Biological Control of *Fusarium* Wilt of Pine Seedlings Using Endophytic Microorganisms and Silicon

By

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DISSERTATION SUMMARY

Globally, pitch canker fungus (*Fusarium circinatum*) poses a serious threat to the softwood forest industry. In South Africa, *F. circinatum* has characteristically been a nursery pathogen, causing a seedling wilt, and has attacked primarily *Pinus patula* and *P. radiata* seedlings and cuttings. With *P. patula* being the most commercially important species in southern Africa, this creates a substantial economic problem. There are no effective control methods to date. The overall study objective, therefore, was to isolate endophytic microorganisms from healthy pine seedling and seeds, and to screen these for activity against *F. circinatum* in pine seedlings. A second primary objective was to test soluble silicon (Si), applied as potassium silicate for its potential to control of *Fusarium* wilt of pine seedlings caused by *F. circinatum*.

For the study to be carried out, standard methodologies and procedures had to be followed, which included the isolation of the pathogen and endophytes that were used in experiments reported in the subsequent chapters. A pathogenic strain of *F. circinatum* was isolated. One hundred and fifty isolates of bacterial and fungal endophytes were also isolated from the seeds of seven different species of pine and 110 seedlings and cuttings of various species of pine.

For the successful screening of resistant cultivars, and control agents against *F. circinatum*, a reliable and representative artificial inoculation technique was needed. A study was undertaken to test various inoculation techniques, aiming to develop a reliable inoculation technique that would mimic the natural infection process of *F. circinatum* in the field, and to investigate the spore load required to initiate disease, without applying an excessive inoculum. Three inoculation techniques were tested

using six *P. patula* hybrids/species. These included drenching with a conidial suspension, injection of the crown with conidia, and a wounding technique as developed by FABI, University of Pretoria, which involves cutting off a few centimetres of the apical shoot and inoculating conidia onto the wounded tissue.

Using a haemocytometer, the spore load was adjusted to two inoculum levels, namely 10^2 and 10^6 conidia ml^{-1}. The concentration of conidia had a significant effect ($p<0.05$) on the Area Under the Disease Progress Curve (AUDPC). A concentration of 10^6 conidia ml^{-1} caused more severe *F. circinatum* symptoms and more severe disease. Inoculation techniques also had a significant effect on AUDPC ($p<0.001$). The highest infection levels were achieved when plants were wounded by cutting of the top or by injection. However, drenching was a simple and reliable inoculation technique. The interactions between concentration of conidia and inoculation techniques was not significant ($p>0.05$). Choice of *P. patula* hybrid had a significant effect ($p<0.05$) on the AUDPC. There was a significant interaction ($p<0.05$) between the hybrids and the inoculation technique, where drenching was more effective at discriminating the different levels of resistance of the six tested hybrids. Wounded seedlings were all equally diseased, which reflects the failure of these two inoculation techniques to provide satisfactory inoculation.

Endophytic microorganisms that were isolated from selected healthy pine seedlings, cuttings and seeds were screened for their potential as biological control agents against *Fusarium* wilt. Young *P. patula* seedlings were drenched weekly for four weeks with 5 ml of each endophyte (10^6 c.f.u ml^{-1}). A pathogenic strain of *F. circinatum* was then inoculated onto the plant and the plants were subjected to drought stress for a

week. The primary screening of the endophytes produced up to 60% reduction of the disease. The eighteen best endophyte isolates were selected for further screening. In the secondary screening, Isolates E56, E8 and E51 were the most effective biological control agents, while Isolate E85 was the least effective. Isolates E 141, E12, E13 and E27 provided a limited but significant level of control.

A further study was undertaken to determine the most effective concentration of silicon (Si), in the form of a 21% potassium silicate solution, to promote growth and reduce *F. circinatum* disease severity. Plants were treated with 100 mg l^{-1}, 200 mg l^{-1} and 400 mg l^{-1} Si for a period of four weeks. The plants were then challenged with a pathogenic strain of *F. circinatum*. The best concentration for disease control was 100 mg l^{-1}. This concentration reduced the final disease level by 53.3%. In the absence of Si, the disease level of *F. circinatum* was higher (1762 AUDPC units and 100% final disease level), but with the application of 100 mg l^{-1} of Si, the disease severity decreased by more than 50% (875 AUDPC units). Any increase in the Si concentration (200 and 400 mg l^{-1}) diminished the effectiveness of the Si, with the levels of disease increasing (1377 and 1435 AUDPC units, respectively). With no Si applied, the dry weight of pine seedlings was low (15.28 g), but this increased to 27.53 g with the application of 100 mg l^{-1} Si. The dry weight of plants treated with 100 mg l^{-1} of Si was greater than the untreated control.

DECLARATION

I, Bomikazi N Gqola, declare that

i. The experimental work in this dissertation was carried out under the supervision of Professor Mark D Laing and Dr Kwasi S Yobo.

ii. The research reported in this dissertation, except where otherwise indicated, and is my original work.

iii. This dissertation has not been submitted for any degree or examination at any other university.

iv. This dissertation does not contain other person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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   a) their words have been re-written but the general information attributed to them has been referenced;

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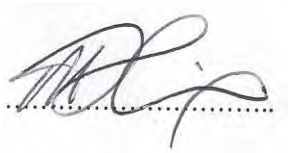
Signed: 

Prof. Mark Laing

Signed: 

Dr. Kwasi Sackey Yobo

PREFACE

This dissertation is made up of six chapters. The dissertation introduction and chapter 1 (literature review) introduce the reader to the fungal pathogen, *Fusarium circinatum,* and the problems it causes in the pine industry of South Africa. The literature review also identifies information gaps in efforts to control the pathogen though methods such as the use of resistant cultivars, endophytes and silicon (Si). This is followed by chapter 2, which describes the methods that were used to isolate and store both the fungal pathogen and the endophytes used in experiments reported in the subsequent chapters. Chapter 3 looks at the various inoculation techniques and sets out to find a technique that mimics the natural infection process of the pathogen in the field. Chapter 4 focuses on the interactions between endophytes and *F. circinatum*, and the use of endophytes as possible biological control agents. Chapter 5 looks at the effects of silicon fertilization on disease incidence and identifies the best Si concentration for the control of *F. circinatum*. Finally, a dissertation overview is given which reviews the outcomes of the research as a whole, and recommends further research derived from the outcomes of this body of research.

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I thank my father and siblings, most specially my youngest brother Dumo, for their love, support and understanding through challenges while working on this research.

DEDICATION

To my late mother, Liziwe Dorothea Gqola, who always aimed to be the best in everything she did.

And to my children, Montle and Nathan Jnr, who are the reason I wake up every morning. They inspires me to reach greater heights.

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GENERAL INTRODUCTION

The forestry industry in South Africa plays an important role in the South African economy. This is achieved mainly through the manufacture of solid timber and paper pulp products (Porter, 2010). About 50% of the timber production is from pine trees. The most important pine species are *Pinus patula* (Schl.et Cham.) and *P. elliottii* (Chapm.). These species are planted mostly in the North and South regions of Mpumalanga, the Eastern Cape and KwaZulu-Natal. Another pine species, *P. radiata* (D. Don.), is planted primarily in the Western Cape (Porter, 2010) and is not a major timber crop today.

Plant pathogenic fungi may cause losses in exotic pine plantations. These pathogens occur naturally in all forest ecosystems but native stands often exhibit very little or no obvious symptoms of tree damage from pathogens. This is partly due to long-term selection for compatible interactions between host and parasite and ultimately, the highly aggressive pathogens are compromised by diminished competitiveness of their preferred host (Aegerter and Gordon, 2006). This natural balance is absent in exotic plantations or in stands where non-native pathogens have been introduced. This results in increased damage and significant economic losses (Aegerter and Gordon, 2006). Such is the case with *Fusarium circinatum* (Nirenberg and O'Donnell) which is a pathogen attacking pine seedlings and adult trees globally. In South Africa, *F. circinatum* is mainly a nursery pathogen, attacking pine seedlings and causing a seedling wilt.

Fusarium circinatum is infectious on more than 47 species of pine, and one host which is not pine, *Pseudotsuga menziesii* (Mirb.) Franco (Douglas-fir) (Wingfield et al., 2008). The pathogen poses a threat in areas where pine species grow naturally, and to plantations in which non-native pine species are grown commercially (Wingfield et al., 2008). In South Africa the first incidence of *Fusarium* wilt was reported by Viljoen et al. (1994) from a nursery in the Mpumalanga Province. Subsequently, pine seedling nurseries across South Africa have experienced pine seedling wilt. Originally, the pathogen did not affect mature trees. However, with time it was noticed in established plantations of *P. radiate* on mature trees in the Western Cape (Coutinho et al., 2007).

Fusarium circinatum is believed to spread primarily by asexual conidia. The conidia can be dispersed by wind, rain, animals, insects or soil. The fungus can also be transmitted via infected seed. Infected seed may exhibit no symptoms until germination (Wingfield et al., 2008). South Africa, Chile and Uruguay are the countries in the Southern Hemisphere growing pine where *F. circinatum* has been encountered. To date, there are no known effective control measures and there are currently no registered fungicides globally. This has led to a demand for alternative control measures for use in South Africa.

Microorganisms that are found freely inside plant tissue without causing any disease are known as endophytes. They can be commensal or beneficial bacteria and fungi that are primarily found in plant intercellular spaces. They are ubiquitous and have been found in all species of plants (Backman and Sikora, 2008).

Some plants take up silicon (Si) in quantities equivalent to some macronutrients, although it is not widely recognized as an essential element. Silicon has emerged as an important mineral in the physiology of many plants, ameliorating a variety of biotic and abiotic stress factors (Bèlanger et al., 2003). It is also known for playing an important role in increasing host resistance to plant diseases and insect pests by priming the host defence reaction mechanisms (Bèlanger et al., 2003).

STUDY OBJECTIVES

The following study objectives were formulated:

1) To isolate one or more pathogenic strains of the causal agent of pine seedlings wilt, *F. circinatum*
2) To isolate a large number of endophytic organisms from a range of healthy pine seedlings, cuttings and seeds
3) To develop a reliable inoculation technique that would mimic the natural infection process of *F. circinatum* in the field
4) To establish an appropriate spore load / inoculum of the pathogen to initiate moderate levels of disease that could discriminate between different control measures;
5) To screen endophytic microorganisms for their potential to reduce the levels of seedling wilt caused by inoculation with *F. circinatum*, and
6) To determine the optimum concentration of silicon fertilizer to promote growth and to reduce the levels of *Fusarium* wilt in pine seedlings.

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CHAPTER 1: LITERATURE REVIEW

1.0 INTRODUCTION

Fusarium circinatum is a pathogen of global importance, attacking pine trees in most countries where they are grown. In South Africa, it is primarily a nursery pathogen affecting germinating seed, causing pre-emergent damping off, and a wilt of established seedlings (Coutinho et al., 2007). The conidia have the ability to remain viable for a minimum of three years within nurseries in the irrigation system, in seedling trays, under the tables and on debris (Viljoen et al., 1994). The fungus causes greatest loss in the pine industry because the pathogen affects the country's most commercially important species, *Pinus patula*. As a result, some nurseries have stopped growing this species. There are no effective control measures to date. The pathogen is becoming more problematic because it has also started to attack mature trees in pine plantations (*P. radiata* (Monterey pine) in the Western Cape and *P. greggii* (Engelm. Ex Parl.) (Gregg's pine) in the Eastern Cape (Coutinho et al., 2007).

In 2010, South African tree plantation occupied a total of 1.3 million ha, which comprised of 54% of pine, 40% eucalyptus, 8.6% to wattle and the remainder to other species (Godsmark, 2010).These are distributed in various Provinces within the country which are the Western Cape, Eastern Cape, KwaZulu-Natal, Mpumalanga and Limpopo (Godsmark, 2010). The 54% comprises of plantations predominantly *P. patula* (approximately 340 000 ha) and *P. elliottii* (approximately 179 000 ha) (Van der Sijde, 1994). Other pine species have been reported as planted on a small scale in the country, for example *P. radiata* (approximately 65 700 ha) in the Western Cape, *P. taeda* (approximately 65 000 ha) and *P pinaster* (approximately 38 000 ha), also in the Western Cape (Van der Sijde, 1994).

Pinus pinaster (laricio Savi, 1798) and *P. radiata* are mainly grown in the winter rainfall areas of the country while *P. patula*, *P. elliottii*, *P. taeda* L. and more recently the *P. caribaea* Morelet x *Pinus elliottii* hybrids are grown in the areas of the country where rainfall is expected in the summer (Gholz and Fisher, 1982). Of these species *P. patula* (45%), *P. elliottii* (27%), and *P. radiata* (9%) are the most important planted species (Hinze, 1993). *Pinus patula*, *P. elliottii* and *P. radiata* occur naturally in Mexico, Southern United States and California, respectively (Wingfield et al., 2008). Pines produce wood which is characteristically resinous, of low to medium density, with fibres that are easy to work with, making pine of economic importance (Gholz and Fisher, 1982).

1.2 THE MORPHOLOGY, ECOLOGY AND USES OF PINE

1.2.1 Morphological features of pine

Pines fall into the family *Pinaceae*. The morphological features of the pine tree have been described previously and extensively by Dvorak and Raymond, (1991). Nevertheless, in introducing the current study, it still appears pertinent to provide a summary of these descriptions. According to Dvorak and Raymond (1991), pines are evergreen and can grow between the range of 3 - 80 m tall, varying in species. Some have thick and scaly bark, while others have thin and flaking bark. The branches are produced as pseudo whorls, with some species producing one whorl of branches annually, while other produce two or more whole branches per season. Pines have both the male and female reproductive organs (cones) on the same tree, with the female cones taking a maximum of 3 years to reach maturity after pollination.

According to Richardson, (1998), pines have four different types of leaves depending on their stages of maturity. Such leaves include seed leaves which are the first 4-24 leaves found on the seedling, juvenile leaves that are produced by 6 months to 5 years old trees, scale leaves which are brown and non-photosynthetic and are arranged in a similar manner as the juvenile leaves, and the adult leaves, which are known as needles, and are produced for 1.5-40 years, depending on species. Once maturity is reached, the cones open to release the seeds (Richardson, 1998).

1.2.2 Ecology of the Pine

Depending on the species, pine trees can tolerate a range of different climatic, soil, and altitude conditions (Richardson, 1998). Certain species are found in soils that are acidic soils and some on calcareous soils (Richardson, 1998). Pine species require good soil drainage and prefer sandy soils, even though some can tolerate poorly drained, wet soils. After forest fires, some species are able to sprout and others need fire to regenerate; without fire, their natural populations slowly decrease. Species such as the Siberian Dwarf Pine, Mountain Pine and White bark Pine are adapted to the extreme conditions caused by elevation and latitude while the Pinyon Pine and several others are well adapted to growth in hot, dry, semi-desert climates (Richardson, 1998).

1.3. THE SOUTH AFRICAN PINE INDUSTRY

1.3.1 The Distribution of Timber Species in South Africa

In South Africa *P. patula* is the mostly planted species and its plantations cover about 338 923 hectares or 52.1% of the area growing softwood (Mitchell et al, 2011). The species is mostly grown in the Northern and Southern parts of Mpumalanga and in

KwaZulu-Natal and the Eastern Cape. *P. elliottii* makes up 27.2% of the softwood plantation area, and is widely distributed all regions except the Western Cape where *P. radiata* is planted almost exclusively (Mitchell et al, 2011). The total *P. radiata* area amounts to 58 127 hectares and is confined to the Western Cape Province. In South Africa, 51% of the timber were pine trees and 40% were *Eucalyptus* spp (Godsmark, 2010).

1.3.2 Revenue generated from the South African forestry industry

In South African plantations, 57% of the area is mostly grown for pulpwood production, 34% for sawlogs, 4% for mining timber and the remaining 4% for other uses (Godsmark, 2010). In the 2008/2009 year, 10 396 380 t were sold as pulpwood, 4 374 799 m³ as sawlogs and 430 788 t as mining timber. The total revenue received from the sale of the different timber products in South Africa is depicted in Fig. 3.

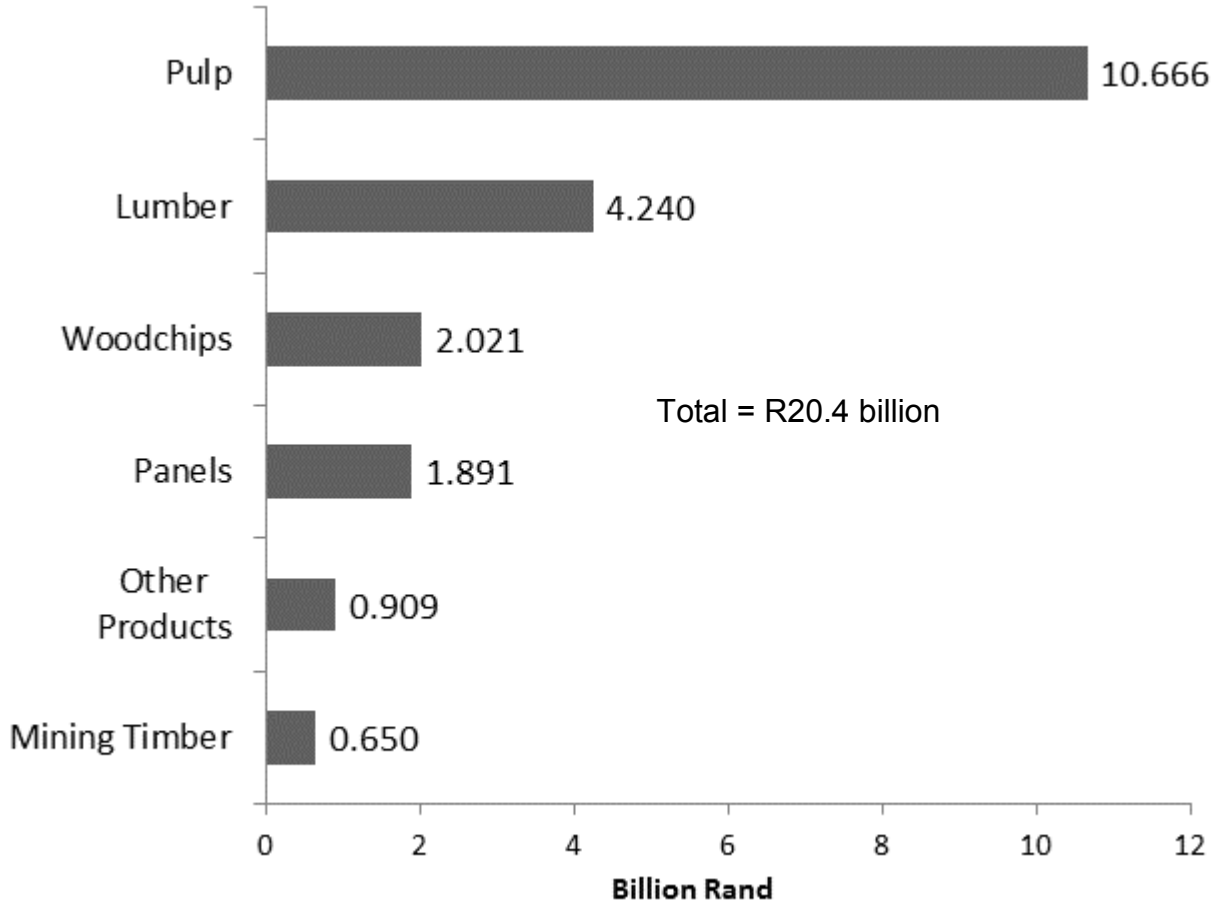

Figure 3: Value of Sales from Primary Processing Plants 2009 (Godsmark, 2010)

To the South African economy and a large number of poor people living in remote rural areas, the forestry industry is vital to their livelihoods. About 9% of the overall country's export of manufactured goods is contributed by the forest products. A total of 169 700 people are dependent on plantation forestry for their livelihoods (Godsmark, 2010).

Table 1: Forest Sector Employment in South Africa 2009 (Godsmark, 2010)

Sub -sector	No. of employees		Total Employment
	Direct	Indirect	
Forestry	66,500	30,000	96,500
Pulp and Paper	13,200	10,800	24,000
Sawmilling	20,000	10,000	30,000
Timber Board	6,000	n/a	6,000
Mining Timber	2,200	n/a	2,200
Other	11,000	n/a	11,000
Total	**118,900**	**50,800**	**169,700**

1.4 *FUSARIUM CIRCINATUM*

1.4.1 Introduction

The fungus, *F. circinatum* (Nirenberg and O'Donnell) is a serious pathogen affecting the pine industry worldwide (Pérez-Sierra, 2007). Hepting and Roth were the first to report the fungus in 1946 in North Carolina, USA. At least 47 *Pinus* species are known to be affected by the disease (Dick 1998). Since the first outbreak in 1946, the disease has spread worldwide. *Fusarium circinatum* is particularly devastating because it affects the tree at all developmental stages, from seeds, to seedlings, right through to mature trees (Pérez-Sierra, 2007).

Fusarium circinatum is a dynamic disease. There is a unique history with each outbreak in a particular area (Dwinell et al., 1985). This is due to several factors such as the pathogen and *Pinus* spp. interaction, and the specific abiotic and biotic environmental conditions encountered at the time of an outbreak. Depending on the outbreak and conditions prevailing at that time, there are different symptoms, varying

from reduction of germination of seeds (Huang and Kuhlman, 1990), seedling damping-off (Viljoen et al., 1994), seedling wilt of established seedlings in nurseries (Carey and Kelly, 1994), to mature tree shoot dieback (Corell et al., 1991), dieback of female flowers and mature cones (Barrows-Broaddus, 1990) and cankers of the trunks and branches mature trees (Dwinell et al., 1985). In several countries around the world, *F. circinatum* has gained global importance as a major pathogen of *Pinus* seedlings (Perez-Sierra et al., 2007).

In South Africa*, F. circinatum* was first identified in a forestry nursery in 1990 when it was observed to cause a root disease on *P. patula* seedlings and cuttings (Viljoen et al., 1994). Since then, the pathogen has spread to most pine-growing forestry nurseries throughout the country. It is currently the most important pathogen of *Pinus* spp. in seedling nurseries and forestry industry at large (Britz et al., 2005). According to Wingfield et al. (2002), *F. circinatum* is believed to have been introduced to both Chile and South Africa from contaminated seed stock.

1.4.2 Economic importance of *F. circinatum*

In the past two decades *F. circinatum* has evolved from a disease restricted to a particular region, to one of national and international importance (Dwinell et al., 1998). Pitch canker of pine causes heavy losses to the forestry industry in the world. Currently, it is regarded as the most serious pathogen of pine in the world (Bloomberg, 1981).

The disease probably gained entry into South Africa via infected seed from Mexico (Britz et al., 2001) because *P. patula* originates in Mexico, and is highly susceptible to

the fungus. All pines grown in South Africa are exotics, and there are three commercially important species planted: *P. patula* (45%), *P. elliottii* (27%), and *P. radiata* (9%) (Hinze, 1993). Since the initial outbreak in Mpumalanga, *F. circinatum* has spread to several other forestry nurseries in South Africa, causing a serious seedling wilt of various *Pinus* spp. (Britz et al., 2001). Wingfield et al. (2008) stated that the impact of the pathogen in the nurseries has extended beyond the direct losses to nursery plants. Such losses are also experienced during establishment of new plantations and this portrays the most important impact of *F. circinatum* in South Africa. The high incidence of *F. circinatum*-associated diseases in South African nurseries could be due to contaminated nursery containers, irrigation water, media and plant debris are also source of infection (Coutinho et al., 2007).

1.4.3 The biology of *F. circinatum*

One of the most complicated issues relating to the pitch canker fungus is that, based on the morphological characteristics alone, it cannot be identified with confidence. For this reason *F. circinatum* has undergone a number of name changes (Dwinell and Nelson, 1978). In 1945 a disease was observed on Virginia near Ashville, North Carolina, with a primary symptom of pitch flowing from cankers on the bark, which soaked into the wood. The disease was called pitch canker because of the flow of gum from the canker. A species of *Fusarium* belonging to the section *Liseola* (Hepting and Roth, 1946) was identified as the causal agent (Dwinell and Nelson, 1978). This was the first report of a *Fusarium* species causing disease in pines.

Due to the lack of chlamydospores and the presence of branching of conidiophores, Snyder et al. (1949) classified the fungus as *F. lateritium* Nees f.sp. *Pini* Heptig . In the

1970's, it was identified as *Fusarium moniliforme* Sheldon var *subglutinans* Wollenweber and Reinking in the section of Liseola (Kuhlman et al., 1978). Its macroconidia typically have three septa and are slender (Figure 1.1). Microconidia are oval to spindle shaped and form on monophialides and polyphialides in false heads, but never in chains (Hammerbacher, 2005). Nirenberg and O'Donnell (1998) described the pitch canker fungus as a distinct species, and therefore they proposed the name *Fusarium circinatum* Nirenberg and O'Donnell. *Fusarium circinatum* can produce distinctive coiled hyphae from which the label is derived. This description is close to that of many related species (Hammerbacher, 2005).

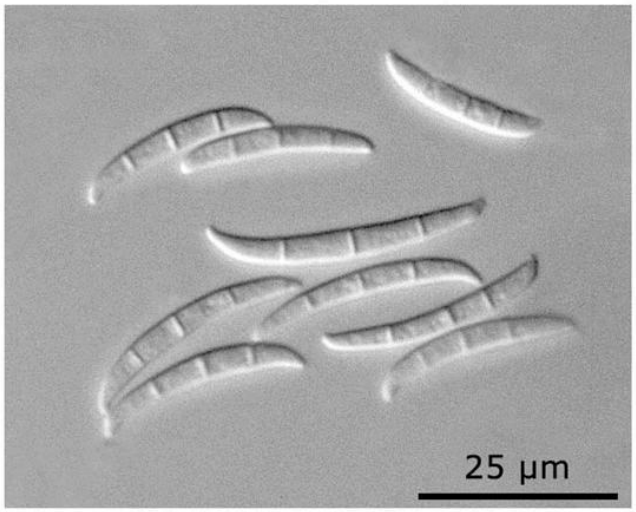

Figure 1: Macroconidia of *Fusarium circinatum*, showing three septa (Leslie and Summerell, 2006)

1.4.4 Disease cycle and epidemiology

Fusarium circinatum is well known to survive in and on pine seeds. Seed seems a likely medium of transport. Wingfield et al. (2008) proposed that *F. circinatum* reached South Africa from Mexico via infected seeds. Infections are initiated by microscopic

spores called conidia. In nurseries, initial inoculum could originate from sources such as wind and irrigation or from reused containers (Coutinho, 2007). Inoculum of *F. circinatum* is generated throughout the year. The pathogen requires a very short inoculation period, following the fungus infecting the host tissue; within four days the pathogen can be isolated from surface-disinfected or artificially inoculated pines (Barrows-Broaddus and Dwinell, 1983). In South Africa, since the problem is encountered at nursery level; airborne spores can enter the nursery through wind dispersal. The infection spreads in the nursery via the irrigation system and the wind, and via contaminated Speedling® trays serving as the primary source of inoculum transferred from one generation to the next (Viljoen et al., 1994). Viljoen et al. (1994) found that *F. circinatum* can survive in soil, and has the ability to infect seedlings at or just above the soil-line.

Fusarium circinatum can reproduce both sexually and asexually, and the alternating cycles of reproduction can affect the structure of the population (Leslie and Klein, 1996). The asexual stage of the fungus is *F. circinatum* and the sexual stage *Gibberella circinata* and is usually cross-fertile (Nirenberg and O'Donnell, 1998). Clonal propagation results from the asexual cycle whereas recombination of the genome in sexual reproduction leads to the creation of new genotypes result (Britz et al., 1998). Like many other ascomycetes, isolates of *F. circinatum* have clearly defined male-female roles, where only female fertile strains or so-called hermaphrodites are capable of reproducing sexual fruiting structures upon fertilisation (Leslie and Summerell, 2006).

South Africa has a low frequency of hermaphrodites of approximately 27%, which was observed during the initial introduction of *G. circinata* population in the country (Britz et al., 1998). For sexual reproduction to take place, both mating types have to be present at that particular location. In South Africa, when Britz et al. (2005) conducted a population study, he found that there was a comparatively high diversity of vegetative compatibility groups (VCG) of *F. circinatum* in the South African population. They found that a high frequencies of fertile female isolates and a reduced population of hermaphrodites.

1.4.5 Symptoms of the pathogen on pine trees

Fusarium circinatum infects both the vegetative and reproductive structures of susceptible pines at any stage of growth. Infection can extend as far as shoots, branches, cones, seeds, stems and exposed roots. At any time during the year, infection can occur. Prevailing biotic, abiotic conditions and geographic area at the time of the fungus occurrence determines the specific symptoms expressed (Gordon, 2006). Affected shoots of inoculated *P. virginiata* showed reddening of needles (Figure 2) and needle fall on infected branches, and also characteristics of shoot dieback. The initial symptoms of *F. circinatum* are usually wilting and discolouration of needles, which with time eventually turn red and fall off (Gordon et al., 2001). Gordon et al. (2001) stated that seedling dieback usually occurs from the tips of branches to the infection sites. This is attributed to the fact that the pathogen has the ability to clog the vascular bundles and in turn obstruct the water flow. The water stress can result in reduced growth due to loss of foliage.

Mortality of female flowers and mature cones and seed deterioration is associated with infection of reproductive structure with *F*. *circinatum* (Barrows-Broaddus, 1990). *F. circinatum* can cause deterioration in infected seed and can significantly reduce seedling emergence (Storer et al., 1998). As has been witnessed in South Africa, *F*. *circinatum* is capable of causing severe and extensive root disease of pine seedlings in nurseries. Viljoen et al. (1994) reported that *F*. *circinatum* causes pre- and post-emergence damping off of seedlings. Should pre- and post- emergence damping off happen, then the seed coats and coleoptiles of germinating seedlings are severely colonised by the pathogen (Viljoen et al., 1994).

Symptoms on young seedling present as pre- and post-emergence damping off; tip die back (Figure 4); roots are frequently underdeveloped (Figure 3) with discoloured lesions and necrosis of the cortex evident; and wilting. These are usually accompanied by a characteristic reddish brownish discoloration of the needles (Figure 2). Asymptomatic seedlings can harbour the pathogen as an endophyte, and this may lead to seedling infection remaining undetected (Storer et al., 1998). Disease caused by *F. circinatum* results in significant economic losses. Inoculation of young seedlings with the pathogen can cause almost 100% mortality (Viljoen et al., 1994). In South Africa where it affects nursery seedlings, the pathogen is associated with extensive seedling mortality (Figure 2).

Figure 2: Diseased seedlings in nurseries showing chlorotic or reddish brown needles (Gordon et al., 2001).

Figure 3: Pine seedlings showing extensive seedling mortality due to the presence of *F. circinatum* in the University of KwaZulu Natal Nursery. Picture taken by author (Ms Gqola).

Figure 4: Healthy roots (left) as compared to the infected roots that are symptomatic and under developed. Picture taken by author (Ms Gqola).

Figure 5: Infected pine seedling with tip dies back (called “shepherd's crook” while the shoot is still green, and the tip bends downwards). Picture taken by author (Ms Gqola).

1.4.6 Management Strategies for *F. circinatum*

1.4.6.1 Cultural control

At present there is no effective means of controlling *F. circinatum* in nurseries or forest plantations. Disease management is especially difficult because the pathogen is both airborne and soil-borne, and because both methods of spore dispersal are very effective. Several control methods have been studied for the control of the *F. circinatum*. The most common method of control is the removal of visibly infected or dying branches to reduce inoculum. In areas like South Africa where *F. circinatum* is problematic in nurseries, sanitation of soil, containers and infected material can be used to help control the disease, combined with the selection of disease tolerant genotypes (Wingfield et al., 2008).

The economic impact of *F. circinatum* can be reduced if an integrated management approach is implemented. Such management can include: quarantine measures, appropriate nursery and silvicultural management, and genetic selection for clones of species that are resistant or tolerant to the pathogen (Wingfield et al., 2008). To prevent the establishment of new strains of *F. circinatum* in areas where the pathogen is already present and entry into places that are currently free, some researchers have posited that quarantine measures are crucial to exclude the fungus (Dick, 1998). However, this is unrealistic in the real world of seedling nurseries, given the many ways that the conidia of the pathogen are spread.

As far as active, direct control of *F. circinatum* in seedling nurseries is concerned, little research appears to have been conducted. Wingfield et al. (2008) suggested that the primary and most important way to prevent infection in nurseries is the use of disease

free seeds. However, in most nurseries around South Africa, where the pathogen is already established, and sound nursery practices and the highest levels of sanitation have been proposed as the best way to prevent further diseases outbreaks in the future. The use of pathogen-free irrigation water, sterile growth media and seedlings trays and farm equipment. Rouging of diseased plants should reduce the level of inoculum of the pathogen (PFWG, 2004). However, these measures are still no guarantee that seedborne, airborne, waterborne or vector-borne inoculum of the pathogen will not enter the nurseries, and cause some level of disease in pine seedlings.

1.4.6.2 Chemical control

A variety of chemical control methods have been studied to control *F. circinatum* in pines. Fungicides that are widely used for the control of *F. circinatum* include prochloraz, tebuconazole and propamocarb (TPCP, 2002). Mitchell et al. (2005) conducted a study, in which he tested these fungicides and found that prochloraz and tebuconazole to be the most effective. During the same year in Chile González, (2005), found that tebuconazole and fludioxinil inhibited *F. circinatum* mycelial growth *in vitro* tests.

There have been reports of the control of *Fusarium* on seed by soaking the seed in diluted ethanol, hypochlorite, hydrogen peroxide, in hot water (90 sec at 55^0C) (Hoefnagels and Linderman, 1999). Dwinell and Fraedrich (1999) confirmed this approach on their study when they soaked *P. radiata* seed for 15 min in a 3% hydrogen peroxide solution and reduced internal seed contamination by *F. circinatum*. Due to

the endophytic characteristics of *F. circinatum* in seeds, no complete means of controlling the fungus has been found (Storer et al., 1998).

1.4.6.3 Biological control

According to Cook and Baker (1983) 'biological control' can be defined as a reduction of the amount of inoculum or disease by the activity of a pathogen, based on the use of natural enemies or the use of compounds derived from its metabolism. Biological control offers an alternative to agrochemical products. Various biological control strategies have been evaluated for the control of *F. circinatum*, involving antagonistic fungi or bacteria. Introduction of biological control agents into nursery production systems is a means of disease control and several commercial products are available.

A lot of research has been conducted, using fungi and bacteria to control diseases caused by *Fusarium* species affecting different crops (Bacon et al., 2001), some studies also include non-pathogenic strains of *Fusarium* spp. (Silva and Bettiol, 2005). To date few studies have been done on biological control of *F. circinatum*, this also include the use of *Trichoderma harzianum* to control the disease in *P. patula* seedlings (Mitchell et al., 2005). Moraga-Suazo et al. (2011) conducted a research on the use of *Trichoderma* spp. and *Clonostachys* spp. strains to control *F. circinatum* in P. *radiata* seedlings and found 15 strains of *Trichoderma* inhibited mycelial growth of the pathogen by more than 60% and one strain of *Clonostachys* showed parasitism of *F. circinatum* hyphae. Endophytes have been shown to confer enhanced growth, herbivore deterrence, and abiotic stress tolerance and disease resistance, although in some instances, the benefits are obtained at a cost in growth (Rodriguez et al., 2009).

1.4.6.4 Breeding for resistance

There is a clear need for the South African timber industry to breed highly resistant *Pinus spp.* as an alternative to the pine species and hybrids currently utilised by commercial forestry (Wingfield et al., 2002). For example, *P*. *elliottii* is less tolerant to *F. circinatum* than *P. taeda* (Hodge and Dvorak, 2000) and it is South Africa's second most important pine crop. It is therefore important to screen the South African *P. elliottii* selections for tolerance to *F. circinatum*. In South Africa there is a growing interest to develop hybrids between *P*. *patula* and other pine species to reduce the susceptibility of young *P. patula* seedlings to *F. circinatum*. Hodge and Dvorak (2006) observed that there is much genetic variation in provenances for tolerance to *F. circinatum* in both *P. patula* and *P. tecunumanii* (Eguiluz & J.P. Perry). Subsequently, hybrids of *P. patula* x *P. tecunumanii* have been bred and screened for resistance to *F. circinatum*.

There have been other successful hybridizations between *P. tecunumanii* and *P. patula* with a number of other species in South Africa. These species include: *P. oocarpa (*Schiede ex Schltdl), *P. elliottii*, *P. pringlei* (Shaw), *P*. *greggii*, *P. taeda*, *P. herrerae (*Martinez), *P*. *maximinoi* (H.E. Moore) and P. *caribaea* (Hodge and Dvorak, 2006). Mitchell et al. (2012) stated that the tolerance of *P*. *maximinoi* and *P. tecunumanii* from low elevation provenances to *F. circinatum* is high and require no screening to identify tolerant families for development. A number of these interspecies *Pinus* hybrids in the future may be planted because of good timber qualities, combined with resistance to *F. circinatum*.

In most cases, these alternative species or hybrids prefer to grow in subtropical and warm temperature locations. This poses a problem in the replacement of *P. patula* as it still remains the best specie for planting in cooler/ colder climates in South Africa (Hodge and Dvorak, 2006). For further use of *P*. *patula* in these regions, without being infected with *F. circinatum*, there would be a need to identify tolerant individuals that can be grafted in new seed orchards, or used in a controlled pollination program (Mitchell et al., 2012).

1.4.6.5 Use of silicon fertilization to control plant pathogens

Mineral nutrients play an important role in the resistance of plants to pests and diseases (Marschner, 2002). The severity of most plant diseases can be reduced by implementing improved nutrition management; this can be achieved by modifying the availability of particular nutrients or improving the efficiency of absorption and utilization by the plants (Hodson et al., 2005). Silicon has been identified as a beneficial nutrient for some, but not all, plants (Epstein, 1994). It is mostly important in reducing the impact of pests and diseases and buffering against abiotic stress (Bèlanger et al., 2003).

Some of the benefits of Si fertilization known for some crops (e.g., rice) include: increased growth and yield, improvement in some morphological characteristics, reduced transpiration and resistance to stress, resistance to salinity and toxic metal, effects on enzyme activity and increased resistance to pests and pathogens (Epstein, 1994) . A notable example of the protection of plants against pathogen due to Si is in rice where resistance is increased against blast, caused by *Magnaporthe grisea* (Datnoff et al., 2007). Walters and Bingham (2007) studied the interaction Si with both

monocots and dicots and confirmed Si's active role in the natural stimulation of defence reaction of the plant. Research on wheat (Bèlanger et al., 2003) and rice (Rodrigues et al., 2004) on the enhancement of defence mechanisms, in the presence of Si and fungal pathogens, indicated the capacity of Si to prime the biological active defence mechanisms of plants (Ton et al., 2009). Induction of the full resistance response itself requires the pathogen or pest to trigger the reaction (Currie and Perry, 2007).

1.5 ENDOPHYTES

Almost all classes of vascular plants researched to date have been found to host endophytic microorganisms. Endophytes are non-aggressive, often adopting a commensal role within their hosts. They colonise the host plant and become solely dependent for nutrients and protection (James and Olivares, 1997). In return they enhance host fitness by producing beneficial metabolites (Tan and Zou, 2001). Furthermore, endophytes can provide their host with protection against nematodes, and bacterial and fungal pathogens (Stone et al., 2004). Mostly studied endophytes are fungal, even though there are also bacterial endophytes. Fungal endophytes have been mostly studied in grasses and trees, on the other hand bacterial endophytes have most frequently been detected in leguminous plants (James and Olivares, 1997). Unlike mycorrhizal fungi that only colonize plant roots and grow largely in the rhizosphere, endophytes reside entirely inside plant tissues, and may be found within roots, stems and leaves (Stone et al., 2004).

1.6 EFFECTS OF INOCULATION TECHNIQUES USED IN *F. CIRCINATUM* EPIDEMIOLOGY ON PINE SEEDLING STUDIES

There is a lack of consensus concerning the most effective inoculation method for *Fusarium* spp. application to their hosts. A number of inoculation techniques have been developed to artificially replicate the many *Fusarium* × host interactions. Some of the widely practised inoculation techniques include: injection of conidia with a syringe, spraying with conidia, drenching with conidia (Kidane, 2004), and application of conidia to wounds. Wounds may be created in several ways, including: apical bud removal, fascicle removal, needle puncture, cutting a slit into a lateral shoot, and cutting off a lateral branch (Kuhlman, 1987). Given the epidemiology of the pathogen, to infect seedlings via their roots, the inoculation approach of drenching is probably the closest to the natural process, and therefore may have the greatest chance of creating representative and reproducible results.

1.7 CONCLUDING REMARKS

The production of pine trees is of importance to the world economy, and South Africa, in particular. The production of pine seedlings in South Africa has been severely affected by the arrival of *F. circinatum*. The pathogen causes mortalities of up to 100% in the seedlings of the pine species currently grown in South Africa. Whilst resistance breeding has been initiated, there are no effective means of controlling the fungus presently. There are no effective fungicides or bio-control agents at the moment. At present, the only control strategy available is avoidance, based on sanitation practices. However, these practices are expensive and difficult for nurseries to apply, and it is difficult to measure their efficacy, especially as the pathogen is often in a latent state with not obvious symptoms. Given the many ways that this pathogen spreads with

great efficiency, locally and globally, prevention alone cannot be expected to be successful. Active control measures are needed, therefore.

In recent years, interest in endophytic microorganisms has increased because they play a key role in the physiology of host crops, and could be manipulated to enhance the productivity of these crops. The use of endophytes and silicon fertilization has not been explored previously as potential control measures to be used against *F. circinatum*.

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CHAPTER 2: ISOLATION AND STORAGE OF ENDOPHYTIC MICROORGANISMS FROM VARIOUS PINE SOURCES AND OF *FUSARIUM CIRCINATUM* FROM DISEASES *PINUS PATULA* SEEDLINGS

Abstract

Standard methodologies and procedures were used to isolate the pathogen and endophytes that were used in experiments reported in the subsequent chapters. Three isolates of *Fusarium circinatum* were isolated using a *Fusarium*- selective medium. One hundred and fifty isolates of bacterial and fungal endophytes were also isolated from seven different species of pine seeds and 110 seedlings and cuttings of various species of pine. Seedlings of a hybrid of *P. patula* x *P. tecumanii* (low elevation) were the richest source of endophytes. All the *F. circinatum* isolates tested for pathogenicity proved to be highly pathogenic and confirmation tests done based on morphological characteristics confirmed the presence of the pathogen.

Keywords: *Fusarium circinatum*, endophytes and pathogenicity

2.1 INTRODUCTION

Fusarium is classified under the phylum Ascomycota. The genus is a ubiquitous fungus found in most environments (Leslie and Summerell, 2006). *Fusarium* species have been reported as endophytes, saprophytes and pathogens of various plants, especially crops of economic importance such as banana, beans and pine trees (Burgess et al., 1994). Endophytes are defined as organisms that at some time of their life cycle lives within plant tissue without producing any symptoms. In plants

endophytes can be bacteria, fungi, actinomycetes and mycoplasma (Burgess et al., 1994). A number of studies have been done on the use of fungi and bacteria to control diseases caused by *Fusarium* species affecting a broad spectrum of different crops (Bacon et al., 2001).

Due to environmental restrictions on fungicide use, this has led to an urgent need for alternative methods of disease control, such as biological control agents. For the purpose of this study which aimed at using endophytes isolated from pine as biological control agents, it was important to isolate endophytes from pine sources, and bulk and store the endophytes. Usually several to hundreds of endophyte species can be isolated from a single plant (Tan and Zou, 2001).

Study objectives:

1. To study endophyte communities within different *Pinus*.
2. To distinguish whether there is a variation in endophyte communities of older pine plants and if different endophytes are found in seed as opposed to cuttings and seedlings.
3. To determine if there is an equal distribution of fungal and bacterial endophytes within a plant tissue.

2.2 MATERIALS AND METHODS

2.2.1 Source of plant material

Pine seedlings of *P. patula*, ranging in age from 6 to 8 months old and showing *Fusarium* symptoms were obtained from a commercial nursery, 18.1 km from Pietermaritzburg, South Africa. Healthy pine seedlings of *P. patula* ranging from 2 to

4 months old were obtained from another commercial nursery, 24.3 km from Pietermaritzburg. Seeds used for endophyte isolation were obtained from Sappi (Pty) Ltd and comprised of the following species and hybrids: *P. patula, P. taeda, P. elliottii, P. tecunumanii, P. greggii, P. patula x P. oocarpa and P. patula x P. tecunumanii*. Various seedlings and cuttings were also obtained from Sappi (Pty) Ltd and these comprised of the following hybrids: *P. patula x P. oocarpa*, *P. patula x P. tecunumanii*, *P. patula* x *P. patula*, *P. elliottii* x *P. caribaea*, *P. patula x P. tecunumanii* (Low Elevation), *P. patula x P. tecunumanii* (High Elevation), *P. patula* x *P. greggii* var. *aus*, *P. patula* x *P. greggii* var. *greggii* and *P. patula x P. oocarpa*.

2.2.2 Isolation of the pathogen

Four *P. patula* seedlings which were showing a tip dieback and discoloration of needles symptoms, were washed to remove any adhered soil, and then the needles were cut off. Samples of 10mm in length were cut 15 mm above the root collar and pieces were surface sterilized in a 1 in 9 solution of 3% sodium hypochlorite (Jik^{R}) for 5 minutes. The samples were then removed from the sterilizing solution and washed three times in sterile distilled water. The samples were then put on sterile filter paper and placed on a laminar flow bench to dry. Both ends of each sample were then cut off and discarded. The samples were then cut into small pieces (approximately 2 mm in length). Four samples of each isolate were firmly placed onto an agar plate of *Fusarium* Selective Media (FSM), the petri dishes were then sealed with parafilm and then incubated for two days at 25 ^{0}C in an inverted position (Kidane, 2008).

2.2.3 Bulking and storage of the pathogen

Pure and dominant colonies from the FSM were re-isolated onto Potato Dextrose Agar (PDA). Conidia from dominant fungal colonies were transferred onto a drop of sterile water on a slide using a sterile transferring needle. The suspension of conidia was then viewed under a light microscope for the presence of typical *Fusarium* macroconidia. *Fusarium* isolates were then grown up on PDA medium. The conidia spores were spread on the plate using a hockey-stick. Blocks were cut from the agar plates and were placed in 10ml of sterile distilled water per agar plate, and stored in McCartney bottles containing double sterilized distilled water.

The pathogenic isolates were also stored on inoculated autoclaved barley seeds in 500 ml Erlenmeyer flasks. The method used was to soak barley seed in tap water over night. Subsequently the water was drained off, and the barley seed was placed in 20ml McCartney bottles and these were autoclaved twice with a 24 h resting period in between. Excised agar blocks of *Fusarium* cultures were inoculated onto the cooled barley seeds and the bottles were incubated at 25-28 ^{0}C. When grains were entirely colonized by the fungus, the bottles were stored in a cool and dust-free cupboard at room temperature (Kidane, 2008).

2.2.4 Preparation of pathogen inoculum

Inoculum of the three *F. circinatum* isolates stored on barley seeds was sub-cultured onto Oat Meal Agar (OMA) and incubated at 25 ^{0}C for 7-10 days. Inoculum, consisting of both conidia and mycelia, was obtained by flooding the agar plate with 150 ml distilled water and hockey sticking the surface. The conidial suspension was sieved

using double layered cheese cloth to remove any mycelium (Leslie and Summerell, 2006).

2.2.5 Pathogenicity test of *Fusarium* isolates

Screening of three *F. circinatum* isolates for their ability to cause disease in pine seedlings was performed in a polycarbonate greenhouse tunnel with temperatures ranging from 26 ^{0}C to 28 ^{0}C. The seedlings were *P. patula* aged three months old and grown in an artificial pine bark medium in sterile Speedling® 24 seedling trays. The seedlings were supplied with irrigation water supplemented with NPK soluble fertilizer (3:1:3) three times daily for 5 minutes. The experiment was arranged in a complete randomized blocks design, with ten replicates for each *Fusarium* isolate and the Control, there were 12 plants per replicate. The pine seedlings were drenched with a 5ml conidial spore suspension of 10^2 conidia/ml of the isolates of pathogen and then subjected to drought stress for 7 days (Kidane, 2008). The Control treatment received 5 ml of sterile distilled water.

2.2.6 Confirmation and identification of pathogen isolated

Post the pathogenicity tests recording of the obtained results occurred. Diseased *P. patula* plants that were showing symptoms that are typical to those caused by *F. circinatum* which are wilting, reddening of needles and the shepherd's crook were tested. The pathogen was re-isolated from these plants following the procedure mentioned above (2.3). Three days later a wet mount was performed by loading the fungal structures growing on the FSM on a microscopic slide, then viewed under a light microscope at a magnification of x1000 and these findings were used for morphological identification of the isolates (Kidane, 2008).

2.2.7 Isolation of endophytes

Seeds were surface sterilized in a 1 in 9 dilution of 3% sodium hypochlorite (Jik^{R}) for 5 minutes. The seeds were then removed from the sterilizing solution and washed thrice in sterile distilled water. The seeds were then placed on sterile filter paper, on a laminar flow bench to dry. The seeds were then cut into two halves. The pieces were placed on Nutrient Agar (NA) media and incubated for seven days at 25 ^{0}C (Bill, 1996). For the isolation of endophytes from seedlings and cuttings, a procedure was followed that was similar to that used for pathogen isolation. However, the samples were plated onto Tryptone Soy Agar (TSA) (Figure 2.1) for bacterial endophytes, while fungal endophytes were cultured in petri dishes containing potato dextrose agar medium (PDA) supplemented with 100 µg/ml of streptomycin. There after the petri dishes were sealed with parafilm and incubated at 30 ^{0}C for 4 days (Hallman et al., 2007).

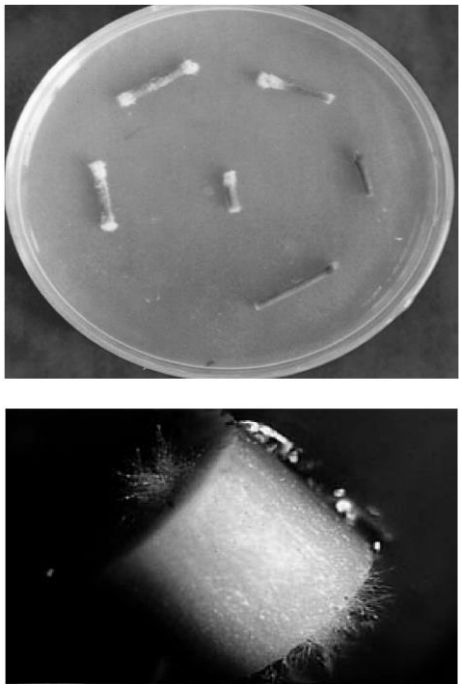

Figure 2.1: Endophytic mycelia growing out from cut areas of surface sterilised plant segments on an antibiotics-supplemented water agar plate. Upper: endophyte on

Artemisia annua L. stems; lower: endophyte on spruce wood (*Picea* sp. Mill.)

(Source: http://www.uni-tuebingen.de/uni/bbm/Forch/OiEndoE.html

2.2.8 Bulking and storage of endophytes

Clearly distinguishable bacterial colonies from both the seeds and seedlings were separated according to their shape, colour and elevation, and were sub-cultured on Potato Carrot Agar (PCA). All bacterial endophytes were stored in 30% glycerol at – 80 ^{0}C. The fungal endophytes were also bulked on PCA and stored in double sterilised distilled water, on half strength PDA agar slants and were placed in a cold room at 4 ^{0}C after the fungal colonies had grown on the agar (Kidane, 2004).

2.2.9 Preparation of inoculum of endophytes

Bacterial endophytes stored in 30% glycerol in water were cultured on PDA plates and incubated at 30 ^{0}C for four days. Colonies were transferred into double sterilized distilled water in 100 ml McCartney bottles and gently mixed by shaking the suspension to allow them to mix. Fungal endophytes stored on half strength PDA slants were cultured on PDA plates and incubated at 22 ^{0}C for 7 days. A conidial inoculum was obtained by flooding the agar plate with 150 ml distilled water. The suspension was sieved using cheese cloth to remove the mycelia (Leslie and Summerell, 2006).

2.3 RESULTS

Looking at the table below, one can notice that some seedlings and cutting produced very few fungal endophytes to none at all. On the other hand, *P. patula x P.*

tecunumanii (Low elevation) was the richest source of both fungal and bacterial endophytes isolated. This is shown in the table below.

Table 2.3.1 showing the number of both bacterial and fungal endophytes isolated from the seeds and the xylem tissue of healthy pine seedlings and cuttings, 6-8 months of age.

Source of Isolation	**Fungal endophytes**	**Bacteria endophytes**
Pine seeds mixture (various species)	5	5
[1]*P. patula*	5	5
[2]*P. taeda*	5	5
[1]*P. elliottii*	0	5
[1]*P. tecunumanii*	0	5
[1]*P. greggii*	0	5
[1]*P. patula x P. oocarpa*	5	5
[2]*P. patula x P. tecunumanii*	5	10
[2]*P. patula* x *P. patula*	0	5
[2]*P. elliottii* x *P. caribaea*	0	5
[1]*P. patula x P. tecunumanii* (Low Elevation)	20	25
[2]*P. patula x P. tecunumanii* (High Elevation)	0	5
[1]*P. patula* x *P. greggii* var. *aus*	0	5
[2]*P. patula* x *P. greggii* var. *greggii*	5	10
Total number	50	100

1 = seedlings; 2 = cuttings

2.3.1 Pathogenicity testing

All the studied *F. circinatum* isolates drenched on three months old *P*. *patula* seedlings were highly pathogenic. Symptoms observed were severe discolouration of needles and branch dieback. On the third week post drought stress, the disease level was up to 80% for all the isolates and there was no disease on the controls.

2.3.2 Confirmation and identification of pathogen isolated

The cultured *Fusarium*-like cultures on FSM isolated from diseased pine seedlings resembled those of *F. circinatum*. A fluffy white fungal structure was produced and the medium at the back of the petri dish produced a distinguished uniform dark pinkish-purple colour. When the microscopic slides carrying these isolates were viewed under a light microscope at a ×1000 magnification, coiled hyphae and macroconidia were observed. These characteristics are typical to those of *F. circinatum* and distinguish this specie from other species of *Fusarium* (Nirenberg and O' Donnell, 1998). With also DNA sequence comparisons for genes encoding translation elongation factor 1-α-and β-tubulin, FABI concluded that the fungus represent *F. circinatum*.

2.4 DISCUSSION

Every plant which grows on the earth is known to host at least one endophytic microbe. From the current study, a total of 150 endophytes were isolated which comprised of 50 fungal endophytes and 100 bacterial endophytes (Table 2.1). However, the table (2) also shows instances where there were no endophytes isolated from various species. The possible reasons to justify this occurrence is that the choice of growth medium which was used was not favourable for all the various types of microorganisms to grow.

On the other hand, research elsewhere has shown that the location of the host plant and the environmental factors surrounding the plant prior to the isolation, does affect the successful colonization and in turn isolation of the endophyte. In a study done by Procópio et al. (2009), on *Eucalyptus* plants, it was observed that there was a difference in the frequency of endophytic bacteria present among the various *Eucalyptus* species. These results then suggested that the host species has a major impact on the colonization and population size of endophytes. Research done by Elvira-Recuenco and Van Vuurde, (2000), Adams and Kloepper, (2002); Raja et al., (2008) on cotton and peas proved the suspicion (that the host does affect the endophytes colonizing it) in their findings that the colonization by endophytic bacteria was affected by the plant genotype. After all has been said, *P. patula x P. tecunumanii* (Low Elevation) could be said to have the necessary genes in this particular study for maximum endophytic population.

During pathogenicity tests, the isolated *Fusarium* species proved to be *F. circinatum*. The morphological characteristics such as coiled hyphae and the presence if macroconidia are those which Britz et al. (2001) states as the major morphological characteristics used to distinguish *F. circinatum* from all the other *Fusarium* species.

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CHAPTER 3: ARTIFICIAL INOCULATION OF *FUSARIUM CIRCINATUM* USING DIFFERENT INOCULATION TECHNIQUES

Abstract

For successful screening of the possible control measures against *Fusarium circinatum*, a reliable and effective artificial inoculation technique of *F. circinatum* is essential. The aim of this study was to develop a reliable inoculation technique that would mimic the natural occurrence of *F. circinatum* in the field, and to investigate the optimum spore load to initiate disease but not to overwhelm the host plant and any control measure. Three inoculation techniques were compared and screened on seedlings of six *Pinus patula* hybrids. This included the drenching of conidial suspension, injection of the crown and the standard wounding technique which is cutting a few centimetres off the growing tip. Using haemocytometer the spore load was adjusted to two inoculum levels namely 10^2 and 10^6 conidia ml^{-1}. The concentration of conidia had a significant effect ($p<0.005$) on the Area Under the Disease Progress Curve (AUDPC). At 10^6 conidia ml^{-1}, *F. circinatum* symptoms developed quicker and more severe disease developed. Inoculation techniques also had a significant effect on AUDPC ($p<0.001$). The highest infection levels were achieved when plants were wounded, by cutting off the top shoot, or by injection of conidia into the crown. Interactions between concentration and inoculation techniques were not significant ($p>0.05$). The host plant had a significant effect on the AUDPC, with *P. patula* seedling being the most susceptible. The interaction of concentration of conidia × pine varieties was not significant ($p>0.05$).

Keywords: drenching, conidia concentration, injection, pine hybrid, wounding

3.1 INTRODUCTION

South African pine nurseries suffer great losses due to the development of a wilt disease caused by the fungus *Fusarium circinatum* Nirenberg and O'Donnell (syn. *F. subglutinans f. sp. pini*; teleomorph: *Giberella circinata* Nirenberg and O'Donnell). This fungal pathogen is known to have a host range of about 47 species in the genus *Pinus*. Pathogenic strains of *Fusarium* have worldwide distribution and are well-known pathogens in forest nurseries in many parts of the world (Viljoen *et al*., 1992).

Pitch canker of pine was first detected in 1946 on Pinus Virginian Mill in North Carolina, USA (Hepting and Roth, 1946), and it later appeared on *P. elliottii* in Florida, *P. taeda* in Mississippi, and on *P*. *radiata* in its native range. In California, it caused a significant epidemic (Gordon et al., 2001). Disease incidence has been reported in Japan (Kobayashi and Muramoto, 1989), South Africa (Viljoen et al., 1994), Mexico (Guerra-Santos, 1999), South Korea (Lee et al., 2000), Chile (Wingfield et al., 2002), Spain (Landeras et al., 2005), and more recently, Italy (Carlucci et al., 2007), Uruguay (Alonso and Bettucci, 2009) and Portugal (Braganca et al., 2009). The movement of infected plant material, especially infected seeds, has led to long-distance dispersal of the fungus. Local dispersal of the pathogen is easily linked to the presence of abundant airborne inoculum (Correll et al., 1991).

A variety of symptoms are caused by *F*. *circinatum* on nursery seedlings. Typically it infects via the roots, occludes the vascular bundles and causes a variety of wilt symptoms, acting as a typical root-infecting pathogen (Dwinell et al., 1985). The fungus can survive in soil and may infect seedlings at or just above the soil-line (Viljoen

et al., 1994). It is essential to develop reliable greenhouse inoculation procedures for the screening control measures against *F. circinatum*.

Numerous techniques have been used to screen trees and seedlings for resistance to *F. circinatum*. The majority of these screening techniques involve wounding of trees. Seedlings have been wounded by cutting off of the growing tip (Hodge and Dvorak, 2000), bark removal (McCain et al., 1987; Viljoen et al., 1995), fascicle removal (Enebak and Stanosz. 2003) and the use of pins or small drill holes to puncture the stem (Barrows-Broaddus and Dwinell 1984; Matheson et al., 2006). Inoculation techniques that avoid wounding have also been tested. One of these studies involved drenching *F. oxysporum* f. sp *phaseoli* onto beans (Kidane, 2008). Smith et al. (1990) investigated the injection of inoculum of *Colletotrichum fragariae* into the crown of strawberries.

The aim of this study was to develop a reliable inoculation technique that would mimic the natural occurrence of *F*. *circinatum* in the field, and to investigate the required spore load to initiate a moderate level of disease to optimize the screening of control measures.

3.1.2 Study objectives

The objectives of this study were as follows:

1. To determine the effects of inoculation technique, pine hybrid type and concentration of inoculum (conidia) for *F*. *circinatum* on disease development in pine seedlings.
2. To determine the nature of the interactions between inoculation technique, pine

hybrid type and concentration of inoculum and effects on disease development in pine seedlings.

3.2 MATERIALS AND METHODS

3.2.1 Plant Material (Pine hybrids)

Six (6) months old seedlings of various hybrids of *P. patula* were obtained from a research nursery 30 km away from Pietermaritzburg. All hybrids were numerically named.

3.2.2 Production of inoculum

F. circinatum mycelium that had been stored on barley seeds was sub-cultured onto Potato Dextrose Agar aseptically using an inoculation loop. The PDA plates were then incubated at 30˚C for 7-10 days. By flooding the agar plates with 150 ml sterile distilled water, both the conidia and mycelium were collected. To remove the mycelium, a double layered cheese cloth was used to sieve the suspension. Conidia were counted using a haemocytometer and the concentration was adjusted to 10^2 and 10^6 conidia ml^{-1}.

3.2.3 Inoculation methods

3.2.3.1 Cutting off the apical shoot

Healthy pine seedlings were transplanted from a Speedling® 128 into a Speedling® 24 tray. The apical shoot of the seedling was then cut off using a pair of sterile scissors. Using a 10 ml sterile syringe, a drop inoculum (20 µl) of a conidial suspension of the pathogen was dispensed onto the wound. Plants were treated with two inoculum doses: 10^2 and 10^6 conidia ml^{-1}.

3.2.3.2 Drenching of plants

Seedlings of the *P. patula* hybrids were drenched with 10^2 and 10^6 conidia suspension of *Fusarium*. This was achieved by slowly drenching the seedlings with 5 ml of the inoculum suspensions using an HF Waly Simplex 2.0 -10 Ml bottle top dispensers. The suspension was applied to the roots of the seedlings. This process was done for both conidial concentrations.

3.2.3.3 Injection of crown

Healthy pine seedlings of the various hybrids were treated with either a 10^2 or a 10^6 conidia ml^{-1} suspension of *Fusarium.* This was achieved by sucking up 5ml of a conidial suspension into a 5 ml syringe with a fine needle. The plant was injected with the conidial suspension at the top of the seedling.

3.2.4 Treatments and Experimental design

The study was carried out as 3 separate experiments to answer Objectives 1 – 3 as follows:

Experiment 1: To determine the effects of inoculation technique and concentration of inoculum on disease development;

Experiment 2: To determine the effects of inoculation technique on disease development in 6 pine hybrids using three replicates.

Experiment 3: To determine the effects of concentration of inoculum on disease development in 6 pine hybrids.

3.2.4.1 Experiment 1

This experiment had two factors namely, inoculation technique and concentration of inoculum. The factor levels and treatment combinations are presented in Table 3.1. Both factors had three levels each, giving a 3 × 3 factorial and 9 treatments which were laid out in a randomized and complete block design.

Table 3.1 Experiment 1 factors, levels and treatment combinations

Inoculation technique	**Concentration of inoculum (conidia ml^{-1})**	Treatment number
Drenching	0	1
	10^2	2
	10^6	3
Wounding	0	4
	10^2	5
	10^6	6
Injection	0	7
	10^2	8
	10^6	9

3.2.4.2 Experiment 2

This experiment had two factors namely, concentration of inoculum and pine variety. The combination of 3 concentration levels and 6 pine varieties gave a total of 18 treatments and these were also laid out in a randomized complete blocks design, using three replicates.

3.2.4.3 Experiment 3

This experiment had two factors namely, pine variety and inoculation technique. They were 3 inoculation techniques and 6 pine varieties with three replicates, giving a total of 18 treatments.

3.2.5 Disease assessment and data analysis

All experimental treatments were replicated three times, with each rep consisting of 3 plants. After inoculation, plants were visually rated for disease symptoms over a period of four weeks, starting after 2 weeks. The rating scale was as described by Kim et al. (2008) whereby:

0 = no symptoms

1 = mild reddening of tips of needles

2 = severe discoloration of needles

3 = branch dieback (shepherd's crook)

4 = resin exudation

5 = plant is dead

Based on the values obtained from the scoring over the different dates, Area Under the Disease Progress Curve (AUDPC) was calculated. The results were analysed using GenStat® Version Twelfth Edition (VSN International, UK).

3.3 RESULTS

3.3.1 Effects of conidia concentration and inoculation technique on disease development in pine seedlings

Concentration of conidia had a significant effect ($p<0.005$) on AUDPC (Table 3.2). The most severe infection occurred at the highest concentration of 10^6 conidia ml^{-1} (Figure 3.1). Infection at 10^2 conidia ml^{-1} was similar to that at 0 conidia ml^{-1} (Control) (Figure 3.1). Inoculation technique also had a significant effect ($p<0.001$) on AUDPC (Table 3.3). The least infection was observed when plants were drenched (Fig 3.2). However, the injection and the wounding techniques gave similar infection results (Figure 3.2). The interaction between concentration and inoculation technique was not significant ($p>0.05$) (Table 3.2).

Table 3.2: Analysis of variance for effects of inoculation technique and conidia concentration on AUDPC

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block	8	68483.	8560.	0.60	
Concentration	2	190671.	95335.	6.65	0.002
Inoculation technique	2	727383.	363691.	25.35	<.001
Concentration × Inoculation technique	4	52195.	13049.	0.91	0.464
Residual	64	918099.	14345.		
Total	80	1956831.			

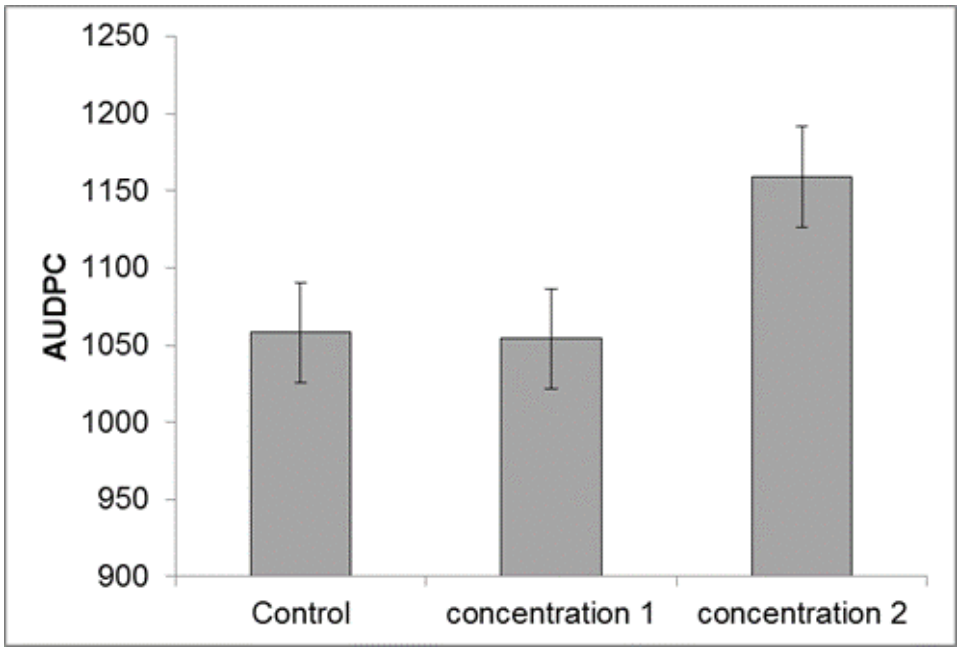

Fig 3.1: The effect of concentration of *F. circinatum* conidia on Area Under Disease Progress Curve in pine seedlings. Control = 0 conidia ml^{-1}, Concentration 1 = 10^2 conidia ml^{-1} and Concentration 2 = 10^6 conidia ml^{-1}

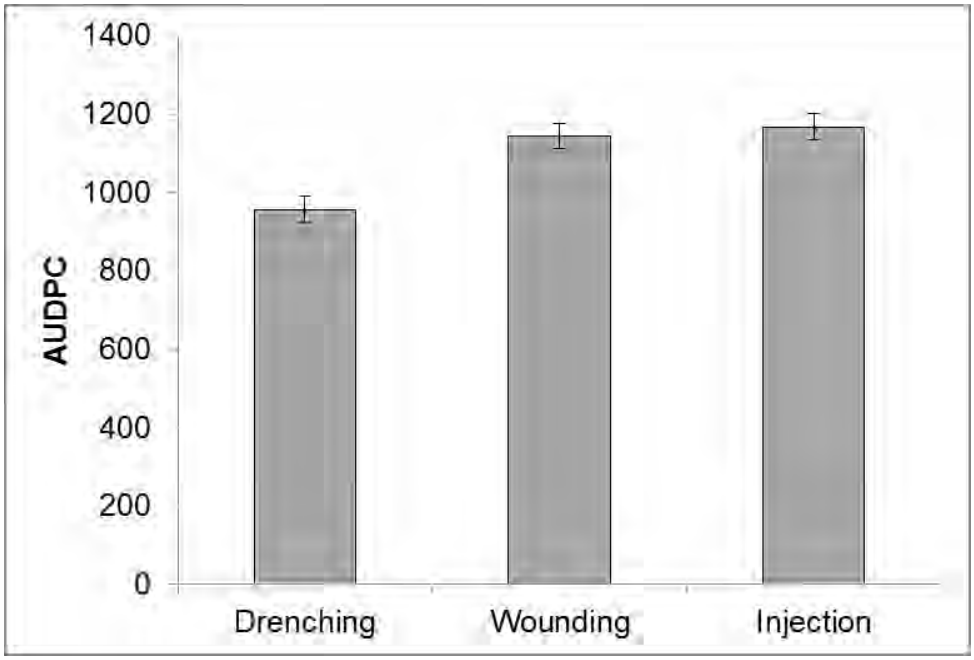

Fig 3.2: The effect of inoculation technique on Area Under Disease Progress Curve in pine seedlings challenged with 10^6 c.f.u.ml^{-1} of *F. circinatum*.

3.3.2 Effects of conidia concentration and pine variety on disease development

Pinus patula hybrid type had a significant ($p<0.01$) effect on the AUDPC (Table 3.3), suggesting that the different pine hybrids had different levels of susceptibility to *F. circinatum.* Mean AUDPC for the six pine families is shown in Figure 3.3. Increase in concentration of conidia significantly increased the AUDPC but the interaction between concentration of conidia and pine variety was not significant ($p>0.05$). This suggests that in all pine families, infection increased significantly when the concentration was increased, regardless of pine family.

Table 3.3: Analysis of variance for effects of pine variety and concentration of conidia on AUDPC

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
Rep stratum	8	109698.	13712.	0.82	
Pine-variety	5	329647.	65929.	3.95	0.002
Concentration	2	408927.	204463.	12.26	<.001
Pine-variety. Concentration	10	390577.	39058.	2.34	0.064
Residual	136	2268221.	16678.		
Total	161	3507070.			

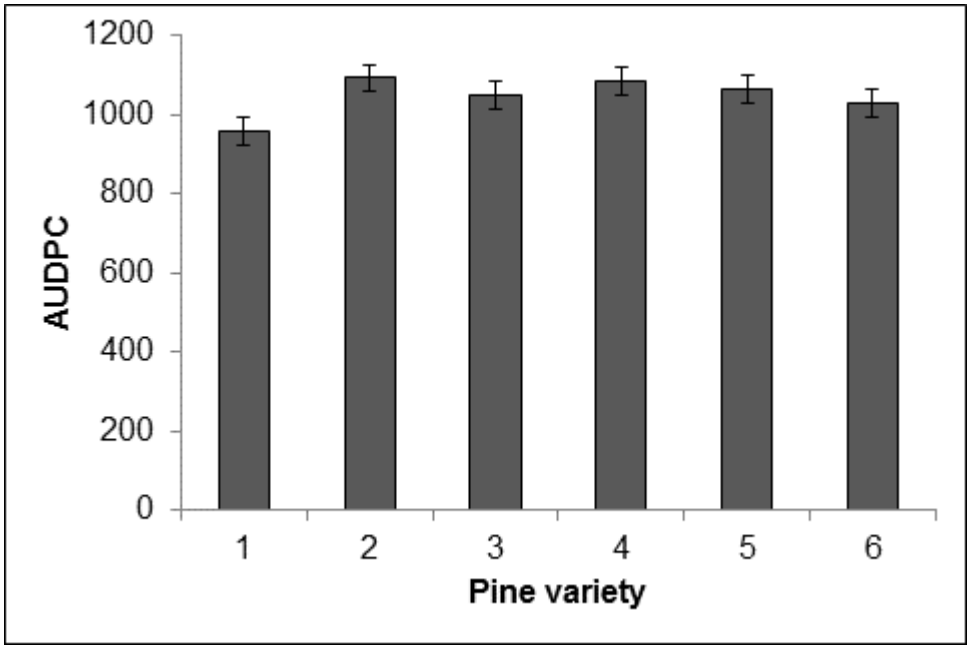

Fig 3.3: The effect of pine hybrid/variety on Area Under Disease Progress Curve in response to *F. circinatum*

3.3.3 Effects of inoculation technique and pine variety on disease development

The interaction between pine variety and inoculation technique was significant ($p<0.01$) (Table 3.4. This interaction is presented in Figure 3.4. It is shown on Figure 3.4, for example, that the drenching technique was more effective for establishing infection on Pine Variety 2 than Pine Variety 1.

Table 3.4: Analysis of variance for effects of pine variety and inoculation technique on Area Under Disease Progress Curve

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	8	174505.	21813.	1.83	
Pine-variety	5	251318.	50264.	4.21	0.001
Inoculation-tech	2	201792.	100896.	8.46	<0.001
Pine-variety. Inoculation-tech	10	362032.	36203.	3.03	0.002
Residual	136	1622569.	11931.		
Total	161	2612217.			

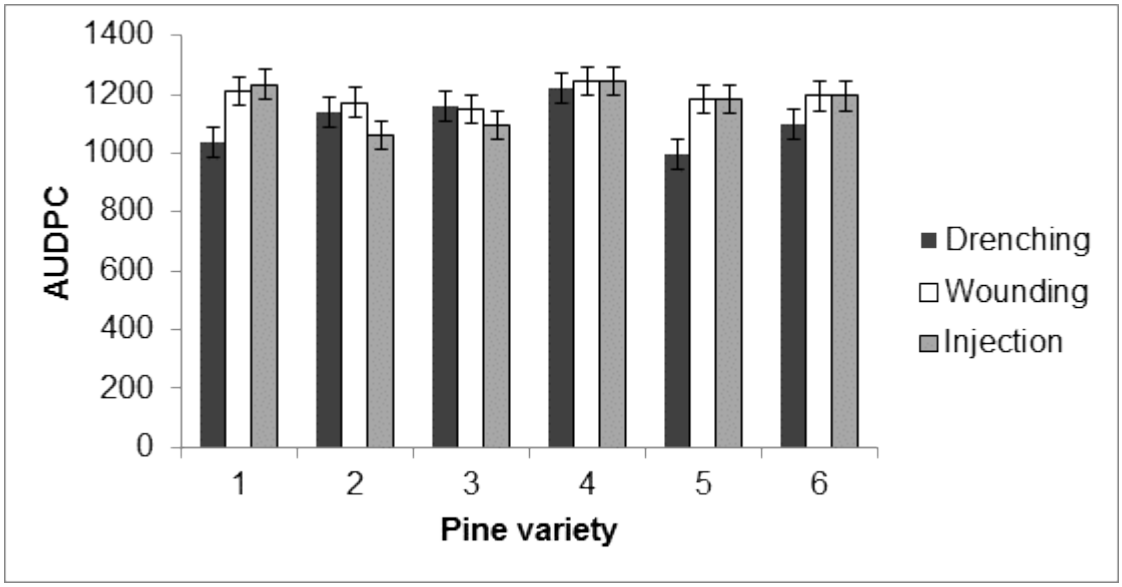

Fig 3.4: The interaction effect of pine variety and inoculation technique on Area Under Disease Progress Curve in response to *F. circinatum*

3.4 DISCUSSION

3.4.1 Effects of conidia concentration on disease development in pine seedlings

Inoculation of pine seedlings with a concentration of 10^6 conidia ml^{-1} resulted in more symptoms and greater disease intensity (AUDPC value) than with the 10^2 conidia ml^{-1} (Fig 3.1). This finding confirms the statement of Agrios (1997); who stated that a higher density of inoculum has a higher potential for causing disease symptoms than a lower level. In research conducted on a different host but related *Fusarium* species, Ben-Yephet et al. (1996) reported that there was a positive correlation between the density of inoculum and the level of *Fusarium* wilt on *Dianthus* sp. Sakamoto and Gordon (2006) also confirmed the above statement that the higher the inoculum concentration, the fewer surviving plants there were.

Hammerbacher et al. (2009) stated that spore concentration is an important factor in the *F. circinatum* infection process. In their study they found that spore concentration had significant effect on the success of inoculations. Hodge and Dvorak (2002) also found that there is no significant difference in infection incidence in plants inoculated with 10^4 and 10^5 c.f.u.ml^{-1} and beyond. This implies that above the spore concentration of 10^4 c.f.u.ml^{-1} and beyond the results remain the same, but with that being said, there is a significant difference between a low spore load and high spore load. Hammerbacher et al. (2009) further describes 10^2 c.f.u.ml^{-1} (0-500) spore load as a low spore load as opposed to 5000-50 000 conidia/ml which is said to be a high spore load. Storer et al. (1999) in his study of spore load ranging from 25-1000 conidia/ml spores, he found significant differences between the diseases severity, which is the case with this study.

3.4.2 Effects of inoculation technique on disease development in pine seedlings

Fusarium circinatum inoculations using both the wounding methods, namely the apical shoot removal and injection of the crown, resulted in the plants showing more symptoms and higher disease intensity than the inoculation using the drenching method (Figure 3.2). Such findings are in line with the research reported by Porter, (2010), where she found that wounding by cutting off the top of pine seedlings was the most effective means to inoculate *F. circinatum*. Kuhlman (1987) also found that wounding was the most effective inoculation method when he did his study on inoculation of loblolly and slash pine seedlings with *F. moniliforme* var. *subglutinans.* Removal of needles and clipping of branches were carried out in this study.

Whether it is an artificial wound or caused by insects or pathogen, wounds play an important role in the introduction of pathogens into a susceptible plant host. With pitch canker of adult pine trees, *F. circinatum* is considered to be a wound pathogen and requires an opening in order to successfully establish in the plant host (Gordon et al., 2001). Wounding is a technique widely used by many researchers to inoculate *Fusarium* species into pine plants (Hodge and Dvorak, 2000; Roux et al., 2007), despite it being highly artificial, especially for the replication of seedling wilt as it occurs naturally in nurseries in South Africa. In contrast, drenching of conidia is an easier technique, and mimics the natural infection event in the nursery. Importantly, drenching allowed for the discrimination between pine varieties with differential resistance, whereas the two wounding inoculations both resulted in an equal level of disease with all pine varieties. This suggests that real resistance mechanisms were

overwhelmed by wounding, which bypassed presumptive physiological and morphological resistance in the roots of plants, which is where pine host resistance needs to be expressed, in practice.

In this study there was no significant difference in the effectiveness of the wounds caused by cutting off the top of seedlings and the wound caused by a needle ($p>0.05$) (Table 3.2).

3.4.3 Effects of the concentration of conidia and pine variety on disease development

The most susceptible variety was pine Variety 1, although a low level of level of resistance was expressed when the plants were drenched with a virulent strain of *F. circinatum* (3.3). Work done by Roux et al. (2007) showed encouraging evidence that variation in susceptibility exists between a limited numbers of *P. patula* selections commonly used in South Africa. This opens up the possibility of developing breeding stock of *P. patula* that is resistant to *F. circinatum*.

3.5 CONCLUSIONS AND RECOMMENDATIONS

Inoculation methods that involve wounding of the pine seedlings, whether by cutting off the top of the apical shoot, or by injecting into the apical shoot with a needle at a concentration of 10^6 c.f.u. ml^{-1} *Fusarium* conidia ml^{-1} reliably causes *Fusarium* wilt of pines. Drenching provided for a higher level of discrimination of differential resistance expressed by the six pine varieties that we screened. Further research is needed to determine the minimal concentration of conidia for drenches that provides the optimal discrimination between pine seedlings with differential resistance.

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CHAPTER 4: SCREENING OF ENDOPHYTIC MICROORGANISMS ISOLATED FROM SELECTED PINE SPECIES TO CONTROL *FUSARIUM CIRCINATUM*

Abstract

Globally, the fungus *Fusarium circinatum* poses a serious threat to the softwood forest industry. In South Africa, *F. circinatum* has characteristically been a nursery pathogen, attacking *Pinus patula, P. elliotii* and *P. radiata* seedlings and cuttings and causing a seedling wilt. The aim of this study was to screen endophytic microorganisms isolated from selected healthy pine seedlings, cuttings and seeds, in order to test their potential as biological control agents against *F. circinatum*. Young *P. patula* seedlings were drenched weekly for four weeks with 5ml of various isolates of endophytes at 10^6 c.f.u ml^{-1}. A pathogenic strain of *F. circinatum* was subsequently inoculated onto the plants as a drench, and the plants were subjected to partial drought stress for a week. Disease was assessed weekly for four weeks. The Area under Disease Progress Curve (AUDPC) data was calculated. In the primary screening of the endophytes, the best treatments produced up to 60% reduction of the disease. From this trial the best 18 endophyte isolates were selected for further screening. In the secondary screening Isolates E56, E8 and E51 were the most effective as biological control agents. Isolates E 141, E12, E13 and E27 were also found to be effective as biocontrol agents ($p<0.001$). Isolate E85 was the least effective.

Keywords: *Fusarium circinatum*, pine seedling, endophytes, biological control

4.1 INTRODUCTION

The pathogen, *Fusarium circinatum,* is increasingly becoming a threat to pine (*Pinus patula*) nursery businesses in South Africa. Crous (2005) stated that the susceptibility of *P. patula* to *F circinatum* is the main reason for poor survival of young seedlings in nurseries from Mpumalanga. Annual surveys from a pine nursery in Mpumalanga revealed a continuous decline in survival of *P. patula* seedlings, from approximately 88% in the year 2000 to approximately 64% in 2007 (Morris, 2011). This rapid decline in *P. patula* production across nurseries calls for urgent action for the effective control of *F. circinatum*.

Endophytes are endosymbionts that live within a plant for at least a part of its life cycle, without appearing to cause disease. They are ubiquitous and have been found in all species of plants. In expanding the definition of the term 'endophyte', Backman and Sikora (2008) stated: "the term endophyte, with exception of the endotrophic mycorrhizal fungi, was always associated with beneficial organisms colonizing the phyllosphere". However, the definition of an endophyte is now broadened by many researchers and can include any organisms that live in plant tissue whether neutral, beneficial or detrimental".

Endophytes are mostly found in the internal parts of plants, while epiphytes colonize the above ground plant parts. Typically, in plant tissue there are likely to be bacterial or fungal endophytes, which may be distinguished into of three types as follows; (1) pathogen of another host that are non-pathogenic in their endophytic relationship; (2) non-pathogenic microbes; and (3) pathogens that have been rendered non-

pathogenic but still capable of colonization by selection methods or genetic alterations (Backman and Sikora, 2008).

In 1926, endophytic growth was recognised as a particular form of infection and as having a close relationship with mutualistic symbiosis (Perotti, 1926). Since then, endophytes have been defined as microorganisms that could be isolated from surface-sterilized plant organs. Bacterial endophytes can be classified as 'obligate' or 'facultative', in accordance with their life strategies. Facultative endophytes have a stage in their life cycle in which they exist outside the plant host. On the other hand, obligate endophytes are strictly dependent on the host plant for their growth and survival and transmission to other plants occurs vertically (via seed or vegetative propagation) or via vectors (Hardoim et al., 2008).

Although the presence of bacterial endophytes in plants is variable and occasionally transient, they are often capable of eliciting physiological changes that modulate the growth and development of the plant (Backman and Sikora, 2008). Todorova and Kozhuharova, (2009) and Chen et al. (2009) stated that *Bacillus subtilis* has been identified as an effective antagonist against numerous fungal pathogens as a result of its ability to produce antifungal compounds, antibiotics and proteases. Selected endophytes could be used to treat seeds or transplants to limit the side-effects of abiotic and biotic factors on the host by their physiological actions from within plant tissue. Endophytic microorganisms isolated from surface-disinfected plant tissues have the potential to serve as a biological control agent against pathogens (Pleban et al., 1995).

The over dose and misuse of chemical products can cause environmental health related risks. Biological control agents offer alternative to the chemical products, contributing to minimize the negative consequences (Kim et al., 2003). Many fungal diseases are found in nurseries and chemical control of these pathogens is the most common practice, very little work has been performed on biological control of forest pathogens (Silva et al., 2012).

4.1.1 Study objectives

The objectives of this study were to screen endophytic microorganisms isolated from selected healthy pine seedlings, cuttings and seeds and to test their potential as a biological control agent against *F. circinatum*.

4.2 MATERIALS AND METHODS

4.2.1 Screening of endophyte isolates against *Fusarium circinatum* on *Pinus patula* seedlings under greenhouse conditions

Twenty four month old *P*. *patula* seedlings were transplanted into Speedling® 24 trays. These trays were randomly placed in a polycarbonate greenhouse with temperatures ranging from 26 to 28^0C and supplied with irrigation water and supplemented with an NPK soluble fertilizer (3:1:3) three time a day for 5 minutes. Each plant was labelled. Then using a 5ml syringe, they were drenched with 5ml of the 10^7 c.f.u. ml^{-1} and 10^6 c.f.u. ml^{-1} endophyte suspensions (conidia or cells for fungi or bacteria, respectively) of the assigned endophyte weekly for four weeks. The same syringe was used for the same isolate throughout the experiment. After colonisation with endophytes, the plants were inoculated with 10^2 CFU per ml *F. circinatum* using the same drenching

technique. Plants were subsequently drought stressed for one week to promote disease development. The plants were then returned to normal irrigated conditions and were monitored for four weeks for symptoms of infection by *F. circinatum*. The study was carried out as two experiments, with findings from the first experiment leading to the design of the second experiment.

4.2.1.1 Experiment 1: treatments and experimental design

One factor was studied, which was to determine the most effective endophyte isolate. There were 172 treatments, the 150 endophytes and two controls (a positive control with no pathogen and no antagonist, and a negative control, inoculated with the pathogen but no antagonist). Six plants were used for each treatment, with plant serving as a replicate. A randomized complete blocks design was used.

4.2.1.2 Experiment 2: treatments and experimental design

Secondary screening of most effective endophyte isolates from Experiment 1, in order to identify the best three endophytes. There were twenty treatments, the 18 best endophytes and two controls (a positive control with no pathogen and no antagonist, and a negative control, inoculated with the pathogen but no antagonist) and only one factor was studied. Ten plants were used per treatment, with each plant serving as a replicate. A randomized complete blocks design was used.

Disease Assessment

Disease incidence and disease severity were recorded, based on visual assessment of the symptoms arising from infection by *F. circinatum* to a five-point key (Kim et al., 2008):

0 = No symptoms

1 = Mild reddening of tips of needles

2 = Severe discoloration of needles

3 = Branch dieback (shepherd's crook)

4 = Resin exudation

5 = Plant is dead

These calculations gave 20%, 40%, 60%, 80% and 100% for the ratings, respectively. The Area Under Disease Progress Curve (AUDPC) values were generated from the four × weekly ratings. The results were analysed using GenStat 12th edition. Treatment means were separated using the Duncan's Multiple Range Test.

4.3 RESULTS

4.3.1 Experiment 1: Treatment of Pine Seedlings with Endophytic Isolates Preceding Inoculation of *F. circinatum*

The various endophytes provided various levels of control of *F. circinatum*. Of the 150 endophytes tested, only 14 provided good control. The other 136 failed to control the pathogen, the disease levels were severe after two weeks and after four weeks, all the plants had died. Isolate E43 controlled the disease best. There was no significant difference in disease levels of plants treated with Isolates E12, E21, E83, E140 and E141 (table 4.1).

Table 4.1: Ratings for the 18 most effective endophytes against *Fusarium circinatum* for a period of four weeks post drought stress and five week post-pathogen inoculation

Isolates	Week1	Week 2	Week 3	Week 4	AUDPC value
E8	1	1	1	1	723.3def
E12	2	2	2	3	1096.7b
E29	1	2	2	3	945bcd
E31	1	2	2	2	777cdef
E33	2	2	2	2	933.3bcd
E34	2	2	2	2	828.3cde
E43	1	1	1	2	536.7f
E51	1	2	2	3	840cde
E56	2	2	2	2	828.3cde
E83	2	2	2	3	991.7bc
E98	1	1	2	3	735def
E99	1	2	2	3	793.3cde
E140	2	2	2	3	910cbd
E141	1	2	2	3	875bcde
CTRL -	2	3	3	3	1761.7a
CTRL +	1	1	1	1	630f

Key Notes:

1. (CTRL -): Pathogen only
2. (CTRL +): Neither antagonist nor pathogen

Table 4.2: Showing the endophytic reaction to *Fusarium circinatum*, illustrates in their ranks from the most effective at the top and least effective at the bottom

Isolate number	Type of endophyte
E43	Bacterial
E8	Bacterial
E 31, 97 and E98	Bacterial, Bacterial, Fungal respectively
E29, 33, 34, 38, 99 and E118	All Fungal and E118 Bacterial
E12, 21, 83, 140 and 141	Bacterial with E83 and 141 Fungal
CTRL (-)	Water

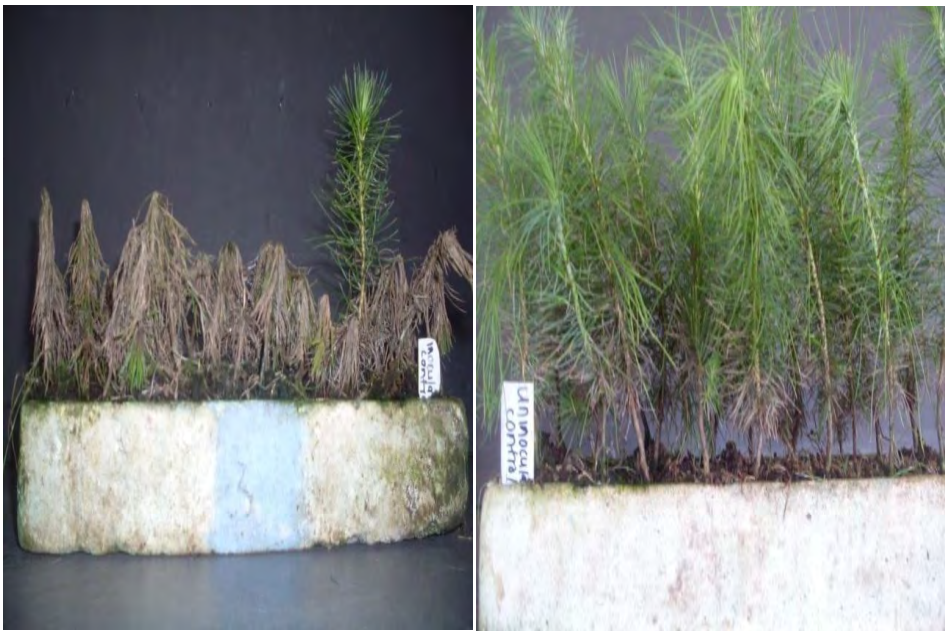

Figure 4.1: Photographs of the disease level in the Inoculated Control caused by *F. circinatum* (left) and the Uninoculated Control (right) (Picture taken at the University of KwaZulu Natal during the experiment).

Figure 4.2: Disease symptoms on plants treated with three endophytes prior to inoculation with *F. circinatum*. Plants treated with Isolate E41 (left) developed more disease than those treated with Isolates E42 (centre) and E43 (right). (Picture taken at the University of KwaZulu Natal during the experiment).

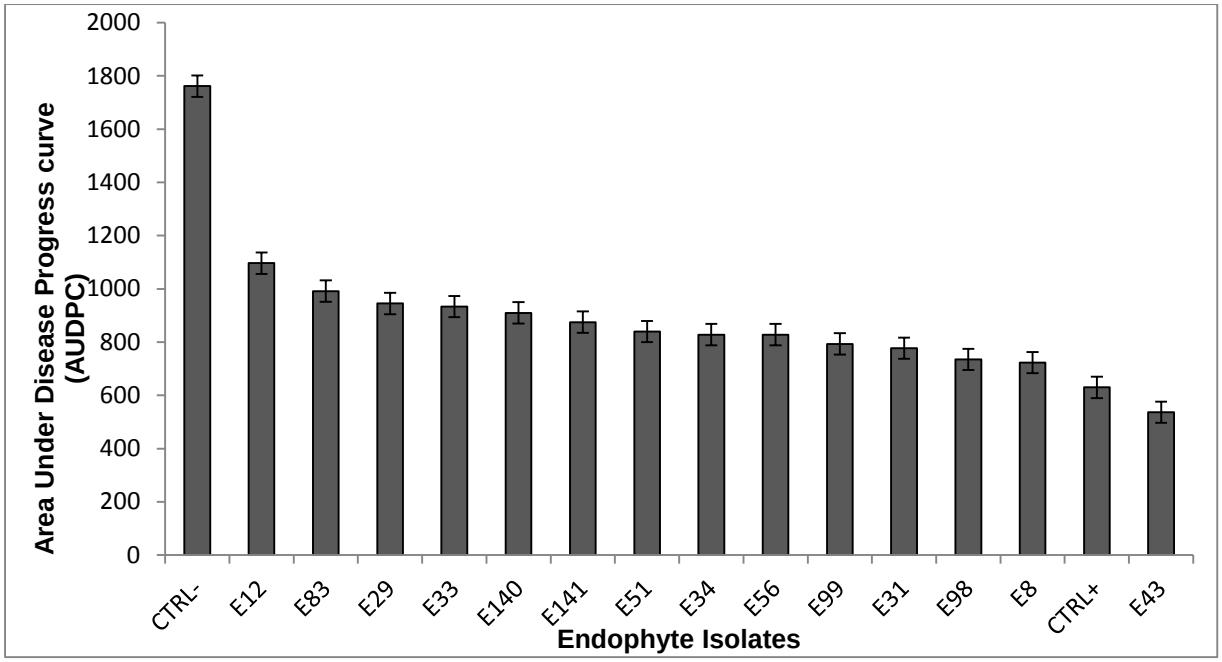

Figure 4.3: Area Under Diseases Progress Curve (AUDPC) values for the most effective endophytes applied to *P. patula* seedlings, prior to inoculation with *F. circinatum*.

Plants treated with Isolate E43 had the lowest AUDPC value, and there was no significant difference between plants treated with this isolate from those of the Uninoculated Control. The Inoculated Control had the highest AUDPC value. There was no significant difference between plants that were treated with Isolates E29, E33 and E140. There was no significant difference with plants treated with Isolates E34, E51 and E99.

4.3.2 Experiment 2

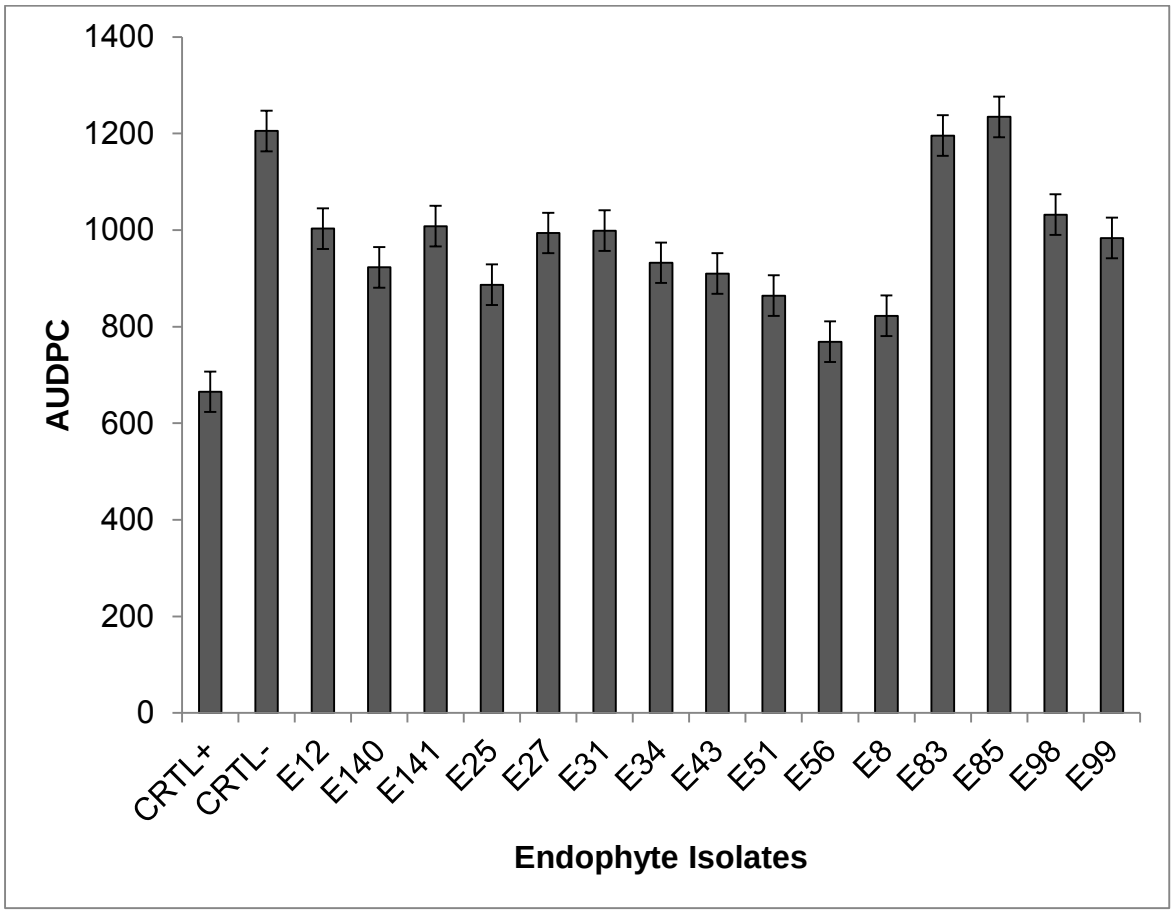

Figure 4.4: Area Under Diseases Progress Curve (AUDPC) values for the most effective endophytes applied to *P. patula* seedlings, prior to inoculation with *F. circinatum*.

Isolates E51, E8 and E56 gave the most effective control of the pathogen with low AUDPC values (864, 822 and 768, respectively). Isolates E141, E12, E13 and E27 were also effective in controlling the pathogen. Isolate E 85 was the least effective endophyte, with an AUDPC value higher than that of the Negative Control (1234>1205), suggesting synergy between the pathogen and this endophyte.

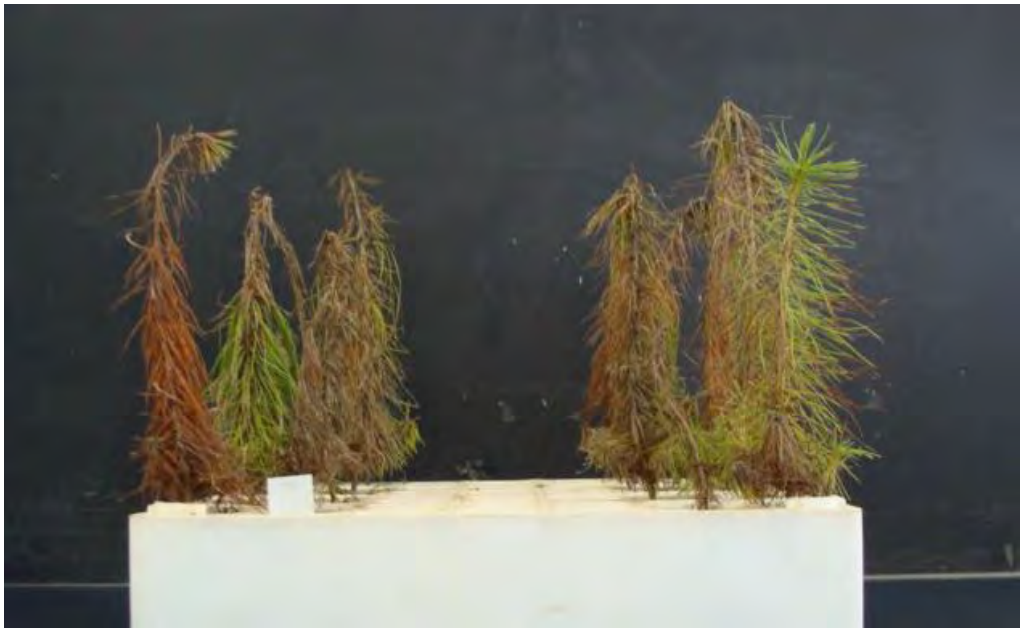

Figure 4.5: A photograph of the failure of Isolate E85 (left) to protect against a virulent strain of *F. circinatum* during secondary screening, compared with the Negative Control (right). Note that the Plants are heavily diseased and show *F. circinatum* symptoms, notably the shepherd's crook and the drooping, wilted appearance. (Picture taken at the University of KwaZulu Natal during the experiment).

Figure 4.6: A photograph illustrating the efficacy of Isolate E56 (left) in its control of *F. circinatum*, compared with the Positive Control (right). (Picture taken at the University of KwaZulu Natal during the experiment). Disease control is not perfect, however, and the plants on the left do show some symptoms of *Fusarium* wilt.

4.4 DISCUSSION

The objectives of this study, which were to screen endophytic microorganisms isolated from selected healthy pine seedlings, cuttings and seeds and to test their potential as a biological control agent against *F. circinatum* have been satisfactorily achieved. The effective endophytes colonised the pine seedlings and inhibited the development of typical wilt caused by *F. circinatum* (Figure 4.4). Sturz et al. (2000) noted that endophytic bacteria may colonize and compete for the same ecological niche as systemic phytopathogens, especially vascular wilt pathogens. It is this characteristic that favours them as biocontrol agents.

Isolation and screening for potential biocontrol organisms are crucial steps in biocontrol research, in order to obtain efficient antagonists for the control of plant diseases. The current study employed a relatively simple, inexpensive and time-efficient procedure, which has repeatable and reliable results. According to Davet (2004), developing, an *in vitro* screening procedure that provides rapid and repeatable results is an important step in the search to find efficient antagonists for biocontrol of plant diseases .

Endophytes naturally colonise plant tissue and many can compete with pathogens to protect their host plants. As seen if Figure 4.4, the best three endophytes controlled *F. circinatum* well, resulting in low AUDPC values. Some isolates failed to control the fungus (Figure 4.3). This may be explained by the fact that plant-species-specific factors such as root architecture, surface structure, and composition of the root exudates sometimes determine the relationship between endophytes and their hosts (Schulz et al., 2006).

Pine seedlings treated with Isolate E56 developed the least severe symptoms (Figure 4.4). This would have been due to the successful colonisation by the endophyte. Biocontrol agents reduce disease severity either by direct or indirect antagonism against the pathogen. Direct antagonism refers to the interaction of two or more microorganisms that share the same ecological niche at the same time and involves antibiosis and competition for nutrients (Alabaouvette and Lemanceau, 2000). Indirect antagonism implies that there is induced host plant's resistance to reduce the disease severity (Fuchs et al., 1997).

Belgrove, (2007) stated that *Fusarium* wilt diseases are reduced by competition for root area. Thus when the *F. circinatum* was inoculated, there would have been competition for space between the endophyte isolate and *F. circinatum*, with the result that *F. circinatum* could not colonize the vascular system. Competition for root area plays a critical role in reducing *Fusarium* wilt. Studies by Olivain et al. (2006) showed that non-pathogenic and pathogenic strains of *F. oxysporum* compete for infection behind the apex of the growing root. A competent endophyte establishes itself in its host with the capacity for optimal nutrient uptake, competition and growth (Davet, 2004).

A lot of mechanisms can be proposed to have played a role in the endophyte- induced reduction of disease severity and disease incidence of *F. circinatum* on pine seedlings. Some microorganisms can produce secondary metabolites that cause adverse effect to other microorganisms (Keel et al., 1996). Such secondary metabolites could have not only attacked *F. circinatum,* but also other endophytes colonising the plant tissue. It is also known that a lot of biocontrol agents induce systemic resistance in plants. It has been proven, in many studies, that a non-pathogenic strain of *F. oxysporum* reduced *Fusarium* wilt through ISR of banana (Gerlach et al., 1999), cucumber (Mandeel and Baker, 1991), watermelon (Larkin et al., 1996) and tomato (Fuchs et al., 1997). A similar reaction could have occurred between the interaction of the endophytes and *F. circinatum*.

4.5 CONCLUSIONS AND RECOMMENDATIONS

Some of the endophytes that were isolated provided good control of the pathogen, suggesting that they had colonised and adapted to the host without showing negative impact. The biological control that they could provide would be an option to reduce the incidence of the pathogen in pine nurseries in South Africa. The biocontrol could be an alternative to reduce the increase of *F. circinatum*.

For future use of the endophytes, it is important to identify the best endophytes. There is also a need for research into their ecological role of the most effective endophytes. A more detailed study on the mode of action of these endophytes might be useful. A molecular study looking at the genotypes and phenotypes of the endophytes may fill some information gaps about the mechanisms involved in endophyte – plant interactions.

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CHAPTER 5: DETERMINATION OF THE OPTIMIMUM CONCENTRATION OF SOLUBLE SILICON FOR THE CONTROL OF *FUSARIUM CIRCINATUM* IN PINE SEEDLINGS

Abstract

The aim of this study was to determine the optimum concentration of soluble silicon to promote growth and reduce *F. circinatum* disease level. Healthy *Pinus patula* seedlings were used. Plants were treated with 100 mg l^{-1}, 200 mg l^{-1} and 400 mg l^{-1} silicon concentration for a period of four weeks by drenching. Plants were then challenged with a pathogenic strain of *F. circinatum* at a concentration of 10^6 c.f.u. ml^{-1}. Visual rating of disease level was done and the data was subjected to analysis of variance. 100 mg l^{-1} was the best concentration for disease control. 100 mg l^{-1} silicon reduced final disease percentage by 53.3%. It was observed that in the absence of Si the disease level of *F. circinatum* is higher with the AUDPC value (1762) and as the concentration increases (100 mg l^{-1}) the diseases severity decreased (875). Any further increase in the concentration (200 and 400 mg l^{-1}) gave a negative response with the disease increasing (1377 and 1435 respectively). When studying the dry weight, at zero concentration, the dry weight was low (15.28 g) and increased (27.53 g) with the application of Si at its optimum concentration (100 mg l^{-1}). It was then concluded that a concentration of 100 mg l^{-1} was the optimum concentration for the control of *F. circinatum* in pine seedling.

Keywords: *Fusarium circinatum,* silicon concentration

5.1 INTRODUCTION

Silicon (Si) is an important element that is mostly used for plant growth. Si helps to overcome various stresses that the plant may be subjected to (Ma, 2004). Research done in the past has shown that Si plays a very significant role in increasing the plant's resistance to unfavourable environmental factors. It is due to this characteristic that Si is known as a beneficial element for plants growing under biotic and abiotic stresses, which could be possibly due to heavy metals, drought, salinity and pathogens (Gao et al., 2006).

As far back in 1940, Wagner showed the efficacy of Si applications in protecting crops against fungal attack (Wagner, 1940). Miyake and Takahashi (1983) also recorded positive results in which the effect of Si on the growth of cucumber. Much research has been done which proves that the uptake of Si can increase the tolerance of rice, sugarcane and other crops to biotic and abiotic factors (Datnoff et al., 2001; Ma and Takahashi 2002). Tesfagiorgis and Laing (2010) studied the control of powdery mildew of zucchini, and proved that Si fertilization resulted in induced plant defence, but only in response to infection with a pathogen. Other successful investigations have been carried out on tomato and grape (Bowen et al, 1992). Germar (1934) reported that wheat (*Triticum aestivum* L.) plants supplied with Si were more resistant to powdery mildew than plants without Si application. Studies done on rice showed that Si is effective in preventing lodging by increasing the thickness of the culm wall and the size of the vascular bundles (Shimoyama, 1958).

It is important to note that Si accumulation varies in plants species based on the ability of plants to absorb it (Chérif et al., 1993). During a study done by Ma and Takahashi,

2002, it was observed that the intake of Si in shoots differed dramatically from one plant species to the other, ranging from 0.1% to 10% Si in the dry weight. In higher plants, only plants in *Gramineae* and *Cyperaceae* show high levels of Si accumulation, while plants in *Commelinaceae*, Urticales and Cucurbitales show medium levels of Si accumulation, whereas most other plants species show low levels of Si accumulation (Takahashi et al., 1990). Limited studies have been carried out previously to determine the effects of Si on resistance to *F. circinatum* in pine tree seedlings.

5.1.1. Research Objectives

1) To study the relationship between the use of silicon fertilization and the levels of damage caused by *F. circinatum.*

2) To determine the optimum usable concentration of soluble silicon for promoting growth and reducing infection by *F. circinatum i*n pine tree seedlings.

5.2 MATERIALS AND METHODS

5.2.1 Source of plant material

Four months old *P. patula* seedlings were obtained from a nursery 35 km outside Pietermaritzburg. These were transplanted into Speedling® 24 trays.

5.2.2 Preparation of pathogen inoculum

Fusarium circinatum previously stored on barley seeds was cultured onto oat meal agar and incubated at 25 ^{0}C for 7-10 days. Inoculum, consisting of both conidia and mycelia, was obtained by flooding the agar plate with 150 ml distilled water. The solution was further sieved using cheese cloth to remove the mycelia (Leslie and

Summerell, 2006). Using a haemocytometer the conidia was counted to get the desired concentration.

5.2.3 Screening for the optimal silicon concentration

Three Si concentrations were tested namely 100, 200 and 400 mg l^{-1}. A 21% w: v solution of potassium silicate was used and calculations were done to obtain the desired soluble silicon concentrations. From the calculations 0.0833 ml, 1.67 ml and 3.33 ml per litre Si solution was added to fertiliser water [NPK (3:1:3), $CaNO_3$ and trace elements] for 100, 200 and 400 mg l^{-1}, respectively. Plants were drenched with the different soluble silicon using a watering can (*ad lib* uptake). Plants were drenched daily after the last irrigation (drip irrigation system) for 4 weeks, and then inoculated with *F. circinatum*. The pathogen was introduced as 5 ml conidial drench (10^6 conidia ml^{-1}) using a 5ml syringe. The treated plants were then subjected to a level of drought stress. Plants were then monitored for four weeks for the typical symptoms of *Fusarium* wilt.

5.2.4 Treatments and experimental design

This research was done under greenhouse conditions at the University of KwaZulu-Natal. One factor was studied, which was the soluble silicon concentration. There were five treatments, 100 mg l^{-1}, 200 mg l^{-1}, 400 mg l^{-1} and two controls (a positive control with no pathogen and no antagonist, and a negative control, which was inoculated with the pathogen but no antagonist. A randomized complete blocks design was used, with three replicates.

5.2.5 Disease assessment

Disease incidence and disease severity were recorded based on visual assessment of the symptoms development from infection by *F. circinatum* on a five-point key (Kim et al., 2008).

0 = No symptoms

1 = Mild reddening of tips of needles

2 = Severe discoloration of needles

3 = Branch dieback (shepherd's crook)

4 = Resin exudation

5 = Plant is dead

An Area Under the Disease Progress Curve (AUDPC) was done using final disease percentage values. The AUDPC results were analysed using GenStat (14th Edition) and means were separated using the Duncan's multiple range test.

5.3 RESULTS

Interactions between the concentrations and the *F. circinatum* pathogen varied.

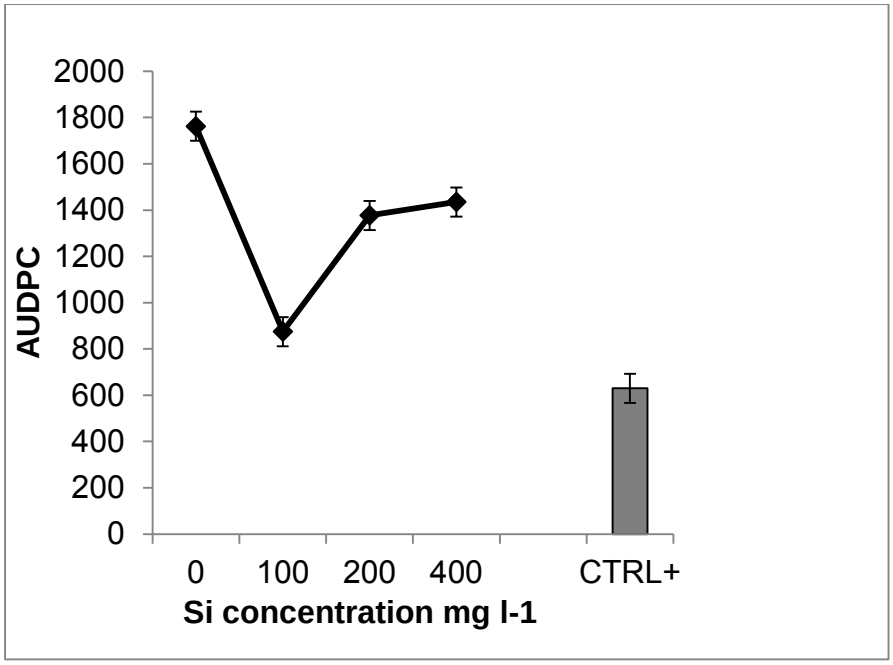
There was a significant difference ($p<0.001$) between treatments for both the AUDPC and FDP. There is no significance between plants treated with 100 mg l^{-1} silicon and the non-inoculated control (Table, 5.1). It was found that AUDPC values were higher in the Inoculated Control plants than those supplemented with silicon. The disease level decreases with the addition of 100 mg l^{-1} silicon, but as the concentration of Si was increased, disease control decreased (Fig. 5.1). When looking at the Negative Control the final disease percentage was high. The FDL (Final Disease Level)

decreased with the application of silicon fertilizer, especially at 100 mg l^{-1} (Fig. 5.2). Towards the end of the study the dry weight was taken, plants treated with 100 mg l-1 soluble silicon had the highest dry weight at 4 weeks after inoculation (Fig. 5.3). Plants that were treated with 100 mg l^{-1} retained their water levels and a higher weight (27 g) than the treatment of silicon at 200 mg l^{-1}, which was less than 15g. Plants that had no silicon treatment had the lowest dry weight. Plants treated with 100 mg l^{-1} retained a greener and healthy appearance as opposed to those that were treated with 400 mg l^{-1} of silicon (Fig, 5.4), which had more needle discoloration, and some plants had died.

Table 5.1: Mean area under the disease (AUDPC) progress curve values and final disease (%) for silicon concentration used on *P. patula* seedlings over a period of 4 weeks to control *Fusarium* wilt

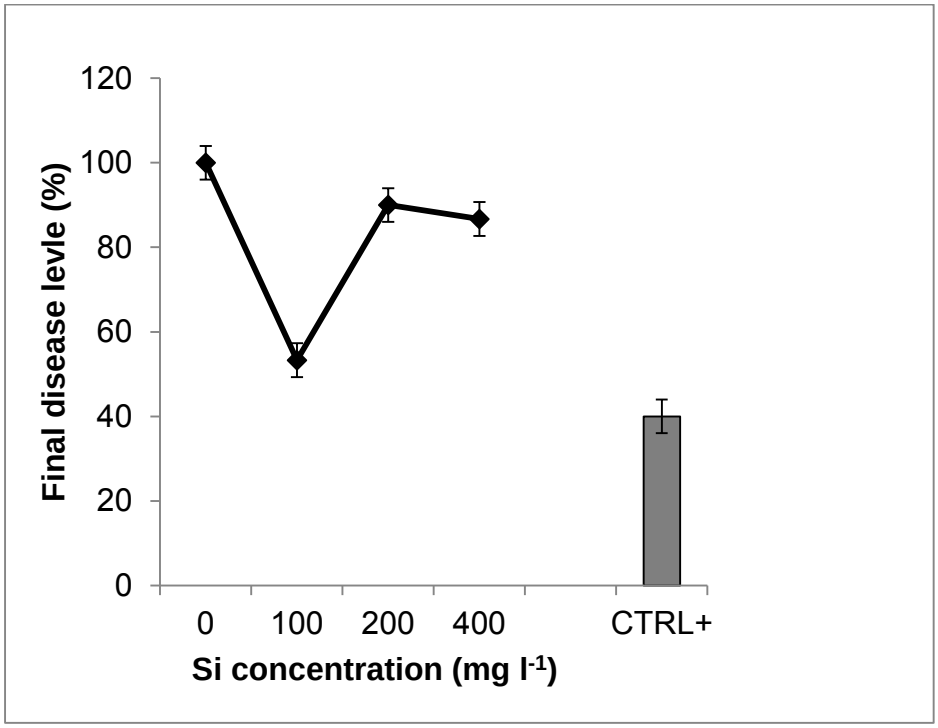
Silicon Concentration (mg l^{-1})	Mean AUDPC value	Mean FDP value
100	875 [a]	53.3[a]
200	1377[b]	90.0[b]
400	1435 [bc]	86.7 [b]
CTRL +	630[a]	40.0[a]
CTRL -	1762[c]	100.0 [b]
Significance (P- value of the F test)	< 0.001	< 0.001
CV %	24.9	17.0
LSD	363.9	15.11
S.E.D	174.4	7.24

Key: CTRL += neither antagonist nor pathogen were applied
CTRL- = pathogen only was applied
AUDPC = area under disease progress curve
FDP = final disease (%)
L.S.D = least significant differences of means (5% level)
CV% = coefficient of variance
S.E.D= standard errors of differences of means



Key: CTRL+: No silicon and no pathogen

Figure 5.1: Area Under the Disease Progress Curve means of the different silicon concentrations with the Control+



Key: CTRL+: No silicon and no pathogen

Figure 5.2: Final Disease Percentage means at the different silicon concentrations.

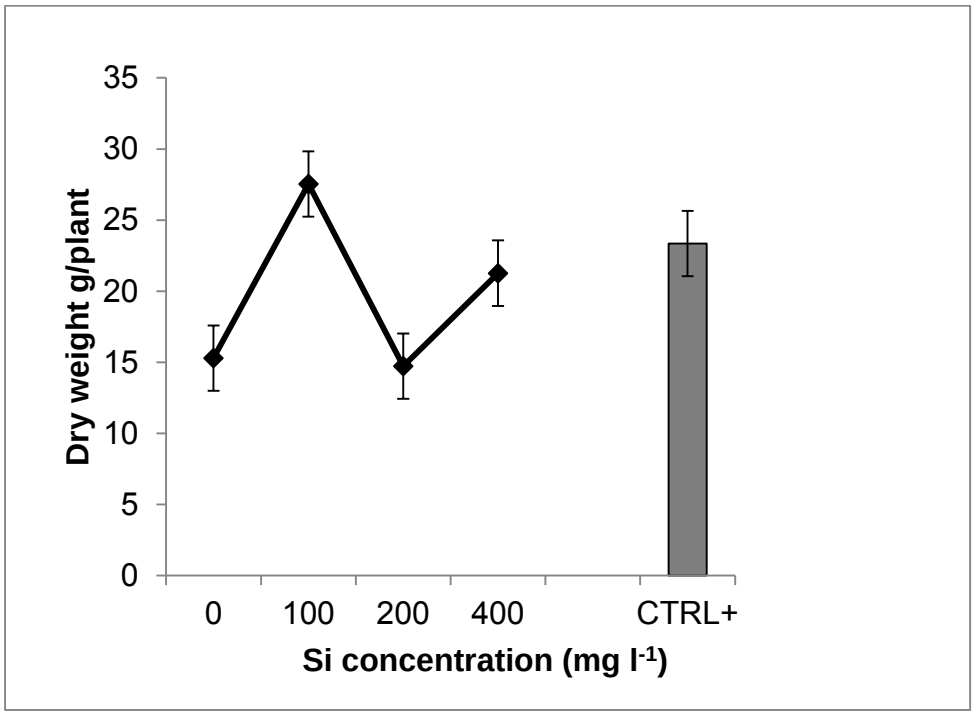


Figure 5.3: Graph showing the dry weight (g) of *P. patula* in response to treatment with different silicon concentration 4 weeks post inoculation with *F. circinatum*.

Figure 5.4: Comparison of plants treated with 100 mg l^{-1} (left) and those treated with 400 mg l^{-1} (right) silicon concentration after 4 weeks post inoculation with *F. circinatum*. Picture taken at the University of KwaZulu Natal, during the experiment).

5.4 DISCUSSION

Different Si concentration had different impacts on the control of the *F. circinatum* fungus. With the treatment of 100 mg l^{-1} silicon concentration was effective at suppressing the symptoms of *F. circinatum* infection, and gave an AUDPC value of 875 and an FDL of 53.3%, compared to the plants that were not treated with silicon (AUDPC value of 1762 and an FDL of 100%).

Silicon is believed to enhance plant resistance to disease in two main ways: creation of a physical barrier and priming of systemic resistance (Datnoff et al., 2001). The element also acts as a growth promoter, and plants treated with silicon had a higher dry weight than the Control plants treated with neither silicon nor *Fusarium*. Alleviation

of abiotic stresses is also equally important especially in relation to plant diseases such as *Fusarium* wilt of pine, because abiotic stresses such as drought result in increased disease levels (Kidane, 2008). When silicon serves as physical barrier it is mostly deposited beneath the cuticle to form a cuticle-silicon double layer, but in this study this could not have been the mechanism of control because the pathogen entered via the roots (Epstein, 1994).

The treatment of 100 mg l^{-1} silicon reduced final disease percentage by 53.3%, and gave the best result. This result is similar to that on cucurbits, where Si treatment reduced the area of the leaves covered with powdery mildew colonies of *S. fuliginea* by up to 98%. Optimum disease control was achieved with concentration of SiO_2 in the nutrient solutions of 100 mg l^{-1} (Menzies et al., 1991).

Vigorously growing plants are more resistant to disease than weak and stressed plants with *Fusarial* diseases. Silicon is known to alleviate some abiotic factors that cause stress to plants (Epstein, 1994). Silicon fertilization at 100 mg l^{-1}, integrated with the most effective endophytes isolate, might provide satisfactory control of *F. circinatum* in pine seedlings. The next experiment would be to integrate the most effect endophytes in controlling *F. circinatum* with the Si treatment in an integrated treatment for the control of seedling wilt caused by *F. circinatum*.

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DISSERTATION OVERVIEW

DISCUSSION

Successful isolation, storage and isolation of both the endophytes and a pathogenic strain of *F. circinatum* were the essential starting point for this study. With an effective and representative inoculation technique and a realistic spore load, researchers can inoculate *F. circinatum* onto seedlings or cuttings of pine species or hybrids. Whilst physical wounding of pine trees prior to inoculation does ensure a consistent level of infection, it is not realistic and does not mimic the actual infection process whereby *F. circinatum* infects pine seedlings, which is via the roots. Furthermore, it results in even infection levels which did not allow for the discrimination between the different levels of disease resistance expressed by the six hybrids on which the different inoculation techniques were tested.

The drenching technique is a superior technique because it mimics the natural event, and because it discriminated between resistant and susceptible pine hybrids amongst the six hybrids tested, even though only two conidial doses were tested. Clearly further research is needed to establish the best conidial dose to provide the maximum discrimination between resistant cultivars, testing 10^0, 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 conidia ml^{-1}. One idea to test is whether a series of conidial concentrations could be used for the inoculum of the pathogen, say 10^3, 10^4, 10^5, 10^6 conidia ml^{-1}. In this way, a differential screen of inoculum pressure can be applied to the pine Germplasm to accurately determine the resistance levels, especially when there is little strong resistance available in the currently available Germplasm.

Hence, the drenching inoculation technique can be recommended to researchers undertaking the screening of new hybrids and crosses of pine genotypes for resistance to *F. circinatum*. It is also recommended as the inoculation of choice for researchers testing agrochemicals and biocontrol agents for the control of *F. circinatum*. Of the many endophytic microorganism screened, only a few showed any useful biocontrol activity against *F. circinatum*. Isolates E56, E51 and E8 were the three most effective endophytes and provided excellent, but not complete, protection against *F. circinatum*. There may be scope for searching for other, more effective biocontrol agents in the many endophytes to be found in pine tissues.

Silicon fertilization (Si) using potassium silicate to provide levels of 100 mg l^{-1} of silicon was remarkably successful at suppressing the level of *Fusarium* wilt expressed in inoculated plants. At this concentration, the Si treatment also stimulated plant growth, and it is therefore a micronutrient treatment that nurseries may consider applying, even in the absence of seedling wilt.

Future Research

- Identify the best endophytes
- Determine their life cycle and ecology and long term competitiveness in the endophytic environment because there is a sequence of endophytic succession that they will need to survive for at least 1-2 years.
- Determine the mode of action of the endophytes
- Determine the mode of action of Si
- Discover why 200 and 400 mg l^{-1} Si is less effective than 100 mg l^{-1} Si

- Optimize the inoculum dosage for drenching at 10^2, 10^3, 10^4, 10^5 or 10^6 conidia ml^{-1}
- Integrate the best of the endophytes with the Si treatment in an integrated treatment for the control of seedling wilt caused by *F. circinatum*.