METHODS OF INOCULATING CYPRESS WITH *SEIRIDIUM* SPECIES TO SCREEN FOR RESISTANCE AND PATHOGEN VARIABILITY

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HELEN TEKULA TSATSIA

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ABSTRACT

The cypress species are grown for their timber value, ornamental beauty and shelter. Their existence is threatened by the presence of cypress canker disease caused by fungal pathogens of the genus *Seiridium*.

The long term solution for controlling this disease is to breed for cypress clones that are resistant to cypress canker. Screening for resistance is conducted by artificially inoculating cypress plants with the pathogen's inoculum.

This study aimed at developing reliable methods of artificial inoculation that are suitable for New Zealand's climatic conditions.

Infection of cypress plants in nature is caused by conidia but mycelial inocula are more commonly used in artificial inoculation. Several methods of inducing sporulation of *Seiridium* species were investigated. Addition of plant substrates was shown to increase sporulation of cultures of *Seiridium* isolates. Studies comparing the two types of inocula (mycelial plugs and conidial suspensions) showed that mycelium inocula caused a higher percentage of canker lesions than spore inocula. Conidial inocula offer a more consistent pathogenicity. Experiments to determine the effective spore load revealed that the percentage of canker increased with the increase of inoculum load. Pathogenicity varied between species and individual colonies of *Seiridium* isolates.

Infection of cypress in nature is thought to occur through wounds and in this work, wounding was required for infection under both glasshouse and outdoor conditions. Inoculation of the main stem and side branches showed disease symptoms develop more rapidly on side branches than on the main stem. Investigations on in vitro inoculation of tissue cultured plants and excised side shoots showed the possibility of screening cypress ramets under different environmental conditions. Temperature and percentage relative humidity were found to influence the percentage of successful inoculations on cypress plants.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Cypress canker is a serious fungal disease of trees belonging to the *Cupressus* family. The disease causes lesions on the bark and cankers on stems and branches. Infection of the branches and crowns of highly susceptible plants can eventually lead to the death of the whole plant. Cypress canker has caused the death of cypress trees in some parts of the Northern Hemisphere during the past years. There is taxonomic evidence that two of the three *Seiridium* species causing cypress canker are present in New Zealand. The disease was first recorded in this country by Birch in 1933. He identified *Seiridium cardinale* (Wagener) Sutton & Gibson= *Coryneum cardinale* Wagener.), as the pathogen responsible for the damages. Beresford & Mulholland (1982) found *S. cardinale* to be predominant in trial plantations. In the later years, it was found that most of the damage was in fact caused by *Seiridium unicorne* (Cooke & Ellis) Sutton= *Monochaetia unicornis* (Cooke& Ellis) Saccardo (Van der Werff 1988; Self 1994).

New Zealand forestry industry supplies 1.1% of the world's total product (Anonymous 2001). At present 95 % of the soft wood timber production is from *Pinus radiata* (Aimers-Halliday et al. 1994). There is an encouraging prospect to develop cypresses as a substitute for the western red cedar and red wood currently being imported for weatherboards and exterior joinery (Miller & Knowles 1996). The *Cupressus* species have advantages over other soft wood tree species such as fast growth, wind resistance, durable heartwood, good machining properties and high quality timber (Franklin 1994).

The cypresses were introduced in to New Zealand in the 1860s from California, Kenya and Guatemala (Miller & Knowles 1996) and were grown mainly as shelterbelts. The four most important cypress species grown in New Zealand include *Cupressus macrocarpa* Hartweg, *Cupressus lusitanica* Miller, *Chamaecyparis lawsoniana* (A. Murray) and the hybrid *Cupressocyparis leylandii* (Jackson and Dallimore). Growers prefer C. *macrocarpa* because of its high quality timber and availability of market. The area planted with the important cypress species as at 1986 is listed in Table1.1. A recent

survey carried out reported an increase in the planting of *C. macrocarpa* and C. *lusitanica* by private growers during the past 10-15 years (Hood et al. 2001). The survey revealed an increase of small to medium sized woodlots, shelterbelts and hedge growers on rural land.

The survey also showed an increase of cypress canker, from South to North through out the country. The disease was more commonly found in young stands.

Cypress canker is identified as one of the major constraints in growing *Cupressus* species especially *C. macrocarpa* (Franklin 1994; Self 2000). Control methods such as the use of fungicides are not practical in forest plantations. The best alternative method for controlling cypress canker is to breed and screen for cypress clones resistant to the disease.

Forest Research in New Zealand requires a reliable method of screening isolates of *Seiridium* species for variations in pathogenicity and for screening cypress clones for resistance to cypress canker.

Table 1.1 Areas of cypress (ha) planted in North and South Island as at 1986

Cypress species	North Island	South Island
Cupressus macrocarpa	439	13000
Cupressus lusitanica	674	92
Chamaecyparis lawsoniana	329	617
Total	1442	2009

Source: Miller & Knowles (1996)

1.2 LITERATURE REVIEW

1.2.1 IMPORTANCE AND DISTRIBUTION OF CYPRESS CANKER DISEASE

The disease is caused by fungal pathogens in the genus *Seiridium*. The genus is classified under the subdivision Deutromycotina and the class Coelomycetes, of the order Blastromatineae (Sutton 1980). Susceptible cypress species become infected through wounds. The pathogens have been found to produce toxins and Graniti (1998) suggested that these could be responsible for the appearance of symptoms on damaged host tissues.

Cypress canker has spread far and wide over the last several decades; California, USA, Africa, New Zealand, Italy and other parts of Europe, and the Mediterranean region. Depending on the type of climate where *Seiridium* species are present, these pathogens can cause major losses. *S. cardinale* was reported as the major cause of serious disease in the Mediterranean and other parts of the world (Xenopoulos 1991). In New Zealand, *S. cardinale* is also reported as attacking *Cupressus macrocarpa* (Beresford & Mulholland 1982; Boesewinkel 1983). Graniti (1998) reported that *S. cardinale* affects several species of *Cupressus*, *Chamaecyparis*, *Cryptomeria*, *Cupressocyparis*, *Juniperus*, *Thuja* and other related genera of *Cupressaceae*.

Seiridium cupressi (Guba) (telemorph: *Lepteutypa cupressi*) has caused a major problem in Europe on *Cupressus* (Graniti 1998). It was also reported in New Zealand (Boesewinkel 1983), but the existence of this species is still being debated. The host range is restricted to the *Cupressacae* family.

S. unicorne on the other hand, attacks *Cupressaceae* and other botanical families (Barnes et al 2001). Boesewinkel (1983), claimed to have isolated *S. unicorne* from *Cryptomeria*. It is of minor importance in the Mediterranean region (Xenopoulous 1991). Graniti (1998) stated that the recent reports restricted the host range of population of *S. unicorne* living on cypress to *Cupressaceae* in countries such as Japan (Tobata et al. 1991) and New Zealand (Beresford & Mulholland 1982; Boesewinkel 1983; Van der Werff 1988). In North America, *S. unicorne* has been found to attack

several *Cupressaceae* and a study carried out revealed no evidence of host specificity (Tisserat et al 1991).

During 1981-1982 periods, *S. unicorne* has spread throughout New Zealand except the coast of the South Island (Van der Werff 1988). Cypress canker disease was reported as widely distributed in younger shelterbelts and woodlots. The disease is also present in older rural stands and forest plantations (Gea & Low 1997; Self 2000; Hood et al. 2001; Hood & Gardner 2002).

1.2.2 TAXONOMY

The disagreement among taxonomists regarding the number of *Seiridium* species causing cypress canker has been a long controversy. There is a history of discussions on the taxonomic species of *Seiridium* (Swart 1973; Boesewinkel 1983; Chou 1989; Viljoen et al. 1993; Graniti 1998; Morrica et al. 1999 & 2000; Barnes et al. 2000, 2001). Conidial morphology; host ranges; cultural characteristics and geographical distributions description were used in distinguishing between the three species.

Swart (1973) suggested one species with variable morphology, while Chou (1989) supported the existence of two species, *S. unicorne* and *S. cardinale*. Boesewinkel (1983) identified 3 distinct species, *S. unicorne*, *S. cupressi* Boes.combi nov.*Cryptostictis cupressi* Guba (teleomorph = *L.cupressi*) and *S. cardinale*. He used the absence of an appendage to distinguish between *S. cardinale* and the other two species and the appendage at an angle of 45° to distinguish *S. cupressi* from *S. unicorne*. Although many workers consider that there are only two species (*S. unicorne* and *S. cardinale*), modern molecular work is providing support for the three species concept. Barnes et al. (2001) found three distinct species on analysis of both β-tubulin and histone sequences.

The results of current studies have reaffirmed the morphological investigations of the three fungi causing cypress canker in New Zealand (Boesewinkel 1983) and biochemical investigations by Graniti (1998) based on appendage angle and toxin production. There is a strong support for the presence of three distinct *Seiridium* species present in different parts of the world. However, as Graniti (1998) pointed out, the existence of races or ecotypes especially with regards to *S. unicorne* cannot be ruled out.

In New Zealand, the general view of cypress canker workers is that only two species exist in the country, *S. cardinale* and *S. unicorne* .The third species identified is thought to be a variation of *S. unicorne*.

1.2.3 SYMPTOMS AND DISSEMINATION OF CYPRESS CANKER DISEASE

The *Seiridium* species that cause canker disease on barks of cypress trees are wound parasites. However, under favourable conditions, the pathogens can enter through the epidermis (Raddi & Panconesi 1981). The canker name itself describes the symptom of the disease. Upon entering through the wounds on the barks of cypress plants, lesions form around the wound as the pathogen progresses though the bark. Resinous canker develops on the bark and in young plants the stems are girdled (Plate 1.1).

Canker formation is found on the tree trunk as well as on the branches. Symptoms of disease include yellowing, browning and wilting of foliage of cankered branches. Infection can result in the loss of foliage and eventually death of susceptible trees. Cankered trunks of mature trees become deformed and reduce the timber quality.

Factors such as cold, wind, insects that cause wounds on bark of cypress plants spread the disease (Raddi & Panconesi 1981). The conidiomata of the *Seiridium* species open wide on canker surfaces under moist conditions (Graniti, 1998). Slimy conidial masses exposed under moist conditions when dry are dispersed by wind. Rain water also disperses conidia from acervuli over short distances. Insects or air borne ascospores could be responsible for spreading the disease on branches high up the trunk of tall mature cypress trees.

Plate 1.1 (a): Wilted side branches (indicated by a white arrow) next to a canker on the main stem of *C. macrocarpa*. (b): Resinous canker on the stem

1.2.4 FACTORS AFFECTING CYPRESS CANKER DISEASE

Cypress canker is believed to spread from one continent to another through the distribution of diseased planting material. However, the establishment of the disease within a locality depends on fungal pathogenicity; environmental conditions such as relative humidity and temperature; and the defence mechanism of the host plant.

1.2.4.1 Fungal pathogencity

Research carried out in Europe revealed that the pathogenicity and host ranges differ between the *Seiridium* species (Barnes et al. 2001). For instance, *S. cupressi* has been found be an aggressive pathogen in Europe on *Cupressus* species. Xenopoulos (1991) reported pathogenicity variation in his study. *S. cardinale* and *S. cupressi* caused bigger and severe cankers than all strains of *S. unicorne*. Initially cankers caused by *S. cardinale* were bigger than those of *S. cupressi* but two years after inoculation, the cankers of *S. cupressi* become larger and more severe than cankers of *S. cardinale* (Xenopoulos 1991). Work done in New Zealand also showed overall higher pathogenicity and less variability among *S. cardinale* isolates than those of *S. unicorne* isolates (Chou 1990). The study also revealed that isolates of *S. unicorne* displayed vast differences in pathogenicity and therefore caused difficulty in determining pathogenicity. Despite the variation in pathogenicity, Chou (1989) reported that apart from the difference in the presence or absence of conidial appendages, *S. unicorne* and *S. cardinale* are similar in many biological characteristics.

1.2.4.2 Host resistance

The susceptibility of the host plant to cypress canker is one of the important factors that influence the rate of spread within a locality. The literature showed that there are considerable differences in susceptibility among cypress species and among individuals within the same species (Raddi & Panconesi 1981; Beresford & Mulholland 1982; Raddi & Panconesi 1984; Xenopoulos 1990). *Cupressus macrocarpa* has been found to be susceptible to cypress canker compared to *C. sempervirens*, *C. arizonica*, *C. lusitanica* and *C. torulsa*. Resistant cypress species are useful for incorporating resistance into breeding programmes.

The resistance mechanism of cypress to attack by *S. cardinale* has been reported to be based on the ability of cypress trees to compartmentalize wounds (Xenopoulos 1990). It also suggested that the resistant mechanism was under apparent polygenic control. This view was also supported by Spanos and co-workers (Spanos et al. 1999). Spanos and co-workers expressed the view that anatomical responses to wounding and infection considered to be stable polygenic process might cause difficulty in manipulation in breeding programmes.

Apart from genetic resistance of certain cypress species, plant maturity appeared to play a role in host resistance. Van der Werff (1988) reported that canker infection on *C. macrcarpa* decreases as the tree age increases. Tree age however, was not correlated with disease incidence on *C. lusitanica* and *Ch. lawsoniana*. A study on the effects of infection and tree age on the progress of *S. unicorne* by Yamada et al (1994) supported the observation by Van der Werff. Yamada & Ito (1995) also found a similar result when inoculations of *S. unicorne* were carried out at heights of 1, 2, 3 metres. The study showed non-wounding inoculations induced infection at height of 3 m and not at height 1 and 2 m. It was concluded that the preformed outer bark was the most responsible factor for fewer infections. However, observation on younger *Cupressus* plants showed a contradicting result. Chou (1990) reported that young seedlings (3-6 months old) of *C. macrocarpa* and *C. lusitanica* were found to be highly resistant but infection occurred when one and half year old plants were used. The study also revealed that the basal part of the stems were infected but not on the inoculations made on the upper green stems of the same plant. A study on *Chamaecyparis obtuse* ("hinoki") showed that infection caused by *S. unicorne* in 3 year old trees spread more rapidly than in younger trees (Kato 1996). It was also noted in Israel that natural infection by *S. cardinale* was observed more frequently on adult trees than on young plants (Solel et.al. 1983).

An artificial study also showed that under greenhouse conditions, the bark maturity prompted infection. These observations were similar to what Chou (1990) observed with cypress plants used in his study. However, the study in Israel also revealed that with artificial inoculation in field conditions, canker development did not differ either between young thin branches and older ones or between inoculations at the base and at the tip of the branches.

1.2.4.3 Environmental factors

Different observations on bark resistance mentioned in the previous section could be related to environmental factors and due to the fact that bark canker resistant trait is not stable as reported by some workers (Casini & Santini 1995; Santini et al 1997).

Geographic-climatic barriers may also responsible for low disease rate among cypresses with no genetic resistance to cypress canker (Santini & Lonardo 2000). The most important environmental conditions critical include relative humidity, water and

temperature. Grantiti (1998) reported that for *S. cardinale*, the conidia failed to germinate when the relative humidity approaches 80 %. On the other hand, Solel et al. (1983) stated that high relative humidity enhanced artificial infection of nursery seedlings.

Water plays the most important role in spreading conidia of cypress canker pathogen (Raddi & Panconesi 1981). Xenopoulos (2000) suggested that drought stress seems to be the main factor for the infection of susceptible host plants.

The optimum temperatures for pathogenicity of each *Seiridium* species differ. The optimum temperature for growth and sporulation of *S. cardinale* and *S. cupressi* is 25°C (Graniti, 1998; Sasaki & Kobayashi 1976). Graniti (1998) reported that *S. cardinale*, is the most thermophilic of the three *Seiridium* species and its conidia can germinate, and colonies can grow up to 35°C. It was also observed that the growth of *S. cardinale* in host tissues is slowed or stopped during the hottest months of the year, resuming again in autumn. The pathogen spreads in the host tissues during winter. For *S. unicorne* the optimum temperature is 20°C (Graniti 1998). Low temperatures during winter or frost can cause damage on the bark of trees and provides an easy entry of the pathogens (Moricca et al. 2000). In New Zealand Van der Werff (1988) reported that infection increased with the increase in temperature across the country.

1.2.5 CYPRESS CANKER DISEASE CONTROL

1.2.5.1 Cultural management

Good management practises can prevent the spread of cypress canker in young plants and small scale plantings such as those used for shelter belts and ornamental cypresses. Selection of clean planting material is always the best option to prevent plant disease spreading within a locality. In the nursery the disease can be controlled by removal of diseased seedlings and obtaining planting material from healthy stock plants. Removal of diseased branches could be easily done with plants grown for ornamental and wind breaks purposes. This practice becomes difficult as the plants grow tall and cannot be applied to forest plantation situations.

1.2.5.2 Chemical control

Studies in Italy have shown that application of benomyl or benomyl and captafol were partly effective in controlling cypress canker in the first stage of infection (Panconesi & Raddi 1986). Work done in New Zealand also showed that *S. cardinale* can be controlled by chemicals such as benomyl and chlorothalonil (McCain 1984). Parrini & Panconesi (1991) stated that chemical trials carried out on *C. sempervirens* in the nursery showed systemic products such as benomyl and thiophanate-methyl proved to be more effective than conventional contact fungicides. However, chemical control is considered as expensive and impractical in the plantation situations.

1.2.5.3 Breeding for resistance to cypress canker

Some cypress species have some level of resistance to cypress canker disease. Some of species include *C. lusitanica*, *C. arizonica* and *C. torulosa*. Breeding for resistant to cypress canker appears to be best alternative of controlling the spread of cypress canker disease. A number of countries in Europe have breeding programmes to screen for resistant clones. It is difficult to develop universally resistance clone due to the fact that the canker resistances has be found to be unstable (Casini & Santini 1995).

1.2.6 ARTIFICIAL INOCULATION METHODS

1.2.6.1 Introduction

Screening for cypress clones resistant to canker disease caused by *Seiridium* species involves artificial inoculation. Inoculations are normally done on young plants in the field and in glasshouse conditions. In vitro inoculation of cypress with *S. cardinale* had been done in Europe and the result has a potential for use in cypress canker screening programmes (Spanos et al. 1997 a). Mycelium is the common type of inoculum used in screening programmes in several countries (Strouts 1973; Raddi & Panconesi 1984; Chou 1990; Xenopoulos 1990; Tisserat et al. 1991; Santini et al. 1997). However, spore suspension was also used in New Zealand by Beresford and Mulholland (1982) and by Ponchete and Andeoli (1989) and Strouts (1973) in England.

1.2.6.2 Wounding technique

The most widely used inoculation technique is to create wounds or insertion on stems or branches of cypress plants. The inoculum, which can be either conidial suspension or agar plugs containing mycelium are placed on the wounds. Wounds are made using cork borers or scalpels. This technique is now commonly used in Europe (Raddi & Panconesi 1984). In New Zealand, the convenient inoculation technique used is reported in Chou (1989, 1990) and Self (1994). Wounds are created by making a V-shape cut on the stem and placing mycelial plugs in the wound. The same technique is used in other, overseas countries (Strouts 1973; Spanos et al. 1999).

1.2.6.3 Fungal inoculum

Infection of cypress plants in nature is by conidia (Raddi & Panconesi 1981). The use of mycelium could mask the type of reaction of the same plant in the natural habitat when it is exposed to conidial inocula. Use of high inoculum could result in early elimination of clones of desirable characters that could be used in the breeding programmes. Raddi & Panconesi (1984) suggested that the variation in screening results could be due to the fact that inoculations might have been carried out with mycelium taken from a surface mutant sector of fungal colony with either higher or lower pathogenicity. Hood & Gardner (2002) expressed a similar concern, that with *S. unicorne* cultures, the ability of cultures to degenerate and loose virulence poses an additional complication as reported by Chou (1989) and Self (1994).

1.2.6.4 Methods of inoculum production for artificial inoculation

1.2.6.4.1 Methods of culturing *Seiridium* species

Isolation is normally done by obtaining small sections of bark or other diseased tissues and culturing on artificial media after surface sterilization using ethanol or sodium hypochlorite. Cultures of *Seiridium* species can be maintained on 1.5- 2.0% potato dextrose agar (Tisserat et al.1991; Graniti et al.1992; Barnes et al. 2001). Other media

being used included malt agar (Viljoen et al 1993; Spanos et al.1997a). Single spores have also been isolated from conidiomata embedded in the bark (Tisserat et al. 1991).

1.2.6.4.2 Methods of inducing sporulation of *Seiridium* species

The use of conidial suspension for artificial inoculation requires adequate supply of conidia. *Seiridium* species in some cases sporulates easily on artificial media and natural substrates such as bark of cypress. However, it has been reported that after series of subcultures or long storage, *Seiridium* isolates often loose their sporulating capability (Tisserat, et al. 1991).

Different methods of spore production have been reported in (Strouts, 1973; Initini & Panconesi, 1974; Sasaki & Kobayashi, 1976; Solel et al. 1983; Chou 1989; Tobata et al. 1991; Sanchez & Gibbs 1995). The length of incubation reported in these references for the appearance of conidia ranged from one week to several weeks. Inoculation of sterilized cypress twigs and exposure of *Seiridium* isolates to near UV light seemed the most common method of inducing spore production. Plant material added to agar has been found to increase sporulation of some fungi (Fisher et al. 1982; Wang et al.. 1985; ; Hu & Wu, 1997; Wyss et al. 2001). .Variability of *Seiridium* isolates was also observed in conidia production.

1.2.6.4.3 Conidial inoculum load

Artificial inoculation with conidia is considered more natural than the use of mycelia. Conidial inocula were used in the past for artificial inoculation. Methods of conidial inoculum applications have been described in (Strouts, 1973; Beresford & Mulholland, 1982; Solel et al., 1983; Ponchet & Andreoli, 1984; Ponchet & Andreoli, 1989; Panconesi & Raddi, 1991). The concentration of conidial suspension varied and in most cases the number of conidia used per wound was not reported. A study by Ponchet & Andreoli (1984) revealed that for *S. cardinale* the minimal effective dose was 50 conidia per wound and the optimum was 500, but it is not known whether there is a difference between *Seiridium* species or isolates of the same species relating to the minimal effective dose.

1.2.7 ASSESSMENT OF CANKER INFECTION IN ARTIFICIAL INOCULATION

From the literature method of assessment carried out in most studies involved measurement of the canker sizes, visual observation of plant growth, disease symptoms and presence of fungal fruiting bodies. The measurements are done from few weeks to few years after the artificial inoculation of cypress plants. For cypress canker disease resistant screening purposes, the method of assessment used is normally based on a descriptive scale. The different methods of assessment are described in various research areas, some of which include (Beresford & Mulholland, 1982; Solel et al. 1983; Xenopoulos 1990; Santini & Lonardo 2000).

1.3 CONCLUSION AND RESEARCH AIMS

Having reviewed work done overseas and New Zealand, it is obvious that screening for cypress canker resistant plants in this country is still at its early stage. A complication is that two of the three pathogenic *Seiridium* species have been confirmed in New Zealand but there is a possibility of existence of a third species. It is important that the number of species is confirmed in order to develop a reliable screening programme. Contradictory results are likely to be related to misidentification of the *Seiridium* isolate used in studies because they may behave differently under the same environmental conditions. Variability in resistance has also been observed between ramets of the same clone growing at the same site (Raddi & Panconesi, 1984). Most of research work on cypress canker in the past decade has been done on *S. cardinale* and *S. unicorne* under overseas climatic condition and the results may not be directly applicable to New Zealand.

Forest Research requires reliable method of artificial inoculation of cypress with *Seiridium* species to screen for canker resistance under New Zealand climatic condition.

The overall goal of the study was to develop reliable methods of artificial inoculation of cypress with *Seiridium* spp. in order to detect any clonal differences in resistance to these pathogens.

The aims were to:

1. Consistently obtain abundant spore production in culture since large numbers of conidia would be required as inoculum.
2. Compare main stem and side branch inoculations.
3. Compare agar plug and conidial suspension inocula.
4. Determine the effective dose for conidial inoculum.
5. Assess inoculation of ramets in vitro and in vivo.
6. Determine whether wounds are required for infection.
7. Assess the pathogenic variability of isolates.
8. Assess the effect of environment on infection.

CHAPTER TWO

THE BIOLOGY OF *SEIRIDIUM* SPECIES

2.1 VARIATION IN SPORULATION AND CULTURAL MORPHOLOGY *OF S. UNICORNE* AND *S. CARDINALE* ISOLATES

2.1.1 INTRODUCTION

Studies on *Seiridium* species in New Zealand showed that there is considerable variation in cultural morphology among *Seiridium* isolates (Chou 1989; Self 1994). It was reported that "degenerative" forms arose from subcultures obtained from sectors of apparently vigorous original cultures. Resulting colonies were morphologically different with an associated reduction in pathogenicity (Chou 1990). Preliminary study showed that "degenerative" forms loose the ability to produce conidia (Tsatsia, 2003, unpublished).

2.1.1.1 Aims

The main aims of the series of studies were to investigate:

1. Colour variation of selected "degenerative" forms of *Seiridium* isolates.
2. Pathogenicity of mutant sector obtained from the standard isolate (SUL101.4*[1])
3. Effect of stock culture media on sporulation of selected *Seiridium* isolates.

*[1] Numbers used for the different *Seiridium* isolates was based on the numbering system used by Forest Research (Rotorua) listed in appendix VII. There were some modifications added to the numbering system. For instance, SU for *S. unicorne*, SC to denote *S. cardinale*, letters a, b or c to represent "degenerative" forms

2.1.2 MATERIALS AND METHODS

2.1.2.1 Study 1: Investigation on the morphological variation of selected *Seiridium* isolates

Different morphology normally arises after serial subcultures of *Seiridium* isolates. For instance isolate SUL175.4 often develops in one of the four colours; grey, orange, creamy white and yellow, examples shown in Plates 2.1, 2.2 and 2.3. Chou (1989) reported that subcultures either sectored out into normal and "degenerative" forms or as pure culture. Two sectors are normally observed, however, in some cases more than three mutant sectors appeared from a single inoculum plug.

- Objective

The objective of this study was to investigate colour variation of selected *Seiridium* isolates with "degenerative" forms under 12 h light/12 h dark cycle.

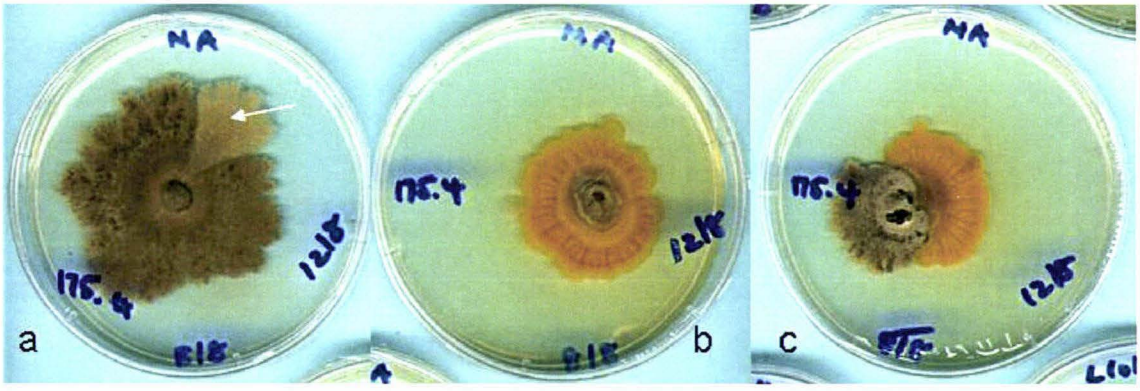

Plate 2.1 Different colour of cultures sub-cultured on malt agar from the same stock culture of *Seiridium unicorne* SUL175.4. (a): Dark grey with a light brown- coloured sector (indicated by an arrow). (b): Extreme "degenerative" form, orange colour. (c): Orange and grey coloured sectors resulted from the same inoculum plug.

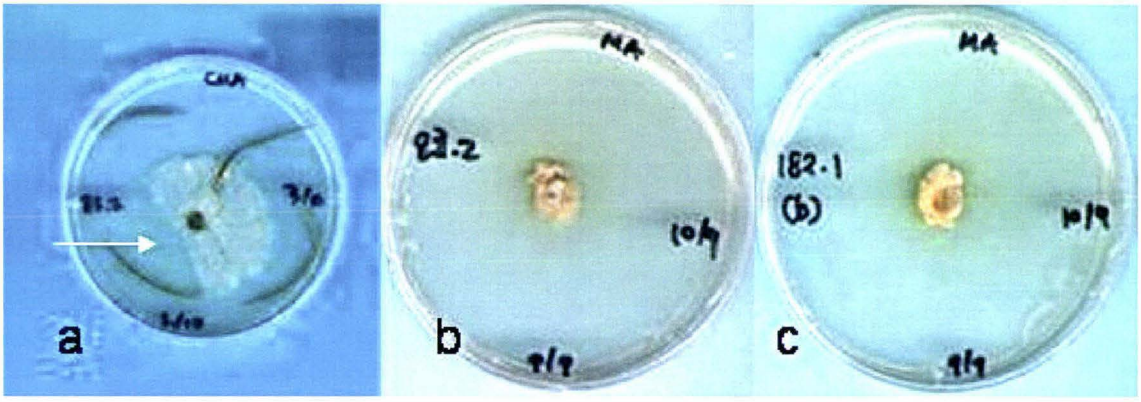

Plate 2.2 (a): *Seiridium unicorne* isolate SUL83.2 developed a 'degenerative sector' (indicated by a white arrow) on corn meal agar (CMA) overlaid with autoclaved carnation leaves (b & c): *Seiridium unicorne* isolate SUL83.2 & *Seiridium cardinale* SCL182.2b subculture resulted in the reduction of in growth rate of the colony.

- Procedure

Five *S. unicorne* isolates selected for this study were maintained initially on 3% malt extract agar (MA). The cultures were incubated under cool white light at 20°C 12 h light/12 h dark cycle for more than 20 days. The colours of the normal cultures of the five *Seiridium* isolates studied were grey.

Inoculum plugs (4 mm diameter.) from four normal cultures of *S. unicorne* isolates (SUL101.4, SUL175.1-sample 1(S1), SUL175.1-sample 2(S2) & SUL175.2); two "degenerative" cultures of isolate SUL175.4 and one "degenerative" cultures of isolate SUL101.4 were subcultured on 3% MA. There were four replicates per *S. unicorne* isolate. The cultures were randomly placed under white light at 20°C on a 12 h light/12 h dark cycle. Resulting colonies four weeks after inoculation were examined and recorded.

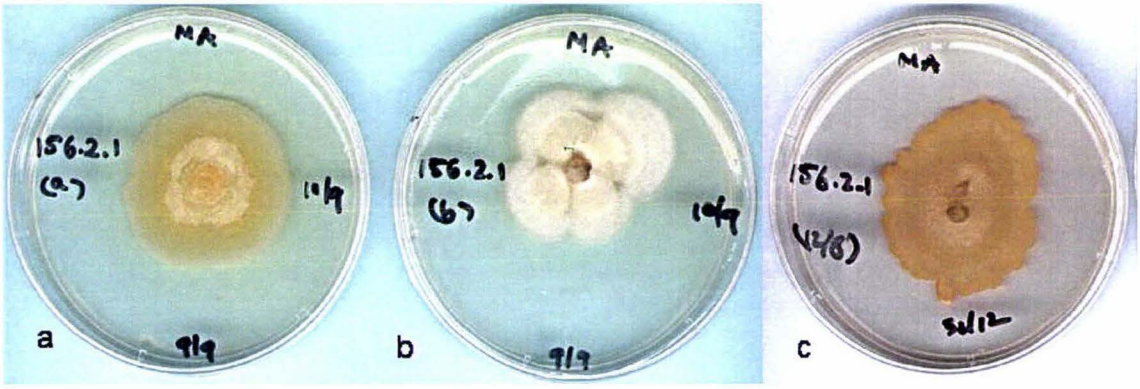

Plate 2.3 *S. unicorne* L156 .2.1 sectored out into three different morphologies

2.1.2.2 Study 2: Investigation on the pathogenicity of a mutant sector of *S. unicorne* isolate SUL101.4

S. unicorne isolate SUL101.4 has been found to be more virulent than other *Seiridium* isolates used in cypress canker disease screening programme in New Zealand (Ian Hood pers. comm.). It is regarded as the standard isolate.

- Objective

The objective of this study was to determine the pathogenicity of a surface mutant sector obtained from *S. unicorne* isolate (SUL101.4)

- Fungal material

Inoculum plugs used were obtained from two sectors (mutant white and "normal" grey colour) from a colony maintained on 3% MA.

- Plant material

The cypress plants used as the host for testing the pathogenicity were from *C. macrocarpa* family No.41 (M41*[1]) a family previously found to be highly susceptible to *S. unicorne* SUL101.4 under glasshouse conditions.

*[1] Numbering system was based on the numbers used by Forest Research. The word "family" was also adopted from that numbering system. M was added to represent *C. macrocarpa* families and L for *C. lusitanica* (Most of the plants used in this research were propagated from seedlings obtained from the different cypress plants or "families").

- Procedure

1. Wounds were created on a side branch of each of 6 plants using a sterilized 3 mm diameter cork borer and inoculum plugs 3 mm diameter were placed on the wound.
2. The wounds and the inoculum plugs were sealed with a strip of parafilm
3. Plants were watered every other day.

- Assessment

The number of side branches that developed canker symptoms was counted 7 weeks after inoculation.

2.1.2.3 Study 3: Investigation on the effect of media used for stock cultures on sporulation

- Objective

The objective of this experiment was to determine the effect media used for maintaining stock cultures on sporulation of *S. unicorne* and *S. cardinale* isolates. Conidia production was not consistent and in most cases failed to produce any conidia even on conidia inducing media described in Chapter 3.

- Fungal material

In the first set, only one *S. cardinale* isolate (SCL152.2.4) maintained on potato dextrose agar (PDA) and MA was used. Three *S. cardinale* isolates (SCL152.2.2a, SCL182.1, and SCL182.4) were used in trial 2.

- Plant material

Autoclaved carnation leaves were placed on the surface of agar to induce sporulation. Carnation leaves have been found to induce sporulation of *Fusarium* sp. (Wang et. al 1995).

- Procedure

Trial 1

1. Petri plates containing water agar overlaid with autoclaved carnation leaves were divided into 2 halves by drawing a line across the upper surface of the lid and at the bottom.

2. Inoculum plugs were cut out from PDA and MA stock cultures using 4 mm diameter cork borer. The two plugs were then placed on each half of the Petri plate and the Petri plates were sealed with strips of glad wrap.
3. Five Petri plates were inoculated and placed randomly under near UV light on a 12 h light/12 h dark cycle.

Trial 2

1. The study was repeated using three *S. cardinale* isolates (SCL152.2.2a, SCL182.1, and SCL182.4).
2. The isolates were inoculated as in the first set but on corn meal agar and carnation leaves.
3. Four replicates per isolate were incubated at 20°C under near UV light on a 12h light/12 h dark cycle.

- Assessment

Visual assessment was carried out 5 weeks after inoculation. Conidia were obtained from the cultures and were identified under a compound microscope (400x magnification).

2.1.3 RESULTS

2.1.3.1 Study 1: Investigation on morphological variation of selected *Seiridium* isolates

Colonies were examined and recorded 4 weeks after inoculation (Table 2.1). Plate 2.4a shows normal colony form of isolate SUL175.4 from the stock cultures. Subcultures from orange colony retained the original colour and morphology. Twenty-five percent of the culture plates from isolate SUL175.2, SUL175.1-(S1) and SUL175.1-(S2) produced degenerative colonies. Degenerative colonies were not produced from subcultures of isolate SUL101.4. Inoculum plugs from white colony of *S. unicorne* SUL175.4 resulted in yellow waxy looking cultures (Plate 2.4b). Subculture of orange coloured colony of *S. unicorne* isolate SUL175.4 produced all orange coloured "degenerative" colonies (Plate 2.4c).

Table 2.1 Colour of surface mutant sectors and resulting colonies sub-cultured on MA under white light at 20°C on a 12 h light/12 h dark cycle

Isolate No.	Original colony colour	Resulting colonies
SUL175.4	White	Yellow and waxy surface (4/4 plates)
SUL175.4	Orange	Orange (4/4 plates)
SUL101.4	Grey	Grey (4/4 plates)
SUL101.4	White	Grey (4/4 plates)
SUL175.2	Grey	1 Petri plate out of 4 produced both grey and white coloured sectors and 3 plates produced only grey colonies
SUL175.1(S1)	Grey	1 Petri plate out of 4 produced both grey and white coloured sectors and 3 plates produced only grey colonies.
SUL175.1(S2)	Grey	1 Petri plate out of 4 produced both slimy orange and grey coloured sectors. The remaining 3 plates produced only grey colonies.

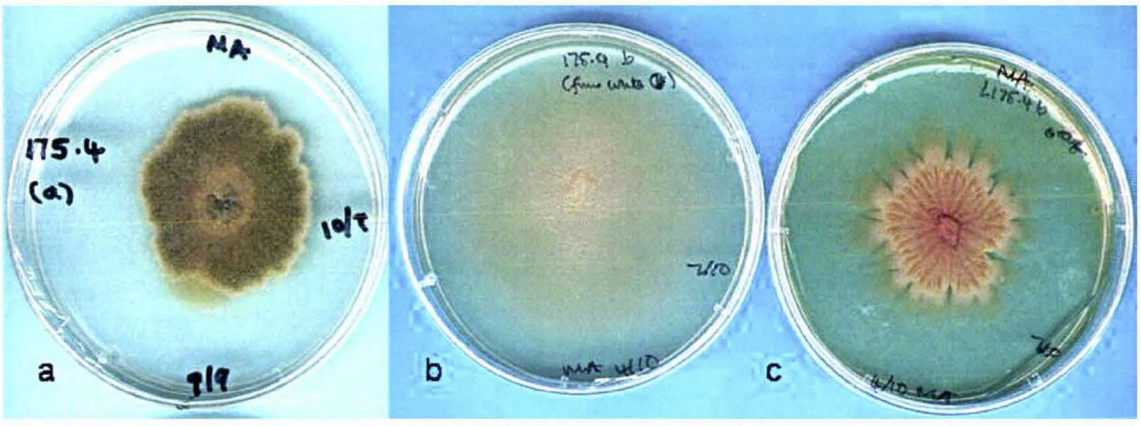

Plate 2.4 (a): "Normal" colony of isolate SUL175.4 (b): Waxy yellow colony subcultured from a white coloured *S. unicorne* SUL175.4 mutant sector; (c): Orange coloured colony subcultured from orange coloured *S. unicorne* SUL175.4 mutant sector.

2.1.3.2 Study 2: Investigation on the pathogenicity of a mutant sector of *S. unicorne* isolate SUL101.4

Four of the six inoculum plugs obtained from the mutant white sector of the colony gave rise to disease lesions; five of the six "normal", grey inoculum plugs did so. This study showed that mutant sectors can cause infection. The pathogen was recovered from 80% of the side branches inoculated with the normal inoculum plugs but only from 50% of the side branches inoculated with the surface mutant sector (Table 2.2).

Table 2.2 Number of inoculated side branches with disease symptoms

Colour of sector	No. of infected side branches out of 6	Remarks
White	4	Fungus found growing on 2 of the 4 Side branches sporulated and the conidia were identical to SUL101.4.* The rest did not develop conidia
Grey	5	Conidia identical to SUL101.4 were found on 4 of the 5 infected side branches

* Spore shape (Appendix VI)

2.1.3.3 Study 3: Investigation on the effect of media used for stock cultures on sporulation

Sporulation started on PDA inoculum plug cultures three weeks after inoculation in triall of the study. Sporulation occurred in all the Petri plates. No sporulation occurred on cultures where inoculum plugs were obtained from MA stock cultures (Table 2.3).

With the second trial of inoculations, all colonies subcultured from PDA stock cultures sporulated. *S. cardinale* isolate SCL182.1 sporulated in all the replicates obtained from both PDA and MA based stock cultures. For those originating from the remaining two isolates SCL152.2.2a and SCL182.4 however, only half of MA inoculum plugs cultures, sporulated.

Table 2.3 Number of cultures with sporulation after five weeks incubation under near UV light on a 12 h light/12 h dark cycle

Isolate No/Set No.	Type of inoculum plug	Number of cultures that sporulated
Trial 1(Inoculation on Water agar and carnation leaves) Max.=5 cultures		
SCL152.2.4	PDA	5
	MA	0
Trial 2(Inoculation on Corn meal agar and carnation leaves) Max.=4 cultures		
SCL152.2.2a	PDA	4
	MA	2
SCL182.1	PDA	4
	MA	4
SCL182.4	PDA	4
	MA	2

2.1.4 DISCUSSION

2.1.4.1 Study 1: Investigation on the morphological variation of Selected *Seiridium* isolates

The study showed that when some *Seiridium* isolates reached a "degenerative" stage, it may be difficult to bring about reversion to "normal" cultures of most isolates. SUL101.4 is fairly stable but continuous subculturing can result in bald orange coloured colonies. This isolate can sometimes revert back into normal cultures spontaneously. The conditions required for reversing it back into normal cultures on artificial medium is not known.

Sectoring out into different morphologies and colour of a fungal colony is quite a common characteristic of most *S. unicorne* isolates; however some isolates appear to have more distinctive degenerative forms than others. *Seiridium* isolate SUL175 is a typical example of those *S. unicorne* isolates that may be difficult to stimulate to revert back into normal cultures.

2.1.4.2 Study 2: Investigation on the pathogenicity of a mutant sector of *S. unicorne* isolate SUL101.4

The grey ("normal") sector appeared to be more virulent resulting in 83% (5/6) infection of the inoculated side branches while inoculum plugs from the white sector resulted only in 66% (5/6) infection of the inoculated side branches. The two sectors were subcultured and the resulting colonies were all normal grey. In this case, the white mutant sector was not a"degenerative" form.

Plants used in this study had previously been inoculated with the same *S. unicorne* isolate SUL101.4 using spores (10,000 spores /wound) obtained from "normal" cultures. That inoculation resulted also in 83 % (5/6) canker infection

This result revealed a variability that might occur when mycelia are being used for inoculation. The mycelial inoculum plugs from the white mutant sector resulted only in 66 % canker infection as compared to 83 % caused by "normal" grey sector from the same fungal colony and conidial inocula from "normal" culture. There was no

difference between mycelium plugs from "normal grey" sector and conidial inocula, however, condial inocula may be more reliable than mycelial inoculum plugs.

2.1.4.3 Study 3: Investigation on the effect of media used for stock cultures on sporulation

PDA-based inoculum plugs inoculated on water agar and carnation leaves sporulated while MA-based inoculum plugs did not sporulate in trial 1. Since the stock cultures were of different ages, there was a possibility that the age of the stock cultures could have had some effect on sporulation. With the second trial where inoculum plugs were placed on corn meal agar, PDA based stock cultures sporulated in all replicates while isolates obtained from MA based stock cultures sporulated in only half of the replicates. Although sporulation occurred in cultures with MA inoculum plugs in trial2, all PDA based inoculum plugs were rated as heavy sporulation while MA-based inoculum plug cultures were either rated as moderate or sparse. Since the cultures were of the same age in the second inoculations, the possibility of stock culture age-related effect is ruled out here. The results supported studies by Chou (1989) where he found that PDA seemed to provide better growth medium than MA. Tabata et al. (1991) also reported good sporulation on PDA cultures compared to other media types.

PDA appeared to be the best medium for maintaining "normal "*Seiridium* stock cultures for conidia production.

2.2 CONIDIUM GERMINATION OF *SEIRIDIUM* SPECIES ON HOST PLANTS

2.2.1 INTRODUCTION

There is very little information available on conidium germination of *S. unicorne*, *S. cardinale* and *S. cupressi* species on the host tissues. Spanos et al (1997a) studied in vitro host expression of resistance responses to *Seiridium* species using mycelia of the pathogen for inoculation.

2.2.1.1 Aim

The aim of this work was to investigate spore germination of *S. unicorne* and *S. cardinale* isolates on host plants with known and unknown resistance to cypress canker infection.

2.2.2 MATERIALS AND METHODS

- *Seiridium* isolates

Conidial suspensions of *Seiridium* isolate SUL101.4, SUL156.2.2 and SCL152.2.4 were used in this study.

- Cypress plants

Side branches were obtained from *C. lusitanica* plant (L2) and 2 *C. macrcocarpa* plants from family M23 and unidentified (UMF) *C. macrocarpa* family. L2 and M23 were previous inoculated for spore load study while the *C. macrcocarpa* plant was not.

- Procedures

Inoculation

1. Side branches (2-5 mm in diameter) were obtained from the selected plants and cut into 5 cm lengths.
2. The cuttings were dipped in 70% ethanol and then were rinsed in sterilised RO water 3 times before drying sterile paper towels.

3. Wounds were made on each cutting at a distance of 1 cm apart using a sterile syringe needle.
4. Ends of the cuttings were sealed using parafilm and were then placed on a moistened paper towels in a sterile Petri plate.
5. A 1 µl conidial suspension (100,000 conidia/ml) droplet containing approximately 500 conidia was the applied to the wounded sites and the lids of the Petri plates were placed on.
6. The cuttings were incubated under room temperature for 1 week.

Preparation of plant tissues for microscopic observation

1. Diseased plant tissues were cut from the inoculation sites using a sterile scalpel blade and soaked in lactophenol.
2. After 24 hours, the tissues were removed, placed on glass slides, stained with lactophenol blue and heated over Bunsen flame until just boiling.
3. When they have cooled, plant tissues were rinsed with water and mounted in Shear's mounting fluid and observed under compound microscope at 400x magnification.

2.2.3 RESULTS

Conidia from the three *Seiridium* isolates (SUL101, SUL156.2.2 & SCL152.2.4) germinated on *C. macrocarpa* M23 and UMF but not on *C. lusitanica*. It was difficult to quantify the number of spores that have germinated due to the presence of thick mycelial growth, however slide photo taken of a section of the infected bark tissue showed at least two fully germinated spores (400 x magnification). There was difference observed between the *Seiridium* isolates in terms of mycelial growth on the inoculation sites. There was more mycelial growth observed on *C. macrocarpa* plants inoculated *S. unicorne* isolate SUL101.4 than SUL156.2.2 and SCL152.2.4. The stem of UMF plant was fully covered with mycelial growth, where as there was very little mycelial growth on M23. Therefore UMF appeared to be more susceptible to the three *Seiridium* isolates used.

2.2.4 DISCUSSION

The *C. lusitanica* (L2) plant used in this study was previously inoculated with SUL156.2.2 and SUL101.4. This plant was found to be resistant to both of the *Seiridium* isolates. This study also produced similar result. Plants from *C. macrocarpa* family M23 showed resistance to cypress canker in the field (Ian Hood pers. comm). However, this particular seedling appeared to less resistant because there was mycelial growth on the inoculated site on the cut stem but growth was slower compared to UMF plant.. Ponchet and Andreoli (1989) reported that the resistance of cypress clones depend on the thickness of Necrophylactic periderm (NP). Resistance clones were found to NP thicker than 100 micron, while susceptible clones have NP less than 60 micron. In this study, no germ tube was observed on spores inoculated on the *C. lusitanica* plant. It appears that antifungal compounds prevented spore germination on the *C. lusitanica* plant. Tissues obtained from inoculated side branches under shade cloth condition also showed similar observation (Appendix VII).

Spore germination on excised host tissue could be a useful method for screening resistance to cypress canker. Detail studies using different staining techniques will give a clearer picture of the host resistance mechanism involved.

CHAPTER THREE

EVALUATION OF A RANGE OF MEDIA FOR SUPPORTING SPORULATION OF *SEIRIDIUM* SPECIES

3.1 EVALUATION OF A RANGE OF AGAR TYPES AND ADDITIVES

3.1.1 INTRODUCTION

Screening for cypress clones resistant to canker disease caused by *Seiridium* species involves artificial inoculation. Mycelium is the common type of inoculum used in screening programmes in several countries (Strouts 1973; Raddi & Panconesi 1984; Chou 1990; Xenopoulos 1990; Tisserat 1991; Santini et al. 1997). Infection of cypress plants in nature is by conidia (Raddi & Panconesi 1981). The use of mycelium could mask the type of reaction of the same plant in the natural habitat when it is exposed to conidia inoculum. Use of high levels of inoculum could result in early elimination of clones of desirable characters that could be used in the breeding programmes. Raddi & Panconesi (1984) suggested that the variation in screening results could be due to the fact that inoculations might have been carried out with mycelium taken from mutant sector of fungal colony with either higher or lower pathogenicity. Hood & Gardner (2002) expressed a similar concern that with *S. unicorne* cultures, the ability of cultures to degenerate and loose virulence poses an additional complication as reported by Chou (1989) and Self (1994).

Sasaki & Kobayashi (1976) reported conidial production by *S. unicorne* was sparse on PDA, MA, V8 juice and Wakman's medium. Tobata et al. (1991) found that PDA and PDA plus 0.4% dried yeast extract induced good sporulation. V8 juice and PDA are examples of nutrient rich media. V8 juice has been reported to increase sporulation of fungal pathogens, for example, *Monilinia oxycocci* (Sanderson & Jeffers 2001), *Alternaria alternata* (Wei et al. 1985) and *Cercospora fijiensis* (Bailey 1995). Malt

extract, a medium commonly used for culturing *Seiridium* species does not support sporulation of the two *S. unicorne* isolates maintained at Massey University. However, Chou (1989) used MA for conidia production. According to Waller (1981), carbohydrate-rich media such as potato dextrose agar delays sporulation. The continuous subculturing of the *S. unicorne* isolates kept at Massey University may be the cause of the reduction of sporulating ability.

Addition of plant extracts to agar has shown to promote fungal growth and sporulation (Wyss et al. 2001). Carnation leaves have been shown to increase sporulation of *Alternaria dianthi* (Hu & Wu 1997) and *Fusarium* (Fisher et al. 1982; Wang et al. 1995) when included in a medium. Cypress plant (*C. macrocarpa* species) is a natural host substrate of the fungal pathogen being studied. Chou (1989) used autoclaved *C. macrocarpa* pieces to induce sporulation of *S. unicorne* and *S. cardinale*.

Preliminary evaluation of four agar types (MA, V8, CMA & WA) and additives (peptone and two plant additives) showed significant differences with additives, isolates (SUL83.2 & SUL101.4) and interaction between agar type and isolates (Tsatsia 2002, unpublished). WA was selected because it is a non-nutrient medium, while CMA is a nutrient poor medium. Carbohydrate starvation may induce asexual sporulation of many fungi (Waller 1981).The plant additives used included autoclaved carnation leaves and side shoots of *C. macrocarpa* seedlings.

The ANOVA results for the preliminary study is shown in Appendix I-A. Sporulation occurred on plant materials added on the agar types (Plate 3.1a-c).

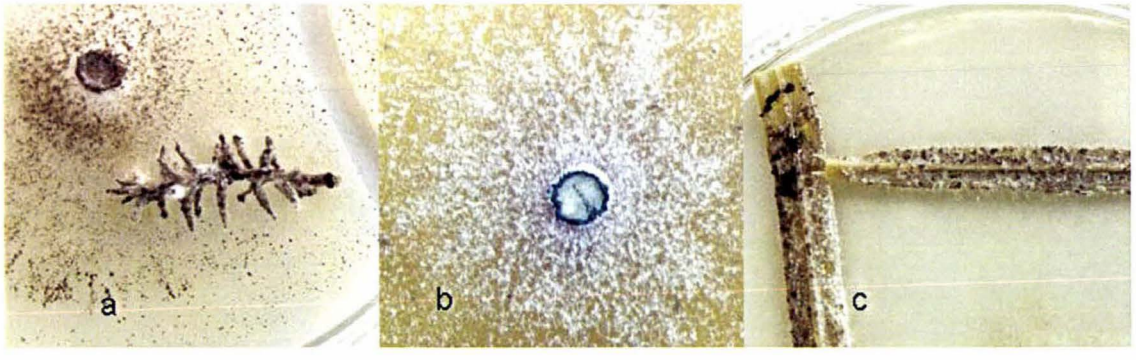

Plate 3.1 (a):Conidia of *S. unicorne* isolate SUL101.4 on *Cupressus macrocarpa* shoots and around original inoculum placed on corn meal agar (b): Conidia of *S. unicorne* SUL83.2 around agar plugs on water agar. (c): Conidia of *S. unicorne* isolate SUL101.4 on carnation leaves shoots placed on water agar.

3.1.1.1 Aims

The overall aim of the experiments was to further evaluate selected agar types with plant material additives (carnation leaves and *C. macrocarpa* side shoots) previously used in the preliminary study.

3.1.2 MATERIALS AND METHODS

3.1.2.1 Experiment 1

- Objective

The objective of this experiment was to compare agar types (CM, V8 and WA) overlaid with plant substrates, with PDA.

- Fungal material

Original stock cultures of *Seiridium unicorne* isolates (SUL101.4 & SUL83.) sent by Forest Research (Rotorua, New Zealand) were grown on 3% MA and incubated under near UV light (two fluorescent tubes, Sylvania – 40 watts) at 20 ± 2°C on a 12 h light/12 h dark cycle. The "normal" appearance of colonies was grey-coloured and fluffy. The morphology of such cultures became "degenerative" (bald and non-fluffy) in appearance after four series of subculturing. "Normal "cultures selected for this study were 8 to 10 weeks old.

- Agar types. The agar types used for the study were:

Potato dextrose agar (PDA), water agar (WA -Agar-granulated-Becton Dickinson), corn meal agar (CMA-DIFCO) and V8 Juice agar (V8A-Campell's Soups).

- Plant material

The plant material additives were autoclaved carnation leaves, autoclaved *C. macrocarpa* shoots and homogenized *C. macrocarpa* shoots (3 g/l). The cypress materials were obtained from *C. macrocarpa* plants supplied by Forest Research and kept in the glasshouse located in the third floor of AGHORT building (Massey University, Palmerston North, New Zealand).

- Experimental Design

A factorial experiment consisting of 2 *S. unicorne* isolates, 3 replicates and 11 media treatments were used.

- Procedure

Shoots were excised, washed before being prepared. Whole shoots were cut up into 15-20 mm sizes, placed in glass Petri plates. For homogenized shoots, 3 grams were weighed and homogenized in 50 ml of reversed osmosis (RO) water using Polytron blender before adding to the allocated agar type and adding more RO water to make up the required amount of media.

Carnation leaves were cut up into 40-50 mm lengths and placed in glass Petri dishes.

RO water was added to each agar type and pH reading to about 7 with the addition of NAOH or HCL as required before autoclaving since the optimum pH range for conidial production of *S. unicorne* was reported to be 6-9.1(Tobata et al.1991). All the agar and plant material were autoclaved for 15 minutes at 120°C. The details for media and additive combinations are shown in Table 3.1. After autoclaving the media were poured into Petri dishes. The plant materials were laid on the surface of the allocated media after the media solidified as shown in Plates.3.2a and b.

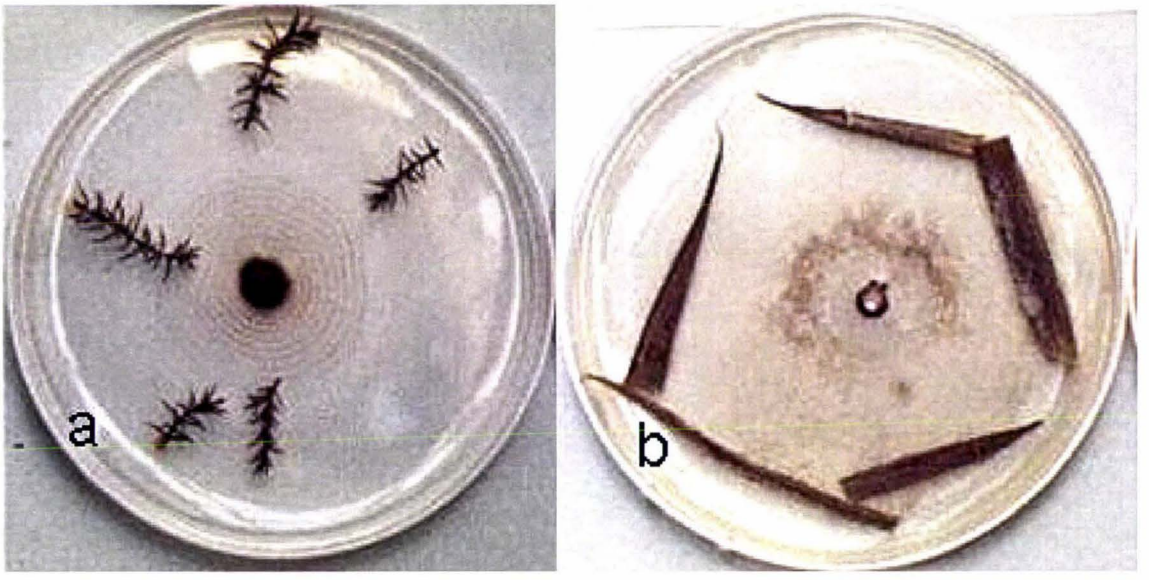

Plate 3.2 (a): 15-20 mm pieces of *C. macrocarpa* shoots placed on the media (b): 40-.50 mm pieces of carnation leaves placed around the original agar plug.

Agar plugs containing mycelium was obtained from the edges of isolates SUL101.4 and SUL83.2 cultures using a 4 mm diameter cork borer and were placed inverted on each agar plate. The plates were sealed with parafilm to prevent the cultures drying up. The treatments for the experiment are given in Table 3.1. The cultures were incubated at 20°C ± 2°C under near UV light on a 12 h light/12 h dark cycle. Tobata et al. (1991) reported light irradiation resulted in good sporulation.

- Assessment

Conidia production in the cultures was assessed after 73 days of incubation under near UV light. A dissecting microscope was used to check for conidia production (40x magnification.). Visual observation was then confirmed and conidia count was carried out using the method described below.

- Procedure

Two ml of RO water containing a drop of 0.1% Tween 80 was added to each culture plate. A glass rod was used to dislodge conidia from the surface of the culture media. The conidia suspension was then poured into McCartney bottles. Conidia on carnation leaves, *C. macrocarpa* pieces and original agar plugs were difficult to remove so these were placed into the McCartney bottles containing the conidia suspension. The suspensions were left over night. The purpose for doing this was to soften dry conidial masses on the plant material and the original agar plugs. The next day the conidial suspensions were agitated for 1 minute and filtered using 20-µm cell strainers. Conidia

concentrations were calculated using a haemocytometer. Analysis of variance was carried out using the SAS Program. Log_{10} (y + 1) transformation was required due to zero values and very high conidia counts in some cultures.

Table 3.1 Ingredients (Media consisting of agar types and plant additives)

Media type	Ingredients
PDA	39 g/l
WAAC	Water agar (15 g/l) and autoclaved carnation leaves
WAHM	Water agar (15 g/l) and homogenized *C. macrocarpa* shoots (3g/l)
CMAC	Corn meal agar (17 g/l) and autoclaved carnation leaves
CMAM	Corn meal agar (17 g/l) and autoclaved *C. macrocarpa* shoots
V8AC	Clarified 5%V8 juice, agar (15 g/l) and autoclaved carnation leaves
V8AM	Clarified 5% V8 juice, agar (15 g/l) and autoclaved *C. macrocarpa* shoots
V8HM	5% V8 juice, agar (15 g/l) and homogenized *C. macrocarpa* shoots (3g/)

3.1.2.2 Experiment 2

Previous evaluation study showed that CM, WA and V8 agar with the addition of plant material induced good sporulation. The variation of sporulation was very high, ranging from no sporulation to very heavy sporulation on cultures inoculated from the same fungal colony.

- Objectives

The objectives of this experiment were:

1. The first objective of this experiment was to compare the two isolates SUL83 and SUL101.4 maintained at Massey University plant pathology laboratory with SUL155, an isolate recently sent from Forest Research. The former two isolates have been subcultured over a period of two years.
2. The second objective was determine the effect of near UV light on the sporulation of the *S. unicorne* isolates cultured on agar types overlaid autoclaved carnation leaves and *C. macrocarpa* twigs.

- Fungal material

Inoculum plugs were obtained from *S. unicorne* isolate SUL83, SUL101.4 and SUL155.7.1 maintained on 3% MA and kept under white light at 20°C on a 12 h light/12 h dark cycle. The age of cultures ranged from 16 weeks to 20 weeks.

- Plant material

Plant additives were autoclaved carnation leaves and twigs from side shoots of *C. macrocarpa* seedlings.

- Experimental design

A randomised complete block design consisting of stacked agar plates with 6 media treatment combinations (3 agar types x 2 plant additives) randomly allocated to four blocks. The bottom was referred as Block 1 and the 4th plate sitting on the top was block 4. Control treatments were not considered as necessary because preliminary evaluation test showed either sparse or no conidia production on these agar types.

- Assessment

Conidia count was carried out as for experiment 1, after 48 days of incubation under near UV light on a 12 h light/12 h dark cycle at 20°C.

The treatment combinations were:

1. WA + Carnation, + SUL83.2
2. WA + Carnation, + SUL101.4
3. WA + Carnation, + SUL155.7.1
4. WA + *C. macrocarpa*, + SUL83.2
5. WA + *C. macrocarpa*, + SUL101.4
6. WA + *C. macrocarpa*, + SUL155.7.1
7. CMA + Carnation, + SUL83.2
8. CMA + Carnation, + SUL101.4
9. CMA + Carnation, + SUL155.7.1
10. CMA + *C. macrocarpa*, + SUL83.2
11. CMA + *C. macrocarpa*, + SUL101.4
12. CMA + *C. macrocarpa*, + SUL155.7.1
13. V8A + Carnation, + SUL83.2
14. V8A + Carnation, + SUL101.4
15. V8A + Carnation, + SUL155.7.1
6. V8A + *C. macrocarpa*, + SUL83.2
17. V8A + *C. macrocarpa*, + SUL101.4
18. V8A + *C. macrocarpa*, + SUL155.7.1

3.1.2.3 Experiment 3

- Objective

The method of inducing sporulation for *S. unicorne* described in experiments 1 and 2 in this chapter failed to produce adequate conidia for other proposed experiments when the method was repeated. Chou (1989) reported method of inducing sporulation of *S. unicorne* and *S. cardinale* by incubating cultures in the dark for two weeks before overlaying with autoclaved *C. macrocarpa* pieces.

The objective of this experiment was to determine whether Chou's method of conidia production will increase conidia production of *S. unicorne* and *S. cardinale* isolates maintained at Massey University.

- Fungal material

The *S. unicorne* isolates selected for this study included SUL101.4, SUL155.7.1, SUL155.7.1a and *S. cardinale* isolate was SCL182.2.2. The stock cultures were maintained on a 3% MA and incubated under near UV light at 20°C on 12 h light/12 h dark cycle. The cultures were 10 weeks old at the time of inoculation. SUL155.7.1a appeared to be a mutant form of SUL155.7.1. The two isolates are very different in the appearance of the colony morphology (Plate.3 3). SUL155.7.1a grow faster and colony size is usually larger than the "normal" grey colonies. No test was done to determine the pathogenicity of the two forms of SUL155.7.1. *S. unicorne* isolate SUL155.7.1a never produce conidia on conidia inducing media described in experiments 1 and 2.

- Agar type used was malt extract (DIFO Lot 139107 x B Exp Jan 01)

MA was selected because it is the standard medium for culturing *Seiridium* species at Forest Research and Chou (1989) also used this agar for conidia production. According to Koch et al. (1988) malt extract supported growth and sporulation of *Collectotrichum trifolii.*

- Procedure

Agar plugs containing mycelium were obtained from the edges of the selected cultures with a 4-mm diameter cork borer and were inoculated on fresh 3% MA. The cultures were placed randomly in a box and incubated in the dark at room temperature for 2 weeks. After two weeks in the dark, the cultures were overlaid with autoclaved cypress

bark, twigs and cone pieces and then placed in a randomized block design under near UV light at 20°C for another 2 weeks.

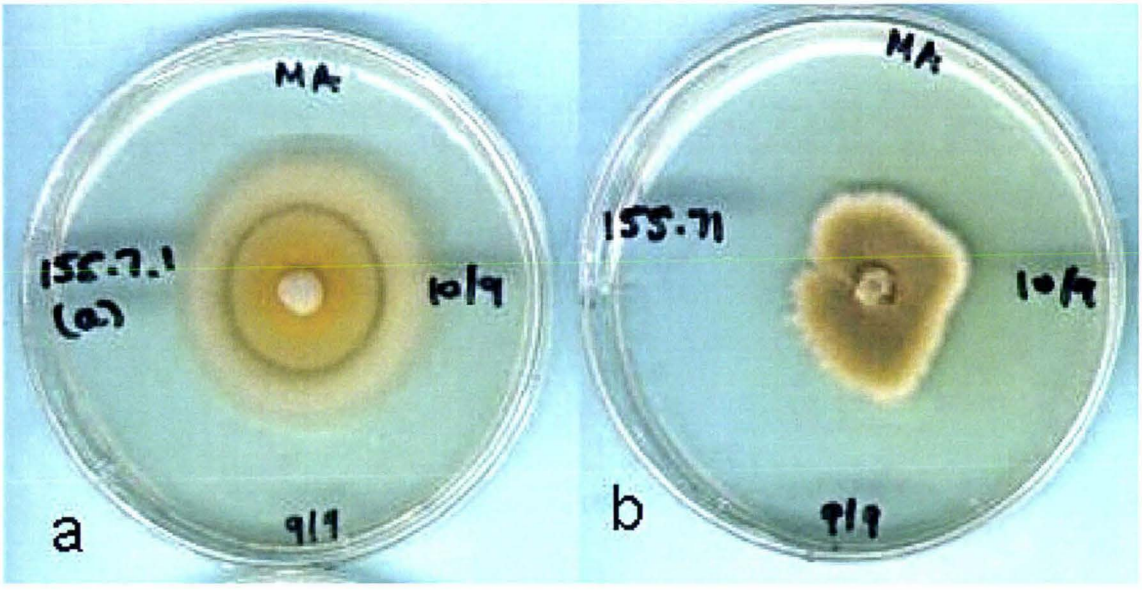

Plate 3.3 Two forms of SUL155.7.1 colonies incubated in the dark at room temperature for two weeks. (a): Mutant form of *S. unicorne* SUL155.7.1; (b): "Normal" form of *S. unicorne* SUL155.7.1.

- Assessment

Spore production was assessed using a scoring system. This system was used because it was found to be easier and quicker than the assessment method used in the previous experiments in this chapter. It was also difficult to remove conidia from the surface of MA using the method described in experiments 1 and 2. The scores were:

1= No spores; 2= Sparse sporulation; 3= Moderate sporulation; 4= Heavy sporulation. Assessment was carried out eight weeks after inoculation (after six weeks of incubation under near UV light).

3.1.2.4 Experiment 4

- Objective

The method of conidia production used in experiment 3 also resulted in poor conidia production of the *S. unicorne* isolates tested.

The objective of this experiment was to test a modified version of the method used in experiment 3. The *S. unicorne* and *S. cardinale* cultures were inoculated on MA overlaid with autoclaved *C. macrocarpa* stem and bark pieces before incubating in the dark for two weeks.

- Fungal material

One *S. cardinale* isolate SCL152.2.2b and one *S. unicorne* isolate SUL101.4 (control) were used for this study. The stock cultures were 22 weeks old and were maintained on 3% MA at room temperature.

- Agar types

Agar types used included MA, and PDA

- Plant material

C. macrocarpa pieces were obtained from side shoots and main stem bark of seedlings. The plant material was autoclaved for 15 minutes at 120°C.

- Procedure

This study differed from the first one in that the *Seiridium* isolates were inoculated and the media overlaid with autoclaved *C. macrocarpa* bark and side shoot pieces before incubating in the dark at 25°C for 2 weeks. After this treatment, the cultures were placed under near UV light for another 2 weeks. The plates were laid out in a randomised complete block design under near UV light. There were five replicates for *S. cardinale* isolate SCL152.2.2 on PDA and MA and three replicates for *S. unicorne* SUL101.4 on PDA and MA.

- Assessment

The assessment was carried out ten weeks after inoculation (after eight weeks incubation under near UV light). The method of assessment was based on scoring system as described for experiment 3.

3.1.3 RESULTS

3.1.3.1 Experiment 1

There was no conidia production on plain PDA. There was sparse sporulation on V8 agar overlaid with autoclaved *C. macrocarpa* shoots and V8 agar over laid with carnation leaves. On WAAC, Isolate SUL101.4 did not produce conidia in all the replicates, but there was good sporulation of isolate SUL83.2, with one of the replicate Petri plates having the highest conidia production of the trial, producing approximately 7,692,000 conidia.

CMAC isolate SUL101.4 failed to produce conidia in two of the culture plates. In the preliminary experiment, isolate SUL83.2 failed to produce any conidia, however, here it produced conidia in all replicates. Isolate SUL101.4 did not sporulate on V8 juice agar plus the additives. Comparing the *Seiridium* isolates, *S. unicorne* isolate SUL83.2 produced more conidia than SUL101.4.

Analysis of variance of transformed conidia count showed significance difference with agar types, isolate and interaction between agar type and isolates (Appendix I-B). *S. unicorne* isolate SUL83.2 the highest mean conidia count and SUL101.4, the lowest (Table 3.2).

Table 3.2 Treatment means for conidia count

Treatments	Log_{10} conidia count mean	Actual conidia count mean
Media Types*		
WA + carnation leaves	3.89a*[1]	2,184,000
CM + carnation leaves	3.88a	1,300,667
CM + *C. macrocarpa* side shoots	3.66a	283,333
V8 + carnation leaves	0.87b	27,334
V8 + *C. macrocarpa* side shoots	0.73b	4,000
WA + homgenised *C. macrocarpa* side shoots	1.68ab	52,667
V8 + homogenised *C. macrocarpa* side shoots	1.68ab	43,33-
PDA	0	0
s.e = 0.8 (n=6)		
Seiridium isolates***		
SUL83.2	3.31	956,000
SUL101.4	0.78	17,834
s.e = 0.4 (n=24)		

Significant *F* test at $P = 0.05$ *** Highly significant *F* test $P = 0.0001$. Interaction is not significant.
*[1]Means followed by the same letters are not significantly different.

3.1.3.2 Experiment 2

Result for the analysis of variance for this experiment is listed in Appendix I-C. Isolate SUL155.7.1 produced the highest mean score for sporulation.V8 juice agar to be more consistent in conidia production with the highest mean of the transformed score data (Table 3.3). CM gave the highest mean for the actual conidia count. Addition of carnation leaves to the agars tested resulted in high conidia production compared with *C. macrocarpa* shoots.

Table 3.3 Treatment means for conidia count

Treatments	Log10 Conidia count mean	Actual conidia count mean
Agar Types**		
V8	5.43 [a*1]	745.266
CM	4.65[ab]	835.475
WA	4.05[b]	508.558
s.e = 0.09 (n=24)		
lsd=0.85		
Additives***		
Carnation leaves	5.4[a]	1,201.650
C. macrocarpa side shoots	4.0[b]	191.216
s.e = 0.06 (n=36)		
lsd=0.70		
Seiridium isolates *		
SUL155.7.1	5.48[a]	1,107.600
SUL83.2	4.35[b]	638.466
SUL101.4	4.30[b]	343.233
s.e = 0.09 (n=24)		
lsd= 0.85		

Significant F test at P = 0.01 ** Significant F test at P = 0.01 *** Highly significant F test P = 0.0001. Interaction is not significant. *[1]Means followed by the same letters are not significantly different.

There was a significant difference between blocking of cultures plates in this experiment. The 4th Petri plates on the top of the stack (4 plates) appear to sporulate better than the lower positions but this also varied among the different treatments.

3.1.3.3 Experiment 3

Isolate SUL155.7.1a and SCL182.2b did not sporulate in any of the Petri plates. For *S. unicorne* isolate SUL101.4, only 20 % produced conidia with score 3. Sixty % of SUL155.7.1 cultures produced conidia with a score of 2, 20 % with score 3 and 20 % with score 4. One of the culture plates of isolate SUL155.7.1 produced the highest score of 3. That isolate also produced the highest mean score of 2.6. The mean score for the standard isolate SUL101.4 was 1.4.

3.1.3.4 Experiment 4

Eighty percent of *S. cardinale* SCL152.2.2 cultures did not sporulate on PDA media (Appendix I-D). The remaining 20% was rated category 3. The mean score was 1.4. On MA media, 40% of isolate SCL152.2.2 cultures were category 1, 60% were category 2 and the mean score MA was 1.6.

Thirty-three percent of *S. unicorne* SUL101.4 cultures were category 2 and 66.7 % were category 4 on PDA. The mean score was 3.3. This same isolate produced a mean score of 2.0 on MA media under the same condition of incubation. Sixty-six % of the cultures did not sporulate on MA media. The remaining 33% sporulated and were assessed as category 4.

3.1.4 DISCUSSION

In experiment 1, there was no sporulation on PDA, contradicting previous reports by Sasaki & Kobayashi (1976). The possible explanations could be due the type and age of PDA used. The result could not be related to degenerative forms because cultures on other agar types sporulated. CM agar and plant additives showed high and consistent result of sporulation.

Isolate SUL155.7.1, an isolate recently sent by Forest Research (Rotorua) produced more spores than isolate SUL101.4 and SUL83.2. The latter isolates have been subcultured for more than a year. However, the mutant form of isolate SUL155.7.1 did not sporulate.

The result from the third experiment again showed SUL155.7.1 with the highest mean score compared with the standard isolate SUL101.4. When this isolate was re-tested in experiment 4, there was an increase in spore production on MA and PDA. Overlaying "normal" *S. cardinale* and *S. unicorne* cultures with plant material before incubation in the dark appeared to increase sporulation.

3.2 EVALUATION OF TREE SHOOTS AND CYPRESS CONES AS SUBSTRATES FOR ACERVULI PRODUCTION OF *SEIRIDIUM* SPECIES

3.2.1 INTRODUCTION

Work done on a Diatrypaceous fungus (*Cryptovalsa* sp.) at Massey University showed that substrates from more than one genus of host plants could be used to induce spore production(Peter Long: pers. Comm.). The fungus was isolated from grape vine but formed ascospores when it was cultured on black current twigs and placed under a sequence of temperature and light regimes to represent substrate colonization, winter chilling and spring days.

Evaluation of different media for supporting sporulation of the two *S. unicorne* isolates (SUL83.2 &SUL101.4) showed that water agar and carnation leaves or *C. macrocarpa* shoots resulted in high production of spores (Section 3.1.2.1 and 3.1.2.2). However, when the two *S. unicorne* isolates were cultured on the agar types overlaid with carnation leaves or *C. macrocarpa* shoot pieces during the winter period (from June to August), conidia production was drastically reduced. In some Petri plates no conidia were produced. Other methods such as those used by Chou (1989) and Intini & Panconesi (1974) were also tested but the results so far showed no increase in spore production. Therefore the method that was developed for ascospore production in Diatrypaceae fungi was tested using plant material from host plants and other tree species.

3.2.2 MATERIALS AND METHODS

- Objective

The objective of the experiments (Trials 1, 2 & 3) was to evaluate twigs from selected tree species and cones from *Cupressus* species as alternative substrates to agar to ensure consistent conidia production.

3.2.2.1 Trial 1

- Objective

The objective of trial 1 was to evaluate twigs of two host and five non host plant species for acervuli production of *S. cardinale* and *S. unicorne isolates.*

- Plant material

Current season's shoots (twigs) of two cypresses (*Cupressus macrocarpa & Chamaecyparis lawsoniana*), radiata pine (*Pinus radiata*), casuarina (*Casuarina* sp.), poplar (*Populus nigra*), alder (*Alnus* sp.), and blackcurrent (*Ribes nigrum*) were evaluated as substrates for supporting sporulation of *Seiridium* isolates.

- Fungal material

S. unicorne isolates (SUL101.4 and SUL175.4) and *S. cardinale* isolates (SCL152.2.2b and SCL182.2.3) were cultured on 3% MA and incubated under near UV light on a 12 h light/12 h dark cycle at 20°C for two weeks. The cultures were stored at 5°C cool room after two weeks under near UV light and were 8 weeks old at the time of inoculation. Mycelium inocula were obtained from the edges of the culture colonies using a 4 mm diameter cork borer.

- Experimental design

The trial was a split plot design with 4 *Seiridium* isolates x 7 substrates x 4 replicates laid out in a randomized complete block design (RBD).

The twigs (9-15 mm diameter) were cut into 50-70 mm pieces and placed into McCartney bottles containing 3 ml of RO water. They were autoclaved for 15 minutes at 120°C.

After autoclaving, the substrates were left to cool, and then inoculum plugs were placed on the upper end of each twig.

The cultures were kept in the dark at 20°C for 2 weeks, and then they were transferred to 1°C for a further 2 weeks. This treatment was followed by incubation in a 10°C dark/15°C light regime on a 12 h /12 h schedule for 8 weeks.

- Assessment

Assessment was carried out by counting the number of acervuli using a dissecting microscope (40x Magnification). A scoring system with scales 1-5 was used where:

1 = 0 acervuli produced, 2 = 1-5 acervuli produced
3 = 6-10 acervuli produced, 4 = 11-20 acervuli produced
5= > 20 acervuli produced.

3.2.2.2 Trial 2

- Objective

Conidia production on agar overlaid with carnation leaves or *C. macrocarpa* shoot pieces was not consistent in the previous experiments. The variability ranged from extreme high production of conidia to no conidia.

The objective of trial 2 was to further assess acervuli production on *C. macrocarpa* and *C. lawsoniana* and *P. nigra*. *C. macrocarpa* produced the highest mean score of conidia production; while *C. lawsoniana* and *P. nigra* produced the lowest acervuli mean score.

- Fungal material

Mycelial inoculum plugs were obtained from *S. unicorne* isolate SUL101.4 and *S. cardinale* SCL152.2.2b incubated under similar condition as trial 1 and were 11 weeks old at the time of inoculation.

- Plant material

Plants substrates used in this trial included twigs of current season of *C. macrocarpa*, *C. lawsoniana* and *P. nigra*.

- Experimental design

A factorial experiment consisting of 2 *Seiridium* isolates x 3 plant substrates x 3 replicate. The experimental design was a completely randomized design.

Preparation of twigs was similar to the first experiment. The inoculated twigs were first incubated in the dark at 25°C for two weeks instead of 20°C as in the first experiment. After two weeks in the dark at 25°C, the cultures were transferred to 1°C cool room for a further two weeks and then followed by incubation at 10°C dark/15°C light regime on a 12 h/12 h schedule for 10 weeks.

- Assessment

The method of assessment of trial 2 was assessed as for trial 1.

3.2.2.3 Trial 3

- Objective

The objective of trial 3 was to assess acervuli production of *S. unicorne* and *S. cardinale* isolates on mature cypress cones using spore inocula.

- Fungal material

Conidia suspensions of *S. unicorne* isolate SUL101, SUL155.7.1, SUL156.2.2 and *S. cardinale* SCL152.2.4 were prepared from 3 month old cultures grown on water agar overlaid with carnation leaves incubated under near UV light on a. 12 h light/12 h dark cycle at 20°C. The conidial suspension had been stored in the cool room at 5°C for a further 5 weeks before the inoculation. Conidial suspension was adjusted to 1,000,000 conidia/ml.

- Plant material

The plant substrates were green mature cones from a *Cupressus* species.

- Experimental design

A completely randomized design consisting of 4 *Seiridium* isolates x 4 replicates was used.

- Procedure

The cones were placed in McCartney bottles containing 3ml RO water and autoclaved for 15 minutes at 120°C. After autoclaving the cones were left to cool down and then 5 µl of conidial suspension droplets were applied on the upper surface of the cone. The lids of McCartney bottles were placed loosely on. The cultures were first incubated in the dark at 25°C for two weeks and then at 1°C cool room for another two weeks. The final treatment was incubation at 20°C on 12 h light/12 h dark cycle for twelve weeks.

- Assessment

Cultures were assessed for acervuli production after 12 weeks incubation at 20°C 12 h light/12 h dark cycle. The method of assessment is described in trial 1.

3.2.3 RESULTS

3.2.3.1 Trial 1

Acervuli production was observed after 8 weeks of incubation under 20°C 12 h light/12 h dark cycle on *R. nigrum* (black current) and *C. macrocarpa* twigs. Examples of some substrates illustrating the rate of acervuli production rate are shown in plates 3.4 and 3.5.

The assessment revealed low production of acervuli. Analysis of variance carried out on the scores revealed no significant differences between the treatments (Appendix I-E). Acervuli production varied among the *S. unicorne* and *S. cardianle* isolates and substrates. *C. macrocarpa* produced the highest mean score and *C. lawsoniana* and *P. nigra* produced the lowest mean score (Table 3.4). One replicate of *R. nigrum* was given a grade of 5 but the overall mean was lower than *C. macrocarpa* (plate 3.5). Mean score for the two *S. cardinale* isolates were a little higher than the *S. unicorne* isolates. Comparing the treatment combinations. *S. cardinale* SCL182.2 inoculated on *Alnus* sp. produced the highest mean score of 2.3, followed by *S. cardinale* SCL152.2.2 inoculated on *Casuarina* sp. and *S. unicorne* SUL175.4 inoculated on *R. nigrum* with a mean score of category 2.

Table 3.4 Treatment means of score for Acervuli production

Treatments	Transformed mean	Actual mean score
Substrates	(Sqrtscore)	
C. macrocarpa	1.17	1.50
Ribes nigrum	1.10	1.31
Alnus sp.	1.11	1.31
Casuarina sp.	1.10	1.31
Pinus radiata	1.05	1.13
Populus nigra	1.02	1.06
C. lawsoniana	1.03	1.06
S.e=0.059 (n=16)		
Isolates		
SCL182.3 (*S. cardinale*)	1.13	1.36
SUL175.4 (*S. unicorne*)	1.11	1.32
SCL152.2.2 (*S. cardinale*)	1.08	1.25
SUCL101.4 (*S. unicorne*)	1.01	1.04
S.e=0.059(=28)		

Non significant *F* test at *P* 0.05

Overall, 87% of the different treatments assessed were category 1, 6% were category 2, 2% were category 3, 3% were category 4 and the remaining 2% were category were category 5.

Sporulation on some of the substrates increased after the cultures were left on the laboratory bench under room temperature for more than 4 weeks. No further assessment was conducted because some of the cultures were discarded earlier.

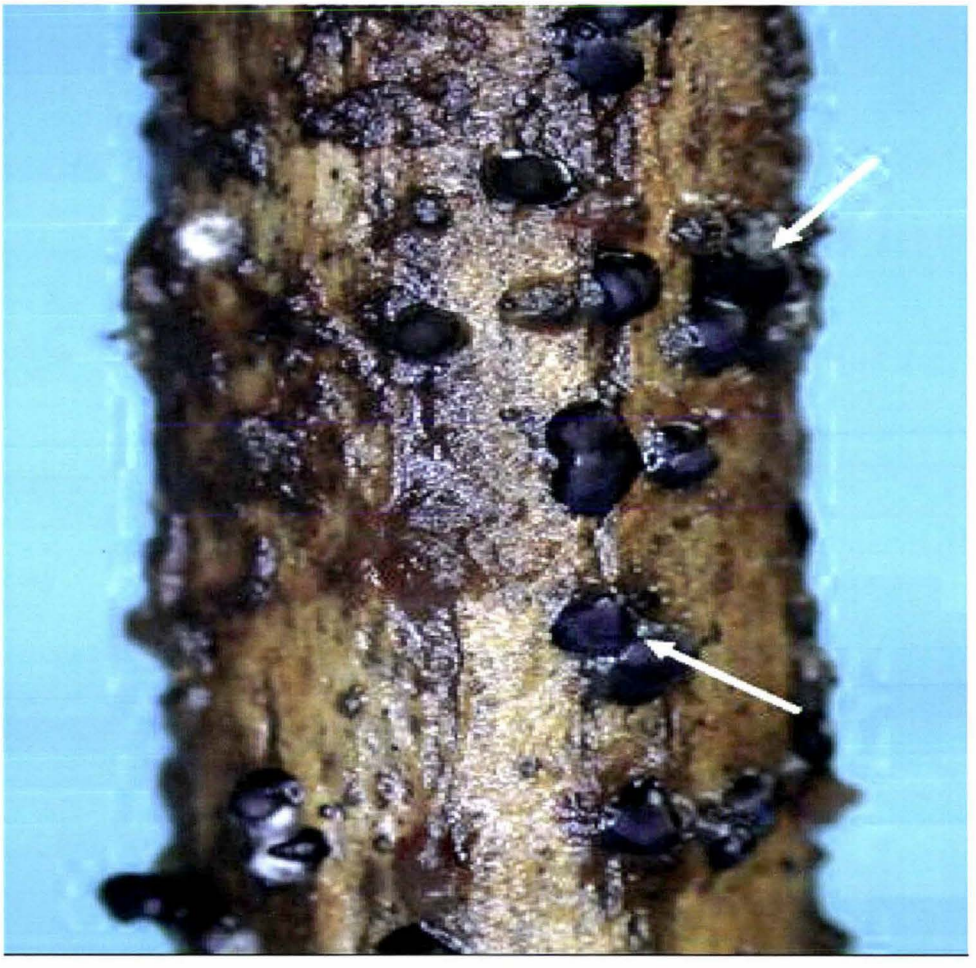

Plate 3.4 Acervuli (indicated by white arrows) formed on the bark of black current twigs. This substrate was given a score of 5 for acervuli production.

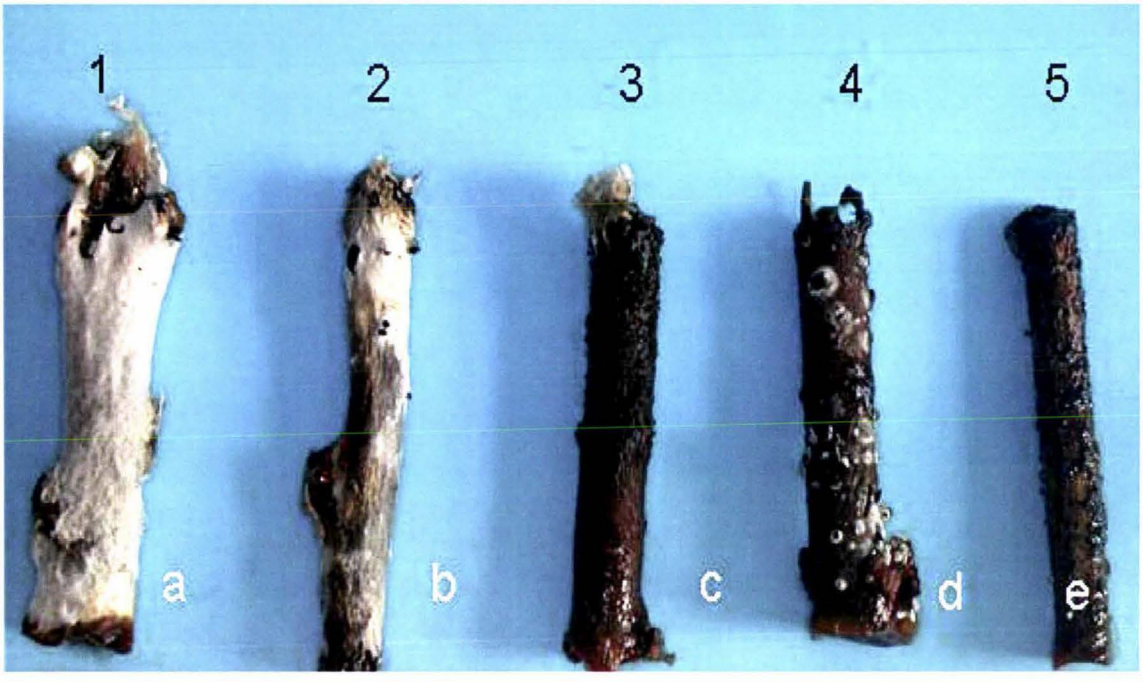

Plate 3.5 (a): *Casuarina* sp. inoculated with *S. unicorne* isolate SUL175.4, (b): *Alnus* sp. inoculated with *S. unicorne* isolate L175.4, (c): *C. macrocarpa* inoculated with *S. cardinale* SCL182.3, (d): *C. macrocarpa* inoculated with *S. unicorne* SUL175.4, (e): Black current inoculated with *S. unicorne* SUL175.4.The visual rating for spore production is above each twig numbered 1-5.

3.2.3.2 Trial 2

In the second trial only three substrates and two *Seiridium* isolates were assessed for acervuli production. There was no acervuli production on *C. lawsoniana* and *P. nigra*. The result also showed that 88% of counts for the different treatments were category 1, 4% were category 2 and 8% were category 5. Analysis of variance carried out showed significance difference between the substrates used (Appendix I-F). Table 3.6 shows the treatment means.

Table 3.5 Treatment score means for acervuli production

Treatments	Transformed mean (Sqrtscore)	Actual mean score
Substrates*		
C. macrocarpa	1.46[a]	2.5
C. lawsoniana	1.00[b*1]	1.00
P. nigra	1.00[b]	1.00
s.e (n=8)=0.14		
lsd=0.4049		
Isolates[NS]		
SUL101.4	1.21	1.67
SCL152.2	1.10	1.33
s.e =0.11(n=12)		

NS Non significant *F* test at $P< 0.05$ * Significant *F* test at $P< 0.05$. *[1]Means with the same letters are not significantly different.

3.2.3.3 Trial 3

Acervuli production results for the *S. unicorne* and *S. cardinale* isolates also varied as in previous trials. Analysis of variance (Appendix I-G) showed significant difference between the *Seiridium* isolates. *S. unicorne* isolate SUL155.7.1 produced the highest score of acervuli production and isolate SUL156.2.2 produced the lowest average score. In this trial only 25% of counts for the different treatments were category 1, 37% were category 2 and 19% were 3 and 5.

Table 3.6 Acervuli production score means

Isolates	Actual mean score
S. unicorne SUL155.7.1	4.00a
S. unicorne SUL156.2.2	1.50b
S. unicorne SUL101.4	2.75ab
S. cardinale SCL152.2.4	2.25ab
s.e=0.55 (n=4)	
Lsd=0.51	

Significant *F* test at $P<0.05$. *1Means followed by the same letters are not significantly different.

3.2.4 DISCUSSION

Evaluation of the seven twigs revealed that non-host plant substrates could be used as alternative substrates for supporting growth and sporulation of *Seiridium* isolates. Poor mycelium growth on *P. nigra* resulted in very low spore production compared with other twigs and cypress cones. Poor sporulation occurred on *C. lawsoniana* and *P. radiata.*

The analysis of variance for trial 1 showed no significance difference between the twigs and isolates under the environmental conditions of the study. However, sporulation on some twigs increased after the cultures were left under room temperature for more than 4 weeks. The increase in acervuli production could be due to the length of time for incubation or the change in environmental conditions. Isolate SUL101.4 was used in all three trials and cultures that were treated in the dark at 25°C (trials 2 & 3) for two weeks produced higher acervuli mean score than at 20°C in the dark. The mean score for acervuli production was higher for cultures that were incubated at 20°C on a 12 h light/12 h dark cycle than those that were incubated at 10°C dark/15°C light regime on a 12 h/12 h schedule. This observation supported work done by Tobata et al (1991) regarding the optimum temperature range (19-22°C) for conidia production of *S. unicorne.*

The use of cypress cones resulted in higher average acervuli production than twigs used in the first two trials. Tisserat et al (1991) reported that serial subculturing of *Seiridium* isolates could result in the reduction of sporulation. Therefore the use of conidial suspension could have also contributed to the increase of acervuli production.

Further investigation is required on evaluation of the substrates under higher light regimes and the optimum length of incubation.

CHAPTER FOUR

METHODS OF ARTIFICIAL INOCULATION

4.1 ARTIFICIAL INOCULATION METHODS FOR INOCULATING CYPRESS SPECIES WITH *SEIRIDIUM* SPECIES

4.1.1 INTRODUCTION

Infection of cypress plants in nature is commonly known to occur through wounds on the bark. There are possibilities that under favourable conditions infection can occur without the presence of wounds. Yamada & Ito (1995) reported non- wounding inoculations induced infection and resinous at a height of 3 m but not below this height. The study also revealed that wounding inoculations induced resin pocket formations most frequently and resinosis at a height of 3 m. No resinosis, however, was observed at heights of 1 and 2 m even when successful infections occurred.

A lot of research has been carried out on cypress canker during the past decades. Some of the research work included that were done by Beresford & Mulholland (1982), Xenpoulos (1990); Casini et al. (1995); Santini et al. (1997) and Spanos et al. (1999). Most of the work was done in European and Mediterranean countries with *S. cardinale*. Environmental factors play a major role in the behaviour of the *Seiridium* species and therefore the results obtained in other countries may not be applicable to New Zealand condition. Resistant clones are screened by artificial inoculation. Mycelium is the common inoculum used for artificial inoculation. Wounds are created on stems to induce infection.

Cypress canker is commonly found in young stands. As the plant ages, its resistance to the disease increases. The resistance may be influenced by the bark maturity and the bulk of the stem and branches. Yamanda & Ito (1995) supported this view stating that the outer bark was responsible for preventing infections. Literature showed that screening programmes takes several months and even years to complete. Inoculation of

side branches could show obvious symptoms within a short period compared to the main stem.

4.1.1.1 Aims

The aims of the work were to:

1. Compare branch and main stem inoculations of cypress plants.
2. Determine the effects of wounding and unwounding of inoculation sites under glasshouse and uncontrolled environmental conditions.
3. Compare mycelial inocula with conidial inocula outside the glasshouse.

4.1.2 MATERIALS AND METHODS

4.1.2.1 Experiment 1: Inoculation of a glasshouse trial

Preliminary study was carried out in the glasshouse to compare main stem and side branch inoculations with *S. unicorne* isolates. This study was conducted in spring and ran through the summer (October 2001 – January 2002).

- Objective The objectives of the study were to:
  1. Determine whether wounds are required for infection.
  2. Compare main stem and side branch inoculation.
- Fungal material

The pathogens were two *S. unicorne* isolates SUL101.4 and I.83. 2 obtained from Forest Research (Rotorua, New Zealand). Twenty days- old cultures of *S. unicorne* isolates (SUL101.4 & SUL83.2) grown on PDA were used for the inoculation.

- Plant material

C. macrocarpa plants 0.4 m-0.9 m high were grown in 0.7 litre plastic pots in the glasshouse on the top floor of the AGHORT building at Massey University. Each plant was treated as a replicate. Five plants were allocated for each of the two *S. unicorne* isolates (SUL101.4 and SUL83.2).

- Experimental design

A factorial experiment consisting of 4 replicates (except for the control) x 2 *Seiridium* isolates x 5 treatments (including control) were used. Two plants were used for the control. The different treatment comparisons were:

1. Main stem inoculation versus side branch inoculation.
2. Wounded side branch versus unwounded side branch and wounded main stem versus unwounded main stem.

- Assessment

Assessment was based on visual observation of symptoms and measurement of canker.

Diseased plant tissue was collected for isolation of the pathogen. The temperature and relative humidity was recorded using a Gemini tiny tag data logger.

4.1.2.1.1 Side branch and main stem inoculations

Four plants were inoculated with mycelial agar plugs on the main stem and on a side branch for each isolate and two plants received agar plugs as controls. The inoculation point for each stem ranged from 18 cm to 29 cm in height above the potting medium, depending on the total height of the plants and where the side branches were located .The main stem inoculations were always positioned above the point of origin of inoculated branches to minimize potential interference between inoculations on the same plant (Figure 4.1).

4.1.2.1.2 The effect of wounding

For each plant wounds were made on the main stem using a 3 mm diameter cork borer. On the side branch a scalpel blade was used to remove a lateral branch and some bark to create the required wound. Agar plugs containing mycelia of the fungal isolates were inverted on the wounds and covered with parafilm (American National Can, Greenwich, CT USA). Separate plants were used for each isolate.

The non-wounding treatment was applied on the same plant but on different side branches to the wounded stem and side branches.

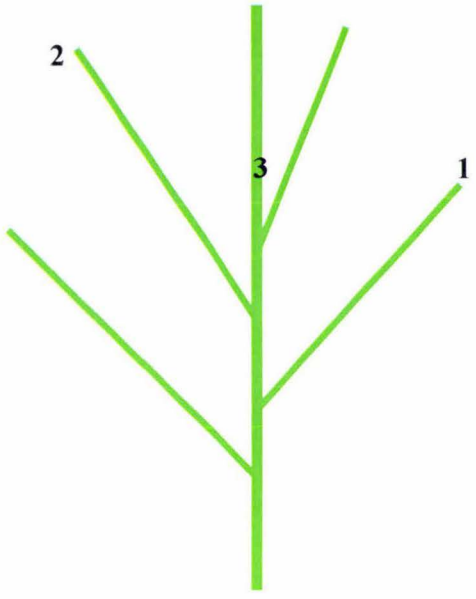

Figure 4. 1 Wounded main stem inoculation site (3) was always positioned above unwounded (1) and wounded (2) side branches

4.1.2.2 Experiment 2: Inoculation carried out under shade cloth

- Objective The objectives of the second experiment were to:
  1. Further compare main stem and side branch inoculations.
  2. Further determine whether wounds are required for infection.
  3. Further compare mycelial agar plugs and conidial suspension inocula.

The study was carried out under shade cloth condition, more or less similar to the field condition.

- Fungal material

Two *S. unicorne* isolates (SUL101.4 and SUL83.2) were cultured on PDA and incubated under near UV light. Conidia were obtained from stock cultures maintained on V8 agar and homogenized C. *macrocarpa* shoots also incubated under near UV light.

- Plant material

C. macrocarpa plants were kept under shade cloth in the standing out area at the Seed Technology Centre of Massey University. The plants were in the range of 30-75 cm in height and stem diameter at the point of inoculation were 1- 1.5 cm. The side branch diameter at the point of inoculation ranged from 0.25 – 0.35 cm. The plants were grown in 3 L plastic pots.

The different treatment comparisons were:

1. Wounding versus non- wounded inoculation sites.
2. Mycelial agar plug versus conidial inocula.
3. Main stem versus side branch inoculation.

- Procedure

1. In the laboratory, agar plugs containing mycelium were cut from 20-days old PDA cultures using a 5 mm cork borer.
2. Conidial suspensions were prepared from *S. unicorne* cultures on V8 and homogenized *C. macrocarpa* shoots. The plates were flooded with 10 ml of sterile RO water and filtered using a cell strainer. A drop of the conidial suspension was withdrawn and spore counts were carried out using a haemocytometer. Isolate SUL101.4 suspension contained 86,000 conidia/ml and Isolate SUL83.2 conidial suspension contained 16,000 conidia/ml.
3. Five (5) mm diameter filter paper discs were made using a 5-mm diameter cork borer. RO water, tissue paper, filter paper discs, cork borer, spatula, glass Petri dishes, McCartney bottles were autoclaved for 15 minutes at 120°C.
4. To apply conidial suspensions, four layers of 1 cm^2 moist tissue paper was placed on a spatula with a 5 mm diameter filter paper disc was laid on top. Twenty microlitre (µl) of conidial suspension was added to the filter paper and the pile was inverted on the inoculation site.
5. The inoculated point containing the inoculum was then enclosed with parafilm.
6. Sterile agar plugs and sterile RO water on filter papers were used for the controls.

4.1.2.2.1 Side branch and main stem inoculations

Inoculations were made on 12 stems of plants, at a height of 20 – 30 cm (depending on the total height of each plant and 12 side branches for each of the *S. unicorne* isolates. Three plants were used for the controls.

4.1.2.2.2 The effect of wounding

Wounds were created on the main stem of 6 plants and 6 side branches from the same 6 plants. A 5-mm diameter cork borer was used to create the wounds. On the selected side branch and on a lateral branch, a piece of bark was removed to create wounds on each selected side branch. Six plants were also used for each *S. unicorne* isolate. For the control plants (wounded and unwounded) sterile RO water and sterile agar plugs were applied instead of the pathogen.

4.1.2.2.3 Comparison between mycelium and conidial inocula

The main stems of 6 plants were inoculated with mycelial inocula and another 6 with conidial inocula from each of the *S. unicorne* isolates.

On each of the 12 plants, a branch from one side of the plant was inoculated with mycelial inocula and one from the opposite side was inoculated with conidial inocula. A total of 12 replicates were used for each type of inocula for the side branch inoculation. Sterile agar plugs and sterile RO water were used on the control inoculation sites.

- Assessment

The method for assessment was similar to that of experiment 1. The number of diseased side branches was counted and Chi-square test described in Mead et al. (1983) was carried out to determine the significance of the different treatments. General observation was carried out every two weeks for a period of 112 days. Conidia were obtained from fruiting bodies on some of diseased tissues and colonies were identical to the isolates used in the experiment.

4.1.2.3 Experiment 3: Glasshouse main stem inoculation trial (MSI)

Previous inoculation studies comparing mycelium and conidial inocula showed that there were differences between these two types of inocula. Whether the differences were due to an inherent difference in the inocula or to the host reaction remains unexplained.

- Objective

The objective of this study was to further determine the differences between mycelium and conidial inocula on unwounded and wounded sites on the same plant.

- Fungal material

Mycelial agar plugs were obtained from 21 days-old cultures of *S. unicorne* isolate (SUL156.2.1) culture grown on 3% MA and incubated at 25°C in the dark.

Conidia were obtained from SUL156.2.1 (100,000 conidia/ml) isolate cultured on water agar and carnation leaves and incubated at 20°C under near UV light 12 h light/12 h dark cycle.

- Plant material

Twelve plants each were selected from four *C. macrocarpa* families (M23, M25, M41 and M50). Four plants each from *C. macrocarpa* family M25, M41 and M50 were inoculated with the fungal pathogen including some control treatments and while only M 23 was used for the rest of the control treatment because there was no other plants available. Each plant was inoculated at six sites on the main stem, (three on each side). Inoculated sites were 5 cm apart.

The six treatments applied to each plant were:

1. Control 1 (sterile RO water).
2. Mycelial plug on a wound.
3. Conidial suspension on a wound.
4. Control 2 (plain MA).
5. Mycelial plug on unwounded stem.
6. Conidial suspension on unwounded stem.

- Experimental Design

The experimental design used was completely randomized design consisting of 12 replicates x 6 treatments.

- Procedure

The procedure used for inoculation was as for experiment 2. For conidial inoculation, only 5 µl of conidial suspension of SUL156.2.1 was used.

- Assessment

Assessment was carried by measuring (cm) the length of lesions (above and below the inoculation point) caused by the pathogen 9 weeks after inoculation. The SAS program was used to carry out ANOVA on the data.

4.1.3 RESULTS

4.1.3.1 Experiment 1

The two *S. unicorne* isolates behaved differently from each under the glasshouse condition. The percentage relative humidity ranged between 30 – 100 %, with an average of about 70%, While the temperature readings recorded ranged between 15-35°C with an average of 25°C (Figure 4.2 & Figure 4.3). There was no evidence of infection at any of the unwounded inoculation sites for isolate SUL101.4 (Table 4.1). Isolate SUL83.2 did not cause any infection at the wounded or unwounded inoculation sites. Obvious symptoms developed on wounded-side branches inoculated with isolate SUL101.4 two to eleven weeks after inoculation, while wounded inoculated stems died within 8-14 weeks.

Table 4.1 Observation results for plants with symptoms inoculated with SUL101.4

Inoculation sties (Number of plants)	Remarks
Main stem (3 out of 4)	The shoot above the inoculation point of one of the 3 plants wilted 9 weeks after inoculation. At 98 days after inoculation, the stem dried up. The initial lesion length was not measured. Another died in less than 14 weeks after inoculation. Lesion length measured was 23 mm at 98 days after inoculation (Plate 4.1a). The shoot above the inoculation of the third plant died after more than 14 weeks. Lesion length measured was 20 mm at 98 days after inoculation
Side branch (3 out of 4)	The first side branch died 2 weeks after inoculation. Second side branch died 3 weeks after inoculation. Third side branch died 11 weeks after inoculation (Plate 4.1b).

Plate 4.1 (a): Wounded main stem, 98 days after inoculation with SUL101.4. The infected stem tissue became brown above and below the inoculation site. (b) Wounded side branch resulted in browning of the foliage.

Resinosis was observed in less susceptible plants inoculated with *S. unicorne* isolate SUL101.4 (Plate 4.1). *Pestalotiopsis* sp. was isolated from one of the main stem inoculations. *S. unicorne* was re-isolated from all side branch and main stem lesions including the one from which *Pestalotiopsis* sp. was isolated.

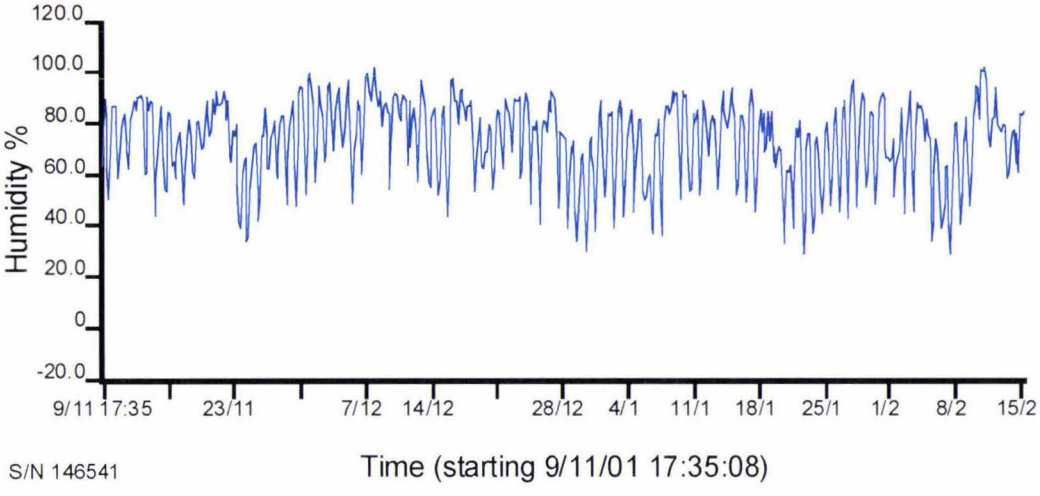

Figure 4.2 Percentage (%) relative humidity recorded from 9/11/01 to 15/2/02

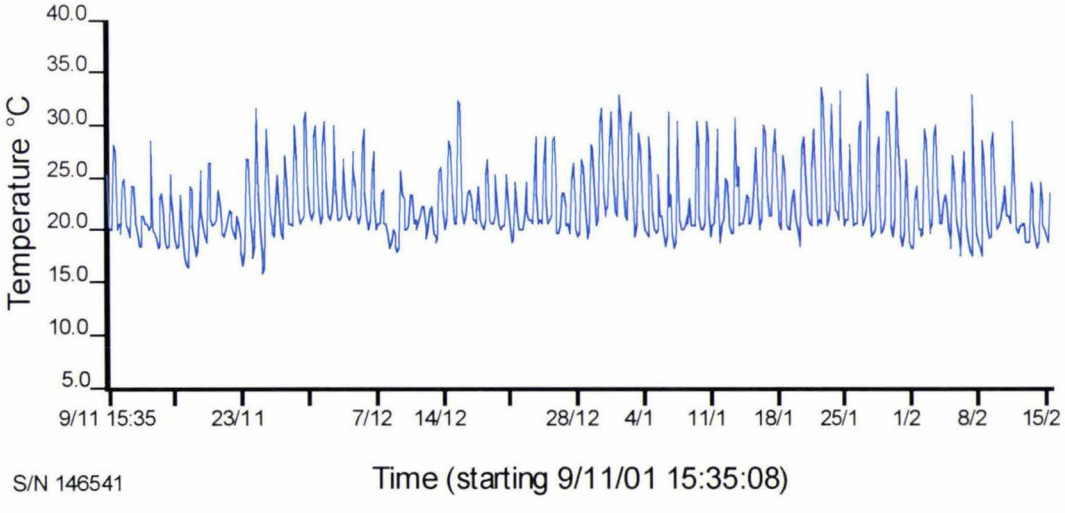

Figure 4.3 Temperature recorded from 9/11/01 to 15/2/02

4.1.3.2 Experiment 2

The Overall percentage of canker caused by the two *S. unicorne* isolates was calculated for the different treatments (Table 4.2). Raw data is listed in Appendix II-A. The result was being discussed under individual sub-sections. The Chi-square test for treatments was listed in Table 4.3.

Table 4.2 Percentages of cankers observed on the different treatments

Treatments	Percentage(%) canker
1. *Seiridium* isolates	
(a) SUL101.4	41.7
(b) SUL83.2	20
2. Inoculation sites	
(a) Wounded	51.7
(b) Unwounded	10
3. Types of inocula	
(a) Mycelium	36.7
(b) Conidium	25
4. Position of inoculation sites	
(a) Main stem	29
(b) Side branch	31

4.1.3.2.1 Side branch and main stem inoculations

Combining the canker results for the two isolates, 29% of inoculation sites on main stems were cankered and 31% on side branches. Canker was formed on 41% (5/12) of the main stems inoculated with isolate SUL101.4. Conidia collected from three of the five infected inoculation sites were identified as those of *S. unicorne* while conidia of *S. cardinale* were obtained from other two sites inoculated with *S. unicorne*. Three of the diseased stems wilted above the inoculation point, while the remaining two developed cankers measuring 4.0 and 4.5 cm in length.

For isolate SUL83.2, only 16 % (2/12) of inoculated sites developed disease symptoms on the main stem inoculations. The lesions measured 5 and 7 cm in length. No wilting

of the shoot was observed at the final assessment. *S. unicorne* conidia were identified from the both cankered sites.

Forty one percent (20/48) of side branches inoculated with isolate SUL101.4 developed canker disease symptoms. Conidia obtained from six branch lesions were identical those of *S. unicorne*.

Disease symptoms developed in 20% (10/ 48) of side branches inoculated with *S. unicorne* isolate SUL83.2 and conidia were from collected from only two inoculation sites. The remaining side branches, inoculated with the two *Seiridium* isolates were highly infested with *Pestalotiopsis* sp. and there was no successful re-isolation on to agar. *Pestalotiopsis* sp. was inoculated on some *C. macrocarpa* seedlings in the glasshouse and no canker disease symptoms developed.

The number of cankers observed depended on the type of inocula and species of *Seiridium* applied (Table 4.3(4)). A Chi-square test showed significance difference between the two types of inocula (mycelium & conidium) applied to the different positions of inoculation sites (main stem and side branch) at $P<0.001$ (3 df). Mycelial plugs of isolate SUL101.4 inoculated on side branches caused 79% (19/24) cankered shoots as compared to 50% (3/6) cankers on main stem inoculations. Inoculation of side branches using conidia of isolate SUL101.4 resulted in 37.5% (9/24) cankered shoots while the main stem inoculations using conidia produced cankers on only 33% (2/6) of the inoculated plants.

4.1.3.2.2 The effect of wounding

None of unwounded sites on main stem inoculated with either isolate developed any disease symptom. However, 16% (4/24) of unwounded side branches inoculated with isolate SUL101.4 developed canker and 8% (2/24) of branches inoculated with isolate SUL83.2. Combing the result for side branch and main stems, there was 10% canker for unwounded and 51.7% for wounded sites.

Wounding caused infection in 83% (5/6) of the main stems of plants inoculated with isolate SUL101.4 and 33% (2/6) of the main stems inoculated with isolate SUL83.2.

Table 4.3 Chi-square test for the different treatments

Treatments	Number of canker (Expected freq)
1. *Seiridium* isolates	
(a) SUL101.4	25 (18.5)*[1]
(b) SUL83.3	12 (18.5)
$\chi^2 = \Sigma \frac{(\text{Observed-Expected})^2}{\text{Expected}}$ =6.602, (1df)*[2] Significant at $P>0.01$	
2.Type of inoculation site	
(a) Wounded	31 (41.5)
(b) Unwounded	6 (41.5)
$\chi^2 = \Sigma \frac{(\text{Observed-Expected})^2}{\text{Expected}}$ =24.44, (1df) Highly significant at $P<0.001$	
3.Type of inocula versus inoculation sites	
(a) Mycelia/wounded site	21(9.3)
(b) Mycelia/unwounded site	1(9.3)
(c) Conidia/wounded site	10(9.3)
(d) Conidia/unwounded site	5(9.3)
$\chi^2 = \Sigma \frac{(\text{Observed-Expected})^2}{\text{Expected}}$ =35.24, (3df), Highly significant at $P<0.001$	
4.Type of inocula versus site position	
(a) Mycelia/main stem	3 (6.16)
(b) Mycelia/side branch	19 (14.8)
(c) Conidia/main stem	4 (16.16)
(d) Conidia/side branch	11 (14.8)
$\chi^2 = \Sigma \frac{(\text{Observed-Expected})^2}{\text{Expected}}$ =10.977, (3df), Highly significant at $P<0.001$	

*[1] Expected frequencies

Sixty-six percent (16/24) of the wounded side branches inoculated with isolate SUL101.4 died, while only 33% of wounded branches inoculated with isolate SUL83.2 died.

Combined data for both isolates showed 51.7 % cankers for wounded sites and only 10% canker for unwounded sites.

A Chi-square test carried out on combined data for the two *S. unicorne* isolates showed that the number of cankered shoots was significant at $P<0.001$ (1df).

4.1.3.2.3 Comparison between mycelial and conidial inocula

Mycelial inocula resulted in 36.7% diseased inoculation sites and conidial inocula in 25%. For isolate SUL101.4, 50% (3/6) of the main stems inoculated with agar plugs/ mycelium died compared with 33% (2/6) of the main stems inoculated with conidial suspensions. For the side branches, the corresponding figures were 45.8% (11/24) and 37.5% (9/24).

None of the main stems inoculated with mycelium of isolate SUL83.2 developed disease symptoms compared with 33% (2/6) inoculated with conidial suspension. Side branches inoculated with mycelium produced infections on 33% (8/24) of side branches compared with 8% (2/24) for conidial inocula.

Wounded sites inoculated with conidial suspensions of isolate SUL101.4 and SUL83.2 resulted in canker development in two plants each inoculated with isolate SUL101.4 and isolate SUL83.2. Cankers caused by isolate SUL83.2 were larger than those caused by isolate SUL101.4.

A Chi-square test showed that the number of cankers were dependent on the type of treatment. The effect of wounding on the type of inocula was highly significant at $P<0.001$ and the effect of inoculation site on the type of inocula was also significant at $P<0.001$.

4.1.3.3 Experiment 3

No disease lesions were observed on control and unwounded treatments (Appendix II-B). Plants from the three cypress families developed disease lesions but no resinosis was observed on the inoculated wounded sites.

Data from wounded stems was analysed using ANOVA (Appendix II C) and the result revealed no significance difference between the two types of inocula and the three *C. macrocarpa* plants tested. There was also no significant difference between the type of inocula and *C. macrocarpa* family interaction (Table 4.4). Mean lesion length for cypress family M50 was longer than for M25 and M41. Mycelial inocula resulted in longer lesion length than conidial inocula.

Table 4.4 Treatment means for canker lengths (cm)

Cypress families	Mean lesion lengths(cm) ^{*NS}
M25	1.35
M41	1.38
M50	1.55
s.e= 0.12 (n=8)	
Inocula	
Mycelium	1.53
Conidium	1.33
s.e= 0.10 (n=12)	

* NS Non significant *F* test at $P=0.05$

4.1.4 DISCUSSION

The experiment conducted in the glasshouse did not result in the infection of unwounded inoculation sites. Interaction between wounded and unwounded inoculation sites on *C. macrocarpa* plants under shade cloth was highly significant. The results of the two experiments showed that the presence of wounds on cypress could lead to fast invasion of susceptible cypress canker pathogen.

There was also a highly significant difference between the two types of inocula applied and the site where they were applied on the *C. macrocarpa* plants. Mycelial inocula

inoculated on side branches resulted in more infections than conidial inocula. The difference was around 40 %. On the main stem, conidial inoculation caused more infections than mycelial inocula but the difference was only 25 %. Since there were fewer main stems than side branches, direct comparisons can not be made here but one observation noted was that disease symptoms appeared faster on side branches than on the main stems. Mycelial inocula are likely to cause more canker infection than those inoculated with conidial inocula. One of the reasons could due to the fact that the agar supplied nutrient for the growth of the pathogen.

Factors such as fungal pathogenicity and host resistance affected canker infection on the plants. The relative humidity and the temperature were not recorded for the out door trial. The study was set up in the late summer (February 2002) and continued through the autumn period (March-May 2003). The average temperature was expected to be lower than for the glasshouse experiment. Therefore under favourable conditions, some infections can occur without wounding. Spanos et al. (1997a) reported that for in vitro inoculations with *S. cardinale*, wounds were not required for infection but wounds did result in a 3 to 4 fold increase in lesion sizes.

One problem encountered in the inoculation studies was difficulty of re-isolating the pathogen used for inoculation. The appearance of *S. cardinale* on sites inoculated initially with *S. unicorne* was interesting. Similar observations were also reported by Hood & Gardner (2002). Chou (1990) also isolated *S. cardinale* from *C. macrocarpa* plant highly infested with *S. unicorne*. The question regarding the number species remains to be answered.

The studies gave an indication that side branches are more susceptible to infection than the main stems. Side branches could be used to test several *Seiridium* isolates at any one time. Mycelium plugs resulted in more infection than conidial suspensions but mutants sectors could be a problem.

4.2 CONIDIAL INOCULUM LOAD REQUIRED FOR CAUSING CYPRESS CANKER INFECTIONS

4.2.1 INTRODUCTION

Cypress canker is spread naturally by conidia. Artificial inoculation is also carried out using conidial suspensions. In New Zealand, Beresford & Mulholland (1982) reported that conidial suspensions were used in artificial inoculation of *Seiridium* species on to cypress plants. The concentration used was 10^6conidia/ ml, however, the amount of conidial inoculum load was not stated. A study carried out on *S. cardinale* by Ponchet & Andreoli (1984) showed that the minimum effective dose was 50 conidia per wound, but the optimum was 500. It is not known whether the same minimum inoculum load is applicable to the *Seiridium* isolates found under New Zealand's climatic conditions. There is no information on the effective minimum dose of *S. unicorne*.

4.2.1.1 Aims

The aims of the studies were to investigate:

1 The relationship between conidial inoculum loads of selected *S. unicorne* and *S. cardinale* isolates and susceptibility of cypress plants.

2 The effect of environment on cypress canker infection.

4.2.2 MATERIALS AND METHODS

4.2.2.1 Glasshouse trial (GT)

- Objective

The objective of this study was to investigate the effective conidial load required to cause cypress canker infection using conidia from two *S. unicorne* isolates under glasshouse condition.

- Fungal material

Conidia of *S. unicorne* isolates (SUL101.4 and SUL83.2) were obtained from 7-week old cultures. Isolate SUL101.4 suspension contained 228,000 conidia/ml while L83.2 contained 374,000 conidia/ml. The different conidium load treatments were:

1. Control (0): Sterile RO water.
2. Conidial load 1: 100 conidia per wound.
3. Conidial load 2: 1,000 conidia per wound.
4. Conidial load 3: 10,000 conidia per wound.

- Plant material

Plants used in the study were selected from four *C. macrocarpa* families (*C. macrocarpa* number 23 (M23); *C. macrocarpa* number 25 (M25); *C. macrocarpa* number 41(M41) and *C. macrocarpa* number 50 (M50). There were six plants from each of the four families.

- Experimental design

The two *Seiridium* isolates were treated as separate experiments. The two experiments were factorial and arranged in a randomized complete block design.

The three conidial loads plus the control were allocated randomly to the side branches. One isolate was inoculated on one side of the stem of each plant and the other on the opposite side. The required conidial load was obtained by adjusting the volume of conidial suspension (Table 4.5).

Table 4.5 Volume of conidial suspension used to obtain the required conidial loads of *S. unicorne* isolate SUL101.4 & SUL83.2

Experiment number and *Seiridium* isolate number.	Volume of conidial suspension(µl)	Estimated conidium load/wound
Expt1a (SUL101.4)	0.5	100
	4.4	1,000
	44.0	10,000
Expt.1b (SUL83.2)	0.3	100
	2.7	1,000
	26.7	10,000

Conidial suspension droplets were applied to sterilized filter paper discs on top of moistened 1-cm^2 discs placed on a parafilm strip. The parafilm containing the conidial suspension on the filter paper was then lifted and placed on the wounded site of the cypress plant. The temperature and relative humidity were monitored inside the glasshouse using a data logger (Gemini Tinytag Plus II ° C/ %RH).

- Assessment

Assessment was carried out by counting the number of side branches with disease symptoms. The data was analysed using a Chi-square test. The final assessment was carried out 8 weeks after inoculations.

4.2.2.2 Shade cloth trial (SCT)

- Objective

The objective of this study was to investigate the effective conidia load required to cause cypress canker infection under outdoor conditions (Shade cloth).

- Fungal material

Conidial suspension was prepared from 12 weeks old cultures of two *S. unicorne* isolates (SUL155.71 and SUL156.2.2) and one *S. cardinale* isolate, SCL152.2.4. The cultures were grown on water agar overlaid with carnation leaves and incubated under near UV light with 12 h light/12 h dark cycle at 20°C.

- Plant material

Healthy looking plants were selected from *C. lusitanica* (from families number L1511 and L1512) grown in 0.7 L plastic pots and *C. macrocarpa* from families M23, M25, M41 and M50, planted in polyethylene planter bags. The average height of *C. lusitanica* plants were 30.8 ± 5.6 cm and *C. macrocarpa* were 46.8 ± 6.8 cm.

- Experimental design

A factorial experiment laid out in a randomized complete block design. There were twelve plants from each cypress family and each plant was treated as a block. The different treatment combinations were allocated randomly per plant.

The treatment combinations were:

1. Control (Sterile RO water) 6. I_2L_2
2. I_1L_1 7. I_2L_3
3. I_1L_2 8. I_3L_1
4. I_1L_3 9. I_3L_2
5. I_2L_1 10. I_3L_3

Where: I_1 = SCL152.2.4; I_2 = SUL156.2.2; I_3 = SUL157.7.1

L_1 = 50 conidia/wound; L_2 = 500 conidia/wound; L_3 = 5,000 conidia/wound

- Procedure

Preparation of conidial suspensions:

(1) Sterile RO water was added to the culture plates and glass rods were used to detach conidia from the surface of agar.

(2) The conidial suspension were agitated and then filtered through cell strainers

(3) Conidia counts were carried out using a haemocytometer. Original conidia counts for the *Seiridium* isolate SCL152.2.4, SUL155.7.1 and SUL156.2.2 were 2,250,000 conidia per ml, 1,790,000 conidia/ml and 1,364,000 conidia per ml respectively.

4. Conidial suspensions were adjusted to obtain the required concentration of 1,000,000; 100,000 and 10,000 conidia per ml.

Inoculation

(1) Wounds were created on the selected side branches by removing a side shoot with a scalpel blade.

(2) Five (5 µl) conidial suspension droplets were applied to filter paper discs on top of moistened 1 cm^2 tissue paper discs using a pipette. The 1 cm^2 tissue paper was first placed on a parafilm strip. The parafilm containing the conidial suspension on the filter paper was then lifted and placed on the wounded site of the plant.

(3) The temperature and relative humidity were monitored using a data logger.

- Assessment

This experiment was set up on 19th October 2002 (spring). It continued through summer and autumn season in 2003. The method of assessment was as described for glasshouse trial. Data was analysed using a Chi-square test. The number of positive infections was assessed at 4-5 week interval throughout the duration of the trial. A final count was done in March 2003, 23 weeks after inoculation.

4.2.3 RESULTS

4.2.3.1 Glasshouse trial (GT)

The relative humidity in the glasshouse was generally between 60 and 100.3 % but on two occasions the record shows it fell to a very low level for a short time - this was when the data logger was removed from the glasshouse and taken to the laboratory for a short time (Figure 4.4). Temperature fluctuated between 9.3°C and 29.1°C with an average of about 17°C (Figure 4 5).

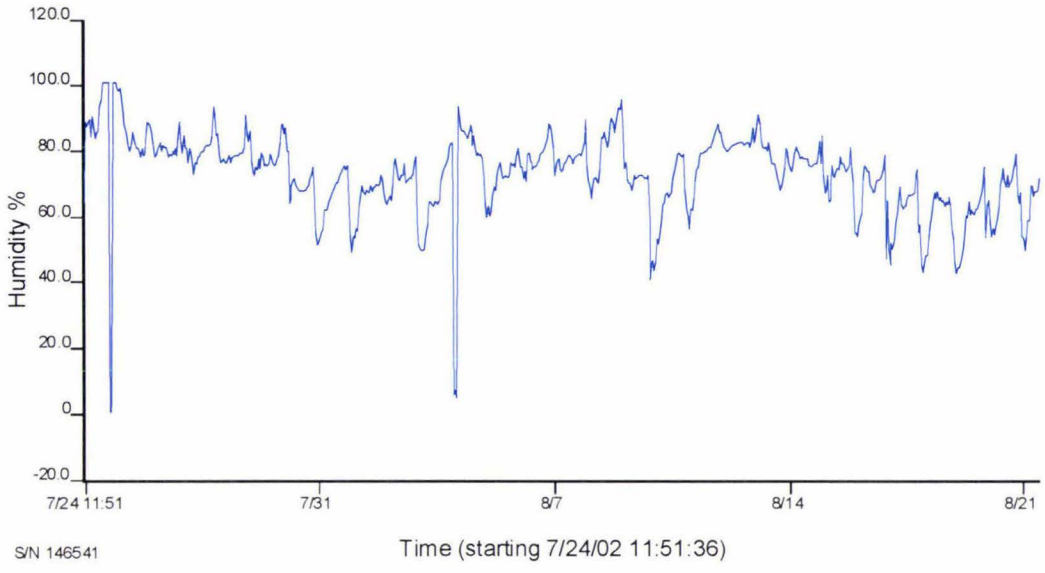

Figure 4.4 Percentage relative humidity recorded during July-August 2002

Disease symptoms became obvious on side branches inoculated with 10,000 conidia per wound 3 weeks after inoculation under the glasshouse condition. Final assessment was carried out 56 days after inoculation (Appendix II-D). When the result for the *S. unicorne* isolates were combined, there were 2% of inoculated side branches cankered for the 100 conidial inoculum load, 8% for the 1,000 conidial load and 25% for the 10,000 conidial load. Sites inoculated with isolate SUL101.4 developed symptoms on 22% (16/72) of the inoculated side branches, while only 1% (1/72) of the side branches inoculated with isolate SUL83.2 developed cypress canker symptoms (Table 4.6).

A Chi-square test showed that the difference in virulence of two the *S. unicorne* isolates is significant at $P<0.01$ (Table 4.6).

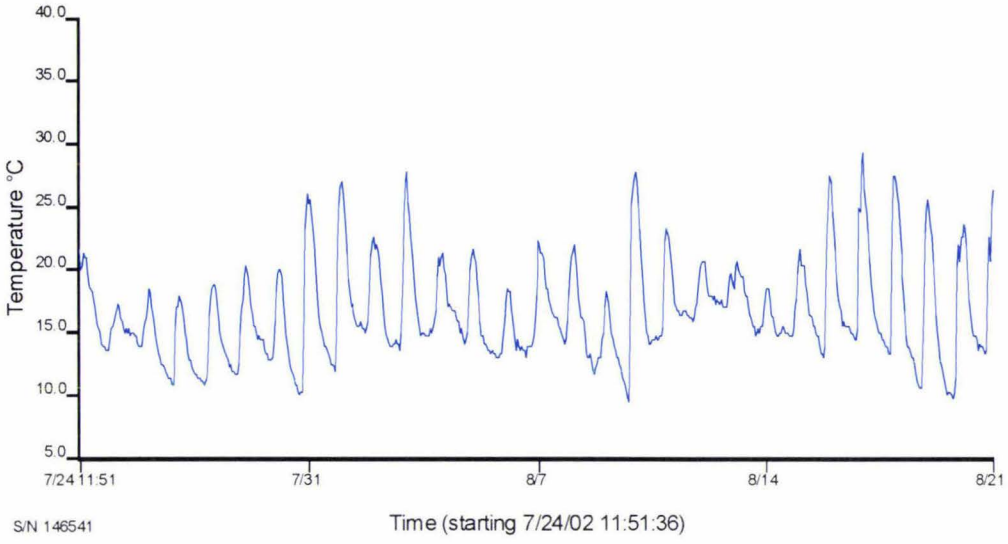

Figure 4.5 Temperature recorded during July-August 2002

The four *C. macrocarpa* families appear to differ in susceptibility to the pathogen under the glasshouse conditions of this experiment but the numbers of infections were too small for a formal statistical analysis to be very meaningful.

Table 4.6 Chi-square test and percentage of side branches with canker symptoms at the three spore loads 56 days after inoculation for the two *S. unicorne* isolates

Conidial load Per inoculation site	Number of shoots with symptoms (maximum= 24 per inoculation sites per *Seiridium* isolate			
	SUL101.4	Percentage	SUL83.2	Percentage
100 conidia (5.7)*[1]	1	4.17	0	0
1,000 Conidia (5.7)	4	16.67	0	0
10,000 " (5.7)	11	45.83	1	4.17
Total	16(8.5)*[1]	22.22	1(8.5)	1.39

(a).Chi square test for the three conidial loads (2df)*[2]

$\chi^2 = \Sigma \frac{(\text{Observed-Expected})^2}{\text{Expected}} = 12.9$ (Significant at P=0.0016)

(b) Chi-square test for the two *Seiridium* isolates (Combining 3 conidial loads) (2df)

$\chi^2 = \Sigma \frac{(\text{Observed-Expected})^2}{\text{Expected}} = 15.01$ (Significant at P=0.0055)

*[1] Expected frequency *[2] = Degrees of freedom

The susceptibility of the four cypress families varied at the three conidial loads but higher canker incidence occurred at a conidial load of 10,000 conidia per wound (Figure 4.6). Family M41 appeared to be more susceptible than the other three families. It was the only family to be infected by isolate SUL83.2 at the conidial load of 10,000 conidia per wound.

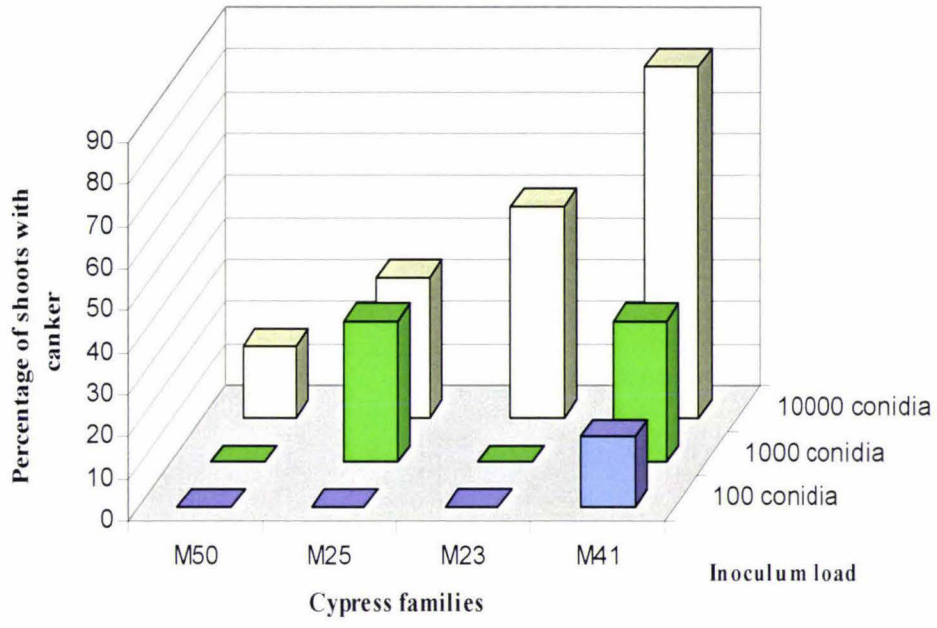

Figure 4.6 Percentages of side branches with cankers for each cypress family caused by isolate SUL101.4 at three conidial load 56 days after inoculation.

4.2.3.2 Shade cloth trial (SCT)

Average percentage relative humidity peaked around January (Figure 4.7). The highest % relative humidity recorded was 67.7% in January 2003.

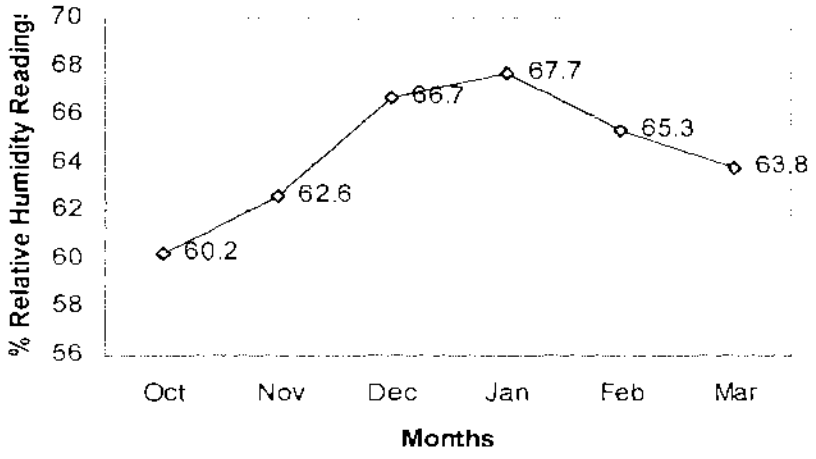

Figure 4.7 Average percentage relative humidity recorded during October 2002-March 2003.

The average temperature increased from 13°C in October 2002 to 16.7°C in March 2003 (Figure 4.8). Disease symptoms appeared on inoculated side branches of plants under shade cloth five weeks after inoculation. Final assessment was carried out 23 weeks after inoculation (Appendix II–E). The canker lesions peaked from January to February (Figure 4.9).

Figure 4.8 Average temperature readings recorded during Oct 2002- March 2003

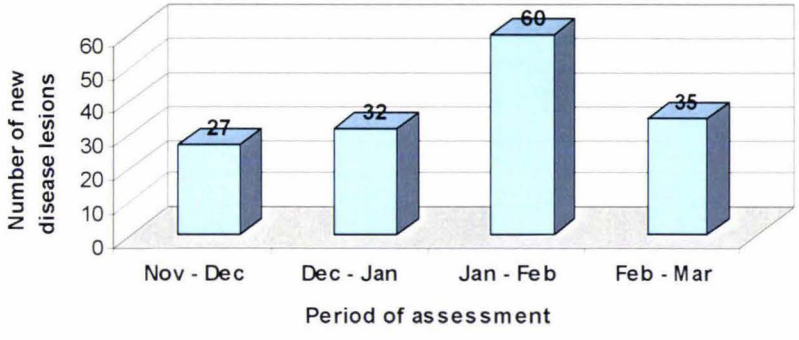

Figure 4.9 Number of new disease lesions observed during November 2002- March 2003

There were differences in the susceptibility of the five cypress families at the three conidial loads of the three *Seiridium* isolates used (Figure 4.10). Isolate SUL156.2.2 caused considerably more cypress canker lesions on side branches at all three spore loads than the other two isolates.

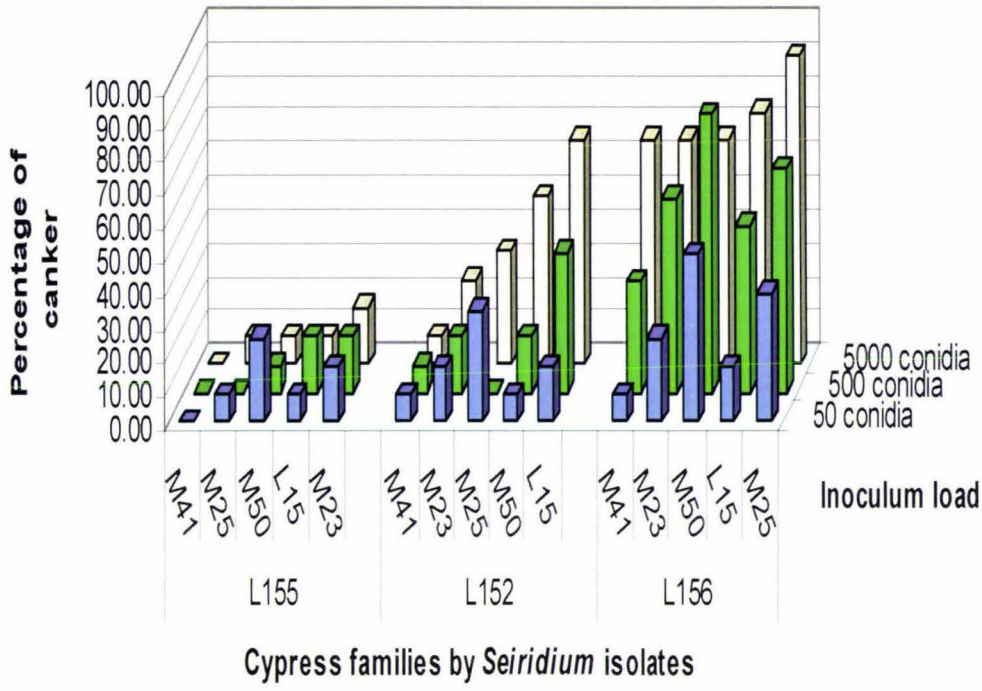

Figure 4.10 Percentage of shoots with canker from each cypress family inoculated with three *Seiridium* isolates at three conidial loads 23 weeks after inoculation.

C. macrocarpa and *C. lusitanica* plants were found to be susceptible to the *Seiridium* isolates used in the study (Plate 4.3). C. *macrocarpa* plants from the four families and *C. lusitanica* plants were equally susceptible to *S. unicorne* isolate SUL156 and *S. cardinale* SUL152.2.4 but less susceptible to *S. unicorne* SUL155.7.1.

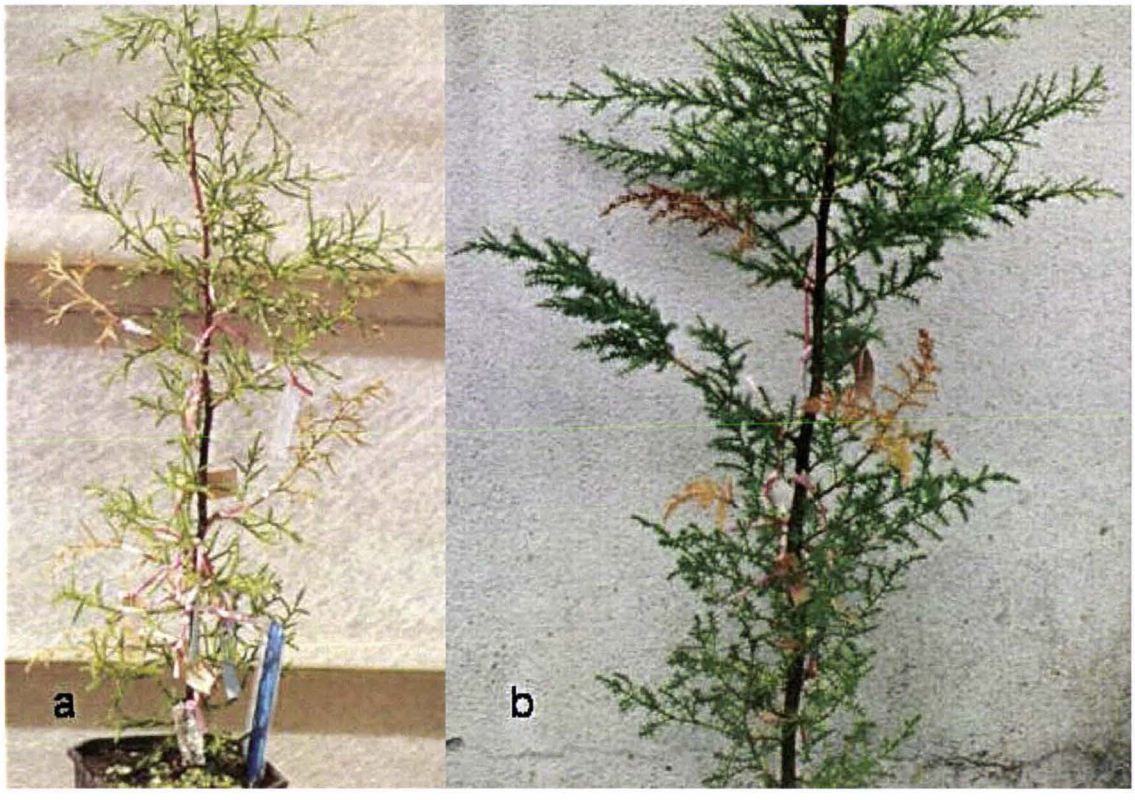

Plate 4.2 (a): *C. lusitanica* (Family no. L15-11); (b): *C. macrocarpa* (Family M50) with diseased side branches more 20 weeks after inoculation.

A Chi-square test carried out was highly significant for the three conidial loads, cypress families, four *Seiridium* isolates and cypress families at the three conidial loads (Table 4.7). When the cypress families were tested at each conidial load, Chi-square test was only significant at conidial loads of 50 and 5,000 conidia per wound.

Table 4.7 Chi-square test for the different treatments

Treatment	Canker	
Conidial loads (2 df)*	Observed frequency	Expected frequency
50	33	51.3
500	50	51.3
5,000	71	51.3
	$\chi^2 = \Sigma \frac{(\text{Observed-Expected})^2}{\text{Expected}} = 19.7$ (Highly significant at $P<0.001$)	
Cypress families(4 df)		
M23	31	30.8
M25	39	30.8
M41	16	30.8
M50	38	30.8
L15	36	30.8
	$\chi^2 = \Sigma \frac{(\text{Observed-Expected})^2}{\text{Expected}} = 13.75$(Significant $P=0.0081$)	
Seiridium isolates(2 df)		
L152.2.4	42	51.3
L156.2.2	95	51.3
L155.7.1	17	51.3
	$\chi^2 = \Sigma \frac{(\text{Observed-Expected})^2}{\text{Expected}} = 86.4$ (Highly significant at $P<0.001$)	
Families/ inoculum loads (14 df)		
M23/50	7	10.3
M25/50	9	10.3
M41/50	2	10.3
M50/50	10	10.3
L15/50	5	10.3
(For inoculum loads of 50 ,4 df, χ^2 =14.9; χ^2 Significant at $P=0.0049$)		
M23/500	11	10.3
M25/500	8	10.3
M41/500	5	10.3
M50/500	13	10.3
L15/500	13	10.3
(For inoculum loads of 500 ,4 df, χ^2=6.6; χ^2 Not Significant at $P=0.159$)		
M23/5,000	13	10.3
M25/5,000	16	10.3
M41/5,000	9	10.3
M50/5,000	15	10.3
L15/5,000	18	10.3
(For inoculum loads of 5,000 ,4 df, χ^2=16.7; χ^2 Significant at $P=0.0022$)		
Overall χ^2 (14 df) = $\Sigma \frac{(\text{Observed-Expected})^2}{\text{Expected}} = 38.31$ (Significant at $P=0.0004$)		

* Degrees of freedom

4.2.4 DISCUSSION

There was a positive correlation between inoculum loading and number of inoculation sites developing symptoms in the outdoor trial under shade cloth. Ponchet & Andreoli (1984) reported an optimum effective dose of 500 conidia per wound. Under the environmental condition of this study and the conidial ranging from 100- 10,000 conidia per wound, number of infections increased with an increase in inoculum load.

In this trial, there was a large difference in virulence of the fungal isolates used. S. *unicorne* isolate SUL156.2.2 was more virulent than *S. cardinale* SCL152.2.4 and *S. unicorne* SUL155.7.1. *S. cardinale* SCL152.2.4 was more virulent than *S. unicorne* SUL155.7.

C. macrocarpa plants from families M23 and M25 were ranked second and third respectively in resistance in this trial but plants from family M41 was most resistant in the shade cloth trial but less resistant in the glasshouse. However, M41 plants under the shade were inoculated with SUL101.4 in March 2003 and 75% of the plants were found to be susceptible 3 months after inoculation. The reverse was true of plants from family M50. These plants were also inoculated with SUL101.4 in March and 72% were susceptible to SUL101.4. The use of different *S. unicorne* isolates and different plants could be some of the factors responsible or the use of. Since the plants were from seedlings, they were not genetically identical therefore they could differ in their response to the attack of cypress canker. Whether this reflects a resistance under different environmental conditions or difference resulting from the use of different fungal isolates requires further investigations.

It appeared that climatic environment also influenced the occurrence of disease lesions. The number of disease lesions increased as the temperature increased. Warmest period during summer in New Zealand usually occurs in January and February. The increase in the % relative humidity also correlated with the increase in the number of new disease lesions. This observation supports the reports by Van der Werff (1988) that cypress canker incidence increases across New Zealand as the temperature increases. Studies carried in other countries also support the fact that environmental factors play an important role in plants resistance to cypress canker (Casin et al. 1995; Santini & Lonardo 2000). Solel et al (1983) reported that high relative humidity prompted infection and development of cypress canker disease.

CHAPTER FIVE

EXCISED SIDE SHOOT INOCULATION TECHNIQUE FOR SCREENING *CUPRESSUS* SPECIES FOR RESISTANCE TO *SEIRIDIUM* SPECIES

5.1 INTRODUCTION

Screening of cypress clones against cypress canker through artificial inoculation takes several weeks to several months to complete. An overseas study has shown the possibility of screening in vitro (Spanos et al. 1997a). Tissue culturing of cypress clones for in vitro screening takes time and requires special facilities.

Cut-shoots have been used for evaluating susceptibility of Scots pine to *Melampsora pinitorqua* (Quencez et al. 2001). Observation at Massey University showed that excised shoots *of Cupressus* spp. could be kept alive in tap water up to 10 weeks. Sanchez & Gibbs (1995) compared inoculation of attached and excised *C. macrocarpa* branches with *S. cardinale* and found that attached branches developed larger lesions than the excised branches. However, there appears to be no comparisons of the susceptibility of excised side shoots from canker resistant and susceptible cypress clones.

5.1.1 Aim

The susceptibility of cypress plants is strongly influenced by environmental conditions such as weather (Casin et al. 1995; Santini & Lonardo 2000). In vitro screening will provide simple screening techniques under different environmental conditions using *Seiridium* species at any one time.

The main aim of this study was to investigate methods of inoculating excised cypress shoots to screen for resistance to cypress canker in vitro to complement screening of young plants in glasshouse and field conditions.

5.2 MATERIALS AND METHODS

5.2.1 Experiment (SSI Trial 1)

- Objective

The objective of this experiment was to determine the effective conidial load for artificial inoculation of excised shoots under glasshouse condition.

- Fungal material

Conidial suspensions for the study were prepared from *S. cardinale* isolate number SCL152.2.4 (I_1) and *S. unicorne* isolate SUL156.2 .2 (I_2) grown on water agar and carnation leaves incubated at 20°C under 12 h light/12 h dark cycle. The concentration of conidia per ml was calculated using a haemocytometer and adjusted to give three inoculum loadings which 50, 500 and 5,000 conidia per wound.

- Plant material

Shoots used in the study were obtained from *C. macrocarpa* plants kept under shade cloth in the standing out area of Seed Technology Department, at Massey University, Palmerston North. The plants had been tested in an earlier trial and found to be susceptible to cypress canker caused by two *S. unicorne* isolates (SUL101.4 and SUL83.2).

- Procedure

(1) Shoots (10-11 cm) in length with side shoots were cut and immediately taken to the laboratory. The shoots were placed in 150 ml flasks containing 75 ml tap water for conditioning.

(2) After one week, each shoot was removed and wounds were created near the apex of the shoot with a sterile scalpel blade.

(3) Conidia from two *Seiridium* isolates (*S. cardinale* SCL152.2.4 and *S. unicorne* SUL156.2.2) were used at three inoculum loadings: 50, 500, and 5,000 conidia per wound. The wounded site was then inoculated with the required inoculum load and sealed with parafilm. There were three replicates per treatment except for the control where only one shoot was not inoculated with the pathogen.

(4) The flasks containing the inoculated shoots were randomly placed in a plastic tray near a glass window.

(5) After 1 week, the parafilm was removed and the tray was transferred to a glasshouse chamber and incubated under Sylvania GRO-LUX (F18W/GRO-TS) lights on a 12 h light/12 h dark cycle. The temperature and humidity were measured inside the growth chamber using a data logger.

- Assessment

Fifty-two days after inoculation, the shoots were assessed for disease symptoms (Table 5.1).

5.2.2 Experiment (SSI Trial 2)

- Objective

The objective of this study was to investigate the use of conidial inocula on unwounded sites of excised shoots under uncontrolled indoor environmental conditions to screen for resistance to cypress canker.

- Fungal material

Conidial suspensions for the study was prepared from *S. unicorne* isolate SUL156.2.2 grown on water agar and carnation leaves incubated at 20°C under 12 h light/12 h dark cycle. The concentration of spores per ml was measured using a haemocytometer and adjusted to 100,000 conidia per ml.

- Plant material

Shoots were excised from *C. macrocarpa* (family M41) and two *C. lusitanica* plants (L1 & L2) in a glasshouse on the third floor of the AGHORT Building at Massey University, Palmerston North. The two *C. lusitanica* plants were also supplied by Forest Research. It was not known whether or not the two *C. lusitanica* plants were of the same family.

- Procedure

(1) Side shoots measuring 4-5 cm in length were cut and taken immediately to the laboratory.

(2) Each shoot was inoculated with 5 µl of conidial suspension midway between the apex and the base. Eight *C. lusitanica* and six *C. macrocarpa* shoots were inoculated. One *C. lusitanica* and one *C. macrocarpa* shoot was used for the control.

(3) The inoculated shoots were placed in McCartney bottles containing 2 mls of tap water and the caps were placed loosely on and the bottles containing the inoculated side shoots were then placed randomly on the laboratory bench.

(4) Tap water was added when the level dropped below 2 ml.

After 30 days, the shoots were visually assessed for infection symptoms.

5.2.3 Experiment (SSI Trial 3)

- Objective

The objective of the study was to further investigate inoculation of excised cypress shoots using conidial inocula of *S. unicorne*.

- Fungal material

Isolate SUL156.2.1 was used for this experiment. Concentration was adjusted to 100,000 conidia per ml.

- Plant material

Cypress shoots were obtained from glasshouse grown plants. The plants were from four *C. macrocarpa* families and two *C. lusitanica* plants.

- Experimental design:

Twenty shoots were obtained from each of the four *C. macrocarpa* families and two *C. lusitanica* plants. The experimental design was a completely randomized design.

The treatments were:

(1) Control plants. (2) *C. lusitanica* L1 (3) *C. lusitanica* L2
(4) *C. macrocarpa* M23 (5) *C. macrocarpa* M25 (6) *C. macrocarpa* M41.
(7) *C. macrocarpa* M50

- Procedure

(1) Side branch tips (4-5 cm) were obtained from healthy *C. macrocarpa* and *C. lusitanca* plants kept under glasshouse condition. The shoot tips were removed and placed under running water for 15 minutes
(2) The shoots were then trimmed to fit into McCartney bottles
(3) A 5µl droplet (500 conidia) of conidial suspension was applied to a 2 mm diameter, sterilized filter paper disc and the disc was placed mid-way between the apex and the base of the shoot using a pair of forceps.
(4) Each inoculated shoot was then placed in a McCartney bottle containing 2 ml of sterile RO water.
(5) The bottle cap was loosely placed on and bottles arranged in a completely randomized design on the bench in the laboratory.
(6) The water was changed once a week.
(7) Assessment was carried out twice a week for 40 days. It had been observed from the previous trials that some shoots began to deteriorate at the cut end when kept longer than 40 days and all became susceptible to the fungal pathogen and saprophytes by that time.

5.2.4 Experiment (SSI Trial 4)

- Objective

In the previous three trials the inoculated shoots were placed in an uncontrolled environment. The relative humidity was maintained by placing the bottle caps loosely on the McCartney bottles in trial 2 and 3. Invasion of saprophytes, under a high relative humidity, such as those in which the shoots were placed in McCartney bottles posed difficulty in the re-isolation of the fungal pathogen.

The objective of this trial was to further investigate inoculation methods of excised cypress shoots with *S. unicorne* and *S. cardinale* isolates using conidial inocula under controlled environmental condition in an incubator.

- Fungal material (conidial suspension of 100,000 conidia per ml)

S. unicorne (SUL83.2, SUL101.4, SUL156.2.2)

S. cardinale (SCL152.2.4)

- Plant material

Four *C. macrocarpa* families (M23, M25, M41, M50) and one *C. lusitanica* family (L15)

- Experimental design

A completely randomize design was used for setting the experiment in an incubator.

There were nine replicates per treatment combination. The treatment combinations were:

(1) M23 & SUL83.2
(2) M23 & SUL101.4
(3) M23 & SUL156.2.2
(4) M23 & SCL152.2.4
(5) M25 & SUL83.2
(6) M25 & SUL101.4
(7) M25 & SUL156.2.2
(8) M25 & SCL152.2.4
(9) M41 & SUL83.2
(10) M41 & SUL101.4
(11) M41 & SUL156.2.2
(12) M41 & SCL152.2.4

(13) M50 & SUL83.2
(14) M50 & SUL101.4
(15) M50 & SUL156.2.2
(16) M50 & SCL152.2.4
(17) L15 & SUL83.2
(18) L15 & SUL101.4
(19) L15 & SUL156.2.2
(20) L15 & SCL152.2.4
(21) M23 & RO water
(22) M25 & RO water
(23) M41 & RO water
(24) M50 & RO water
(25) L15 & RO water

- Procedure

1. Side branch tips (4-5 cm) were obtained from healthy *C. macrocarpa* and *C. lusitanica* plants kept under shade cloth conditions. The shoot tips were removed and placed under running water for 15 minutes.
2. The shoots were then trimmed to fit McCartney bottles and rinsed in 3 changes of sterilized RO water.
3. Wounds were created on the shoot (Mid way between the shoot apex and the cut end of the stem by removing a side shoot. This is to allow rapid infection by the pathogen.
4. A 5µl droplet of conidial suspension was applied to a 2 mm diameter sterilized filter paper disc that was placed on a strip of parafilm. The pile was then left and placed on the wounded site. The parafilm was used to hold the filter paper discs in place.
5. Each inoculated shoot was then placed in a McCartney bottle containing 2 ml of sterile water .The bottle cap was loosely placed on and bottles arranged in a completely randomized design in plastic trays. The plastic trays were placed in the incubator set at 10°C dark/15°C light on a 12 h/12 h schedule.
6. The bottle caps were removed after 1 week and water was replaced once a week and 1 ml added every other day when the volume dropped below 2ml.
7. Assessment was carried out three times a week for 40 days.

5.3 RESULT

5.3.1 Experiment (SSI Trial 1)

The relative humidity in the chamber ranged from 40-80 % (Figure 5.1) and the temperature range was 15°C to 35°C (Figure 5.2).

Two shoots (66.7%) inoculated with conidial suspension of the two *Seiridium* isolates (SCL152.2.4 & SUL156.2.2) at the three conidial loadings of 50, 500 and 5,000 conidia per wound developed browning of foliage. Percentage of shoots that developed browning symptoms at the three conidial loads were similar for both *Seiridium* isolates (Table 5.1). An inoculum load of 5,000 conidia per wound resulted in disease symptoms on one shoot (33.3%) each inoculated with SCL152.2.4 or SUL156.2.2. Browning symptoms occurred only on a few side shoots above the inoculation points (Plate 5.1). There were no disease symptoms in the control treatment (Plate 5.1). There was no difference in the virulence of the two *Seiridium* isolates under the conditions of the trial (Figure 5.1 & 5.2).

This is the first experiment where there was no positive relationship between inoculum load and number of infections.

Table 5.1 Percentage of *C. macrocarpa* shoots with browning symptom 52 days after inoculation with three conidial loads for the two *Seiridium* isolates

	Number of shoots with brown lesions (Maximum=3 shoots per isolate)		
	Seiridium isolates		% with brown
Conidial load/wound	SCL152.2.4	SUL156.2.2	lesions
50	2	2	66.7
500	2	2	66.7
5,000	1	1	33.3
Total	5(max.=9)	5(max.=9)	

Conidia obtained from the fruiting bodies found on disease lesions were identified to be of the same as those of *S. cardinale* and *S. unicorne* used for the inoculation.

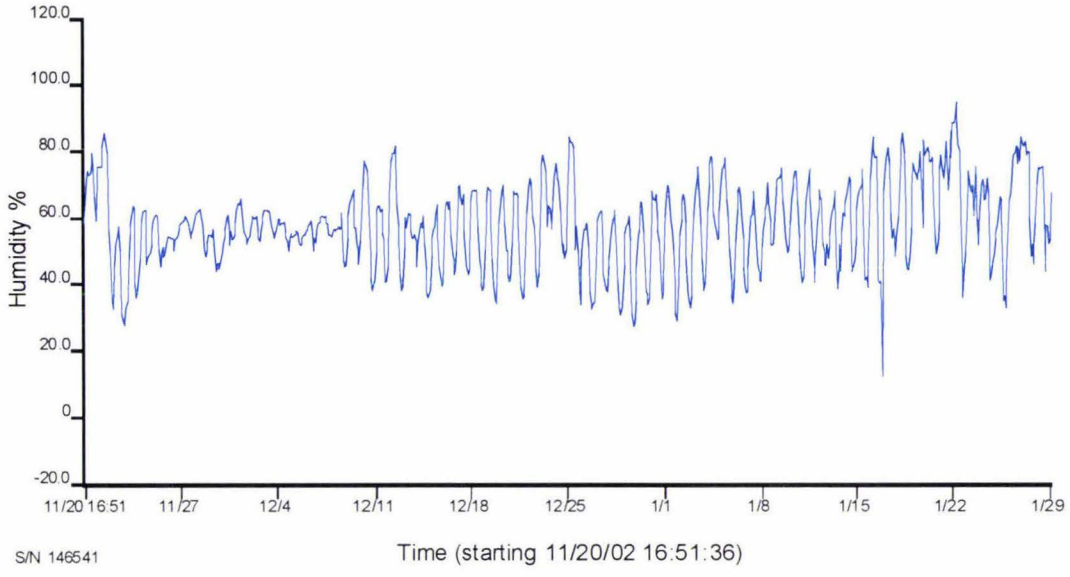

Figure 5.1 Percentage relative humidity recorded during November 2002- January 2003

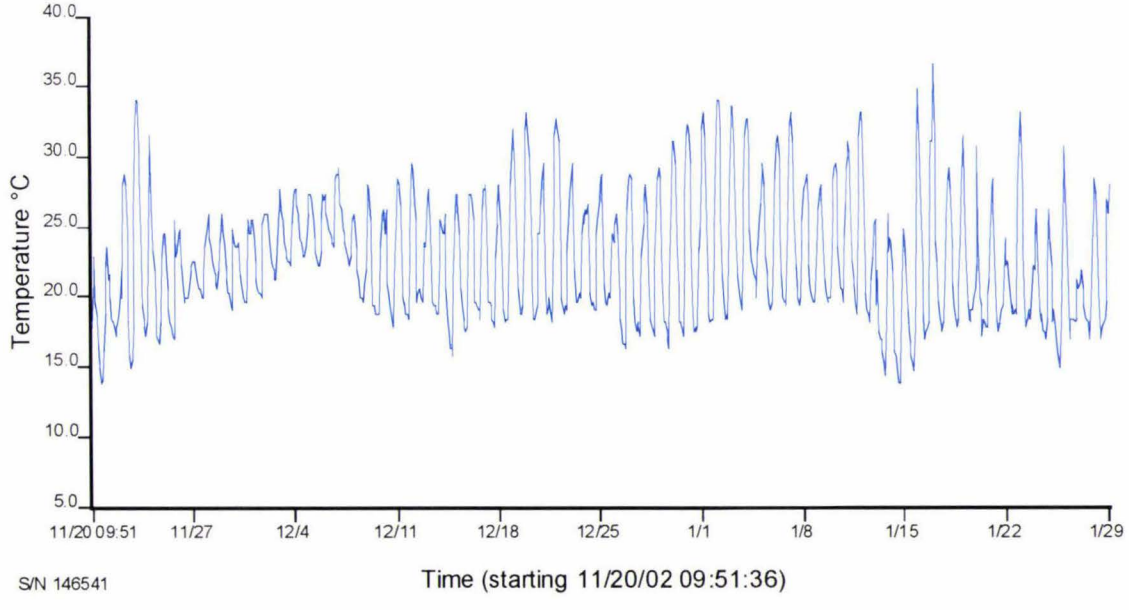

Figure 5.2. Temperature recorded during November 2002- January 2003

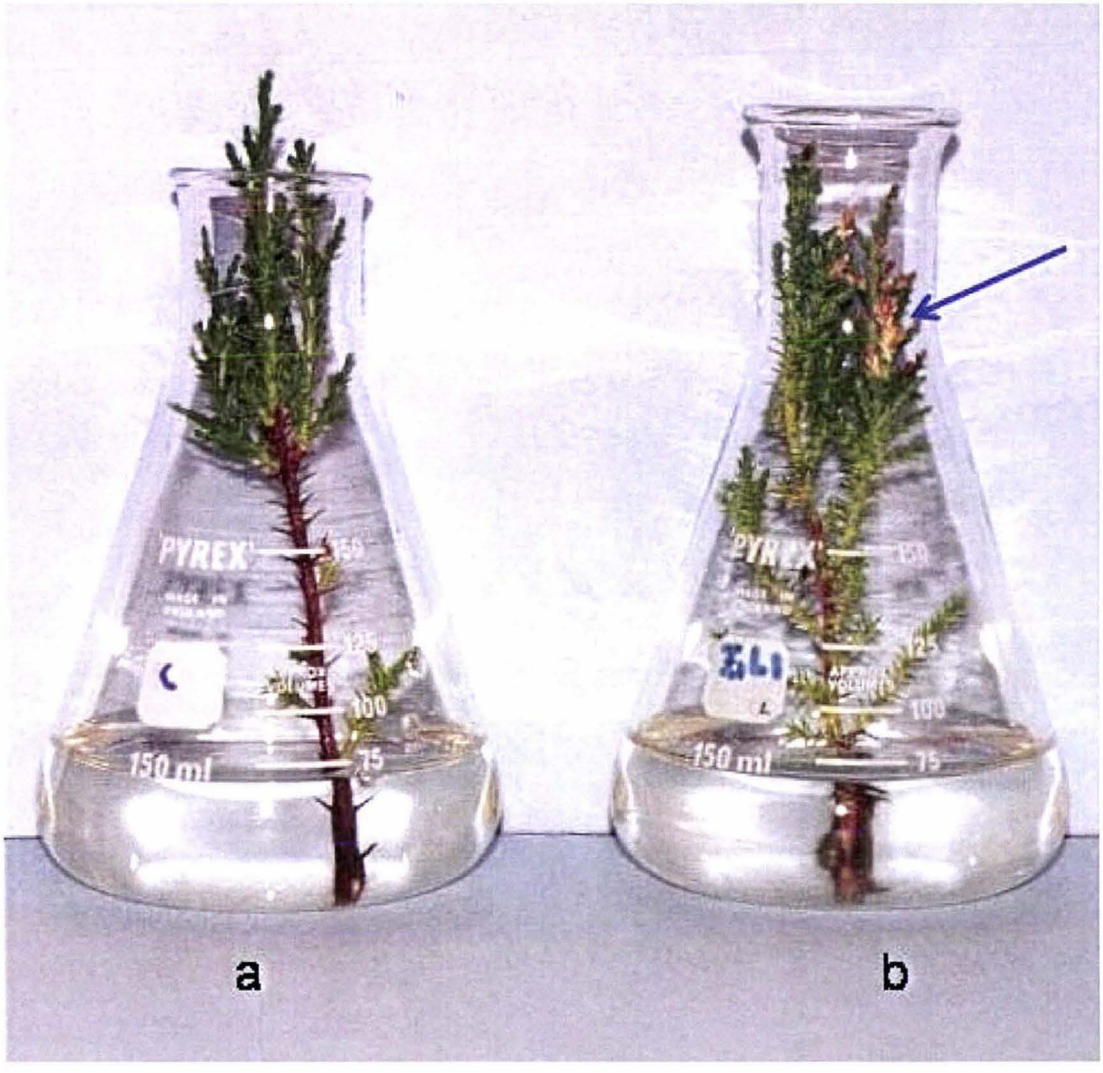

Plate 5.1 (a): Control shoot; (b): Shoot with browning symptom of the foliage (indicated by an arrow).

5.3.2 Experiment (SSI Trial 2)

All of the *C. macrocarpa* and four *C. lusitanica* shoots had become brown 30 days after inoculation (Table 5.2). Disease development was faster on *C. macrocarpa* shoots with symptoms first appearing after 14 days while the *C. lusitanica* did not develop symptoms until 21 days after inoculation. No disease symptom was observed on the control shoots. Shoots obtained from *C. lusitanica* plant L1 was susceptible and L2 plant was resistant (Plate 5.2). *C. macrocarpa* (family M41) shoots were susceptible (Plate5.3).

Table 5.2. Percentage of *C. macrocarpa* and *C. lusitanica* shoots with canker symptoms 30 days after inoculation.

Cypress species	Number of shoots with canker symptom	% of shoots with canker symptoms
C. macrocarpa	6 (Maximum= 6)	100
C. lusitanica	4 (Maximum=8)	50
Total	10 (Maximum=14)	70

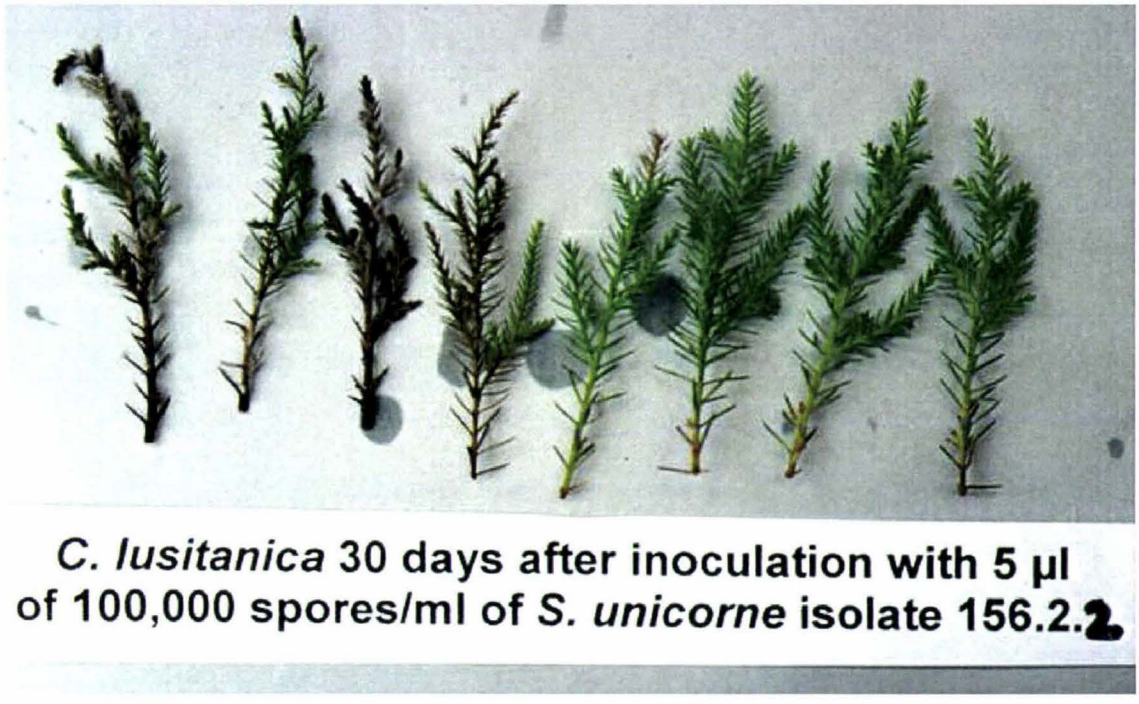

Plate 5.2 Four *C. lusitanica* shoots (left) with canker symptoms and inoculated healthy shoots (right) after 30 days incubation in McCartney bottles at room temperatures.

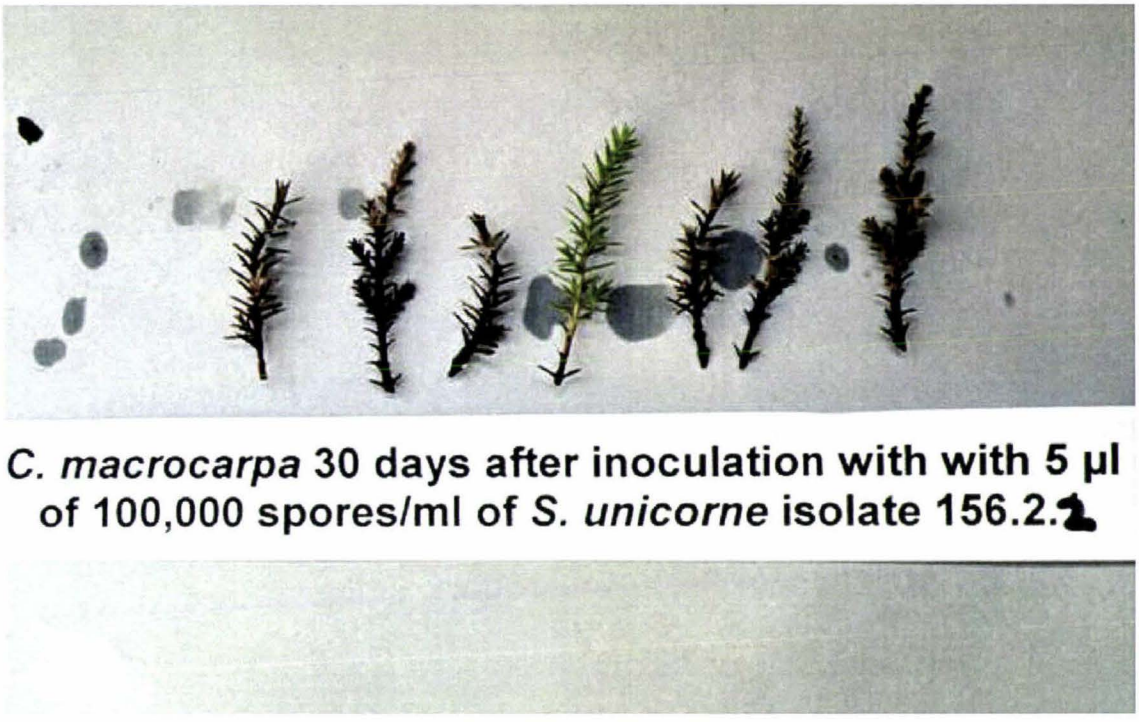

Plate 5.3 Six *C. macrocarpa* shoots with canker symptoms and control shoot (centre) after 30 days incubation in McCartney bottles at room temperatures.

Conidia obtained from a random sampling of conidia formed on cankered shoots were similar to those of isolate SUL156.2.2 which was used for inoculation.

5.3.3 Experiment (SSI Trial 3)

Canker symptoms on the most susceptible shoots appeared within 2- 3 weeks after inoculation. The inoculated site appeared brown initially and then spread both upward and below the inoculated site (Plate 5.4). The whole shoot became completely brown within 3 days if the caps of the bottles were not removed.

C. macrocarpa families M25 and M41 were susceptible to S. *unicorne* SUL156.2.1 (Table 5.3). Seventy (70) % of M25 shoots inoculated and 55 % of M41 developed canker lesions. The pathogen was able to infect only 20% of C. *macrocarpa* families' number M23 and M50 forty days after inoculation. *C. lusitanica* L1 appeared less susceptible and L2 was resistant. Shoots from L1 that were not infected and all the shoots from L2 remained healthy for more than forty days after inoculation. L2 was previously inoculated with isolate SUL101.4 and found to be resistant to the isolate.

Chi-square test carried out showed high significance between the cypress families at $P<0.001$ (Table 5.3).

Table 5.3 Chi- square test result and overall percentages of *C. macrocarpa* and *C. lusitanica* shoots with canker 40 days after inoculation.

Cypress species	Number of shoots with canker symptom and (Expected frequency)	% of shoots with canker
C. macrocarpa		
M23	4 (5.7)	20
M25	14(5.7)	70
M41	11(5.7)	55
M50	4(5.7)	20
C. lusitanica		
L1	1(5.7)	5
L2	0(5.7)	0
$\chi^2 = \Sigma \frac{(\text{Observed-Expected})^2}{\text{Expected}} = 38.6$ (5 df) significant at $P<0.001$		

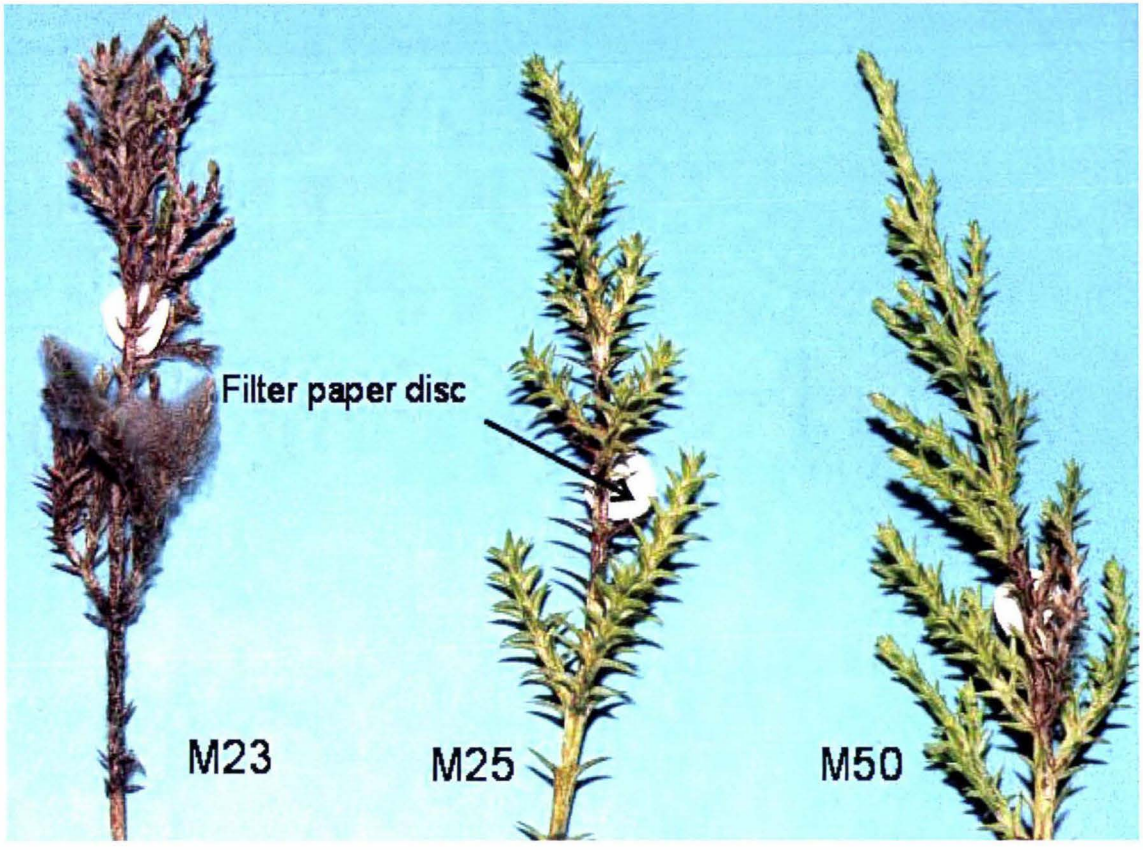

Plate 5.4 Shoots from *C. macrocarpa* families M23, M25 and M50 with browning symptom after 51 days incubation in McCartney bottles at room temperatures

Diseased shoots were randomly checked for the presence of spores. Spores were identical to SUL156.2.1 used in the inoculation (Appendix VI)

5.3.4 Experiment (SSI Trial 4)

Disease symptoms were observed 7 days after inoculation. Some shoots wilted and became brown. Appendix III-A shows the raw data for the experiment collected 40 days after inoculation. Isolate SUL101.4 caused more damaged compared to other isolates. It caused the highest % of cankered shoots; 100% in M25, 88% in M50, 77% in M23, 66% in M41 and 33 % in L15 (Figure 5.3 and Appendix III –B). Isolate SUL156.2.2 did not cause any canker in L15 forty days after inoculation.

The overall percentage of cankered shoots caused by the *Seiridium* isolates is listed in Table 5.4. Chi-square test revealed significance difference between the cypress families at $P<0.001$ (Table 5.4). Cypress families M50 and M41 ranked first and second in

susceptibility. Cypress families M25. M23 and L15 were less susceptible. The percentage of cankered shoots for each family was below 50 %.

Table 5.4 Chi-square test result and overall percentages of *C. macrocarpa* and *C. lusitanica* shoots with canker 40 days after inoculation

Cypress species	Number of shoots with symptom and (Expected frequency)	% of shoots with canker symptoms
M23	14 (16.5)	38.89
M25	18(16.5)	50.00
M41	14(16.5)	38.89
M50	13(16.5)	36.11
L15	7(16.5)	19.44
$\chi^2 = \Sigma \frac{(\text{Observed-Expected})^2}{\text{Expected}} = 15.00$ (4 df) Significant at $P<0.001$		

Sporulation occurred on most of the diseased shoots. Conidia found on the shoots were identical to *Seiridium* isolates (SCL152.2.4 and SUL101.4) used in the inoculation (Appendix IV).

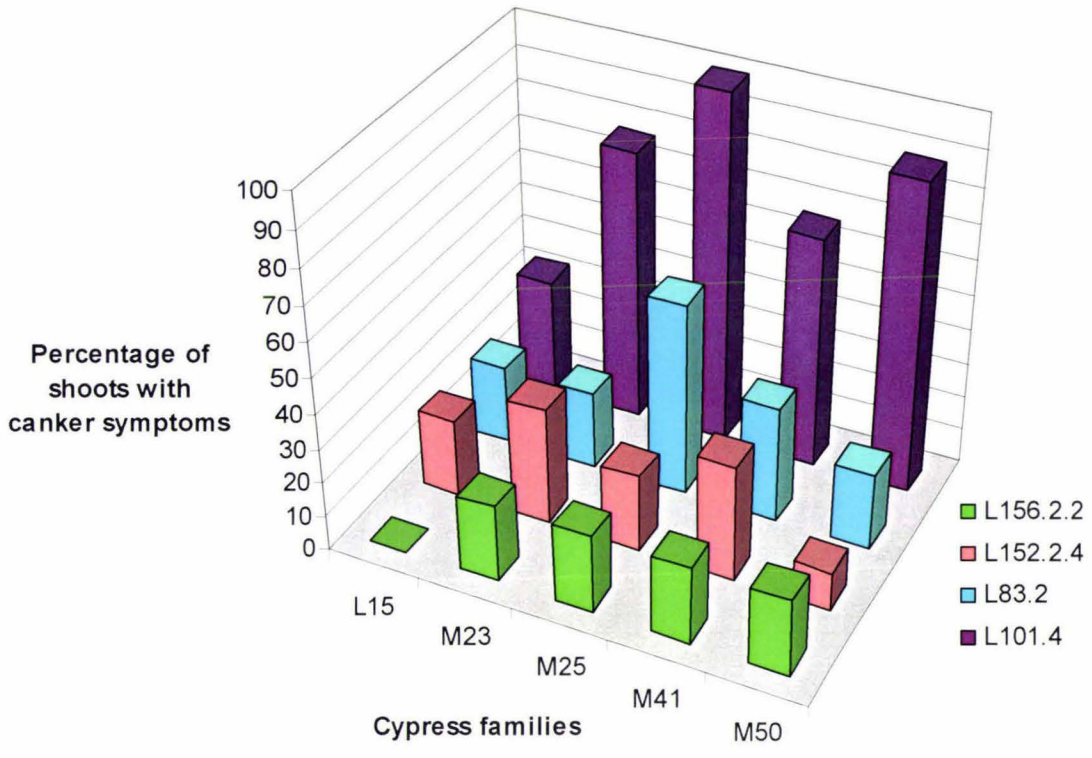

Figure 5.3 Percentage of shoots from each cypress family with canker symptoms caused by four *Seiridium* isolates 40 days after inoculation

5.4 DISCUSSION

The result of SSI Trial 1 showed that 66 % infection occurred when the inoculum load was 50 and 500 conidia per wound, while only 33 % infection occurred with inoculum loads of 5,000 conidia per wound. The data collected was not enough to carry out a statistical analysis on it but the result indicated the importance of using optimum amount of inoculum load under controlled conditions.

The shoots of the *C. macrocarpa* plants (family M41) were susceptible to the *Seiridium* isolates under the conditions of the experiment. The plants were also found to be susceptible to *S. unicorne* isolates SUL101.4 and SUL83.2 in the previous trial under the shade cloth.

In the second experiment, the shoot tips of *C. macrocarpa* become brown within two weeks. Thirty days after inoculation all the *C. macrocarpa* shoots were covered with mycelium and four of the *C. lusitanica* shoots also developed similar symptoms.

Disease symptoms developed faster in *C. macrocarpa* compared to *C. lusitanica.* The *C. lusitanica* shoots were taken from two different plants and it appears that one of the plants could be more resistant to the pathogen. Cypress family M41 has been found to be highly susceptible to isolate SUL101.4 in the glasshouse conidial load trial.

With the third experiment (SSI Trial 3) shoots from family M25 was the most susceptible of the six cypress families tested. Shoots from family M41 ranked second, followed by M23. All shoots from M50 and L2 remained healthy 40 days after inoculation.

The *C. macrocarpa* plants from which the shoots were obtained were previously inoculated with *S. unicorne* isolate SUL101.4 under glasshouse condition. The result showed that with inoculum load of 10,000 conidia per wound, M41 was the most susceptible of the four *C. macrocarpa* families. Plants from family M25 ranked second and M23 ranked third. Plants from family M50 were resistant in the glasshouse experiment.

All the four trials showed high variability in pathogenicity of the *Seiridium* isolates under different environmental conditions. Isolate SUL101.4 appears to be less affected by the change in environmental condition though. Isolate SUL156.2.2 was virulent under shade cloth conditions but become less virulent under temperatures of 10- 15°C.

Susceptibility to the four *Seiridium* isolates varied among the cypress families. The results of susceptibility and resistance of the four *C. macrocarpa* families reflects field reports of the parent plants. Cypress family M41 and M50 were susceptible and M25 and M23 were more resistant.

Variability in pathogenicity among *Seiridium* isolates also been reported (Barnes et al, 20001) and environmental factors have been found to play an important role in the host resistance to cypress canker (Casin et al 1995; Santini & Lonardo 2000). Beresford & Mulholland (1982) also found that there are differences in susceptibility to *S. cardinale* between cypress species.

CHAPTER SIX

METHODS OF IN VITRO INOCULATION OF TISSUE CULTURE CYPRESS PLANTS WITH *S. UNICORNE* ISOLATES

6.1 INTRODUCTION

Tissue culture or in vitro propagation provides a means by which host and pathogen interaction can be studied in simplified experimental systems where the physical and chemical environments are precisely controlled (Ingram et al. 1980).

Research on the micropropagation of cypress has already been carried out at New Zealand Forest Research Institute (Aimers-Halliday et al.1994). Shoot tips from mature cypress plants were used and there were difficulties with rooting of the explants (Kathy Horgan: pers. Comm.). Cypress plants are currently propagated using conventional methods. Sturrock & Ferguson (1989) previously carried out an investigation on the micro-propagation of Leyland Cypress. The results were encouraging but further work was required. Spanos et al. (1997b) carried out research on *Cupressus sempervirens* and *Chamaecyparis lawsoniana*. Information on tissue culture of different conifers and other woody plants carried out in different countries has been documented and most research results indicated a high potential for micropropagation.

Studies on in vitro inoculation of various fungal pathogens have been carried out on different tissue cultured plants during the past decades (Cutter 1960; Diner & Mott 1982; Diner & Mott 1985; Pei 1989; Spanos et al. 1997a; Spanos & Woodward 1997).

6.1.1 Aim

The main advantages of in vitro inoculations over conventional method includes handling of a large number of cypress clones in a small space, screening through out the year and exposure of clones to a more stable controlled environmental condition. In vitro inoculation methods could be used to complement screening work in the

glasshouse and in the field. This is to ensure that cypress clones are thoroughly tested for susceptibility to cypress canker under different environmental condition and to obtain information on the variability in their susceptibility.

The aim of this study was to investigate methods of in vitro inoculation of tissue cultured cypress plants.

6.2 MATERIALS AND METHODS

- Pathogen establishment

Seiridium fungus can be easily isolated from cankered plant material. The cankered materials are normally surfaced sterilized with 70% ethanol and rinsed in sterile distilled water. Sterilized plant material are cut up into thin slices and inoculated on the culture medium. The most common widely used medium is malt extract.

- Host establishment

The tissue culture medium used by Spanos et al. (1997b) for culturing *Cupressus sempervirens* and *Chamaecyparis lawsoniana* was modified Murashige and Skoog medium (MSM) at pH 5.7. MSM medium (Sigma) 2.2g/l + supplements (2 mg glycine, 100mg inositol, 0.5mg nicotinic acid, 0.5mg pyridoxine.Hcl, 0.1 mg thiamine-Hcl and 30 g sucrose was used as the standard culture medium. The type of agar used was bacteriological agar (0.6%). For elimination of contaminated cultures, explants were initially cultured on the standard medium for 10 days. MSM medium without BA or + 0.001 to 1.0mg/l BA was used for proliferation of axillary shoots. The condition medium was without growth regulator for 28 days. For root initiation explants were grown on ½ (half) strength MSM medium + 1% sucrose + 0.5mg/l IBA (for *C. sempervirens*) and ½ (half) strength MSM medium + 1% sucrose + 1mg/l IBA (*Chamaecyparis lawsoniana*). Type of explants used was: shoot tip 50mm from 18 month old seedlings maintained in glasshouse with no over head watering for 14 days. Surface sterilization procedures involved immersing of shoot tips in solution containing H2O2 (30% V/V) + 0.025% Tween 20 as a wetting agent for 10 minutes. The shoot tips were then trimmed to 20 mm length before placing on the growth medium.

Recipes used for the tissue culture medium were modified version of MSM (Spanos & Woodward 1997b) and Quoirin and Lepoivre (LPch) medium.

6.2.1 Experiment 1 In vitro inoculation of *C. macrocarpa* shoots with mycelia of *S. unicorne* on agar plugs

- Objective

The objective of this work was to investigate the interaction between *S. unicorne* isolates (SUL101.4 and SUL83.2) and the host, *C. macrocarpa* under tissue culture conditions. *S. unicorne* isolate SUL101.4 was virulent in both the glasshouse and outdoor conditions. Isolate SUL83.2 was less virulent under both conditions but the cankers caused by this isolate are more severe than those caused by SUL101.4.

- Fungal material

The two *S. unicorne* isolates (SUL101.4 and SUL83.2) were cultured on PDA and they were 18 days old at the time of inoculation.

- Plant material (Host establishment)

C. macrocarpa has been identified to be one of the most susceptible cypress species. The host material (*C. macrocarpa* family M25) used in this work has been identified to be resistant in the field (Ian Hood; pers. comm.). For this study, seedlings of height between 13- 25 cm were obtained from the nursery in the open at Forest Research (Rotorua). The diameters of the main stem were 3-4 mm and the side shoots were between 1-2 mm.

Shoots half the length of each seedling were cut and placed in bleach solution 50% v/v and agitated on the magnetic stirrer for 10-15 minutes. A drop of a wetting agent "Citowet" was added also added. The shoots were put in to jars containing sterile water and then transferred into a lamina air- flow cabinet. After the shoots were rinsed in the sterilized distilled water, each shoot was placed on a sterilized paper towel and dried using another paper towel. Shoots from the main stem and side branches were trimmed down to 5-10 mm and placed on the medium prepared. Leaves were removed from a shoot from each clone to expose tissue bigger than the meristem. An average of 6 pieces per type of plant material was placed in each Petri dish containing the culture medium. Each shoot produced sufficient pieces for at least 3 Petri dishes.

- Tissue culture media

The medium used was modified Quoirin and Lepoivre (LPch)* medium used for radiata pine (Appendix IV-A). For shoot proliferation, growth regulator was added and charcoal was not included. The lids of the Petri dishes were sealed with a plastic wrapper. The cultures were then placed in the incubator at 20°C ±2°C under continuous cool fluorescent light illumination. Shoots were removed and subcultured when they were 10-20 mm in height after 4 months. The shoots were subcultured on modified Murashige and Skoog (Appendix IV-B). The pH of the media was adjusted to 5.7 before autoclaving.

- Procedure

Single shoots were grown on modified MS media (Appendix IV-B) in McCartney bottles for 4 weeks before inoculating them with two *S. unicorne* isolates SUL101.4 and SUL83.2. Shoots selected from *C. macrocarpa* family M25 were used for the inoculation.. The shoots were 10- 20 mm in height and without roots.

Two mm discs of agar plugs were obtained from the edge of 18- day old *S. unicorne* isolate SUL101.4 and SUL83.2 cultures and with a pair of sterile forceps. The agar plugs were placed on the stem of the shoot mid-way between the shoot apex and the base of each shoot. The plugs were pressed firmly with a pair forceps. For the controls sterile agar plug was used. Two isolates x four replicates were used in the study. The cultures were incubated at 20 ± 2°C under 12 h light/12 h dark cycle.

- Assessment

Assessment was carried out 10, 20 and 30 days after inoculation.

The assessment was based on visual observation of the canker symptoms on each shoot and the growth of the fungus.

6.2.2 Experiment 2: In vitro inoculation of *C. macrocarpa* shoots with conidial suspension of *S. unicorne*

- Objective

The objective of this experiment was to investigate methods of applying condial inocula on tissue cultured cypress plants in vitro to screen for resistance to cypress canker.

- Fungal material

S. unicorne isolate SUL101.4 was cultured on water agar and *C. macrocarpa* shoots. The culture was incubated under UV light on a 12 h light/12 h dark cycle at 20 ± 2° C. Spore suspension was prepared from 20 days-old cultures by flooding with 5 ml of sterile RO water and then poured into a McCartney bottle. After shaking for a few minutes, the suspension was filtered using a 20-µm-cell strainer. The inoculum concentration was adjusted to 136,000 conidia per ml.

- Plant material

Shoots selected from *C. macrocarpa* family (M25) used for the inoculation were maintained on modified MS medium as described in experiment 1. The shoots were 10-20 mm in height and without roots.

- Procedure

A sterile syringe and needle was used to withdraw 20 µl from the spore suspension. A drop was placed on the stem mid-way from the apex and the base. Sterile water was used for the controls. Lids were placed on and the edges of the McCartney bottles were sealed with strips of glad wrap. The cultures were arranged in a randomized complete block design on a bench under white lights on a 12 h light/12 h dark cycle at 20°C.

Assessment were based on visual observation was carried out 10 and 20 days after inoculation.

6.2.3 Experiment 3: In vitro inoculation of *C. macrocarpa* shoots with conidial suspension of *S. unicorne* on filter paper discs

The method used for inoculating tissue cultured *C. macrocarpa* in the previous study (section 6.2.3) resulted in contamination of the tissue culture media. The pathogen grew faster on the media than it would do on the host. Contamination problem caused difficulty in the assessment of inoculated tissue cultured *C. macrcarpa* plants.

Previous inoculations of tissue cultured *C. macrocarpa* plants showed similar responses of *S. unicorne* isolates SUL101.4 and SUL83.2 to that of glasshouse and outdoor studies. Isolate SUL101.4 was more virulent than SUL83.2. The two isolates were capable of infecting the plantlets under tissue culture condition. However, symptoms of canker developed faster on plantlets inoculated with SUL101.4 than on plantlets inoculated with SUL83.2. The cypress plantlets were of the same genetic makeup, therefore the results were consistent.

- Objectives

The objectives of experiment were to investigate:

(1) The use of filter discs to apply conidial inocula on tissue culture cypress plants to screen for resistance to cypress canker.

(2) The susceptibility of tissue cultured cypress plants to three *S. unicorne* isolates.

- Fungal material (conidial suspensions adjusted to 100,000 conidia per ml) of SUL83, SUL101.4 and SUL156.2.2. Isolates SULL101.4 and SUL156.2.2 had been observed to be highly virulent in the glasshouse and shade cloth condition.
- Plant material

Plant material included a mixture of plantlets from *C. macrocarpa* family M50.6, M50.4, M23.5 and M (numbers after the family name stands for the seedling number from which shoots were cultured from, e.g M50.6= plant no. 6 from family M50) and *C. lusitanica* (L1 and L2) because there was not adequate plant material from each clone. The explants were obtained from the seedling batch from which the explants for the previous two experiments were obtained. The *C. macrocarpa* seedlings were about one year old and the height of plants ranged from 40- 70 cm. The side shoots were 3-4 mm

in diameter. The method for culture preparation was as described for the previous two experiments.

- Tissue culture media (Modified version of Spanos et al (1997b)

The standard media used for growing the shoot tips was half strength MS (premixed 1 L packet 4.3 g/l). The other ingredients included 5,000 g/ charcoal and the vitamins listed in (Appendix IV-B). For shoot proliferation, 0.1mg/l BA (6 Benzylamino purine – SIGMA) was added and charcoal was not included. The tissue cultured cypress plants were kept on multiplication medium for 14 days before transferring on the standard medium. The plantlets for this study had been on the standard medium for 5- 7 months and incubated at 20°C under white light on a 12 h light/12 h dark cycle. They were transferred to fresh media at least every four weeks. The latest subculture was carried out in March 2003. The height of plantlets ranged from 10 to 30 mm at the time of inoculation.

- Procedure

Five (5 µl) microlitre of conidial suspension (= 500 conidia) was applied on to sterile 3 mm diameter filter paper discs using a micropipette. The filter paper discs containing the spore suspension were then placed on leaves close to the stem of each plantlet. Sterile RO water was used in the place of conidial suspension in the control treatments. The lids of McCartney bottles were placed on before sealing with strips of glad wrap.

- Experimental design

The McCartney bottles were placed on a bench in a completely randomized design under a white light on a 12 h light/12 h dark cycle. There were 10 replicates per *S. unicorne* isolate (x 3 isolates) and control.

- Assessment

Visual assessment based on canker symptoms was carried out at 10 and 20 days after inoculation. The assessment scale was as follows:

1 = No fungal growth on filter paper disc.

2 = Fungal growth on filter paper disc only.

3 = Fungal growth on filter paper disc and the plant; browning of inoculation point.

4 = Dead plant or plant completely covered by mycelial growth.

NB. This assessment scale excluded fungal growth on the media due to contamination. Plants were transferred to fresh media in the case where the media was contaminated but there was no fungal growth on the filter paper discs or the plant.

6.3 RESULTS

6.3.1 Experiment 1

There was fungal growth on shoots inoculated with *S. unicorne* SUL101.4 ten days after inoculation but no obvious symptom of disease was observed on these shoots. No fungal growth was observed on shoots inoculated with SUL83.2 tens after inoculation.

Twenty days after inoculation, 50% (2/4) of plants inoculated with isolate SUL101. 4 became brown and were completely covered with mycelia of the pathogen. One shoot was completely covered but remained green. The fourth plant developed browning on the site where the agar plug containing mycelia was inoculated.

Isolate SUL83.2 grew and mycelium covered most of the leaves of the four shoots inoculated. One plant developed browning at the site of inoculation. With the exception of a leaf from a shoot, all the leaves remained green. The two fungal pathogens grew on the surface of the medium and penetrated in the medium around the base of the shoots.

All *C. macrocarpa* shoots inoculated with isolate SUL101.4 became brown 30 days after inoculation and the tops of shoots inoculated with isolate SUL83.2 also became brown compared to the control (Plate 6.1).

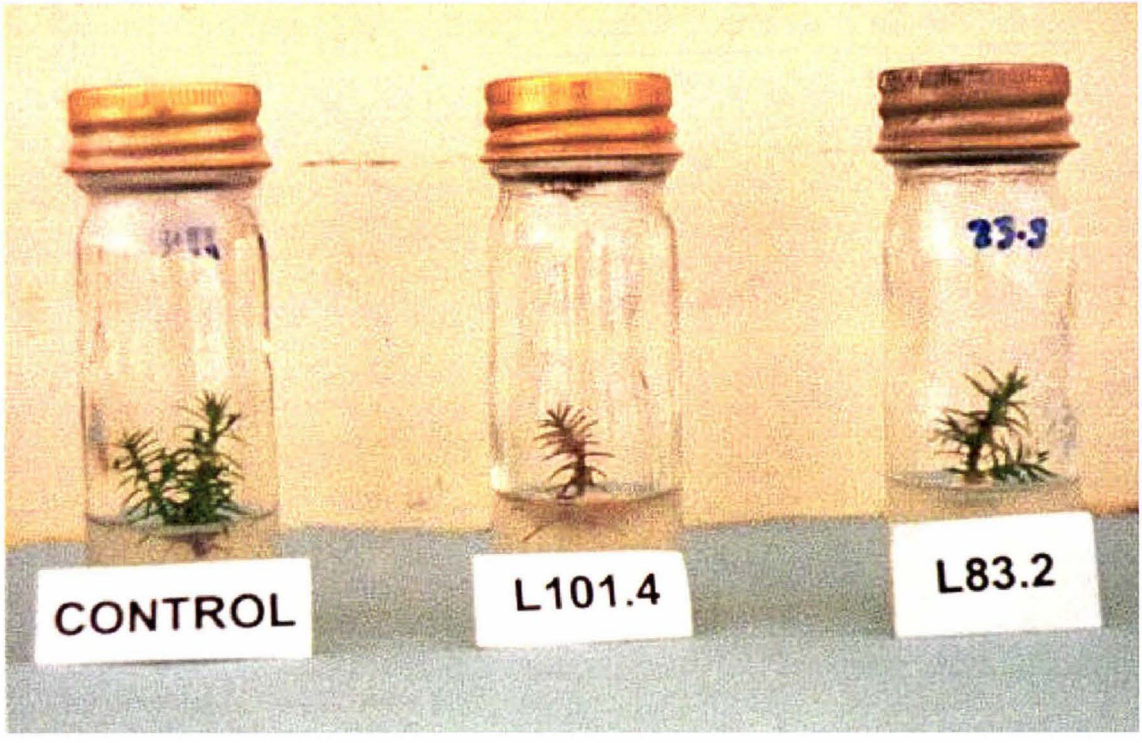

Plate 6.1 Symptoms observed on two plants inoculated with *S. unicorne* isolates SUL101.4 and SUL83.2 twenty days after inoculation.

6.3.2 Experiment 2

Ten days after inoculation the fungus grew on the tissue culture medium and covered the surface completely. There was no fungal growth on the *C. macrocarpa* shoots where they were inoculated and the shoots appeared normal.

All the shoots were dead and covered with the fungal mycelia 20 days after inoculation. The fungus grew upwards from medium and on to the shoots. Browning of leaves started at the base of the shoot where the fungus was advancing as it grew upwards.

6.3.3 Experiment 3

Three *C. macrocarpa* plants (two from family M50.6 and one from family M23.5) which were 10 mm in height were dead and completely covered with fungal mycelia 10 days after inoculation. Two of these plants were inoculated with *S. unicorne* isolate SUL101 and one was inoculated with SUL156.2.2. All the control plants remained healthy 20 days after inoculation. Plants inoculated with isolate SUL101 developed more severe browning symptoms compared to plants inoculated by the other two isolates. Isolate SUL83.2 was less virulent compared with SUL156.2.2 and SUL101.4. Analysis of variance (Appendix IV-C) carried out on the infection scores showed no significance difference between the three *S. unicorne* isolates. Isolate SUL101.4 ranked first with the highest mean score (Table 6.1) and SUL83.2 the lowest.

Table 6.1 Canker mean score for three *S. unicorne* isolates

Isolate No.	No. of replicates	Mean score	Std Dev
SUL83.2	10	1.60	0.84
SUL101.4	10	2.70	1.06
SUL156.2.2	10	2.30	1.25

The plants used in this study were tissue cultured from plants identified to be resistant to cypress canker under glasshouse conditions. Most of the plants developed disease symptoms as a result of fungal growth on media and not from the filter paper discs soaked with spore suspension. Those cultures with no fungal growth on the media remained healthy more than 20 days after inoculation (Plate 6.2). There was no fungal growth on the filter paper. After two months, fungal growth occurred on filter inoculated with isolate SUL101.4 and placed on *C. macrocarpa* M50 plantlet (Plate 6.3).

Plate 6.2 *C. mcarocarpa* family M50 inoculated with three *S. unicorne* isolates (L83.2, L101.4 and L156.22) 20 days after inoculation.

Plate 6.3 Mycelia of the fungal pathogen (SUL101.4), growing on filter paper disc 2 months after inoculation.

6.4 DISCUSSION

Experiment 1 showed that plants cloned from *C. macrocarpa* family M25 seedling were susceptible to *S. unicorne* isolates in vitro. Disease symptoms developed within 1 week after inoculation. Two shoots inoculated with isolate SUL101.4 were dead by the second week. Inoculation of *C. macrocarpa* family M25 in the glasshouse in the previous study showed that this family is susceptible to *S. unicorne* isolate SUL101.4 Under glasshouse conditions, isolate SUL83.2 appeared to be non pathogenic during the length of time allocated for the study and in the field the pathogen is weak compared to isolate SUL101.4. Given the optimum conditions such as in vitro culture, isolate SUL83.2 could cause similar infections to isolate SUL101.4 but it takes a longer time for symptoms to develop.

The method used in applying the conidial suspension did not work out as expected. This was due to the fact that plants used in the study were too small. The droplet of conidial suspensions applied was too big to remain on the stem of the plants. Plants died as a result of fungal growth upward from the media on to the plants. The media supported the growth of the pathogen; therefore no valid conclusion could be drawn on the observation. However, inoculation with mycelium showed that *C. macrocarpa* family M25 was susceptible to *S. unicorne* isolate SUL101.4.

There was difficulty as in the previous study with the application of pathogen inocula due to size of plants (10 – 30 mm), however there was a clear demonstration of pathogenicity of the three *S. unicorne* isolates tested. The result correlated with observation in the glasshouse condition.

C. macrocarpa and *C. lusitanica* plants have been found to grow well under white light on a 12 h light/12 h dark cycle at 20°C. The plants grew better when the MS was reduced to half strength. Further investigation is required on the growth medium and light hours.

The use of filter paper discs soaked with conidial suspensions appeared to be the best method appropriate for in vitro inoculation of tissue cultured cypress plants.

The studies showed that unrooted cypress shoots can be used for in vitro inoculation. In vitro inoculation of unrooted shoots of apple (*Malus domestica* Mill) were used in evaluation of scab (*Venturia inaequalis*) and found to be useful in testing resistant and susceptible cultivars (Ivanicka et al. 1996). Unrooted shoots would reduce the length of

time of culturing the plants in vitro. Plant heights greater than 30 mm would be much easier to handle than heights below 30 mm. Spanos et al. (1997a) inoculated rooted cypress shoots and there is a need to investigate if there are differences between rooted and unrooted shoots of cypress clones in the reaction to the infection of *S. unicorne* isolates.

CHAPTER SEVEN

OVERVIEW OF ALL EXPERIMENTAL WORK

7.1 INTRODUCTION

Experiments conducted in this study covered all the eight aims of the project. However, there were problems with uniformity. For example, the best method of producing conidia was not found until the final stages of the project and in several experiments, some isolates had not sporulated well and others had to be substituted so that isolates used were not consistent from one trial to another. There was also a limited amount of plant material that could be obtained from any one ramet. This chapter covers a summary of inoculation results from the various experiments described in previous chapters.

7.2 MATERIALS AND METHODS

The materials and methods for this section have been described in Chapters Four and Five.

- Fungal material

Different fungal isolates were used in more than half of the experiments and details were given in each materials and methods.

- Plant material

Cypress plants used in this study were mostly seedlings of four *C. macrcoarpa* and two *C. lusitanica* clonal families.

7.3 RESULTS

7.3.1 Effective inoculum load

There were two studies to determine the effective conidial load for causing infection of cypress canker under glasshouse and shade cloth conditions. Two S. *unicorne* isolates (SUL101.4 and SUL83.2) were used in the glasshouse, while three *Seiridium* isolates (*S. unicorne* L 155.7.1 and L156.2.2, *S. cardinale* isolate L152.2.4) in the shade cloth trial. Canker incidence increased with the increase in the spore load in both environmental conditions (Figure 7.1).

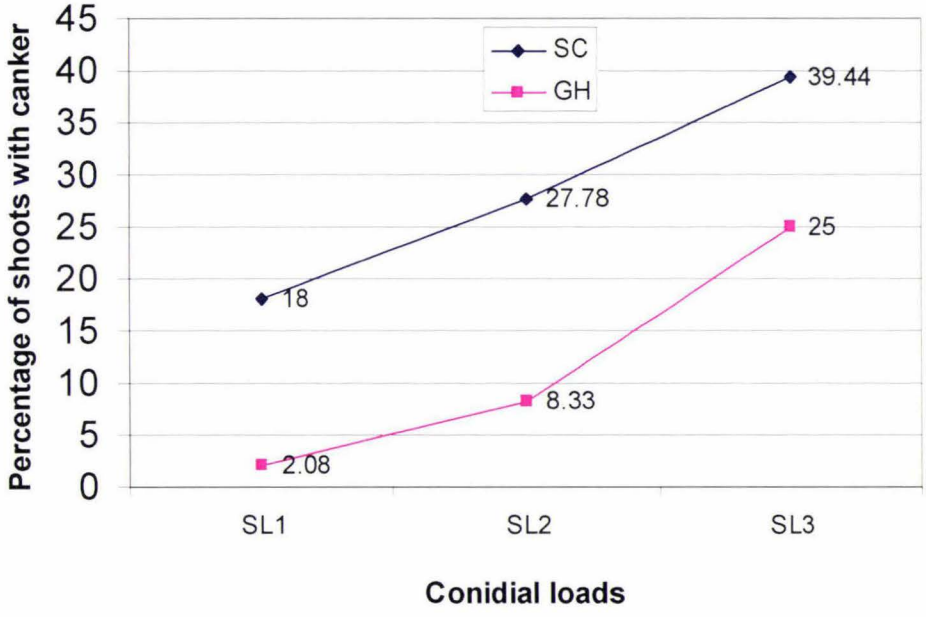

Figure 7.1 Percentage of shoots with canker at three conidial loads under shade cloth (SC) condition (where SL1=50 conidia per wound; SL2= 500 conidia; SL3= 5,000 conidia) and glasshouse (GH) condition (where SL1= 100 conidia per wound; SL2= 1,000 conidia; SL3= 10,000 conidia).

The average temperature in the glasshouse was (16.5°C) higher than the shade cloth trial (14.4°C) but since different conidial loads and different fungal isolates were used in the two trials the difference can not be solely be attributed to temperatures.

7.3.2 Susceptibility of cypress families under different environmental conditions

Five cypress families were tested under two environmental conditions with different fungal isolates. The susceptibility of each cypress families varied under different environmental conditions (Figure 7.2). Results of % canker incidence for other trials conducted under different environmental conditions are listed in appendix V.

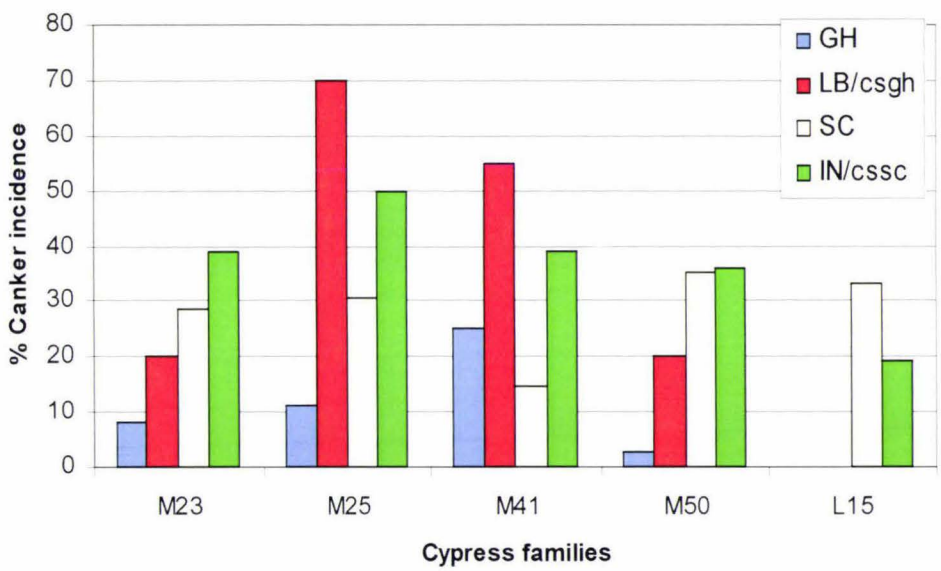

Figure 7.2 Susceptibility of cypress families under different environmental conditions (GH=glasshouse, LB/csgh=Laboratory bench using cut shoots from plants maintained in the glasshouse, SC=shade cloth; IN/cssc= Incubator using cut shoots obtained from plants maintained under the shade cloth).

In general, *C. macrocarpa* M23, M50 and *C. lusitanica* L15 were less susceptible than M25 and M41 with a canker incidence less than 40 %. The pathogenicity of *Seiridium* isolates used also influenced the susceptibility of the cypress families (Figure 7.3). In this trial the same cypress families were inoculated with the same *Seiridium* isolates under shade cloth and incubator conditions. *S. unicorne* isolate SUL1562.2 was considerably more virulent than SUL152.2.4 in the shade cloth trial but there was little difference between the two in the incubator trial.

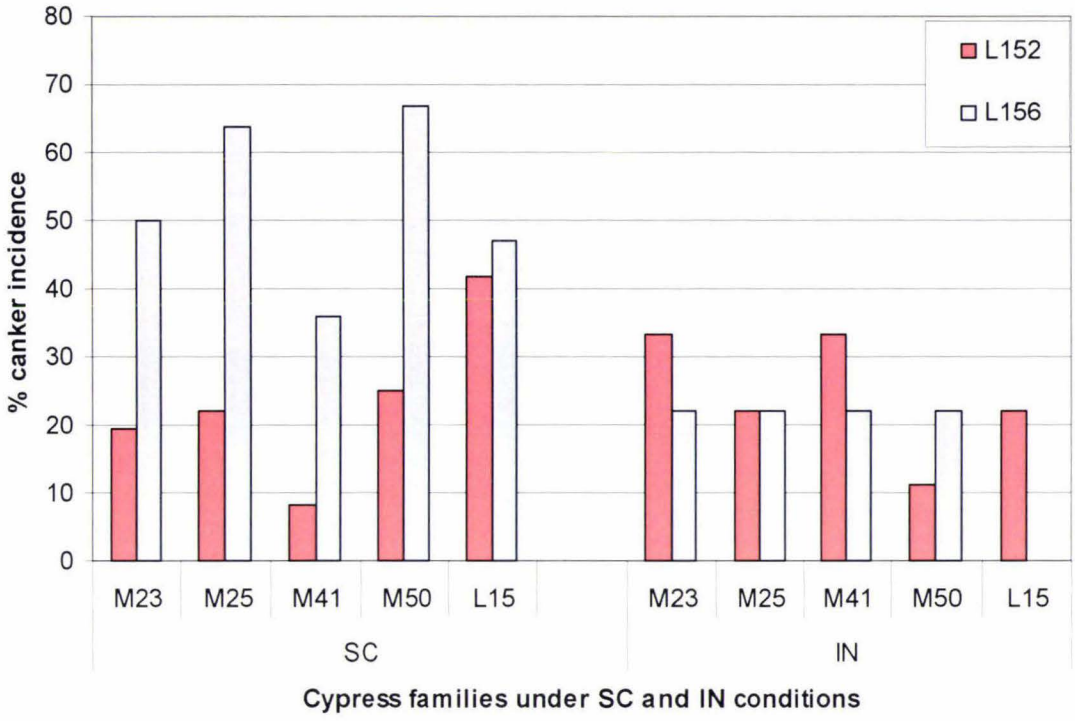

Figure 7.3 Susceptibility of cypress families inoculated with *S. cardinale* SCL152.2.4 and *S. unicorne* SUL156.2.2 under shade cloth (SC) and incubator conditions (IN) on a 10°C dark/15°C light cycle)

The cypress families were more susceptible to cypress canker infection under shade cloth than in the incubator conditions where the temperature was controlled. The average in the shade cloth was (14.4°C) and in the incubator the temperature was at 10°C dark/ 15°C light. The shade cloth trial was set up in spring of 2002 and continued through summer and autumn of 2003. During the summer months, the average temperature was 16.1°C.

7.3.3 Pathogenicity of *Seiridium* species under different environmental conditions

The virulence of each *Seiridium* isolate varied under different environmental conditions (Figure 7.4). *S. unicorne* isolate SUL101.4 was more virulent than other *S. unicorne* isolates and *S. cardinale* SCL152.2.4.

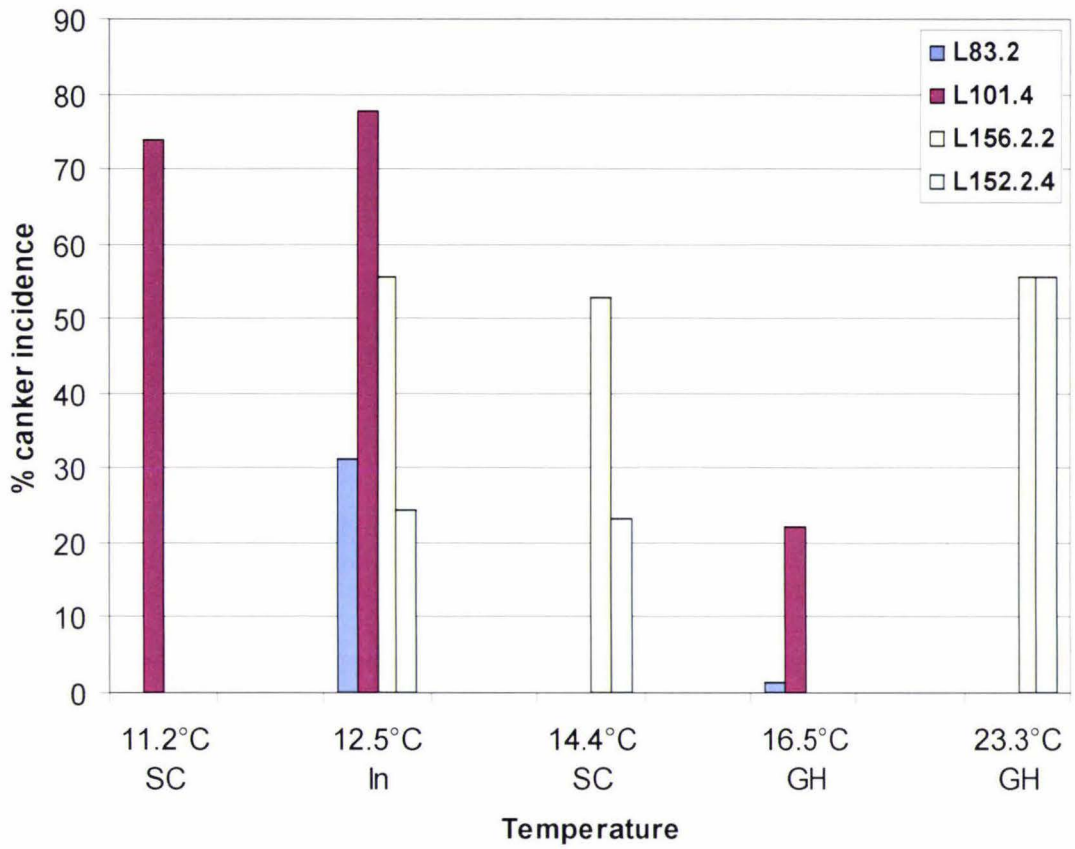

Figure 7.4 Percentage canker incidence caused by *Seiridium* isolates under different temperatures (SC= Shade cloth, In= incubator and GH= Glasshouse)

S. unicorne isolates SUL83.2 and SUL101.4 caused a higher % canker incidence at lower temperatures and the reverse is true of *S. cardinale* SCL152.2.4. *S. unicorne* SUL156.2.2 was not affected by the changes in temperature.

7.4 DISCUSSION

The inoculum load study showed a positive relationship between percentage canker incidence and conidial load. Since different isolates, conidial loads and different cypress seedlings were used in the two studies, a direct comparison of the effect of environmental conditions in which the trials were carried out cannot be made.

Environmental conditions and pathogenicity of *Seiridium* isolates affected the infection of cypress families. Similarly, the pathogenicity of the *Seiridium* isolates varied under different environmental conditions. Sole et al (1983) reported that susceptibility of cypress plants correlated with active growth in autumn and resistance corresponded to dormancy-like periods during summer and end of winter.

Pathogenic variation in *Seiridium* spp. have been reported in studies conducted in New Zealand over the past decades (Beresford & Mulholland, 1982; Chou 1990; Self 1994).These studies showed that there was a wider variation among *S. unicorne* isolates than among *S. cardinale*. Chou (1990) reported that *S. cardinale* showed an overall higher pathogenicity compared to *S. unicorne*. Environment, the third component of the disease triangle (Agrios 1988) was not clearly reported in these studies.

Results from this study clearly showed that environmental conditions play an important role in the degree of pathogencity of *Seiridium* isolates tested. *S. unicorne* isolates SUL 101.4 and SUL83.2 appeared to be more virulent at lower temperatures while isolate *S. cardinale* isolate SCL152.2.4 caused more damage at higher temperatures. Graniti (1998) reported the optimum temperature for germination and growth of *S. unicorne* to be around 20°C and 25°C for *S. cardinale* and *S. cupressi.*

CHAPTER EIGHT

GENERAL DISCUSSION

8.1 INTRODUCTION

The research aimed at investigating methods of inoculation cypress species with *Seiridium* spp. to screen for resistance and pathogen variability. The preliminary studies involved looking at methods of artificial inoculations reported in previous studies conducted in the Northern Hemisphere countries as well as in New Zealand (Beresford & Mulholland 1982; Solel et al. 1983; Xenopoulos 1990; Ponchete & Andreoli 1989; Tisserat et al. 1990). Environmental factors such as temperature and relative humidity play an important role in the pathogenicity of *S. unicorne* and *S. cardinale* and in the susceptibility of cypress families. The type of inoculum and species of *Seiridium* used in artificial inoculation also affected the reliability of screening results.

Seiridium species are known as wound pathogens but the question is whether these pathogens are capable of causing infections without wounding outside the natural environment such as glasshouse conditions. Experiments conducted in this study showed that wounding is required for causing infection in glasshouse trials, and under shade cloth, only 10% of inoculated unwounded side branches and main stems became infected. Therefore, in practice it is best to wound the inoculation sites.

The common type of inocula used in artificial inoculations is mycelium cut from colonies grown on agar. The agar supplies nutrient for the growth of the pathogen unlike the situation in natural conditions and the pathogen may overcome the resistance of plants with canker resistance trait. This kind of screening may lead to early discarding of plants with high potential for timber that may perform well in other climatic conditions. Growing of cypress plants with very high resistance may also lead to the emergence of new virulent *Seiridium* strains.

Conidial inocula were also used in artificial inoculation studies by several workers (Strouts 1973; Beresford & Mulholland 1982; Solel et al. 1983; Pochete & Andreoli 1989; Panconesi & Raddi 1991). There is not much information on the standard amount of inoculum load to be applied under different environmental conditions. To provide supply of conidial inocula for comparing mycelium and conidial inocula, various methods of conidia production are required. A number of methods of inducing sporulation of *Seiridium* isolates have been described (Strouts 1973; Initini & Panconesi 1974; Sasaki 1976; Solel et al. 1983; Chou 1989; Tobata et al.1991; Sanchez & Gibbs 1995). The *Seiridium* isolates used in the study at Massey University were supplied by Forest Research (Rotorua, New Zealand). After two series of subcultures, spore production was drastically reduced. Some of the methods described in the literature were tested but results obtained were not consistent. It is possible that spore production depends on the *Seiridium* isolates used and environmental conditions of incubation. If spores are to be used in artificial inoculation, then there is a need to investigate the required spore load for causing infection. The results of inoculum study conducted in New Zealand should be compared to similar studies conducted overseas.

The range of pathogenic variability in *Seiridium* isolates and the effect of environment were also determined in the various experiments carried out. The possibility of using cut shoots of cypress was also tested under uncontrolled and in incubator conditions. The pathogenicity of the *Seiridium* isolates varied under the different conditions tested. There is a possibility that the age of conidial inocula contributed to the reduction of pathogenicity in some isolates.

8.2 MAIN STEM AND SIDE BRANCH INOCULATION

Inoculation of main stem of cypress seedlings is the common method practised in both the Northern Hemisphere countries and New Zealand. Side branch inoculation on the same plant would eliminate the variation within individual plants. Different treatments for instance wounding and non-wounding, agar plugs and spore suspension could be done on the same plant. Side branches have a smaller bulk than the entire plant so results may appear more rapidly. Experiments conducted showed that cypress canker symptoms are more likely to appear earlier on side branches than main stem inoculations. Under natural conditions, Raddi & Panconesi (1981) reported that in the

case of *S. cardinale*, more infections are observed at the insertion of small branches on the trunk. Several *Seiridium* isolates were tested on the same plant when side branches were used and reliable result on pathogenicity of each isolate were obtained.

8.3 METHODS OF INDUCING SPORULATION OF *SEIRIDIUM* SPECIES.

Observation with *Seiridium* isolates kept at Massey University showed that the isolates are capable of producing conidia on MA, PDA,WA and V8 juice agar with or without addition of natural substrates occasionally. Addition of carnation leaves have resulted in higher conidia production compared with other natural substrates. However, conidia production depends on the type of *Seiridium* isolates. Acervuli formation has been reported to be continuous under natural environment but maturation and sporulation depends on climatic factors (Raddi & Panconesi 1981; Xenopoulos 1990; Graniti 1998). Isolates with normal morphology are more likely to sporulate than "degenerative"forms. Frequent subculturing appears to be the main cause of reduction in sporulation. Natural substrates such as tree twigs from non-host plants have been found to support sporulation of *Seiridium* isolates.

8.4 AGAR PLUGS AND CONIDIAL SUSPENSION INOCULA

Mycelial plugs are commonly used in artificial inoculation. However, under natural conditions, infection is caused by conidia. There is a view that mycelial plugs cut from agar may provide nutrients for the growth of *Seiridium* pathogen. This could lead to extreme pathogenicity and the resistance of promising cypress clones could be masked.

Studies carried out to compare the two types of inocula showed that in general, plugs cut from colonies of fungi growing on agar media resulted in higher number of canker infections than conidial inocula. A study conducted under shade cloth conditions showed a difference of 11% canker incidence between mycelial plugs and conidial inocula. This difference however, was also influenced by the type of inoculation sites (main and side branches, wounding or non- wounding) used in this experiment.

Repeated study in a glasshouse using only the main stem for inoculation revealed no significant difference between the two types of inocula

Another trial where the same *C. macrocarpa* plants were inoculated with the two types of inocula at different times with *S. unicorne* isolate L101.4 produced similar results.

The main problem with the use of mycelial plugs is related to a possibility of "degenerative" form and conidial inocula offers a more natural means of infection.

8.5 CONIDIAL LOAD REQUIRED FOR CAUSING INFECTIONS

Artificial inoculations were also conducted using spore suspensions. There is very little information available on the required conidial load for causing infection in artificial inoculations. According to Ponchet & Andreoli (1984) that the minimum effective dose for *S. cardinale* was found to be 50 conidia and the optimum was 500 conidia per wound. Two studies using cypress seedlings conducted in the glasshouse and shade cloth conditions on inoculum load showed that infection increased with the increase of number of conidia per wound.

Inoculum loads tested ranged from 50- 10,000 conidia per wound for *S. unicorne*. The third study using cut shoots was carried out in a humid chamber with relative humidity between 40% and 60% and temperature range from 15°C to 35°C with an average of 25°C, showed an optimum load of 500 conidia per wound.

8.6 ASSESSMENT OF BRANCH INOCULATIONS OF RAMETS IN VITRO AND IN VIVO

Ostry et al. (1990) reported correlations between responses of in vitro inoculated adventitious shoot culture of *Larix* species with *Mycospherella laricina* and known levels of field resistance.

Studies conducted on side branch inoculation in vivo and cut side branches inoculation in vitro have been successful. Inoculation of adventitious shoot culture of cypress ramets in vitro could offer an alternative method of screening. It provides an easy method of testing the same plants under more than one environmental condition.

Susceptibility of cypress canker depended on the type of *Seiridium* isolate used and the environmental conditions under which plants are tested. In vitro inoculation may indicate the likely pathogenicity of *Seiridium* isolates under a particular environmental conditions.

8.7 ASSESSMENT OF WOUNDING AND NON-WOUNDING OF INOCULATION SITES

As mentioned earlier that *Seiridium* isolates gain entry to plants via wounds on the bark. Host plants respond naturally to any type of wounds. *Seiridium* pathogens are able to overcome host resistance in susceptible cypress plants and continue to grow in the bark of the host. Experiments conducted revealed that wounds are required for infection in the field and glasshouse conditions. However, wounding is not required under optimal conditions such as those in tissue culture and cut shoots placed in McCartney bottles tested in this study. Under optimal conditions, the pathogen enters through the epidermis (Raddi & Panconesi 1981). High percentage relative humidity is necessary for causing infection without wounds.

8.8 RANGE OF PATHOGENIC VARIABILITY IN *SEIRIDIUM* ISOLATES

Available literature on studies conducted in various countries in the past decades showed considerable variation in virulence between isolates of the same species and between tests conducted at different times with the same isolates. Results from this research showed that virulence of isolates differ between isolates of the same species and the same species used for testing under different environmental conditions. However, *S. unicorne* SUL101.4 has been found to maintain its virulence under different environmental conditions. Isolates of *S. unicorne* have been found to be more pathogenic than *S. cardinale* isolate tested in the studies conducted. Findings by Chou (1990) showed that isolates of *S. cardinale* displayed overall higher pathogenicity than those of *S. unicorne*.

8.9 THE EFFECT OF ENVIRONMENT (Temperature and % relative humidity) ON CYPRESS CANKER INFECTION AND DISEASE DEVELOPMENT)

Environment is the third component of the 'disease triangle' (Agrios 1988). The series of experiment showed that with some *Seiridium* isolates, warmer temperatures favour development of cypress canker and with some they do not. More infection occurred under relative humidity between 80-100%.

8.10 CONCLUSION AND FUTURE CONSIDERATIONS

Wide variation in *Seiridium* isolates and use of different cypress seedlings makes any firm conclusion from this kind of work difficult. However, these findings could be useful as a guideline for future research on screening programmes. For instance the use of side branches or excised side branches provides a reliable method of screening with several *Seiridium* isolates at any one time under different environmental conditions. In this way screening could be done thoroughly and only the potential clones are planted out in the field conditions for further screening.

Environmental factors should be given more consideration in screening programmes. Screening of ramets should be conducted under different environmental conditions and the best clones should be selected for a specific site that a clone is performing well under. The research was carried out using seedlings from cypress seedlings and there is genetic variability among the seedlings. Therefore the results were not consistent for the same cypress family.

There is also a need to study individual isolates found under different parts of the country and the number of species found in New Zealand should be confirmed using molecular studies. Further research is required on the inoculation methods tested in this study using ramets or clones of cypress families. Now that problems with conidia production have been identified, the effect of media type on the viability and germination of conidia should be investigated in future studies.

REFERENCES

Agrios, G. N. 1997: Plant Pathology. Fourth edition. Academic Press, Inc: San Diego.

Aimers-Halliday, J.; Miller, J.; Shelbourne, T.; Menzies. 1994: Genetically improved planting stock for *Cupressus macrocarpa* and *Cupressus lusitanica*. *New Zealand Tree grower 15*: 40-42.

Anonymous. 2001: New Zealand Forestry Industry Facts and Figures. 2000 / 2001.

Bailey, C. W. W. 1995: Sporulation of *Cercospora fijiensis* in different environments. [Spanish] <Esporulacion de *Cercopora fijiensis* en diferentes ambientes.> *Manejo Intergrato de Plagas* 37: 16-20. (CAB Abstract-English summary).

Barnes, I.; Coetzee, M. P. A.; Coutinho, T. A; Roux, J.; Wingfield, B. D.; Wingfield, M. J. 2000: Characterisation of *Seiridium* isolates associated with cypress canker based on molecular sequence data. *Phytopathology 90 (6)*: S5.

Barnes, I.; Roux, J.; Wingfield, M. J. 2001: Characterisation of *Seiridium* spp. associated with Cypress canker based on B-Tublin and Histone Sequences. *Plant Disease 85(3)*: 317-321.

Beresford, R. M.; Muholland, R. I. 1982: Susceptibility of farm shelter cypresses to 3 fungi associated with cypress canker disease. *New Zealand Journal of Forestry Science 12*: 7-13.

Birch, T. T. C. 1933: Gummosis diseases of *Cupressus macrocarpa*. *New Zealand Journal of Forestry 3*: 108-113.

Boesewinkel, H. J. 1983: New Recordings of the three fungi causing cypress canker in New Zealand, *Seiridium cupressi*(Guba) comb.Nov and *S. cardinale* on *Cupressocyparis* and *S .unicorne* and *Cryptomenia* and *Cupressus*. *Transaction of the British Mycological Society 80(3)*: 544-547.

Casini, N; Santini, A. 1995: Stability of resistance to *Seiridium cardinale* canker in some clones of common cypress. (editors: Capretii, P et al.) *In*: Shoot and Foliage Diseases in Forest Trees. Proceedings of a Joint Meeting of the IUFRO Working Parties S2.06.02 and S2.06.04. Vallombrosa, Firenze, Italy, 6-11 June, 1994. Pp 118-125.

Chou, C. K. S. 1989: Morphological and Cultural variations of *Seiridium* spp. from cankered Cupressaceace hosts in New Zealand. *European Journal of Forest Pathology 19*: 435-445.

Chou, C. K. S. 1990: Pathogenic Variation of *Seiridum* spp. isolated from cankered Cupressaceace hosts in New Zealand. *European Journal of Forest Pathology 20* 32-43.

Cutter, V. M. Jr. 1960: Studies on the isolation and growth of plant rusts in host tissue cultures and upon synthetic media. II. *Uromyces ari-triphylli*. *Myocologia 52*: 726-742.

Diner, A. M.; Mott. R. L. 1982: Direct inoculation of five-needle pines by *Cronartium ribcola* in axenic culture. *Phytopathology 72*: 1181-1184.

Diner, A. M.; Mott, R. L. 1985: In vitro inoculation of western white pine tissue culture propagules with vegetative hyphae of *Cronartium ribicola*. *Phytopathology 75*:1130-1131.

Fisher, N. L.; Burgess, L. W.; Toussoun, T. A.; Nelson, P. E. 1982: Carnation leaves as a substrate and for preserving cultures of *Fusarium* species. *Phytopathology 72 (1)*: 151-153.

Franklin, D. 1994: Update on Macrocarpa. *New Zealand Forestry 39(1)*: Pp 46 & 48.

Gea, L. D.; Low, C. B. 1997: Genetic Parameters for growth, form and canker resistance of *Cupressus macrocarpa* in New Zealand. *New Zealand Journal of Forestry Science 27 (3)*: 245-254.

Graniti, A. 1998: Cypress canker: A pandemic in Progress. *Annual Review of Phytopathology 36*: 91-114.

Hood, I. A.; Gardner, J. F. 2002: Inoculation studies with the cypress canker fungus *Seiridium unicorne*. 31 p.

Hood, A.; Gardener, J. F.; Kimberly, M. O.; Gatenby, S. J.; Cox, J. C. 2001: A survey of Cypress Canker disease. *New Zealand Tree Grower 22(1)*: 38-41.

Hu, C. F.; Wu, Q. H. 1997: Biological characteristics of *Alternaria dianthi* Stevens & Hall. *Journal of Fujian Agricultural University* 26 (3): 308-312. (CAB Abstract-English summary).

Ingram, D. S. 1980: Tissue culture methods in plant pathology. *In*: Tissue culture methods for plant pathologists ((ed.) Ingram, D. S.; Helgeson, J. P.). Blackwell Scientific Publications. Pp 41-49.

Intini, M.; Panconesi, A. 1974: Research on fructifiction in vitro of *Coryneum cardinale*[Italian] *Rivista di Patologia Vegatale,IV 10(3)*: 337-342 (CABI Abstract-English).

Ivanicka, J.; Kellerhals, M.; Theiler, R. 1996: Evaluation of scab (*Venturia inequalis* (Cooke) G. Wint.) on shoots and detached leaves from in vitro and greenhouse grown plants of the apple (Malus *domestica* Mill.) cultivars Golden Delicious and Florina.*Gartenbauwissenschaft 61(5)*: 242-248. (CAB Abstract-English summary).

Kato, K. 1996: Comparison of resistance in elite hinoki trees (*Chamaecyparis obtuse*) (Sieb.et Zucc.) Endl.) to *Seiridium unicorne* (Cooke et Ellis) Sutto. *Bulletin of the National Forest Tree Breeding Center 14*: 145-157. (Abstract).

Koch, S. H.; Knox-Davies, P. S. 1988: Partially selective medium for isolating *Collectotrichum trifolii* from Lucerne tissue. *Phytophylactica 20 (4)*: 333-336. (CAB Abstract-English summary).

McCain, A. H. 1984: Cypress canker control with fungicides. *Journal of Arboriculture 10 (7)*:212-214.

Mead, R.; Curnow, R. N.; Hasted, A. M. 1983: Statistical methods in agriculture and experimental biology. Second edition. Chapman and Hall .415 p.

Miller, J. T; Knowles, F. B. 1996: Introduced Forest trees in New Zealand; Recognition, Role and Seed Source .9. The Cypresses. FRI Bulletin No. 124. 33 p.

Morrica, S.; Raddi, P. 1999; Novel assays for the diagnosis and differentiation of species of *Seiridium* associated with cypress canker diseases. Methodology of forest insect and disease survey in Central Europe: Proceedings of the second workshop of the IUFRO working party. Pp 205-210.

Morrica, S.; Borja, I.; Vendramin, G. G. 2000: Differentiation of *Seiridium* species associated with virulent cankers on cypress in the Mediterranean region by PCR-SSP. *Plant Pathology 49*: 774-781.

Ostry, M. E.; Pijut, P.; Skilling, D. D. 1990: Screening larch in vitro for resistance to *Mycosphaerella laricina*. *Plant Disease 75 (12)*:1222-1224.

Panconesi, A;, Raddi, P. 1986: The influence of some chemical treatments on cypress canker diseases development. *European Journal of Forest Pathology 16(2)* 83-86 (CAB abstract –English summary).

Parrini, C.; Panconesi, A. 1991: Methods to control cypress canker. In: "IL CIPRESSO". Proposals of environmental and productive valorization in the Mediterranean countries of the European Community. Pp 106-109.

Pei, M. H. 1989: Inoculation of callus tissues of Scots pine, Corsican pine and three varieties of mountain pine with *Peridermium pini*. *Mycological Research 93(4)*: 503-507.

Ponchet, J.; Andreoli, C. 1984: Host-parasite relations in the pair *Cupressus-Coryneum cardinale* Wag.(French). *Agronomie 4(3)* 245-255. (CAB Abstract-English summary).

Ponchet, J.; Andreoli, C.1989: Histopathology of cortical canker of cypress caused by *Seiridium cardinale*. *European Journal of Forest Pathology 19(4)*: 212-221.

Quencez, C.; Desprez-Loustau, M. L.; Bastien, C. 2001: Reliability of field, greenhouse and cut-shoot screening procedures for evaluating susceptibility of Scots pine to *Melampsora pinitorqua*. *Forest Pathology 31 (4)*: 193-208.

Raddi, P.; Panconesi, A. 1981: Cypress canker disease in Italy biology control possibilities and genetic improvement for resistance. *European Journal of Forest Pathology 11*: 340-347.

Raddi, P.; Panconesi, A. 1984: Pathogenicity of some isolates of *Seiridium* (*Coryneum*) *cardinale*, agent of cypress canker disease. *European Journal of Forest Pathology 14(6)*: 348-354.

Sanchez, M. E; Gibbs, J. N. 1995: The ecology of fungal cankers on *Cupressus macrocarpa* in Southern England. *European Journal of Forest Pathology 25*:266-273.

Sanderson, P. G.; Jeffers, S. N. 2001: Vegetative growth and conidium production by *Monilinia oxycocci* in vitro. *Mycologia 93(1)*: 9-16.

Santini, A.; Lonardo, V. di. 2000: Genetic variability of the 'bark canker resistance' character in several natural provenances of *Cupressus sempervirens*. *Forest Pathology 30(2)*: 87-96.

Santini, A.; Casini, N.; Di Lonardo, V.; Raddi, P. 1997: Canker resistance stability of some *Cupressus sempervirens* clones to *Seiridium cardinale*. *Journal of Genetics and Breeding 51*:269-277.

Sasaki, K. Kobayashi, T. 1976: Resinous canker disease of Cupressaceae caused by *Monochaetia unicornis* (Cke. & Ell.) Sacc. II. Physiologic characters of the casual fungus. *Bulletin of the Government Forest Experiment Station 280*: 57-68.

Self, N. M 1994: Pathogenicity of *Seiridium unicorne* produced by simultaneous inoculation with normal and degenerate isolates. *New Zealand Journal of Forestry Science 24(1)*: 78-84

Self, N. M. 2000: Cypress Canker. *New Zealand Tree Grower* 21(2):31-32.

Solel, Z.; Messinger, R.; Golan, Y.;Madar, Z. 1983: *Coryneum* canker of cypress in Israel *Plant Disease 67 (5)*: 550-551.

Spanos, K. A.; Woodward, S. 1997: Responses of *Cupressus* and *Chamaecyparis* callus tissues to inoculations with *Seiridium cardinale*. *European Journal of Forest Pathology 27*: 13-21.

Spanos, K. A.; Pirrie, A.; Woodward, 1997a: In vitro expression of resistance responses to *Seiridium* species in micropropagated shoots of *Cupressus sempervirens* and *Chamaecyparis lawsoniana*. *Canadian Journal of Botany 75*: 1103-1109.

Spanos, K. A.; Pirrie, A.; Woodward, S. 1997b: Micropropagation of *Cupressus sempervirens* L. and *Chamaecyparis lawsoniana* (A. MURR.) PAR. *Silvae Genetica 46(5)*: 291-295.

Spanos, K. A.; Pirrie, A.; Woodward, S.; Xenopoulos, S. 1999: Responses in the bark of *Cupressus sempervirens* clones artificially inoculated with *Seiridium cardinale* under field conditions. *European Journal of Forest Pathology 29*: 135-142.

Strouts, R. G. 1973: Canker of cypresses caused by *Coryneum Cardinale* Wag. in Britian. *European Journal of Forest Pathogology 3*: 13 –24.

Sturrock, J. W.; Ferguson, J. D. 1989: Macro and micro propagation of Leyland cypress. *The international Plant Propagators Society 39*: 285-290.

Sutton, B. C. 1980: The Coelomycetes. Commonwealth. Mycological Institute, Kew.

Swart, H. J. 1973: The fungus causing cypress canker. *Transaction of British Mycological Society 61(1)*: 71-82.

Tisserat, N. A.; Nus, A.; Barnes, L. W. 1991: A canker disease of the Cupressaceae in Kansas and Texas caused by *Seiridium unicorne*. *Plant Disease 75(2)*:138-140.

Tobata, M.; Sakamoto, Y.; Yokouchi, H. 1991: Effects of media, temperature, pH and light on sporulation of *Seiridium unicorne*. *Transaction of the Mycological Society of Japan 32*: 523-528.

Tsatsia, H. 2002: Methods of inoculating cypress with *Seiridium*- Progress report for Forest Research, July 2002. 34 p (unpublished).

Tsatsia, H. 2003: Methods of inoculating cypress with *Seiridium*- Progress report for Forest Research, April 2003. 30 p (unpublished).

Van der Werff, H. S. 1988: Cypress canker in New Zealand plantations. *New Zealand Journal of Forestry Science* 18(1):101-102.

Viljoen, C. D.; Wingfiled, B. D.; Wingfiled, M. J. 1993; Comparison of *Seiridium* isolates associated with cypress canker using sequence data. *Experimental Mycology 17*:4 Pp 323-328.

Waller, J. M. 1981: Fungal pathogens in the air and plant shoots. *In*: Burchill, R.T. Methods in Plant Pathology. Commonwealth Mycological Institute, Kew, Surrey , England. Pp 9-16.

Wang, G. C.; Zhang, C. L.; Zheng, Z. 1995: Studies on the section Liseola of *Fusarium*: The plant tissue promoting sporulation of the section of Liseola. *Journal of Zhejiang Agricultural University 21 (*6): 599 – 602.(CAB Abstract-English summary).

Wei, C. I.; Swartz, D. D.; Cornell, J. A. 1985: Effects of culture media, exposure time and temperature on near – ultraviolet- induced sporulation of *Alternaria alternata*. *Journal of Food Protection 48 (* 4): 316 –319.

Wyss, G. S.; Charudattan, R.; DeValerio, J. T. 2001: Evaluation of agar and grain media for mass production of conidia of *Dactylaria higginsii*. *Plant Disease 5*: 1165-1170.

Xenopoulos, S. G. 1990: Screening for resistance to cypress canker (*Seiridim cardinale*) in three Greek provenances of *Cupressus sempervirens*. *European Journal of Forest Pathology 20(3)*: 140-147.

Xenopoulos, S. G. 1991: Pathogenic variability isolates of *Seiridium cardinale*, *S. cupressi* and *S. unicorne* inoculated on selected Cupressus clones and seedlings. *European Journal of Forest Pathology 21*:129-135.

Xenopoulos, S.; Tsaopelas, P. 2000: Sphaeropsis canker, a new disease of cypress in Greece. *Forest Pathology 30(3)*: 121-126.

Yamanda, T.; Ito, S. 1995: Factors affecting the age- related resistance of hinoki stem to *Seiridium unicorne* infection. *Journal of the Japanese Forestry Society* 77(1):67-71.

Yamanda, T.; Ito, S.; Shiomi, S.; Kokuby, Y. 1994: Effects of infection rate and tree age on the progress of *Seiridium unicorne* canker in young hinoki plantations. *Journal of the Japanese Forestry Society 76(3)*: 270-275.

APPENDICES

Appendix I Raw data and analysis results for Chapter Three

(A) Analysis of variance for log spore count for Preliminary Experiment on evaluation of agar types for supporting sporulation of *Seiridium* isolates.

Source	DF	Type III SS	Mean Square	*F* value	*Pr*> *F*
Agar Type	2	8.73456531	4.36728266	1.86	0.1697
Additive	2	22.48901200	11.24450600	4.80	0.0142
Isolate	1	15.76577617	15.76577617	6.73	0.0136
Ag x Ad	4	30.77471826	7.69367957	3.28	0.0215
Ag x Is	2	22.93499736	11.46749868	4.89	0.0132
Ad x Is	2	2.33352335	1.16676167	0.50	0.6119
Ag x Ad x Is	4	47.06582680	11.76645670	5.02	0.0025

(B) Analysis of variance for log spore count for Experiment 1 on evaluation of agar types for supporting sporulation of *Seiridium* isolates.

Source	DF	Type III SS	Mean Square	*F* value	*Pr*> *F*
Block	2	16.9503644	8.47518220	1.96	0.1605
Media type	6	72.80381529	12.13396922	2.81	0.0303
Isolate	1	88.21327097	88.21327097	20.45	0.0001
Me x Isolate	6	29.37947917	4.89657986	1.13	0.3703

(C) Analysis of variance for log spore count for Experiment 2 on evaluation of agar types for supporting sporulation of *Seiridium* isolates.

Source	DF	Type III SS	Mean Square	*F* value	*Pr> F*
Block	3	22.22073763	7.40691254	3.45	0.0233
Agar Type	2	23.01898007	11.50949003	5.36	0.0077
Additive	1	34.10288048	34.10288048	15.87	0.0002
Isolate	2	21.58097126	10.79048563	5.02	0.0102
Ag x Ad	2	11.41617496	5.70808748	2.66	0.0799
Ag x Is	4	11.50244951	2.87561238	1.34	0.2666
Ad x Is	2	0.53816083	0.26908041	0.13	0.8826
Ag x Ad x Is	4	12.44617558	3.11154390	1.45	0.2318

(D) Experiment 4: Percentage of counts for each sporulation rate based on visual category for *Seiridium* isolates tested

	Counts per treatment combinations (Maximum 5 for I.152.2.2b and 3 for I.101.4)							
	Seiridium isolates							
	I.152.2.2b				I.101.4			
Visual Category	Agar Types							
	PDA		MA		PDA		MA	
	Counts	%	Counts	%	Counts	%	Counts	%
1	4	80	2	40	0	0	2	66.7
2	0	0	3	60	1	33.3	0	0
3	1	20	0	0	0	0	0	0
4	0	0	0	0	2	66.7	1	33.3

(E) Evaluation of tree shoots as substrates for spore production of *Seiridium* spp Trial 1: The completed analysis of variance SqScore (Transformed score)

Source	DF	Type III SS	Mean Square	*F* value	*Pr> F*
Block	3	0.705	0.235	3.47	0.038
Substrate	6	0.271	0.045	0.67	0.676
Blk*Sub	18	1.218	0.068		
Isolate	3	0.222	0.0.74	1.31	0.278
Iso *Sub	18	1.189	0.066	1.17	0.311
Split plot error	63	3.550	0.056		
Total	111	7.155			

(F) Evaluation of tree shoots as substrates for spore production of *Seiridium* spp Trial 2: Analysis of variance for the spore production score

Source	DF	Type III SS	Mean Square	*F* value	*Pr > F*
Substrates	2	12.000	5.000	3.86	0.0404
Isolates	1	0.667	0.667	0.43	0.5210
Sub*Iso	2	1.333	0.667	0.43	0.6579

(G) Evaluation of cypress cones as substrates for spore production of *Seiridium* spp Trial 3: Analysis of variance for the spore production score

Source	DF	Sums of Squares	Mean Square	*F value*	*Pr > F*
Model	3	13.2500	4.4167	3.66	0.0443
Error	12	14.5000	1.2083		
Corrected Total	15	27.7500			

Appendix II Raw data and analysis results for Chapter Four

(A) Raw data for the number of inoculation sites with disease symptoms for both of the *Seiridium* isolates (L101.4 & L83.2) conducted under shade cloth.

Inoculation Sites	Mycelium				Spores suspension			
	Wounded site		Unwounded site		Wounded site		Unwounded site	
	Seiridium isolates				*Seiridium* isolates			
	L101.4	L83.2	L101.4	L83.2	L101.4	L83.2	L101.4	L83.2
Main stem (3 sites)	3	0	0	0	2	2	0	0
S/branch (12 sites)	10	8	1	0	6	0	3	2
Total (15 sites)	13	8	1	0	8	2	3	2

(B) Raw data for measurements (cm) of disease lesions 9 weeks after inoculation (Experiment 3 Main stem inoculation: Glasshouse trial)

		Canker length caused by spore inoculated on		Canker length caused by mycelium inoculated on	
Macrocarpa families	Replicate	Wounded stem	Unwounded stem	Wounded stem	Unwounded stem
M25	1	1.0	0	1.4	0
	2	1.5	0	1.5	0
	3	1.2	0	1.5	0
	4	1.3	0	1.4	0
M41	1	1.0	0	2.0	0
	2	1.4	0	1.0	0
	3	1.6	0	1.5	0
	4	1.5	0	1.0	0
M50	1	1.5	0	1.5	0
	2	1.5	0	2.5	0
	3	1.0	0	1.5	0
	4	1.4	0	1.5	0

(C) ANOVA table for main stem inoculations (Experiment 3: Glasshouse trial)

Source	DF	Type III SS	Mean Square	*F* Value	*Pr* > *F*
CLONE	2	0.19000000	0.09500000	0.87	0.4340
INOCULA	1	0.24000000	0.24000000	2.21	0.1545
CLONE*INOCULA	2	0.16000000	0.08000000	0.74	0.4926

(D) Raw data for the number of *C. macrocarpa* side branches with canker symptoms 56 days after inoculation with *Seiridium* isolate I.101.4 and I.83.2 (Spore load experiment:Glasshouse trial)

		Number of side branches with canker symptoms (maximum= 6 per load per cypress family)		
Cypress family	Spore loads	*Seiridium* isolates		
	per wound	I.101.4	I.83.2	Total
M23	100	0	0	0
	1,000	0	0	0
	10,000	3	0	3
	Total	3	0	3
M25	100	0	0	0
	1,000	2	0	2
	10,000	2	0	2
	Total	4	0	4
M41	100	1	0	1
	1,000	2	0	2
	10,000	5	0	5
	Total	8	1	9
M50	100	0	0	0
	1,000	0	0	0
	10,000	1	1	1
	Total	1	1	1
	Grand total	16	1	17

(E) Raw data for the number of disease lesions on cypress plants 23 weeks after inoculation with three spore loadings of three *Seiridium* isolates (Spore load experiment: Shade cloth trial).

Cypress family	Spore loads per wound	Number of shoots with symptoms (Maximum= 12 per cypress family per spore load) *Seiridium* isolates L152.2.4 (*S. cardinale*)	L156.2.2 (*S. unicorne*)	L155.7.1 (*S. unicorne*)	Total
C. lusitanica L15-11/15-12					
	50	2	2	1	5
	500	5	6	2	13
	5000	8	9	1	18
	Total	15	17	4	36
C. macrocarpa M23					
	50	2	3	2	7
	500	2	7	2	11
	5000	3	8	2	13
	Total	7	18	6	31
M25					
	50	4	4	1	9
	500	0	8	0	8
	5000	4	11	1	16
	Total	8	23	2	33
M41					
	50	1	1	0	2
	500	1	4	0	5
	5000	1	8	0	9
	Total	3	13	0	16
M50					
	50	1	6	3	10
	500	2	10	1	13
	5000	6	8	1	15
	Total	9	24	5	38
	G total	42	95	17	154

Appendix III Raw data and analysis results for Chapter Five

(A) Number of shoots with disease lesions caused by four *Seiridium* isolates 40 days after inoculation (SSI trial 4)

Cypress family	Number of shoots with symptoms (Maximum= 9 per cypress family per *Seiridium* isolate)				
	Seiridium isolates				
	L83.2 (*S. unicorne*)	L101.4 (*S. unicorne*)	L152.2.4 (*S. cardinale*)	L156.2.2 (*S. unicorne*)	Total
C. lusitanica					
L15	2	3	2	0	7
C. macrocarpa					
M23	2	7	3	2	14
M25	5	9	2	2	18
M41	3	6	3	2	14
M50	2	8	1	2	13
Total	14	33	11	8	66

(B): Percentage of shoots with canker symptoms caused by four *Seiridium* isolates (SSI trial 4)

Host	Percentage of cankered shoots caused by four *Seiridium* isolates			
	Seiridium isolates			
	L83.2	L101	L152.2.4	L156.2.2
L15	22.22	33.33	22.22	0
M23	22.22	77.78	33.33	22.22
M25	55.56	100	22.22	22.22
M41	33.33	66.67	33.33	22.22
M50	22.22	88.89	11.11	22.22

Appendix IV Tissue culture media ingredients and analysis results for Chapter Six

(A): Composition of modified LPch medium (mg/l)

Compound	Amount mg/l
Major Elements	
KNO_3	1,800.00
$Ca(NO_3)2.4H2O$	1,200.00
NH_4NO_3	400.00
$MgSO_4.7H_2O$	360.00
KH_2PO_4	270.00
Iron	
$FeSO_4.7H_2O$	30.00
Na_2ETDA	40.00
Minor	
$ZnSO_4.7H_2O$	8.60
H_3BO_3	6.20
$MnSO_4.4H_2$	*20.00
$CuSO_4.5H_2O$	*0.25
KI	0.08
$Na_2MoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.025
Vitamins	
Thiamine HCL	0.40
Inositol	1,000.00
Other supplements	
Sucrose	30,000.00
Difco Bacto agar	8,000.00
Activated charcoal	5,000.00

(* Modification by K.Horgan and Aitken-Christie on July 17, 1985, based on analysis of tissue culture shoots and normal levels expected in *radiata* foliage from nursery grown seedlings and trees in the forest).

(B) Modified Murashige and Skoog medium composition

Compound	Amount mg/l
Major elements	
NH_4NO_3	1,650.00
KNO_3	1,900.00
Ca ($NO_3.4H_2O$)	-
K_2SO_4	-
$MgSO_4.7H_2O$	370.00
Minor Elements	
$MnSO_4.H_2O$	16.90
$ZnSO_4.7H_2O$	8.60
$CuSO_4.5H_2O$	0.025
NH_4SO_4	-
$CaCl_2.2H_2O$	440.00
K I	0.83
$CoCl_2.6H_2O$	0.025
KH_2PO_4	170.00
H_3BO_3	6.20
$Na_2MoO_4 H_2O$	0.25
$NaH_2PO_4.H_2O$	-
Iron	
$FeSO_4.7H_2O$	27.84
$Na_2.EDTA$	37.24
Vitamins	
Thiamine.HCL	0.1 0*
Nicotinic acid	0.50
Pyridoxine.HCL	0.50
Glycine	2.00
Myo-inositol	100.00
Agar (Bacto)	6,000.00
Sucrose	30,000.00

(* Modified)

(C) Analysis of variance of canker score caused by three *S. unicorne* isolates

Source	DF	Sum of squares	Mean square	*F* value	*Pr*> *F*
Model	2	6.2	3.1	2.74	0.0829
Error	27	30.6	1.13		
Corrected total	29	36.8			

R-Square	Coeff var	Root MSE	Score mean
0.168478	48.39006	1.064581	2.20

Appendix V Result of % canker incidence recorded on each cypress family inoculated with *Seiridium* isolates under different environmental conditions

Cypress families	Percentage of canker under different environmental conditions				
	Location	Type of plant material	Average % Relative Humidity	Average Temperature °C	% Canker
M23	G/house	*[1]Sidebranches	72.5	16.5	8.3
M25	G/house	*[1]Sidebranches	72.5	16.5	11.1
M41	G/house	*[1]Sidebranches	72.5	16.5	25
M50	G/house	*[1]Sidebranches	72.5	16.5	2.8
M23	Lab bench	Cut-shoot	54.3	20	20.0
M25	Lab bench	Cut-shoot	54.3	20	70.0
M41	Lab-bench	Cut-shoot	54.3	20	55.0
M50	Lab-bench	Cut-shoot	54.3	20	20.0
L1	Lab bench	Cut-shoot (T1)	54.3	20	100
L2	Lab bench	Cut shoot (T1)	54.3	20	0
L1	Lab bench	Cut-shoot (T2)	54.3	20	5
L2	Lab bench	Cut shoot (T2)	54.3	20	0
L1 (1 shoot)	S/cloth	Cut shoot (T3)	45.1	11.2	Infected
L2 (1 shoot)	S/cloth	Cut-shoot (T3)	45.1	11.2	Healthy
M23	S/cloth	*[1]Sidebranches	58.3	14.4	28.7
M25	S/cloth	*[1]Sidebranches	58.3	14.4	30.6
M41	S/cloth	*[1]Sidebranches	58.3	14.4	14.8
M50	S/cloth	*[1]Sidebranches	58.3	14.4	35.2
L15	S/cloth	*[1]Sidebranches	58.3	14.4	33.3
M23	Incubator	Cut-shoot	35	13.3	38.9
M25	Incubator	Cut-shoot	35	13.3	50.0
M41	Incubator	Cut-shoot	35	13.3	38.9
M50	Incubator	Cut-shoot	35	13.3	36.1
L15	Incubator	Cut- shoot	35	13.3	19.4

NB: 1. Colour code shows plant materials used were from the same stock plant

2. Different *Seiridium* isolates were used in the different trials in most cases

Appendix VI Spore shapes of *Seiridium* isolates used in this study

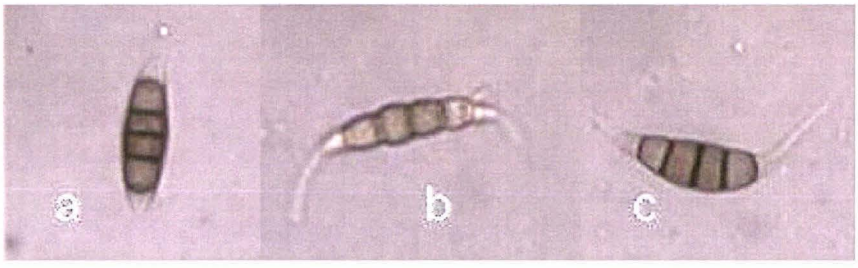

Plate VI Light microscopic image of *Seiridium* spores of (a): L152.2.4 (*S. cardinale*) (b) L101.4 (*S. unicorne*) (c) L156.2.1 (*S. unicorne*?) (200x magnification)

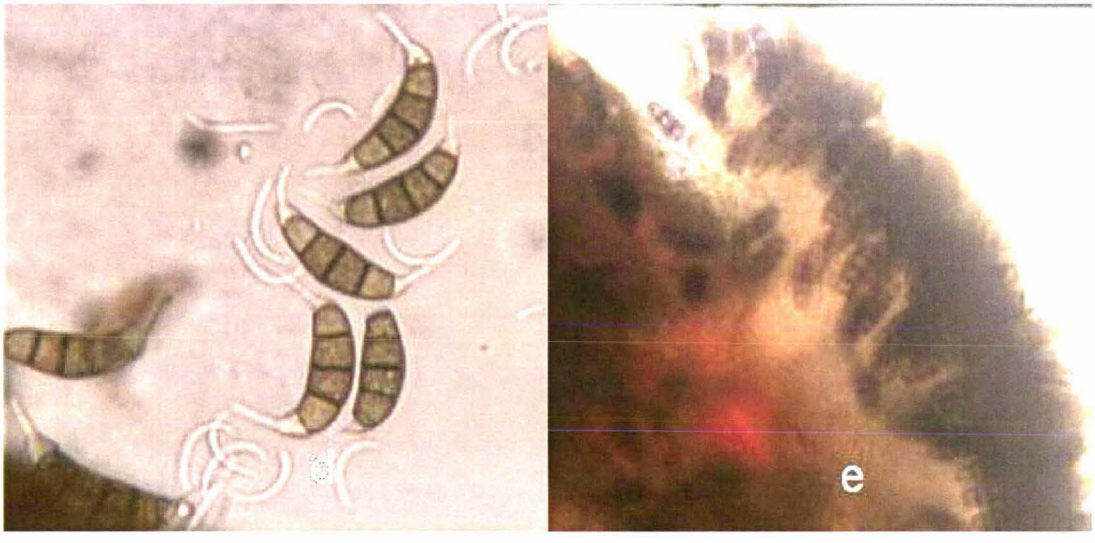

Plate VI (d) Normal spore and spermatia of *S. unicorne* L156.2.1(?) 200x magnification (e) Acervulus found on inoculated excised shoot. (100x magnification)

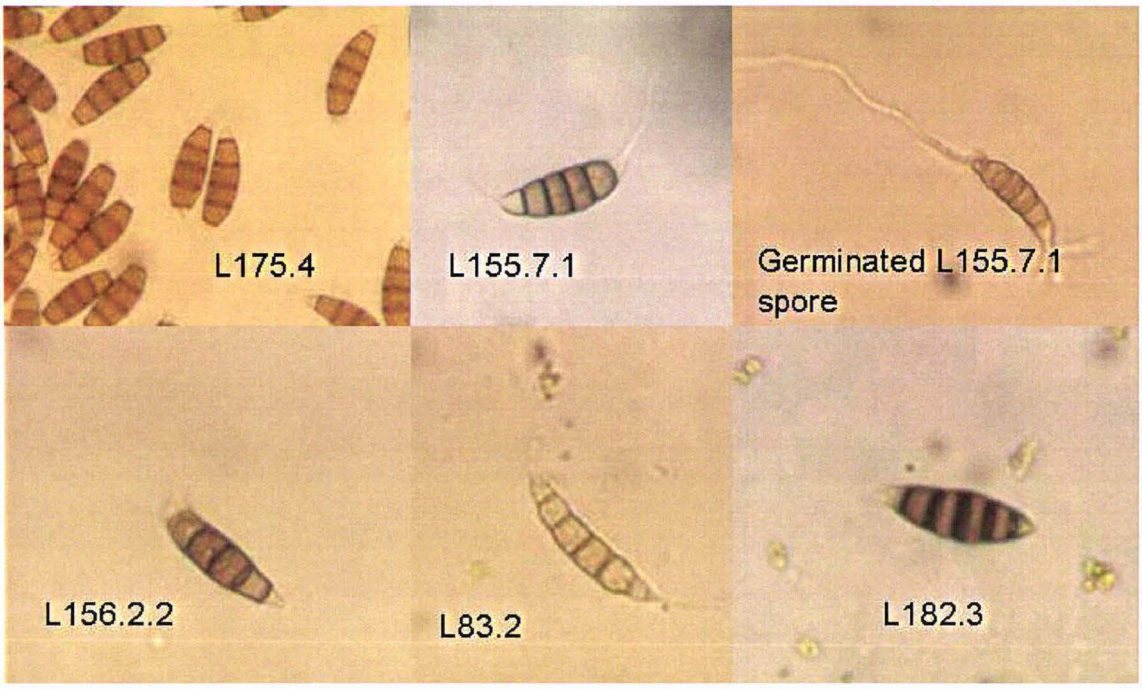

Plate VI (f) Light microscopic image of *Seiridium* spores from cultures at Massey University (400x magnification except for L175.4)

Appendix VII Microscopic observation of susceptible and resistant host tissues

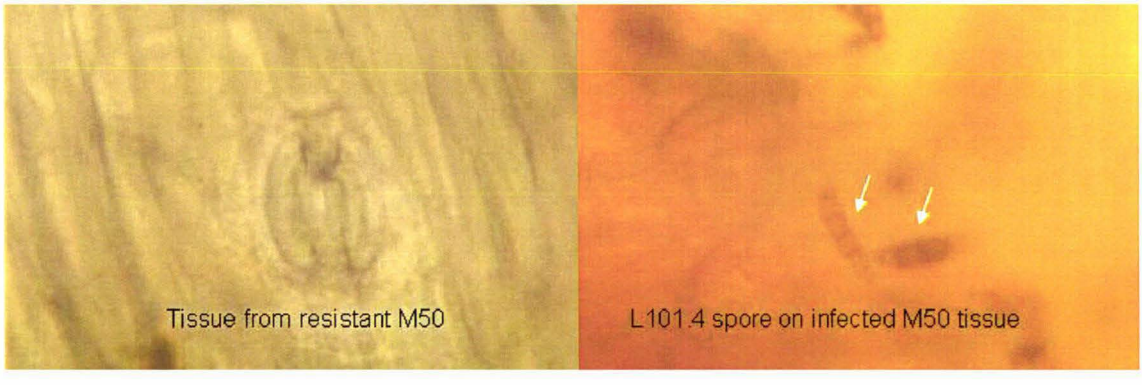

Plate VII (a): Light microscopic surface view of longitudinal section through the bark obtained from inoculated site of resistant *C. macrocarpa* and susceptible plant from family M50 two months after inoculation.

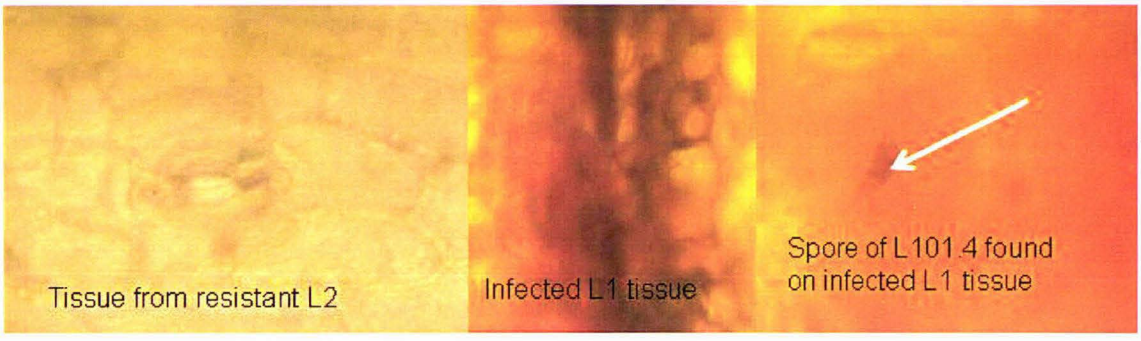

Plate VII (b) Light microscopic surface view of longitudinal section through the bark obtained from inoculated site of resistant *C. lusitanica* (L2) and susceptible *C. lusitanica* (L1).

Appendix VIII List of *Seiridium unicorne* and *Seiridium cardinale* maintained at Massey University

Isolate number	*Seiridium* species	Host	Origin
L83.2*[1]	*Seiridium unicorne*	-	-
L101.4	*Seiridium unicorne*	*C. macrocarpa*	Otturoa Rd
L155.7.1	*Seiridium unicorne*	*C. macrocarpa*	Hawkers property
L156.2.1	*Seiridium unicorne*	*C. macrocarpa*	West of Palmerston North
			towards Bulls
L156.2.2	*Seiridium unicorne*	*C. macrocarpa*	West of Palmerston North
			towards Bulls
L175.1	*Seiridium unicorne*	Chamaecyparis sp.	Timaru
L175.2	*Seiridium unicorne*	Chamaecyparis sp	Timaru
L175.3	*Seiridium unicorne*	Chamaecyparis sp	Timaru
L175.4	*Seiridium unicorne*	Chamaecyparis sp	Timaru
L152.2.2	*Seiridium cardinale*	*C. macrocarpa*	Rangiora
L152.2.3	*Seiridium cardinale*	*C. macrocarpa*	Rangiora
L152.2.4	*Seiridium cardinale*	*C. macrocarpa*	Rangiora
L182.1	*Seiridium cardinale*	*Cupressus leylandii*	Burn Military Camp
L182.2	*Seiridium cardinale*	*Cupressus leylandii*	Burn Military Camp
L182.3	*Seiridium cardinale*	*Cupressus leylandii*	Burn Military Camp
L182.4	*Seiridium cardinale*	*Cupressus leylandii*	Burn Military Camp

*[1] Original numbering system used by Forest Research. Numbers after the point represents samples /subsamples.