BIOLOGY AND EPIDEMIOLOGY OF

Fusarium circinatum

This dissertation is presented in partial fulfilment of the requirements of the degree

Magister Scientiae (Agric)

in the Faculty of Agricultural and Natural Sciences, Department of Microbiology and Plant Pathology, University of Pretoria

by

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August 2005

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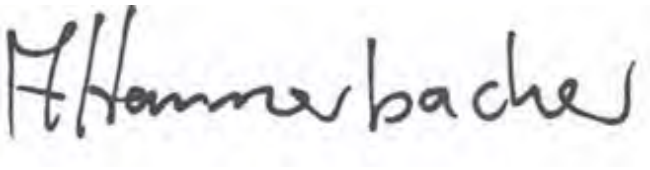
Declaration

I, the undersigned, hereby declare that the thesis submitted herewith for the degree *Magister Scientiae (Agric)* to the University of Pretoria contains my own independent work. This work has hitherto not been submitted for any degree at any other University.



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August 2005

“Existence is a strange bargain. Life owes us little; we owe it everything. The only true happiness comes from squandering ourselves for a purpose.”

WILLIAM COWPER (1731-1800)

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ACKNOWLEDGEMENTS

I acknowledge with gratitude the following people and institutions:

- The Tree Protection Co-operative Programme, the National Research Foundation and the THRIP program funded by the Department of Trade and Industry for financial support.
- Prof Teresa Coutinho for support, loyalty and sound guidance during the course of my studies.
- My Co-promotors Profs Mike and Brenda Wingfield for their excellent academic input.
- Dr Ben Eisenberg and Dr Louwrance Wright for their assistance with the statistical analysis of my data.
- Dr Emma Steenkamp and Dr Martin Coetzee for their assistance with the analysis of my phylogenetic data.
- Albe van der Merwe who taught me basic molecular techniques and has given his time freely whenever I had need for assistance.
- Richard Parker (Top Crop Nursery) who taught me much about pine seedling growing.
- All members of FABI who contribute in the running and maintenance of this institution.

PREFACE

Fusarium circinatum is the causal agent of the disease known as pitch canker of pine. The fungus causes resinous cankers on stems and branches of mature trees, dieback of female flowers and cones, as well as root rot and pre- and post emergence damping off of seedlings. Little is known regarding the epidemiology and biology of *F. circinatum* in South African pine seedling nurseries, where the fungus has been causing major economic losses since its introduction into the country in the early 1990s. The objectives of this study were, therefore, to study the infection biology and epidemiology of *F. circinatum* on pine seedlings, the organism's saprophytic biology. I also considered approaches to rapidly diagnose plants infected by *F. circinatum* and its relatedness to other species.

Much research has been done on the pitch canker disease and the causal agent *F. circinatum*. Chapter 1 of this thesis aimed to summarize the available knowledge on the pitch canker fungus and its biology, ecology and epidemiology.

Trials to screen for resistance of *Pinus* spp. to the pitch canker fungus have been conducted by many research groups and also by industries that rely on *Pinus* spp. for pulp and wood production. In Chapter 2, parameters for such trials, including optimal wounding methods, spore concentrations, plant physiological considerations and time elapsed between wounding and inoculation, were investigated.

Temperature and ambient humidity are considered important factors in plant disease epidemiology. The effect of these factors on pitch canker epidemics has not yet been studied. In Chapter 3, a survey of disease incidence in pine nurseries from different geographic areas in South Africa with different climates is presented. This was done by correlating disease incidence data from the nurseries with temperature and humidity measurements.

The saprophytic biology of a plant pathogen is of great importance in its epidemiology. The extent of any plant pathogen's saprophytic survival determines the initial inoculum levels at the onset of an epidemic. In Chapter 4, I investigated the saprophytic growth and survival of *F. circinatum* in various substrates, temperatures and moisture regimes.

Fusarium circinatum is morphologically similar to fungi referred to as *Fusarium subglutinans sensu lato*. Distinguishing *F. circinatum* from other species in this group has in the past required pathogenicity tests and sexual crosses, which are labour intensive and time consuming. In Chapter 5, a molecular diagnostic technique, based on real-time PCR, with

which identification of *Fusarium* spp. commonly occurring in South African nurseries is possible, was developed.

Fusarium circinatum and other fungi referred to as *F. subglutinans sensu lato* are members of the *Gibberella fujikuroi* species complex. Molecular taxonomic studies have shown that *F. subflutinans sensu lato* is a polyphyletic taxon. The objective of the study presented in Chapter 6 was to resolve the taxon phylogenetically with the use of multiple loci.

The studies in the individual chapters of this thesis present individual aspects of the biology, ecology, epidemiology and molecular ecology of *Fusarium circinatum*. Each chapter represents an independent entity and consequently repetition between chapters has been unavoidable.

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Ecology and Epidemiology of *Fusarium circinatum*

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Abstract: *Fusarium circinatum*, the causal agent of pitch canker, is one of the most important pathogens of *Pinus* species. Sporadic outbreaks and epidemics caused by this fungus have been reported from numerous countries. Symptoms differ depending on host species, geographical region, climatic conditions and associated insects. Pitch canker represents a significant threat to countries where non-native and susceptible *Pinus* spp. are grown intensively in plantations and a thorough understanding of the ecology and epidemiology of the causal agent is an important component to manage this threat. The aim of this review is to summarise contemporary knowledge relating to the pitch canker pathogen, with a particular focus on its threat to plantation forestry in the southern hemisphere.

Contents

1. Introduction

Pitch canker, caused by *Fusarium circinatum* Nirenberg and O'Donnell (teleomorph *Gibberella circinata* Nirenberg and O'Donnell) (Nirenberg and O'Donnell 1998), is an economically important disease of pine. The disease is reported to occur on 47 *Pinus* species (Dick 1998) and Douglas-fir (Storer et al. 1994). Depending on the host, and the prevailing biotic and abiotic conditions, damage caused by the pathogen includes resin soaked cankers on tree trunks and lateral branches (Dwinell et al. 1985), shoot dieback (Corell et al. 1991), dieback of female flowers and mature cones (Barrows-Broaddus, 1990), reduced germination of seeds (Huang and Kuhlman 1990), seedling damping-off (Viljoen et al. 1994) and mortality of established seedlings in nurseries (Carey and Kelly 1994b).

Pitch canker has been reported from the south eastern United States (Hepting and Roth 1946), Haiti (Hepting and Roth 1953), California (McCain et al., 1987), Japan (Muramoto and Dwinell 1990), South Africa (Viljoen et al. 1994), Chile (Wingfield et al. 2002b) and Spain (Dwinell et al. 2001). The disease is endemic in the south eastern United States, where severe outbreaks occur only under conditions of abiotic stress (Blakeslee et al. 1979). In California, *F. circinatum* has caused severe damage to native *P. radiata* in natural stands and landscape plantings (Gordon et al. 1996). The severity of the disease on Californian *P. radiata* has been attributed to favourable environmental conditions for infection (Gordon et al. 2001) and to mutualistic interactions of the indigenous pine pests with this exotic pathogen (Storer et al. 1999c).

In South Africa and Chile, non-native *Pinus* species are widely planted in an environment in which few associations have developed between native biota and these plantation trees. Here severe outbreaks of infection by *F. circinatum* on seedlings and cuttings have been reported, after accidental introduction of the pathogen. Symptoms typical of pitch canker on older trees have not been observed in these two countries (Viljoen et al. 1994; Wingfield et al. 2002a).

Pitch canker is a dynamic disease. Each outbreak in a particular area has a unique case history (Dwinell et al. 1985). This is primarily due to the interaction between the pathogen, the *Pinus* spp. or clone affected and the unique abiotic and biotic environmental conditions prevailing at the time of an outbreak. This review focuses mainly on these fundamental aspects that contribute to pitch canker outbreaks and epidemics. These are considered with

particular reference to the threat of the pathogen to intensively managed plantations of non-native trees in the southern Hemisphere.

2. Global Importance of *F. circinatum*

Pitch canker is endemic in the south-eastern United States where it was first recorded in 1946 (Hepting and Roth 1946). The disease occurs throughout this region from Florida in the south, northward through Virginia and as far east as Texas (Dwinell et al. 1985). In these areas, the disease occasionally becomes epidemic causing cankers, shoot dieback and in some cases tree death (Barrows-Broaddus and Dwinell 1985). Usually the disease causes economic losses only in managed stands such as in plantations and seed orchards, but is rarely a problem in native stands of *Pinus* spp. (Blakeslee et al. 1979).

The Pitch canker fungus was first identified in California in 1986 (McCain et al. 1987). The most severe incidence of the disease was in Santa Cruz County, where it was identified as a cause of branch dieback in *Pinus radiata, Pinus muricata* and *Pinus halepensis*. The disease was at first confined to landscape plantings, but by 1992 it was known to occur in native populations of *Pinus radiata* on the Monterey peninsula. Pitch canker is now in evidence throughout coastal California, from San Diego, in the south, to Mendocino County, north of San Francisco (Gordon et al. 1996).

Until the 1980s, the only report of pitch canker from outside the United States was that of Heptig and Roth (1953) who noted that the disease was abundant in Haiti on *Pinus occidentalis*. Since then, *F. circinatum* has spread to three continents. In the late 1980s, pitch canker was identified causing trunk cankers and dieback of *Pinus luchuensis* on the islands of Amamiooshima and Okinawa in Japan (Muramoto and Dwinell 1990). *F. circinatum* first appeared in South Africa in a single forestry nursery in 1990, causing a root disease on *P. patula* seedlings (Viljoen et al. 1994). The disease was probably introduced via infected seed from Mexico (Britz et al. 2001). Since this initial outbreak, *F. circinatum* has spread to several other forestry nurseries in South Africa, causing serious root and collar disease of various *Pinus* spp. (Britz et al. 2001, 2005). Stem cankers on mature trees, typical of those found elsewhere, have not been observed in South Africa (Viljoen et al. 1997b; Wingfield et al. 1999). In 1995, pitch canker was confirmed to occur in Mexico. The disease was prevalent on planted *P. radiata* and *P. halepensis* and in natural stands of *P. douglasiana, P.*

leicophylla, *P. durangensis* and other pine species (Santos 1991 as cited by Dwinell et al. 2001). By 2002, it was confirmed that the disease had been introduced into Chile, where a similar disease situation to that occurring in South Africa was encountered (Wingfield et al. 2002a). The pathogen has also been introduced into Spain, where it is causing mortality of *P. radiata* seedlings (Dwinell 1999b). Further spread of *F. circinatum*, is of great concern to many countries such as Australia and New Zealand, where the highly susceptible *P. radiata* is grown extensively in plantations (Dick 1998).

3. The Disease

3.1 Symptoms

Fusarium circinatum infects vegetative and reproductive plant parts of susceptible pine hosts of all ages. Shoots, branches, cones, seeds, stems and exposed roots may all become infected. In addition, the symptoms of pitch canker vary from one pine host to another (Dwinell et al. 1985). Trunk and branch cankers are most common on *Pinus radiata, P. palustris, P. luchuensis* and *P. strobus* (Correll et al. 1991; Dwinell et al. 1985; Muramoto and Dwinell 1990). Trunk cankers are also common on *P. elliottii* in seed orchards and are usually associated with the use of tree shakers for cone removal (Dwinell and Phelps 1977). Infected tissue on the cankers becomes resin soaked and typically, copious resin bleeding occurs at the point of infection. Spread of cankers in trees is usually arrested at the branch nodes (Dwinell et al. 1985) due to the formation of tyloses in the vascular bundles (Barrows-Broaddus and Dwinell 1984). Cankers on exposed roots are common on *P. radiata* (Adams 1999). Shoot dieback is common on *P. elliottii* , *P. taeda, P. echinata, P. clausa* and *P. radiata* (Correll et al. 1991; Dwinell et al. 1985). Witches brooms develop in some cases, when adventitious buds form in response to repeated shoot infections and dieback (Dwinell et al. 1985). To date, infection of Douglas-fir has been characterized by twig dieback only, with swollen resinous tissue apparent at the infection point on the twigs (Storer et al. 1994).

Fusarium circinatum has been shown to cause severe physiological damage in some pine species. Reddening of needles and needle fall on infected branches, characteristic of shoot dieback, were shown in affected shoots of inoculated *P. virginiata*. This symptom was as a result of invasion by *F. circinatum* hyphae from the adjacent cortex into the xylem (Barrows-Broaddus and Dwinell 1984). Solel and Bruck (1990) have observed wilting of

young shoots both under natural infection and artificial inoculation of *P. taeda* and *P. elliottii*. This was attributed to the pathogen's ability to obstruct water flow through the vascular bundles. It was also observed that infections by the pitch canker fungus result in reductions in volume growth in *P. elliottii*i. Reduction in growth can be attributed to loss of foliage due to shoot dieback and water stress (Arvanitis et al. 1984; Bethune and Hepting 1963).

The pitch canker fungus can infect the reproductive structures of *Pinus* spp. The infection causes mortality of female flowers and mature cones and seed deterioration of several pine species (Barrows-Broaddus 1990). Wounded and artificially inoculated *P. radiata* cones abort prior to reaching maturity (Correll et al. 1991). Infected *P. taeda* cones are misshapen and smaller than normal. Some infected *P. taeda* cones have necrotic tips, characterized by internal resin pockets (Dwinell et al. 2001). Dwinell et al. (2001) further concluded that interior contamination by the pitch canker fungus was not correlated with external and internal symptoms on the cone, and that cones appearing to be healthy, may contain the fungus. *F. circinatum* may even be present in cones from healthy branches (Storer et al. 1998) usually contaminating seeds only superficially. In this case, the pitch canker fungus can move from unshed seed coats to colonize cotyledons and cause seedling death (Huang and Kuhlman 1990). The fungus can also colonize seeds systemically, reducing seedling emergence significantly (Storer et al. 1998). The ability of the pathogen to infect and survive in/on the reproductive organs of *Pinus* spp. could facilitate its spread into new areas (Dick 1998).

Fusarium circinatum causes pre-and post emergence damping off of seedlings (Huang and Kuhlman, 1990; Viljoen et al. 1994), as well as mortality of established seedlings (Carey and Kelly, 1994; Viljoen et al. 1994). If pre-emergence damping off occurs, seed coats and coleoptiles of germinating seedlings are heavily colonized by the pathogen (Viljoen et al. 1994). Storer et al. (1998) found that internal seed contamination by *F. circinatum* causes higher rates of pre-emergence damping off than superficially contaminated seed. In the case of post-emergence damping off caused by *F. circinatum*, root collars of seedlings are girdled (Barnard and Blakeslee, 1980). Inoculations of young seedlings with the pathogen caused almost 100% mortality (Viljoen et al. 1994). Mortality of established seedlings is lower than that of newly germinated seedlings. The disease causes wilting of established seedlings with subsequent needle discoloration. Seedlings seldom collapse, and roots are underdeveloped, with discoloured lesions and necrosis of the cortex is evident (Viljoen et al. 1994). Seedlings from 7-10 months of age in the south eastern United States become diseased, primarily from

the tips downwards (Carey and Kelly 1994b; Rowan 1982), while in California and South Africa, seedlings are mainly affected at the lower part of the stem, root collar and roots (Storer et al. 1998; Viljoen et al. 1994). Seedling infections may remain undetected, since asymptomatic seedlings may harbour the pathogen as an endophyte (Storer et al. 1998).

3.2. Control

3.2.1. Cultural Control

At present there is no absolute means of controlling pitch canker in nurseries and forest plantations (Wilson et al. 1995). An integrated management approach (Dwinell et al. 2001) can, however, reduce the economic impact of the disease. Integrated management includes adequate quarantine measures (Dick 1998), appropriate nursery and silvicultural management (Dwinell et al. 1985) and selection for more resistant clones of species that are susceptible to the pitch canker pathogen (Wingfield et al. 2002a; Gordon et al. 2001).

Quarantine measures should be in place to exclude the fungus from areas that are currently free of pitch canker (Dick 1998) and to prevent the establishment of new vegetative compatibility groups in areas where the pathogen is already present (Gordon et al. 2001). In New Zealand, stringent regulations have been established to limit importation of live plant material (Dick 1998) and seed is screened for the pitch canker fungus prior to planting (Dick 1998). These measures can be effective in excluding *F. circinatum* propagules from New Zealand, but great concern exists about the accidental introduction of the fungus via wood products such as packaging material and contaminated insects and plant material harboured in second-hand vehicles, used logging equipment or in camping equipment (Dick 1998).

Little research has been done on the control of *F. circinatum* in pine seedling nurseries. Presumably the most important control measure of the fungus in nurseries is the use of disease-free seed (Dwinell et al. 1985). However, if a pathogen is already established in a nursery, sound nursery practices are of great importance in preventing disease outbreaks. The use of pathogen-free irrigation water and growth media and sterile containers as well as rouging diseased plants can reduce the population size of the pathogen within a nursery. Measures to control the activity of vectors and wounding agents may also be economically feasible in nurseries.

In pine plantations and seed orchards it is possible to avoid disease outbreaks with proper silvicultural management. Environmental stress increases the susceptibility of *Pinus* spp. to the pitch canker pathogen (Blakeslee and Rockwood 1999). Planting sites, therefore, should be suited for pine production, planting density should not be inordinately high (to avoid drought stress), trees should be thinned to appropriate stocking levels, and the site should have adequate drainage (to avoid water logging) (Blakeslee and Rockwood 1999). High fertilization increases the susceptibility of pine to pitch canker (Fisher et al. 1981) and should therefore be avoided. Because *F. circinatum* is a wound infecting pathogen, wounding of trees should be avoided during cone harvesting and other forest management practices (Dwinell et al. 1985). Branch tip cankers have been pruned out of landscape trees in California, but this practice has not been successful in slowing the progress of the disease or in increasing the lifespan of a tree (Gordon et al. 2001).

Long-term management of pitch canker of *Pinus* spp. grown in plantations can only be achieved by planting resistant selections or species (Wingfield et al. 2002a). Variation in susceptibility is very common in *P. elliottii* (Dwinell et al. 2001; Rockwood et al. 1988) and in native *P. radiata* forests (Gordon et al. 2001). It is, therefore, feasible to develop resistant planting stock, which can be used for commercial forestry and for replanting of native forests (Gordon et al. 2001; Gordon et al. 1998b). Planting of resistant species can be considered as an alternative to improving the existent planting stock, which is currently utilized by commercial forestry (Wingfield et al. 2002a).

3.2.2. Chemical Control

Thiabendazole (2-(4-thiazolyl) benzimidazole) is a systemic and residual fungicide that has been tested in various concentrations *in vitro* and *in vivo* and found to limit growth of *F. circinatum* and to prevent pitch canker symptom expression (Runion and Bruck, 1988b). These authors recommended 21,3g/l of the fungicide for disease management in pine nurseries, which would provide disease control for 12 weeks. Unfortunately these positive results were negated in subsequent studies. Runion et al. (1993) found that although thiabendazole reduced the incidence of infection by *F. circinatum* on terminal shoots, the reduction in disease incidence observed was not considered sufficient to warrant its use. It was also noted that higher rates of thiabendazole, result in larger seedlings. Larger seedlings

may provide more area for infection by *F. circinatum*, therefore, increasing susceptibility to the disease (Runion and Bruck 1988b). In addition, it has been reported that relatively high levels of resistance to benomyl, a thiabendazole derivative, exist in populations of the *Gibberella fujikuroi* species complex (Yan and Dickman 1993), making the use of thiabendazole and its derivatives problematic. Furthermore, use of such chemicals is strongly discouraged by certification programmes such as FSC and they are unlikely to provide solutions for nursery infestations.

Seed is a potential source of inoculum and a vehicle for the dispersal of the pitch canker fungus. Levels of contamination of seed can be tested by embryo dissection, crushing seeds on *Fusarium* selective medium or crushing seeds on blotting paper and subsequently examining them for fungal growth (Anderson 1986). On potentially contaminated seed, a number of seed treatments have been tested for the control of *F. circinatum* seedling disease. Runion and Bruck (1998a) found that thiabendazole suspended in 10% dimethyl sulphoxide was an effective seed treatment for the control of the pitch canker fungus. Treating seed prior to stratification with sodium hypochloride, after stratification with sodium peroxide or ethanol, or after stratification in hot water (55.5 C) significantly reduced seedborne *Fusarium* spp. (Dumroese 1988). In contrast, Storer et al. (1999) found that sodium hypochloride was ineffective in reducing *F. circinatum* contamination of *P. radiata* seed due to high levels of internal seed contamination by the pathogen. Dwinell (1999a) found that internal seed contamination by *F. circinatum* could be reduced by soaking *P. radiata* seed for 15 minutes in a 30% hydrogen peroxide solution. Hydrogen peroxide can penetrate the seed coat, thereby scarifying the seed, which increases germination. Due to the endophytic nature of *F. circinatum* in seeds, no absolute means of controlling the fungus has been found (Storer et al. 1999a).

3.3. Host-Pathogen Interactions

Trees that are considered susceptible to pitch canker include 47 species of *Pinus* (Dick 1998). Douglas-fir (*Pseudotzuga menziesii*) is the only recorded non-pine host (Storer et al. 1994). Indications are that *P. radiata* could be the most susceptible of all pine species thus far exposed to the pathogen (Dick 1998; Hodge and Dvorak 2000). Barrows-Broaddus and

Dwinell (1980) suggested that the pitch canker fungus is pathogenic to gladiolus corms, but this was later refuted by Viljoen et al. (1995b) who showed that the fungus on gladiolus was *F. proliferatum*. *Fusarium circinatum* is, therefore, exclusively a pathogen of coniferous trees.

Although the host range of *F. circinatum* extends to most *Pinus* spp., some species appear to be resistant and quantitative differences have been documented within species (Rockwood et al. 1988; Viljoen et al. 1995a) that are susceptible. *Pinus brutia* is reported to be resistant to pitch canker (McCain et al. 1987), as is *P. thunbergiana* (Gordon et al. 1998a). A number of species have been found to have relatively high levels of resistance to the pitch canker fungus. These include *P. pringlei*, *P. jaliscana*, *P. oocarpa*, *P. carribea*, *P. taeda* and improved families of *P. elliottii* (Hodge and Dvorak 2000); *P. canariensis* and *P. pinea* have also been shown to sustain relatively little damage from pitch canker (Gordon et al., 1998a). Species thought to be relatively resistant, frequently develop a high incidence of disease under the influence of environmental stress and inoculum pressure (Dwinell et al. 1985; Blakeslee and Rockwood 1999). This is underscored by the fact that plants derived from seeds harvested from an area of low disease incidence in the south eastern United States develop far more pitch canker than plants derived from native seeds (Dwinell et al. 1985). Relative resistance of different *Pinus* sp. is thus controlled by genetic as well as environmental factors.

Within species variation has been reported for pines grown in the south-eastern US (Barrows-Broaddus and Dwinell 1984; Blakeslee and Rockwood 1978; Bronson et al. 1992), and in California (Storer et al. 1999a). Blakeslee and Rockwood (1999) found variation in resistance to the pitch canker pathogen in breeding families of *P. elliottii* and *P. taeda*. Families in which resistance persists in adverse environmental conditions have been selected and are now used in operational plantations, advanced breeding programs and in research on mechanisms of resistance (Blakeslee and Rockwood 1999).

In 1988, it became apparent that some Monterey pines in California were unaffected by pitch canker, in spite of their proximity to heavily infected trees. Direct inoculations of these asymptomatic trees showed that they supported a significantly slower rate of lesion expansion than susceptible trees (Correll et al. 1991). This suggests a genetic basis for the observed differences in disease severity. Subsequent tests on seedlings under greenhouse conditions confirmed variation in the extent of lesion development within all five native populations of *Pinus radiata* (Schultz et al. 1990). There is thus a genetic basis for resistance to the pitch canker fungus within the native *P. radiata* population. This supports the view that

indigenous Monterey pine forests will survive the onslaught of pitch canker and should ensure the continued existence of this valuable resource.

Spore loads have an effect on tree resistance to the pitch canker fungus. Gordon et al. (1998) observed in trials testing the resistance of *P. radiata*, that tree resistance is not dependent on spore dosage when low spore dosages were used for inoculation. There was no significant difference in mean lesion length resulting from high and low spore loads. Thus, spore loads in the range of 30-140, was not a limiting factor in lesion development. Similar results have been obtained by Hodge and Dvorak (2000), who did not obtain significant differences in disease severity on plants inoculated with concentrations of 50 000 and 100 000 spores per inoculation. In contrast, Storer et al. (1999) found increasing disease severity with increasing spore loads, when spore loads between 25 and 1000 spores/inoculation were used. This indicates that the effect of inoculum level on disease severity is sigmoidal rather than linear.

A histopathological study showed that resistance to the pitch canker fungus is partially based on the speed and degree of host response to wounding (Barrows-Broaddus and Dwinell 1983). Davis et al. (1999) successfully identified pine genes which are up-regulated in response to *F. circinatum* challenge. In California it has been found that susceptibility is not necessarily a static characteristic, but that it can change over time and with exposure to the disease. This has been attributed to a systemic induced resistance (SIR) host response, the first report of this phenomenon in coniferous trees (Bonello et al. 2001a). Disease remission presumably due to SIR has now been observed in most *P. radiata* stands since the onset of the pitch canker epidemic in California (Gordon et al. 2001).

The durability of genetic resistance over time may be affected by the facility with which the *F. circinatum* population can generate novel pathotypes (Gordon et al. 1998b). The capacity to undergo sexual reproduction (Viljoen et al. 1997b) may enhance prospects for the appearance of pathogen genotypes capable of overcoming disease resistance. In South Africa, Mexico and the south eastern United States, *F. circinatum* populations are genetically diverse (Wikler and Gordon 2000), whereas in California and Japan there is little evidence that sexual reproduction is occurring. Breeding for resistance to the pitch canker fungus will only prove effective if multi-gene resistance is employed, which can meet the challenge of novel pathotypes that might appear due to sexual recombination (Gordon et al. 2001).

4. The pathogen

4.1. Taxonomy

Fusarium circinatum Nirenberg and O'Donnell (teleomorph *Gibberella circinata* Nirenberg and O'Donnell) has undergone a number of name changes. In 1946, when pitch canker was first described, it was referred to as an undescribed species of *Fusarium* belonging to the section Liseola (Heptig and Roth 1946). Three years later, it was designated by Snyder et al. (1949) as *F. lateritium* Nees f. sp. *pini* Heptig. In the 1970s, based on the fact that *Fusarium* isolates from pitch canker-affected tissue had abundant microconidia on sympodially branching conidiophores and no chlamydospores, they were identified as *Fusarium moniliforme* Sheldon var *subglutinans* Wollenweber and Reinking in the section Liseola (Dwinell and Phelps 1977; Kuhlman et al. 1978). In 1983, the variety was raised to species level by Nelson et al. as *F. subglutinans* (Wollenweber and Reinking) Nelson, Toussoun and Marasas. Later, based on differing RFLP patterns of mitochondrial (mt) DNA and host specificity, Correll et al. (1992), concluded that there was considerable justification for assigning strains of *F. subglutinans* pathogenic to pines to a *forma specialis*. They thus provided the pitch canker fungus with the name *F. subglutinans* f. sp. p*ini* Correll, Gordon, McCain, Fox, Koehler, Wood and Schultz. The unique nature of the pitch canker fungus was further supported by isozyme analysis (Huss et al. 1996), RAPD profiles (Viljoen et al. 1997a) and by the reproductive biology of the fungus (Britz et al. 1999).

In 1998, Nirenberg and O'Donnell described the pine pitch canker fungus as a distinct species, for which they provided the name *Fusarium circinatum* Nirenberg and O'Donnell. The authors differentiated the fungus from *F. subglutinans* (Wollenweber and Reinking) Nelson, Toussoun and Marasas pathogenic to maize, by its polyphialides with more than three openings (as opposed to fewer than three conidiogenous openings in *F. subglutinans*), proliferating conidiophores (those of *F. subglutinans* are strongly branched) and hyphal coils (absent in *F. subglutinans*, but present in *F. pseudocircinatum*). The teleomorph was given the name *Gibberella circinata* Nirenberg and O'Donnell. This separation of the pine pitch canker fungus as a distinct taxon was supported by phylogenetic lineages based on the beta-tubulin gene- and alpha-translation elongation factor gene sequences (O'Donnell et al. 1998; 2000). Subsequently questions were raised concerning the characterization of the type specimen of *F. circinatum* (*G. circinata*). Only a small number of isolates were used in the morphological descriptions and a single cross was used to describe the sexual stage, *G.*

circinata (Nirenberg and O'Donnell 1998). Britz et al. (2002) re-characterized the pine pitch canker fungus and found that the morphological characteristics amongst a large collection of *F. circinatum* and *G. circinata* was consistent with those given in the original description, with some minor differences. The distinctiveness of the taxon was further supported by phylogenetic data based on sequence analysis of the histone H3 gene (Steenkamp et al. 1999), the mating type gene (Steenkamp et al. 2000) and the rDNA intergenic spacer (Schweigkofler et al. 2004).

Fusarium circinatum is capable of sexual reproduction and the species was initially assigned to the mating population B within the *G. fujikuroi* complex (Kuhlman 1982). Successful crosses with B tester strains failed in later studies (Correll et al. 1992; Viljoen et al. 1994), which lead to doubt as to whether the pitch canker fungus should reside in the B mating population. Britz et al. (1999) found that self-fertilization occurs in the B mating population, thus providing an explanation for ascospore formation in the crosses performed by Kuhlman (1982). Furthermore, results of that study confirmed that the pitch canker fungus resides in a unique mating population, designated mating population H (Britz et al. 1999). The fungus is thus heterothallic (Leslie 1995) and mating is controlled by two alleles at a single mating type locus. The Mat 1 and Mat 2 idiomorphs have been sequenced and characterized by Steenkamp et al. (2000). *F. circinatum* strains of opposite mating type can thus recombine, leading to the formation of sexual structures (Britz et al. 1998; Viljoen et al. 1997b) or teleomorph, designated as *Gibberella circinata* by Nirenberg and O'Donnell (1998).

4.2. Biology and Ecology

Relatively little is known regarding the biology and ecology of *F. circinatum* but studies have been conducted on inoculum dynamics (Blakeslee et al. 1978; Fraedrich and Dwinell 1997), pathogen dispersal (Fraedrich and Dwinell 1997; Hoover et al. 1995), infection (Kuhlman et al. 1982) and colonisation (Barrows-Broaddus et al. 1985; Kuhlman et al. 1987) of *F. circinatum*. Mechanisms by which the pathogen infects seeds (Storer et al. 1998; 1997), sexual reproduction (Wikler et al. 2000) and the saprophytic survival of the pathogen (Mc Nee et al. 2002) have also been studied, although not in great detail.

Inoculum of *F. circinatum* is available during all seasons of the year. In Florida, Blakeslee et al. (1978) reported that sporodochia containing macroconidia occurred commonly on infected branches in the upper crown of infected trees during the entire year. Sporodochia were also commonly observed on dead needles attached to infected shoots, which are shed into the litter, where they possibly continue to serve as an inoculum source (Barrows-Broaddus and Dwinell 1984). In a loblolly seed orchard, Kuhlman et al. (1982) found spores of the pitch canker fungus were produced throughout the growing season on dead branches in the crown and could be recovered from rainwater falling through infected trees and in the air. Fraedrich and Dwinell (1997) reported that the fungus was primarily detected in spore traps beneath asymptomatic longleaf pine trees during the nights when it rained. In California, airborne inoculum of *F. circinatum* was detected in abundance throughout the year in an area with a high incidence of pitch canker on *P. radiata*, but not in areas where the disease was absent (Correll et al. 1991). A bark wash survey conducted on the central coast of California revealed that where the pathogen was present, both symptomatic and asymptomatic trees could test positive for the presence of spores of the pitch canker fungus (Adams 1989).

Dispersal of *F. circinatum* spores occurs through wind (Fraedrich and Dwinnel 1997), insect vectors (Blakeslee et al. 1978; Hepting and Roth 1953; Hoover et al. 1995) and movement of infected plant materials to new areas by man (Storer et al. 1998; Wikler and Gordon 2000). Dispersal mechanisms are very effective, as was demonstrated by Correll et al. (1992), who found that a single vegetative compatibility group (VCG) which was present in a single native stand in Florida in 1977, was recovered from almost all sites surveyed in 1988.

In the south-eastern United States, natural infection of wounds by air-borne spores was demonstrated by Kuhlman et al. (1982) in a study where 52% of wounded *P. elliottii* and *P. taeda* seedlings placed under infected trees in a seed orchard developed symptoms of pitch canker. No infection was observed in the absence of wounds, whether experimentally inflicted or of abiotic origin. For this reason, Dwinell et al. (1985) suggest that pitch canker epidemics may be a consequence of intensive forest management practices. By contrast, no association of the disease with artificial wounding and hence abiotic damage was observed in California (Correll et al. 1991). Thus, in the western United States, insects are considered to be the primary, if not the only, agents of transmission of the fungus, while in the south eastern United States abiotic as well as insect inflicted damage plays a role in the infection cycle of the disease.

Conflicting reports exist regarding the colonization of wounds by *F. circinatum*. Barrows-Broaddus et al. (1985) reported that one-day-old wounds were highly susceptible to colonisation by the pathogen while 7 day-old wounds were no longer susceptible. Kuhlman (1987), on the other hand, found no significant differences in disease severity between plants wounded 3 and 21 days before inoculation. After inoculation, infection occurs rapidly in the presence of free atmospheric moisture (Gordon et al., 2001). Barrows-Broaddus and Dwinell (1983) reported that the incubation time of the fungus following infection of host tissue is very short, and that *F. circinatum* can be isolated from surface-disinfected, artificially inoculated loblolly pine stems after four days. These authors also observed that infections in pine tissues are often latent, and that infected pines harbour the pathogen until environmental conditions permit a disease outbreak.

The mechanisms by which the pitch canker pathogen infests seed are not well studied. Storer et al. (1998) hypothesised that superficial seed infestation occurs by airborne propagules that enter the cone during periods when they are open. For systemic infestations, fungal hyphae from lesions on the tree may grow through the cone stalk into the seed (Storer et al. 1998). The pitch canker fungus has been isolated from the surface of *P. radiata* seed collected from healthy cones: both internally (where it can remain dormant until seed germination) and externally on living seeds from infected cones (Storer et al. 1997). *F. circinatum* has been isolated from seedlings germinating from seed collected from cones on healthy branches (Storer et al. 1998). This fungus could not be isolated from this seed prior to germination, thus supporting the hypothesis of dormancy and cryptic or endophytic development stages of *F. circinatum*.

The saprophytic survival of *Fusarium circinatum* is not well understood. Viljoen et al. (1994) found that the pathogen can survive in soil, and may infect seedlings at or just above the soil-line. The fungus has been found to act as a typical soil inhabitant and root-infecting pathogen (Dwinell and Barrows-Broaddus 1978; Dwinell et al. 1985). The pathogen is also able to survive in dead branches and wood chips for up to three years (Mc Nee et al. 2002). Survival of the pathogen is higher in wood stored in cooler areas than in hotter areas (Mc Nee et al. 2002). It was also reported, that spores and other infectious propagules can enter the soil from dead infected tissue (Dwinell and Barrows-Broaddus 1978).

Fusarium circinatum has been observed to reproduce sexually *in vitro* on agar medium (Britz, 1999) and on surface sterilized host tissue (Wikler et al. 2000). Sexual structures have not been observed in the field. This might be due to the fact that sexual reproduction is

favoured at temperatures lower than those that are optimal for disease outbreaks (Covert et al. 1999). Sexual structures might, therefore, easily escape observation. Both mating types must be present at a location for sexual reproduction to occur. This effectively limits the sexual reproduction of the fungus, since both mating types are seldom observed in the same locality in California (Wikler et al. 2000).

4.3. Population genetics:

Fusarium circinatum can reproduce both asexually and sexually. Each of these cycles affects the population structure differently (Britz et al. 1998; Leslie and Klein 1996). The asexual cycle results in clonal propagation, whereas the sexual cycle results in recombination leading to new genotypes (Leslie and Klein 1996). The population structure and the evolution of new genotypes by out crossing have implications for disease control using chemicals as well as for tree breeding for resistance against the pathogen.

Correll et al. (1992) found by means of VCG (Vegetative Compatibility Group) studies that the *F. circinatum* population in Florida is very diverse (41 VCGs among 106 isolates). This is consistent with the assumption that pitch canker is endemic in the South Eastern United States. In contrast, only 5 VCGs were identified in the Californian population among 209 isolates, with one VCG accounting for 70% of the population (Correll et al. 1992). This low diversity is indicative of a recent introduction of the fungus into California and the absence of sexual reproduction. In a survey by Gordon et al. (1996) of the Californian pitch canker fungus population, the highest diversity in the fungal population was found to be in Christmas tree nurseries (4 VCGs out of a total of 8). The authors suggest that these nurseries served as sites of introduction of the pathogen, and contributed to its subsequent movement within California.

Wikler and Gordon (2000) compared allelic and genotypic frequencies based on eight polymorphic genetic markers, and found that genetic diversity was greatest in Mexico. The authors therefore speculated that Mexico might be the centre of origin of *F. circinatum*. The only country into which the fungus was probably directly introduced from Mexico is South Africa (Wikler and Gordon 2000). From studies conducted with microsatellite markers as well as using mitochondrial DNA, it was confirmed that the South Eastern United States was almost certainly the source of the fungus which was introduced into California and Japan.

Although none of the VCGs found in California are found in Florida, the similarity of mitochondrial DNA between *F. circinatum* isolates from California and Florida led Corell et al (1992) to suggest that both populations have a recent common origin. These results were confirmed by Wikler and Gordon (2000). They reported a close relationship between isolates from the South Eastern United States, California and Japan. These authors, therefore, suggested that the South Eastern United States is the point of common origin and migration.

Fusarium circinatum was first described in South Africa in 1994 (Viljoen et al. 1994). In a population study conducted by Viljoen et al. (1997), a relatively high VCG diversity was found in the South African *F. circinatum* population. The large number of vegetative compatibility groups was inconsistent with the evidence that this was a recently introduced species. Usually, an accidental introduction is associated with a low genetic diversity. A study by Britz et al. (2005) however, suggests that multiple genotypes have been introduced into South Africa and that sexual reproduction occurs in this country, resulting in the segregation of multiple vegetative incompatible loci. Studying South African mating type distribution of *F. circinatum*, Britz et al. (1998) found a high number of female sterile isolates and a low number of hermaphrodites. This indicates that the population is evolving towards an asexual state. The population might thus be evolving towards a stable clonal state, which is favourable for future plant resistance breeding efforts.

5. Interactions of the Pathogen with the Environment

5.1 Abiotic factors:

A number of abiotic factors contribute to wounding trees and thus allowing entry to the pitch canker fungus. Bole cankers infected with *F. circinatum* on slash pine in the south-eastern United States are usually caused by the use of mechanical tree shakers for cone harvest. In loblolly pine seed orchards in the south-eastern United States, shoot dieback caused by *F. circinatum* is associated with wounds caused by twisting and tearing cones off branches (Dwinell and Barrows-Broaddus 1982). Adverse weather conditions such as hurricanes and tornadoes also cause wounds that can be infected by *F. circinatum* (Dwinell and Barrows-Broaddus 1982). It, therefore, becomes obvious, that mechanical wounding associated with human activities, wind and hail damage is of primary importance in the infection cycle of *F. circinatum* in the south-eastern United States (Dwinell et al. 1985).

Edaphic factors such as soil structure and fertility affect host susceptibility to the pitch canker pathogen. Applications of high rates of nitrogen, potassium and phosphorus have been found to increase disease induced mortality (Fisher et al. 1981) especially in more susceptible breeding families (Blakeslee and Rockwood 1999). It has also been found that late summer applications of ammonium nitrate have especially devastating effects. Choice of planting site also has a pronounced effect on pitch canker epidemiology. Susceptibility to pitch canker increases during drought stress or water logging (Dwinell et al. 1985). Shallow soils, especially when trees are planted at high stand densities, increases the susceptibility of pines to the pitch canker fungus (Blakeslee and Rockwood 1999). The stability of the resistance of pines to the pitch canker fungus is very dependant on edaphic factors and should be considered when planting pine plantations (Blakeslee and Rockwood 1999).

It has been shown that air pollution plays a role in development of disease caused by the pitch canker fungus. High ambient ozone concentrations were found to enhance canker development in susceptible trees, whereas in resistant trees, the interaction of the pathogen and the air pollutant caused stunted growth and decreased root mass (Carey and Kelly 1994a). This might provide the explanation for the predominant pitch canker incidence in California along highways and landscape plantings (Correll et al. 1991).

Abiotic factors that have been identified as components of pitch canker epidemics contribute to disease by wounding pine hosts or in placing these trees under environmental or plant physiological stress. Inclement weather or human activities can cause wounding, effectively providing entry to the pathogen (Dwinell and Barrows-Broaddus 1982). Edaphic factors such as inordinately high fertilisation and the utilisation of poor planting sites (Blakeslee and Rockwood 1999) as well as air pollution (Carey and Kelly 1994a) can contribute in reducing resistance of pine to the pitch canker pathogen.

5.2. Climatic conditions:

Temperature affects infection, colonisation of tissues and survival of *F. circinatum.* The rate of lesion expansion has been reported to increase with temperatures between 14 and 26 ˚C (Gordon et al. 1998a). Huang and Kuhlman (1990) found that *F. circinatum* causes seedling damping off between 20-30 ˚C, while *F. proliferatum* associated with pines, only causes seedling damping off at temperatures close to 30 ˚C. The pitch canker fungus appears

to prefer warmer climates for infection and colonization of host tissue, since it has not been reported in areas where average temperatures fall below 0 ˚C for some part of the year (Gordon et al. 2001). High temperatures are also unfavourable, and *F. circinatum* propagules have been eliminated at a temperature of 50 ˚C (Gordon et al. 2001). The pitch canker fungus thus appears to be limited by its temperature requirements to mild, frost-free environments.

Some seasonal variation can be detected in the pathogenicity of the pitch canker fungus. In the south-eastern United States symptom expression associated with *F. circinatum* begins in autumn and continues through winter and spring (Dwinell et al. 1985). In contrast, branch tip cankers progress faster in spring inoculations than in autumn inoculations in California's Mediterranean climate (Correll et al. 1991). However, variation in lesion sizes on the same inoculated clone between experiments conducted in late autumn and spring could only be detected in the more susceptible *Pinus radiata* clones (Gordon et al. 1998b). Gordon et al. (1998b), therefore, concluded that lesion size in controlled inoculation trials is primarily influenced by the interaction between the host and the pathogen, and less by environmental conditions. Similar conclusions were drawn from experiments conducted in field and greenhouse microclimates, where differences in lesion size were more pronounced in the more susceptible *P. radiata* clones (Gordon et al. 1998b). Presumably intervals of higher temperature in the greenhouse relative to the field, would contribute to more rapid growth of lesions. Thus, in resistant trees, development of the fungus is restricted by the host and is more or less independent of the ambient conditions. In the case of trees prone to more extensive lesion development, growth of the pathogen and hence susceptibility to pitch canker may be significantly affected by the environmental and seasonal variations (Gordon et al. 1998b; Storer et al. 1999a).

High ambient humidity seems to be advantageous to the pitch canker fungus. It has been observed that plants are more severely affected by the pathogen in coastal regions, than in inland regions in California (Gordon et al. 2001). Up until 1999 the disease was found only in California in locations having a maritime climate (Adams et al. 1999). Furthermore, if *F. circinatum* is vectored by insects, feeding only superficially on *Pinus* spp., free atmospheric moisture must be present for infection to occur (Gordon et al. 2001). Ambient humidity was not reported to have an effect on the epidemiology of the pitch canker fungus in the south-eastern United States. Humidity, therefore, seems to have an effect only in the unique biotic system, which contributes to the severe pitch canker epidemic in California.

According to Dwinell et al. (1985), various stress factors may predispose trees to infection by the pitch canker fungus. Moisture deficiency has been associated with pitch canker epidemics. High stand densities at crown closure, when competition for moisture is high, increase the susceptibility of pines to the pitch canker fungus in the south-eastern United States (Blakeslee and Rockwood 1999). California's protracted drought from 1987 to 1992 might, therefore, have been of great importance in the Californian pitch canker epidemic, especially, since high mortality stands were situated on soils with poor water holding capacity (Owen and Adams 2001). The extent to which stress factors including early frost (Dwinell et al. 1985) are involved in pitch canker epidemics is not clear, since epidemics have also occurred in their absence.

5.3. Associations with Insects:

Insects serve as wounding agents of trees or as vectors of *F. circinatum* both in the south-eastern United States (Blakeslee et al. 1978) and in California (Gordon et al. 2001). When discussing different insect-pathogen associations it is necessary to differentiate between insects associated with different *Pinus* spp. and insects endemic to specific areas. Insect species associated with pines, that are indigenous in California, such as *Pinus radiata*, have not been reported to be endemic in the south-eastern United States. They have also not been reported to play a role in dissemination of the disease in this area. The same is true for insects endemic to the south-eastern United States, which appear to be less important associates of the disease in California.

5.3.1. *Insects Endemic to the South-Eastern United States, implicated as vectors*:

In the south-eastern United States, the Deodar weevil (*Pissodes nemorensis* Germar) (Blakeslee et al. 1978) and the subtropical pine tip moth (*Rhyacionia subtropica* Miller) (Matthews 1962 as cited by Dwinell et al., 1985) have been reported to be the most important insects for the creation of wounds that might become infected by airborne spores of *F. circinatum*. The deodar weevil has frequently been observed to carry propagules of the pitch canker fungus and infection of pine seedlings has been clearly linked with the presence of

artificially contaminated weevils in greenhouse trials (Blakeslee et al. 1978). A direct relationship has also been observed between pine tip moth damage and the incidence of pitch canker in North Carolina, although the extent of this relationship is unknown (Runion 1993). The percentages of tip moth infested *P. elliottii* terminals infected with *F. circinatum* were consistently high in unsprayed plantations in North Carolina. However, *F. circinatum* infection persisted, even when tip moth damage was reduced by the use of an insecticide. This may indicate that the primary wounding agent associated with *F. circinatum* infection shifts from pine tip moths to some other biotic or abiotic agent (Runion 1993).

A number of insects of secondary importance have been identified as possible vectors of *F. circinatum* in the south eastern United States. Wounds caused by the needle midge (*Contarinia* sp.) are common on pine in seed orchards and plantations and are often colonised by the pitch canker pathogen (Dwinell et al. 1985). Cone and seed feeding insects have also been identified, which may contribute to pitch canker dissemination and infection. These include seedbugs (*Leptoglossus corculus* Say, *Tetrya bipunktulata* H.S.), seed worms (*Laspreyresia* spp) and seed chalcids (*Megastionus atedius* Walker) (Dwinell et al. 1985). However, in the south eastern United States, mechanical transmission of the pathogen is considered to be a more important means of dissemination of pitch canker than these insect vectors (Dwinell et al. 1985).

5.3.2. *Insects endemic to California, which are implicated as vectors*:

In California, where mechanical injury due to, for example, cone harvesting is not common, insect vectors are thought to be primarily responsible for the spread of the pathogen (Hoover et al. 1995). Three species of bark beetle are common in Santa Cruz, California: *Ips mexicanus* (Hopkins), *Ips paraconfusus* (Lanier), and *Ips plastographus* (LeConte). All three species rapidly colonize suitable host material (usually slash created by wind-thrown trees and logging debris) and the insects are associated with mortality in stressed or declining trees. *Ips paraconfusus* also attacks apparently vigorous trees (Schultz and Bedard 1987). Fox et al. (1991) reported that these beetles transmit the pitch canker pathogen. By causing shoot dieback, branch decline and tree mortality, *F. circinatum* provides stressed trees which can be colonized by *Ips* spp. Beetles can cohabitate with *F. circinatum* and in this way a new

association has developed between these insects and the pathogen (Fox et al. 1990; Storer et al. 2002a).

Five species of twig beetles (*Pityophthorus* spp) naturally associated with stressed trees and dying branches in the lower canopy, have been shown to carry *F. circinatum* (Dallara et al. 2000; Hoover et al. 1996). The pathogen has been isolated from *P. carmeli* Swaine, *P. setosus* Blackman, *P. nitidulus* Mannerheim, *P. pulchellus tuberculatus* Eichoff and *P. californicus* Bright (Storer et al. 1994). Twig beetles attack smaller branches, and are for this reason considered very important in the establishment and early intensification of pitch canker disease (Gordon et al. 2001). *Pityophthorus* spp. are closely associated with *Lasoconotus pertenuis* Casey and *L. nucleates* Casey (Storer et al. 1999b). *F. circinatum* has been isolated from these *Lasoconotus* spp., but no transmission studies involving the beetles have been conducted (Storer et al. 1999b).

The cone beetle, *Conophthorus radiatae* Hopkins is found commonly on *P. radiata* and was shown to vector the *F. circinatum* (Hoover et al. 1995). The young beetles attack healthy one-year-old cones, in which they overwinter. In spring, matured beetles emerge to attack second year cones, where eggs are laid. Since as much as 50% of initial infections of pitch canker in California originate at the cone whorl, it has been surmised, that *Conophthorus radiatae* contributes substantially to the pitch canker epidemic in this region (Hoover et al. 1995). The dry twig and cone beetle, *Ernobius punctulatus* Fall, which was found to vector *F. circinatum* (Hoover et al. 1996) is a common associate of *C. radiatae* and colonizes galleries inhabited by *C. radiatae*. The beetle is reported to oviposit in these galleries. Elkinton and Wood (1980) have suggested, that dry twig and cone beetles probe trees by excavating entrance tunnels and abandon them if resin flow is prolonged and abundant. This 'tasting' phenomenon may enhance transmission of the disease by the beetles to apparently healthy trees (Fox et al. 1990; Bonello et al. 2001b). Transmission of inoculum between *C. radiatae* and *E. punctulatus* has been suggested by Hoover et al. (1995), since higher levels of inoculum of *F. circinatum* were found on *C. radiatae,* when found in the same cone with *E. punctulatus*, than when found in cones not containing *E. punctulatus*. Transmission of inoculum may occur while beetles use the same galleries to move about, to feed or to exit the cone.

In addition to the above-mentioned beetles, Fox et al (1991) found the pitch canker fungus on the Monterey pine weevil (*Pissodes radiatae* Hopkins), *Hylastes nigrinus* Mannerheim, the western pine spittlebug (*Aphrophona permutata* Uhler) and the western

pine leafhopper (*Koebelia california* Baker). Fox et al. (1991) also found the pitch canker fungus on common houseflies, wasps and beetles not known to feed on *Pinus* spp. In addition, the spittlebug *Aphophora canadensis* Walley has been shown to act as a wounding agent capable of initiating infections in the internodal regions on succulent shoots during late winter and early spring (Storer et al. 1998).

Most insects implicated in vectoring the pitch canker fungus in California, attack weakened hosts (Storer et al. 1998). Infection of pines by *F. circinatum* weakens trees making them more susceptible to insect attack. The introduction of *F. circinatum* into California has, therefore, modified the ecology of insect-plant interactions, and has caused a disturbance in natural and artificial Californian ecosystems, by making *P. radiata* and other *Pinus* spp. more prone to insect attack (Storer et al. 1999c; 2002).

5.4. *Associations with Micro-organisms:*

Fusarium circinatum has been shown to be associated with fusiform rust, an important fungal disease of southern pines. The pitch canker fungus is frequently an ecological component of fusiform rust galls. Hepting (1971 as cited by Dwinell et al. 2001) suggested, that some of the greatest damage resulting from fusiform rust, caused by *Cronartium querquum* f. sp *fusiforme*, resulted from the rust damage being followed by *Dioryctria* spp., the southern pine cone worm, and the pitch canker fungus. Dwinell and Barrows-Broaddus (1985) found that *F. circinatum* rapidly colonized rust infected tissue and hastened mortality of *P. taeda* and *P. elliottii* seedlings. Infection of rust galls by the pitch canker fungus further weakens stems of mature trees and increase chances of breakage and tree mortality (Dwinell and Barrows-Broaddus 1985).

Various biological control strategies have been proposed for the control of the pitch canker fungus involving antagonistic fungi or bacteria. Doemroese et al. (1988) noted the benefits of *Trichoderma* spp. on the seed coats of conifer seed to control seedling diseases caused by *Fusarium* spp. Unfortunately, *Trichoderma* spp. have in a later study been found to be ineffective against *F. circinatum* (Mitchell et al. 2004). Dwinell et al. (1985) in contrast, proposed the use of avirulent competitors such as *F. monilliforme* as competitors to *F. circinatum* but the efficacy of such competitors has never been demonstrated. Certain strains of *Arthrobacter* spp. are antagonistic to the pitch canker pathogen *in vitro* (Barrows-

Broaddus et al. 1983). During *in vivo* testing, it has been found, that the bacterium is not effective for the control of *F. circinatum*, because it does not protect wounds from colonisation of the pathogen and is not adapted for survival on wounded pines (Barrows-Broaddus et al. 1985). Biocontrol of *F. circinatum* has to date not been shown to be effective, due to the unique nature of the pathogen's ecology.

6. Conclusions

Since its discovery in 1946, pitch canker has gradually become one of the most important diseases of pines in the world. There is little doubt that the disease seriously threatens plantation forestry in southern hemisphere countries, especially those with a strong reliance on highly susceptible species such as *P. radiata* and *P. patula*. Some of the most important forest industries in the world rely on these species. The fact that the fungus has already appeared in South Africa and Chile substantially enhances this threat.

Much has been learned about *F. circinatum* in recent years and particularly in the last 15 years, subsequent to its appearance in California and South Africa. After many years of confusion, the taxonomy of the fungus has been clarified and there is a relatively clear understanding of its area of origin. Our understanding of the susceptibility of pine species has improved greatly and the epidemiology of the pathogen is better understood than has been true in the past.

Despite a substantially improved knowledge of pitch canker and its causal agent, there are many questions that remain to be answered. Many of these relate to the threat of the disease to plantation forestry in the southern hemisphere. For example, the fact that the pathogen has remained restricted to nurseries in Chile and South Africa is an enigma. It is possible, that the absence of certain insects has influenced this situation (Wingfield et al., 1999). However, in both these countries insects similar to those associated with the disease in various parts of the United States are present. Furthermore, there is good evidence to show that disease on large trees can occur in the absence of insect damage. Might it then merely be a matter of time before inoculum levels become high enough for the disease to appear on established trees in South Africa and Chile?

Although a great deal is known regarding the means in which the pitch canker pathogen can spread, its recent appearance in new areas suggests that efforts to exclude it have not

been very effective. Despite very intensive quarantine efforts focused specifically on the pitch canker pathogen, it was recently encountered on a shipment of Douglas fir cuttings introduced into New Zealand. Clearly, substantially enhanced research is required to limit the appearance of the pathogen in new areas.

Most of the research conducted on tolerance to the pitch canker pathogen has been with species that are not commonly planted in the southern hemisphere. Furthermore, because of the absence of the pathogen in field situations in the southern hemisphere, there has been little work done to consider the impact that pitch canker could have on, for example clonal forestry. Means to limit its impact through the propagation of hybrid pines needs urgent attention. DNA based methods such as the development of rapid screening techniques, quantitative trait linked markers and genetically modified trees resistant to the pathogen also deserve attention.

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2

Effect of inoculum density, plant vigour and wounding on *Fusarium circinatum* infection of *Pinus patula* seedlings.

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Abstract: *Fusarium circinatum* is an important pathogen of pine seedlings and cuttings in South Africa. The fungus results in plant death in nurseries as well as during establishment of pine plantations. Little research has been conducted on the epidemiology of the pathogen in South Africa. The aim of this study was to consider the effects of wound type, spore concentration and environmental stress on disease development and symptom expression. Pine seedlings were inoculated using three wounding methods and five spore concentrations. Inoculated seedlings were incubated under optimal environmental conditions, sub-optimal conditions as well as sub-optimal conditions combined with a fungicide treatment. Trials were also undertaken to consider the effect of different time intervals between wounding and inoculation. Shoots of *P. patula* saplings were inoculated with six spore concentrations at nine intervals of six hours after wounding. Results of this study showed that the mean percentage disease caused by increasing spore concentrations can be described by the Michaelis-Menten function. The gradient of the function as well as the asymptotic maximum level of disease was dependant on the environmental and plant physiological state of the host as well as the wounding method. The highest rate at which spore concentration influenced disease incidence was observed in seedlings that were physiologically stressed. Stressed seedlings reached the same maximum disease incidence as seedlings incubated at optimal conditions after inoculation. Fungicide treatment did not influence the rate of disease incidence in comparison with the treatments conducted under optimal environmental conditions, but significantly lowered the asymptotic maximum level of disease incidence. Seedlings wounded on the stems had the highest disease incidence, when compared to other wounding methods. Lower disease incidence was observed in treatments where longer time intervals between wounding and inoculation were allowed to elapse.

INTRODUCTION

Pitch canker, caused by *Fusarium circinatum* Nirenberg and O'Donnell (teleomorph *Gibberella circinata* Nirenberg and O'Donnell) (Nirenberg & O'Donnell, 1998), is a serious pathogen of pine trees. The disease has been reported from the south eastern United States (Hepting & Roth, 1946), Haiti (Hepting & Roth, 1953), California (McCain et al., 1987) Mexico (Santos & Tovar, 1991) and Japan (Muramoto & Dwinell, 1990). Damage caused by *F. circinatum* in these regions includes resin soaked cankers on the trunks and lateral branches of diseased trees (Dwinell et al., 1985). Shoot dieback (Correll et al., 1991) has also been reported. Dieback of female flowers and mature cones (Barrows-Broaddus, 1990), reduced germination of seeds (Huang & Kuhlman, 1990), as well as pine seedling mortality (Huang & Kuhlman, 1990; Carey & Kelly, 1994) is commonly caused by this pathogen. Severe outbreaks of *F. circinatum* infection of nursery plants have been reported in South Africa and Chile (Viljoen et al., 1995; Viljoen et al., 1994; Wingfield et al., 2002). Typical pitch canker symptoms on older trees have not been observed in these countries, where plantations of exotic pine species support important industries (Viljoen et al., 1994; Wingfield et al., 2002).

Fusarium circinatum causes a seedling disease resulting in both pre- and post emergence damping off (Huang & Kuhlman, 1990: Viljoen et al, 1994), as well as mortality of established seedlings (Carey & Kelly, 1994; Viljoen et al, 1994). Where pre-emergence damping off occurs, the seed coats and coleoptiles of germinated seeds are heavily colonized by the fungus. In the case of post-emergence damping off, root collars are girdled. Infection also results in chlorosis of the needles and wilting of the seedling tips (Viljoen et al., 1994). Pitch soaked lesions usually occur at or near the soil surface, but are occasionally found in the region of the cotyledonary node (Barnard & Blakeslee, 1980) or in the region of the bud (Carey & Kelley, 1994). The pitch canker pathogen has also been reported to cause severe root rot in established seedlings. Viljoen et al. (1994) noted that established seedlings infected with the pitch canker pathogen had underdeveloped roots with multiple pitch soaked lesions. In South Africa root rot caused by *F. circinatum* is most common on *Pinus patula* seedlings (Viljoen et al., 1994).

The pitch canker fungus infects pines through wounds (Barrows-Broaddus et al., 1985; Kuhlman, 1987). Wounds can be of abiotic origin (Kuhlman et al., 1982) or as the result of insect feeding (Blakeslee et al., 1978). Abiotic wounds such as those caused by wind damage or silvicultural practices are the primary points of infection in the southeastern United States (Dwinell et al., 1985). In California, insects are considered to be the main wounding agents (Correll et al., 1991). In South African pine seedling nurseries, a number of horticultural practices, such as pricking out, weeding, and rough handling of seedlings during planting, may provide wounds, which can serve as infection sites.

Conidia of *Fusarium circinatum* are abundant in areas where the pitch canker disease occurs (Dwinell et al., 1985). Inoculum can be present during all seasons of the year (Correll et al., 1991; Fraedrich & Dwinell, 1997). Furthermore, Blakeslee et al. (1978) showed, that sporodochia-containing macroconidia occurred abundantly on infected branches and dead needles attached to diseased shoots. Infected and dying plant organs are shed into the litter where they serve as a source of inoculum (Barrows-Broaddus & Dwinell, 1984). A bark wash survey conducted in California showed that both symptomatic and asympomatic trees could harbour inoculum (Adams, 1989). There is some debate as to the minimum number of spores needed for infection to occur, but studies on tree resistance report that resistance is not dependant on spore dosage (Gordon et al. 1998a; Hodge and Dvorak, 2000).

Various stress factors may predispose trees to infection by *F. circinatum*. Moisture deficiency and early frost have been associated with pitch canker epidemics (Dwinell et al., 1985). Barrows-Broaddus and Dwinell (1983) observed that infections are often latent, and that infected pines also harbour the pathogen until environmental conditions permit a disease outbreak. The extent to which environmental stress is involved in pitch canker epidemics is not clear, since epidemics have also occurred in the absence of stress (Dwinell et al., 1985).

The aim of this study was to determine the effect of various wound types and different spore concentrations on disease development and symptom expression in pine seedlings. We also considered the role played by environmental stress conditions such as heat and water stress.

MATERIALS AND METHODS

Preparation of inoculum and plants

An isolate of *F. circinatum* (MRC 6213) previously shown to be highly pathogenic (Viljoen et al., 1994) was grown on Potato Dextrose Agar (PDA, Biolab) in the dark for 2 weeks. Five healthy 16-month-old *Pinus taeda* saplings were inoculated with agar discs bearing the fungal isolate. *F. circinatum* was reisolated from trees 12 weeks after inoculation on *Fusarium* Selective Medium (FSM; Nash & Snyder, 1962) and identified from cultures grown on Synthetic Nutrient Agar (SNA; Nirenberg & O'Donnell, 1998). Single conidial cultures were made from isolates of *F. circinatum* reisolated from inoculated trees on PDA, lypophillized in a 15% glycerol solution and maintained at -80°C. These cultures were used in later inoculations.

Pinus patula seeds were surface disinfested in a 1% solution of NaHClO and sown into a peat seedbed. Six weeks after sowing, seedlings were transplanted into steam sterilized composted bark medium in 26 cm^3 removable plugs in planting trays. Within the trays, seedlings were arranged in blocks of six, with at least one space (25 mm) between the blocks. Eight blocks of six seedlings were fitted into a seedling tray. Seedlings were grown in a greenhouse at an average temperature of 24°C for 4 months prior to conducting inoculation experiments.

Experimental design of pathogenicity trial

Pinus patula seedlings were arranged in three greenhouse units. In each greenhouse unit, seedlings were arranged in a randomised block design of four blocks. Each block was made up of 24 groups of 6 seedlings. Each group of 6 seedlings was considered as one experimental entity and received the same treatment. Four wounding treatments were tested in combination with 6 spore concentrations. Treatments were replicated in each

block and in each greenhouse unit. In each greenhouse unit different incubation conditions were tested and are, therefore, sub-trials of each other.

Wound types

Three different wounding techniques were used prior to inoculations. These included i) root wound (severing a few roots with a sharp scalpel), ii) stem wound (shallow 1mm cut into the phloem on the lower part of the seedling stem), and iii) a wound simulating rough handling (ripping out a few needles). Within one experimental block, 6 experimental entities of 6 seedlings each were treated with each wounding method and 6 experimental entities per block were not wounded to serve as controls.

Conidial concentrations

Conidial suspensions of 5, 50, 500, 5000 and 50000 spores/ml were prepared by placing 15ml of sterile water on cultures of *F. circinatum* and rotating the Petri dishes to dislodge spores. The spore bearing liquid was removed from the Petri dishes and placed in Erlenmeyer flasks. Conidial concentrations in these flasks were adjusted with sterile water and the number of conidia were counted using a haemocytometer.

Within each block of seedlings, each experimental entity, which received a different wounding treatment was inoculated with a different spore concentration. For inoculations, 1 ml spore suspensions were applied to non wounded seedlings and wounds of wounded seedlings with a pipette. Within each block, one experimental entity of each wounding treatment was inoculated with sterile water. Non-wounded seedlings were also inoculated with the 5 spore concentrations and sterile water. The non-wounded treatment inoculated with sterile water served as the negative control.

Incubation conditions

Inoculation trials were incubated under three different sets of treatment conditions. These included optimal conditions for the pine seedling growth, sub-optimal conditions for the pine seedlings as well as sub-optimal conditions in combination with a fungicide treatment. In the sub-trial conducted under optimal conditions, seedlings were maintained at an average temperature of 22 °C. The seedlings were watered three times daily at eight-hour intervals for 4 minutes. In the sub-trial conducted under sub-optimal conditions, seedlings were placed under water and heat stress. In this sub-trial, seedlings were maintained after inoculation at an average temperature of 32 °C, and were watered three times daily at eight-hour intervals for 1 minute. In the sub-trial conducted under sub-optimal conditions for pine seedlings in combination with a fungicide treatment, seedlings were treated with a 24g/l benomyl drench (Runion et al., 1993; Runion and Bruck, 1988) prior to inoculation. The benomyl treated plants were maintained under the same conditions as the sub-trial conducted under sub-optimal conditions. Plants were acclimatized to the three different treatment conditions two weeks prior to inoculation.

Pathogen re-isolation

Three months after inoculation, isolations were made from small pieces of wood from the area immediately surrounding the inoculation sites. Pieces of wood were removed under near sterile conditions and plated out onto *Fusarium* Selective Media (FSM). Cultures were incubated for five days at 25 °C in white light. Isolates with white or pink mycelium were subsequently plated out on SNA and identified after 7 days under a light microscope.

Effect of spore concentration and wound age on susceptibility

Eighteen, 20-month-old potted *P. patula* saplings were randomly selected from a nursery stand of about 800 plants. All selected saplings had a minimum of 12 lateral branches. The growing tips of twelve lateral branches of each tree were severed. Branches on two saplings were inoculated immediately after wounding. Thereafter, branches of two trees were inoculated every six hours up to 48 hours after wounding.

For inoculations, fresh spore suspensions were prepared for each inoculation period. Inoculum was prepared to provide five different spore concentrations (5, 50, 500, 5000 and 50000 spores/ml) and a control (0 spores/ml). Two shoots on each sapling were inoculated with the same spore concentration, by immersing the cut shoot into the spore suspension or into the water control for 30 seconds. Trees were placed in a greenhouse at an average temperature of 28 °C and watered for 1 min, three times each day.

Plants were evaluated for symptom development after ten weeks. Lesion lengths on the inoculated shoots were measured, and re-isolations were made from tissue at two points, 1cm and 5cm behind the severed tip

Data analysis

The percentage of diseased plants was calculated for each unit of six seedlings comprising the various treatments. Mean-to-variance plots for the data demonstrated some heterogeneity of variance and thus data were normalized by arcsine transformation. A standard analysis of variance was done with the data from each of the three individual sub-trials conducted under different conditions, in order to investigate the effects of spore concentration and wounding under each different treatment condition. The statistical analyses were done using the Statistical Analysis Systems (SAS) software. Two factors (wounding and spore concentration) and one variable (mean percentage disease) were used in this analysis. Significance was tested using Tukey's T-test at the 1% significance level. The least significant difference (LSD) was calculated at a 1% significance level.

Data from the three sub-trials were combined and subjected to the same statistical tests as the data from each individual experiment. This was done in order to obtain greater resolution of the data for spore concentration and wounding due to the larger sample size. This was also done to determine how the different environmental conditions under which seedlings were grown, influenced the mean percentage disease incidence for the spore concentrations and wounding methods tested. The analysis was done with three factors (spore concentration, wounding and environmental conditions tested) and one variable (mean percentage disease incidence). Regression analysis was done on putative interactions using the Pearson correlation coefficient, and correlations were tested with the r (n) test for significance. For regressions, means from the three-factor analysis were used.

In the experiment studying the effect of spore concentration and wound age, data obtained from lesion length measurements were subjected to a standard Analysis of Variance using SAS. Significance tests were conducted using Tukey's honestly significant test at the 1% significance level. A Chi square test was done on the qualitative data (presence/absence of disease from 1cm or 5 cm behind the inoculation point) in order to determine whether differences in treatments were significant.

RESULTS:

Effect of wounding

Different wounding methods in the experiment conducted under optimal conditions for pine seedlings had an effect on mean percentage disease development. However, this effect was not significant ($P>0.01$). The highest disease incidence was found in seedlings with root wounds, followed by those with stem wounds (Fig. 1). Non-wounded and plants that had been handled roughly had comparatively similar mean percentage disease incidence (MPDI) (Fig. 1). The pathogen was reisolated consistently from the different treatments including those that had not been wounded prior to inoculation (Fig. 1). The least significant difference at a 1% significance level (LSD) for wounding method under optimal conditions was 18.2%.

Stem wounding had in the experiment conducted under water and heat stress the highest MPDI (Fig. 1), which differed significantly ($P<0.01$) from the other wounding methods. The effects of rough handling wounds and root wounds were comparatively similar (Fig. 1). Plants that were not wounded had the lowest MPDI (LSD=11.4%). In the experiment where heat and water stressed plants were treated with fungicide, differences in the MPDI between different wounding methods were not significant ($P>0.01$). Stem wounds resulted in the highest MPDI, with non-wounded plants having the least (Fig. 1) (LSD=11.7%).

Effect of spore concentration

Spore concentrations had a highly significant effect on the MPDI under optimal conditions for pine seedling growth. This is evident from standard analysis of variance (ANOVA) of the mean percentage pine seedlings from which *F. circinatum* could be reisolated for each treatment. There was no significant difference ($P>0.01$) in levels of disease development on seedlings inoculated with different spore concentrations up to 500 spores (Fig. 2). However, where 5000 and 50000 spores were inoculated onto plants, there was a highly significant increase ($P<0.01$) in mean % disease development (Fig2). The calculated LSD at a 1% significance level for spore concentration under optimal conditions was 18.3%.

In the sub-trial conducted under water and heat stress, there were highly significant differences for spore concentration. The MPDI using spore concentrations of 500, 5000 and 50 000 spores per inoculation was significantly higher ($P<0.01$) than those with 5 and 50 spores per inoculation (LSD for spore concentration under suboptimal conditions=14%). No significant differences in disease incidence between spore concentrations in the range of 500-50 000 spores per inoculation and beween treatments inoculated with 5 and 50 spores were observed (Fig. 2). The negative controls where no spores were inoculated showed no signs of disease (Fig. 2).

In the sub-trial testing the effect of water and heat stress in combination with a fungicide treatment, there were highly significant differences in the mean percentage disease obtained from inoculations with different spore concentrations ($P<0.01$). The

mean percentage disease occurrence in plants with 50 000 spores/inoculation differed significantly from treatments with 50, 5 and 0 spores/inoculation (Fig. 2). Similarly the MPDI of 500 and 5000 spores/inoculation differed significantly from that of 5 and 0 spores/treatment (Fig. 2). There were, however, no significant differences between MPDI in the treatments with 0, 5 and 50 spores per inoculation. There were also no significant differences in disease occurrence between treatments of 50, 500 and 5000 spores per inoculation (Fig. 2). Also, no significant differences could be detected between the MPDI of treaments with 500, 5000 and 50 000 spores per inoculation (LSD=11.7%).

In the experiment conducted to determine the effect of wound age on susceptibility, there were highly significant differences in lesion length for the different spore concentrations with which pine shoots were inoculated. Mean lesion lengths obtained from treatments with 50 000 and 5000 spores per inoculation differed significantly from treatments with 500, 50, 5 and 0 spores per inoculation (Table 2).

Combined effect of spore concentration, wounding and environment

When data from the three sub-trials conducted under different environmental/plant physiological states were combined in a three-factor analysis of variance, the MPDI of plants wounded at the stem was significantly higher ($P<0.01$) than that of plants with no wounds or with wounds simulating rough handling. The MPDI of stem wounded plants did not differ significantly from treatments with root wounds ($P>0.01$). The MPDI of root wounded plants did not differ significantly from all the other treatments (LSD=11.0%) (Fig. 1).

Considering the effect of spore concentrations with the three-factor combined analysis of variance, the MPDI of the negative control (0 spores per inoculation) differed significantly from all other treatments ($P<0.01$). The MPDI of plants treated with 5 spores per inoculation differed significantly from that of plants treated with 500 or more spores per inoculation. The MPDI of plants treated with 500 spores per inoculation differed significantly from that of plants treated with 5 000 or more spores per inoculation ($P<0.01$). The MPDI of plants inoculated with 5000 and 50000, however, did

not differ from each other (P>0.01). Similarly, the mean percentage disease values obtained for 500 and 50 spores per inoculation as well as 50 and 5 spores per inoculation did not differ significantly from each other (LSD=13.4%) (Fig. 2).

The MPDI of the different environmental/plant vigour states tested, differed significantly from each other (P<0.01). Plants placed under water and heat stress after inoculation had the highest MPDI (39.1%). Plants incubated at optimal environmental conditions after inoculation had a MPDI of 27.3%. Seedlings treated with benomyl had the lowest (8.0%) MPDI (LSD=6.1).

Highly significant interactions between spore concentration and wounding method were oberved. The interaction between spore concentration and MPDI for the different wounding methods could be plotted on a Michaelis-Menten function $f(x)=\mu_{max}x/(K_s+x)$. The regression for the combined data points of all four wounding methods yielded the function f(x)=44.5x/(134+x), which had a correlation coefficient of 0.894, that was significant at a 1% significance level. For the four wounding methods tested, 89% of the variation in the MPDI can thus be explained by the spore concentration. Correlations between the MPDI for the individual wounding methods and spore concentrations (Fig. 3) were significant at P<0.01, with correlation coefficients ranging from 0.8 to 0.96. The largest μ_{max} -value was observed in the Michaelis-Menten function describing stem wounds (65.5%) followed by root wounds (42.0%), rough handling wounds (38.3%) and non wounded seedlings (32.3%). The gradients of the curves describing the different wounding methods as indicated by the K_s-value, did not differ significantly from each other. This indicates, that wounding method had little effect on the rate of increase of the MPDI at increasing spore concentrations.

Highly significant interactions between spore concentration and environmental conditions/plant vigour states were oberved. These interactions become evident when plotting the Michaelis-Menten function of MPDI and spore concentration for the different environmental conditions/plant vigour states. The regression for the combined datapoints of the three environmental/plant physiological conditions yielded the function f(x)=44.5x/(134+x), which had a correlation coefficient of 0.894, that was significant at a 1% significance level. Therefore, 89% of the variation in the MPDI for the data from the three environmental conditions/plant vigour states tested can thus be explained by the

spore concentration. Correlations between spore concentrations and MPDI for the individual conditions with spore concentrations (Fig. 4) were significant at $P<0.01$, with correlation coefficients ranging from 0.92 to 0.97. The μ_{max}-value of the trial incubated under optimal conditions (64.3%) did not significantly differ from that observed in the trial conducted under water and heat stress (62.8%). The μ_{max}-value of the trial incubated under water and heat stress in combination with a fungicide treatment was significantly lower (20.3%) than that of the other two trials. The K_s-value of the experiment conducted under optimal conditions (1218.6) and that of the experiment conducted under water and heat stress in combination with a fungicide treatment (1186.7) did not differ significantly from each other, but differed significantly from that of the experiment conducted under water and heat stress without the fungicide treatment (40.8).

Effect of wound age on susceptibility

There were no significant differences in the susceptibility of shoots left for different periods of time after wounding, before inoculation ($p=0.47$). There were no significant interactions between different spore concentrations and different time intervals between wounding and inoculation ($p=0.98$). However, the Null Hypothesis that the same amount of disease would be expected from all time intervals between wounding and inoculation could be rejected when considering the scoring data of disease present/absent at 1cm/5cm from the shoot tip. The raw data obtained from this experiment clearly showed a directional decrease of disease over increasing time intervals between wounding and inoculation (Table 1). For example, the pathogen was reisolated from an average of 33% of the shoots inoculated at 0 hours after wounding. From the shoots inoculated 48 hours after wounding, the pathogen could only be isolated from 8.3% of inoculated shoots.

Discussion:

Results of this study provide strong evidence that plant vigour in a genetically non-uniform pine seedling population has a major effect on the pine seedling disease caused by *F. circinatum*. It was also evident that the type and the age of wounds affect the infection and colonisation by the pathogen. Our findings also suggest that spore concentration is an important factor in the *F. circinatum* infection process. There were also strong interactions between spore concentration and plant vigour as well as interactions between wounding method and spore concentration. These interactions could be described with the Michaelis-Menten function.

Where the effect of different wounding methods and spore concentrations were tested under optimal conditions for the pine seedlings, a small percentage of uninoculated seedlings were infected by the pathogen. *F. circinatum* is an airborne pathogen, that can be disseminated by air currents (Fraedrich and Dwinell, 1997) and splashed water (Kuhlman et al., 1982). Since all treatments were inoculated in the same greenhouse, and different treatments were placed in close proximity to each other, it is probable that disease on control plants resulted from cross-contamination from inoculated plants.

Fusarium circinatum is a wound infecting pathogen. Kuhlman et al. (1982) demonstrated natural infection by placing wounded seedlings under infected mature trees. These researchers showed that no disease was observed in the absence of wounds. Our studies have shown that *F. circinatum* can infect seedlings and cause disease in the absence of a wounding agent. We consistently reisolated *F. circinatum* from inoculated non-wounded seedlings conducted in a greenhouse in the absence of insects and nematodes. Infections arising in non-wounded plants originated in all instances from the root collar. Upon closer inspection of the root collar, we found microscopic growth cracks in the periderm, which might have served as points of infection for the fungus. A possible explanation for the occurrence of these cracks might lie in the fertilization program used in our experiments.We applied relatively high rates of potassium, phosphorous and nitrogen. Growth cracks might thus have originated in the seedlings due to rapid cell elongation that took place in response to high mineral nutrition in the absence of cell division, cell wall thickening and lignification.Other researchers have also

found, that application of high rates of mineral nutrition to pines increases pitch canker induced mortality (Fisher et al., 1981), but these studies were conducted on older pine trees.

There were highly significant interactions between wounding method and environmental conditions. Wounding had little effect under optimal conditions except for increased infection of root wounds, but had considerable effects in the experiment conducted under water and heat stress, where stem wounds were significantly more susceptible. These results are consistent with different epidemiological patterns observed in different climatic regions in the United States. Dwinell et al. (1985) suggested that mechanical damage of abiotic origin was the main driving force of pitch canker epidemics in the south-eastern United States. In contrast, no association with abiotic damage was observed in California, where insect inflicted damage plays a major role in the infection cycle of the pitch canker disease (Correll et al., 1991). Climatic conditions thus appear to be an important factor relating to the susceptibility of wounds to infection by *F. circinatum*.

Results of our study showed that infection occurred less readily where longer time intervals between wounding and inoculation were allowed to elapse. These findings are consistent with those of Barrows-Broaddus et al. (1985), who found that 1-day-old wounds are highly susceptible to colonisation by *F. circinatum*, while 7-day-old wounds were no longer susceptible. In contrast, Kuhlman (1987) found no significant differences in disease severity between plants wounded 3 and 21 days before inoculation. This can be explained, if increased susceptibility occurs only in the first hours after wounding.

In our study, spore concentrations had a significant effect on the success of inoculations. In inoculations conducted under optimal environmental conditions, there were no significant differences in disease incidence in plants inoculated with low spore loads (0-500). This result is consistent with those by Gordon et al. (1998a), who found no significant differences in lesion length between *P. radiata* clones inoculated with spore concentrations in the range of 50-140 spores. Our results showed that there are no significant differences in disease incidence in plants inoculated with high spore loads (5000-50 000). This supports findings by Hodge and Dvorak (2000), who failed to find significant differences in disease severity for spore concentrations between 50 000 and

100 000. We have however, shown that there are significant differences between the MPDIs caused by high and low spore loads. Storer et al. (1999), tested a range of spore concentrations between 25 and 1000 spores, and found significant differences between disease severity, which is consistent with the results of the present study.

Interactions between spore concentration and the MPDI of the individual environmental/plant physiological states tested were highly significant. Correlation coefficients of the Michaelis-Menten function plotted for the interactions were high, ranging from 0.92 and 0.97 showing that the Michaelis-Menten function can effectively model the effect of spore concentration on the MPDI in a genetically diverse seedling population inoculated with *F. circinatum*.

The Michaelis-Menten function of the experiments under optimal conditions and under water and heat stress did not differ significantly in their μ_{max}-values, but the K_s-value was considerably smaller for the stressed plants. Water and heat stress thus act as a competitive activator for infection. The stress conditions lowered the resistance of plants to infection. This can be seen in the higher percentage disease at lower spore concentrations as compared to infection under optimal environmental conditions. However, the maximum percentage disease occurring at higher spore concentrations still remained the same for both stressed and non-stressed plants. Gordon et al. (1998b) suggested that pitch canker of pine is primarily controlled by the interaction between the host and the pathogen, and less so by the environmental conditions. The fact, that 89% of the variation in our experiments can be accounted for by spore concentation supports this view. Nevertheless, the significant differences between the gradients of the Michaelis-Menten function plotted for the interaction between spore concentration and environment prooves that the interaction between the pathogen and the host is also strongly influenced by the environment. This finding is supported by observations made by Dwinell et al. (1985) who showed that various stress factors such as moisture deficiency may predispose pine trees to infection by the pitch canker fungus.

Our results showed that fungicide treatment can act as an uncompetitive inhibitor to infection, since the benomyl treatment lowered the μ_{max}-value of the Michaelis-Menten curve considerably, when compared with the other two sub-trials, while the K_s-value is similar to that of the experiment conducted under optimal conditions. Benomyl is a

systemic residual fungicide and its application effectively lowered the possible sites for infection. This resulted in a lowering of the percentage disease inflicted. The fungicide also appeared to counteract the decreased seedling resistance under stress conditions. The low K_s-value shows that at lower spore concentrations the stressed plants have a similar resistance to fungal attack, than that of plants grown under optimal environmental conditions. Although reduction in disease was significant due to fungicide application, disease incidence was still sufficiently high to have an economic impact in a commercial situation.

The Michaelis-Menten function has not been used previously to model the effect of fungal spore concentration on disease incidence in plant pathology. Nonetheless, in our study, the function was useful in modelling various responses of a genetically non-uniform seedling population confronted with a pathogen. We believe, that modelling plant disease development with the Michaelis-Menten function might be usefull in determinig the optimal spore concentration in pathogenicity trials conducted under different abiotic conditions. The function might also be useful in determining the importance of different environmental conditions on pathogen attack and might, therefore, aid in devising disease management programmes.

Acknowledgements:

We are grateful to the members of the Tree Pathology Co-operative Programme (TPCP), the National Research Foundation (NRF) and the TRIP support programme of the Department of Trade and Industry for finantial support. We also thank L.P. Wright for his assistance in analyzing the data of this work, as well as A.E. Hammerbacher for her assistance in cultivating the plants for this study.

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Table 1: Inoculations on *P. patula* tree branch tips with specific time lapses between wounding and inoculation. Values are the means of the different results obtained for the spore concentrations between 0 and 50 000 spore

Time intervals between wounding and inoculation	Mean lesion length (cm)	Percentage branches infected with *F. circinatum*	
		1cm Behind point of inoculation	5cm Behind point of inoculation
0 HRS	4.42	29	38
6 HRS	5.33	33	25
12 HRS	3.08	33	25
18 HRS	3.96	8	17
24 HRS	3.46	25	21
30 HRS	4.46	17	8
36 HRS	2.42	17	21
42 HRS	3.54	17	4
48 HRS	2.08	8	4

Table 2: Reisolation of *F. circinatum* from *P. patula* branches at 1cm and 5cm behind the point of inoculation. Table values are the means of the results of all time intervals tested between 0 and 48 hours

		Percentage branches infected with *F. circinatum*	
Spore concentrations	**Mean lesion length (cm)**	**1cm behind point of inoculation**	**5cm behind point of inoculation**
0 SPORES	**0.28**	**0**	**0**
5 SPORES	**0.89**	**3**	**3**
50 SPORES	**0.92**	**0**	**0**
500 SPORES	**2.47**	**14**	**14**
5 000 SPORES	**6.78**	**41**	**28**
50 000 SPORES	**10.75**	**58**	**58**

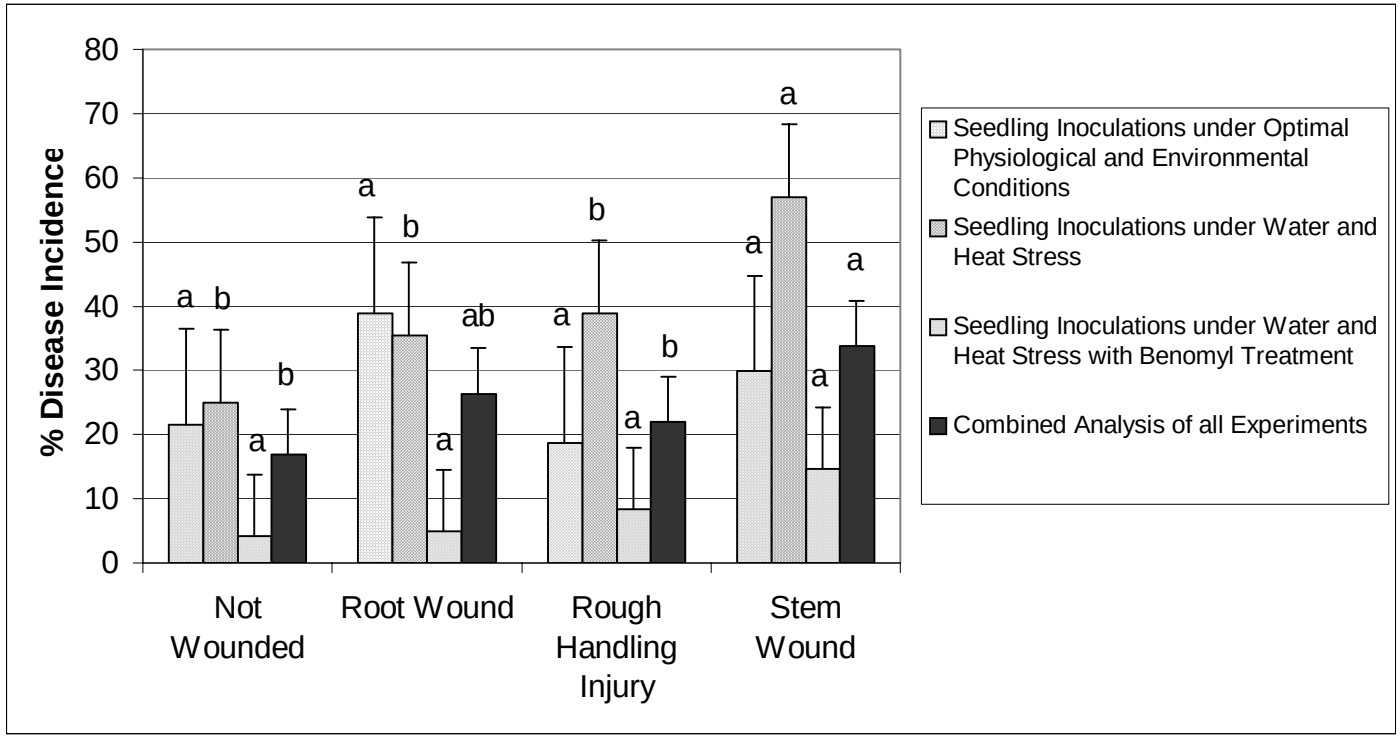

Fig 1: Mean percentage disease incidence of pine seedlings that were wounded prior to inoculation. Error bars represent the Least Significant Difference at a 1% significance level (Tukey's T-method). Lower caste letters represent statistical difference calculated by using Tukey's T-method at a 1% significance level.

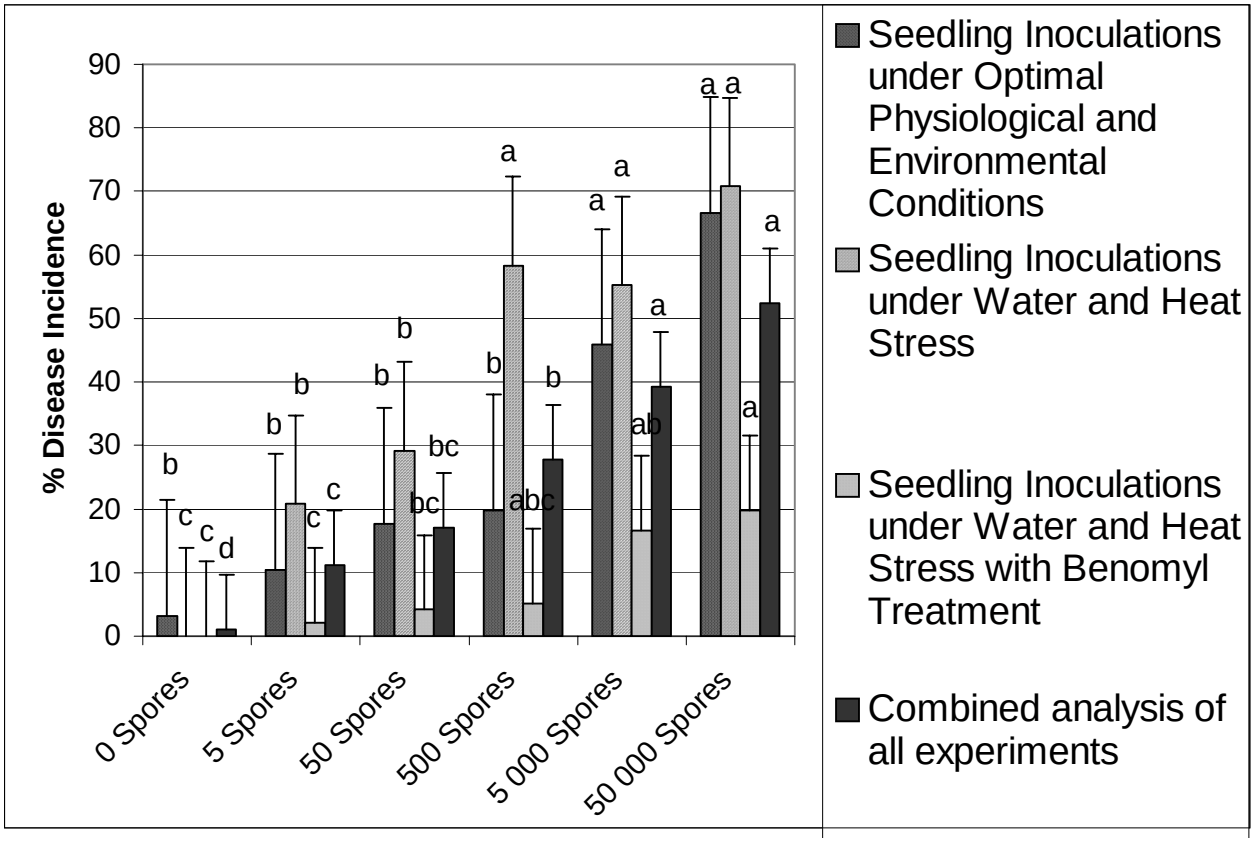

Fig 2: Mean percentage disease incidence of pine seedlings inoculated with 0, 5,50, 500, 5 000 and 50 000 spores. The error bars represent the Least Significant Difference at a 1% significance level (Tukey's T-method). Lower case letters above bars represent statistical differences calculated using Tukeys T-method at a 1% significance level for each individual treatment.

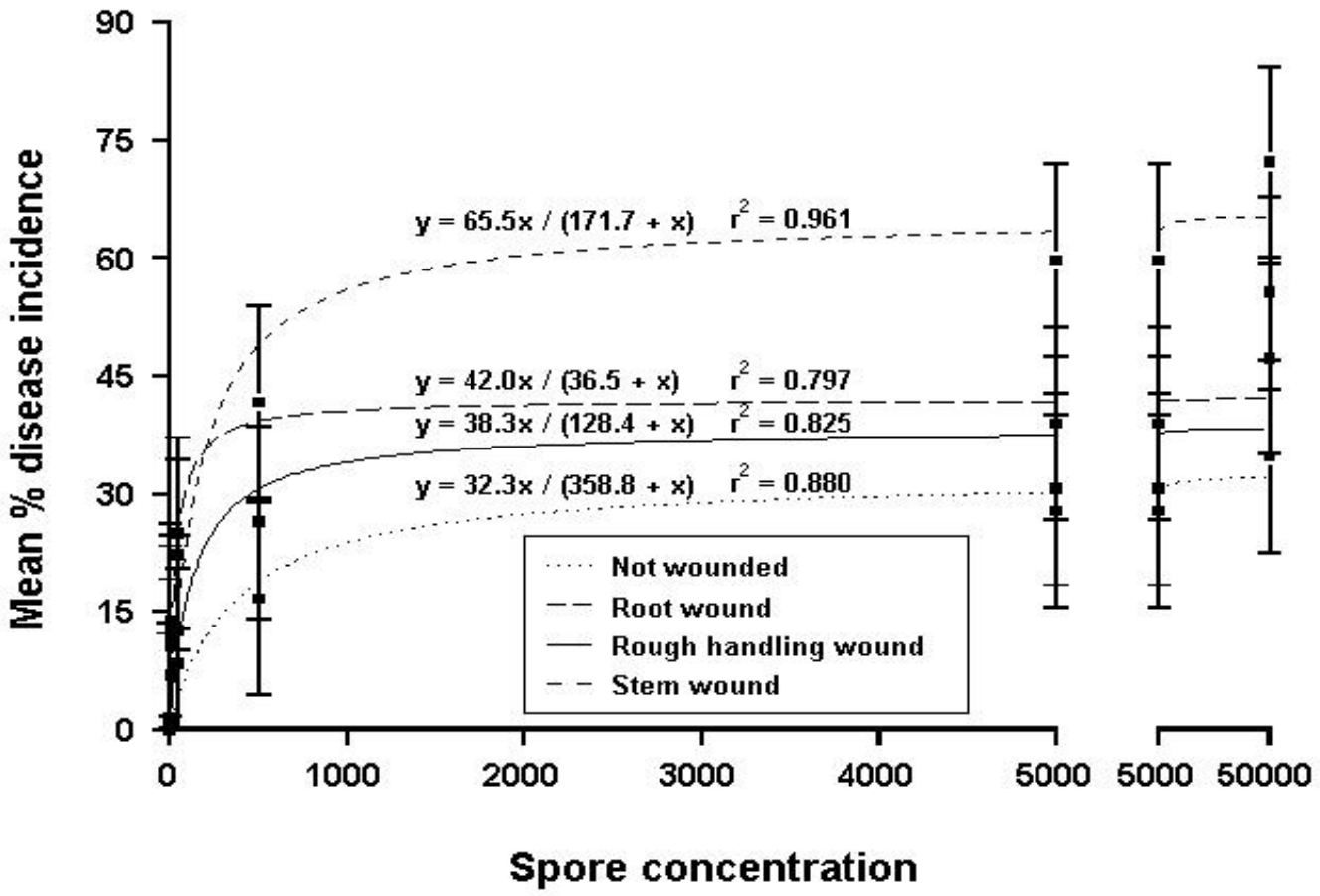

Fig 3: Graph showing the correlations of mean percentage disease incidence with spore concentrations for different wounding methods. Data from the different sub-trials were combined. Error bars represent the LSD.

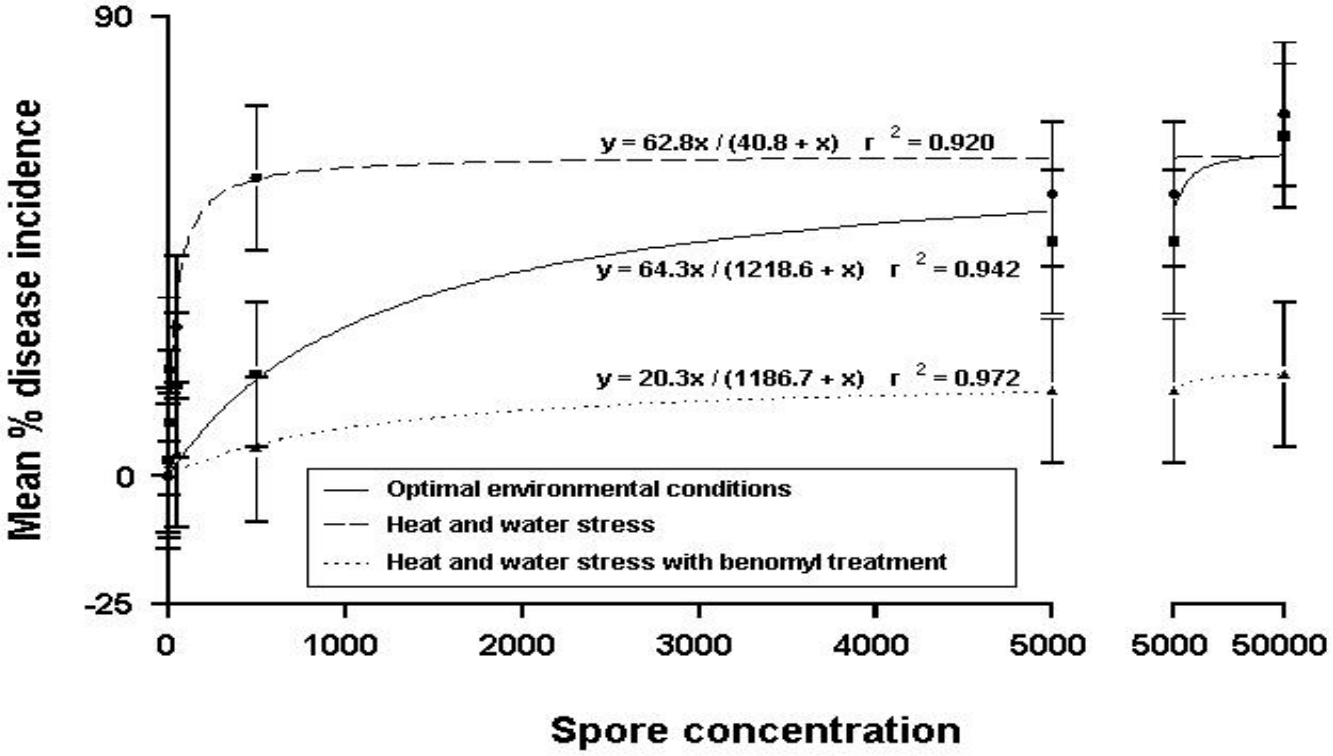

Fig 5: Graph showing the correlations of the mean percentage disease incidence with spore concentration for different environmental conditions and plant vigour states. Data from the different sub-trials were combined. Error bars represent the LSD.

3

Influence of ambient temperature and humidity on infection by *Fusarium circinatum* in South African pine nurseries

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Abstract: *Fusarium circinatum* is an economically important pathogen of pine seedlings and vegetatively propagated plants in South Africa. The fungus causes root disease of seedlings in nurseries as well as of plants during establishment of pine plantations. Despite it's importance little is known regarding the biology of *F. circinatum* in South Africa. The aim of this study was thus to consider the effect of ambient temperature and humidity on disease development and symptom expression. A survey of *F. circinatum* seedling disease incidence was conducted in three commercial pine seedling nurseries. Disease incidence was correlated with the ambient temperature and humidity in the nurseries. Results showed that humidity had no quantifyable effect on *F. circinatum* infection. There was an indication that temperature and seasonal changes had an effect on the infection process, but this effect was not statistically significant.

INTRODUCTION

Pitch canker, caused by *Fusarium circinatum* Nirenberg and O'Donnell (teleomorph *Gibberella circinata* Nirenberg and O'Donnell) (Nirenberg & O'Donnell, 1998), is one of the most important diseases of *Pinus* spp. The pathogen has been reported to infect 47 *Pinus* spp. (Dick, 1998) in at least 6 countries. In countries where *Pinus* spp. are native, such as the United States of America (Hepting & Roth, 1946; McCain et al., 1987), Haiti (Hepting & Roth, 1953), Mexico (Santos & Tovar, 1991) and Japan (Muramoto & Dwinell, 1990), symptoms commonly include stem, bole and branch cankers on mature trees (Dwinell et al., 1985) as well as shoot dieback (Correll et al., 1991), dieback of female flowers and mature cones (Barrows-Broaddus, 1990), reduced germination of seeds (Huang & Kuhlman, 1990) and pine seedling mortality (Huang & Kuhlman, 1990; Carey & Kelly, 1994). In countries where *Pinus* spp. are exotic, such as South Africa and Chile, the fungus does not cause a stem canker disease on mature trees but rather causes a serious root disease on seedlings and cuttings in nurseries (Viljoen et al., 1995; Viljoen et al., 1994; Wingfield et al., 2002).

In nurseries, *F. circinatum* causes both pre- and post emergence damping off (Huang & Kuhlman, 1990: Viljoen et al, 1994), as well as mortality of established plants (Carey & Kelly, 1994; Viljoen et al, 1994). Pre-emergence damping off occurs usually due to high inoculum densities, which can result from contaminated seed (Viljoen et al., 1994). Post-emergence damping off occurs when root collars are girdled. Infection of established seedlings results in chlorosis of the needles and wilting of the seedling tips (Viljoen et al., 1994). Pitch soaked lesions usually occur at or near the root collar, but are occasionally found in the region of the cotyledonary node (Barnard & Blakeslee, 1980) or in the region of the bud (Carey & Kelley, 1994). The pitch canker pathogen has also been reported to cause severe seedling root rot. Viljoen et al. (1994) reported that established seedlings infected with the pitch canker pathogen had underdeveloped roots with multiple pitch soaked lesions.

In South Africa root rot caused by *F. circinatum* is most common on *Pinus patula* seedlings (Viljoen et al., 1994). The disease was first detected in the early 1990s in a single forestry nursery. Since then, it has spread throughout the country. The severity of

the disease varies between nurseries, and ranges from very low incidences to almost epidemic levels. The disease also causes seedling mortality at varying degrees of severity, shortly after they are planted out into plantations. This is mainly due to the fact that seedlings may harbour the pathogen in the absence of symptoms and thus seedling infections may remain undetected (Storer et al., 1998).

Temperature and humidity are known to have an effect on the infection rate and expression of disease caused by *F. circinatum* (Gordon et al., 2001). Seasonal temperature and humidity fluctuations have been reported to affect symptom expression (Dwinell et al., 1985). In the south eastern United States, symptom expression begins in autumn and continues through winter and spring (Dwinell et al., 1985). In California, branch tip cankers have been found to progress faster in spring inoculations, than in autumn inoculations (Correll et al., 1991). The rate of lesion expansion has been shown to increase in controlled environments with average temperatures between 14 ºC and 26 ºC (Gordon et al., 1998). In addition Gordon et al. (1998) have suggested that high ambient humidity is advantageous to the pitch canker fungus. However, the extent to which temperature and humidity contributes to the development of pitch canker remains unclear.

Very little is known regarding the biology of *F. circinatum* in South African nurseries. The aim of this study was, therefore, to consider the effect of ambient temperature and humidity on the development of seedling disease caused by *F. circinatum* in local nurseries.

MATERIALS AND METHODS

Nurseries

Three commercial pine-growing nurseries were selected for this study. These included the following: a nursery near Sabie (25° 10' S; 30° 48' E), in Mpumalanga Province situated in a temperate, high altitude area, where temperatures rarely drop below zero or rise above 34 °C. A nursery near Nelspruit (25° 29' S; 30° 59' E), in Mpumalanga Province situated in a sub-tropical climatic zone characterized by high humidity, where

temperature extremes range between 4 °C and 38 °C and a nursery near Pietermaritzburg (29° 35' S; 30° 25' E), situated in the coastal area of Kwazulu Natal with a temperate climate with high humidity and warm temperatures ranging on average between 8 °C and 39 °C in summer. Severe outbreaks of seedling disease caused by *F. circinatum* infection have been reported to occur in all three nurseries.

Sampling and climate monitoring

At each nursery, randomly assigned blocks of approximately 2000 seedlings were selected. These represented all the different ages of seedlings present in the nurseries. All seedlings showing signs of infection by *F. circinatum* were removed from the selected block, every two weeks for ten months. These plants were transported to the laboratory, where pieces of symptomatic wood were removed under near sterile conditions and plated out onto *Fusarium* Selective Media (Nash and Snyder, 1962). Cultures were incubated for five days at 25 °C in white light. Isolates with white or pink mycelium were subsequently placed on Synthetic Nutrient Agar (Nirenberg and O'Donnell, 1998) and identified after 7 days using a light microscope to confirm the presence of the pathogen. The percentages of plants (from a total of 2000 plants), yielding cultures of *F. circinatum* were calculated for each seedling age group within each nursery.

Temperature and humidity were measured hourly at the nurseries using Hobo Centre™ (Onset Computer Corporation, Bourne, MA) data loggers. The data loggers were positioned approximately 2 m above soil level in the nurseries under a Styrofoam roof. Mean temperature and humidity was calculated for each sampling period (approximately two weeks).

Data analysis

The mean temperature and relative humidity calculated for each sampling period were correlated with the percentage of plants of each seedling age group from which *F. circinatum* was isolated (PDI) within the sampling period, using Principal Components Analysis (PCA). Two to three principal components from the correlation matrix were retained. Factor pattern matrices were rotated with the VARIMAX procedure. The correlations of retained principal components and the actual data were tested with the regression procedure of SAS using the Pearson correlation coefficient. The p-value and R^2-value for each model was calculated. p-values were also calculated for the parameter estimates of the regression functions.

RESULTS

Principal Component Analysis

In a principal component analysis on the temperature and disease incidence data for plants aged 3 to 7 months, two principal components (PC's) were retained. These described 68% and 75% of the variation in the data for the Sabie and Nelspruit nursery respectvely. Three PC's were retained for the Pietermaritzburg nursery, which described 82% of the variation in the sampling data. No PC's describing any significant percentage of the variation in the data could be extracted from the correlations between relative humidity and disease incidence data for all three nurseries included in this study. Humidity, therefore, had no effect on the variation in percentage disease incidence (PDI).

For the Sabie nursery the factor pattern matrix before rotation and after rotation (VARIMAX) showed that PC 1 (Fig. 1) described the variance of PDI of pine seedlings

aged 3 and 5 months. PC 2 (Fig. 1) had high loadings for seedlings aged 4 and 6 months. PC 1 and PC 2 did not describe the variance of PDI of plants aged 7 months.

The factor pattern matrix for the Nelspruit nursery showed that PC 1 (Fig. 2) described the variance of the PDI of pine seedlings aged 3 to 6 months. PC 2 (Fig. 2) described the variance of PDI of pine seedlings aged 7 months. After rotation, PC 1 had high loadings for plants aged 3 to 5 months and PC 2 had high loadings for plants aged 6 to 7 months.

The factor pattern matrix for Pietermaritzburg nursery before and after rotation showed that PC 1 (Fig. 3) described the variance of PDI of pine seedlings aged 4 to 5 months. PC 2 (Fig. 3) had high loadings for seedlings aged 7 months. PC 3 (Fig. 3) had high loadings for plants aged 6 months. None of the PCs could adequately describe the variance in the mean percentage disease incidence of plants aged 3 months.

Verification of model

Regression analysis for the disease incidence data at the three nurseries was done with PC 1 and PC 2 (and PC 3 for the Pietermaritzburg nursery) as the dependant variables and temperature as the independent variable (Figs. 1 - 3). The calculated p-value for the F test as well as the p-values for the Students t-tests done for the parameter estimates were non-significant. The correlation coefficients for the functions plotted for the PCs were small. The PCs extracted from the data obtained from the three nurseries surveyed are thus unreliable estimates and describe only a small fraction of the variation in the data sampled.

A regression analysis for all three nurseries with the PDI of each individual sampling event as the dependant variable and temperature as the independent variable had correlation coefficients that were not significant. The plotted regression functions showed that disease occurred in the Sabie nursery between 9 °C and 24 °C (Fig. 1). Disease in the Nelspruit nursery occurred between 12 °C and 26 °C (Fig. 2), and in the Pietermaritzburg nursery, disease occurred within the temperature range of 10 °C and 25 °C (Fig. 3).

There were seasonal trends in the PDI at the Sabie and Nelspruit nurseries (Fig. 4). The PDI at the Sabie nursery increased sharply from October 2002 to November 2002. From November 2002 to February 2003 the mean percentage disease decreased sharply. The mean percentage disease remained fairly constant between February 2003 to July 2003. The PDI at the Nelspruit nursery increased between October 2002 until December 2002. Between December 2002 and January 2002 the PDI decreased very rapidly. The PDI at the Nelspruit decreased constantly between January 2003 to July 2003. There were no clear seasonal trends in the mean percentage disease data for the Pietermaritzburg nursery, except for a small increase in disease over February to April 2003 (Fig. 4).

DISCUSSION

This study represents the first attempt to analyze the effect of environmental conditions on the impact of infection of pine seedlings by *F. circinatum* in South African nurseries. Although the results were not conclusive in terms of statistical significance, there was an indication that temperature and seasonal changes have an effect on PDI in pine seedling nurseries. In contrast, relative humidity, had no quantifyable effect on PDI.

The average percentage disease incidence at the three nurseries having different overall environments was low during the 10 months in which this study was undertaken. The low disease incidence is likely to have influenced the low resolution of the data. Normal nursery practices such as removal of dying plants and chemical disease control continued throughout the survey period. This probably also reduced our ablity to model the interactions between climate and PDI.

We were not able to use PCA to identify any principal components that accounted for a large fraction of the variation in the data from the three nurseries. The regression analysis with the functions of the extracted principal components showed that none of the PCs accounted for the variation in the data. However, our analyses did provide an indication that temperature may contribute to *F. circinatum* infection. While we were not able to quantify the importance of this abiotic factor, temperature ranges in which we

encountered the disease were similar to those reported by Gordon et al. (1998). The disease thus appears to be most severe when temperatures range from 14 °C to 26 °C.

Gordon et al. (2001) suggested that high ambient humidity might be advantageous to the pitch canker fungus. These authors observed that plants are more severely affected by the pathogen in coastal regions than in inland regions in California. Our results show that the effect of relative humidity on infection and colonisation of the pitch canker fungus is negligible under nursery conditions. We found no correlation in our principal component analysis.This difference might be due to the fact that climatic conditions in nurseries are very different to those in California, which has a mediterranean climate, and where Gordon et al. (2001) conducted their study. Clearly, conditions in nurseries and associated with nursery plants where there is a continuous high humidity, are very different to those found in stands of mature trees, and comparison of our results and those of Gordon et al. (2001) might be futile.

Results of this study illustrated trends in the seasonal occurrence in PDI in two of the three nurseries surveyed. The PDI at the Sabie and Nelspruit nurseries increased sharply in late spring and into early summer. The mean percentage disease decreased over the rest of the season and reached a minimum in mid-winter. Seasonal trends of *F. circinatum* disease expression have also been observed in the United States (Dwinell et al., 1985; Corell et al., 1991). In the south-eastern USA, symptom expression associated with *F. circinatum* begins in autumn and continues through winter and spring (Dwinell et al., 1985). In contrast, branch tip cankers in California were observed to progress faster in spring than in autumn (Correll et al., 1991).

In this study, we attempted to elucidate the role of temperature and humidity on *F. circinatum* seedling disease. The resolution of the overall data was low, but we were able to show that temperature had some effect on the infection biology of *F. circinatum*. In order to clarify the role of ambient conditions on the infection and colonization of pine seedlings by *F. circinatum*, studies in controlled environments under a gradient of conditions will need to be undertaken.

ACKNOWLEDGEMENTS:

We are grateful to the owners and managers of the three nurseries for their assistance in conducting this research. In this regard we would especially like to thank Gail Allwood, Piet Bredenkamp, Eric Msomi and Richard Parker. We are also grateful to Dr Ben Eisenberg for his assistance with the analysis of the data. We also acknowledge the financial support of the members of the Tree Protection Co-operative Programme (TPCP), the National Research Foundation (NRF) and the THRIP initiative of the Department of Trade and Industry for financial support.

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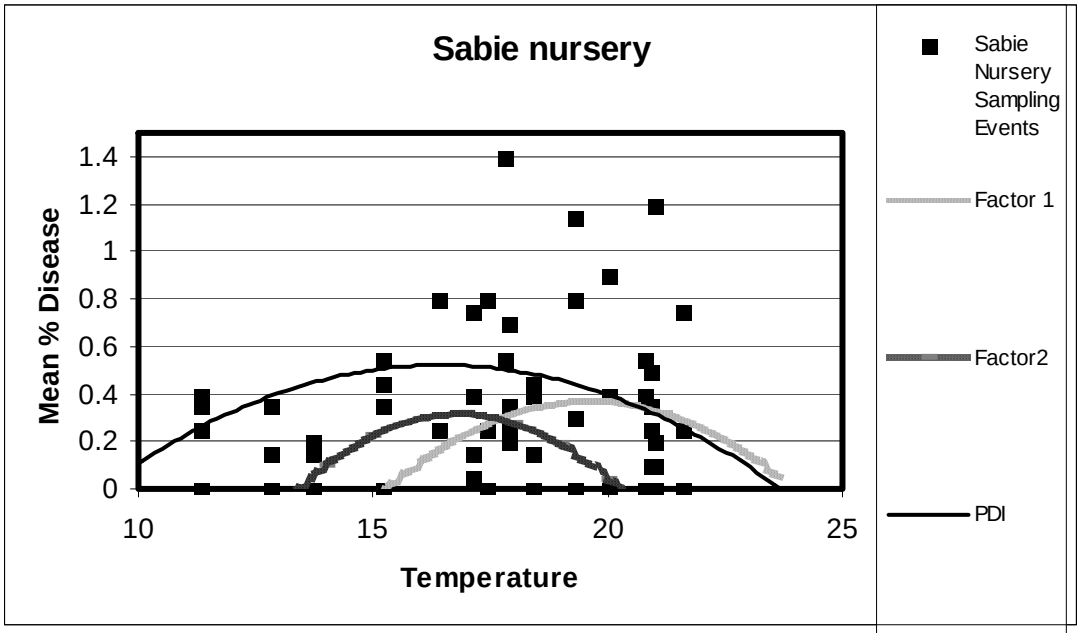

Fig 1: Mean percentage disease incidence of pine seedlings of the Sabie nursery grown under field conditions at different mean temperatures (°C). The y-axis represents the mean percentage disease incidence. The x-axis represents the mean temperatures over the sampling period. Each data point represents a sampling event. The regression functions represent the percentage disease incidence (PDI) vs. temperature of the Sabie nursery ($y=-0.02719x^2+0.3273x-2.1702$; $R^2=0.0637$), factor 1 that was extracted by PCA ($y=-0.01974x^2+0.77392x-7.23085$; $R^2=0.3074$) and factor 2 that was extracted by PCA ($y=-0.02719x^2+0.19621x-7.39391$; $R^2=0.1338$).

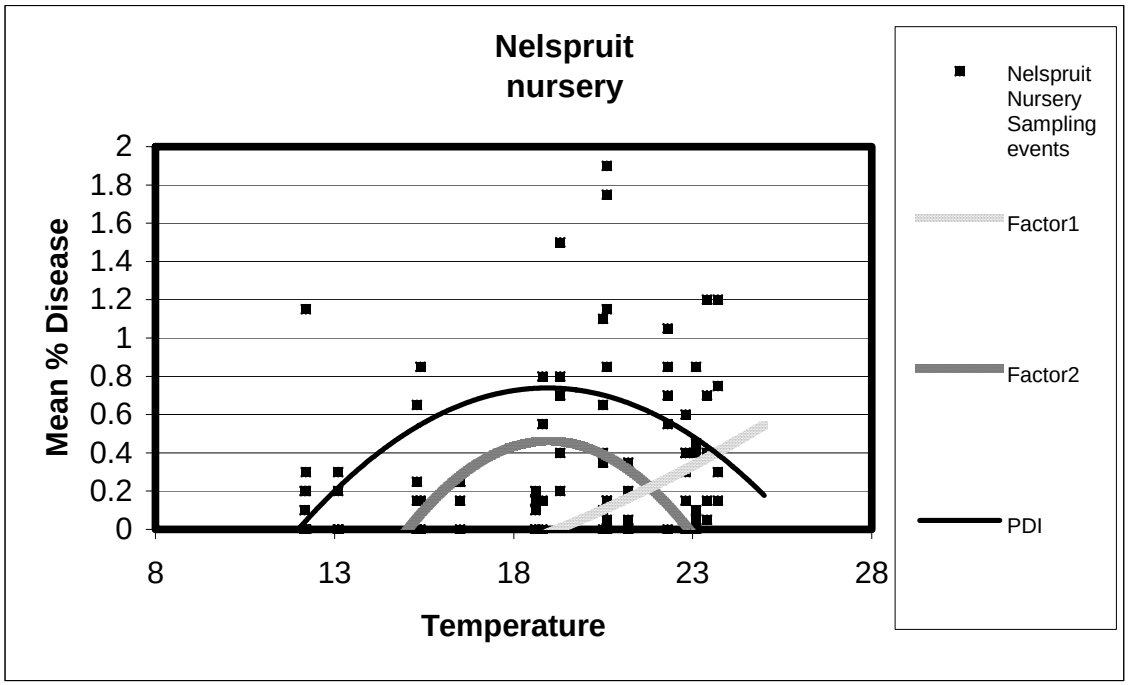

Fig 2: Mean percentage disease incidence of pine seedlings of the Nelspruit nursery grown under field conditions at different mean temperatures (°C). The y-axis represents the mean % disease incidence. The x-axis represents the mean temperatures over the sampling period. Each data point represents a sampling event. The functions represent the regression function of percentage disease incidence (PDI) vs. temperature of the Nelspruit nursery ($y=-0.0153x^2+0.5792x-4.7454$; $R^2=0.0814$), factor 1 that was extracted by PCA ($y=0.00328x^2-0.05072x-0.23624$; $R^2=0.0718$) and factor 2 that was extracted by PCA ($y=-0.03003x^2+1.13935x-10.34513$; $R^2=0.2087$).

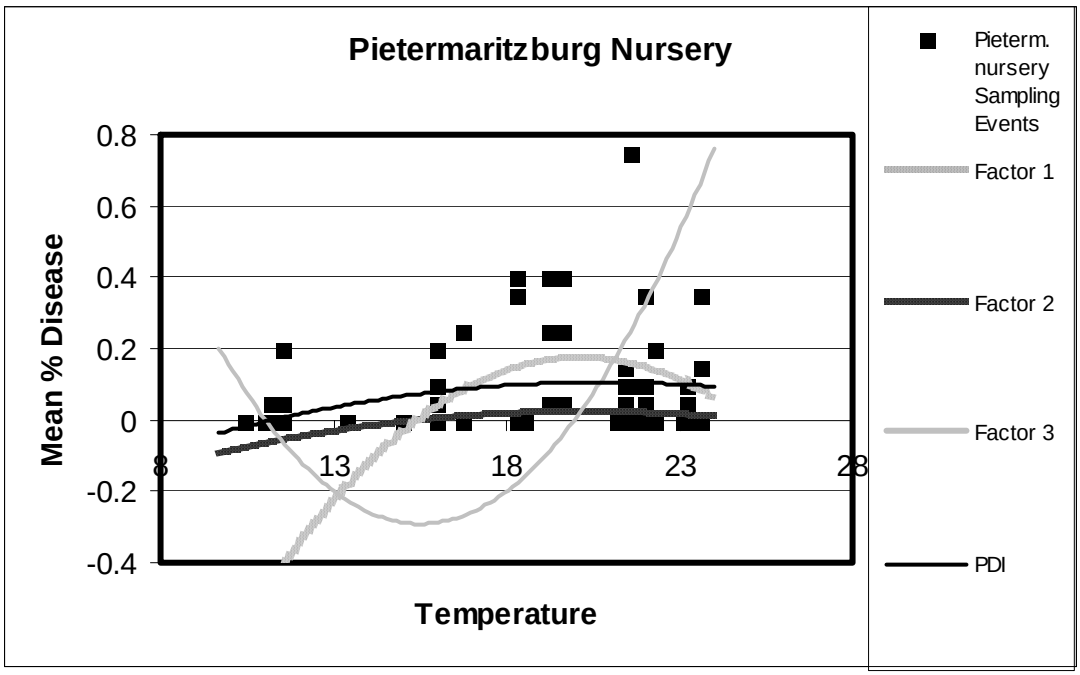

Fig 3: Mean percentage disease incidence of pine seedlings of the Pietermaritzburg nursery grown under field conditions at different mean temperatures (°C). The y-axis represents the mean percentage disease incidence. The x-axis represents the mean temperatures over the sampling period. Each data point represents a sampling event. The functions represent the regression function of percentage disease incidence (PDI) vs. temperature of the Pietermaritzburg nursery ($y=-0.0012x^2+0.0484x-0.3967$; $R^2=0.0339$), factor 1 that was extracted by PCA ($y=-0.0078x^2+0.31506x-3.00326$; $R^2=0.0605$), factor 2 that was extracted by PCA ($y=-0.00111x^2+0.04344x-0.40054$; $R^2=0.0009$) and factor 3 that was extracted by PCA ($y=0.01387x^2-0.44505+3.22088$; $R^2=0.0643$).

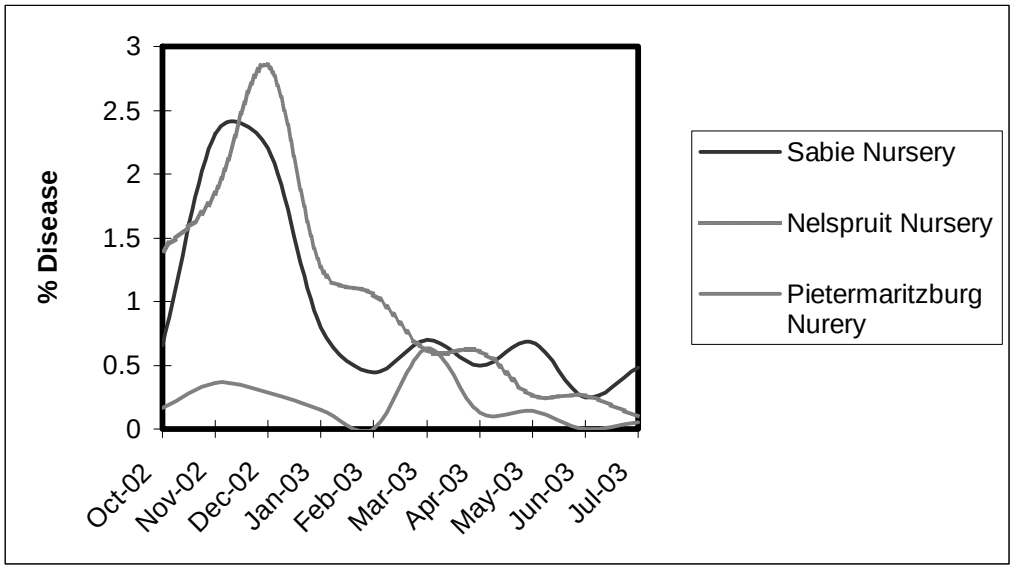

Fig 4: Seasonal dynamics of absolute percentage disease incidence of pine seedlings at three different nurseries over a period of 10 months.

4

Saprophytic Survival of *Fusarium circinatum* in Soil and Organic Matter

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Abstract: *Fusarium circinatum* is the causal agent of pitch canker of pine. This fungus is a primary pathogen but it can also survive saprophytically. However, little is known regarding the saprophytic survival of *F. circinatum*. Understanding the ability of this fungus to survive saprophytically is essential if this disease is to be effectively managed, particularly in pine nurseries. The objective of this study was to characterize the saprophytic growth and survival as well as nitrogen mineralisation and immobilization of *F. circinatum* in sterile soil and organic matter at different moisture levels and temperatures. Growth and survival as well as nitrogen mineralisation and immobilization was optimal in substrates containing high mineral nutrient concentrations. Saprophytic fungal activity was favoured by low temperatures. Differences in moisture content had the least effect on the saprophytic survival of the fungus, but there was an indication that 40%-80% of the substrate water holding capacity was optimal for fungal growth. Results suggest that *F. circinatum* can grow prolifically in sterile soil containing organic matter and can survive for long periods of time, especially under cool conditions.

INTRODUCTION

Fusarium circinatum Nirenberg and O'Donnell (teleomorph *Gibberella circinata* Nirenberg and O'Donnell) (Nirenberg & O'Donnell, 1998), is the causal agent of pitch canker, an economically important disease of pine trees. Pitch canker has been reported from many parts of the world, including the United States (Hepting & Roth, 1946; McCain et al., 1987), Haiti (Hepting & Roth, 1953), Mexico (Santos & Tovar, 1991) and Japan (Muramoto & Dwinell, 1990). In these regions the disease commonly causes resin soaked cankers on the trunks and lateral branches of diseased pine trees (Dwinell et al., 1985), shoot dieback (Correll et al., 1991), dieback of female flowers and mature cones (Barrows-Broaddus, 1990), reduced germination of seeds (Huang & Kuhlman, 1990), as well as pine seedling mortality (Huang & Kuhlman, 1990; Carey & Kelly, 1994). In South Africa and Chile, *F. circinatum* causes a severe seedling disease (Viljoen et al., 1995; Viljoen et al., 1994; Wingfield et al., 2002). Typical pitch canker symptoms on older trees have not been observed in the latter countries, where plantations of non-native *Pinus* spp. have been established for timber and pulp production (Viljoen et al., 1994; Wingfield et al., 2002).

The saprophytic growth and survival of *F. circinatum* is not well understood. The fungus is known to survive in soil and nursery growth media (Viljoen et al., 1994) and has been reported to act as a typical soil inhabitant and root-infecting pathogen (Dwinell & Barrows-Broaddus, 1978; Dwinell et al., 1985). *F. circinatum* is also able to survive in infected dead branches and wood chips for up to three years (Mc Nee et al., 2002). Climatic conditions seem to be important for survival of *F. circinatum*, since survival of the fungus is higher in wood stored in cool rather than hot areas (Mc Nee et al., 2002).

During saprophytic microbial activity in soil, organic matter is broken down and nitrogen is mineralised or immobilized by soil microbes (Paul & Clark, 1989). Mineralisation or ammonification of organic nitrogen refers to the degradation of proteins, amino sugars and nucleic acids to soluble ammonium. Nitrogen immobilization involves incorporation of ammonium into microbial biomass. Whether ammonium is immobilized or accumulates in the soil depends on the requirement for nitrogen and carbon for growth of specific micro organisms (Paul & Clark, 1989). The extent of

immobilization and mineralisation by soil microbes is strongly dependant on the C:N ratio of the soil organic matter, as well as on the carbon and nitrogen requirements of the saprophytic soil microbes. Soil microbes with high carbon requirements mineralise nitrogen more readily than soil microbes with high nitrogen requirements (De Neve & Hofman, 1996).

Since the pitch canker fungus has not yet been observed to cause mortality of older trees in countries such as South Africa and Chile, it is important to understand the saprophytic ecology of the fungus particularly as it applies to nurseries. Such knowledge will serve to prevent the spread of the pathogen from nurseries into pine plantations. In this way, it might be possible to avoid epidemics similar to those observed in California (Gordon et al., 2001) and in the southeastern United States (Dwinell et al., 1985). Knowledge regarding the survival of *F. circinatum* in growth media and seedbeds is also important for developing effective management strategies for seedling disease caused by *F. circinatum*.

The objective of this study was to assess the saprophytic growth and survival of *F. circinatum* in artificial planting medium. In addition we considered nitrogen mineralisation and immobilization by the pathogen in soil and organic matter under different moisture concentrations and temperatures.

MATERIALS AND METHODS

Saprophytic growth and survival

Preparation of growth medium

Saprophytic survival and growth of *F. circinatum* was tested using four different growth media or substrates. These included composted bark, typical of the growth medium used for commercial pine seedling production, *Pinus patula* wood chips with an average volume of 5mm^3, typical red topsoil obtained from non-cultivated South African

grassland (veld) and red subsoil obtained from 1m under the veld surface, and which was poor in organic matter.

The water retention of each substrate was measured by moistening 100g of dried substrate with 200ml water. The substrate-water mixture was then filtered for 24 hours in a cool place. The volume of filtrate was measured, and the water retention capacity of the substrate was calculated by deducting the amount of filtrate from the original 200ml water added to the substrate.

Substrates (250g dry weight) were weighed and placed in 500ml Erlenmeyer flasks, which were closed with thick wads of cotton wool and aluminium foils. Substrates in Erlenmeyer flasks were autoclaved twice with a 14-day interval. Substrates were moistened with distilled sterile water up to 20%, 40%, 60% or 80% of their water retention capacity. The volume of *F. circinatum* spore suspension, which was to be added later, was deducted from this volume.

Inoculation of substrates and incubation

A spore and mycelium suspension was prepared by scraping vegetative growth from the surface of ten-day old *F. circinatum* cultures (Isolate no: MRC 6213 maintained in the Forestry and Agricultural Biotechnology Institute Culture Collection and the culture collection of the Medical Research Council (Tygerberg, South Africa) grown on Potato Dextrose Agar (PDA). The resultant fungal mycelium and spore material was placed in a beaker with sterile, distilled water and stirred continuously for 30 minutes. The number of propagules present, was counted using a haemocytometer. Haemocytometer counts were also verified by performing a dilution series on PDA plates. In this way the number of viable propagules could be measured with a high degree of accuracy.

Substrates were inoculated with the spore/mycelium suspension. A spore suspension containing 100 000 propagules per gram of soil was used. A control flask was prepared for each treatment using sterile substrate and water. Erlenmeyer flasks were incubated after inoculation at 15ºC (±2ºC), 25ºC (±2ºC) and 35ºC (±2ºC), respectively. All treatments were done in triplicate.

Assessment of survival and data analysis

A dilution series was prepared for each treatment replica and for the control flasks every 28 days for 15 months. A volume of 1cm³ substrate was removed from each treatment flask and mixed with 9ml sterile water using a vortex mixer. Dilution series were prepared to 10^{-6}. A volume of 0.1ml of each dilution was plated onto Fusarium Selective Medium (FSM) (Nash and Snyder, 1962) and incubated for four days at 25ºC in the dark. Colonies were counted after four days incubation and from these data, the viable population numbers in the treatment flasks were calculated in colony forming units (cfu) per gram of substrate. Colony identity was verified by growing 0.01%-1% of colonies obtained on Synthetic Nutrient Agar (SNA) (Nirenberg and O'Donnell, 1998) after which they were identified based on morphological characteristics.

A standard analysis of variance was done using the Statistical Analysis Systems (SAS) software with the colony count data from each of the different treatment conditions. Four factors (substrate, water content, incubation time and incubation temperature) and one variable (colony forming units - cfu) were used in the analysis. Significance was tested using Tukey's T-test at the 5% significance level. The least significant difference (LSD) was calculated at a 5% significance level. The individual factors were tested for their significance including the means of all factors studied.

***Fusarium circinatum* activity in soil**

Preparation of growth medium

Subsoil (50g) which was amended with 10%, 5% or 0% dry weight organic matter (Kikuyu grass cuttings) was weighed and placed into 250ml Erlenmeyer flasks. Erlenmeyer flasks were closed with thick wads of cotton wool and silver foil prior to autoclaving. The water retention capacity of the soil was determined as described previously. Soil in Erlenmeyer flasks was moistened to 20%, 50% or 80% of its water

retention capacity, with sterile distilled water. The amount of liquid needed for inoculation with a spore suspension was deducted from the amount of water added to flasks.

Inoculation of substrates and incubation

A spore and mycelium suspension was prepared as described above using the same isolate as noted previously. The spore concentration was adjusted to 100 000 propagules/ml. Five millilitre of spore suspension was inoculated into each treatment flask and mixed. Control flasks were prepared for each treatment using sterile soil and water. Erlenmeyer flasks for all the treatments and controls were incubated at 15ºC (±2ºC), 25ºC (±2ºC) and 35ºC (±2ºC) respectively. After inoculation, flasks were incubated for either 1, 3, 7 or 14 days after inoculation.

Ammonium measurements and data analysis

After incubation, soluble ammonium was extracted from the inoculated soil and uninoculated controls using potassium chloride (KCl) (Keeney and Nelson, 1982). Soil (50g) from treatment flasks was mixed with 100ml of 1M KCl solution and shaken for 30 minutes. The solution was then filtered and the ammonium content in the filtrate was determined using an auto analyser.

A standard analysis of variance, using SAS software, was done with the ammonium content from each of the different treatment conditions. Four factors (organic material content in substrate, water content, incubation time and incubation temperature) and one variable (soluble ammonium content) were used in this analysis. Each treatment had three replicates. Significance was tested using Tukey's T-test at the 5% significance level. The least significant difference (LSD) was calculated at a 5% significance level. The effects of individual factors were tested for their significance by including means of all factors studied.

RESULTS

Saprophytic growth and survival

Saprophytic growth

In the first 28 to 56 days after inoculation of the growth media with *F. circinatum*, there was an exponential increase in population size in all the inoculated treatment flasks (Fig. 1,2,3). The largest increase in population size was observed in the inoculated pine bark compost. After 56 days of incubation, the inoculum density in the pine bark treatments had increased to more than 60 times (6 million cfu) the original inoculated population. Treatments conducted in topsoil reached a maximum population density of 32 times (3.2 million cfu) the initial inoculated population density after 28 days of incubation. Inoculated wood chips and subsoil reached a maximum population density of 14.5 times (1.45 million cfu) and 8 times (0.8 million cfu) the initial population density within the first 28 days after inoculation ($p<0.0001$) (Fig. 1).

There were significant differences ($p<0.0001$) in *F. circinatum* growth under different incubation temperatures (Fig. 2). The largest increase in population size was observed in the treatments incubated at 15 ºC. The population density increased to 60 (6 million cfu) times the original inoculated population density after 56 days of incubation. Treatments incubated at 25ºC reached the highest population density of 41 times (4.1 million cfu) the initial population density after 28 days. Treatments incubated at 35ºC reached a maximum population density of 8 times (0.8 million cfu) the original population after the first 28 days of the incubation period.

The inoculated *F. circinatum* population expanded at different rates at different water contents ($p<0.0001$). The fastest growth rate was observed at 40% moisture content, where the population size increased to 37 (3.7 million cfu) times the original inoculum density within the first 28 days of the incubation period. Treatments with 60% moisture content reached a maximum population density of 30 times (3million cfu) 28 days after inoculation. Treatments with 80% and 20% moisture content reached a maximum population density of 28 (2.8 million cfu) and 21 (2.1 million cfu) times the initial population density after 56 days of the incubation period (Fig. 3).

Saprophytic survival

After the initial growth phase, the *F*. circinatum population in all the treatments entered a decline and different survival rates were observed (Fig. 1,2,3). Analysis of variance showed that there were significant differences ($p<0.0001$) in the survival of *F. circinatum* in different growth media. Bark compost resulted in the highest survival rate, with over 17% of the maximum population size still viable after 13 months of incubation. In the topsoil 9,9% of the maximum population size was viable after 13 months of incubation. In the wood medium and subsoil, 7% and 3.5%, respectively, of the maximum population size survived after 13 months of incubation (Fig. 1).

There were significant differences ($p<0.0001$) in the survival of *F. circinatum* at different incubation temperatures (Fig. 2). At 15ºC, 17% of the maximum population size was viable after 13 months of incubation. Treatments that were incubated at 25ºC had a survival rate of 0.24% of the maximum population density after 13 months of incubation. At 35ºC, no *F. circinatum* propagules survived an incubation period of three months (Fig. 2).

At different water contents, survival of *F. circinatum* was significantly different ($p<0.0001$). After 13 months of incubation, survival in treatments with 40% and 60% moisture content was 9% and 20% of the maximum population size respectively. Treatments with 80% and 20% moisture content had survival of 41% and 10.75% of the maximum population density respectively after 13 months of incubation.

Fusarium circinatum *activity in soil*

Ammonium immobilization

After inoculation, there was an initial phase of ammonium immobilization in all the treatments (Fig. 4,5,6). This phase had a duration of 3 to 7 days. There were differences ($p<0.0001$) in nitrogen immobilization by *F. circinatum* at different levels of soil organic matter. In soil that was amended to 10% organic matter content, 39% of the free ammonium in the soil solution was immobilized within the first 7 days after inoculation.

In the soil amended with 5% organic matter, 46% of the available ammonium was immobilized 7 days after inoculation. *Fusarium circinatum* utilized 25% of the available ammonium in soil, which was not amended with organic matter within the first 3 days after inoculation (Fig. 4).

Nitrogen immobilization by *F. circinatum* differed significantly ($p<0.0001$) between different incubation temperatures. At an incubation temperature of 15ºC, the fungus utilized 64% of the free ammonium in the soil solution within the first 7 days of the incubation period. At 25ºC *F. circinatum* immobilized 70% of the free ammonium in the soil solution after 3 days of incubation. Nitrogen immobilization at 35ºC took place only in the first 3 days after inoculation, where 21% of the available nitrogen was utilized by the fungus (Fig. 5).

The capacity of *F. circinatum* to immobilize nitrogen differed ($p<0.0001$) in soils with different moisture contents. The fungus utilized 42% of the available ammonium in the soil with 80% water content 3 days after inoculation. In the soil moistened to 50% water content, the fungus immobilized 48% of the available ammonium in the soil solution 7 days after inoculation. In the soil with 20% moisture, *F. circinatum* utilized 36% ammonium 3 days after inoculation (Fig. 6).

Nitrogen mineralisation

After an initial phase of ammonium immobilization, the fungus began to mineralise ammonium under all treatment conditions (Fig. 4,5,6). In soils amended with different contents of organic matter, there were differences ($p<0.0001$) in the duration and extent of nitrogen mineralisation or ammonification by *F. circinatum*. In soil amended with 10% organic matter, an increase of 11% ammonium was measured between day 7 and day 14 of the incubation period, which presumably originated from the nitrogen fixed in the soil organic matter, and that was mineralised due to higher carbon requirements relative to nitrogen requirements of the fungus. In the soil amended with 5% organic matter, no measurable nitrogen mineralisation took place over the incubation period. In non-

amended soil, a 25% increase in soluble ammonium was detected between day 3 and day 14 of the incubation period (Fig. 4).

There were significant differences ($p<0.0001$) in ammonification by *F. circinatum* in soils incubated at different temperatures. In the soils incubated at 15ºC, an increase of 12% in the soil ammonium concentration was measured between day 7 and day 14 of the incubation period. In the treatments incubated at 25ºC, the ammonium concentration increased by 9% between day 3 and day 14 of the incubation period. In the treatments incubated at 35ºC, an increase in soluble ammonium of 29% was detected between the third and fourteenth day of incubation (Fig. 5).

There were differences ($p<0.0001$) in nitrogen mineralisation from soil organic matter in soils with different water contents. In soil with 80% moisture content an increase in soluble ammonium of 19% was measured between day 3 and day 14 of the incubation period. In the treatments conducted at 50% moisture content, the soluble ammonium concentration increased by only 7% between day 7 and day 14, while no increase in soluble ammonium was detected in the treatments with 20% moisture content (Fig. 6).

DISCUSSION

Results of this study considering survival and growth, as well as ammonium mineralisation and immobilization, provide evidence that the saprophytic phase of *F. circinatum* is favoured by growth media high in mineral nutrients. The saprophytic processes studied were optimal at lower incubation temperatures. Differences in substrate moisture content had the least effect on the saprophytic phase of the fungus. There was, however, an indication that moisture contents above 50% of the substrate water holding capacity, are optimal.

All experiments in this study demonstrate that *F. circinatum* can grow prolifically in sterile soils and in organic matter. Dwinell and Barrows-Broaddus (1978) reported that spores and other infective propagules of *F. circinatum* can enter the soil from dead infected pine tissue. Our findings, therefore, suggest that when propagules enter the soil,

a build-up of inoculum will occur. Since *F. circinatum* can be a root infecting pathogen (Viljoen et al, 1994), its active growth and survival in soil could explain why it has been so damaging in South African pine seedling nurseries.

In this study we have shown that *F. circinatum* can survive for long periods in sterile soil and in organic matter. This finding was unexpected because the fungus does not produce survival structures such as chlamydospores, which are found in many other soil inhabiting *Fusarium* spp. Mc Nee et al. (2002) reported similar results, where they demonstrated that *F. circinatum* propagules can survive in wood chips for up to three years.

Organic matter with a low C:N ratio enhanced both *F. circinatum* growth and survival. The fungus grew significantly better in bark compost, which is amended with mineral nutrients during the composting process. There were also high levels of immobilization of ammonium under all treatment conditions. These findings lead us to believe that the limiting factor for growth of *F. circinatum* is the nitrogen source. Similar results have been obtained by Schisler and Linderman (1989), who showed that growth and survival of *Fusarium* spp. is favoured by mineral nutrition. In contrast, Couteaudier and Alabouvette (1990) have demonstrated that nitrogen is not limiting to *F. oxysporum* survival. *Fusarium oxysporum* is limited by carbon concentration and is, therefore, better adapted to growth and survival in soil than *F. circinatum*, due to its ability to survive on nutrient poor substrates.

Fusarium circinatum growth in wood chips was poor. This can be explained by the fact that high levels of nitrogen are needed for digestion of celluloses. Garrett (1976) showed that *F. oxysporum* could colonize wheat straw only in the presence of high soil nitrogen levels. Our results suggest that *F. circinatum* behaves in the same manner as *F. oxysporum* and is not adapted to growth in nutrient poor substrates such as pine wood.

Survival and growth of *F. circinatum* in subsoil not amended with organic matter, was low. This could be due to the low carbon and nitrogen levels in subsoil. However, soil structure and composition might also have had an influence on the fungal growth. It has been shown that soils rich in clay have fungistatic characteristics (Amir and Alabouvette, 1993). Organic carbon decomposition in soils with high clay content has

also been shown to be lower due to the high cation exchange capacity of such soils (Ladd et al., 1993).

Fusarium circinatum growth and survival in this study was favoured by low temperatures. This is consistent with the results of Bolkan et al. (1979) who showed that *F. guttiforme*, a close relative of *F. circinatum*, survived better at 4ºC and 18ºC than at 25ºC and 30ºC. Survival of *F. oxysporum* has also been shown to be higher at 10ºC than at 25ºC (Vakalounakis and Chalkias, 2004). The high final population density at 15ºC relative to the 25ºC treatments in our experiments can, therefore, be attributed to the higher rate of death at 25ºC. This finding is also in agreement with those of Mc Nee et al. (2004), who suggested that *F. circinatum* survives for longer periods in cooler areas.

Soil water content had the least significant effect on the survival of *F. circinatum* of any of the other factors studied. Studies on *Ganoderma* spp. and other wood inhabiting fungi have shown that water potential does not have significant effects on pathogen survival (Tun Tschu Chang, 2003). In our study, the best growth of the fungus was observed with moisture contents between 40% and 60%. This is in contrast to studies on *F. guttiforme*, showing that this fungus is favoured by low moisture contents (Bolkan et al, 1979). Survival of *F. guttiforme* was higher in air-dried soil than in soils with 10%, 25% and 35% water. *Fusarium circinatum* does not appear to behave in the same manner as *F. guttiforme* with respect to soil water.

Fusarium circinatum grew prolifically in sterile soils amended with organic matter and survived for long periods of time. Numerous studies have demonstrated that pathogen decline is much higher in non-sterile soil than in pure culture. This is generally attributed to microbial competition that is deleterious to the pathogen (Bonde et al., 2004; Hildebrand et al., 2001; Marios and Mitchell, 1981). We, therefore, anticipate, that under non-sterile conditions, the saprophytic ability of *F. circinatum* will be greatly reduced in comparison to the results we present here.

Fusarium circinatum has a significant saprophytic phase, enhanced by the presence of organic matter and low temperature. The survival of the fungus was greater than expected although it is likely that in the presence of other micro organisms, this fungus would not survive well. Yet it is probable that *F. circinatum* can survive for long periods of time in soils with high organic content and that this survival will lead to an increase in

disease development in pine-growing nurseries. This is likely to be especially true during cooler times of the year and management strategies to contain the disease will need to consider these factors.

ACKNOWLEDGEMENTS

We are grateful to the members of the Tree Pathology Co-operative Programme (TPCP), the National Research Foundation (NRF) and the THRIP support programme of the Department of Trade and Industry for financial support.

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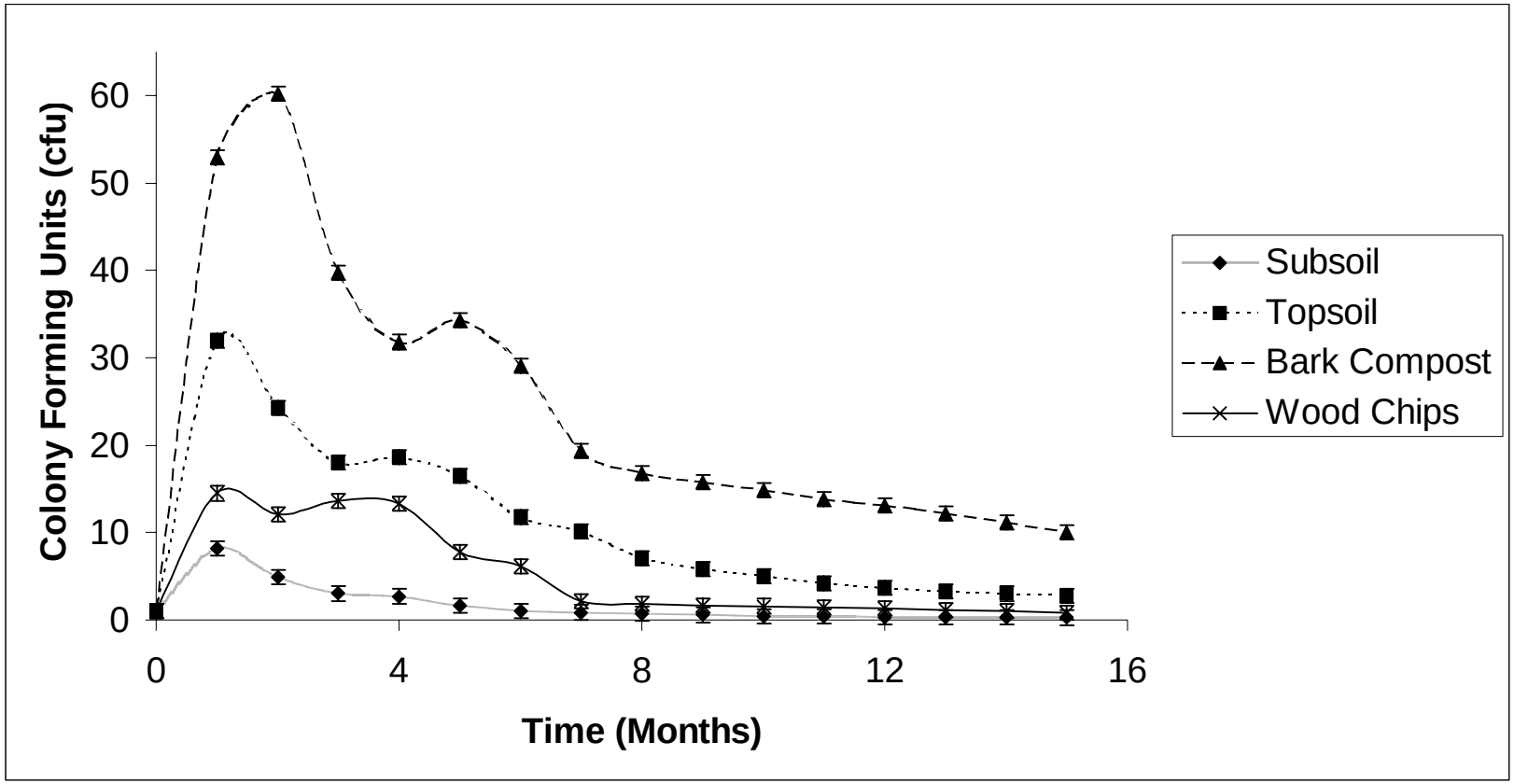

Fig 1:Mean effects of substrate on *Fusarium circinatum* growth and survival.

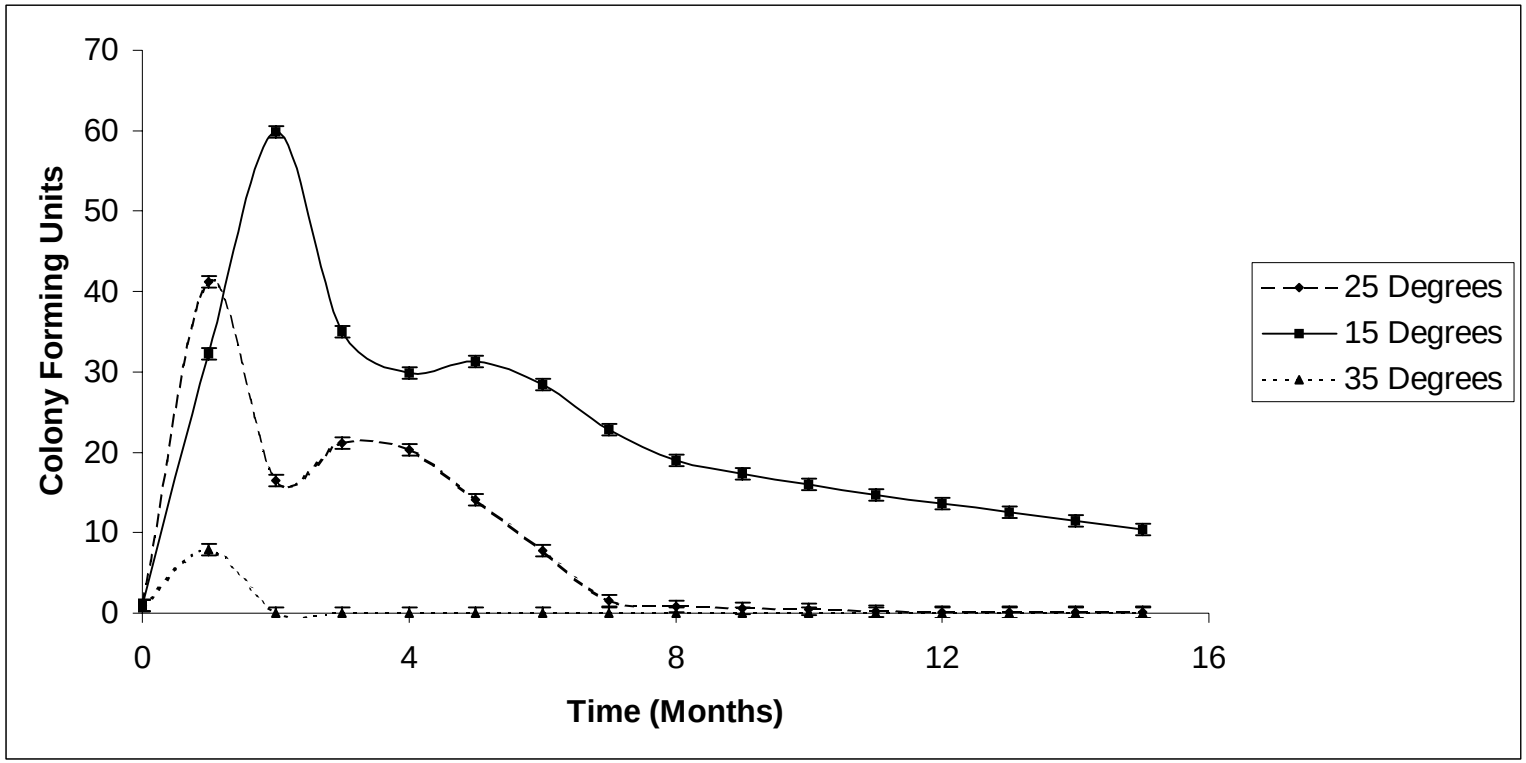

Fig 2: Mean effects of temperature on *Fusarium circinatum* growth and survival.

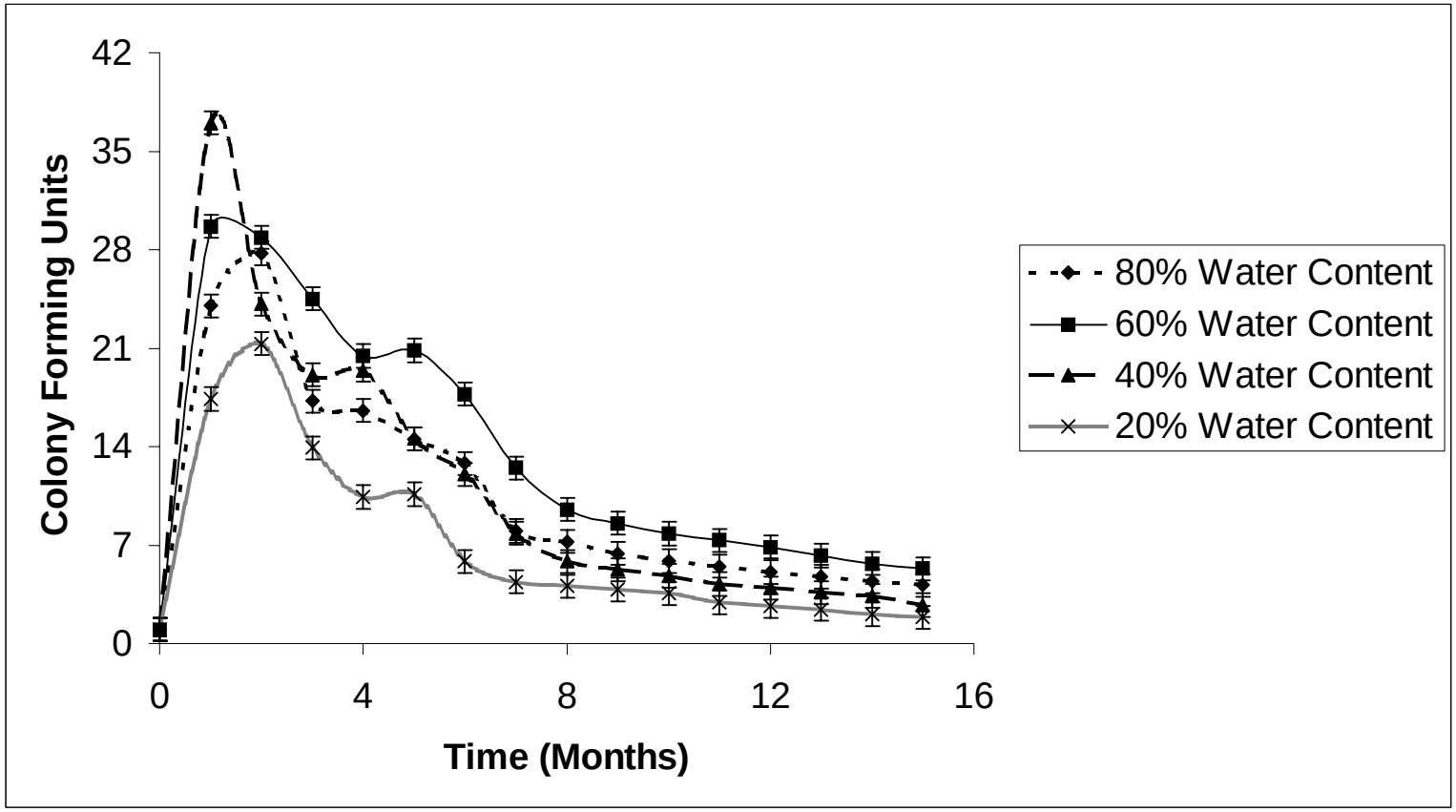

Fig 3: Mean effects of moisture content on *Fusarium circinatum* growth and survival.

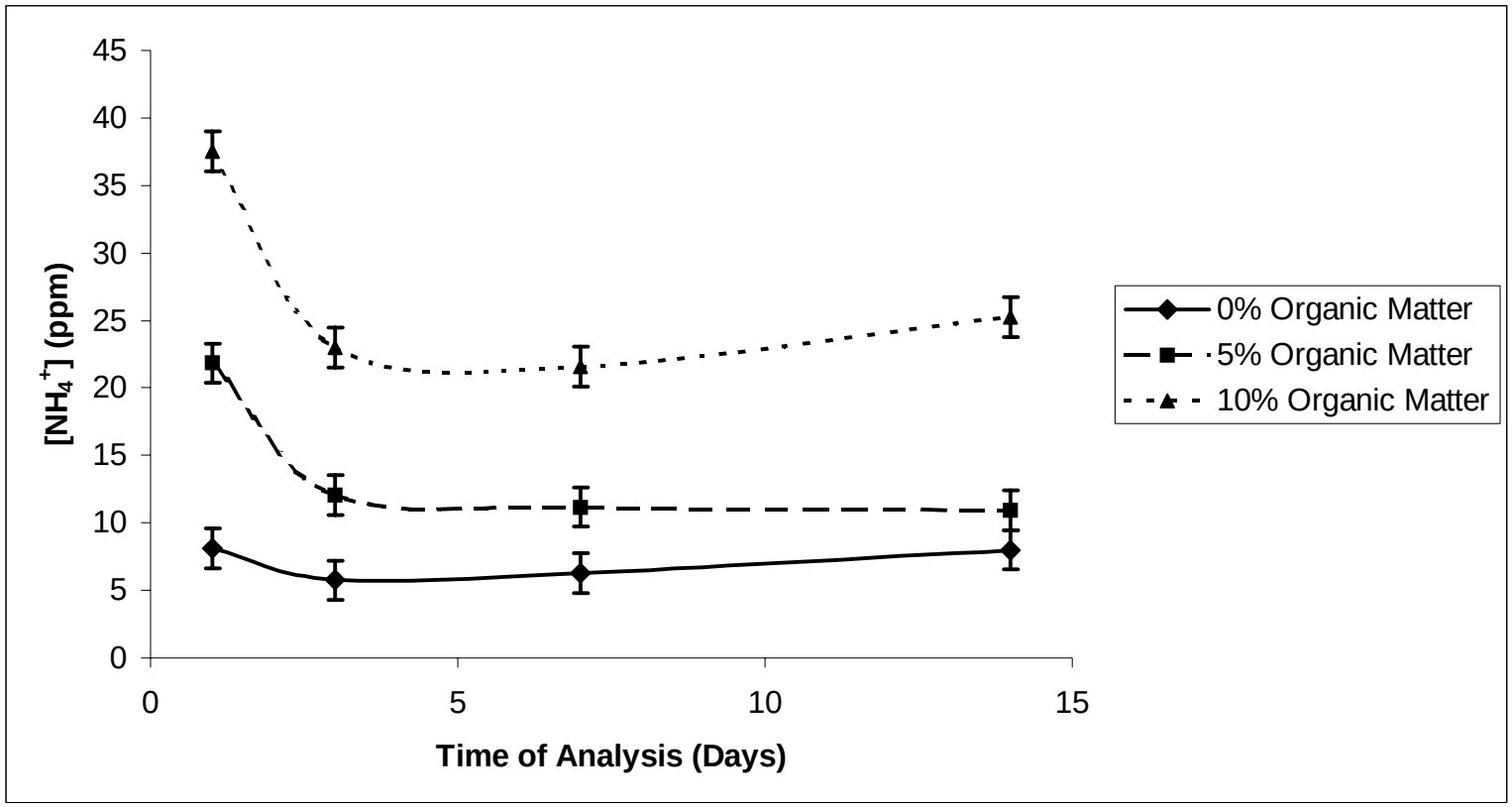

Fig. 4: Mean effects of organic material amendment on *Fusarium circinatum* ammonium immobilization and mineralisation.

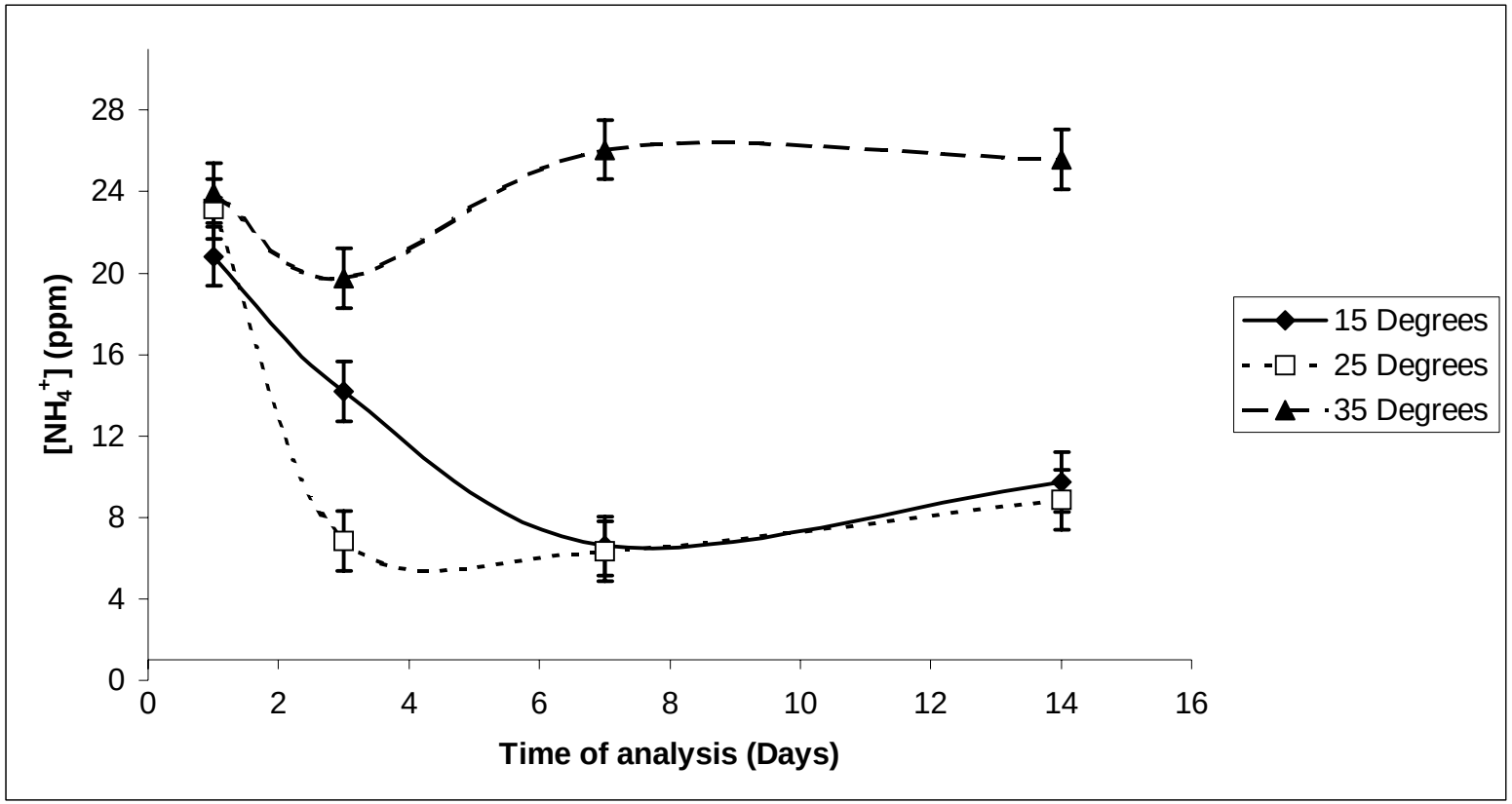

Fig. 5: Mean effects of temperature on *Fusarium circinatum* ammonium immobilization and mineralisation.

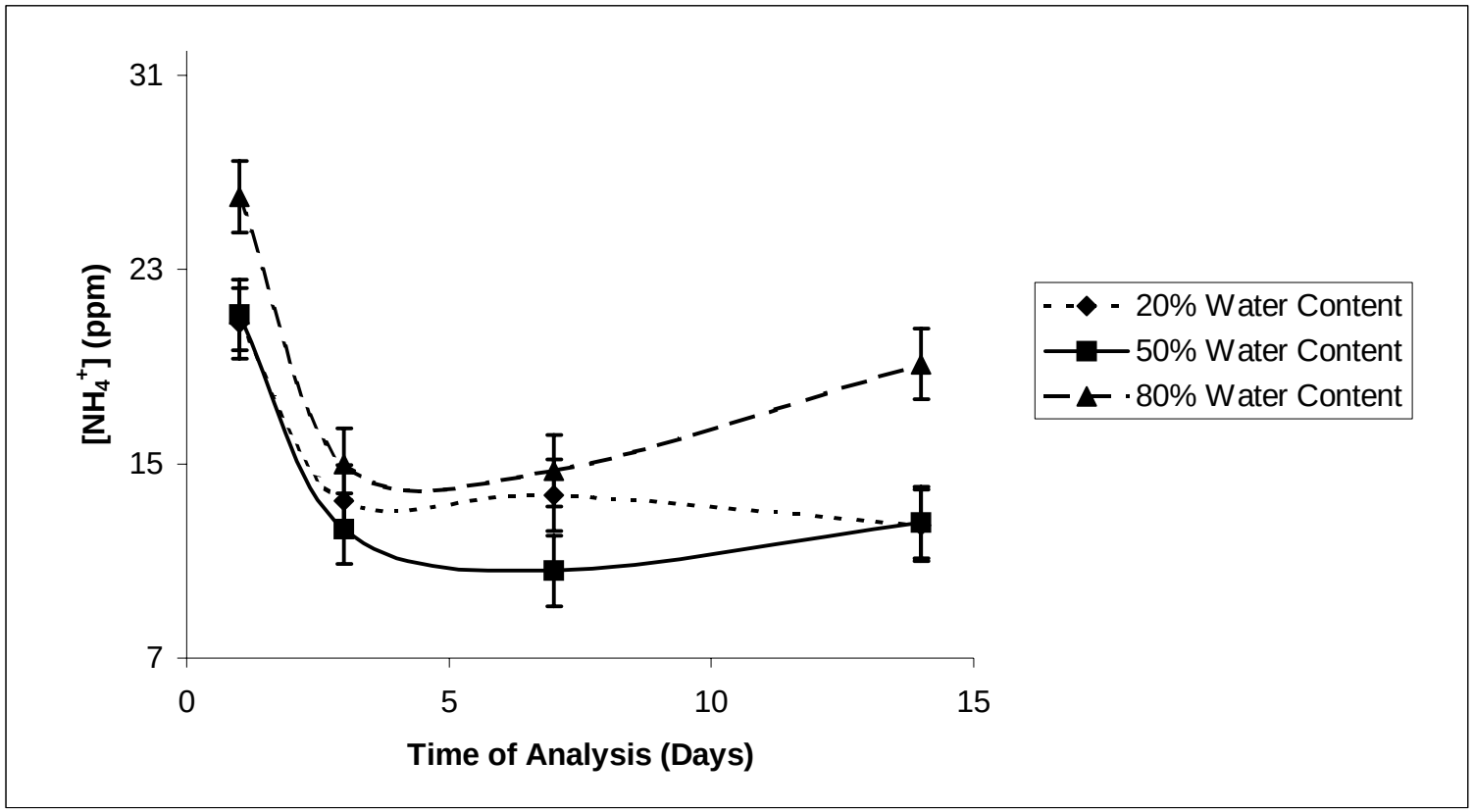

Fig. 6: Mean effects of moisture content on *Fusarium circinatum* ammonium immobilization and mineralisation

5

DNA based diagnostic technique developed for rapid identification of the pine pitch canker fungus, *Fusarium circinatum*.

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Abstract: *Fusarium circinatum* is an important pathogen of pine seedlings and cuttings in South Africa. The fungus is difficult to distinguish from related species based on morphological characteristics and this hinders rapid and accurate diagnosis required in nursery management. The objective of this study was to develop a DNA based diagnostic technique for the rapid and accurate identification of *Fusarium* species associated with root disease in South African pine nurseries. A LightCycler probe pair was thus developed for a variable region on the *histone 3* gene. By comparing the melting curves of the probe to PCR amplified *histone 3* DNA, we could distinguish *F. circinatum* from four closely related *Fusarium* species encountered in South African pine nurseries.

INTRODUCTION:

Fusarium circinatum (Nierenberg & O'Donnell, 1998) is the causal agent of pitch canker, which is one of the most important diseases of *Pinus* spp. On mature trees the pathogen causes resinous cankers on stems and lateral branches (Dwinell et al., 1985). Affected trees die back from the branch tips (Correll et al., 1991). Female flowers and mature cones can also be infected by the fungus (Barrows-Broaddus, 1990), which can occur within seeds or on the seed coats (Storer et al., 1998). The pathogen causes damping off of germinating seedlings and die-back of established seedlings in South African pine nurseries (Viljoen et al., 1994). During the course of the last decade, this disease has become one of the most important impediments to pine production in the country.

Fusarium circinatum has been reported to be endemic in the south-eastern United States of America (Dwinell et al., 1985) and in Mexico (Santos & Tovar, 1991). The fungus has been introduced into California (McCain et al., 1987), Japan (Muramoto & Dwinell, 1990), South Africa (Viljoen et al., 1994) and into Chile (Wingfield et al., 2002). Dispersal *F. circinatum* into new areas occurs through insect vectors (Blakeslee et al., 1978), infected plant material, contaminated soil and water (Wikler & Gordon, 2000; Gordon et al., 2001), air (Fraedrich and Dwinell, 1997) and seed (Storer et al., 1998). To prevent human dispersal of *F. circinatum* into new areas, and to preclude the introduction of new strains into areas where the fungus already occurs, it is important to rapidly screen seed, vegetative plant material and other products for its presence.

Identification of *F. circinatum* based on morphological characteristics is complicated, prone to error and is typically time-consuming (Steenkamp et al., 1999). DNA based techniques have, therefore, been employed to detect the fungus. PCR-RFLPs based on the *histone 3* gene have been shown to be effective in distinguishing *F. circinatum* from related species (Steenkamp et al., 1999). Species specific primers based on the sequence of the nuclear ribosomal *intergenic spacer* gene region have also been shown to be reliable in identifying the pitch canker fungus (Schweigkofler et al., 2004).

The objective of this study was to develop a diagnostic tool that could be used to rapidly distinguish between *F. circinatum* and other related *Fusarium* spp. that are commonly encountered in South African forestry nurseries.

MATERIALS AND METHODS:

Fungal isolates:

Fusarium species selected for this study included *F. circinatum*, *F. proliferatum*, *F. oxysporum* and an undescribed *Fusarium* spp. isolated from pine (Table 1). These species were included based on their prevalence in South African pine seedling nurseries. The following *Fusarium* spp. were included in the phylogenetic analysis: *F. concentricum* (MRC 7541), *F. begoniae* (MRC 7542), *F. bulbicola* (MRC 7534), *F. guttiforme* (MRC 7539), *F. pseudocircinatum* (MRC 7536), *F. sacchari* (MRC 6525), *F. subglutinans* (MRC 6512), *Fusarium* spp. (MRC 1077, MRC 6748, MRC 2382), *F. sterillihyphosum* (MRC 2802), *F. mangiferae* (MRC 2730), *F. thapsinum* (MRC 6536), *F. verticillioides* (MRC 6155), *F. nygamai* (MRC 7549), *F. fujikuroi* (MRC 6570) and *F. konzum* (KSU 10653). Two *Fusarium solani* isolates (MS 48A and MS 107 from Ethiopia) were selected as outgroups for the phylogenetic analyses. All isolates used in this study are maintained in the culture collections of the Forestry and Agricultural Biotechnology Institute at the University of Pretoria, as well as in the Medical Research Council (MRC) at Tygerberg, South Africa.

DNA isolation:

For DNA extractions, fungal cultures were grown on Potato Dextrose Agar (Biolab Diagnostics, Halfwayhouse, SA) at 25°C in the dark for seven days. Mycelium was harvested from agar plates, and DNA was extracted using a modification of the method of Murray and Thompson (1980).

PCR amplification:

PCR amplification of the *histone 3* gene was achieved using primers H3 1a (5'-actaagcagaccgcccgcagg-3') and H3 1b (5'-gcgggcgagctggatgtcctt-3') designed by Glass and Donaldson (1995) for amplification of approximately 450 base pairs of the *Neurospora crassa histone3* gene. PCR amplification was preformed using a Roche LightCycler™ (Roche Diagnostics Pty Ltd, Randburg, SA). Each PCR reaction contained 1mM deoxyribonucleotide triphosphates (dNTP's) (0.25 mM of each), 7.5mM $MgCl_2$, 0.4µM H3 1a, 0.4µM H3 1b, 0.2ng/µL genomic DNA, 100 ppm CybrGreen (Southern Cross Biotechnology Pty Ltd, Cape Town, SA), 0.05U/µL Super-Therm DNA polymerase (Southern Cross Biotechnology Pty Ltd, Cape Town, SA) and 1X Super-Therm reaction buffer. The PCR reactions were conducted with the following cycling parameters: 1 denaturation cycle at 95°C for 1 minute, 25 cycles of 95°C for 10s, 56°C for 15s and 72°C for 20s. A final extension was preformed at 72°C for 1 minute.

DNA sequencing and phylogenetic analysis:

PCR products were precipitated with 0.1µL of 3M sodium acetate and 10µL 100% ethanol per µL PCR reaction volume. *Histone 3* gene fragments from the *Fusarium* isolates included in this study were sequenced in both directions using the primers H3 1a and H3 1b. Cycle sequencing was preformed with AB BigDye™ (Applied Biosystems, Johannesburg, SA) in an AB Gene Amp 9700 thermo cycler as follows: 25 cycles of 96°C for 10s, 50°C for 5s and 60°C for 4 minutes. Sequencing reactions were purified by precipitation with sodium acetate and ethanol (described above) and separated on an AB Prism 3100 genetic analyzer. DNA sequences were manually edited and aligned with Sequence Navigator™ version 1.0.1 (Perkin Elmer, Foster City, CA). Phylogenetic analyses were preformed with Phylogenetic Analysis using Parsimony (PAUP) version

4.0b1 (Swofford, 1998). Gaps were treated as a fifth character, and bootstrap analyses were based on 10000 replications.

Diagnostic PCR:

Two oligonucleotide hybridisation probes were designed for a region in the *histone 3* gene of *F. circinatum*, which differed for *F. proliferatum*, *F. oxysporum* and the undescribed *Fusarium* sp. isolated from pine in South African nurseries (Table 1). The 5' oligonucleotide (21 bases) was designed to have a Fluorescin (Roche Diagnostics, Switzerland) label (donor) at its 3' end. The 3' oligonucleotide (19 bases) was designed to have a LightCycler Red (Roche Diagnostics, Switzerland) label (acceptor) at its 5' end. The two probes were designed to bind in close proximity to each other (3 base pair gap) so that there was transfer of fluorescent resonance energy between the fluorescent labels when bound to the DNA during the primer-annealing step of the PCR reaction.

The diagnostic PCRs were performed with the LightCycler. Each PCR reaction contained 1mM dNTP's, 2.5mM $MgCl_2$, 0.4µM of each of the H3 primers, 0.2µM of each probe, 5ng/µL genomic DNA and 0.05U/µL Super Therm polymerase and 1X Super Therm PCR reaction buffer. Cycling parameters for the PCR reaction were, an initial denaturation cycle of 1 minute at 95°C, 40 cycles of 95°C for 10s, 52°C for 20s and 72°C for 30s, with a final cycle of 72°C for 2 minutes.

After completion of the PCR reaction, analysis of melting curves was done on the LightCycler. The temperature of the PCR reaction was raised to 95°C after which the temperature was rapidly reduced to 48°C for 2 minutes. The reaction temperature was then raised stepwise at a rate of 0.1°C/s to 85°C. The negative first derivative of the melting curve of the probe was calculated with LightCycler software version 3.5 (Roche Diagnostics Pty Ltd, Randburg, SA), and the melting point (T_M) of the probe, when annealed to the DNA of the species included in this study, was determined manually.

RESULTS:

DNA sequencing:

For all the *Fusarium* sp. included in this study, a *histone 3* gene fragment of approximately 520 bp with 2 introns (Steenkamp et al., 1999) was obtained. Phylogenetic analysis with PAUP generated a most parsimonious tree from 506bp of aligned DNA sequence. This tree (Fig. 1) had four sub-clades, and was very similar to trees published previously (O'Donnell et al., 1998, 2000). One clade (Bootstrap: 93%), referred to as the 'American Clade' by O'Donnell et al. (1998) included the *F. circinatum* isolates. The second clade (Bootstrap: 60%), referred to as the 'Asian Clade' by O'Donnell et al. (1998), included the *F. proliferatum* isolates as well as the undescribed species from pine. The third clade (Bootstrap: 63%) included isolates that have been placed by O'Donnell et al. (1998) into an 'African Clade'. *F. oxysporum* was the only species in the fourth clade (Bootstrap: 100%).

Diagnostic PCR:

There were sufficient differences between the *histone 3* DNA sequences of *F. circinatum*, *Fusarium* sp. from pine, *F. proliferatum* and *F. oxysporum* to design a LightCycler probe with differing annealing temperatures for all the species prevalent in SA pine nurseries. The fluorescent probe labelled with the fluorescin dye (donor) was designed to be specifically complementary to the *F. circinatum* gene sequence. The other nursery pathogens included in this study differed at this locus by three or four base pairs (Table 1). The T_m of the fluorescin labelled probe was unique for *F. circinatum* (58°C), the undescribed *Fusarium* sp. isolated from pine (52°C), *F. proliferatum* (56°C) and *F. oxysporum* (54°C) (Table 1). It was, therefore, possible to distinguish between the DNA of different species by comparing the melting curves of the probe (Fig.2).

DISCUSSION:

In this study we developed a DNA-based diagnostic technique, which enabled us to distinguish between *Fusarium* species that are prevalent in South African pine nurseries. By making use of Roche LightCycler technologies, and a LightCycler probe designed for a variable region on the *histone 3* gene, we were thus able to distinguish between *F. circinatum*, an undescribed *Fusarium* sp. isolated from pine tissue, *F. proliferatum* and *F. oxysporum*, by comparing melting curves of hybrids between the probe and the *Fusarium* DNA.

Identifying *Fusarium* spp. using DNA based methods is considerably less time consuming and labour intensive than identification based on morphology and cross-fertility tests (Steenkamp et al., 1999). It is also more accurate and negates problems linked to lack of taxonomic experience required for reliable identification, particularly where fungi are morphologically cryptic. In order to address this problem as it relates to the pitch canker fungus in South African pine nurseries, Steenkamp et al. (1999) proposed identification of *F. circinatum* by PCR-RFLP. That technique requires a double restriction enzyme digest with a duration of approximately 8 hours. Using the approach developed in this study, positive identification is possible with a single PCR reaction requiring less than 45 minutes.

A number of researchers have used species specific primers to distinguish between *Fusarium* spp. Nicholson et al. (2004) distinguished between fungi causing *Fusarium* head blight in small grain cereals with a PCR assay using multiple primers. Multiple species specific primers, based on the calmodulin gene, have also been used to identify *F. verticillioides*, *F. proliferatum* and *F. subglutinans* (Mule et al., 2004). Waalwijk et al. (2004) employed multiple primers and probes to distinguish between *Fusarium* species causing head blight of small grain cereals. Using the technique characterized in this study, we could distinguish between *Fusarium* species infecting pine seedlings in South Africa by the use of a single primer and probe pair.

Since species identification in the diagnostic technique developed in this study is based on DNA-DNA hybridisation, results are strongly influenced by salt concentration in the PCR reaction mix. It is, therefore, important to standardize DNA isolation

techniques and reagents used in the PCR reactions. Positive controls for all the species included in this study must also be included in a diagnostic PCR reaction batch to prevent misidentification that may result due to variation in the salt concentration of the reaction mix.

In this study, we have presented a diagnostic technique making it possible to distinguish *F. circinatum* from pathogenic *Fusarium* spp. commonly encountered in South African forestry nurseries. The technique is rapid, can be used in real time and does not require expensive reagents. The intention is that this technique will be employed to rapidly detect the presence of *F. circinatum* in South African nurseries. This will make it possible to implement necessary control measures in a timely way.

ACKNOWLEDGEMENTS:

We are grateful to the members of the Tree Pathology Co-operative Programme (TPCP), the National Research Foundation (NRF) and the THRIP support programme of the Department of Trade and Industry for financial support.

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Table 1:
Species and isolates included in this study. Gene sequences represent actual histone 3 gene sequences at the hybridisation point. Probe sequence is homologous to the F. circinatum gene sequence. The TM is the approximate melting temperature of the probe when annealed to DNA of the Fusarium species at a 2.5 mM $MgCl_2$ concentration.

Species	Isolate Number	Host	Origin	Fluorescin Probe Sequence	LC Red Probe Sequence	TM
F. circinatum	MRC7541	Pine	USA	CATTTGACAAACAGGTCCGCG	TTGCCCAGGACTTCAAGTC	58
	MRC 6216	Pine	RSA			
	MRC 7454	Pine	RSA			
Fusarium spp.	MRC 7151	Pine	RSA	CATTCGACGAACAGGTTCGTG	TTGCCCAGGACTTCAAGTC	52
F. proliferatum	MRC 5659	Maize	RSA	CATTCGACGAACAGGTCCGTG	TTGCCCAGGACTTCAAGTC	56
	MRC 6568	Maize	RSA			
F. oxysporum	MRC 6212	Pine	RSA	CATTAGACAAACAGGTTCGTG	TTGCCCAGGACTTCAAGTC	54
	MRC 5049	Pine	RSA			

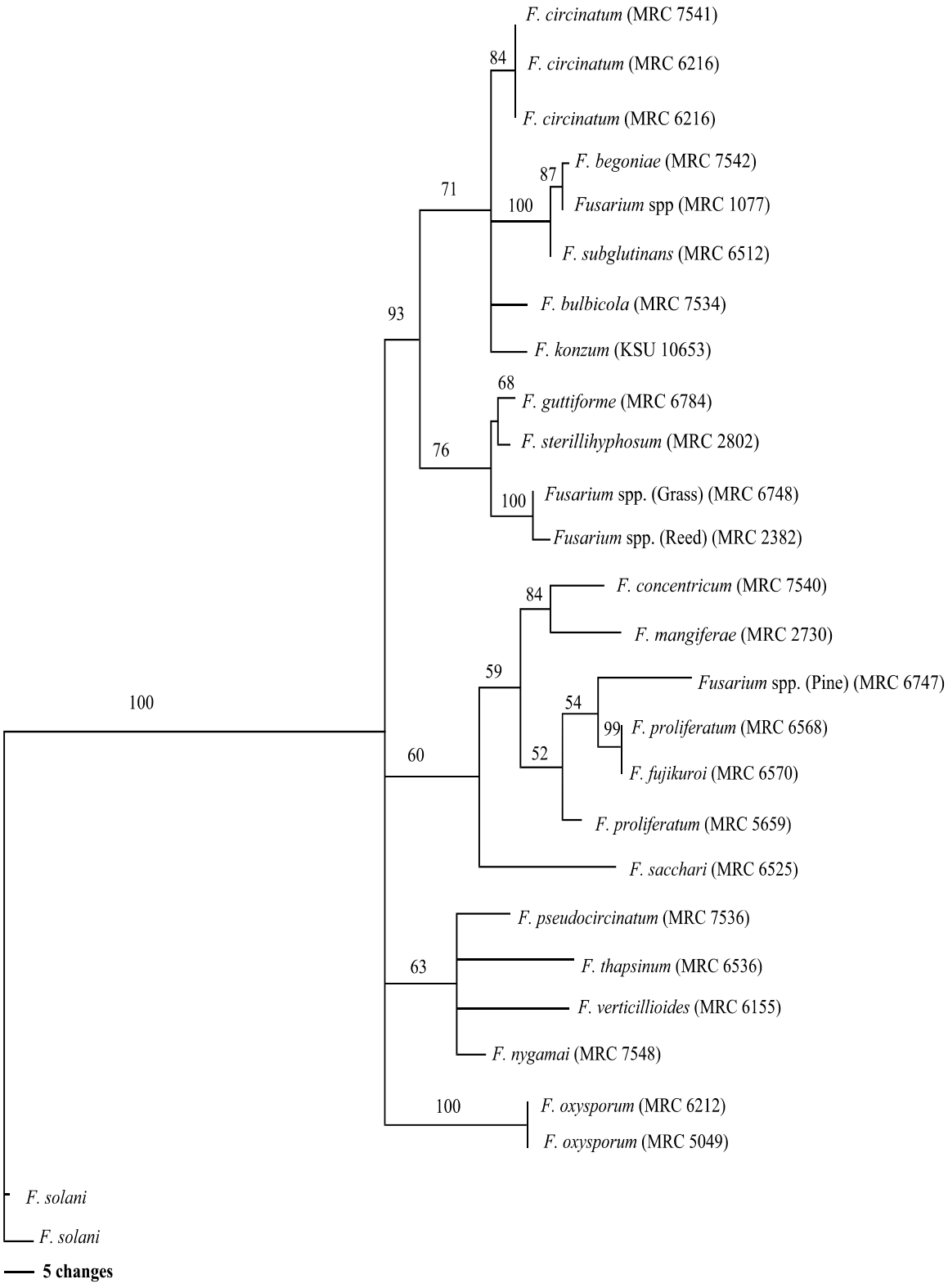

Fig. 1: Phylogenetic tree with bootstrap values for the histone 3 sequences of the species and isolates included in this study. Gaps were treated as a fifth character, and bootstrap analyses were based on 10000 replications.

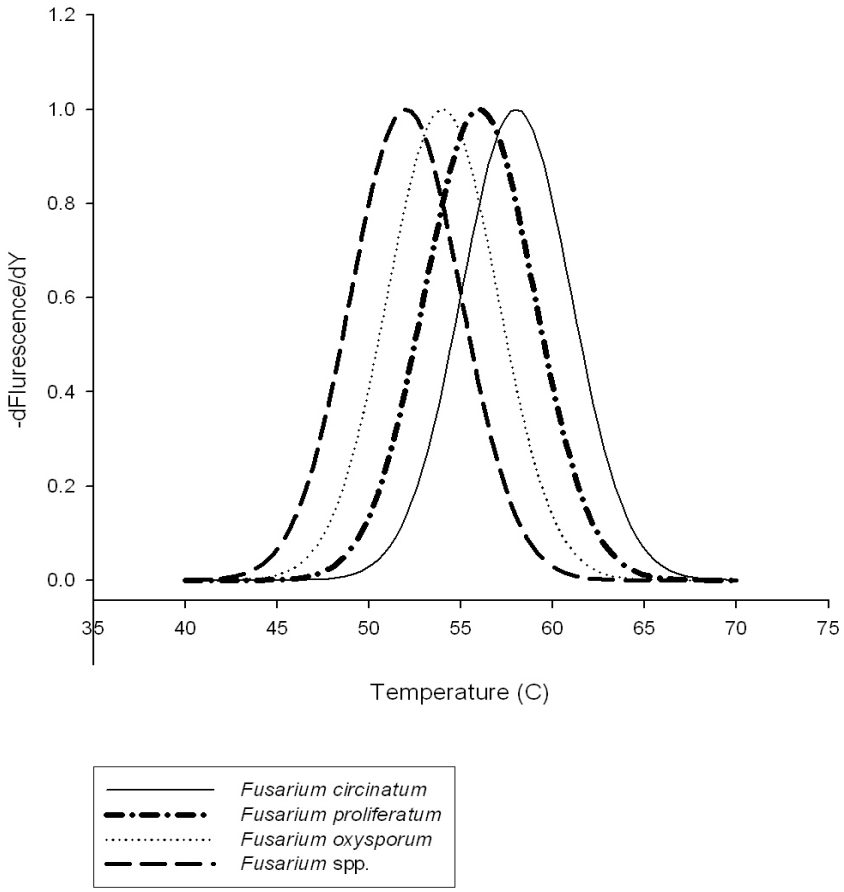

Fig.2: Positioning of negative derivatives of LightCycler annealing curves to allow species identification.

6

Molecular taxonomy of the *Fusarium subglutinans sensu lato* species complex.

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Abstract: *Fusarium subglutinans sensu lato* represents a unified group of species sharing similar morphological characteristics. Phylogenetic analyses have shown that this is a species complex that can be separated into at least 13 distinct groups. The objectives of this study were to resolve *F. subglutinans sensu lato* and related *Gibberella* mating populations phylogenetically using sequence data for multiple loci. For this purpose, part of the ribosomal DNA intergenic spacer (IGS), β tubulin, translation elongation factor 1α and histone 3 genes were sequenced. Parsimony analysis of single and combined datasets yielded trees comprised of three major clades. Bootstrap support for the minor clades was low. The IGS region was phylogenetically most informative and yielded a well resolved tree. The combined dataset yielded a well resolved tree, where bootstrap support of major and minor clades were generally higher. This study confirms species concepts proposed for this complex.

INTRODUCTION:

Species in the *Gibberella fujikuroi* complex (Sections Liseola, Dlaminia and Elegans) have a worldwide distribution (O'Donnell et al. 1998). These species are causal agents of important plant diseases on a wide variety of agronomic and horticultural crops (Nirenberg and O'Donnell, 1998). Some species in the complex are also producers of mycotoxins and secondary metabolites that pose a health risk to animals and humans (Nelson et al., 1992).

The taxonomy of the *G. fujikuroi* complex is not yet fully resolved and members can be classified as morphological (Nelson et al., 1983), biological (Leslie, 1991) or phylogenetic species (O'Donnell et al., 1998a). During the 1980's, two conflicting morphological species concepts were widely applied to this group. Gerlach and Nirenberg (1982) recognized seven species in the section *Liseola*, while Nelson et al. (1983) recognized only four species. Attempts to resolve the taxonomy of these species by cross-fertility testing among strains and thus applying the biological species concept have also been made (Leslie, 1991). In this way, nine biological species (mating population A to I), which have the ability to form perithecia and to produce fertile ascospores, when mated with members of the same mating population have been identified (Leslie, 1995). However, many taxa in the *G. fujikuroi* species complex have no known sexual stage and cannot be classified using the biological species concept (O'Donnell et al., 2000). Isolates in some mating populations are also homothallic (Britz et al., 1999) and members of mating populations are also not always reproductively isolated, giving rise to hybrids (Desjardins et al., 2000). Thus, the biological species concept is not uniformly useful for these fungi.

Utilisation of DNA sequence has made it possible to show that the *G. fujikuroi* species complex includes at least 36 distinct phylogenetic species (O'Donnell et al., 1998a; 2000). Based on these phylogenies, a new morphological species concept has been established in which 29 species within the *G. fujikuroi* species complex have been formally described (Nirenberg & O'Donnell, 1998; Zeller et al., 2003).

A *Fusarium* species in the *G. fujikuroi* complex that has received much taxonomic scrutiny is *F. subglutinans* (Wolleweber & Reinking) Nelson, Toussoun and Marasas

(Nelson et al., 1983). This taxon has traditionally been associated with a wide variety of plant hosts and with three biological species: mating populations B, E and H, pathogenic on sugarcane, maize and pine, respectively. Phylogenetic analysis of multiple unlinked loci, however, showed that *F. subglutinans* represents at least 13 distinct taxa (= *F. subglutinans sensu lato*), which are associated with specific plant hosts (O'Donnell et al., 1998a; 2000; Steenkamp et al., 2000; 2002). Furthermore, Nirenberg and O'Donnell (1998) described six new species within *F. subglutinans sensu lato* using a combination of morphological and molecular characteristics. Currently, 10 species are recognized in this complex, namely, *F. circinatum, F. concentricum, F. begoniae, F. bulbicola, F. guttiforme, F. pseudicircinatum, F. sacchari, F. subglutinans, F .sterillihyphosum* and *F. mangiferae*.

Several DNA-based and biochemical methods have been used in addition to morphological characteristics and sexual mating tests to distinguish between the different species in *F. subglutinans sensu lato*. These include electrophoretic karyotyping (Xu et al, 1995), differential benomyl and hygromycin B sensitivity (Yan and Dickman, 1993), Fumosin production (Leslie et al., 1992) and isozyme patterns (Huss et al., 1996), which allowed discrimination between mating population B, E and H. These populations could also be distinguished by different randomly amplified polymorphic DNA (RAPD) profiles (Amoah et al., 1996; Voigt et al., 1995), polymerase chain reaction restriction fragment length polymorphisms (PCR-RFLP) based on the histone 3 gene (Steenkamp et al., 1999) and species specific PCR assays based on RAPD primers (Moeller et al., 1999) and the IGS region (Schweigkofler et al., 2004).

The phylogenetic species concept as it is applied to *F. subglutinans sensu lato* is based on partial sequence data from protein coding genes (O'Donnell et al., 1998a; 2000; Steenkamp et al., 1999; 2000). It has been shown that the β tubulin, translation elongation factor 1α (EF1α), calmodulin and histone 3 genes are sufficiently variable to infer some phylogenetic lineages for these fungi. However, these were not sufficiently variable to resolve closely related species. In contrast, ribosomal DNA sequences have been shown to be inordinately conserved and inferred trees were not well resolved (O'Donnell et al., 1998a; 2000; Peterson and Logrieco, 1991). Studies done for the development of PCR based diagnostics have, however, shown that the intergenic spacer (IGS) region of taxa

making up the *G. fujikuroi* species complex is highly variable (Hinojo et al., 2004; Schweigkofler et al., 2004). Using this region for phylogenetic studies might, therefore, yield more conclusive results concerning the taxonomy of *F. subglutinans sensu lato*.

The objectives of this study were to evaluate the utility of combined DNA sequence data from protein coding genes and a ribosomal DNA region in differentiating between the 13 species included in *F. subglutinans sensu lato*. A further aim was to test the utility of the IGS region in resolving phylogenetic lineages within *F. subglutinans sensu lato*.

MATERIALS AND METHODS:

Fungal isolates:

Twelve of the thirteen *Fusarium* spp. recognized as belonging to *F. subglutinans sensu lato* were included in this study. Isolates from *Bidens pilosa* described by O'Donnell et al. (2000) were not available (Table 1). The strains included six of the species described by Nirenberg and O'Donnell (1998) as well as one of the mating type tester strains from *F. sacchari* (mating population B) and *F. subglutinans sensu stricto* (mating population E). In addition, representative strains of *F. sterillihyphosum* and *F. mangiferae* were included. Strains from two *Fusarium* spp. associated with *Zea* spp. (O'Donnell et al., 2000; Steenkamp et al., 2002) and ornamental grass and reed (O'Donnell et al., 2000) were also included. Representatives of mating populations A, C, D, F, G and I were included for comparative purposes. A single *F. oxysporum* isolate was used as outgroup in the phylogenetic analyses.

Isolates used in this study are maintained on 15% glycerol at -80°C in the *Fusarium* collection of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, South Africa (SA), as well as in the culture collection of the Medical Research Council (MRC) at Tygerberg, SA. Details of culture numbers and other collection data are presented in Table 1.

DNA isolation and PCR amplification:

For DNA extractions, fungal cultures were grown on Potato Dextrose Agar (Biolab Diagnostics, Halfwayhouse, SA) at 25°C in the dark for seven days. Mycelium was harvested from agar plates, and DNA was extracted by using a modified CTAB method (Murray and Thompson, 1980; Steenkamp et al., 1999).

The four loci that were PCR amplified and sequenced in this study are partial regions of the IGS, β tubulin, the translation elongation factor 1α (EF1α) and the histone 3 gene (H3). IGS was amplified with primers NL 11 and CNS 1 (Schweigkofler et al., 2004), β tubulin was amplified with primers Bt 1b and Bt 2a (Glass and Donaldson, 1995), the EF1α region was amplified with primers Ef 1 and Ef 2 (O'Donnell et al., 1998b) and H3 was amplified with primers H3 1a and H3 1b (Glass and Donaldson, 1995) (Table 2).

PCR mixtures contained 1 to 2 U *Taq* Polymerase, 1X PCR buffer, 0.2mM of each dNTP, 1 - 2.5mM $MgCl_2$ (Table 2), 0.005 mM of each primer and 10 to 0.8 ng/µl of genomic DNA. PCR was performed on an Applied Biosystems (AB) Gene Amp 9700 thermal cycler as follows: an initial denaturation step at 95°C for 1min, 30 cycles of 95°C for 10s, 30s at 54 - 62°C (Table 2) and 1s for each 10 base pairs to be amplified at 72°C, with a final elongation step of 72°C for 2 min. Where PCR amplifications produced double bands, those with the expected sizes where purified with the QIAquick gel purification kit (Qiagen GmbH Hilden, Germany). All other PCR products were precipitated with 0.1 volume 3M sodium acetate and 10 volumes 100% ethanol.

DNA sequencing and phylogenetic analysis:

Purified PCR products were sequenced in both directions using the initial PCR primers as well as internal PCR primers in the case of the IGS and β tubulin (Table 2). Cycle sequencing was performed with ABI BigDye™ (Applied Biosystems, Johannesburg, SA) in an AB Gene Amp 9700 thermal cycler as follows: 25 cycles of 96°C for 10s, 50°C for 5s and 60°C for 4 minutes. Sequenced products were purified by

precipitation with sodium acetate and ethanol as described above and separated on an ABI Prism 3100 genetic analyzer in 50cm or 80cm capillaries, depending on fragment size.

DNA sequences were manually edited and aligned with Sequence Navigator™ version 1.0.1 (Perkin Elmer, Foster City, CA). Phylogenetic analyses were performed with PAUP version 4.0b1 (Swofford, 1998) where gaps were treated as fifth characters. Most parsimonious trees were calculated using heuristic searches, with tree bisection-reconnection branch swapping effective. Bootstrap analyses were based on 10000 replications. Pairwise partition homogeneity tests were performed for all partitions (genes) included in this study.

RESULTS:

PCR amplification:

The PCR products of the IGS region had an approximate length of 2500 base pairs (bp), while those for β tubulin, the EF1α gene and the H3 gene were approximately 1000, 700, 550 bp, in length respectively. For some taxa (*F. proliferatum*, *F. sacchari* and *F. konzum*) the PCR of the IGS region yielded double bands. In such cases the amplicon having the expected size (2500 bp) was gel-purified for subsequent sequencing.

DNA sequence analysis:

The manually adjusted alignment for the partial IGS region contained 19 taxa (including the outgroup) and 2768 characters including the gaps that were included for alighnment purposes. Of the 2768 characters used in the analysis, 526 were parsimony informative. From these data two most parsimonious trees (MPT's) were generated. Both MPT's were 2348 steps steps long, with a consistency index (CI) of 0.65 and retention index (RI) of 0.55 (Fig. 1). Trees included three well supported clades, which are

consistent with the three clades described by O'Donnell et al. (2000; 1998). Clade 1 [100% bootstrap support(BP)] contained *F. circinatum*, *F. subglutinans*, *F. begoniae*, *F. bulbicola*, *Fusarium* spp. from *Zea mays*, *F. guttiforme*, *F. sterillihyphosum*, *Fusarium* spp. from ornamental grass and *F. konzum*. BP's for subclades in Clade 1 ranged from 69% to 83%. Clade 2 (100% BP (Bootstrap support)) contained *F. verticillioides* and *F. psudocircinatum* clustering together (100% BP). This clade clustered with *F. nygamai* (51% BP) and *F. thapsinum*. Clade 1 and Clade 2 grouped together (63% BP) and separate from Clade 3. Clade 3 (100% BP) contained *F. concentricum*, *F. proliferatum* and *F. fujikuroi* which clustered together (56% BP) as well as *F. sacchari* and *F. mangiferae* clustering together (62% BP).

Of the 1262 aligned β tubulin characters used, 85 were informative and resulted in 123 MPT's (TL = 245 steps, CI = 0.8, RI = 0.86) (Fig. 2). These data also separated the isolates into three well supported clades (<99% BP). Clade 1 and Clade 3 grouped together (91% BP). All three clades contained the same taxa as those in the IGS MPT's.

The EF1α dataset consisted of 750 characters including alignment gaps of which 94 characters were parsimony informative. Analysis of this dataset yielded 13 MPT's (TL = 370 steps, CI = 0.69, RI = 0.67) (Fig. 3). Overall groupings among the taxa were similar to those inferred from the IGS and β tubulin data. Clade 1 (100% BP) again consisted of the same species as in Figs 1 and 2. However, based on the EF1α sequences *F. konzum* grouped closely together with *F. fujikuroi* (90% BP) in Clade 3. Apart from this, Clade 3 (73% BP) contained the same species as in Figs 1 and 2. Furthermore, in contrast to the IGS and β tubulin, Clade 2 could not be inferred from the EF1α data. A clade somewhat resembling the IGS and β tubulin Clade 2 could be reconstructed (69% BP), but it excluded *F. thapsinum*.

The histone 3 dataset consisted of 500 aligned characters of which 68 were informative. Parsimony analysis of this dataset yielded 12 MPT's (TL = 221 steps, CI = 0.69, RI = 0.71) (Fig. 4). The basic clustering pattern of these trees corresponded with those of the IGS MPT's. Clade 1 (94% BP) was separated into two subclades. The first subclade (75% BP) contained *F. circinatum*, *F. begoniae*, *Fusarium* sp. (MRC 1077), *F. subglutinans*, *F. konzum* and *F. bulbicola*, while the other subclade (73% BP) contained *F. guttiforme*, *F. sterillihyphosum* and *Fusarium* sp. (MRC 6747). Clade 2 (86% BP) and

Clade 3 (98% BP) contained the same taxa as the respective clades of the IGS and β tubulin MPT's. However, different to the IGS topology, Clades 2 and 3 grouped together, albeit with low support (64% BP).

The partition-homogeneity test showed that data from the loci sequenced in this study could not be combined ($p<0.05$). Some authors, however, suggested that the partition-homogeneity and the Templeton tests are inordinately stringent, and that trees from combined data can still be phylogenetically accurate where $p>0.001$ (Cunningham, 1997; O'Donnell et al., 2000; Dettman et al., 2003). The p-value for combinations of various partitions in this study ranged between $0.03>p>0.001$. Furthermore, since the overall topologies of single locus trees were highly similar a combined analysis of the four datasets was undertaken.

The combined dataset consisted of 5549 characters of which 773 were informative. From this data a single MPT (TL = 3190 steps, CI = 0.66, RI = 0.62) was inferred (Fig. 5). In the combined analysis Clades 1, 2 and 3 are supported by 100% BP values. Clade 1 and 3 grouped together (69% BP), which is similar to the results obtained from the β tubulin dataset (Fig. 2). In general, the groupings received significant support values (>70%). The only exceptions were the support values for the clade joining Clades 1 and 3 , *F. sacchari* and *F. mangiferae* grouping together and *F. concentricum* grouping together with *F. proliferatum* and *F. fujikuroi*.

DISCUSSION:

In this study we have shown that the MPT generated from the combined sequence information for four regions (IGS, β tubulin, EF1α and H3) was sufficiently informative to separate species in the *G. fujikuroi* species complex and *F. subglutinans sensu lato*. The phylogeny inferred from combined data indicates that Clades 1 and 3 share a recent common ancestor. Within the *G. fujikuroi* complex, our data, therefore, suggests that Clade 2, the so-called 'African Clade' of O'Donnell et al. (1998) represents an ancestral linage.

In previous studies, protein coding gene sequence data have been used to show that the species in *F. subglutinans sensu lato* group naturally into three clades with high bootstrap support (O'Donnell et al., 1998a; 2000; Steenkamp et al., 1999; 2000). The phylograms presented in this study for the partial β tubulin, EF1α and histone 3 genes show similar groupings, although in some cases the clades had low bootstrap support. This implies that single protein coding genes on their own are not sufficient to fully resolve groups in the species complex.

For this study one ribosomal DNA locus was selected for DNA sequence comparisons. In previous studies it has been shown that ribosomal DNA is unsuitable for phylogenetic studies of the *G. fujikuroi* species complex, due to low transition/transversion ratios (O'Donnell et al., 1998a; 2000; Peterson and Logrieco, 1991) as well as multiple alleles (e.g. the internal transcribed spacer region) in ribosomal sequences (O'Donnell et al., 1997). However the IGS locus is highly variable and our results show that it is the most effective locus that can be used in separating lineages of *F. subglutinans sensu lato*. However, when applied alone, this DNA region is not sufficient to reconstruct the evolutionary history of this complex. This is because the tree generated using IGS data is not entirely similar to that inferred from the combined data and BP's are lower.

Results of this study show that there are highly variable regions within the IGS of *F. subglutinans sensu lato* and PCR based diagnostics have already been developed based on this region. Schweigkofler et al. (2004) and Hinojo et al. (2004) used this region to develop highly effective diagnostic primers for the identification of *F. circinatum* and *F. verticillioides* respectively. Our IGS data suggest that it would be possible to develop species-specific diagnostic PCR assays for all species within the *F. subglutinans sensu lato* species complex including all the defined biological species included in this study (Fig. 6). Furthermore our study shows that the diagnostic tests developed for *F. circinatum* (Schweigkofler et al., 2004) will accurately differentiate this important pathogen from all other phylogenetic species in *F. subglutinans*. This was not conclusively shown in previous studies because not all *F. subglutinans sensu lato* species were included in the analyses.

According to O'Donnell et al. (1998; 2000), the three clades into which taxa in the *G. fujikuroi* species complex are grouped, represent an 'American clade' (corresponds to Clade 1 of the IGS tree in this study), an 'African clade' (Clade 2) and an 'Asian clade' (Clade 3). Based on this hypothesis, *F. konzum*, which was isolated from North American prairie grass (Zeller et al., 2003), should fall into the 'American clade'. When part of the EF 1α is analyzed, *F. konzum*, however, falls into the 'Asian clade'. In support of our data, Leslie et al. (2004) showed that *F. proliferatum* which falls into the 'Asian clade' is the predominant species on North American prairie grass and, therefore, probably native to that area. The designation of the different clades into biogeographic regions is, therefore, misleading and should be used with caution or abandoned altogether.

In this study we were able to demonstrate that the phylogenetic species concept is useful in the resolution of taxa in the *F. subglutinans sensu lato* species complex. In some cases neither the biological nor the morphological species concepts are sufficient to differentiate between these taxa. The IGS is a long non-coding region with a large number of characters that differ between the species studied. It would, therefore, provide an ideal region from which to develop species specific PCR primers for rapid and reliable diagnostic tools. Given the importance of many of the fungi as pathogens, these diagnostic tools should be actively pursued and tested.

ACKNOWLEDGEMENTS:

We are grateful to the members of the Tree Pathology Co-operative Programme (TPCP), the National Research Foundation (NRF) and the THRIP support programme of the Department of Trade and Industry for financial support.

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Table 1: Isolate numbers, specific relevance, host and geographic origin of the species included in this study.

Species	Relevance	Isolate Number*	Host	Geographic origin
F. circinatum	*F. subglutinans sensu lato* / MP H	MRC 7541 / FCC 2954	*Pinus radiata*	United States
F. concentricum	*F. subglutinans sensu lato*	MRC 7540 / FCC 3106	*Musa sapientum*	Costa Rica
F. begoniae	*F. subglutinans sensu lato*	MRC 7542 / FCC 2414	*Begonia* hybrid	Germany
F. bulbicola	*F. subglutinans sensu lato*	MRC 7534 / FCC 2951	*Nerine bowdenii*	Germany
F. guttiforme	*F. subglutinans sensu lato*	MRC 7539 / FCC 2994	*Ananas comosus*	Brazil
F. pseudocircinatum	*F. subglutinans sensu lato*	MRC 7536 / FCC 2949	*Solanum* sp.	Ghana
F. sacchari	*F. subglutinans sensu lato* / MP B	MRC 6525 / FCC 713	Laboratory cross	
F. subglutinans sensu stricto	*F. subglutinans sensu lato* / MP E	MRC 6512 / FCC 712	*Zea mays*	United States
Fusarium spp.	*F. subglutinans sensu lato*	MRC 1077 / FCC 1117	*Zea mays*	South Africa
F. sterillihyphosum	*F. subglutinans sensu lato*	MRC 2802 / FCC 2955	*Mangifera indica*	South Africa
F. mangiferae	*F. subglutinans sensu lato*	MRC 2730 / FCC 2956	*Mangifera indica*	South Africa
Fusarium spp.	*F. subglutinans sensu lato*	MRC 6747 / FCC 1093	Ornamental grass	South Africa
F. thapsinum	Mating population F	MRC 6536 / FCC 2428	*Sorghum bicolor*	South Africa
F. verticillioides	Mating population A	MRC 6155 / FCC 2432	*Zea mays*	United States
F. nygamai	Mating population G	MRC 7548 / FCC 4161	*Oryza sativa*	Unknown
F. proliferatum	Mating population D	MRC 6568 / FCC 2427	*Sorghum bicolor*	United States
F. oxysporum	Outgroup	MRC 6212 / FCC 3098	*Pinus radiata*	South Africa
F. fujikuroi	Mating population C	MRC 6570 / FCC 2426	*Oriza sativa*	Taiwan
F. konzum	Mating population I	FCC 3267	Prairie grass	United States

* MRC: Stored in the culture collection of the Medical Research Council, Tygerberg, SA
FCC: Stored in the culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, SA

Table 2: Primers and PCR conditions used for the four loci analyzed in this study.

Locus	External Primers	Internal Primers	$MgCl_2$	Annealing Temperature	Reference
B Tubulin	Bt 2A Bt 1B	Bt 2B Bt 2BR	2.5mM	56°C	Glass and Donaldson, 1995
Elongation Factor 1α	Ef-1 Ef-2		1.5mM	62°C	O'Donell et al., 1998
Histone 3	H3 1A H3 1B		2.5mM	56°C	Glass and Donaldson, 1995
Intergenic Spacer Region	NL11 CNS1	GCNS7f GCNS7r	2.5mM	55°C	Schweigkofler et al., 2004

Fig. 1: One of two most parsimonious trees (MPT's) inferred from the IGS nucleotide dataset for *F. subglutinans sensu lato*. Bootstrap values based on 10 000 replications are indicated above the internodes. The tree is rooted to *F. oxysporum*.

Fig. 2: One of 123 MPT's inferred from the β tubulin nucleotide dataset for *F. subglutinans sensu lato*. Bootstrap values based on 10 000 replications are indicated above the internodes. The tree is rooted to *F. oxysporum*.

Fig. 3: One of 13 MPT's inferred from the EF 1α nucleotide dataset for *F. subglutinans sensu lato*. Bootstrap values based on 10 000 replications are indicated above the internodes. The tree is rooted to *F. oxysporum*.

Fig. 4: One of 12 MPT's inferred from the histone 3 nucleotide dataset for *F. subglutinans sensu lato*. Bootstrap values based on 10 000 replications are indicated above the internodes. The tree is rooted to *F. oxysporum*.

Fig. 5: The single MPT inferred from the combined IGS, β-tubulin, EF 1α and histone 3 nucleotide datasets for *F. subglutinans sensu lato*. Bootstrap values, based on 10 000 replications are indicated above the internodes. The tree is rooted to *F. oxysporum*.

	761	821
F. circinatum	GGTAGAA---TCTC-A-----------GTTTC-GTCGCT-ACAGTTT-GCCATAGGTGT	
F. begoniae	GGTAGAA---TCTC-A-----------G-TTCTGTCGCT-ACGGTTT-GCTATAGGTGT	
F. bulbicola	GGTAGAA---T-TCCA-----------ATTTC-GTCGCT-ACATTTT-GCTATAGGTGT	
F. guttiforme	G---GAAGAGTCTC-A-----------ATATC-GTCACT-ACAGTTT-GTCATAGGTGT	
F. subglutinans	GGTAGAA---TCTC-A-----------ATTTC-GCTGCT-ACAGCTT-GCCATAGGTGT	
Fusarium sp.MRC 1077	GGTAGAA---T-TCCA-----------GTTT-TGTTGCT-ACA-TTTGGCTATAGGTGT	
F. sterillihyphosum	GGTAGAA---TCTC-A-----------ATTTC-GTCGCT-ACAGTTT-GCCATGGGTGT	
Fusarium sp. MRC 6747	GGTAGAA---TCTC-A-----------ATTTC-GTTGCT-ATAGCTT-ACCATAGGTGT	
F. konzum	GGTAGAA---TCTC-A-----------ATTTC-GTTGCT-ATAGCTT-ACCATAGGTGT	
F. thapsinum	GTAGAA----T-TCCA-----------GTTTC-GTCGCGGAAAGATC-GCAGCAGGTGT	
F. pseudocircinatum	G--CGA---GTTTCCA-----------GTTTC-GTCGCT-ATGGATT-GCCATAGGTGT	
F. verticillioides	G--CGA--GTTTCCA------------GTTTC-GTCGCTGATGGATT-GCCATAGGTGT	
F. nygamai	GTAGAA----TTCGA------------GTTTC-GTCGCT-ACAGTTT-GCCATAGGTGT	
F. proliferatum	G--CAA---GTTC-TTTCTCTACCAGATCGATTGGC--AATAA--ATAT---AGA-GTCT	
F. concentricum	GG-CAA---GTTC--TTTACCA-GAAATCGATTGGC--AGTA-AGATAT---AT-GGTCT	
F. sacchari	GG-CAA---GTTC--TTTACCA-GAAATCGATTGGC--AGTA-AGATAT---AT-GGTCT	
F. mangiferae	GG-CAA---GTTC--TTTACCA-GAAATCGATTGGC--AGTA-AGATAT---AT-GGTCT	
F. fujikuroi	GG-TAA---GTTCTCTCTACCA-GAAATCGATTGGC--AATA-GGATAT---ATA-GTCT	
F. oxysporum	AGTAGAA----TCCGA------------GTTTC--GTCGCCGACAGTTTTCTGTGGTGT	

	1427	1474
F. circinatum	TGAATGTATGATTATGTAAGCTGTATAGCTCTAGGGTAGGTAAAAAT	
F. begoniae	TGAATCTATGATTATGTAAGCTGTGCTACTCTAGGGTAGGTAAAAAT	
F. bulbicola	TGATTTTATGATTGTGTACGCTACATTACTCTAGGGTAGGTGAAAAT	
F. guttiforme	TGAATCTACGATTATGTAAGCTGTGCTACTCCAGGGTAGGTAAAAAT	
F. subglutinans	TGAATGTATGATTATGTAAGCTGTATAGCTCTAGGGTAGGTAAAAAT	
Fusarium sp.MRC 1077	TAAATATGTGATTATGTAAGCTGTATAGCTCTAGGGTAGGTAAAAAT	
F. sterillihyphosum	TGAATCTATGATTATGTAAGCTGTATAGCTCTAGGGTAGGTGAAAAT	
Fusarium sp. MRC 6747	TGAATCTATGATTATGTAAGCTGTGCTACTCCAGGGTAGGTAAAAAT	
F. konzum	TGGATTAATGATTATGTAAGCTATATAACTCTAGGGTAGGTGAGAAT	
F. thapsinum	TGAATTTATGAATATATAAGCTGTATTACTCTAGGGTAGGTGAAAAT	
F. pseudocircinatum	TGAATCTATGATTATATAAGCTATACTGCTCTAGGGTAGGTGAAAAT	
F. verticillioides	TGAATCTATGATTATATAAGCTATACTGCTCTAGGGTAGGTGAAGAT	
F. nygamai	TGAATCTATGATTATATAAGCTGTATTTCTCTAGGGTAAGTGAAAAT	
F. proliferatum	TGAATTTATGATTATATAAGCTGGATAGCTCTAGGGTAGTTGAAAAT	
F. concentricum	TGAATTTATGATTATATAAGCTGGATAGCTCTAGGGTAGTTGAAAAT	
F. sacchari	TGAATGTATGATTATGTAAGCTGTATAGCTCTAGGGTAGGTAAAAAT	
F. mangiferae	TGAATATATGATTACATAAGCTGTACTGCTCTAGGGTAGGTGAAGAT	
F. fujikuroi	TGAATCTATAATTGTATAAGCTAGACTACTCTAGGGTAGGTGAAAAT	
F. oxysporum	TTAATTTACGATTACATGATCTGCGTCACTCTAGGGTAGGTGAAAAT	

Fig. 6: Alignments showing sequence differences between isolates of the IGS gene at position 761 to 821 and 1427 to 1474 in the alignment used for phylogenetic analysis.

SUMMARY

Pine seedling root disease and damping off is one of the most important diseases affecting the South African forestry industry. The primary goal of this project was to address different questions regarding the ecology and epidemiology of *Fusarium circinatum*, which is the primary pathogen responsible for this problem. Although intensive research has treated the taxonomy of the fungus, very little is known regarding the biology and epidemiology in South Africa. This is also particularly important given the unusual fact that *F. circinatum* is found only in nurseries in the country and not on mature trees, as is the case elsewhere in the world. In the introductory chapter of this thesis, a review of the current knowledge of the pitch canker fungus is presented. This review focuses primarily on the biology, ecology and epidemiology of *F. circinatum*. Gaps in research are identified and considered as a prelude to studies presented here. The effect of increasing *F. circinatum* spore concentrations was shown to follow the Michaelis-Menten function. Environmental and plant physiological factors as well as different wounding methods influence the gradient of the curve as well as the maximum level of disease that can occur in any genetically heterogenous subset of hosts. In addition the effect of temperature and ambient humidity on the epidemiology of *F. circinatum* in South African pine nurseries has been investigated. The effect of humidity on disease incidence was minimal, however, results from this study indicate that temperature might influence the infection process. *Fusarium* circinatum was shown to survive and grow as a saprophyte in soil. Parameters such as content of organic matter in the substrate, growth temperature as well as humidity of the substrate influences growth and survival of the fungus. A real-time PCR based technique was developed to allow rapid identification of *F. circinatum* and other *Fusarium* spp. that are prevalent in South African pine seedling nurseries. In addition, a multi-gene phylogeny of the species represented by *Fusarium subglutinans sensu lato* as well as the *Gibberella* mating populations is presented. For this investigation four loci were sequenced and analysed. Results clearly show that the phylogenetic species concept is the most appropriate for classifying taxa in the *F. subglutinans sensu lato* species complex. *Fusarium circinatum* is an interesting and versatile tree pathogen. Research presented in this thesis contributes towards a better understanding of the epidemiology, biology and ecology of this fungus.

University of Pretoria etd – Hammerbacher, A (2006)

OPSOMMING

Denneboom saailing wortel siekte en verwelking is een van die mees belangrike siektes in die Suid Afrikaanse bosbou industrie. Die primere doel van hierdie projek was om verskillende vrae oor die ekologie en epidemiologie van *Fusarium circinatum*, die patogeen wat verandwoordelik is vir die probleem, te beantwoord. Ten spyte van die feit dat die taksonomie van die fungus in diepte behandel is, is daar min kennis oor sy biologie en epidemiologie in Suid Afrika. Die navorsingsdoel is spesifiek van belang omdat net saailinge in Suid Afrika geaffekteer word deur die siekte ten spyte van die feit dat in ander wereld dele ouer bome deur die patogeen bedryg word. In die inleidene hoofstuk van hierdie tesis word ‘n samevatting van die huidige kennis oor *F. circinatum* gegee waar spesifiek gekonsentreer word op die biologie, ekologie en epidemiologie van die swam. In die eerste navorsings hoofstuk word gewys dat die effek van toenemende spoor konsentrasies deur die Michaelis-Menten vergelyking beskryf kan word. Verder is gewys dat biotiese en abiotiese faktore aanleiding gee tot verskille van die gradient van hierdie kurve. Bestudeering van die effek van temperatuur en humiditeit op *F. circinatum* siekte ontwikkeling toon ‘n geringe verband met humiditeit maar ‘n moontlike temperatuur invloed. In die tesis word daar gewys dat *F. circinatum* as ‘n saprofiet in grond kan groei en oorleef. Parameters soos organisie materiaal in die substraat, groei temperatuur en voginhoud van die groei medium het ‘n duidelike invloed op die saprofietiese fase van die swam. ‘n PKR tegniek is ontwikkel vir vinnige en doeltreffende identifikasie van *F. circinatum* en ander *Fusarium* spesies wat gereeld in denneboom kweekerye aangetref word. Spesies in die *Fusarium subglutinans sensu latu* en die seksueel vrugbare populasies in die *Giberella fujikuroi* spesies kompleks, word ook in hierdie tesis deur multigeen filogenie ge-analiseer. Die resultate van hierdie werk wys dat die filogenetiese spesies konsep die mees doeltreffende klassifikasie methode is vir taxa in *F. subglutinans sensu lato*. *Fusarium circinatum* is ‘n interessante en veelsydige boom patogeen. Navorsing in hierdie tesis behoort bytedra tot groter kennis van die biologie, epidemiologie en ekologie van hierdie swam.