Minko, 1969), a root disease (Wingfield & Knox-Davies, 1980) and blue stain of timber (Da Costa, 1955; Eldridge, 1961). The expression of these disease symptoms is associated with unfavourable environmental conditions or physical factors that reduce defences in the host (Laughton, 1937; Waterman, 1943; Marks & Minko, 1969; Brown, Bevege & Stevens, 1981). Despite its opportunistic nature, *S. sapinea* causes serious economic losses to forestry industries world-wide, especially where pines are grown as exotics in plantations (Laughton, 1937; Waterman, 1943; Buchanan, 1967; Peterson, 1977; Swart *et al.*, 1985; Swart & Wingfield, 1991b).

Management of damage due to *S. sapinea* is largely dependent on planting resistant pine species in high risk areas and pruning at times when infection is not likely to occur (Lückhoff, 1964; Wright and Marks, 1970; Gibson, 1979; Burdon, Currie & Chou, 1980; Swart *et al.*, 1985; Swart & Wingfield, 1991a). In South Africa, the susceptible *P. patula* Schl. et Cham. and *P. radiata* D. Don. are restricted to the summer rainfall areas where hailstorms rarely occur, while the more resistant *P. elliottii* Engalm. et Vasey. and *P. taeda* L. are planted in regions where hail and other well-recognised predisposition factors are more common (Laughton, 1937; Lückhoff, 1964; Zwolinski, Swart & Wingfield, 1990). Despite the implementation of these management strategies, losses due to *S. sapinea* infections still account for approximately 55% annual loss in the potential production value of *P. radiata* (Zwolinski *et al.*, 1990).

Biological control through dsRNA-mediated hypovirulence is a new, potentially useful strategy to reduce the impact of *S. sapinea*, following the recent discovery of dsRNA fragments in isolates of this fungus (Wu, Palmer & Adams, 1989; Steenkamp *et al.*, 1998; Preisig, Wingfield & Wingfield, 1998). Knowledge regarding the genetic structure of the pathogen population must, however, form an essential part of such a management strategy. The level of genetic variation in populations of *S. sapinea*, as in other plant pathogens, influences the ability of the fungus to overcome disease management strategies (Caten, 1972; Perkins, 1991; Leslie, 1993). A highly heterogeneous genetic structure enhances the adaptability of the fungus and, therefore restricts options for resistance breeding and the optimal deployment of cultivars (Perkins, 1991). A highly diverse fungal population furthermore, restricts the effective

transmission of biocontrol agents, such as virus-like dsRNA fragments, making biocontrol through hypovirulence impractical (Caten, 1972; Anagnostakis, Hau & Kranz, 1986).

Sphaeropsis sapinea lacks a sexual state and the degree of diversity in populations of this fungus is assumed to be relatively low, at least where it is introduced. Isozyme analysis has, however, shown that a high degree of heterogeneity exists in the gene pool of this pathogen (Palmer, Stewart & Wingfield, 1987; Swart *et al.*, 1991). Smith *et al.* (2000) also found that the genetic diversity in the introduced *S. sapinea* population from South Africa is much higher than that of an apparently native population from Northern Sumatra, Indonesia. The higher level of diversity in the South African population was thought to have arisen due to multiple introductions of the fungus, with seed imports, over an extended period of time.

The objective of this study was to determine and compare the diversity for five geographically distant *S. sapinea* populations, using vegetative compatibility tests. The results obtained regarding the population structure add significantly to our knowledge of distribution of this fungus. These results should also be useful in the establishment of effective and sustainable management programmes for *S. sapinea* diseases.

MATERIALS AND METHODS

Sampling and maintenance of isolates

Pine cones and infected pine shoot tips were collected from five regions namely, Mexico, Colombia, California, Switzerland and Madagascar (Table 1). The Mexican and Californian *S. sapinea* populations were presumed to be native, since collections were made from natural *P. patula* and *P. greggii* Engelm. stands in Mexico, and *P. radiata* in California. The Swiss population was also presumed to be native, since natural pine stands occur in Switzerland although, collections were made from introduced *P. sylvestris* L. and *P. nigra* Arnld. The Colombian and Malagasy *S. sapinea* populations would have been introduced along with their respective pine hosts.

Sphaeropsis sapinea isolates from Mexico, Switzerland and Madagascar were obtained by direct isolation from the pith tissue of *P. patula*, *P. greggii*, *P. sylvestris* and *P. nigra* cones. Colombian *S. sapinea* isolates were obtained from pycnidia on *P. patula* and the Californian isolates from *P. radiata* shoots showing die-back symptoms. All dark-coloured fungal colonies were transferred to 2% Malt Extract Agar (MEA) (2% m/v Biolab malt extract; 2% m/v Biolab agar) and incubated at 25°C in the presence of a mixture of white and near-ultraviolet light set to provide 12 hour light/dark cycles.

Positive identification of *S. sapinea* isolates was based on conidial and pycnidial morphology as described by Sutton (1980). To induce sporulation, all dark-coloured colonies were transferred to 2% Water Agar (WA) (2% m/v Biolab agar), supplemented with sterile pine needles, incubated at 25°C and exposed to a mixture of white and near-ultraviolet light set to provide 12 hour light/dark cycles. Single conidial cultures were generated from 152 *S. sapinea* isolates by spreading conidia, released from broken pycnidia, onto the surface of 2% WA in Petri dishes. The Petri dishes were incubated at 25°C for 16 hours, or until germination was observed. A single germinating conidium was then transferred to fresh 2% MEA and allowed to grow at 25°C.

For long-term storage and conservation single conidial cultures were transferred to McCartney bottles containing 2% MEA slants and stored at 4°C. All isolates are maintained in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Vegetative compatibility tests

Tests to determine the number of vegetative compatibility groups (VCGs) were done as described by Anagnostakis (1977). Small blocks (2 mm) of mycelium, from the margins of one-week old, single conidial *S. sapinea* isolates, were paired in all possible combinations on Oatmeal agar (OMA) (6% m/v Oatmeal) in Petri dishes. The oatmeal was steamed for two hours at 70°C and filtered through a double-layered cheese-cloth. The oatmeal filtrate (600ml) was then added to a melted agar suspension (1.6% m/v

Biolab agar, final concentration) and autoclaved. After inoculation, cultures were incubated at 25°C in the dark. The compatibility between the isolates was assessed after seven days through the occurrence of black barrage lines (incompatibility) or confluent mycelial growth (compatibility) between isolates (Fig. 1).

Assessment of diversity

Vegetative compatibility tests were done on 153 *S. sapinea* isolates representing the various test populations included in this study (Table 1). The number of VCGs and the frequency of isolates in these VCGs were determined for each of the five populations. The degree of diversity in each of these populations was then assessed based on VCGs as a phenotypic character. The different VCGs were all weighted as equal characters and the possible involvement of different loci in the VCG reactions was ignored.

Three different parameters of diversity were used to assess the degree of diversity within each of the five (Mexico, California, Switzerland, Madagascar and Colombia) populations. The first indicator was calculated by dividing the number of VCGs (S) by the sample size of each population (N). The second parameter was the Shannon index (SI) (Bowman *et al.*, 1971) calculated as: $SI = -\sum p_i \ln p_i$ (p_i = the frequency of isolates with the i^{th} phenotype, k = the number of phenotypes in the population). The Shannon index was converted to a normalised Shannon diversity index (H_s) calculated as: $H_s = SI/H_{max}$ (SI = Shannon index, H_{max} = the maximum diversity for a sample of size N calculated as the natural logarithm of N). Another parameter of analysis was genotypic diversity (G) (Stoddart & Taylor, 1988) calculated as: $G = 1/\sum p_i^2$ (p_i = observed frequency of a specific phenotype). The estimation of G was standardised by determining the percentage of maximum diversity as: $\% G_{max} = \% G/N$ (G = genotypic diversity, N = population size). This standardisation allows comparison of genetic diversity between populations of different sample size. The mean degree of diversity for each of the five *S. sapinea* populations was assessed based on S/N ratios, H_s and the maximum percentage of genotypic diversity (Table 2). These values were then used to test the null hypothesis that no significant differences in the degree of diversity exist between the five populations.

RESULTS

Vegetative compatibility tests

All *S. sapinea* isolates included in this study showed strong interaction when paired on OMA that could easily be visually assessed to distinguish between compatible and incompatible isolates. Dark barrage lines were formed between incompatible *S. sapinea* isolates (Fig. 1), and those isolates were assigned to different VCGs. Compatible isolates and isolates paired against themselves as controls were characterised by a confluent lawn of mycelium without the formation of barrage lines (Fig. 1) and those isolates were assigned to the same VCG.

The three presumed native *S. sapinea* populations, Mexico (26 isolates), California (22 isolates) and Switzerland (22 isolates) were represented by 5, 8 and 6 VCGs, respectively (Table 1). The Mexican population had a single dominant VCG representing 58% of the total number of isolates, while dominant VCGs in the Californian and Swiss populations were represented by 27% and 37% of the total number of isolates, respectively (Table 1).

The two presumed introduced *S. sapinea* populations from Madagascar (15 isolates) and Colombia (68 isolates), were represented by 10 and 13 VCGs, respectively (Table 1). The most dominant VCG in the Malagasy population was comprised of 20% of the total number of isolates, while the Colombian population had a dominant VCG comprising 22% of all isolates.

Assessment of diversity

The S/N ratios (S = number of VCGs, N = total number of isolates) assessed for each of the five *S. sapinea* populations range from 0.2 to 0.7 (Table 2). The lowest S/N ratio was obtained for both the Mexican (0.2) and Colombian (0.2) populations, while the highest ratio obtained was for the Malagasy (0.7) population. The Californian and Swiss populations had relatively low S/N ratios of 0.3 each.

The Shannon diversity index (H_s) assessed for each of the five *S. sapinea* populations was higher than the S/N ratios for these populations and ranged from 0.3 to 0.8 (Table 2). The lowest H_s was found for the Mexican population (0.3) and the highest for the Malagasy population (0.8). H_s obtained for the Californian, Swiss and Colombian populations was 0.6, 0.5 and 0.5, respectively.

Genotypic diversity (G) assessed for each of the five *S. sapinea* populations was the lowest in the Mexican population (2.4) and the highest in the Malagasy population (7.8) (Table 2). Genotypic diversity assessed for the Californian, Swiss and Colombian populations was 5.8, 4 and 7.3, respectively. When G was standardised by assessing the maximum percentage of genotypic diversity, the Mexican population had the lowest maximum percentage (9%), while the Malagasy population had the highest percentage (52%) (Table 2). The maximum percentage of genotypic diversity for the Californian, Swiss and Colombian populations was 26%, 18% and 11%, respectively.

The mean degree of diversity derived for each of the five *S. sapinea* populations was lowest in the Mexican population (20%) and highest in the Malagasy population (66%), while those of the Californian, Swiss and Colombian populations were assessed as 38%, 32% and 27%, respectively (Table 2). The mean degree of diversity in the presumed native populations (Mexico, California and Switzerland) were not significantly different to those of the Colombian population ($df = 3$; $F = 0.580$; $p = 0.644$). Significant differences were, however, found between these populations (Mexico, California, Switzerland and Colombia) and the Malagasy population ($df = 3$; $F = 4.771$; $p = 0.034$) (Fig. 2).

DISCUSSION

The degrees of diversity estimated for five, geographically distant *S. sapinea* populations from Mexico, California, Switzerland, Colombia and Madagascar, included in this study, were highly variable. These estimates were based on the number, distribution and frequency of specific phenotypic characteristics (=VCGs) for each population and were expressed using four parameters (N/S, H_s , G and max % of genotypic diversity). Isolates in the most common VCG for the Malagasy population

had a low frequency of occurrence, indicating to a high degree of diversity for this population. Populations from Mexico, California, Switzerland and Colombia had higher frequencies of isolates in individual VCGs, indicating to lower degrees of diversity. The degree of diversity was significantly higher for the introduced Malagasy population than those of the three presumed native populations (Mexico, California and Switzerland). The population from Colombia, which like the Malagasy population is presumed to be introduced, had a lower degree of diversity and was more like those populations where *S. sapinea* is native.

Different statistical parameters for assessing the degree of diversity in fungal populations can reflect the level of diversity in a fungal population differently. The best choice of statistical parameter will thus give the most reliable assessment of the degree of diversity in the population. In this study, the degree of diversity assessed for each of the five *S. sapinea* populations was found to be lower when using genotypic diversity (G) (Stoddart & Taylor, 1988) compared to the number of VCG to isolate ratio (S/N) and the Shannon diversity index (Hs). In this study, the estimation of G is based on the number of VCGs, and since each VCG might represent more than one genotype, the degree of diversity observed in these populations could have been an under-estimation. Lower than expected degrees of diversity can, furthermore, be a consequence of the inability of this parameter (G) to distinguish between highly repeated phenotypes and rare phenotypes (Stoddart & Taylor, 1988). The Shannon diversity index (Hs) is probably the most accurate reflection of the degree of diversity in these populations, since it is based on the frequency of a particular phenotype and is reported to be stable when comparing populations with different sample sizes (Sheldon, 1969). The small sample sizes of the introduced Malagasy and native Californian populations, on the other hand, might also have lead to higher than expected degrees of diversity in those populations.

Sphaeropsis sapinea isolates from Colombia and Madagascar, represent exotic populations of the fungus and were probably introduced into these regions, together with *P. patula* seed. Repeated introductions of seed from different origins into Madagascar could account for the high degree of diversity assessed for this population. This would be similar to results of Smith *et al.* 2000 that showed lower levels of diversity in a presumably native Northern Sumatra *S. sapinea* population than in the

introduced South African population. The lower level of diversity in the Colombian population which, like that from Malagasy, could be due to a very limited introduction of the fungus into the former country.

The three presumed native populations (Mexico, California and Switzerland) considered in this study had equally low levels of diversity. This finding might also be contradictory to expectation. Generally, native populations of fungi would be more diverse than introduced populations (Nei, 1972; Coates, 1988; Perkins, 1991). However, *S. sapinea* is apparently restricted to asexual reproduction, and genetically fit genotypes might preferentially occupy the niche and the result is a relatively uniform population of the pathogen.

Sample size can significantly influence results of studies such as ours. In this study, we have attempted to gather the largest samples possible. This effort is, however, limited when making collections from a wide diversity of geographic origins, and with trees occurring in remote localities. Although we believe that our results contribute new knowledge to the understanding of *S. sapinea* distribution, it will be desirable to analyse larger sets of isolates in the future. This would be especially true in the case of Madagascar where a very small collection of isolates was available to us.

The degree of diversity estimated for the five *S. sapinea* populations considered in this study, enabled us to predict what influence the genetic structure of the pathogen might have on management of the pathogen. The highly diverse structure of the Malagasy population will impair breeding for resistance in pine species (Perkins, 1991) and might also restrict the effective transmission of biocontrol agents such as dsRNA (Caten, 1972; Anagnostakis *et al.*, 1986; Leslie, 1993). These control strategies could potentially be more sustainable and viable options to control *S. sapinea* from Mexico, Switzerland, California and Colombia where less genetic variability was observed. The estimation of the degree of diversity in *S. sapinea* populations such as those considered here, not only adds knowledge to our understanding of the genetic structure of the pathogen in various parts of the world, but also contributes to programmes aimed at effective management of *S. sapinea* die-back.

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Table 1. Summarised vegetative compatibility groups (VCG) results for five *Sphaeropsis sapinea* populations.

VCG nr.	Frequency of VCGs ^{a/}				
	Native populations			Introduced populations	
	Mexico ^{b/}	California	Switzerland ^{c/}	Madagascar	Colombia
1	15	6	8	3	15
2	7*	5	6	3	12
3	2	3	4*	2	11
4	1*	2	2	1	7
5	1*	2	1	1	6
6	0	2	1*	1	5
7	0	1	0	1	3
8	0	1	0	1	3
9	0	0	0	1	2
10	0	0	0	1	1
11	0	0	0	0	1
12	0	0	0	0	1
13	0	0	0	0	1
Number of isolates	26	22	22	15	68

^{a/} The frequencies of the VCGs represent the number of isolates in each VCG.

^{b/} All but four Mexican isolates were collected from *P. patula* and the remainder, indicated with an asterisk (*), were from *P. greggii*.

^{c/} All but two Swiss isolates were collected from *P. sylvestris* and the remainder, indicated with an asterisk (*), were from *P. nigra*.

Table 2. Summarized population data for five populations of *Sphaeropsis sapinea*.

Region ^{a/}	Host ^{b/}	Nr of isolates (N) ^{c/}	Nr of VCGs (S)	Population diversity			Max % of genotypic diversity ^{g/}	Mean % of diversity ^{h/}
				S/N ^{d/}	Hs ^{e/}	G ^{f/}		
Mexico	<i>P. patula</i> , <i>P. greggii</i>	26	5	0.2	0.3	2.4	9	20
California	<i>P. radiata</i>	22	6	0.3	0.6	5.8	26	38
Switzerland	<i>P. sylvestris</i> , <i>P. nigra</i>	22	6	0.3	0.5	4.0	18	32
Colombia	<i>P. patula</i>	68	13	0.2	0.5	7.3	11	27
Madagascar	<i>P. patula</i>	15	10	0.7	0.8	7.8	52	66

^{a/}The *S. sapinea* isolates obtained from Mexico, California and Switzerland are native, while isolates obtained from Colombia and Madagascar are exotic.

^{b/}All but four Mexican isolates were collected from *P. patula* and the remainder from *P. greggii*, while all but two Swiss isolates were collected from *P. sylvestris* and the remainder from *P. nigra*.

^{c/}The total number of isolates considered as the sampled population.

^{d/}Diversity of the population expressed by the number of VCG's divided by the number of isolates.

^{e/} Diversity expressed by the Shannon diversity index (Bowman *et al.*, 1971).

^{f/}Genotypic diversity (Stoddart and Taylor, 1988).

^{g/}Percentage of the genotypic diversity (G) of the population divided by the total number of isolates in that population (N).

^{h/}Mean percentage of S/N ratio, Hs and the maximum percentage of genotypic diversity.

Figure 1. *Sphaeropsis sapinea* vegetative compatibility groups on oatmeal agar. **(A)** Incompatible reactions between isolates belonging to different VCGs. **(B)** Compatible reactions between isolates belonging to the same VCG. Arrows indicate to barrage lines between isolates of different VCGs or to confluent mycelial mats between isolates of the same VCG.

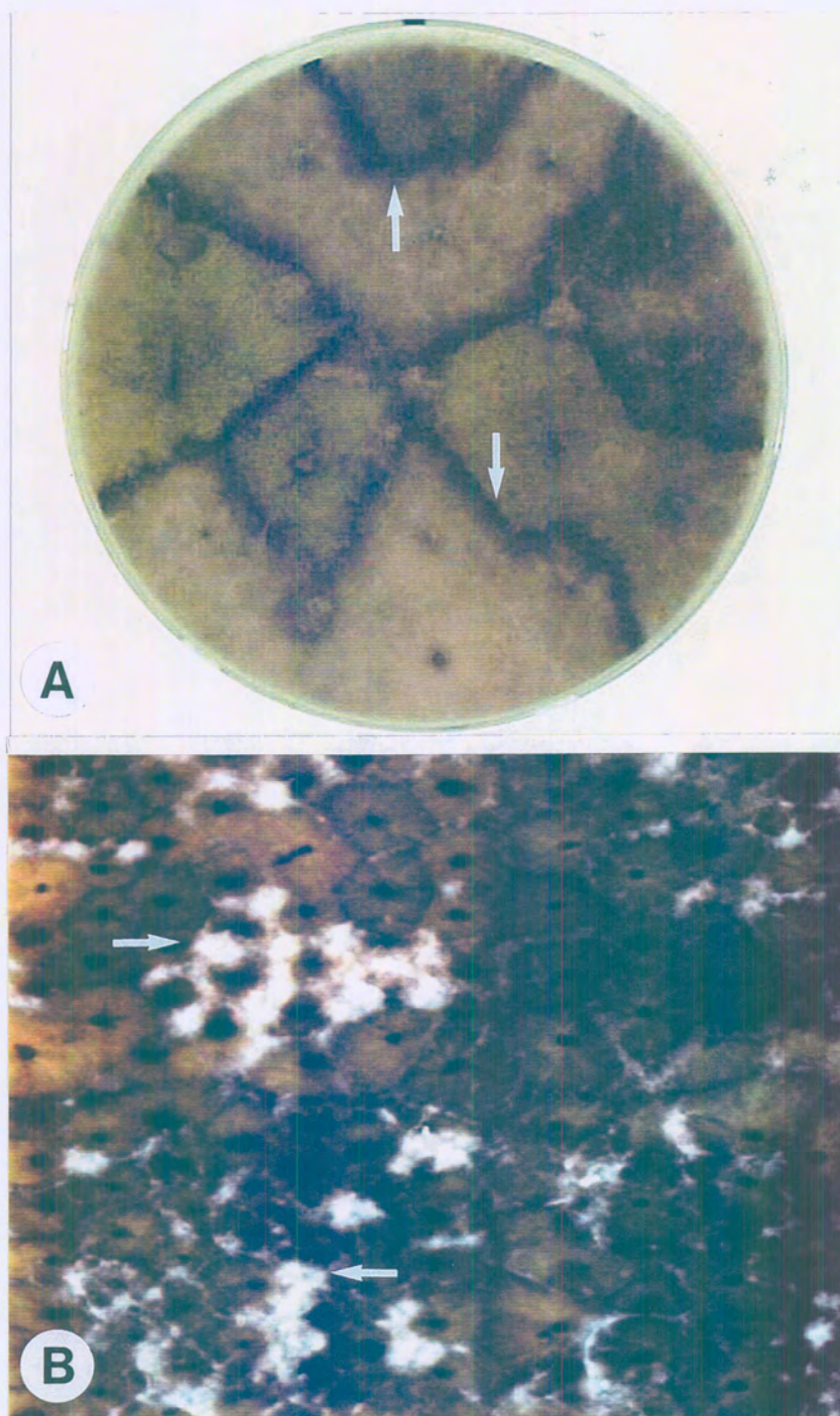
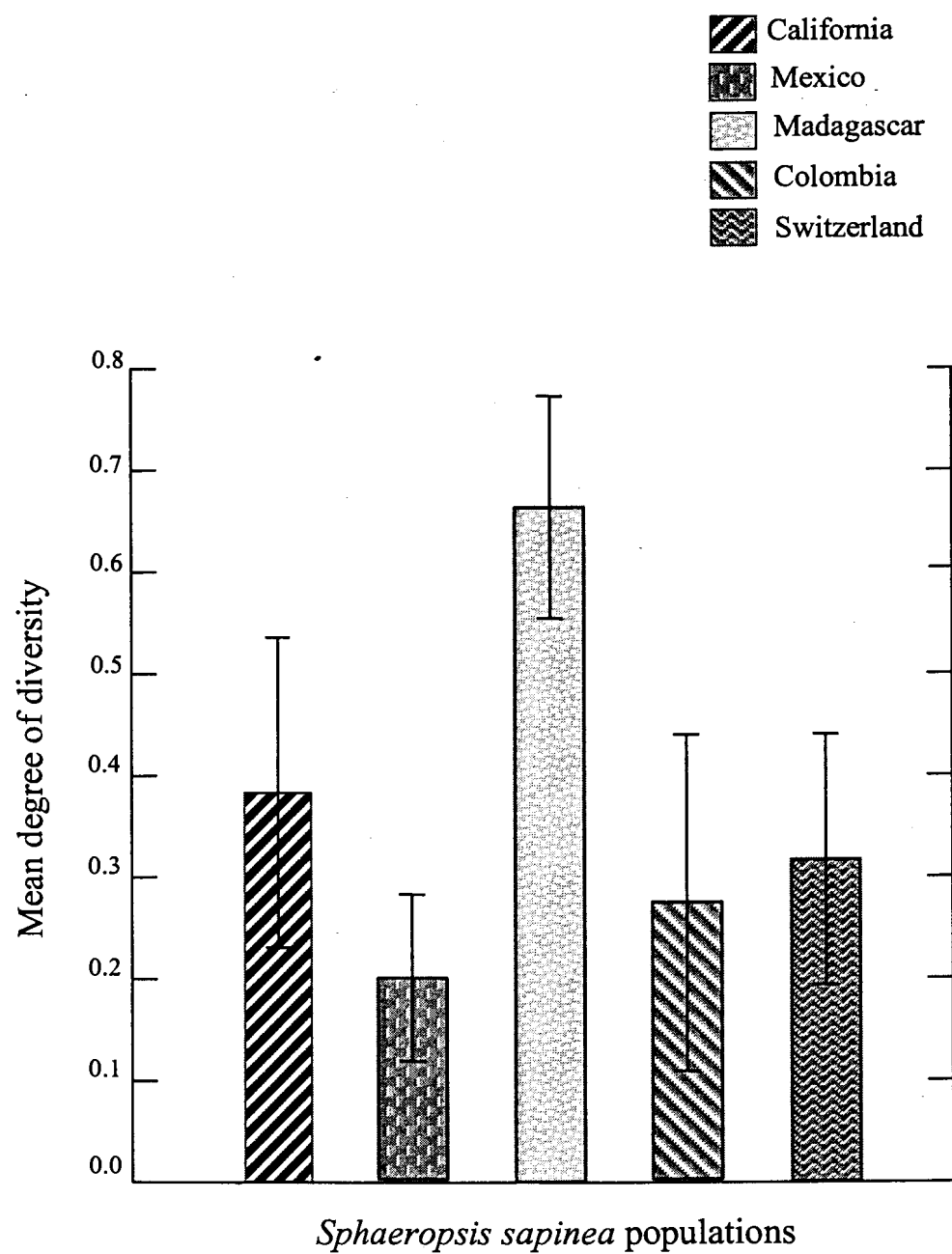
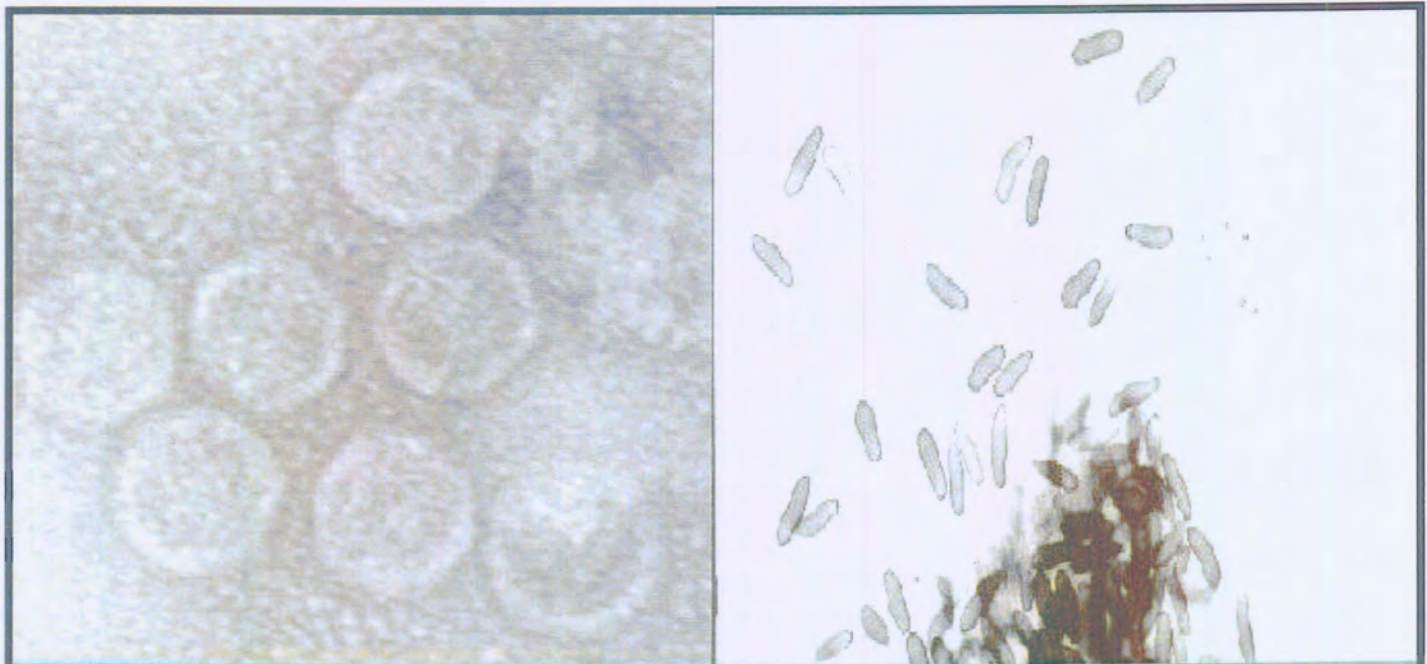


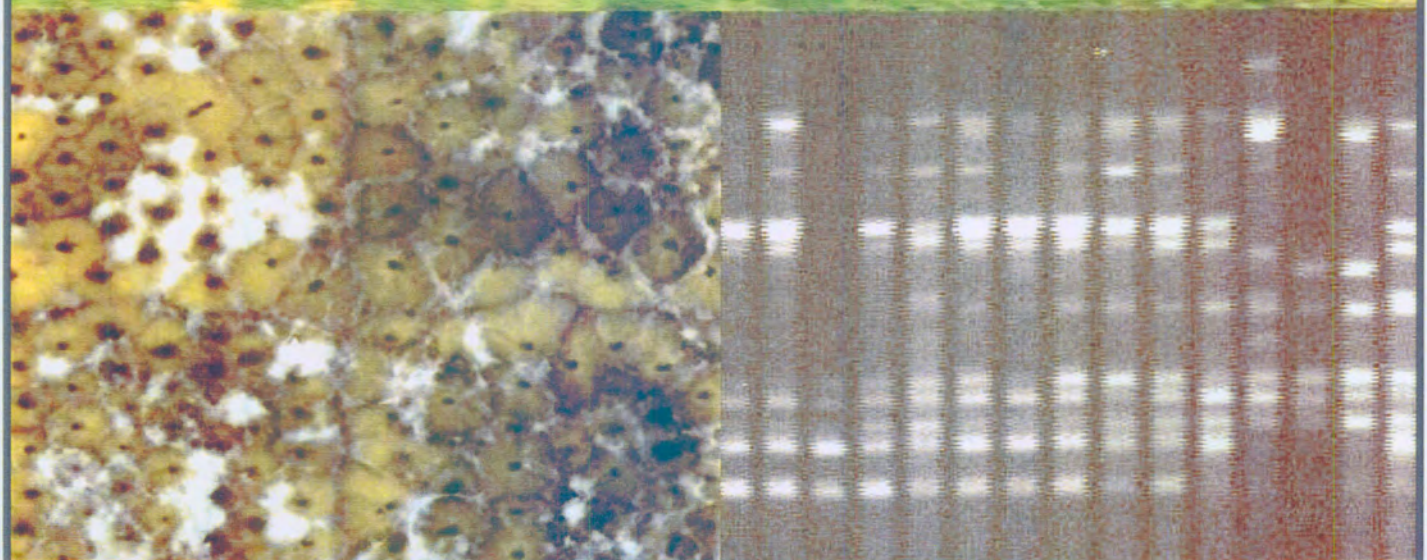
Figure 2. Mean degree of diversity for five *Sphaeropsis sapinea* populations from geographically distant regions (Mexico, California, Switzerland, Colombia and Madagascar). The bars represent the mean value ($\pm$ S.E.M.) of diversity for each population.





CHAPTER 5

dsRNA FRAGMENTS IN *SPHAEROPSIS* *SAPINEA* ISOLATES AND THEIR EFFECT ON MORPHOLOGY AND PATHOGENICITY



ABSTRACT

Two distinct double-stranded RNA viruses (SsRV1 and SsRV2), belonging to the family Totiviridae, are known to co-infect South African isolates of the pine pathogen, *Sphaeropsis sapinea*. These two dsRNA viruses have no obvious effect on the morphology or pathogenicity of the fungus. The objective of this study was to screen *S. sapinea* isolates from a wide range of geographical regions for the presence of dsRNA fragments and to compare these with SsRV1 and SsRV2. *In vitro* growth studies and pathogenicity tests were done in order to determine whether the presence of dsRNA fragments has an effect on the fungus. *Sphaeropsis sapinea* isolates (104) from Switzerland, Madagascar, Mexico, Indonesia, Colombia and California were screened for the presence of dsRNA fragments using CFII cellulose extraction chromatography. The isolated dsRNA fragments were compared based on electrophoretic mobility in agarose gels. DsRNA fragments of similar size to the genomes of SsRV1 and SsRV2 were subjected to RT-PCR amplification using SsRV1- and SsRV2-specific primers. DsRNA isolated from Malagasy and Colombian isolates were similar in size to SsRV1 and SsRV2, while those from the Mexican and Californian isolates differed from the SsRVs. No significant differences were found in cultural characteristics, growth rate or virulence for dsRNA-containing and dsRNA-free isolates of *S. sapinea*. The results of this study indicate that dsRNA fragments are common in *S. sapinea* isolates from a wide range of regions. Five different dsRNA fragment sizes are recognised, of which one represents two previously characterised genomes (SsRV1 and SsRV2). The additional dsRNA fragments in *S. sapinea* now deserve characterisation at the molecular level.

INTRODUCTION

Sphaeropsis sapinea (Fr.:Fr.) Dyko & Sutton (= *Diplodia pinea* (Desm.) Kickx.) is a widely distributed pathogen of conifers (Eldridge, 1961; Buchanan, 1967; Punithalingam & Waterston, 1970; Gibson, 1979; Swart, Knox-Davies & Wingfield, 1985). It is an opportunistic pathogen that is dependent on host stress in order to

manifest itself as pathogen (Laughton, 1937; Waterman, 1943; Buchanan, 1967; Marks & Minko, 1969; Brown, Bevege. & Stevens, 1981). Many disease symptoms have been associated with this fungus and these include top die-back, shoot blight, stem cankers, collar rot, a root disease and blue stain (Da Costa, 1955; Millikan & Anderson, 1957; Purnell, 1957; Eldridge, 1961; Buchanan, 1967; Marks & Minko, 1969; Wingfield & Knox-Davies, 1980). Recently, *S. sapinea* has also been recognised as an endophyte, causing latent and symptomless infections in the tissue of healthy pine trees and seedlings (Smith *et al.*, 1996; Stanosz *et al.*, 1997).

Subsequent to the discovery of virus-like particles (VLPs) in the commercial mushroom, *Agaricus bisporus* (Hollings, 1962), VLPs have been found in more than 100 fungal species belonging to 50 different genera. These mycoviruses typically have simple isometric protein structures that encapsulate the genetic material which is predominantly double-stranded RNA (dsRNA) (Hollings, 1962; Hollings & Stone, 1971; Buck *et al.*, 1981; Buck, 1986; Travantzis & Bandy, 1988; Chen *et al.*, 1996). The dsRNA genomes usually include two genes, one coding for the coat protein and the other for a RNA dependent RNA polymerase (RDRP) (Bruenn, 1993).

The occurrence of multiple viral infections, including more than one dsRNA fragment, are very common in fungi (Hollings, 1962; Buck, 1986; Ghabrial & Havens, 1992; Bottacin, Lévesque & Punja, 1994). Most viral infections are usually symptomless but can potentially also have altered effects on the expression of fungal genes, resulting in various phenotypic changes (Hollings, 1962; Hollings & Stone, 1971; Buck, 1986; Anagnostakis, 1988; Nuss & Koltin, 1990; Chen *et al.*, 1996). These can include modifications to the growth rate and pigmentation of the pathogen (Van Alfen *et al.*, 1975; Chen *et al.*, 1996), a reduction or increase in its virulence (Day *et al.*, 1977; Elliston, 1981; Nuss & Koltin, 1990; Boland, 1992) and the production of toxins and antibiotics (Lemke & Nash, 1974).

Naturally occurring dsRNA fragments of different sizes have been found in the cytoplasm of *S. sapinea* isolates (Wu, Palmer & Adams, 1989; Steenkamp *et al.*, 1998; Preisig, Wingfield & Wingfield, 1998). Since the initial discovery of these fragments, the genomes of two distinct dsRNA mycoviruses, known as SsRV1 and SsRV2 (*Sphaeropsis sapinea* RNA virus 1 and 2), have been completely sequenced at the

cDNA level (Preisig *et al.*, 1998). These two mycoviruses have linear dsRNA genomes, with SsRV2 having a slightly bigger genome (5202 bps) than SsRV1 (5163 bps) (Preisig *et al.*, 1998). Furthermore, SsRV1 and SsRV2 are known to belong to the family Totiviridae based on their amino acid homologies. Unlike the Hypoviruses, members of this virus family do not mediate hypovirulence in fungal pathogens (Preisig *et al.*, 1998).

The viral infections found in *S. sapinea* have, thus far, been found to be symptomless (Wu *et al.*, 1989; Steenkamp *et al.*, 1998; Preisig *et al.*, 1998; G.C. Adams, personal communication). The B morphotype of *S. sapinea* is slower growing and less virulent (Wang *et al.*, 1985; Palmer, Stewart & Wingfield, 1987), that can potentially be the result of viral infections. However, various attempts to correlate the unusual phenotype of the B morphotype isolates to the presence of dsRNA fragments have been unsuccessful (Wu *et al.*, 1989).

Management of *S. sapinea* diseases, is mainly through breeding and selection for disease tolerance in *Pinus* spp., planting of tolerant trees in areas prone to environmental stress conditions, and the restriction of pruning to times when the pathogen is not likely to infect (Lückhoff, 1964; Wright and Marks, 1970, Gibson, 1979; Burdon, Currie & Chou, 1980; Swart *et al.*, 1985; Swart & Wingfield, 1991). Despite careful management, *S. sapinea*-induced diseases remain important in South Africa, where post-hail associated die-back of *Pinus radiata* D. Don. has resulted in losses of up to 2000 ha. of trees in a single year (Zwolinski, Swart. & Wingfield, 1994). A contemporary approach to the management of fungal pathogens could be through hypovirulence (reduce virulence)-mediated dsRNA viruses (Day *et al.*, 1977; Elliston, 1981; Boland, 1992). The Hypoviruses associated with *Cryphonectria parasitica* (Murr.) Barr. are effective as biological control agents in reducing the impact of this pathogen (Anagnostakis, 1982; Nuss, 1992). DsRNA fragments associated with *S. sapinea* should also have the potential of being exploited as biocontrol agents particularly in areas where the pathogen has a limited diversity.

The objective of this study was to screen *S. sapinea* isolates from native and introduced populations for the presence of dsRNA fragments and to consider the distribution of the SsRVs in these populations. We, furthermore, considered differences in cultural

characteristics, *in vitro* growth rate and pathogenicity for dsRNA-free and dsRNA-containing *S. sapinea* isolates.

MATERIALS AND METHODS

Fungal isolates

Sphaeropsis sapinea isolates from Mexico, Switzerland and Madagascar were obtained by direct isolation from the pith tissue of *P. patula* Schl. et Cham., *P. greggii* Engelm., *P. sylvestris* L., and *P. nigra* Arnld. cones (Table 1). Colombian and Indonesian *S. sapinea* isolates were obtained from pycnidia on *P. patula* and Californian isolates from *P. radiata* shoots showing die-back symptoms (Table 1). All dark-coloured fungal colonies were transferred to 2% Malt Extract Agar (MEA) (2% m/v Biolab malt extract; 2% m/v Biolab agar), as well as to 2% Water Agar (WA) (2% m/v Biolab agar), supplemented with sterile pine needles to induce sporulation. All Petri dishes were incubated at 25°C under mixture of white and near-ultraviolet light set to provide 12 hour light/dark cycles.

Identification of *S. sapinea* isolates was based on conidial and pycnidial morphology (Sutton, 1980). Single conidial cultures were generated by spreading conidia, released from excised pycnidia, onto the surface of 2% WA in Petri dishes. The cultures were incubated at 25°C in a mixture of white and near-ultraviolet light set to provide 12 hour light/dark cycles until germination was observed. Four germinating conidia for each isolate were then transferred to 2% MEA and were incubated at 25°C.

For long-term storage and conservation, single conidial isolates were transferred to McCartney bottles containing 2% MEA slants and kept at 4°C. All isolates are maintained in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DsRNA extraction

Sphaeropsis sapinea isolates (104) obtained from Madagascar, Colombia, California, Switzerland, Mexico and Indonesia were screened for the presence of dsRNA (Table 1). These isolates were grown in 250 ml Erlenmeyer flasks containing 2% ME (2% m/v Biolab Malt Extract) broth, incubated at 25°C with shaking (200 rpm) for at least two weeks or until sufficient biomass was produced for dsRNA extraction.

Mycelium was harvested through centrifugation and then lyophilised. The lyophilised mycelium was ground to a fine powder using a mortar and pestle in the presence of liquid nitrogen. The ground mycelium from four isolates of the same population was pooled to accelerate the screening process. All the pooled sets that showed positive results for the presence of dsRNA were then individually screened. DsRNA-infected isolates were included in the extraction procedures as positive controls. These three control isolates from South Africa (CMW4254 and CMW4241) and North America (BOT299) have previously been shown to contain dsRNA (Van der Merwe, 1997).

Ground mycelium was suspended in 2x STE buffer (10x STE: 0.5 M Tris-HCL, 1 M NaCl 10 mM EDTA, pH 6.8) and 1% SDS, followed by incubation at 60°C for 10 min. An equal volume of phenol was added and incubated in a shake incubator at 37°C for 30 min. This was followed by centrifugation at 10 000 rpm and 4°C for 20 min to separate the nucleic acids from the proteins and cell debris (organic phase). Proteins were extracted with an equal volume of chloroform and centrifugation at 10 000 rpm for 20 min. The nucleic acid-containing aqueous phase was equilibrated to 16% ethanol and centrifuged at 5000 rpm for 10 min.

CFII cellulose columns (Valverde, Nameth & Jordan, 1990) were prepared to separate the dsRNA from the other nucleic acids. Syringes (2 ml) were plugged with glass wool and filled with the CFII cellulose suspension (0.5 g cellulose, 2x STE, 16% ethanol). The columns were equilibrated with 15 ml 2x STE containing 16% ethanol, after which the nucleic acid samples were applied. This was followed by washing of the columns with 15 ml 2x STE containing 16% ethanol. Ten dsRNA fractions (1 ml each) were eluted from the columns using 10 ml 2x STE and were collected in 1.5 ml microcentrifuge tubes. The dsRNA of each fraction was precipitated with 0,7 volumes

isopropanol and 0,1 volume 3 M sodium acetate and overnight incubation at -20°C . The dsRNA was collected through centrifugation at 13 000 rpm for 30 min at 4°C , washed with 70% ethanol, dried in a Speedy-Vac and the ten fractions were then re-suspended to a final volume of 100 μl with DEPC-treated double sterilised water. DNase I (Boehringer Mannheim) and RNase A (Boehringer Mannheim) digestions were done at 37°C for two hours to verify the nature of the isolated nucleic acids.

The undigested dsRNA fragments, DNase- and RNase-digested dsRNA fragments were separated on a 1% agarose (w/v) gel (Promega) containing ethidium bromide (1 μl /100 ml). From each sample, 10 μl was electrophoresed in a 0.5x Tris-boric acid-EDTA (TBE) (pH 8,0) electrophoresis buffer at 80 Volts for one hour. The size-separated fragments were visualised under UV light and stored at -20°C . All glassware and solutions were treated to eliminate the presence of RNase.

RT-PCR reactions

RT-PCR reactions were performed using the TitanTM One Tube RT-PCR System (Boehringer Mannheim) and two primer pairs. Primer pair 1 (Oli3: 5'-CGCGTGAGTACCGGAGGAT-3' and Oli31: 5'-CATTGACAGGGCCGAACAATTCC-3') is specific to the RNA-dependent RNA polymerase of the SsRV1 genome (Fig. 2). Primer pair 2 (Oli32: 5'-CACGCCATCGAGTACCCAACCTT-3' and 5'-RDRP2: 5'-CCGCAATGAGTAACGACAGCTCC-3') is specific to the RNA-dependent RNA polymerase of the SsRV2 genome (Fig. 2). DsRNA fragments extracted from all the isolates, as well as the dsRNA of two South African isolates (CMW4254 and CMW4241) and an isolate from the United States (BOT299) that were included for comparative purposes, were subjected to these RT-PCR reactions using both primer pairs (Table 2).

The RT-PCR reaction mixture consisted of: 5 U RNase inhibitor, 5 mM DTT, 5x RT-PCR buffer, 2.5 mM dNTP (each), 15 μM of each primer, 3 μl of the dsRNA template and 0.5 μl of the heat-sensitive enzyme mix. The reaction volume was adjusted to 25 μl with DEPC-treated double sterilised water. The dsRNA template, together with both

primers and the DEPC-treated double sterilised water were incubated at 98°C for three min. The rest of the RT-PCR reaction was then added and subjected to the following temperature profile: 30 min at 50°C, 2 min at 94°C, 10 cycles of 30 s at 94°C, 30s at 58°C and 45 s at 68°C, the last three temperature intervals were repeated for another 30 cycles with a 5 s increase per cycle.

The RT-PCR products (cDNA) were separated on a 1% agarose (w/v) gel (Promega) containing ethidium bromide (1 µl/100 ml). From each sample, 10 µl was electrophoresed in a 0.5x Tris-boric acid-EDTA (TBE) (pH 8,0) electrophoresis buffer at 80 Volts for one hour. The size-separated fragments were visualised under UV light.

The cDNA fragments in the desired size range, compared to a molecular weight marker, were excised from the gel and purified using the Boehringer Mannheim PCR Product Purification System (Boehringer Mannheim). The purified cDNA products were stored at -20°C.

Growth in culture

Colony morphology and colour of eight *S. sapinea* isolates from South Africa, United States, Mexico, Columbia and Madagascar (Table 2) were noted on 2% MEA after 15 days of incubation at 25°C in the dark. The mycological colour chart of Rayner (1970) was used to assess colony colour. An *in vitro* growth study was also performed on these eight isolates. Five of these isolates contained dsRNA fragments of varying size and the remaining three isolates were free of any dsRNA fragments. We included isolates that did not contain dsRNA fragments and did not cure the dsRNA-infected isolates of their dsRNA fragments due to the difficulty of curing dsRNA-infected isolates (Boland, 1992) and the inability to retain the viability of cured isolates (Steenkamp, 1996).

Mycelial plugs (10 mm) from the margins of one-week old, single conidial isolates were transferred to 2% MEA in Petri dishes (9 cm). Four petri dishes per isolate were incubated at each of the four temperatures (10, 15, 25 and 30°C) in the dark. The growth rate of these isolates was determined by measuring colony diameters at the four

temperatures after 2, 4, 8 and 15 days. Growth rates (mm/day) were statistically analysed using mean separation procedures (SYSTAT, version 7.0.1). Differences in growth rate among dsRNA-containing and dsRNA-free *S. sapinea* isolates were analysed using the Analysis of Variance and the Tukey pairwise comparisons test (SYSTAT, version 7.0.1). The null hypothesis that no significant differences in growth rate exist between these isolates, was also tested. In addition, significant differences in growth rate among isolates representing the four classes of dsRNA infection (no dsRNA infections, single SsRV-infections, co-infections with SsRV1 and SsRV2 and infections with the unique 8 kb fragment) were determined.

Pathogenicity tests

The pathogenicity of eight *S. sapinea* isolates from the United States, South Africa, Mexico, Madagascar and Colombia, used in cultural studies, was tested (Table 2). The pathogenicity of each isolate was determined by measuring the surface area of infection on Granny Smith apples, as well as, lesion lengths on two-year-old *P. patula* seedlings. Inoculations of apples have, in the past, been effectively used to predict the virulence of isolates of *C. parasitica* (Elliston 1985), as well as for isolates of *S. sapinea* (Steenkamp, 1996). The inoculum was prepared by growing single conidial isolates on 2% MEA at 25°C, one week prior to inoculation. Sterile 2% MEA plugs were inoculated as controls. A completely randomised block design was used and all tests were repeated once.

Tests on Granny Smith apples. Prior to inoculation, the apples were surface sterilised with 70% ethanol and 1% NaClO. A wound (10 mm wide and 5 mm deep) was made in each apple using a cork borer. Mycelial plugs of the same size from the margins of actively growing cultures were placed, mycelium-surface down, in the wound. Four apples were inoculated for each isolate. The wounds were sealed with masking tape to prevent desiccation and incubated at room temperature. After two weeks lesion lengths, widths and surface areas were measured and means compiled.

Tests on *P. patula* seedlings. Ninety, two-year-old *P. patula* trees (ca. 1 m in height) were inoculated with the same *S. sapinea* isolates used for the tests on apples (Table 2). Mycelial plugs (10 mm) from the margins of actively growing cultures were placed

mycelium-side down in wounds of the same size, made on the stem. Ten seedlings were inoculated for each isolate. The wounds were sealed with masking tape to prevent desiccation. The tests were performed in a controlled environment greenhouse with an average daily temperature of 25°C. Lesion lengths were measured after four weeks.

Results for both the tests, on apples and pine seedlings, were statistically analysed using mean separation procedures (SYSTAT, version 7.0.1). Differences in virulence among dsRNA-containing and dsRNA-free *S. sapinea* isolates were analysed using the Analysis of Variance and the Tukey pairwise comparisons test (SYSTAT, version 7.0.1). The frequency distribution in these data sets was skewed and a log (10) transformation was done to normalise the distribution frequencies and to meet assumptions of parametric testing. The null hypothesis that no significant differences in virulence among dsRNA-infected and dsRNA-free *S. sapinea* isolates exist, was also tested. In addition, significant differences in virulence among isolates representing the four classes of dsRNA infections (no dsRNA infections, single SsRV-infections, co-infections with SsRV1 and SsRV2 and infection with the unique 8 kb fragment) were determined.

RESULTS

Presence of dsRNA

DsRNA fragments were isolated from 28% of the 104 single conidial *S. sapinea* isolates collected from six different geographical regions (Table 1). The occurrence of dsRNA fragments in isolates from Colombia and Madagascar was greater (ca. 33%), while fewer isolates from California and Mexico contained dsRNA (ca. 10 - 27%). No dsRNA fragments were found in isolates from Switzerland and Indonesia. The nature of all these fragments was verified as dsRNA by performing RNase A and DNase I digestions (Hansen *et al.*, 1985).

Based on their relative mobility against DNA molecular weight markers, dsRNA fragments could be grouped in five size classes, ca. 8 kb, 5 kb, 4 kb, 1.5 kb and 1.3 kb (Fig. 1). Isolates that contained more than one of these five dsRNA fragments were

common and these represented 62% of the isolates with dsRNA (Table 2). Three of four dsRNA-containing Malagasy isolates, six of nine dsRNA-containing Colombian isolates and one of three dsRNA-containing Californian isolates contained multiple fragments. Multiple occurrences of the 5 kb, 4 kb, 1.5 kb and 1.3 kb dsRNA fragments were found in isolates from Colombia, while multiple occurrences of only the 4 kb, 1.5 kb and 1.3 kb dsRNA fragments were found in isolates from California. In isolates from Madagascar, co-infections with the two 5 kb fragments (SsRV1 and SsRV2) were found. Single infections with the 8 kb dsRNA fragment were unique to two of the Mexican isolates.

Previously characterised *S. sapinea* dsRNA viruses (SsRV1 and SsRV2), have genome sizes in the 5 kb range. DsRNA fragments in the same size range were extracted from two South African, one United States, eight Colombian and four Malagasy *S. sapinea* isolates (Table 2). The dsRNA of all these isolates were successfully transcribed to 23 cDNA (compliment DNA) products through RT-PCR amplification (Fig. 3). RT-PCR products of *ca.* 1 kb were obtained from 10 isolates using SsRV1-specific primers, while RT-PCR products of *ca.* 800 bp were obtained from 13 isolates using SsRV2-specific primers. Eight of these 15 isolates were thus co-infected with both SsRV1 and SsRV2 (BOT796, BOT798, BOT835, BOT777, BOT899, BOT893, CMW54254 and BOT299), while the other seven isolates were infected only with either SsRV1 or SsRV2 (BOT760, BOT793, BOT785, BOT801, BOT812, BOT892 and CMW4241) (Table 2). No dsRNA fragments in the 5 kb size range were extracted from the Californian and Mexican isolates and thus no RT-PCR products were generated from any of these isolates.

Effect of dsRNA on culture characteristics and growth rate

The colour and appearance of the mycelium of eight dsRNA-containing and dsRNA-free *S. sapinea* isolates, included in the study, was unaffected by the presence of dsRNA fragments (Table 3, Fig. 4). These isolates also had optimal growth rates at 25-30°C and slower growth at 15°C and 10°C, irrespective of the presence of dsRNA (Table 3). After 8 days, all the isolates incubated at 25°C had completely covered the surface of the medium in Petri dish. Based on the mean growth rate at 25°C, the South African isolate (CMW4254) had the slowest growth (11 mm/day), while the Colombian

isolate (BOT760) had the fastest growth (21 mm/day) (Table 3). No significant differences in mean growth rates (mm/day) for dsRNA-containing and dsRNA-free *S. sapinea* isolates were observed at any of the four temperatures tested (Table 4).

A comparison of mean growth rate at 25°C for isolates co-infected with both SsRV1 and SsRV2, those infected only with SsRV2, those containing the larger dsRNA fragment (8 kb) and those free of any dsRNA did revealed significant differences. Isolates (CMW4254, BOT299 and BOT777) infected with both SsRV1 and SsRV2 had slower growth than those infected only with SsRV2 (BOT760) (Table 4). The growth rate of the Mexican isolate (BOT769), which contained a 8 kb dsRNA fragment, was not significantly different to those co-infected with both SsRVs or those without dsRNA (BOT770, BOT848 and BOT768) (Table 4).

Effect of dsRNA on pathogenicity

Tests on Granny Smith apples. Inoculation of five dsRNA-containing and three dsRNA-free *S. sapinea* isolates into Granny Smith apples all resulted in lesions, whereas control inoculations with sterile MEA plugs showed no lesion development (Table 5, Fig. 5). Surface areas of infection by *S. sapinea* isolates varied from small (*ca.* 27mm²) to large (*ca.* 800mm²) (Table 5). Based on the mean surface area of infection, a slightly smaller area was induced by dsRNA-infected isolates when compared to dsRNA-free isolates (Table 6). There was, however, no significant difference in pathogenicity between dsRNA-infected and dsRNA-free isolates.

Isolates (CMW4254, BOT299 and BOT777) co-infected with both SsRV1 and SsRV2 and the one (BOT769) containing the 8 kb dsRNA fragment produced significantly smaller areas of infection than those where a single SsRV-infection (BOT760) or no dsRNA (BOT770, BOT848, BOT768) occurred (Table 6).

Tests on *P. patula* seedlings. Inoculation of pine seedlings with *S. sapinea* isolates all resulted in lesions whereas control inoculations with sterile MEA plugs led to no lesion development (Table 5, Fig. 5). Lesion lengths varied between *ca.* 16 mm and *ca.* 57 mm (Table 5). The longest lesions were produced by the Colombian isolate (BOT760) and the smallest lesions by the South African (CMW4254) isolate (Table 5). The

lesion lengths among the isolates were significantly different but there was no obvious correlation among isolates containing dsRNA and those without dsRNA (Table 7).

Isolates (CMW4254, BOT299 and BOT777) co-infected with SsRV1 and SsRV2 produced significantly smaller lesions than the isolate (BOT760) with only a single SsRV2 infection (Table 7). No differences in pathogenicity were found between isolates (BOT770, BOT848 and BOT768) with no dsRNA infections and those co-infected with SsRV1 and SsRV2 or the one (BOT769) containing the 8 kb dsRNA fragment (Table 7).

DISCUSSION

In this study, dsRNA fragments were associated with more than a quarter of the total number of *S. sapinea* isolates screened. These isolates were collected from Colombia, Madagascar, Mexico and California. This indicates that such dsRNA fragments are common in these fungi and have previously also been reported from the north Central United States and South Africa (Wu *et al.*, 1989; Steenkamp *et al.*, 1998; G.C. Adams, personal communication). The occurrence of dsRNA fragments in the populations from Madagascar and Colombia (*ca.* 30% each) was higher than in those from Mexico (10%) and California (27%). The isolates from Madagascar and Colombia are introduced while those from Mexico and California are presumed to be native, as *Pinus* spp. are native to those countries. No dsRNA fragments were extracted from isolates from Switzerland and Indonesia where native *Pinus* spp. also occur, although our collections were made from introduced species. These results clearly show that dsRNA fragments are being introduced into new environments together with *S. sapinea*.

In this study, the occurrence of dsRNA fragments was higher in the more diverse populations from Colombia and Madagascar (as determined in Chapter 4) than in those from Mexico and California, that were less diverse. A greater number of VCGs (higher diversity) is generally seen as a barrier against the effective spread of VLPs (Caten, 1972; Anagnostakis, Hau & Kranz, 1986; Leslie, 1993). These results, however, indicate that the higher degree of diversity in *S. sapinea* populations did not impair the transmission of the dsRNA fragments.

All *S. sapinea* isolates used in this study originated from single conidia. Our results, therefore, show dsRNA fragments are transmitted through the conidia. This finding contrast to reports for other fungi showing that viral fragments do not enter the conidia (Shain & Miller, 1991). Repeated monoconidial culturing can, however, result in the loss of dsRNA fragments and this technique can be used to generate dsRNA-cured isolates (G.C. Adams, personal communication). Steenkamp (1996), however, made repeated monoconidial transfers of dsRNA-infected isolates without eliminating dsRNA.

During this study, multiple infections with two or more dsRNA fragments were found in the majority of *S. sapinea* isolates (57%). A high degree of size variation (*ca.* 8 kb, 5 kb, 4 kb, 1.5 kb and 1.3 kb) was furthermore, found among these fragments. DsRNA fragments of eight different sizes were also found in *S. sapinea* isolates from the north central United States (G.C. Adams, personal communication). The smaller bands could be deletion derivatives of the larger bands but these bands were consistently isolated from the same isolates which suggests that they are distinct and representing discrete VLPs. In a previous study, Van der Merwe (1997) was also unsuccessful in linking some of the smaller dsRNA bands with the SsRVs, based on amplification with specific SsRV-derived primers.

The characterisation of SsRV1 and SsRV2, and the construction of specific primer pairs (Preisig *et al.*, 1998) made it possible to determine the identity of these two viruses in a wide range of *S. sapinea* isolates. In this study, SsRV1/SsRV2 co-infections and single SsRV infections with predominantly SsRV2, were common in isolates from Colombia and Madagascar, but did not occur in the isolates from Mexico and California. Various pine species planted in South Africa and Madagascar are native to Mexico and California. The absence of the SsRVs in *S. sapinea* isolates from these regions might be due to relatively small sample sizes or the fact that SsRVs might have originated elsewhere. DsRNA fragments other than SsRVs deserve further characterisation. This will also expand available knowledge regarding the movement of *S. sapinea* around the world.

In this study, no phenotypic characteristics observed in dsRNA-containing *S. sapinea* isolates could be positively correlated to the presence of dsRNA fragments. Previous attempts have also failed to link the presence of dsRNA fragments to alterations in the normal phenotype of *S. sapinea* (Wu *et al.*, 1989; Steenkamp *et al.*, 1998; Preisig *et al.*, 1998; G.C. Adams, personal communication). The type of dsRNA fragment present, and whether co-infections with SsRV1 and SsRV2 or single infections with one of them occur did, however, have an effect on the growth rate and pathogenicity of the fungus. Isolates infected with both SsRVs had significantly slower growth and were less virulent than those with only SsRV2 infections. There was, however no significant differences in growth rate and pathogenicity of isolates with no dsRNA and those with only SsRV2 infections, or among isolates co-infected with both SsRVs and those containing an 8 kb dsRNA fragment. Although, the presence of dsRNA can result in reduced virulence (Day *et al.*, 1977), there is thus far no evidence to link the presence of dsRNA in *S. sapinea* with hypovirulence.

In this study, we have included a relatively large collection of *S. sapinea* isolates from countries with substantial geographic separation. Through considering the occurrence of SsRV1 and SsRV2, interesting information pertaining to the international spread of *S. sapinea* has emerged. However, dsRNA infections are likely to be present in a relatively small number of isolates, particularly where pines are grown as natives. Thus, very large collections of isolates need to be studied in order to obtain a more complete view of the distribution of dsRNA fragments in *S. sapinea*.

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Table 1. *Sphaeropsis sapinea* isolates from six geographical regions screened for the presence of dsRNA.

Origin	Number of isolates screened	% of isolates infected with dsRNA	Host	Collector
Colombia	24	33.3	<i>P. patula</i>	M.J. Wingfield
Madagascar	16	31.3	<i>P. patula</i>	O. Preisig
California	11	27.3	<i>P. radiata</i>	M.J. Wingfield
Mexico	20	10	<i>P. patula</i> , <i>P. greggii</i>	M.J. Wingfield
Switzerland	18	0	<i>P. sylvestris</i> , <i>P. nigra</i>	O. Preisig
Indonesia	15	0	<i>P. patula</i>	M.J. Wingfield
TOTAL:	104	28%		

Table 2. *Sphaeropsis sapinea* isolates used in this study and the dsRNA fragments associated with them.

Isolates ^{a/}	Origin	Host	Type of dsRNA ^{b/}	
			SsRV1, SsRV2 or co-infection	Non-SsRV dsRNA fragments
CMW4254*	Gauteng, South Africa	<i>P. roxburghii</i>	SsRV1, SsRV2	1.5 kb, 1.3 kb
CMW4241	Western Cape, South Africa	<i>P. radiata</i>	SsRV1	-
BOT299*	Michigan, United States	<i>P. banksiana</i>	SsRV1, SsRV2	-
BOT760*	Rancho Grande, Colombia	<i>P. patula</i>	SsRV2	-
BOT793	"	"	"	-
BOT785	"	"	"	4 kb, 1.5 kb, 1.3 kb
BOT796	"	"	SsRV1, SsRV2	1.5 kb, 1.3 kb
BOT797	"	"	-	"
BOT798	"	"	SsRV1, SsRV2	-
BOT801	"	"	SsRV2	1.5 kb, 1.3 kb
BOT800	"	"	-	-
BOT848*	Besteppo Lab, Colombia	<i>P. patula</i>	-	-
BOT812	"	"	SsRV1	-
BOT806	"	"	-	-
BOT835	"	"	SsRV1, SsRV2	1.5 kb, 1.3 kb
BOT777*	Madagascar	<i>P. patula</i>	"	-
BOT899	"	"	"	-
BOT893	"	"	"	-
BOT892	"	"	SsRV2	-
BOT894	"	"	-	-
BOT770*	"	"	-	-
BOT769*	Mexico	<i>P. greggii</i>	-	8 kb
BOT960	"	"	-	"
BOT768*	Mexico	<i>P. patula</i>	-	-
BOT868	Pebble beach, Congress Road, California	<i>P. radiata</i>	-	8 kb, 4 kb, 1.5 kb, 1.3 kb
BOT869	"	"	-	4 kb
BOT858	Pebble beach, Spoustbay, California	"	-	"

^{a/} Isolates marked with an asterisk (*) were included in comparisons of cultural characteristics, *in vitro* growth rate and pathogenicity.

^{b/} SsRV1 and SsRV2 have dsRNA genomes of *ca.* 5 kb each (Preisig *et al.*, 1998).

Table 3. Culture characteristics and mean growth rate at four different temperatures (10°, 15°, 25° and 30°C) for dsRNA-containing and dsRNA-free *Sphaeropsis sapinea* isolates.

Isolates	Presence of dsRNA fragments	Colony morphology ^{a/}	Mean growth rate (mm/day) ^{b/}			
			10°C	15°C	25°C	30°C
CMW4254	SsRV1 and SsRV2	Dark mouse grey-mouse grey, fluffy (+)	3.6 a	4.7 a	10.6 b	8.1 b
BOT299	SsRV1 and SsRV2	Dark mouse grey, fluffy (+)	3.9 a	5.6 b	12.8 ab	11.9 ab
BOT760	SsRV2	Dark mouse grey-mouse grey, fluffy (++)	4.9 b	6.9 c	21.1 c	13.5 a
BOT777	SsRV1 and SsRV2	Dark mouse grey, fluffy (++)	4.2 ab	6.4 bc	19.5 c	19.8 c
BOT769	8 kb fragment	Dark mouse grey, fluffy (+)	4.0 ab	5.3 ab	13.7 a	10.3 ab
BOT770	No dsRNA	Dark mouse grey-mouse grey, fluffy (++)	4.0 ab	6.4 bc	20.5 c	20.1 c
BOT848	No dsRNA	"	4.8 b	7.2 c	20.8 c	20.4 c
BOT768	No dsRNA	Dark mouse grey, fluffy (+)	3.4 a	5.2 ab	11.7 b	10.3 ab

^{a/} Cultural characteristics are based on the morphology and colour of the mycelia after 15 days incubation at 25°C. Colony colour was assessed according to the mycological colour chart of Rayner (1970). "Fluffy" refers to aerial mycelia with a cotton wool appearance. The fluffy appearance is rated on a scale between (+) (less fluffy) and (++) (more fluffy).

^{b/} Growth rates of isolates (BOT760, BOT777, BOT769, BOT770, BOT848 and BOT768) incubated at 25°C, and isolates (BOT777, BOT770 and BOT848) incubated at 30°C were calculated after 8 days since the mycelia had covered the surface area of the MEA in the Petri dish. The growth rates of all the isolates incubated at 10° and 15° were calculated only after 15 days. Values within a column followed by the same letter do not differ significantly with the Tukey pairwise comparisons test ($p < 0.05$).

Table 4. Analysis of variance for the growth rate of dsRNA-containing and dsRNA-free *Sphaeropsis sapinea* isolates from various geographical regions.

Source of variation ^{a/}	Sum of squares (SS)	Degrees of freedom (df)	Mean of squares (MS)	F-ratio	Probability (p)
Temp – 10°C	5.3	7	0.8	9	0.55
Error	9.5	11	0.9		
Temp – 15°C	20.5	7	2.9	11.7	0.00
Error	2.7	11	0.2		
Temp – 25°C	346.3	7	49.5	29.6	0.00
Error	18.4	11	1.7		
Temp – 30°C	424.7	7	60.7	22.5	0.00
Error	29.6	11	2.7		
Type of dsRNA*	108.6	3	36.2	2.1	0.14
Error	256.0	15	17.1		

Source of variation ^{b/}	Least squares means (LSM) ^{c/}	Standard error (SE)	Population size (N)
SsRV1 and SsRV2	13.6 a	1.5	3
SsRV2 infection	21.090 b	2.921	1
8 kb dsRNA	13.7 a	2.9	1
No dsRNA	16.805 ab	1.562	3

^{a/} Growth rates for eight *S. sapinea* isolates at four different temperatures (Table 4). The asterisk (*) represents the comparison in mean growth rate at 25°C for *S. sapinea* isolates infected with four types of dsRNA (SsRV2, SsRV1 and SsRV2, 8 kb fragments and no-dsRNA).

^{b/} Mean growth rates for *S. sapinea* isolates representing one of the four dsRNA infections.

^{c/} Values within a column followed by the same letter do not differ significantly with the Tukey pairwise comparisons test ($p < 0.05$).

Table 5. Mean lesion lengths, widths and surface areas on Granny Smith apples and mean lesion lengths on *Pinus patula* seedlings after inoculation with dsRNA-containing and dsRNA-free isolates of *Sphaeropsis sapinea*.

Isolates	Presence of dsRNA fragments	Granny Smith apples ^{a/}			<i>P. patula</i> seedlings ^{b/}
		Lesion length (mm)	Lesion width (mm)	Area of lesion (mm ²)	Lesion length (mm)
CMW4254	SsRV1 and SsRV2	44.8 b	45.7 a	436.3 b	15.6 ab
BOT299	SsRV1 and SsRV2	12.7 d	12.0 c	27.5 c	17.5 ab
BOT760	SsRV2	54.6 ab	53.7 a	711.8 a	57.1 c
BOT777	SsRV1 and SsRV2	36.3 bc	36.9 ab	325.3 b	27.8 b
BOT769	8 kb fragment	31.3 c	31.6 b	306.5 b	20.2 ab
BOT770	No dsRNA	51.5 ab	50.9 a	635.3 a	47.8 c
BOT848	No dsRNA	52.3 ab	51.6 a	645.3 a	16.9 ab
BOT768	No dsRNA	56.3 a	53.5 a	798.8 a	39.6 c
CONTROL	-	0	0	0	0

^{a/} Mean lesion lengths, widths and the area of the lesion were determined after two independent inoculation trials in which four Granny Smith apples per isolate were inoculated. The area of the lesion was determined by calculating the number of 1 mm x 1 mm squares represented by the infected area. Values within a column followed by the same letter do not differ significantly with the Tukey pairwise comparisons test ($p < 0.05$).

^{b/} Mean lesion lengths was determined after two separately conducted green house pathogenicity trails with ten replications of each isolate. Values within a column followed by the same letter do not differ significantly with the Tukey pairwise comparisons test ($p < 0.05$).

Table 6. Analysis of variance for the pathogenicity of dsRNA-containing and dsRNA-free *Sphaeropsis sapinea* isolates after inoculation of Granny Smith apples.

Source of variation ^{a/}	Sum of squares (SS)	Degrees of freedom (df)	Mean of squares (MS)	F-ratio	Probability (p)
Length	1.4	7	0.2	9.0	0.000
Error	0.6	24	0.023		
Width	1.5	7	0.2	8.5	0.000
Error	0.6	24	0.025		
Area	7.1	7	1.0	9.1	0.000
Error	2.7	24	0.1		
Type of dsRNA*	3.9	3	1.3	6.4	0.002
Error	5.8	28	0.2		

Source of variation ^{b/}	Least squares means (LSM) ^{c/}	Standard error (SE)	Population size (N)
SsRV1 and SsRV2	2.1 a	0.1	3
SsRV2 infection	2.8 b	0.2	1
8 kb dsRNA	2.1 a	0.2	1
No dsRNA	2.8 b	0.1	3

^{a/} Lengths, widths and surface areas of infection for eight *S. sapinea* isolates (Table 6). The asterisk (*) represents the comparison in virulence for *S. sapinea* isolates infected with four types of dsRNA (SsRV2, SsRV1 and SsRV2, 8 kb fragments and no-dsRNA).

^{b/} Mean surface areas of infection for *S. sapinea* isolates infected with three types of dsRNA, as well as isolates without dsRNA.

^{c/} Values within a column followed by the same letter do not differ significantly with the Tukey pairwise comparisons test ($p < 0.05$).

Table 7. Analysis of variance for the pathogenicity of dsRNA-containing and dsRNA-free *Sphaeropsis sapinea* isolates after inoculation of *Pinus patula* seedlings.

Source of variation ^{a/}	Sum of squares (SS)	Degrees of freedom (df)	Mean of squares (MS)	F-ratio	Probability (p)
Lesion length	0.7	7	0.1	7.2	0.006
Error	0.1	8	0.014		
Types of dsRNA*	0.4	3	0.1	3.5	0.049
Error	0.4	12	0.037		

Least squares			
Source of variation ^{b/}	means (LSM) ^{c/}	Standard error (SE)	Population size (N)
Co-infected with SsRV1 and SsRV2	1.3 a	0.078	3
SsRV2 infection	1.8 b	0.1	1
8 kb dsRNA	1.3 a	0.1	1
No dsRNA	1.5 ab	0.078	3

^{a/} Lesion lengths for eight *S. sapinea* isolates (Table 6). The asterisk (*) represents the comparison in virulence for *S. sapinea* isolates infected with four types of dsRNA (SsRV2, SsRV1 and SsRV2, 8 kb fragments and no-dsRNA).

^{b/} Mean lesion length for *S. sapinea* isolates infected with three types of dsRNA, as well as isolates without dsRNA.

^{c/} Values within a column followed by the same letter do not differ significantly with the Tukey pairwise comparisons test ($p < 0.05$).

Figure 1. DsRNA fragments isolated from *Sphaeropsis sapinea* isolates from various geographical regions separated on an ethidium bromide-stained 1% agarose (w/v) gel (80 V). Lane 1 = molecular weight marker (λ DNA cut with *HindIII* and *EcoRI*); lane 2-3 = isolates from South Africa; lane 4-5 = isolates from Mexico; lane 6-7 = isolates from Colombia; lane 8-9 = isolates from Madagascar. Arrows indicate to the five different sizes (in kilobases) of dsRNA fragments.

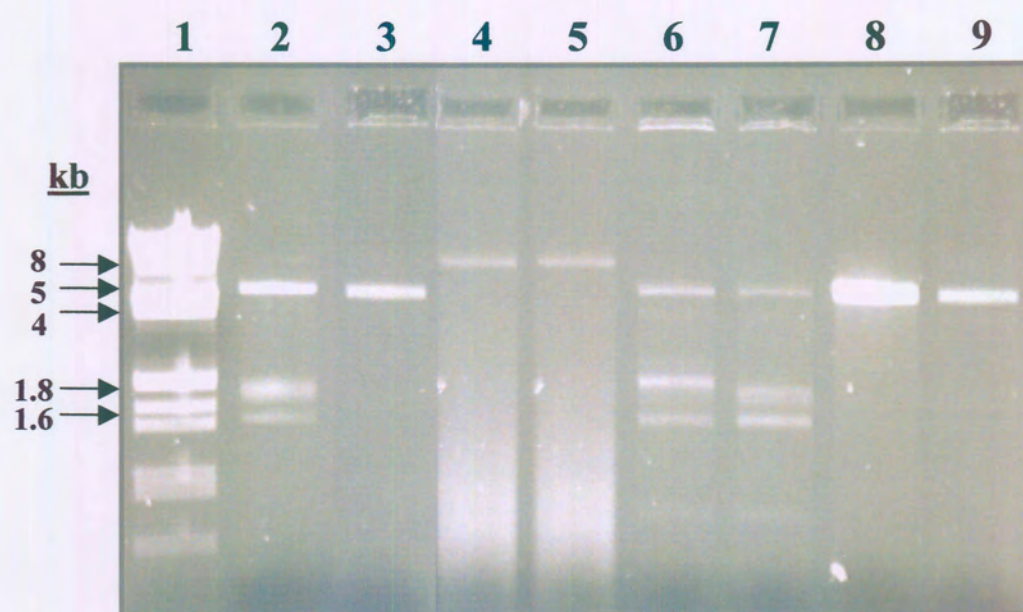


Figure 2. Schematic representation of the SsRV1 (5163 bps) and SsRV2 (5202 bps) genomes showing primer binding sites. **(A)** SsRV1: primers Oli3 and Oli31 amplify a 1 kb fragment. **(B)** SsRV2: primers 5'-RDRP2 and Oli32 amplify a 800 bp fragment.

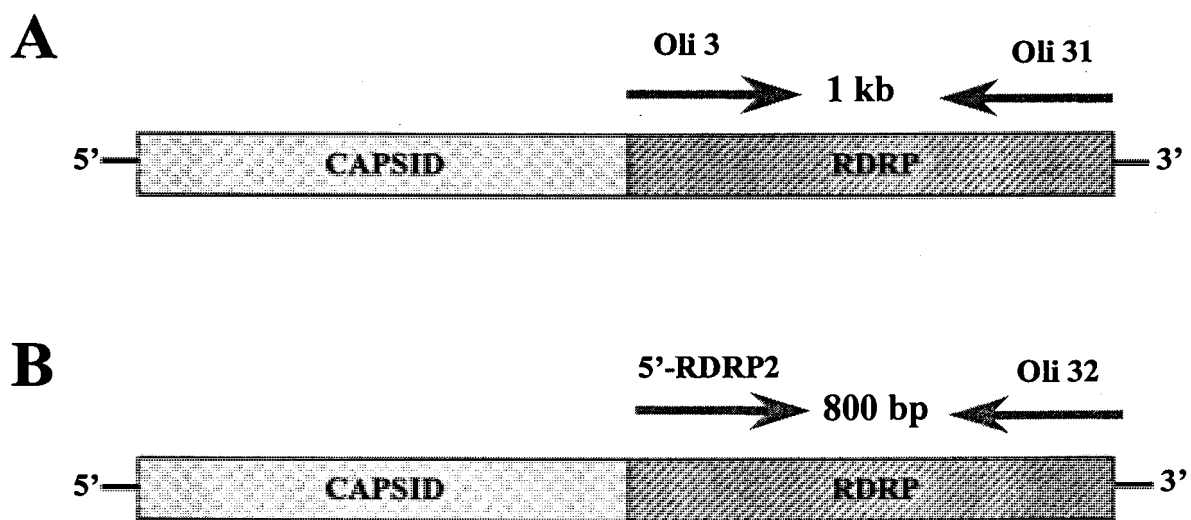


Figure 3. RT-PCR products (cDNA) generated from dsRNA with SsRV-specific primers (SsRV1: Oli3 & Oli31, SsRV2: 2RDRP-5' & Oli32) separated on an ethidium bromide-stained 1% (m/v) agarose gel (80 V). Lane 1 = molecular weight marker (λ DNA cut with *HindIII* and *EcoRI*), lanes 2 & 3 = CMW4254 (SsRV1 & SsRV2), lanes 4 & 5 = BOT777 (SsRV1 & SsRV2), lanes 6 & 7 = BOT894 (SsRV1 & SsRV2), lanes 8 & 9 = BOT835 (SsRV1 & SsRV2), lane 10 = BOT798 (SsRV1), lane 11 = Molecular weight marker (λ DNA cut with *HindIII* and *EcoRI*). Arrows indicate to the sizes of the cDNA products (in kilobases) generated with SsRV1-specific and SsRV2-specific primers.

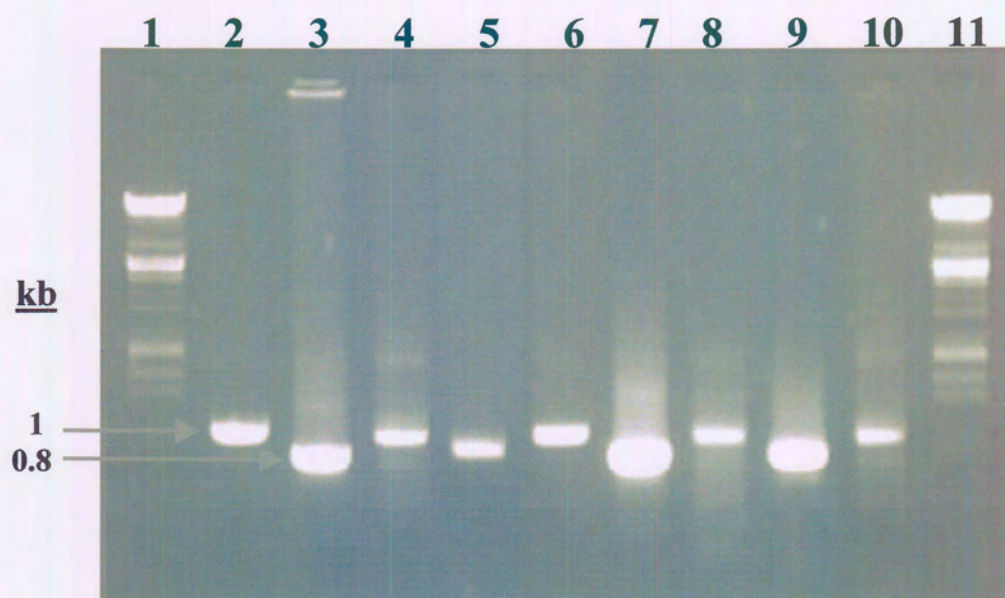


Figure 4. Cultural characteristics of dsRNA-containing and dsRNA-free *Sphaeropsis sapinea* isolates on 2% MEA after 15 days incubation at 25°C in the dark. (A) BOT299+dsRNA, USA; (B) BOT777+dsRNA, Madagascar; (C) BOT769+dsRNA, Mexico; (D) BOT848-dsRNA, Colombia; (E) BOT776-dsRNA, Madagascar; (F) BOT768-dsRNA, Mexico.

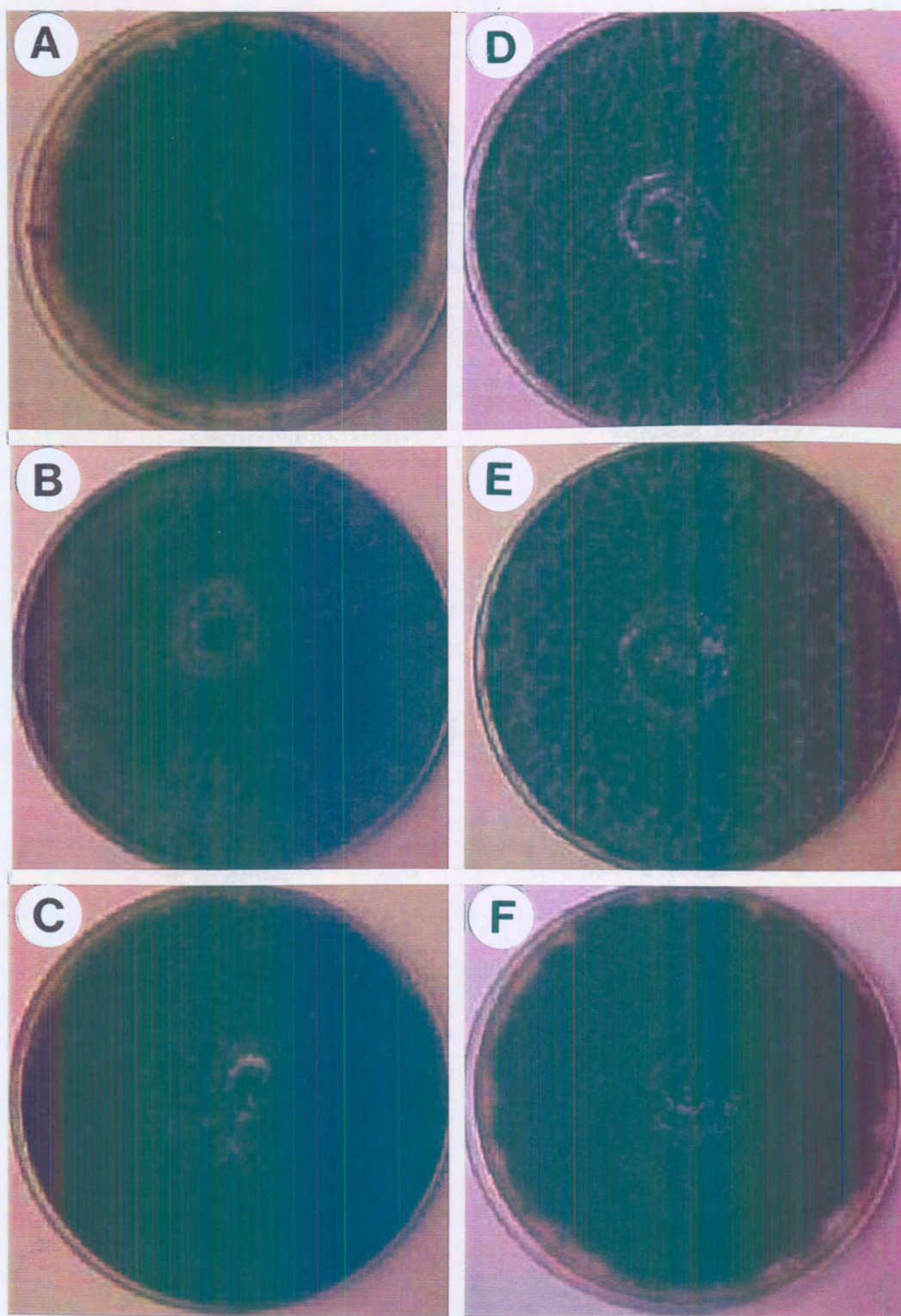
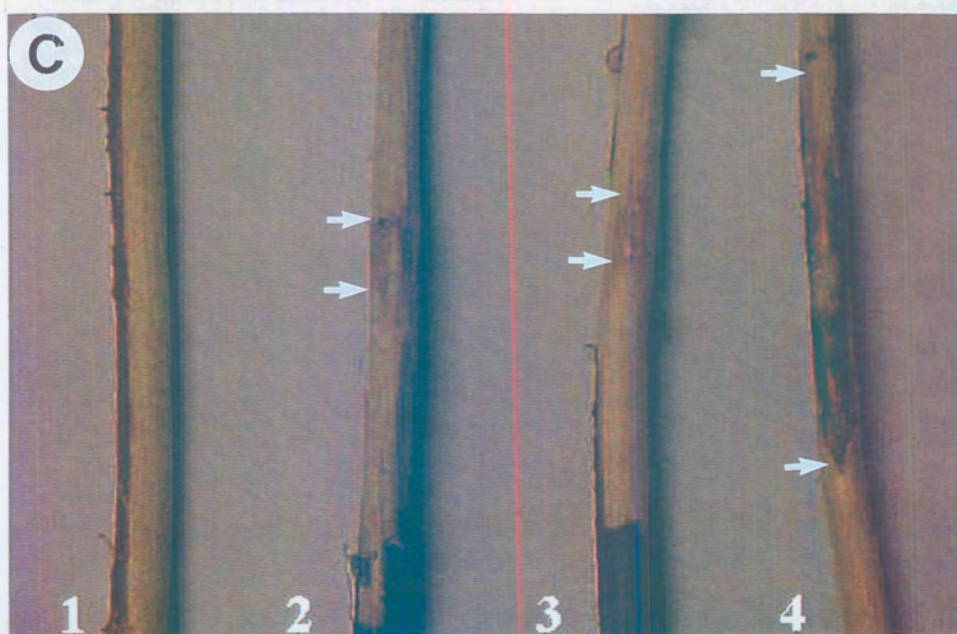
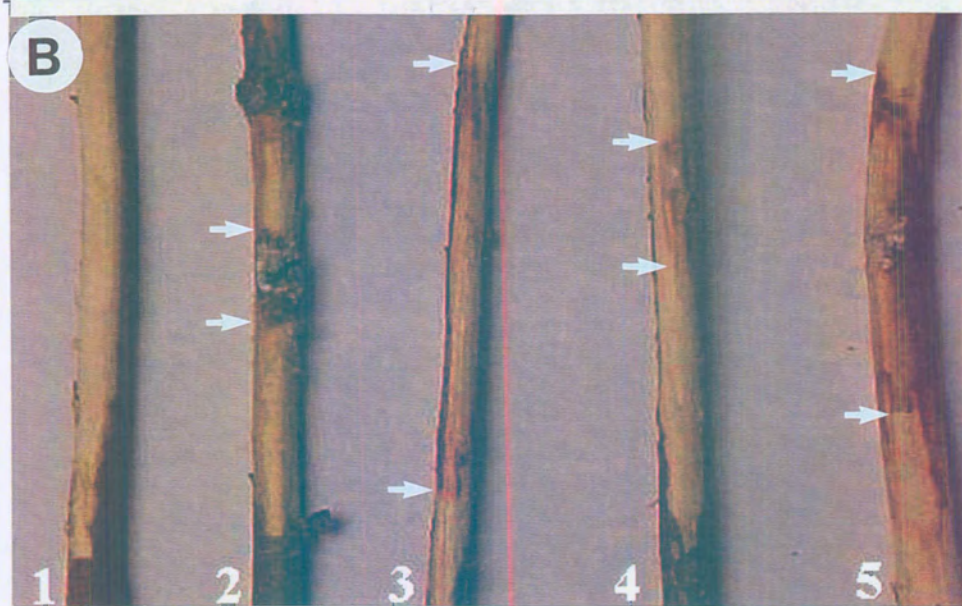
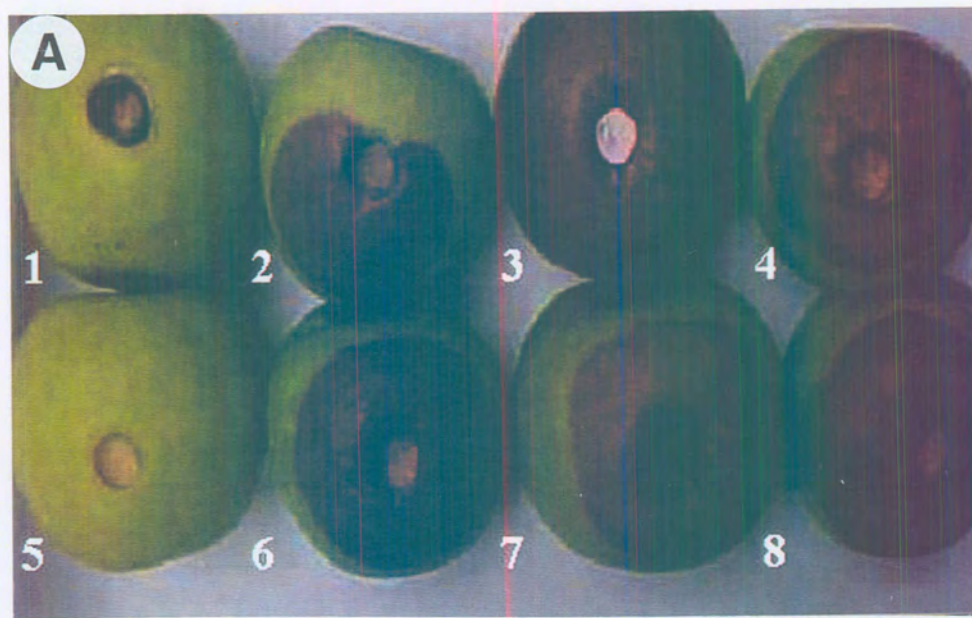


Figure 5. Lesions formed after inoculation with dsRNA-containing and dsRNA-free *Sphaeropsis sapinea* isolates. **(A)** Granny Smith apples: 1-4 = BOT299+dsRNA, BOT777+dsRNA, BOT760+dsRNA, BOT769+dsRNA), 5 = sterile control, 6-8 = BOT770-dsRNA, BOT848-dsRNA, BOT768-dsRNA. **(B)** *Pinus patula* seedlings: 1 = sterile control, 2&4= BOT848-dsRNA, 3 = BOT768-dsRNA, 5 = BOT770-dsRNA. **(C)** *P. patula* seedlings: 1 = sterile control, 2 = CMW4254+dsRNA, 3 = BOT299+dsRNA, 4 = BOT769+dsRNA. Arrows indicate the length of the lesions, four weeks after inoculation.



SUMMARY

Post-hail associated die-back of *Pinus radiata* is associated with the opportunistic pine pathogen *Sphaeropsis sapinea*. This disease has a serious impact on forestry, especially in regions such as South Africa where *Pinus* spp. are planted as exotics. Current management strategies are based on the selection of disease tolerant *Pinus* spp. and the planting of these pines in regions less affected by adverse environmental conditions. A possible management strategy for *S. sapinea* is biocontrol through dsRNA-mediated hypovirulence. Improvement of current management strategies and the implementation of biocontrol, however, require a re-evaluation of the taxonomic status of the pathogen and the assessment of genetic variability in populations. Chapter One of this thesis reviews knowledge on *S. sapinea* in order to obtain the ultimate goal of formulating an integrated management strategy for *S. sapinea*, especially in South Africa and other regions dependent on exotic pines for commercial forestry.

Successful management of *S. sapinea* in exotic forestry plantations has not, as yet, been fully achieved. This is probably due to a lack of knowledge of the current taxonomy of the fungus, the genetic status of *S. sapinea* populations and the association with dsRNA viral fragments that could be exploited for biocontrol.

In Chapter Two, three morphotypes (A, B and C) of *S. sapinea* are reported from diverse geographical areas. This also includes the discovery, for the first time, of a third morphotype of the fungus. Isolates of the C morphotype are characterised based on RAPD analysis and ITS sequences, as well as cultural and conidial characteristics. Isolates of the C morphotype were found to be more similar to those of the A morphotype based on cultural characteristics, texture of their conidial walls and ITS sequences. The three morphotypes are, however, clearly distinguishable based on their RAPD banding profiles and conidial size.

Chapter Three represents the characterisation of the C morphotype of *S. sapinea* based on morphology and pathogenicity. Based on cultural and conidial characteristics, as well as growth rate the A and C morphotypes are very similar. Isolates of the C morphotype were, however, found to be much more virulent than either those of the A

or B morphotype. This could have a great impact on the management of *S. sapinea* die-back, as well as on quarantine practices.

In Chapter Four, I present an assessment of the diversity for *S. sapinea* populations from various regions. The diversity in the different populations was found to be highly variable, despite the fact that only an asexual state is known for this fungus. The diversity in presumed native populations from Mexico, California and Switzerland was relative low. In the introduced Malagasy population the diversity was very high, probably due to many introductions of the fungus with seed imports. Due to the high variability of the fungus in the Malagasy population, management of *S. sapinea* from this region will be extremely difficult.

In Chapter Five, dsRNA fragments of different sizes were commonly found to occur in isolates of *S. sapinea*. Two previously described dsRNA viruses (SsRV1 and SsRV2) from South Africa were found to also occur in isolates from Madagascar and Colombia. It shows that these dsRNA fragments have been introduced with *S. sapinea* into new environments. The type and number of dsRNA fragments was found to have some effect on the growth rate and pathogenicity of the fungus. These alterations in the normal phenotype of the fungus could, however, not be correlated to the presence of dsRNA fragments. The presence of dsRNA fragments does, however, still have the potential to be implemented in future management programmes for *S. sapinea*.

OPSOMMING

Terugsterwing van *Pinus radiata* na haelstorms is geassosieerd met *Sphaeropsis sapinea*, 'n opportunistiese patogeen. Hierdie na-hael geassosieerde terugsterwing het 'n belangrike invloed op bosbou, veral in lande soos Suid-Afrika waar *Pinus* spp. eksoties is. Huidige beheermaatreëls van hierdie fungus is gebaseer op die seleksie van siekte-tolerante *Pinus* spp. en die plant van hierdie spesies in gebiede wat minder geaffekteer word deur ongewone omgewingstoestande soos haelstorms. 'n Alternatiewe beheermaatreël teen *S. sapinea* is biologiese beheer deur dsRNS-bemiddelde hipovirulensie. Verbetering van die huidige beheermaatreëls en die implementering van biologiese beheer vereis egter die herevaluering van die taksonomiese stand van die patogeen en die bepaling van die genetiese variasie in populasies. Hoofstuk Een van hierdie skripsie is 'n opsomming van alle kennis rakende *S. sapinea* ten einde 'n geïntegreerde beheerprogram teen *S. sapinea*-siektes te implementeer, veral in Suid-Afrika en ander lande waar kommersiële bosbou afhanklik is van eksotiese denne.

In Hoofstuk Twee word melding gemaak van drie morfotipes (A, B en C) van *S. sapinea* vanuit verskeie geografiese streke. Dit verwys verder na die ontdekking, vir die eerste keer, van 'n derde morfotipe van hierdie patogeen. Hierdie morfotipe is gekarakteriseer deur middel van RAPD (lukraak geamplifiseerde polimorfiese DNS) analise, basisvolgordes van die ITS (nie-getranskribeerde streek) asook kulturele en konidiale eienskappe. Daar is gevind dat isolate van die C morfotipe nader verwant is aan die van die A morfotipe gebaseer op kulturele eienskappe, tekstuur van konidiale wande en ITS basisvolgordes. Die drie morfotipes verskil van mekaar ten opsigte van hul RAPD profiele en die grootte van hul konidia.

Hoofstuk Drie handel oor die karakterisering van die C morfotipe van *S. sapinea* gebaseer op morfologie en patogenisiteit. Isolate van die A en C morfotipes toon baie ooreenkomste ten opsigte van hul kulturele en konidiale eienskappe, asook groeitempo. Daar is egter gevind dat isolate van die C morfotipe baie meer virulent is as isolate van die A of B morfotipes. Dit kan 'n groot invloed hê op die beheer van *S. sapinea*, asook op die toepassing van kwarantyn maatreëls.

In Hoofstuk Vier is die diversiteit van vyf *S. sapinea* populasies van verskillende streke bepaal. Daar is gevind dat die diversiteit in hierdie populasies hoogs varieerbaar is ten spyte daarvan dat slegs 'n ongeslagtelike fase vir hierdie fungus bekend is. Daar is verder gevind dat die diversiteit in drie inheemse populasies van die patogeen, van Mexiko, Kalifornië en Switserland relatief laag is. Die diversiteit in 'n uitheemse populasie van Madagaskar was egter baie hoog. Die hoër diversiteit van *S. sapinea* in Madagaskar is waarskynlik die resultaat van herhaalde vrystellings van die fungus, na hierdie streek, tesame met ingevoerde saad. Die hoë vlak van variasie in die populasie van Madagaskar, maak beheer van *S. sapinea* in hierdie streek baie moeilik.

In Hoofstuk Vyf is daar gevind dat dsRNS fragmente met verskillende groottes algemeen voorkom in isolate van *S. sapinea* van verskeie streke. Twee dsRNS virusse (SsRV1 en SsRV2), wat voorheen beskryf is van Suid-Afrika, is ook gevind in isolate van Madagaskar en Columbia. Dit dui aan dat hierdie dsRNS fragmente saam met die fungus ingevoer word na nuwe omgewings. Daar is verder gevind dat die tipe en aantal dsRNS fragmente 'n invloed het op die groeitempo en patogenisiteit van die fungus. Afwykings in die normale fenotipe van die fungus kon egter nie gekoppel word aan die teenwoordigheid van dsRNS fragmente nie. Die teenwoordigheid van die hierdie fragmente het steeds die potensiaal om geïmplementeer te word in toekomstige beheerprogramme teen *S. sapinea*.