Development of a pathogenicity testing system for *Dothistroma pini* infection of *Pinus radiata*.

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Abstract

Dothistroma pini is a fungal pathogen of pine species around the world and can be found in most parts of New Zealand. Infection by *D. pini* causes a disease commonly known as Dothistroma needle blight. Dothistroma needle blight has a significant financial impact on New Zealand's forestry industry. Although control of infection by *D. pini* is currently very successful there is a possibility that a new strain introduced from another country could be a lot more damaging and overcome current control measures. In recent years both the incidence and severity of the disease have increased in the northern hemisphere and other parts of the world.

A distinctive characteristic of *Dothistroma* needle blight is the production in the infected needle of a toxic red pigment called dothistromin. Dothistromin is produced as a secondary metabolite by *D. pini* and has known phytotoxic properties as well as clastogenic and mutagenic properties towards human cells. Purified dothistromin toxin injected into pine needles has been shown to reproduce symptoms similar to those observed during *D. pini* infection. Because of this production, dothistromin is thought to play an important role in the infection process. Mutants of *D. pini* that are deficient in dothistromin production have been made recently that will allow this role to be investigated.

The aim of this study was to develop a pathogenicity testing system under PC2 containment (required for dothistromin deficient mutant) and to develop microscopy methods required to monitor both epiphytic and endophytic growth of the fungus on the needle. *D. pini* requires high light intensity, continuous leaf moisture and a specific temperature range in order to infect pine needles. Progress was made towards developing a robust pathogenicity testing system.

This study has also developed several microscopy techniques for the visualisation of epiphytic growth including a fluorescent microscopy technique. Other bright field and fluorescent staining techniques were investigated with some success.

Staining techniques were not successful for the visualisation of endophytic *D. pini* growth but a green fluorescent protein (*sgfp*) reporter construct was obtained and two

gfp plasmid constructs were developed for the transformation of *D. pini* for use as biomarkers. Successful introduction of the *gfp* constructs into *D. pini* will allow *in situ* visualisation of endophytic and epiphytic *D. pini* growth.

The work done in this study will be useful for the further investigation into the role of dothistromin toxin, which may lead to new or more efficient methods of controlling *D. pini* as well as possibly providing information about other polyketide molecules of economic or medical significance.

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1.1 Introduction to Dothistroma needle blight

1.1.1 General Introduction

Dothistroma needle blight (red band disease) of pines is caused by the filamentous fungus *Dothistroma pini* and has several distinctive characteristics making diagnosis relatively easy. Dothistroma needle blight affects a wide range of the *Pinus* species to varying degrees with *Pinus radiata* being highly susceptible to infection. *P. radiata* is a very important forestry tree making up a large proportion of New Zealand's exotic forest industry. As a result *D. pini* infection has a large economic impact in terms of lost wood production and control measures. Currently *D. pini* is successfully controlled in New Zealand but there is the possibility that if a new strain of *D. pini* were to be introduced into the country that current control measures may prove inadequate. The incidence of *D. pini* disease around the world is increasing and *D. pini* disease is starting to have more of an impact in the northern hemisphere making *D. pini* a fungus of widening global interest.

D. pini produces a toxin (dothistromin) that is thought to play a key role in pathogenicity. Dothistromin shows similarities in structure and biosynthesis to the medically important aflatoxin as well as other polyketide molecules, making study of dothistromin relevant to other areas of science as well as plant pathology. There is a need to confirm the role of dothistromin in the infection process of *D. pini*. In other toxin-producing fungal phytopathogens where it was thought the toxins played a major role in infection, knockout of toxin production has been shown to result in both pathogenic and non-pathogenic fungal strains. This study investigates the role of dothistromin toxin by studying the pathogenicity of a *D. pini* mutant deficient in the production of dothistromin. Further investigation of the role dothistromin has in *D. pini* infection may lead to new, more specific, targets for control and may shed light on the function of other similar polyketides.

D. pini infection levels are largely dependent on environmental factors and favourable conditions needed to be reproduced *in vitro* in order to study the dothistromin deficient mutant. Microscopy methods used to follow *D. pini* infection of *P. radiata* were published in the late 1960s. This study investigates new developments in fluorescent staining and imaging technology to try and identify more effective and simple methods for the visualisation of epiphytic and endophytic *D. pini* growth.

1.1.2 Disease symptoms

Dothistroma needle blight has several distinctive characteristics. The first visible symptom is the development of brick-red tinged bands (1-3 mm) on the infected needles caused by high levels of the toxin dothistromin being present. This colouring can normally still be seen after the needle has died (Shain and Franich 1981; Gadgil 1984). Adjacent to these red bands are yellow necrotic lesions that are sometimes bordered by dark green areas of needle tissue also known as 'green islands'. These green islands have been shown to contain highly lignified cells (Franich, Carson et al. 1986). Asexual black fruiting bodies (stromata) develop within the red bands during the later stages of infection. The stromata initiate below the surface of the needle before swelling and eventually breaking through the epidermis and cuticle of the needle (Edwards and Walker 1978; Evans 1984).

The necrosis of diseased needles results in needle death and premature defoliation of the tree. This can produce a 'lion tailing' effect whereby new young disease free needles are the only remaining needles on the branch.

Infection of a tree normally initiates on needles at the base of the crown before working its way up. However, it is also possible for infection to start on the inner needles at the base of the crown before moving up the innermost foliage and finally moving out along the branches (Marks, Smith et al. 1989).

1.1.3 Economic impact on New Zealand's Forest Industry

Infection caused by *D. pini* has a large financial impact on New Zealand's plantation forestry. *P. radiata* makes up 89.8% (as of April 2000) of New Zealand's plantation forest area, and is highly susceptible to infection by *D. pini* at a young age (less than 10 years old). The most significant impact *D. pini* infection has in terms of lost revenue is reduced wood production on trees as a result of premature defoliation. Lost wood yield is proportional to the severity of infection. If 20% of a tree's foliage is diseased then a reduction in wood yield of approximately 20% is observed. This relationship is the same for trees exhibiting 40% diseased foliage (Gibson 1974; Woods 2003) with more severe levels of infection possibly resulting in tree death. The most resent survey investigating the impact of *D. pini* was carried out in 1989 for the North Island. Residual growth loss per year was estimated at $4,500,000 and the direct cost of control per year was $1,600,000. This was calculated to be $7.02/ha/year making *D. pini* the most economically significant disease in New Zealand's plantation forestry (New 1989).

Dothistroma needle blight has historically been more of an economic concern for forestry in the southern hemisphere with pine species being planted outside of their native areas. In addition to becoming more prevalent in the northern hemisphere Dothistroma needle blight has been infecting trees in their native areas more frequently as well as those planted as exotics. A survey carried out in 2002 found 93% of 700 stands of young (<20 years old) lodge pole pines (*Pinus contorta* var. *latifolia*) showed disease symptoms in North-Western British Columbia, Canada covering an area of 21 000 ha. 6.2% of this surveyed area showed mortality (Woods 2003).

1.1.4 Control

Control of *D. pini* disease in New Zealand is the result of indirect as well as direct measures taken by forest management. Pruning of tree branches means that there is a high, acceptable level of foliage loss and infected branches can sometimes be pruned off. Thinning of stands of trees ensures ample room for tree growth and means fungal spores aren't as easily transferred from one tree to another. Aerial spraying of copper fungicides is currently used in areas where the infection level is higher than 25%. This

has proved successful in controlling *D. pini* but the cost effectiveness of aerial spraying has been debated (Chou 1991). *Pinus radiata* breeding programs selecting for *Dothistroma* resistance have also had some success and will be discussed in Section 1.2.4.

1.2 Introduction to *Pinus Radiata*

1.2.1 Classification

Pinus radiata originates from three locations on the mainland of California (Ano Nuevo, Monterey, and Canbria) and from two Mexican islands (Cedros and Guadalupe) off the coast of Baja California. Seeds were introduced to New Zealand in the 1850s to 1880s (Maclaren 1993). *P. radiata* developed as a forestry stock due to its ability to outperform other tree species on a wide range of soil types and environments (Kininmonth and Whitehouse 1991). Due to intense breeding programs current seedlings differ genetically from the trees originally introduced and the wild population from which their seeds were sourced.

Active seed selection started in the 1960s from outstanding mature trees. This led to the development of seed orchards where quality trees were grown especially for seed production. Genetic selection has seen several advancements over the years and four main breeds have been developed, each with its own improvement rating. These four breeds are 'Growth and Form' (GF), 'Long Internode' (LI), 'Dothistroma Resistant' (DR) and 'High Wood Density' (HD). Seed lots are given a rating based on one or more of these characteristics (Maclaren 1993). Due to the inability of seed orchards to keep up with the demand for the most advanced seed lots, plant subculturing techniques, such as taking cuttings and shoot development from callus tissue, have become useful tools for the large scale production of genetically superior tree lines. This has produced a large monoculture component to New Zealand's exotic forests introducing a very real disease risk to the industry. If a disease emerges that significantly damages one tree the low genetic diversity present in a monoculture will mean the disease may rapidly spread due to other trees in the monoculture also being

susceptible. *P. radiata* remains in use despite this risk because of its aforementioned out performance of other tree species over a wide range of environmental conditions.

1.2.2 Dothistroma resistance

There is natural variation in *Dothistroma* disease resistance within the *P. radiata* species (Wilcox 1982). As mentioned *D. pini* resistance has been actively selected for in *P. radiata* breeding programs for application in areas with high *D. pini* disease risk. Field trials have shown *Dothistroma* resistance to show moderately high heritability while also being a polygenic trait (Wilcox 1982). This breeding program has resulted in trees with an estimated 15% less disease and 56% less chemical spraying costs compared to control trees (Carson 1989; Dick 1989; Carson and Carson 1991). In recent times, New Zealand has been focused on improved growth and wood quality but *Dothistroma* resistance has still been assessed during field trials (Jayawickrama and Carson 2000).

Pinus radiata also exhibits increased *Dothistroma* resistance as the tree ages (Gibson 1972; Kershaw, Gadgil et al. 1988). This can be exploited to provide increased Dothistroma resistance by using cuttings from more mature trees and planting them out rather than using seedlings (Ades and Simpson 1990).

While a lot of work has been done to develop *Dothistroma* disease resistance in *P. radiata* the mechanism for this heritable resistance still remains elusive, however some possible contributing factors to age related resistance in *P. radiata* have been identified and some ruled out. Buffer capacity of needle homogenates has been shown to increase with tree age but did not relate to the resistance seen in older trees (Franich and Wells 1977). Needle monoterpenes vary in composition and amount with age but no clear association was observed with resistance (Franich, Gaskin et al. 1982). A study was also done investigating resinous material that occludes the stomatal opening in needles of mature trees . It was found that the resinous material provided a physical barrier to penetration and that oxidised resin acid derivatives found on the surface of the needles were capable of inhibiting fungal growth *in vitro* and prevented spore germination (Franich, Gadgil et al. 1983).

1.3 Introduction to *Dothistroma pini*

1.3.1 Classification

D. pini is an ascomycete fungus that has been known by several taxonomic names in the past for both its anamorphic and teleomorphic forms (Sutton 1980; Evans 1984; Roux 1984). The anamorphic form was originally known as both *Cytosporina septospora* Dorog, and as *Dothistroma pini* (Hulbary 1941). After it was realised that these two fungi were the same a newcombined name was assigned, *D. septospora* (Dorog.) Morelet (Morelet 1968). Both *D. septospora* and *D. pini* are in use today. The teleomorphic form was first named *Scirrhia pini* Funk and Parker but was renamed *Mycosphaerella pini* E. Rostrup apud Monk and both names are still used. Based on the diversity of the genus *Mycosphaerella* an attempt was made to reclassify the teleomorphic form into a new genus *Eruptio* (Barr 1996). However phylogenetic analysis suggested that *Mycosphaerella* was a more appropriate classification (Goodwin, Dunkle et al. 2001).

The anamorphic form has been divided into three varieties based on conidiospore length. *D. pini* Hulbary var. *pini* (syn. *D. septospora* var. *septospora*) is characterised as having conidial lengths of 15.4-28.0 µm with a mean of 22.4 µm. *D. pini* Hulbary var. *linearis* (syn. *D. septospora* var. *lineare*) is characterised as having conidial lengths of 23.0-42.0 µm with a mean of 31.9 µm (Thyr and Shaw 1964). An intermediate variety was later proposed, *D. pini* Hulbary var. *keniensis* (syn. *D. septospora* var. *keniense*) with conidial lengths ranging from 13.0-47.5 µm with a mean of 28.9 µm (Ivory 1967). Several leading authorities have questioned the division of *D. pini* into different varieties based on a single characteristic (Funk and Parker 1966; Gadgil 1967; Sutton 1980; Evans 1984) and subsequent characterisation of isolates into the different varieties has proven difficult (Edwards and Walker 1978; Roux 1984; Bradshaw, Ganley et al. 2000). More recent work, involving ribosomal ITS sequencing of isolates from different countries, did not support distinction of different varieties based on geographical isolation. However this work also recognised the need for further sampling and more comprehensive genetic analysis before a more

definitive decision could be made about dividing *D. pini* into varieties (Bradshaw, Ganley et al. 2000).

1.3.2 Life cycle of *D. pini*

Needle blight caused by *D. pini* is spread by the dispersal of its asexual spores via a 'rain splash' mechanism. Drops of water dissolve the 'slime' around the spores in the stromata (fruiting body) and pick the spores up into suspension. The aerosols produced by the water droplets splashing on different surfaces carry the spores a short distance, which normally results in them being deposited on a near by branch or tree. Another source of inoculum is the production of secondary conidia by mycelium. Once on the needle surface the spore germinates and the germ tube grows across the surface of the needle before forming an appressorium-like structure above a stomatal opening. An infection peg then penetrates through to the substomatal chamber. Following penetration, mycelium exhibits intra- and inter-cellular growth throughout the mesophyll. Resin canals are also penetrated and occasional but rare infection of endodermal cells is observed. Plant tissue is degraded in advance of hyphal invasion forming the visible lesion on the needle. Following plant cell necrosis and hyphal growth, stromata develop to complete the life cycle (Gadgil 1967).

1.3.3 Origin and distribution

The contentious nature of *D. pini*'s classification has already been discussed in Section 1.3.1, which makes it difficult to make clear delineations concerning the origin and spread of the disease. The review by Bradshaw (2004) gives a good overview of the different opinions on the matter.

D. pini is thought to have originated in the high altitude cloud-covered forest regions of South America (Evans 1984). There are some reports of *D. pini* or its teleomorphic form in the USA and Europe prior to the 1920's. Widespread reports of disease did not occur until the large scale exports and plantations of different pine species outside their native areas into Africa, Australasia, America and Europe (Cobb and Miller 1968; Gibson 1972; Evans 1984). More recently Dothistroma needle blight has been

reported in Germany (Maschning and Pehl 1994), Portugal (Fonseca 1998), and Canada (Woods 2003).

D. pini was first discovered in New Zealand forests in 1964 where it soon spread rapidly to affect most of the *P. radiata* forests of the country. The most recent thorough survey documenting *D. pini*'s distribution in New Zealand was done in 1984. *D. pini* was found in all parts of the North Island except the northern most areas (Mangonui country) and Great Barrier Island. In the South Island it can be found in Nelson, Marlborough north of the Wairau River, Westland, Southland, and Otago (Gadgil 1984).

The New Zealand population of *D. pini* has been shown to have very little genetic diversity, implying that a single strain has been introduced into the country, which has reproduced asexually since (Hirst, Richardson et al. 1999).

1.4 Effect of environmental conditions on *D. pini* growth and infection of *P. radiata*.

1.4.1 Seasonal effects on *D. pini* infection levels

Environmental factors such as temperature, leaf wetness and light intensity have been shown to be important in determining the severity of *D. pini* disease of *P. radiata* seen in the field. This means seasons have a large impact on Dothistroma infection levels. *D. pini* infection mainly takes place in the warmer summer months of November to February with another less substantial period during March-April. Infection is not common in the cooler months of autumn, winter and early spring (May-September) (Gilmour 1981).

1.4.2 Effect of light intensity

Light intensity has been shown to have a significant influence on disease symptom severity but no impact on spore germination or early growth (Gadgil 1976). Foliage in experiments that have been shaded for 5-20 days following inoculation show no

symptoms but disease symptoms are quick to appear following removal of the shading (Ivory 1972). It has been suggested that this effect is the result of the plant host response and not a direct function of *D. pini* or its toxin dothistromin(Gadgil 1976). There is no direct evidence to support this but there is some evidence to suggest that photosynthetically active plant tissue is involved in dothistromin's toxicity (Section 1.6).

1.4.3 Effect of humidity and free water

Spore trap experiments have shown that wet weather is necessary for the dispersal of large numbers of spores (Peterson 1973). Wet spores are capable of germinating and penetrating the surface of needles even in dry conditions but high humidity is re quired for further disease development. Continuous leaf moisture results in high levels of infected foliage (Gadgil 1977).

1.4.4 Effect of temperature

D. pini is capable of growth over a temperature range of 5°C to 26°C but extended periods of high humidity or leaf wetness is necessary for disease at temperatures below 7°C (Gilmour and Crockett 1972). High temperatures are capable of shortening the pre-reproduction period in the field and in controlled experiments (Gadgil 1974; Gilmour 1981). This is a possible explanation of why higher levels of infection are seen in the warmer months of the year (Gilmour 1981). A linear increase in spore germination is observed with increasing temperature (Gadgil 1974).

1.5 Microscopy methods to visualise *D. pini* infection

In order to investigate the infection process of *D. pini* it is necessary to have techniques to observe the different stages of infection. Two main problems are faced when using microscopy to study pine needles. The first is the high curvature of the needle surface. This makes the depth of field very small under high magnification making it difficult to obtain a detailed view of the surface of the needle. The second problem is the density of the needle tissue. This becomes a problem when using bright

field microscopy as light does not penetrate the tissue of older larger needles. This problem is not so evident on younger needle tissue. When a high density of cells is studied using bright field microscopy a cluttered image is observed that is often difficult to interpret.

Before 1967 there were reports that *D. pini* penetrated the needle surface of several pine species through stomata but (Gadgil 1967) was the first to look at the actual mode of infection in detail. The paper describes three bright field methods for the visualisation of hyphae growing on and in the pine needle structure.

The first method utilised acidified sodium chlorite to clear whole needles before processing them through an alcohol series to dehydrate them. The needle was then immersed in methyl salicylate to render it transparent before staining in crystal violet (Hering and Nicholson 1964). Normal microscopy could then be used to visualise these needles. This technique cleared pigment from the needle tissue making bright field microscopy possible. It may not be suitable however for investigating the early stages of infection as the clearing treatment may dislodge *D. pini* from the surface of the needle.

The second method used whole needles that had been killed and fixed in FAA before being stained with the periodic acid-Schiff technique (Preece 1959). These needles could then be visualised using inverted microscopy. The use of an inverted microscope means the pine needle tissue does not need to be cleared and only the top layer of cells is being observed. The use of FAA to fix the plant tissue is also beneficial to ensure the integrity of the sample although the FAA treatment may disrupt *D. pini* on the surface of the needle.

The third technique used whole needles that had been killed and fixed in FAA and then dehydrated in a tertiary butyl alcohol series. The needles were then embedded in paraffin and transverse and longitudinal sections were taken using a microtome. The sections were then stained with Conant's quadruple-staining schedule (Johansen 1940). This technique was successful for the visualisation of *D. pini* penetration and growth inside the needle tissue. The draw back of this technique is the large amount

of time needed to prepare the sample and the large number of sections that need to be viewed to obtain an image of a penetration event.

While these techniques were successful for the detailed investigation of *D. pini* infection of pine needles, this work had been done some time ago and it was deemed desirable to investigate different microscopy techniques and apply new technology to see if a more effective method could be identified while still supplying detailed information about the infection process.

1.6 Dothistromin toxin

1.6.1 Description

Dothistromin was first identified as a pigment obtained from an ethyl acetate extraction of a *D. pini* culture. Two related compounds were identified in the red pigment using mass spectroscopy, $C_{18}H_{12}O_9$ and $C_{18}H_{12}O_8$. The first was named dothistromin and the second, deoxydothistromin. The structure and chemical make up of dothistromin was solved using mass spectroscopy and various chemical analysis techniques and was proven to be a difuranoanthraquinone (Bassett and Buchanan 1970; Gallagher and Hodges 1972).

The dothistromin molecule is very similar in structure to the aflatoxin precursor versicolorin B and the dothistromin biosynthetic pathway shares similar intermediates with the aflatoxin and sterigmatocystin pathways of *Aspergillus* spp. ^{13}C labelling was used to investigate the biosynthesis of dothistromin and it was discovered that the final molecule is synthesised from 9 acetate units and shares a unique labelling pattern of the bistetrahydrofurano side chain with sterigmatocystin and aflatoxin B_1 (Shaw, Chick et al. 1978). The two main structures making up the dothistromin molecule are the anthraquinone and difuran moieties (Jones, Harvey et al. 1995).

Dothistromin's structural similarity to the known hepatotoxin and human carcinogen aflatoxin, sparked concern about potential health risks in contaminated forestry and the potential to contaminate water supplies. A series of studies revealed dothistromin

was strongly cytotoxic and was a weak mutagen (Elliott, Mason et al. 1989). Human exposure studies were also done, which found small traces of dothistromin in hair, clothing, and in the air breathed by forestry workers. The amount of dothistromin present in these samples increased in wet weather and the season was also a contributing factor. This led to a study looking at the incidence of cancer in forestry workers and a small increase in the cases of lung cancer was observed. The increase in lung cancer was said to be more likely a result of dusty work conditions rather than dothistromin but dothistromin couldn't be ruled out as a contributing factor (Elliott, Mason et al. 1989)

It has also been shown that the strain of *D. pini* present in New Zealand produces relatively low levels of dothistromin compared to some strains in other countries (Bradshaw, Ganley et al. 2000).

1.6.2 Evidence suggesting a biological role

Dothistromin is classified as a secondary metabolite. Secondary metabolites are not essential for growth of an organism but are normally important for the organism to occupy a specific environmental niche. They require a substantial energy input by the organism so theoretically should provide the organism with some type of selective advantage.

It is thought that dothistromin plays a primary role in *D. pini* infection of pine needles. It has been shown that *Dothistroma* needle blight symptoms, i.e. death of host tissue, occur before hyphae penetrate the tissue, which implies that some sort of toxin is excreted by *D. pini* (Gadgil 1967). It has also been shown that needle blight symptoms can be reproduced by injecting pine needles with purified dothistromin toxin (Shain and Franich 1981).

However, previous genetic studies of other fungal toxins thought to be involved in virulence or pathogenicity, have yielded very different outcomes. *Cercospora kikuchii* is a fungal soybean pathogen that produces a photosensitising toxin, cercosporin. Treatment of soybean tissue with cercosporin was shown to reproduce symptoms commonly seen as a result of *C. kikuchii* infection. Knockout mutants deficient in

cersosporin production were unable to form lesions defining cercosporin as a pathogenicity factor (Upchurch, Walker et al. 1991). Treatment of Elm seedlings with purified cerato-ulmin toxin from the Dutch elm disease fungus *Ophiostoma ulmi* was also shown to reproduce disease symptoms (Takai 1974). However a cerato-ulmin deficient knockout mutant showed no reduction in virulence compared to the toxin producing wild type strain of *O. ulmi* (Bowden, Smalley et al. 19 96). These two cases highlight the importance of confirming a toxin's role using toxin deficient mutants. While evidence such as the reproduction of disease symptoms by injection of the dothistromin into pine needle tissue (Shain and Franich 1981) suggests that dothistromin is a pathogenicity or virulence factor this is by no means conclusive.

Although the mode of action of dothistromin is not fully understood a closer investigation of needle blight lesions has provided a small amount of insight. Lesions caused by injection of dothistromin and as a result of natural infection commonly have dark green areas of tissue at each end known as 'green islands'. It has been shown that the larger the green island on either end of the lesion correlates to shorter lesion length. This suggests these green islands act to contain the infecting fungi. Closer examination of the tissue making up these green islands revealed they have relatively high levels of lignin of the type formed in response to stress (Franich, Carson et al. 1986). The lignin in this tissue was also found to have benzoic acid immobilised in its structure. The work done by Franich, Carson et al (1986) showed dothistromin was metabolised or degraded by the plant tissue. The dothistromin was oxidised to CO_2 and oxalic acid as well as other compounds in much smaller amounts that couldn't be identified. None of the identified 'breakdown' components of dothistromin were shown to exhibit toxicity to the plant tissue but lesion extension continued despite the dothistromin degradation. When extracts from the lesions were run on TLC plates, a spot was seen to increase as time of lesion sample increased. This spot was identified as benzoic acid and was produced by the plant tissue. Benzoic acid was shown to have fungistatic effects but was also toxic to plant tissue causing necrotic lesions very similar to those observed in *Dothistroma* needle blight. The outcome of the work done by Franich, Carson et al (1986) identified benzoic acid as a phytoalexin produced by pine cells neighbouring those cells that had been destroyed by dothistromin.

Dothistromin was originally thought to act as a photosensitising agent and have a mode of action similar to cercosporin. This was shown not to be the case. Instead of light providing the energy for dothistromin to produce reactive oxygen molecules, the plant actually reduces dothistromin in a NADPH dependent system. The reduced dothistromin is then capable of reacting with O_2 and producing O_2^- and H_2O_2 by autooxidation (Youngman and Elstner 1984; Heiser, Koehl et al. 2002). This action of producing reactive oxygen molecules as well as other toxic activities of dothistromin kills plant tissue. The plant cells neighbouring the killed cells then produce benzoic acid, which then contributes to lesion extension. Benzoic acid production slows fungal growth and prevents the spread of the fungal hyphae. It is also possible that t he benzoic acid provides a signal to form lignin walls (green islands) to contain the fungi. It can be interpreted that the pine tree produces benzoic acid to sacrifice its needles in an attempt to isolate and contain the damage caused by the infection.

Dothistromin has also been observed to have other biological activities. Administration of dothistromin to peanut leaves induces a high rate of electrolyte leakage and when 100 μg/ml was applied dothistromin was visibly toxic (Ramanujam and Swamy 1985). Dothistromin inhibits RNA synthesis in *Chlorella pyrenoidosa* and *Bacillus megaterium*. The inhibition of RNA synthesis in these organisms is thought to be a key factor in the growth inhibition observed for these two organisms when dothistromin is present (Harvey, Batt et al. 1976). Dothistromin has also been shown to be clastogenic and mutagenic towards human peripheral blood lymphocyte cultures. Higher concentrations of dothistromin caused haemolysis of the culture cells (Ferguson, Parslow et al. 1986).

As mentioned previously (Section 1.6.1) the two main structures making up the dothistromin molecule are the anthraquinone and difuran moieties (Jones, Harvey et al. 1995). The difuran part of the dothistromin molecule is thought to be the part of the molecule responsible for dothistromin's toxicity (Jones, Harvey et al. 1995).

More recently specific binding sites of dothistromin have been identified in plant tissue. Dothistromin has been observed to specifically bind to small vesicles in pine embryos. This binding was later narrowed down, by Western blotting, to a 40 kDa protein (Jones, Harvey et al. 1995). The same paper mentioned in the discussion that

they had started looking at binding sites in mature pine needles and had found them to be associated with chloroplasts.

If dothistromin is shown to be a primary virulence factor, and a new strain of *D. pini* that produces high levels of dothistromin is introduced to New Zealand, it is possible that the new strain will have a much larger impact on New Zealand's forestry and current control methods may prove inadequate. Furthermore it may be possible to develop new control methods specifically targeted at dothistromin or its biosynthesis.

1.7 Generation of a dothistromin deficient mutant (*ΔdotA*)

To further investigate the role of dothistromin in the life cycle of *D. pini* a gene replacement mutant was made that is deficient in dothistromin production. Dothistromin genes were identified on the basis of their similarity to those involved in the biosynthesis of aflatoxin. One of the genes identified (*dotA*) showed a high level of amino acid identity to the *ver-1* ketoreductase required for aflatoxin biosynthesis in *Aspergillus parasiticus*. A gene replacement vector was then developed and used to knock out the *dotA* gene in *D. pini* using homologous recombination. ELISAs showed that the *dotA* knockout mutant did not produce a detectable amount of dothistromin. Growth studies of the *dotA* mutant showed a small but significant reduction in colony diameter growth compared to the wild type (Bradshaw, Bhatnagar et al. 2002).

1.8 Research objectives

- Develop a pathogenicity testing system for *D. pini* infection of *P. radiata* under controlled PC2 containment conditions.

- Develop new and more efficient methods for visualisation of the *D. pini* infection process by microscopy.

- Determine the relative virulence of the dothistromin deficient *dotA* knockout mutant compared to wild type *D. pini* to provide information about the role of dothistromin in the infection process of *P. radiata*.

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 Biological Strains

2.1.1 Bacterial strains

Bacterial strain	Genotype	Source, Reference material
XL-1 *(E. coli)*	*sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A46 *thi rel*A1 *lac*⁻F' [*pro*AB *lacq*Δ(*lacZ*) M15 Tn10(*tet*^{r})]	(Bullock, Fernandez et al. 1987)

Table 2.1: Table of bacterial strains used in this study.

2.1.2 Fungal strains

Fungal strain	Relevant Features	Source, Reference material
NZE5 *(D. pini)*	Single spore isolate of wild type	(Bradshaw, Ganley et al. 2000)
NZE7 *(D. pini)*	Single spore isolate of wild type	Obtained from Te Matai Forest in the Bay of Plenty. The stand of trees was on the eastern side of the Mamaku Plateau from trees that were about 4-5 years old. (correspondence from Margaret Dick, FR).
32 *(D. pini)*	*dot*A knockout of NZE5	(Bradshaw, Bhatnagar et al. 2002)
34C1 *(D. pini)*	*dotA* knockout of NZE5	(Bradshaw, Bhatnagar et al. 2002)
34Y1 *(D. pini)*	*dot*A knockout of NZE5	(Bradshaw, Bhatnagar et al. 2002)
8A1 *(D. pini)*	*Ectopic dotA*$^+$ *of NZE5*	(Bradshaw, Bhatnagar et al. 2002)

Table 2.2: Table of fungal strains used in this study.

2.1.3 Plant material

Rament Number	Parents with (Dothistroma resistance scores)	Source
60/5	268.429(8) x 883.4	Forest Research, Rotorua
40/8	875.80(12) x 288.8	Forest Research, Rotorua
18/19	880.732(23.7) x 268.350(19)	Forest Research, Rotorua
13/11	885.382 x 268.228(17.1)	Forest Research, Rotorua
Short internode	Unknown	Dr Peter Long, Palmerston North

Table 2.3: Ramet numbers of groups used in pathogenicity test and parent clone numbers. Available *Dothistroma* resistance scores are in brackets. The higher the score the higher the level of resistance. The scores are arbitrary with complete resistance not achieved.

2.2 Growth Medium

All media were prepared using milli-Q water and sterilised by autoclaving at 121°C for 15 mins. Media were cooled to approximately 55°C before addition of antibiotics.

2.2.1 Aspergillus minimal medium (AMM)

$NaNO_3$ (Univar) 6 g/l, $MgSO_4$. $7H_2O$ (Merck) 0.52 g/l, KCl (BDH) 0.52 g/l, KH_2PO_4 (BDH) 1.52 g/l, $FeSO_4$. $7H_2O$ (Univar) 2 or 3 crystals, $ZnSO_4$. $7H_20$ (BDH) 2 or 3 crystals, H_2O to 950 ml and pH 6.5.

25 ml/l of sterile 40% glucose was added after autoclaving.

2.2.2 Dothistroma medium (DM)

Malt extract (Oxoid) 5% (w/v), Nutrient agar (Oxoid) 2.3% (w/v)

2.2.3 Dothistroma sporulation medium (DSM)

Malt extract (Oxoid) 20 g/l, Yeast extract (Oxoid) 5 g/l, Bacteriologial agar (Oxoid) 15 g/l

2.2.4 LB agar

Tryptone (Difco) 10 g/l, Yeast extract (Difco) 5 g/l, NaCl (Merck) 5 g/l, Bacteriologial agar (Oxoid) 15 g/l

2.2.5 LB broth

Tryptone (Difco) 10 g/l, Yeast extract (Difco) 5 g/l, NaCl (Merck) 5 g/l

2.2.6 Potato Dextrose Agar (PDA)

Potato dextrose agar (Merck) 39 g/l

2.3 Buffers and Solutions

2.3.1 Hexadecyl Trimethyl-Ammonium Bromide buffer (CTAB buffer)

CTAB (Sigma) 0.8 g, PVP40 (Sigma) 0.4 g, 5 M NaCl (Merck) 11.2 ml, 0.5 M EDTA $Na_2.H_2O$ (BDH) 1.6 ml, 1 M tris HCl (Invitrogen) pH 8 4 ml, milli-Q water 22.4 ml

2.3.2 Ethidium bromide staining solution

1 mg/ml ethidium bromide in milli-Q water

2.3.3 Gel loading dye

Sucrose (BDH) 20% (w/v), EDTA $Na_2.H_2O$ (BDH) 5 mM, SDS (BDH) 1% (w/v), bromophenol blue (J. T. Baker Chemical Co) 0.2 % (w/v), xylene cyanol (Sigma) 0.2% (w/v)

2.3.4 Fluorometer working solution

Hoechst H 33258 stock solution (Section 2.3.5) 10 µl, 10X TNE (Section 2.3.9) 10 ml, milli-Q water 90 ml

2.3.5 Hoechst dye stock solution

Ten ml of milli-Q water was added to 10 mg of Hoechst H 33258 (Amersham Biosciences). Stored at 4°C for up to 6 months. Protected from light

2.3.6 Fluorometer DNA standard

100 ng/µl calf thymus DNA (Amersham Biosciences) in 1X TNE (Section 2.3.9)

2.3.7 TBE buffer (10X)

Tris (Invitrogen) 108.0 g/l, EDTA $Na_2.H_2O$ (BDH) 9.3 g/l, boric acid (Univar) 55 g/l. Added milli-Q water to make up to just below 1 litre and adjusted pH to 8.2 using concentrated HCl (BDH). Made up to 1 litre with milli-Q water.

2.3.8 TE buffer (1X)

Tris (Invtrogen) 10 mM, EDTA $Na_2.H_2O$ (BDH) 1 mM pH 8.5

2.3.9 TNE buffer (10X)

Tris (Invitrogen) 12.11 g/l , EDTA $Na_2.H_2O$ (BDH) 3.72 g/l, NaCl (Merck) 116.89 g/l. Dissolve in roughly 800 ml of milli-Q water. Adjust pH to 7.4 using concentrated HCl (BDH) before making volume up to 1 litre.

2.4 Culturing techniques

2.4.1 Maintenance of *D. pini* stocks

Initial cultures were subcultured onto PDA (Section 2.2.6) from AMM (Section2.2.1) plates and kept in the cold room at 4°C. Stock cultures were maintained on AMM plates sealed with parafilm incubated at 22°C with sub-culturing carried out as required or every 6 months.

2.4.2 Obtaining and quantifying *D. pini* spore suspensions

A 1 cm^2 section was cut from the edge of the stock *D. pini* colony on AMM (Section 2.2.1). This was combined with 1 ml of sterile H_2O in a sterile micro-centrifuge tube and a plastic pestle was used to grind the agar section until it was dispersed in the water. 100 µl of this solution was plated out onto a DSM (Section 2.2.3) containing petri dish. The cultures on DSM were stored at 22°C for 10-16 days before spores were collected.

To collect the spores from the DSM plates, 4 ml of sterile H_2O was pipetted onto the plates and spores suspended by gently rubbing with a glass spreader. Once the spores were suspended in the water, the water was drawn off using a 5 ml pipette. The plate

was then rinsed with 1 ml of sterile H_2O, which was also collected along with the rest of the spore suspension (Rack. K 1973).

The concentration of spores was quantified using a cytometer and was sometimes diluted to obtain a standardised suspension before being used.

2.4.3 Inoculation of *P. radiata* seedlings with *D. pini* spore suspensions

Approximately half of the spore suspension was applied to the needles of the trees and allowed to dry before applying the remainder of the spore suspension. A house-hold trigger action atomiser previously sterilised with a 70% ethanol solution and rinsed with sterile water was used to apply the spore suspension. All inoculations were carried out in a PC2 containment flow hood that was sterilised with ethanol in between inoculations of the different treatments.

2.5 Bacterial plasmid DNA preparation

2.5.1 Preparation of electroporation competent *E. coli* cells (Dower, Miller et al. 1988)

Two 5 ml LB broths (Section 2.2.5) containing tetracycline (Tet) (Sigma) (10 µg/ml) were inoculated with the XL-1 strain of *E. coli* and grown overnight on a shaker at 37°C.

One litre of LB broth (Section 2.2.5) containing Tet (Sigma) (10 µg/ml) was inoculated with 10 ml of the overnight culture of *E. coli* and grown at 37°C, with vigorous shaking, to mid-log phase (OD_{600} 0.5-1.0). The cells were then chilled on ice for 20 minutes before being harvested by centrifugation at 4000 g (GSA rotor-5000 rpm) for 10 min (All centrifugations were carried out at 4°C). The cells were then washed by, resuspension, centrifugation to pellet the cells, and removing the supernatant. These washes were done in succession with 1 litre and 0.5 litres of ice cold sterile water, 20 ml of ice cold 10% (v/v) glycerol. The final wash containing 20 ml was centrifuged in a SS34 rotor at 6000 rpm before the cells were finally resuspended in 4 ml ice cold 10 % (v/v) glycerol. The cells were then frozen in dry ice before being transferred to a -80°C freezer in smaller aliquots.

2.5.2 Electroporation of competent *E. coli* XL-1 cells

Competent *E. coli* cells (Section 2.5.1) and DNA were thawed. The cells andplasmid DNA along with the cuvettes to be used were all placed on ice. A Bio-Rad Gene Pulser and Pulse controller were used for the electroporation. The settings were as follows; 25 µF, 2.5 Volts, 200-600 Ohms. Forty µl of competent cells were added to the cuvette on ice before adding 2 µl of DNA. The DNA and competent cells were then mixed by gently pipetting up and down several times. The pulse was applied to the cuvette immediately followed by the addition of 220 µl of LB broth. The cells and broth were then removed to a 1.5 ml micro centrifuge tube and incubated at 37°C for 10-30 mins before being plated out onto LB agar (Section 2.2.4) containing the appropriate antibiotic (Dower, Miller et al. 1988).

If blue/white selection was being used, 60 µl of IPTG (Promega) (10 mg/ml) and 30 µl of X-gal (Promega) (40 mg/ml) was spread onto the LB plates (~20 ml) before they were inoculated.

Final antibiotic concentrations used were ampicillin (Sigma) 100 mg/ml, spectinomycin (Sigma) 100 mg/ml, chloramphenicol (Sigma) 30 µg/ml

2.5.3 Bacterial plasmid DNA extraction

For small-scale preparations (1-5 ml) the 'Qiagen QIAprep Spin Miniprep Kit' was used according to manufacture's instructions. Medium sized preparations (25 ml) were done using the 'Qiagen Plasmid Midi Kit' according to manufacture's instructions. Extractions were mainly quantified using fluorometric assay and occasionally by gel electrophoresis.

2.6 Genomic DNA preparation (Van Burik, Schreckhise et al. 1998)

Mycelium was grown on cellophanes laid over DM petri dishes (Section 2.2.2). The mycelium was then harvested and ground in liquid nitrogen using a mortar and pestle. Six hundred µl of CTAB buffer (Section 2.3.1) was added to each sample as well as 2 µl of RNAse (Sigma) (20 mg/ml) before incubating the samples at 37°C for 5 to 10 minutes. The tubes were then heated in a 65°C water bath for 30-45 minutes with

occasional inversion. The tubes were taken out of the water bath and allowed to cool to 60°C before adding 600 µl of chloroform (Merck). The tubes were then mixed gently and left to stand for 2 minutes to let the aqueous and organic phases separate. Next the tubes were briefly centrifuged at 6000 rpm in a microcentrifuge ('Biofuge *pico*' Heraeus Instruments) and then the upper aqueous phase was transferred to clean tubes. 600 µl of isopropanol (BDH) was then added to the aqueous phase and mixed gently by a single inversion before standing the tubes on ice for 5-10 minutes. The DNA formed a stringy precipitate. Excess liquid was drawn off taking care not to disrupt the precipitated DNA until there was about 300 µl left as well as the DNA. 600 µl of 70% ethanol (BDH) was then added to the tubes before gentle mixing being careful not to collapse the DNA on itself. Once the DNA settled to the bottom of the tube (sometimes a brief centrifuge was needed if the DNA floated) the fluid was removed and two more washes were done using 70% ethanol (BDH). Once the last wash had been done the ethanol was decanted off and the pellet left to air dry before being resuspended in 50-100 µl TE buffer (Section 2.3.8).

2.7 DNA quantification

2.7.1 Fluorometric assay

Fluorometric analysis was the most commonly used method of DNA quantification. Quantification was done using a 'Hoefer DyNA Quant 200' fluorometer produced by Amersham Biosciences. The fluorometer was calibrated by adding 2 µl of 100 ng/ml calf thymus DNA standard (Section 2.3.6) to 2 ml of fluorometer working solution (Section 2.3.4). The value however was entered as 120 ng/ml to allow for the difference in AT% between calf thymus DNA (42%) and fungal (50%) and bacterial plasmid DNA (50%).

2.7.2 Gel electrophoresis assay

Samples of DNA mixed with gel loading dye were loaded onto a 1.5% LE agarose (BMA) gel (1X TBE, Section 2.3.7) and run alongside lambda DNA standards of 10 ng, 25 ng, 50 ng and 100 ng. A current was applied to the gel (30 -100V) until the dye front neared the end of the gel. The gel was then removed from the current and stained with ethidium bromide (Section 2.3.2) for 10 -20 mins. The stained DNA bands in the

gel were then visualised under UV light and photographed. A comparison of the intensities for the standard and sample bands was then done to estimate the amount of DNA contained in the band and hence the amount of DNA in the original sample.

CHAPTER 3: METHODS TO VISUALISE INFECTION

3.1 Introduction

Before a pathogenicity trial was set up it was necessary to develop methods to monitor the growth of *D. pini* on the surface and inside *P. radiata* needles. The infection cycle of *D. pini* was described by (Gadgil 1967) who used the microscopy techniques of (Johansen 1940; Preece 1959; Hering and Nicholson 1964). While these techniques provided detailed information about the infection process of *D. pini*, new techniques and imaging technology have become available which may provide better images and require less sample preparation. Other pine pathogens have been investigated using modern as well as traditional microscopy techniques to visualise the infection process. These studies provided detailed information about the fungal host interaction but still involved lengthy and complicated sample preparation (Franz, Grotjahn et al. 1993; Deckert, Melville et al. 2001; Moricca, Ragazzi et al. 2001; Pirttila, Pospiech et al. 2003). Attempts were made to modify proven techniques to allow a large number of samples to be examined over a time series while providing as much information about the infection process as possible. In this way it was hoped to make observations about differences in *D. pini* fitness between the *dotA* knockout mutant and wild type at different stages of infection.

The thickness of *P. radiata* needles presents difficulties when trying to visualise them using bright field microscopy. High light intensities are required to penetrate the needle. A method was needed to clear the pigment from the pine needle tissue and at the same time, leave the structure of the pine needle and the *D. pini* cells intact. Sodium hypochlorite is an oxidiser, which clears pigments from plant tissue allowing the use of bright field light microscopy. This method was used by (Gadgil 1967), however the original paper outlining the method was (Hering and Nicholson 1964). This method was adapted to allow for a high throughput of samples and to determine whether a more readily available sodium hypochlorite, in the form of bleach, could be used.

FAA is normally used as a fixative in plant tissue including *D. pini* infected pine needles (Gadgil 1967) but also was observed to clear some pigment from the pine needles (personal observation) so FAA was included in the pigment clearing trial. (Preece 1959) mentions the use of methanol in a clearing method that was very quick and simple which would suit the purposes of high throughput needed for this study so this technique was also investigated.

The high curvature of the needle surface means only a small area can be seen under high magnifications due to the small depth of feild. Sectioning using a microtome can be used to eliminate this problem but the time series nature of this study made this technique impractical due to the large amount of time that would be required to prepare all the sections.

Epi-illumination microscopy was also trialled in an attempt to obviate the need to clear pigments from the plant tissue. Epi-illumination involves a normal light microscope but with the light source coming from above the microscope stage rather than penetrating through the sample.

Epi-illumination fluorescent imaging can provide several benefits in this study that normal light microscopy cannot. The first is that the excitation light comes from the top of the sample providing the same benefits seen with epi-illumination microscopy but without the difficulty of setting up the light source. This eliminates the need to clear pigment from the pine needle and should reduce the amount of time needed for preparation. Fluorescent imaging is also often more sensitive than normal light microscopy techniques because the stained tissue is more easily distinguished from the dark background providing a higher level of contrast.

Calcofluor white is a non-specific fluorescent stain that binds a wide range of glucans including chitin and cellulose. It has been shown to effectively stain a wide range of fungi, tissue elements and to a lesser extent even some bacteria (Monheit, Cowan et al. 1984). Calcofluor white has proved to be fast and sensitive stain of fungi in different tissue types and has especially been useful in medical applications(Monheit, Brown et al. 1986; Thomson and Robertson 1989).

Gluteraldehyde is commonly used as a fixative of both plant tissue (Deckert, Melville et al. 2001; Moricca, Ragazzi et al. 2001) and mammalian cells (Gilbert and Parmley 1998) often in preparation for electron microscopy. One of the problems caused by using glutaraldehyde as a fixative is that it causes background fluorescence when exposed to excitation wavelengths in the realm of blue light. However, work has previously been done to develop this trait as a useful staining method for visualisation of the Camellia flower blight fungus (Vingnanasingam 2002).

3.2 Materials and Methods

3.2.1 Plant material and inoculation procedures

3.2.1.1 Pigment clearing trials

Plant material used was a detached branch from a short internode tree (Section 2.1.3) 1-2 years old. Needles were cut into ~2 cm sections. Some sections were cut longitudinally to aid in the clearing while others remained whole.

3.2.1.2 Cytoplasmic stain comparison

Plant material used was ramet 18/19 (Section 2.1.3) less than 1 year old. The seedling was inoculated with a NZE7 (Section 2.1.2) spore suspension (4.38×10^7 spores/ml). The inoculated seedling was left outside in an uncovered concrete tub and sample collection and staining was done after 56 days.

3.2.2 Fungal strains and inoculation

Three ml of NZE5 (Section 2.1.2) spore suspension (Section 2.4.2)(4.2×10^6 spores/ml) was used to inoculate the pine needles (Section 2.4.3, NB: Spore suspension was applied in one application not over two applications). The detached branch was left on a sunny windowsill covered with a clear plastic bag to maintain humidity. Needles were collected four days after inoculation.

3.2.3 Microscopy Stains

3.2.3.1 Cytoplasmic stains

Acid fuschin

Acid fuschin powder (BDH) 2% (w/v) was dissolved in 70% ethanol 30 % milli-Q water (Bradbury 1973)

Aniline blue

Aniline blue-water soluble (BDH) 0.4 % (w/v) in milli-Q water.

Coomassie blue

Coomassie-R powder (BDH) 10 mg, 95% ethanol (BDH) 5 ml, phosphoric acid (BDH) 10 ml. Made up to 100 ml with milli-Q water.

Lactophenol cotton blue

Phenol (BDH) 20 ml, lactic acid (BDH) 20 ml, glycerol (BDH) 20 ml, aniline blue water soluble (BDH) 0.05 g. Heat phenol, lactic acid and glycerol together at 70°C and add aniline blue.

Schiff's reagent

Schiff's reagent (BDH).

Toluidine blue

Toluidine blue (Raymond A. Lamb waxes and General laboratory supplies, Alperton, Middx) 0.25 g, 70% ethanol (BDH) 100 ml, concentrated HCl (BDH) 0.5 ml.

Trypan blue

Trypan blue (Sigma) 1 % (w/v) in milli-Q water.

3.2.3.2 Fluorescent stains

Calcofluor white.

Calcofluor white M2R (Sigma) 0.01% (w/v) in milli-Q water.

Gluteraldehyde solution (GA-PBS)

Glutaraldehyde 3% (v/v) (BDH) in 1X PBS pH 7.2 (Section 3.2.5.1). Solution was stored at 4°C wrapped in tin foil.

3.2.4 Solutions

3.2.4.1 Acetic acid

Acetic acid (glacial) (Rhône-Poulene Chemical Limited) 10% (v/v) in milli-Q water.

3.2.4.2 Formic acetic alcohol (FAA)

Acetic acid (glacial) (BDH) 5 ml, 70% ethanol (BDH) 90 ml, 40% formaldehyde (BDH) 5 ml.

3.2.4.3 Sodium hypochlorite solutions

Sodium hypochlorite in the form of household bleach (Budget) 31.5 g/l. Serial 2 fold dilutions were done using milli -Q water to produce concentrations of 15.8 g/l, 7.9 g/l, 3.9 g/l and 2 g/l.

3.2.4.4 Shear's mounting fluid

Potassium acetate (BDH) 10 g, 95% ethanol (BDH) 300 ml, glycerol (BDH) 200 ml, milli-Q water 500 ml.

3.2.5 Buffers

3.2.5.1 Phosphate buffered saline (PBS) (10 X)

NaCl (Univar) 8% (w/v), KCl (Univar) 0.2% (w/v), Na_2HPO_4 (BDH) 5.75 g in 500 ml of milli-Q water. A 1X working solution was made from this stock and the pH was adjusted to pH 7.2 using 0.1 M NaOH (BDH) and 0.1 M HCl (BDH).

3.2.5.2 Sucrose in phosphate buffered saline (S-PBS)

Sucrose (BDH) 6.5% (w/v) in 1 X PBS at pH 7.2. Solution was stored at 4°C.

3.2.6 Clearing and staining procedures

3.2.6.1 FAA pigment clearing method

Halved inoculated pine needles as well as whole ones (Section 3.2.1.1) were kept in Universal bottles with 20 ml FAA (Section 3.2.4.2) overnight at 4°C.

3.2.6.2 Methanol pigment clearing method

Longitudinally halved needles as well as whole ones (Section 3.2.1.1) were stored in Universal bottles containing 20 ml methanol (BDH) and were stored overnight at 4 °C (Preece 1959).

3.2.6.3 Sodium hypochlorite pigment clearing method and the effect of heat and acetic acid

The method used deviated from the original paper (Hering and Nicholson 1964) in several respects. Needles (Section 3.2.1.1) were cut in half longitudinally to aid in the clearing of host tissue and this also made the preparations easier to visualise due to the thinner layer of cells being observed. Sodium hypochlorite was used instead of sodium chlorite because sodium hypochlorite was easily and cheaply obtained in the form of bleach. 'Budget' bleach (Section 3.2.4.3) was used in this experiment. Also methyl salicylate was not used to further clear the tissue because no additional clearing was required to visualise the tissue.

Stock dilutions of sodium hypochlorite were made (Section 3.2.4.3) before decanting 20 ml into individual Universal bottles. Half a ml of 10 % acetic acid (Section 3.2.4.1) was added to half the tubes. Half a ml of milli-Q water was added to the remaining bottles. Whole needles as well as halved needles that had been sprayed with spore suspension the day before were added to the bottles at this point. Bottles undergoing heat treatment were placed in a water bath calibrated to 60°C and those not undergoing heat treatment were left on a bench out of the sun. Observations of bleaching of needles were made every few minutes although gaps between observations increased as time went by. Once needles were cleared they were

removed and examined for spore adherence and fungal growth by staining for 50 min in Schiff's reagent (Section 3.2.3.1) and visualising using bright field microscopy (Section 3.2.7.1).

3.2.6.4 Cytoplasmic staining comparison and the effect of heat

The collected needles (Section 3.2.1.2) were cut into ~2 cm sections and stained with 4-5 drops of different cytoplasmic stains for 10 minutes (Aniline blue, lactophenol cotton blue, coomassie blue, acid fuschin, Schiff's reagent, toluidine blue and trypan blue (Section 3.2.3.1). The effect of heat was assessed by heating in a water bath at 60°C for 5 mins. Room temperature staining was used as a control. All stained needles were re-stained with calcofluor white (Section 3.2.6.5) to confirm the presence of spores and mycelium.

3.2.6.5 Calcofluor white staining method (Monheit, Cowan et al. 1984)

Pine needles were sectioned into ~2 cm lengths and placed on a glass slide. Five to six drops of calcofluor white stain (Section 3.2.3.2) were then dropped onto the needle sections. The needle sections were turned to ensure complete coverage of the needle. The needle sections remained in the stain for ~1 min before being removed and rinsed in RO water. Needle sections were mounted onto glass slides and covered with glass cover slips before being visualised under wide band UV excitation fluorescent microscopy (Section 3.2.7.2).

3.2.6.6 Gluteraldehyde staining and de-staining method

Needle sections about 1.5 cm in length were left in ~3 ml of 3% GA-PBS overnight at room temperature. The following day the needles were mounted on a glass slide in Shear's mounting fluid (Section 3.2.4.4) and a cover slip was sealed onto the slide using clear nail polish to prevent the needles drying out. The needles were observed using an epi-fluorescence (Section 3.2.7.3) and confocal microscopes (Section 3.2.7.4).

Some glutaraldehyde samples underwent a de-staining process which involved two changes of 3 ml S-PBS (Section 3.2.5.1) over a period of at least 3 hours (Vingnanasingam 2002).

3.2.7 Microscopy

An Olympus BX51 combined bright field and fluorescent microscope was used for all microscopy. Photographic images were taken using an Olympus digital camera (model number C-4040ZOOM).

3.2.7.1 Bright field microscopy

Bright field microscopy was used for samples treated with cytoplasmic stains.

3.2.7.2 Epi-illumination microscopy

An addition cold light source was used for epi-illumination work. Manufactured by 'Schott', model number KL150B. The cold light source was manually focused onto the sample from above the microscope stage.

3.2.7.3 Fluorescent microscopy

Samples stained with calcofluor white were visualised under wide band UV. Samples stained with Gluteraldehyde were visualised using wide band blue light. Excitation wavelengths for fluorescent microscopy are as followswide band ultra violet (WUV) = 330-385 nm, wide band blue (WB) = 460-490 nm.

3.2.7.4 Confocal Microscopy

Pre-examined gluteraldehyde treated specimens were examined under a Leica TCS/NT confocal laser scanning microscope using filters with exciter wavelengths of 488 and 568 nm and imaging wavelengths between 530 and 590 nm. Confocal images were produced by depth scanning to create optical sections taken 5 μm apart and combined to produce the final 3D image.

3.3 Results

3.3.1 Clearing of pigment from Pine needles

3.3.1.1 Sodium hypochlorite with combinations of acetic acid and heat

Background

At the time when this trial was done staining methods had not been optimised and did not show much fungal material. Staining using Schiff's reagent (Section 3.2.3.1) had shown some success so was used for this test.

Results

Clearing of pigment was complete in the halved needles but clearing of pigment from the whole needles started at the cut ends and took some time to completely clear the pigment from the ~2 cm sections. The data in Table 3.1 shows the time needed to clear pigments from halved needles. Increased temperature and increased sodium hypochlorite concentration both reduced the amount of time needed to completely clear the needle tissue of pigments. Addition of acetic acid increased the time needed to clear the needle tissue. Cell wall structure and stomata appeared to remain intact but staining with Schiff's reagent preferentially stained damaged needle tissue including cut ends of the sections and longitudinally cut sections.

No spores or fungal growth were observed on any of the cleared needles following treatment with sodium hypochlorite (Fig 3.2). One possible spore was seen on the negative control, which had been treated with water (Fig 3.1).

	Sodium hypochlorite concentration (g/l)				
	31.5	**15.8**	**7.9**	**3.9**	**2**
Room temp only	<30 mins	40 mins	60 mins	+2 hrs	
Room temp + 0.5ml of 10% acetic acid	40 mins	40-60 mins	60 mins	+2 hrs	
60°C only	10 mins	19 mins	24 mins	30 mins	60 mins
60°C + 0.5ml of 10% acetic acid	40 mins	40-60 mins	40-60 mins	40-60 mins	60 mins

Table 3.1: Length of time before halved needles were observed to be clear of pigments.

3.3.1.2 FAA treatment

Overnight treatment of needle samples with FAA partially cleared pigments from the needle sections. Halved needle sections were more effectively cleared compared to whole sections. Germinated *D. pini* spores were seen on the surface of several needles when stained with Schiff's reagent (Fig 3.3) but stain was also taken up by damaged needle tissue making it impossible to distinguish fungal material on halved needle sections.

3.3.1.3 Methanol treatment

Overnight treatment of needle samples with methanol was more effective than FAA treatment in clearing pigments from the pine needles to allow the use of bright field microscopy (Fig 3.4). Again halved needles were cleared more effectively than whole needle sections. Pigment clearing was less effective in older/thicker needles. Spores and fungal growth were observed on the surface of several needles using normal light microscopy.

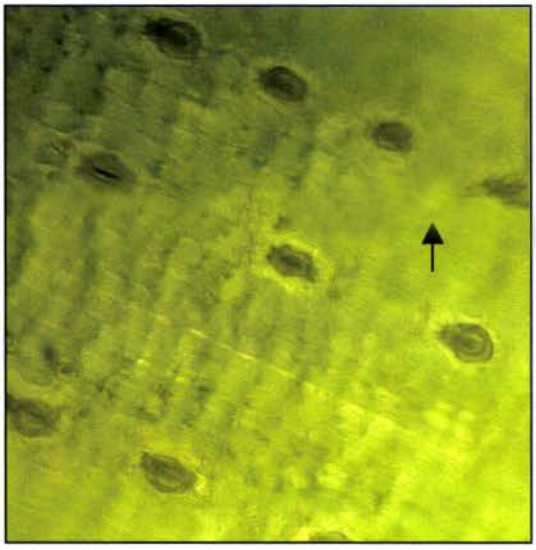


Fig 3.1: Whole pine needle section treated with water overnight as a control. Stained with Schiff's reagent. Arrow indicates possible *D. pini* spore. Bright field 200X magnification.

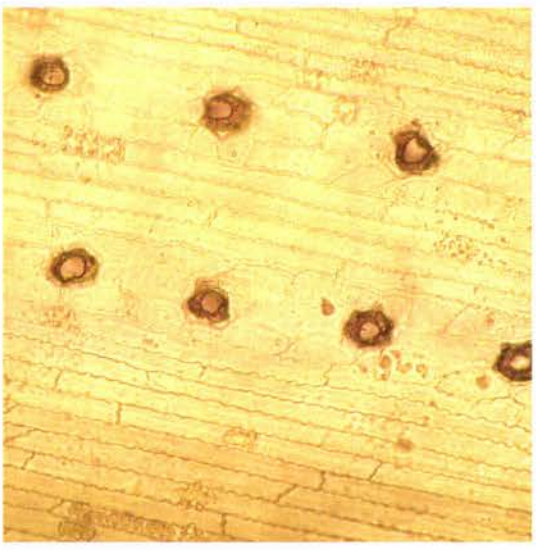


Fig 3.2: Whole pine needle section treated with sodium hypochlorite (15.8 g/l) for 40 mins at room temperature. Stained with Schiff's reagent. Bright field 200X magnification

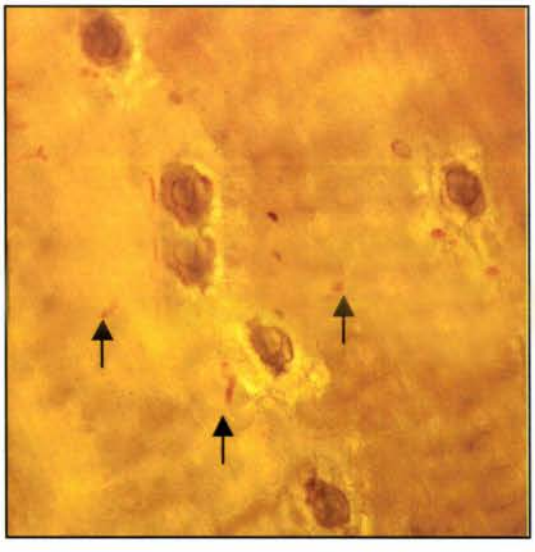


Fig 3.3: Whole pine needle section treated with FAA overnight. Stained with Schiffs reagent. Arrows indicate *D. pini* spores. Bright field 200X magnification.

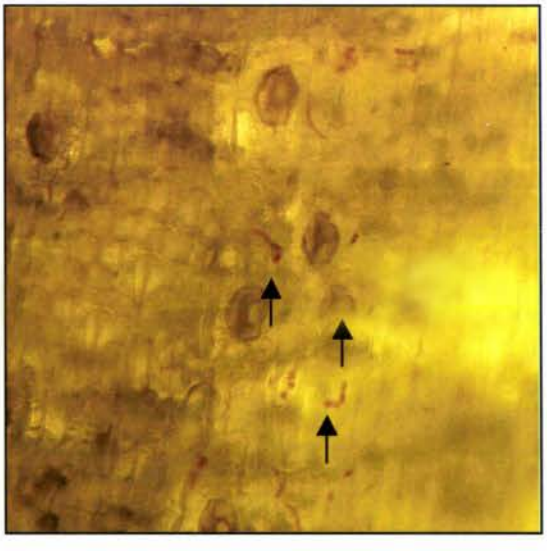


Fig 3.4: Whole pine needle section treated with methanol overnight. Stained with Schiff's reagent. Arrows indicate *D. pini* spores. Bright field 200X magnification.

3.3.2 Schiff stained needles visualised by epi-illumination

3.3.2.1 Background

As mentioned previously the thickness of pine needles makes it difficult for light to penetrate through, limiting the effectiveness of bright field microscopy. The high light intensity required to penetrate the sample made visualisation of tissue near the edge of the needle impossible due to the inability to close the iris diaphragm of the microscope enough. Also because of the large number of cell layers the image often appeared cluttered making distinction between cells difficult. The clearing of pigment from needles in Section 3.3.1 was shown to be effective at reducing the problem, allowing light to penetrate the sample but this required time and had mixed success depending on the thickness of the needle being treated. Treating the needle with chemicals also raised the spectre of the treatment possibly washing fungal material from the surface of the needle, giving a false impression of *D. pini* growth. In an attempt to negate the need to clear pigment from the pine needles it was decided to try epi-illumination microscopy to visualise stained needles. Epi-illumination involves the light source coming from above the object rather than penetrating through. Also because only the top layer of cells is being looked at the image obtained tends to be less cluttered when compared to that of bright field microscopy of thick specimens.

3.3.2.2 Results

Figures 3.5 and 3.6 show the comparison between brightfield and epi-illumination of the same frame under 200X magnification. In the epi-illumination image (Fig 3.6) the cells in the top layer of the needle were more distinct but the shiny surface of the needle produces a 'halo' effect whereby it is difficult to see tissue at or near points of reflecting light. Both methods of microscopy were sufficient to visualise spores and fungal growth on the surface of pine needle. Images obtained by epi-illumination provided a similarly low level of contrast to that of bright field microscopy. Another problem faced by this method was focusing the epi-illumination light source on the stage so the sample could be visualised using high-powered objectives. The 20X objective was the highest able to be used while maintaining enough light to see a clear image.

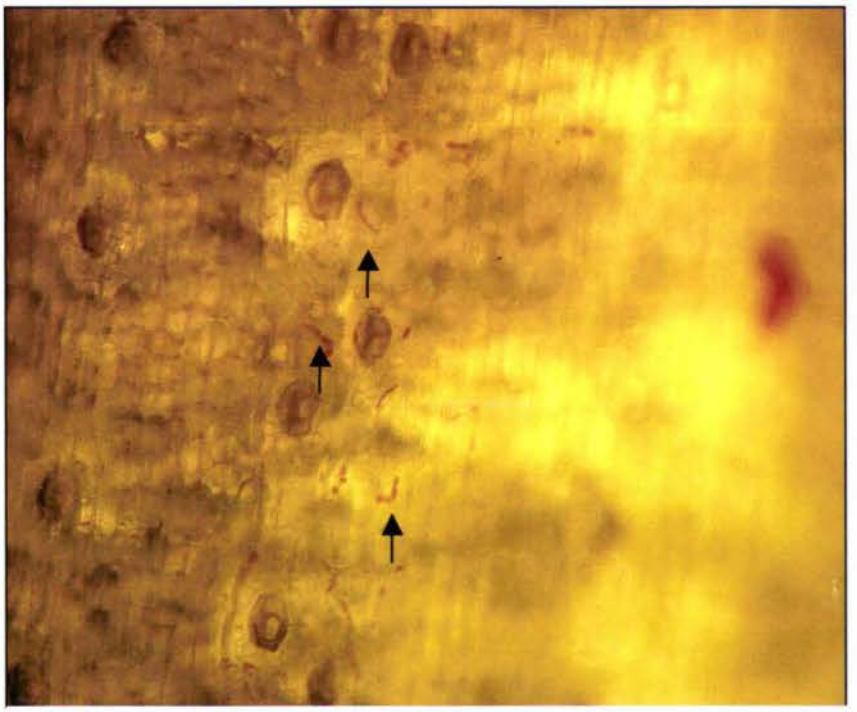


Fig 3.5: Pine needle treated with methanol overnight. Stained with Schiff's reagent four days after inoculation with a NZE5 spore suspension. Arrows indicate *D. pini* spores. Bright field 200X magnification

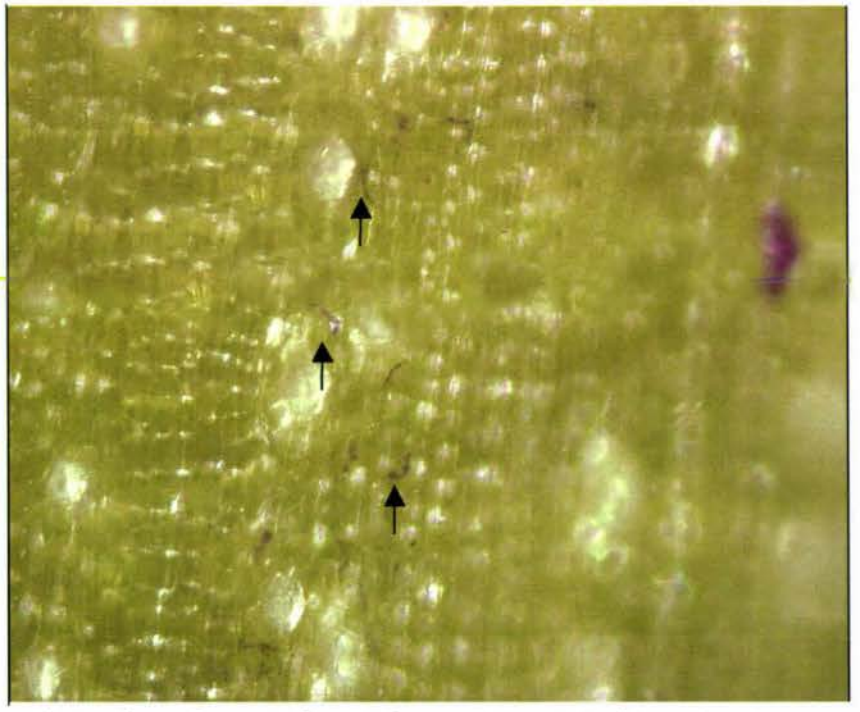


Fig 3.6: Same frame as fig 3.5. Pine needle treated with methanol overnight. Stained with Schiff's reagent four days after inoculation with a NZE5 spore suspension. Arrows indicate *D. pini* spores. Epi-illumination 200X magnification

3.3.3 Cytoplasmic staining and the effect of heat

3.3.3.1 Background

A pilot trial, done as part of this study, found calcofluor white staining, in combination with fluorescent microscopy, to be a sensitive technique for the visualisation of fungal material on the surface of pine needles. However an efficient staining technique for use with bright field or epi-illumination was desirable because of the ability to see necrotic tissue that was not visible under the excitation wavelengths needed for calcofluor white visualisation. Therefore an experiment was set up to investigate whether other cytoplasmic stains were more sensitive and faster to use than Schiff's reagent. Heat treatment was also investigated. The use of fluorescent microscopy following staining with calcofluor white was used as a control for this experiment.

3.3.3.2 Results

All cytoplasmic stains worked successfully at similar levels to Schiff's reagent but none were as sensitive as calcofluor white staining (Table 3.2). Trypan blue staining with heat treatment was second best compared to calcofluor white providing good contrast between the fungal cells and the plant tissue (Fig 3.14). Bright field microscopy was effective on thinner needles (Fig 3.14) but epi -illumination was more effective than bright field on thicker needles (Fig 3.9) or near the edge of a needle (Fig 3.10). Calcofluor white staining was was less effective on fungal material that had been previously stained with any of the coomassie blue, trypan blue, or toluidine blue stains (Fig 3.14).

Stain	Room temp	60°C	Calcofluor white (Counter Stain)	Images
Aniline blue	+	+	+++	Fig 3.7
Acid Fuschin	+	+	+++	Fig 3.8
Coomassie blue	+	+	++	Fig 3.9
Trypan blue	+	++	++	Fig 3.14
Toluidine blue	+	+	++	Fig 3.12
Schiff's reagent	+	+	+++	Fig 3.11

Table 3.2: Comparison of different stains. – indicates no fungal material was observed. The number of +'s indicates the relative effectiveness of the staining with +++ being the highest.

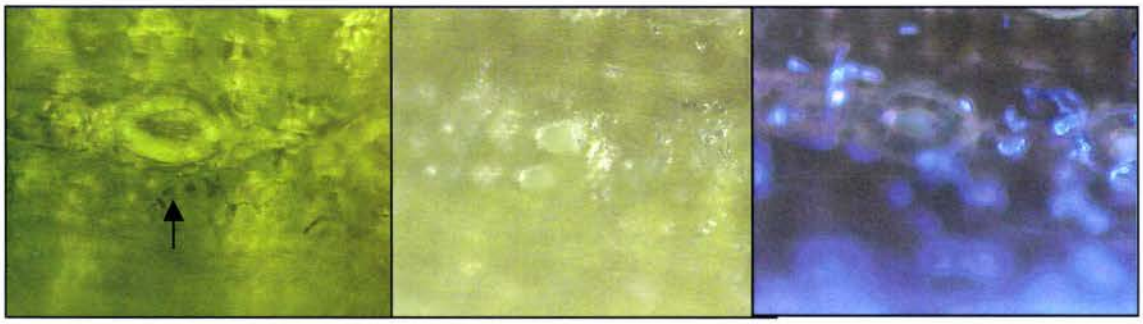


Fig 3.7: Aniline blue stained followed by staining with calcofluor white. Left: Bright field. Middle: Epi-illumination. Right: Wide band UV excitation. All pictures are the same frame of a needle inoculated with a NZE7 spore suspension (Section 3.2.4.4).

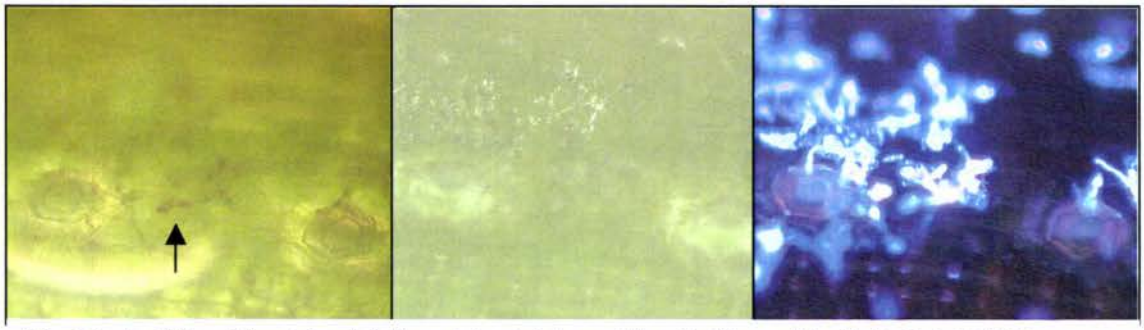


Fig 3.8: Acid Fuschin stained followed by staining with calcofluor white. Left: Bright field. Middle: Epi-illumination. Right: Wide band UV excitation. All pictures are the same frame of a needle inoculated with a NZE7 spore suspension (Section 3.2.4.4).

Fig 3.9: Coomassie blue stained followed by staining with calcofluor white. Left: Bright field (Image is dark because needle is thick and exposure was short due to the edge of the needle being in frame of uncropped image). Middle: Epi -illumination. Right: Wide band UV excitation. All pictures are the same frame of a needle inoculated with a NZE7 spore suspension (Section 3.2.4.4).

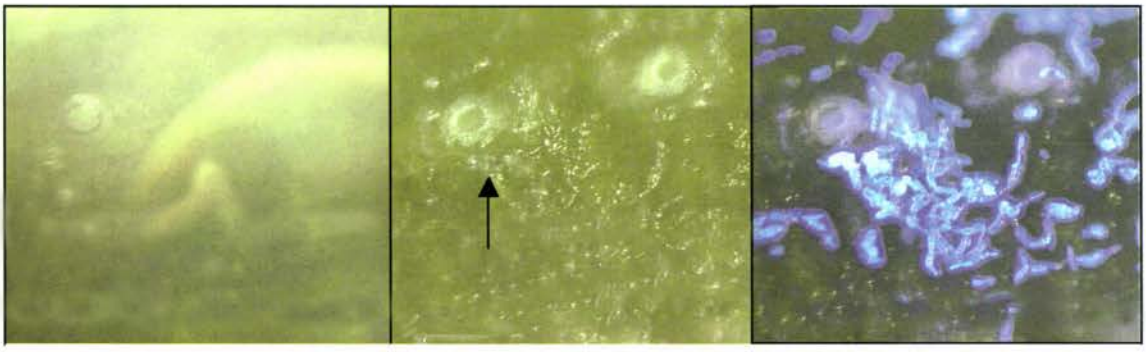


Fig 3.10: All pictures are the same frame of a needle inoculated with a NZE7 spore suspension (Section 3.2.4.4). Lactophenol cotton blue stained followed by staining with calcofluor white. Left: Bright field (Image is hazy due the glare caused by the edge of the needle being in frame of uncropped image). Middle: Epi-illumination. Right: Wide band UV excitation.

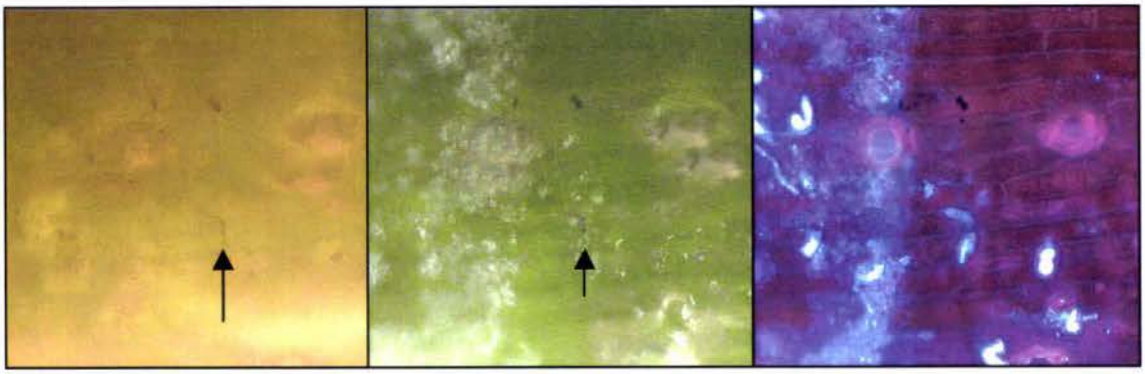


Fig 3.11: All pictures are the same frame of a needle inoculated with a NZE7 spore suspension (Section 3.2.4.4). Schiff's reagent stained followed by staining with calcofluor white. Left: Bright field. Middle: Epi-illumination. Right: Wide band UV excitation.

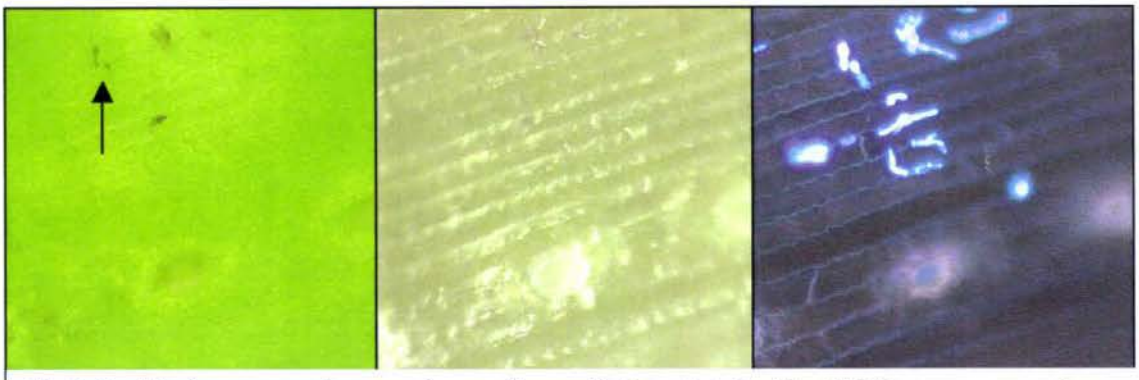


Fig 3.12: All pictures are the same frame of a needle inoculated with a NZE7 spore suspension (Section 3.2.4.4). Toluidine stained followed by staining with calcofluor white. Left: Bright field. Middle: Epi-illumination. Right: Wide band UV excitation.

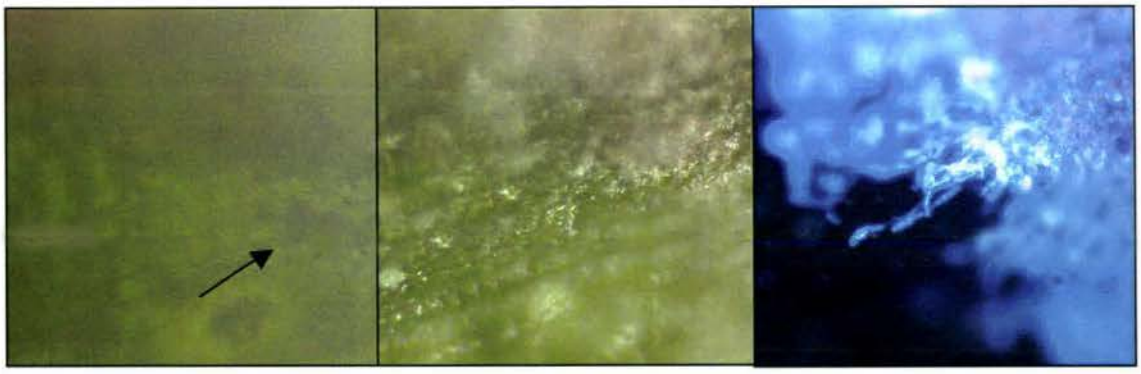


Fig 3.13: All pictures are the same frame of a needle inoculated with a NZE7 spore suspension (Section 3.2.4.4). Trypan blue (room temperature) stained followed by staining with calcofluor white. Left: Bright field. Middle: Epi-illumination. Right: Wide band UV excitation.

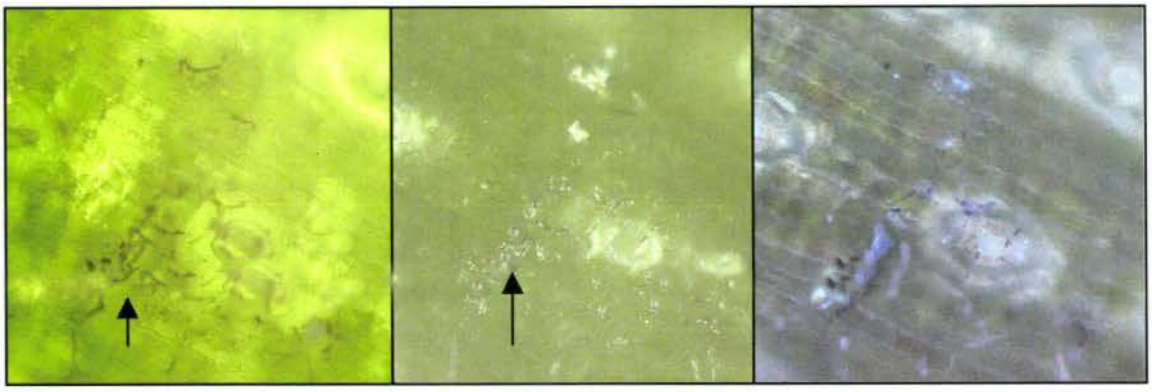


Fig 3.14: All pictures are the same frame of a needle inoculated with a NZE7 spore suspension (Section 3.2.4.4). Trypan blue (heated 60°C) stained followed by staining with calcofluor white. Left: Bright field. Middle: Epi-illumination. Right: Wide band UV excitation.

3.3.4 Fluorescent Stains

3.3.4.1 Gluteraldehyde

Background

This experiment was designed to assess the usefulness of glutaraldehyde staining to visualise *D. pini* growth within the needle tissue using confocal microscopy.

Results

Staining with glutaraldehyde proved to be inconsistent. In some samples (Fig 3.15), *D. pini* growth was clearly visible but in others, that had gone through the same staining process, no *D. pini* was observed despite its presence being confirmed by staining with calcofluor white. Different staining times and a de-stain period using PBS-sucrose (Section 3.2.5.1) were investigated with no improvement to the results (Results not shown). No internal growth of mycelium was observed using this technique but no disease symptoms were seen on the needles to confirm invasive growth.

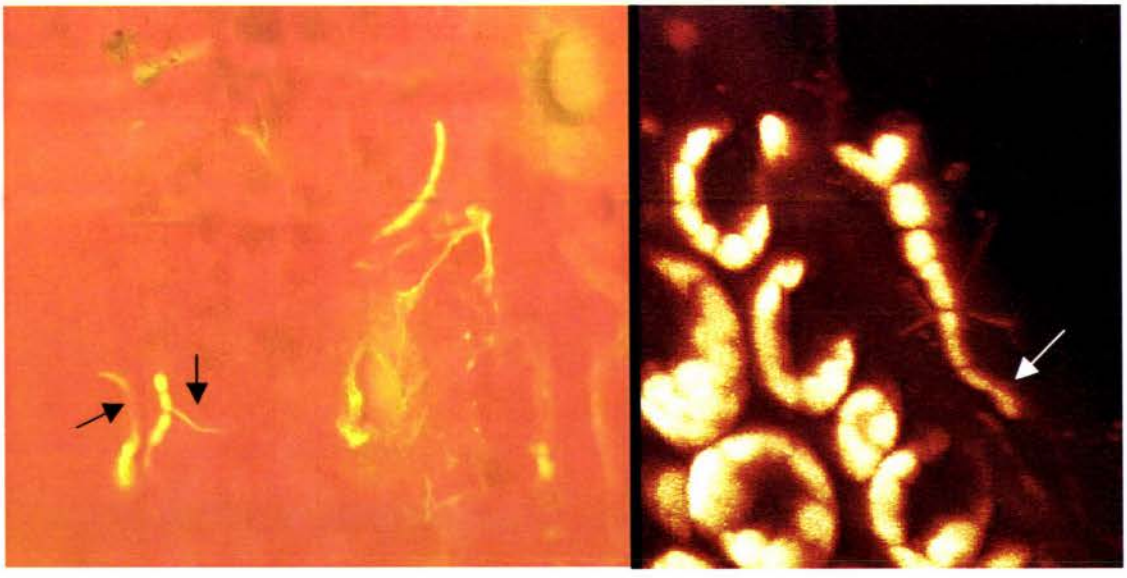


Fig 3.15: Pine needle stained with 4% gluteraldehyde PBS solution three days after inoculation with a NZE5 spore suspension. Left: Wide band blue excitation 200X magnification. Arrow indicates germ tube extending from spore. Right: Confocal image of same needle as left panel. 400X magnification. Spore with septate germ tube extending out along the surface of needle. Fluorescing chloroplast tissue can be seen below the germinating spore. Arrow indicates germ tube extending from spore.

3.3.4.2 Calcofluor white

Results

As shown in Section 3.3.3 above, fungal material was observed on calcofluor white stained tissue when other staining methods had shown no fungal material at all. Both spores and germ tubes were clearly visible with a high degree of contrast. No orientation of germ tubes towards stomata was observed but branching was seen (Fig 3.16). Plant cell walls were also stained to a lesser extent, which provided a useful reference for fungal growth (Fig 3.16). The staining process (Section 3.2.6.5) was the shortest of the staining techniques trialled in this study.

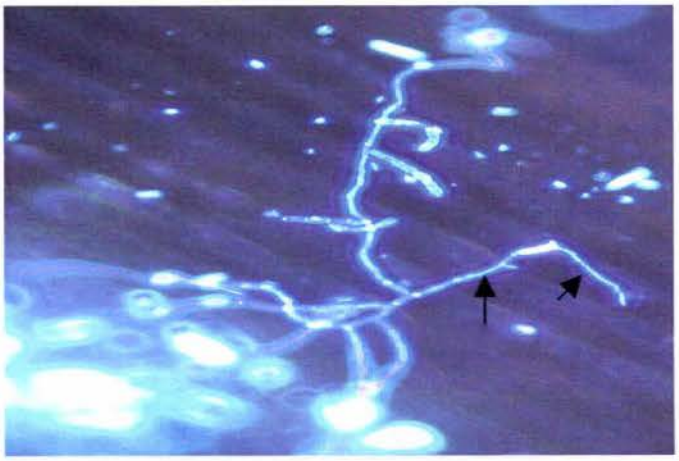


Fig 3.16: Pine needle from short internode tree less than one year old inoculated three days earlier with a NZE5 spore suspension stained with calcofluor white. Arrow indicate germ tubes extending from spores and branching. Wide band UV excitation. 200X magnification.

3.4 Discussion

When deciding the most effective clearing method it is also necessary to take into account the quality of staining following the pigment clearing. While the sodium hypochlorite method was the most effective at clearing pigment from the needles the fact that no fungal material was observed following this treatment makes the method inappropriate for this study. While it is possible that there were no spores present on the needles prior to treatment, this explanation is unlikely due to the high occurrence of spores being observed following the other two treatments. Repeating the clearing process used by Gadgil (1967) exactly may have improved this technique but

meanwhile fast effective methods were developed in the form of methanol treatment and therefore this was not attempted.

Spores were visualised equally well following both the methanol and FAA treatment suggesting that the fixative properties of FAA had minimal effect on the integrity of the sample. Because methanol cleared pigment from the needles more effectively then the FAA it was decided that methanol was the most effective method for preparing samples for bright field microscopy. Bright field microscopy worked well on thin needles without chemical treatment so it may be an option for treating older, thicker, needles where pigment clearing is required.

The other problem faced when imaging pine needles was that the high curvature of the needle surface meant only a very small area could be observed under high-powered objectives because of a small depth of field and indeed anything over 200X magnification was very difficult to interpret. The dense nature of the pine needle and high level of pigmentation generally meant bright field microscopy was not practical on untreated needles unless the needle was relatively thin. Attempts to clear pigment from the needles to make bright field microscopy possible were successful but required a significant amount of time that was not required by epi-illumination and epi-fluorescent microscopy techniques.

Epi-illumination microscopy was by far the fastest method to visualise epiphytic *D. pini* growth on thick needles using cytoplasmic stains. Insufficient light intensity at magnifications above 200X was not a problem because images at these magnifications were not useful due to the lack of depth of field.

The use of calcofluor white was the best method of those trialled to visualise epiphytic *D. pini* growth. Staining required less time and was shown to be more sensitive when compared to the cytoplasmic staining methods. The method used for this study (Section 3.2.6.5) used a somewhat shorter staining period than in some publications but was still very effective. A lower concentration of calcofluor white was also used than by some authors (Monheit, Brown et al. 1986; McIntyre, Dynesen et al. 2001) although the 0.01 % concentration used in this study has been used previously (Narisawa, Kageyama et al. 1996). A lower concentration is likely to limit

the osmotic stress placed on the fungal and plant cells. Higher concentrations or longer staining periods using more stain volume maybe required for samples other than pine needles where surrounding tissue takes up more stain.

The calcofluor white method provided a lot of information about the plant/fungal system. Spores and germ tubes were easily distinguished. Plant cell fluorescence provided clear reference points to fungal growth due to the stain not penetrating any further than the first layer of cells. However, the non-specific staining of calcofluor white remains a draw back, which is also faced with the other staining methods investigated. This means identification of *D. pini* by its morphological features is still required. *D. pini* spores are characterised as being long narrow and oval, ranging in length from 15 – 47.5 μm (Bradshaw 2004) but when grown in culture *D. pini* spores can lose this characteristic shape and appear in a range of morphologies from the traditional long narrow shape to an almost round structure (Personal correspondence with Dr Peter Gadgil, Forest Research, and personal observation). The inability to distinguish between *D. pini* and other fungi is not normally a problem as the fungal population on new healthy pine seedlings is relatively low so inoculation with *D. pini* spore suspensions results in *D. pini* outnumbering native fungal microflora by a huge margin. Relative spore size, germ tube growth characteristics and hyphal morphology are still useful in identifying *D. pini* on the needle surface.

Gluteraldehyde showed early promise for the visualisation of endophytic *D. pini* growth but early success was not reproducible. The early success of this technique suggests further development and optimisation may yield a useful staining method. If this could be done *D. pini* penetration into the pine needle could be visualised and in combination with confocal microscopy useful 3D images of growth within the needle itself could be obtained. However I think to develop this technique to useful levels of reliability and sensitivity may take some time. A lot of time was needed to develop a technique for use with Camellia petals infected with the Camellia flower blight fungus (Vingnanasingam 2002). Possible factors to investigate would be different concentrations of gluteraldehyde and a more extensive trial of staining times and destain periods. Before this work is done however it will be necessary to have a proven infection of *P. radiata* needles *in vitro* within our laboratory.

Sectioning of the needle as outlined in (Gadgil 1967) could provide detailed information about the infection process of the *dotA* mutant and may be something to consider once conditions for infection are proven to be duplicated within our laboratory. It is also necessary to check that *D. pini* kept in culture for extended periods of time are still capable of infecting *P. radiata* trees.

In summary, a fast and sensitive technique has been developed for visualisation of epiphytic growth of *D. pini* by staining with calcofluor white. This technique will be very useful for investigation of the early stages of *D. pini* infection. It now remains to develop a technique for the visualisation of endophytic *D. pini* growth.

CHAPTER 4: PATHOGENICITY TEST

4.1 Introduction

As mentioned previously (Section 1.6.2) dothistromin is thought to play a key role in *D. pini* infection of *P. radiata* (Shain and Franich 1981). A *dotA* gene knockout mutant has been produced that is deficient in dothistromin production (Bradshaw, Bhatnagar et al. 2002). By comparing this dothistromin deficient mutant to wild type *D. pini* in a pathogenicity test, we will obtain more detailed information regarding dothistromin's role in the infection process and test the hypothesis that dothistromin is a pathogenicity factor for *D. pini* infection of *P. radiata*.

Current pathogenicity tests for *D. pini* on *P. radiata* consist of field trials of young trees as part of a breeding program developing *Dothistroma* blight resistance (Carson and Carson 1991). It is not possible to use a similar system for this test because of the need to contain the *dotA* mutant under PC2 containment conditions. Favourable conditions for infection needed to be reproduced in a controlled environment. Correlation of field trials results with infection of seedlings following inoculation with *D. pini* in a controlled environment has been described as 'moderate' (Carson and Carson 1991). This work shows that comparisons will be able to be made between results obtained in a controlled environment and infection observed in the field, but that the accuracy of the results obtained are somewhat limited.

While PC2 conditions will not fully represent conditions present in the field there is the benefit of allowing for a certain degree of environmental control. This will allow the reproduction of environmental conditions favourable for high *D. pini* infection levels. Optimum conditions should highlight any difference in pathogenicity and possibly virulence. Conditions favourable for high infection levels by *D. pini* have already been investigated, with high light intensity, continuous leaf moisture and temperature being shown to be important (Section 1.4).

With high light intensity being a key factor for symptom development it is important to consider the different types of auxiliary lighting available. Metal halide bulbs emit

a larger percentage of their light in the photosynthetically active (PAR) wavelength range than do incandescent or High Pressure Sodium (HPS) bulbs. Both metal halide and HPS bulbs are significantly more efficient at converting electrical energy into light than incandescent bulbs. Metal halide and HPS bulbs convert 30 to 40% of the electrical energy they are supplied with into light whilst incandescent bulbs convert about 6%.

Modern air conditioning units make temperature control relatively straight forward in a controlled environment. Sunlight shining through windows as well as the temperature differential across windows can introduce slight variations in temperature control. The air conditioning unit itself can also introduce variation when switching between cooling or warming cycles.

Misting systems are commercially available for green houses and garden hobbyists making it possible to maintain continuous leaf wetness.

While it is important to view the infection process from the fungal side and develop environmental conditions favourable for *D. pini* to infect, it is equally important to look at things from the side of the *P. radiata* host in terms of resistance. *Dothistroma* resistance level is an important factor to consider when determining the plant material to be used in a pathogenicity test. As mentioned above *Dothistroma* resistance is a heritable trait (Section 1.2.2) so a seedling's level of resistance is determined largely by the resistance levels of the parents, but there are exceptions to this rule (Dick 2003). *P. radiata* develops significant resistance to *D. pini* infection at about 10 - 20 years of age (Gibson 1972). This makes seedlings suitable for a pathogenicity test, which also reduces the amount of space needed to set up a trial making it feasible to carry out a pathogenicity test in a relatively confined environment.

Dothistroma needle blight is easily confirmed in the field by its characteristic red banding on the pine needle and the production of dark fruiting bodies within the red bands. Darker green edges or 'green islands' are also sometimes observed on the edges of the necrotic lesion (Franich, Carson et al. 1986). Tip browning can often occur with *D. pini* infection but it is important to note tip browning can also be the result of drying or magnesium deficiency (Maclaren 1993).

Some of the *D. pini* strains used for inoculation in this test had been maintained in culture for several years. The strains showed variation in their appearance and it was deemed prudent to confirm their identities. When grown in culture *D. pini* showed colony morphology variation with a surface ranging from smooth to fluffy. Colony growth was roughly circular with irregular edges. The colony colour varied from a very dark red/brown to light pink and also appeared green once. The *dotA* mutant colonies sometimes showed yellow colouration. Purple and brick red pigments were observed being extruded into media from the wild type *D. pini* and production varied from undetectable to high. *DotA* knockout mutants were only observed to produce a yellow pigment (versicolorin A) but production of this pigment showed similar variation in quantity to the wild type *D. pini* ranging from undetectable to high production. As well as colony and pigment production variation, variation is also observed in secondary conidial morphology when *D. pini* is grown in culture (Section 3.4).

No symptoms characteristic of *D. pini* infection had been observed in previous staining trials in which pine seedlings were inoculated with *D. pini* spores. The long length of time the *D. pini* strains had been maintained in culture raised the possibility that they had become attenuated and were no longer able to cause disease. However conditions during those trials were far from ideal for infection. To investigate this potential problem a new wild type *D. pini* needed to be isolated.

Growth studies during the characterisation of the *dotA* mutants showed a small, but significant reduction in growth rate compared to the wild type (Bradshaw, Bhatnagar et al. 2002), but spore viability wasn't investigated. To ensure pine seedlings in the different treatments received the same amount of viable spores, spore viability would need to be tested.

4.2 Materials and Methods

4.2.1 Plant Material

Groups of ramets were used in the pathogenicity test. Ramets are groups of cuttings from the same tree so are genetically identical (Table 2.3). Ramets cut from one tree were used to provide replication within the pathogenicity test but the same four sets of ramet groups were used in each different treatment. *Dothistroma* resistance scores for the ramet groups were not available but some of their parents scores were. Available scores are listed in Table 2.3. All of the ramet groups are cuttings taken from plants grown from seed sown in May 2002. Cuttings were taken when the plants were 1 year old.

4.2.2 *D. pini* strains used

NZE7 (fresh wild type), 8A1 (ectopic), 34C1 (*dotA* knockout mutant) were used (Table 2.2). NZE5 (original wild type) was planned to be included but sporulation cultures failed to grow and due to time restraints were not included in the pathogenicity test.

4.2.3 Isolation of fresh wild-type *D. pini*

Pine needles containing stroma were incubated for two days in humidity chambers at room temperature (Paul Hirst 1997). Stroma from a single needle were cut away from the surrounding needle tissue being careful to remove as much of the tissue as possible. The stroma were then ground up in 100 µl of sterile milli-Q water with a plastic pestle in a micro-centrifuge tube to release the spores. Five serial two-fold dilutions of the spore suspension were done and 100 µl from each of these was spread out onto separate DM plates (Section 2.2.2). Due to the origin of the spores from an infected needle, some bacterial contamination was observed. A section of a *D. pini* colony that was visibly free of any contamination was cut from the plate using a scalpel and ground up and processed as mentioned above. The suspensions were then spread onto DM plates containing oxytetracycline hydrochloride (Sigma) (10 µg/ml) and ampicillin (Sigma) (100 µg/ml). Individual colonies were cut from these DM plates, processed as mentioned previously and plated out onto DSM plates (Section 2.2.3). In addition to this dilution plating a sterile wire loop full of sterile milli-Q

water was rubbed over a colony from one of the DM plates containing antibiotics. A classical streak was then done onto DSM to obtain a single spore isolate.

NB: It may be possible to cut out several of these steps by using the antibiotics early on and by using the loop full of sterile water to rub spores from the first colony that looks free of any other and streak them out.

4.2.4 PCR amplification

PCR amplification of the *D. pini* ITS region between the nuclear 18S and 5.8S rRNA genes was carried out with primers ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3')(White, Bruns et al. 1990). Each 50 µl reaction contained a final concentration of 1 X PCR buffer (Roche), 0.3 units Taq polymerase (Roche), 2 µM of each primer and 20 ng of genomic template. The PCR reaction was carried out in a 'Genius' thermal cycler (Techne) with an initial step of 95°C for 4 mins followed by 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C and then a final step of 72°C for 6 mins before holding at 4°C.

4.2.5 DNA sequencing

DNA sequencing was carried out by the 'Allan Wilson Centre Genome Sequencing Service' (AWCGS), Massey University, Palmerston North using a capillary ABI3730 Genetic Analyzer (Applied Biosystems Inc.). Genomic DNA from the different *D. pini* strains along with ITS5 primer (Appendix 1) were supplied to the AWCGS and an electronic nucleotide sequence was obtained.

4.2.6 GMO suite

The GMO suite in the Institute of Molecular Biosciences (IMBS), Massey University , Palmerston North was used to contain the pathogenicity test. It is classified as having Physical Containment level 2 (PC2) and is capable of housing genetically modified plants. The GMO suite is constructed with large glass windows on one side of the bench, a solid wall opposite the windows and a tinted glass roof. Environmental factors that can be controlled in the GMO suite are temperature (+/- 3°C), humidity, CO_2 levels, and to a certain extent light, using shade cloths and auxillary lighting. Day/night period can also be controlled via the auxillary lighting.

4.2.7 Lighting

The desired light intensity for the pathogenicity test was 133 W m^2 (Gadgil 1976) Based on an article written by Sri Rahm obtained on the internet (http://www.venturelighting.com/WhatsNew/Lighting_for_plant_growth.htm; 4/6/03) the wattage of lights needed to obtain a minimum light intensity of 133 W/m^2 over the area of the construct to contain the *P. radiata* seedlings was calculated.
Area of growth rack = 0.878 m^2 multiplied by the desired light intensity of 133 W/m^2 = 117 Watts. 117 Watts multiplied by 3 to obtain the bulb wattage rating required for a metal halide of HPS bulb working at ~ 33% efficiency gives 351 Watts.

The large glass windows and tinted glass ceiling of the GMO suite means natural light supplied some of the lighting (Section 4.2.6). A 400W HPS bulb was already in place in the GMO glasshouse which provided a light intensity of 120 W/m^2. This is the expected output of the 400 W HPS bulb based on 30 % efficiency. As bulbs age they tend to lose some of their efficiency. To safe guard against this future problem and allow for differences in bulb efficiency two additional 100W met al halide bulbs were chosen to supply the additional required light intensity to reach 133 W/m^2. Metal halide bulbs were chosen for the benefit of the plants growth and two bulbs would ensure a more even distribution of light as opposed to one. The three lights were on from 8 am to 6 pm.

4.2.8 Light intensity measurements

Photosynthetically active radiation (PAR) and light radiation (LR) readings were taken at three time points during the day, 10 am, 1 pm and 4 pm. Readings were taken on a sunny day (21/5/04) and an overcast day (23/5/04), with and without the auxiliary lights on. Three positions inside the growth rack were monitored which were termed left, middle and right. The sensor was always placed in the same position at the height of the divisions separating the treatments within the growth construct. A 'Quantum/Radiometer/Photometer' Model number 'LI-250' (Li-Cor) light meter was used to measure PAR and a 'Quantum/Radiometer/Photometer' Model number 'LI-185' (Li-Cor) was used to measure LR.

4.2.9 Misting system

The misting system contained an 'Exal Balance Arm Mist Controller' (Exal Industries Ltd, Christchurch, New Zealand), which controled the water flow. 13 mm garden PVC pipe with 'Hardie Pope, microjet mistspray' nozzles were attached to a steel frame above the seedlings. The misting nozzles faced downwards (Fig 4.1). Local potable water was used as the water supply.

4.2.10 Watering system

Watering was controlled by a 'C1030 Plus Water Computer' (Gardena, Germany), which was connected to a pipe system made up of 13 mm and 4 mm inner diameter poly-tubing. 'Adjustable Flow Drippers – Stake' (Neta) were connected to the 4 mm piping and staked into the polythene bags containing the pine seedlings. The drippers were adjusted to deliver roughly the same amount of water. Watering period was 1 min with a frequency of once every twelve hours. Local potable water was used as the water supply.

4.2.11 Temperature control

Temperature was controlled by an air conditioning unit that is part of the IMBS GMO suite. The day temperature was set to 20°C and the night was set to 16°C (Section 1.4.4). The day period extended from 8 am to 6 pm.

4.2.12 Growth rack

The growth rack was comprised of a painted rectangular steel frame sitting inside a stainless steel tray. The bottom and top of the rack is spanned by wire mesh. The mesh on the bottom prevented the seedlings from sitting in the run off water contained by the tray. The mesh on the top was used to fasten the misting nozzles. The two short sides of the rack were covered with clear polythene to contain the water from the misting nozzle. The long side facing the window of the GMO suite was not covered but the opposite side was covered with white nylon frost mesh to contain the mist while allowing air movement. For a schematic representation see Fig 4.1.

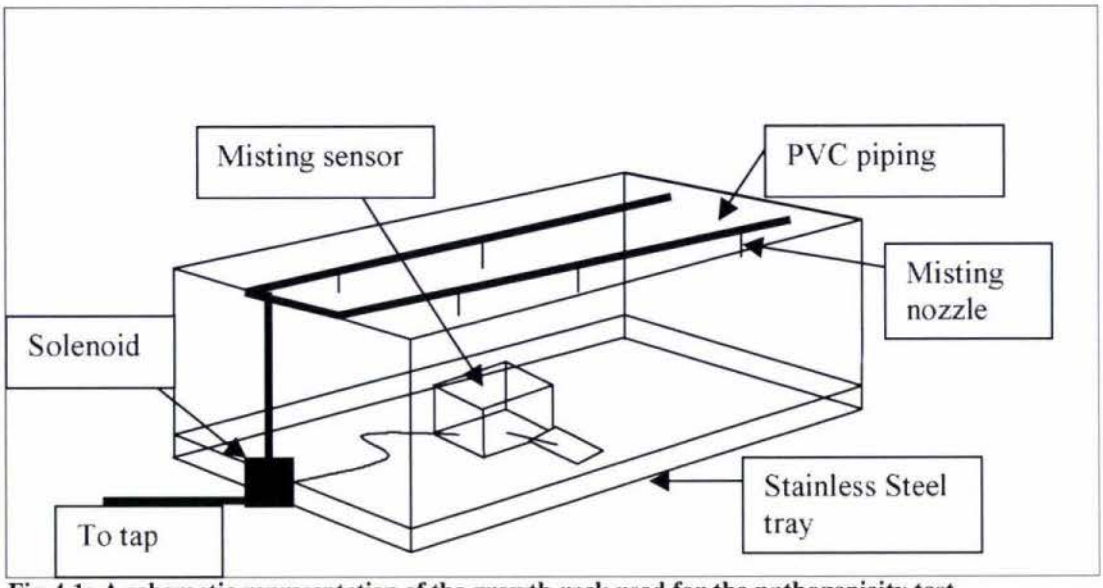


Fig 4.1: A schematic representation of the growth rack used for the pathogenicity test.

4.2.13 Treatments and tree layout

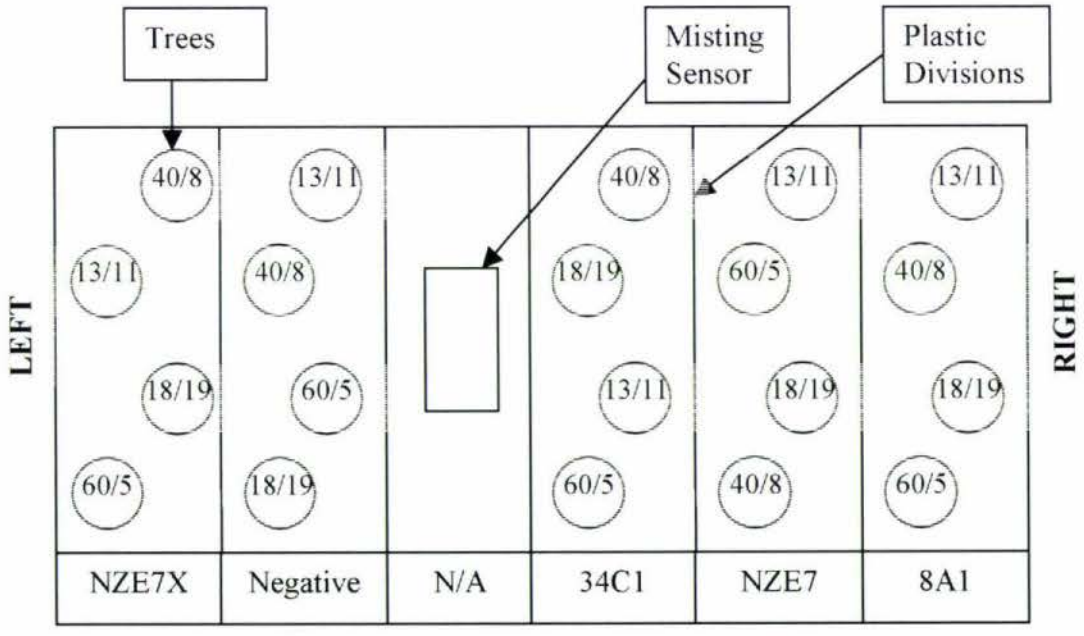


Fig 4.2: Schematic representation of treatment and tree layout showing ramet numbers of trees. NZE7X contains a higher concentration of spores than NZE7 treatment.

NZE7X treatment was placed next to the negative control to investigate possible cross contamination. Other treatment placements were arbitrarily chosen. Tree layout was selected randomly by drawing pieces of paper with the ramet numbers on from a pocket.

4.2.14 Inoculation of *P. radiata* trees

Spore suspensions of the different *D. pini* strains were obtained (Section 2.4.2). 4 ml of each spore suspension was sprayed onto each ramet (Section 2.4.3). The final concentrations of the spore suspensions used to inoculate the ramets in the different treatments were as follows. NZE7X = 5.76×10^7 spores/ml

NZE7, 8A1 and 34C1 were all adjusted to 7.33×10^6 spores/ml with milli-Q water.

NB: A change was made to the protocol in Section 2.4.2 when obtaining the spore suspension. The spores were centrifuged at 3000 rpm in a SS-34 rotor for 5 mins and washed once by resuspension in milli-Q water to remove any dothistromin that may have been in solution with the spores. This was the first time this had been done.

4.2.15 Sampling of needles from treatments

Needles were collected from each tree in each treatment every two days for the first two weeks following inoculation and twice weekly thereafter. The trees were removed from the growth rack and visually assessed before collecting the samples. Two needles from each tree were collected at each of the time points from random locations of the tree. Two dice were used to ensure randomness of the location the needles were selected from. One of the dice was used to identify the vertical region to collect the needle from and the other dice the horizontal. Each tree was divided into 6 vertical sections of equal size by eye. Rolling the number one on the dice corresponded to the bottom section and six corresponded to the top section. Horizontally the tree was split into two sections. Rolling a number between one and three corresponded to the left section and four to six was the right. By combining the numbers from the two dice the location on the tree from which to collect the sample was decided.

At the end of three months following inoculation of the trees all needles were stripped from the trees and counted. Each needle was also inspected for any *D. pini* disease symptoms as well as any other damage.

4.2.16 Microscopy

All microscopy used to visualise epiphytic *D. pini* growth was carried out using calcofluor white staining (Section 3.2.6.5).

4.2.17 Spore viability on PDA and AMM

Spore suspensions were obtained (Section 2.4.2). The spore suspensions were then quantified and standardised to 1 x 10^6 spores/ml before spreading 100 µl of each onto petri-dishes containing PDA (Section 2.2.6) and AMM (Section 2.2.1). Inoculations were done in triplicate. Sections ~1.5 cm x ~1.5 cm were cut from each of the triplicate plates at time intervals of 0, 6, 16, 24 and 48 hours. Two drops of lactophenol cotton blue (Section 3.2.1.1) were pipetted onto the section and covered with a glass cover slip. Roughly 200 spores from each section were counted, noting if the spores had germinated or not. These counts were combined to calculate a mean spore germination percentage. Multiple germ tubes or germ tube length were not investigated.

4.2.18 *D. pini* spore density calculations on inoculated *P. radiata* seedlings

Five randomly selected sections, 5 µm across, were chosen from two needles obtained from each tree contained in a treatment every two days for the first two weeks following inoculation. Measurements of the sections length were made using a calibrated eyepiece micrometer and the number of spores within this section counted. The number of *D. pini* spores contained per mm^2 was calculated. The spore densities of all the sections counted within a treatment were combined and mean and standard errors were calculated for the treatment using Microsoft Excel.

4.3 Results

4.3.1 *DotA* knockout mutant and wild type *D. pini* spore viability

4.3.1.1 Background

Before setting up the pathogenicity test it was necessary to check that the levels of spore viability between the *dotA* knockout mutant and the wild type were similar. This was to ensure that similar numbers of viable spores were inoculated onto the *P. radiata* seedlings. NZE7 (fresh wild type) was not included because it was isolated after this test. Moreover, spore viability on the needles themselves would also be calculated and this would provide a more accurate evaluation when setting up the pathogenicity test. It was assumed NZE7 would have a similar viability to NZE5 (original wild type) as all wild type isolates in New Zealand are thought to be genetically identical (Hirst, Richardson et al 1999).

4.3.1.2 Results

At the time of obtaining the *D. pini* spore suspensions a low level of germination was already present (Day 0, Figures 4.3, 4.4). On both PDA (Section 2.2.6.) and AMM (Section 2.2.1) strains 32 (Δ*dotA*), 8A1 (ectopic) and NZE5 (wild type) showed longer lag times in their germination compared to 34C1 (Δ*dotA*) and 34Y1 (Δ*dotA*). Other interesting features include the significantly higher, 48 hour final levels of spore germination observed on AMM than PDA ($P< 0.001$, $F= 4.20$ with 1, 28 df). At 48 hours there was no significant difference between the germination percentage of the 32, 34Y1, 34C1 and 8A1 strains when grown on PDA ($P= 0.189$, $F= 2.03$ with 3, 8 df), but the wild type NZE5 showed significantly lower levels of spore germination compared to the rest ($P< 0.001$, $F= 14.34$, with 4, 10 df). There was no significant difference between the spore germination of the different strains at 48 hours when grown on AMM ($P= 0.006$, $F= 6.87$, with 4, 10df).

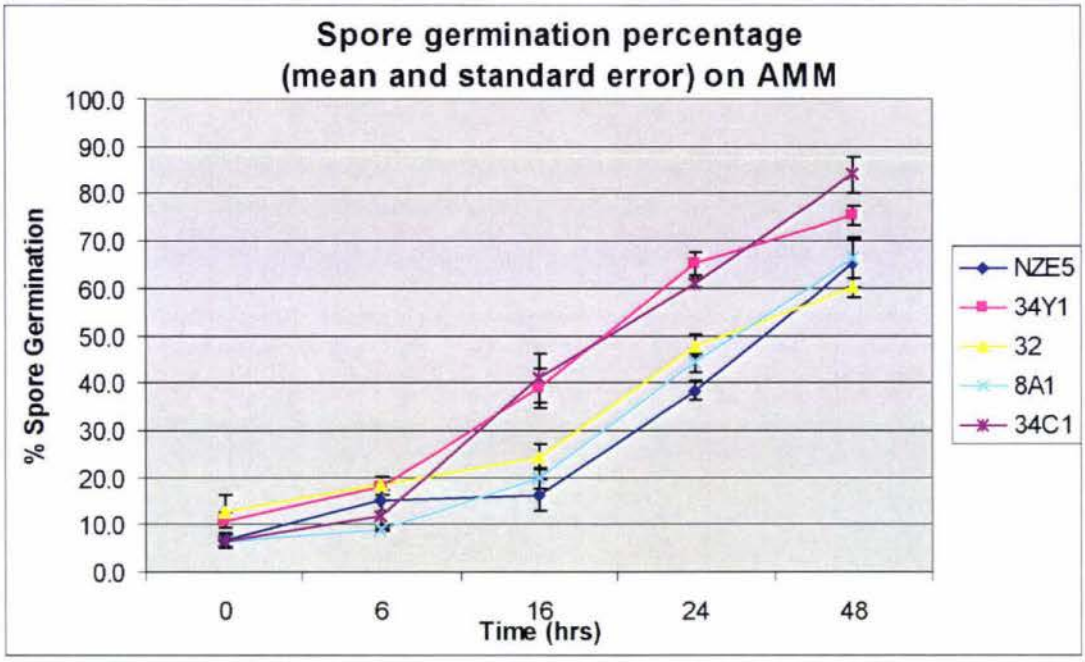


Fig 4.3: Spore germination (mean and standard error) on PDA media was similar for the first 16 hours after which time the wild type *D. pini* showed less of an increase compared to the *dotA* mutants and ectopic *D. pini* strains.

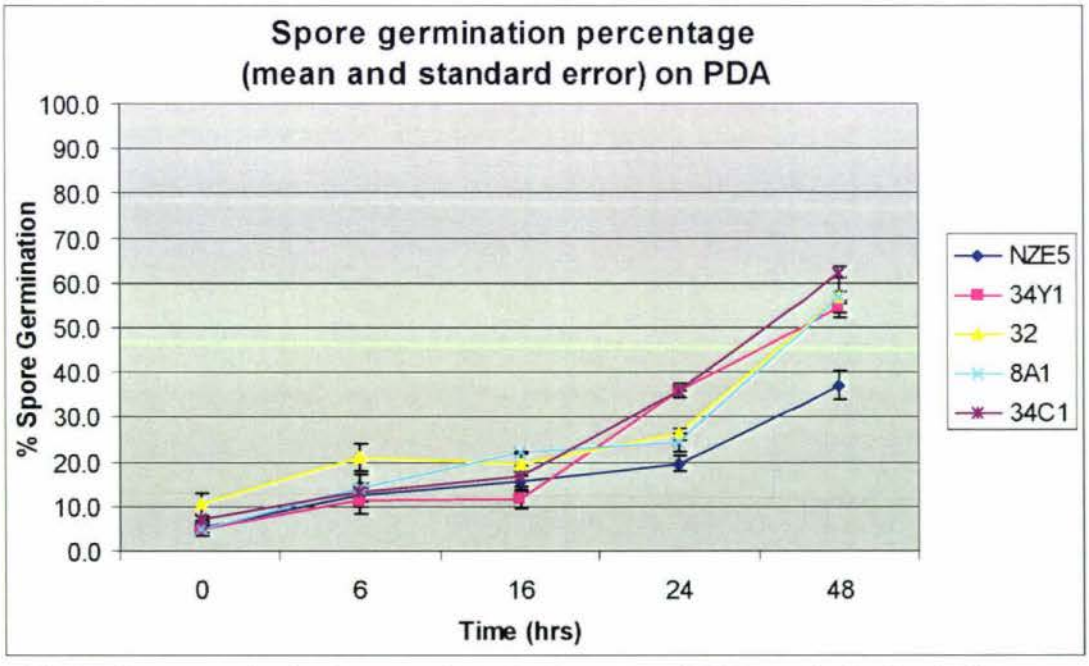


Fig 4.4: Spore germination (mean and standard error) on AMM was similar for the first 6 hours after which time the various strains diverged with 34Y1 and 34C1 showing the highest level of percentage germination.

4.3.2 Development of conditions favourable for *D. pini* infection of *P. radiata*

4.3.2.1 Background

Initial attempts to develop favourable environmental conditions presented problems. To rent space, for the length of time required, in a PC2 room in the 'Climate Control Laboratory', Palmerston North was far too expensive so attempts were made to modify existing constructs present on the Massey University campus. Several constructs and locations were trialled until a metal frame in a stainless steel tray was obtained and space became available in the Institute of Molecular Biosciences (IMBS) GMO Suite.

4.3.2.2 Lighting

Background

Fluorescent grow bulbs were trialled and found not to provide enough light intensity so Metal Halide (MH) and High Pressure Sodium (HPS) lamps were investigated (Section 4.2.6).

Results

Sun shone through the window of the GMO suite in the morning but not in the afternoon due to shading by the building housing the GMO suite. This high light intensity in the morning is reflected in the light measurements taken (Tables 4.2 – 4.5). PAR was measured in μmol s^{-1} m^{-2} and LR in W/m^2. The auxillary lights supplied an additional ~ 250 μmol s^{-1} m^{-2} (107 W/m^2) to the left side, ~ 330 μmol s^{-1} m^{-2} (150 W/m^2) to the middle and 130 μmol s^{-1} m^{-2} (57 W/m^2) to the right hand side on a sunny day.

On a cloudy day the auxillary lights supplied an additional ~ 285 μmol s^{-1} m^{-2} (127 W/m^2) to the left side, ~ 377 μmol s^{-1} m^{-2} (161 W/m^2)to the middle and ~ 145 μmol s^{-1} m^{-2} (61 W/m^2) to the right hand side. Because of the afternoon shading and due to natural fluctuations some of the overcast day light intensities exceed those on a sunny day.

The auxillary lights were effective at maintaining a level of light intensity close to the desired 133 W/m^2 but the right side did not receive as much light from the auxillary light as did the left and middle. Incorrect installation of the middle 400W HPS bulb resulted in a slight orientation of the bulb towards the left side altering the distribution of light. This problem was found at the end of the pathogenicity test so the light levels seen in Tables 4.2 – 4.5 are the light levels that were present during the pathogenicity test.

Sunny (µmol s^{-1} m^{-2})				
	Time	Left	Mid	Right
Lights on	10:00 a.m.	762.7	897.2	662.1
	1:00 p.m.	288.1	391.0	211.7
	4:00 p.m.	274.0	355.7	165.94
Lights off	10:00 a.m.	519.1	570.8	591.7
	1:00 p.m.	35.39	36.87	33.03
	4:00 p.m.	14.51	14.94	13.66

Table 4.1: PAR in growth rack on a sunny day.

Overcast (µmol s^{-1} m^{-2})				
	Time	Left	Mid	Right
Lights on	10:00 a.m.	331.7	430.1	191.2
	1:00 p.m.	325.0	429.8	188.1
	4:00 p.m.	308.4	391.8	172.0
Lights off	10:00 a.m.	50.14	55.02	55.40
	1:00 p.m.	39.69	50.60	42.05
	4:00 p.m.	12.78	13.68	11.90

Table 4.2: PAR in growth rack on an overcast day.

Sunny (Watts/m^2)				
		Left	Mid	Right
Lights on	10:00 a.m.	340	410	320
	1:00 p.m.	125	165	80
	4:00 p.m.	115	155	70
Lights off	10:00 a.m.	230	260	270
	1:00 p.m.	13	13	13
	4:00 p.m.	4.6	4.9	4.6

Table 4.3: LR in growth rack on a sunny day.

Overcast (Watts/m^2)				
		Left	Mid	Right
Lights on	10:00 a.m.	150	190	85
	1:00 p.m.	150	190	85
	4:00 p.m.	130	160	65
Lights off	10:00 a.m.	27	27	27
	1:00 p.m.	18	19.5	18.5
	4:00 p.m.	4	4.2	3.5

Table 4.4: LR in growth rack on an overcast day.

4.3.2.3 Leaf wetness

During early trials involving the misting system a lot of secondary fungal growth was observed on the *P. radiata* seedlings. The high humidity produced by the misting system combined with a lack of air movement was thought t o be responsible. The lack of air movement was caused by the construct having clear polythene sheets on all sides. Air movement needed to be improved with the hopes of reducing the likelihood of this secondary fungal growth. To achieve this, the polythene from the back of the construct was removed and frost mesh replaced the polythene on the front. This combined with the high level of air movement in the GMO suite, due to the fan forced air from the air conditioning unit, was sufficient to reduce humidity while maintaining leaf wetness.

Several other problems were faced once the misting system was set up in the GMO suite. Two months into the pathogenicity trial it was noticed that the air movement over the misting sensor, caused by the air conditioning unit, occasionally prevented the swing arm moving to the up position, preventing the misting system being turned on. This was remedied by placing a plastic division at the back of the section containing the misting sensor preventing air movement disrupting the sensor. The sensor was also adjusted to provide continuous leaf wetness at this point because the sensor took a lot longer to dry out with reduction the in air movement.

Another malfunction of the misting system was caused by the mesh of the misting system sensor getting caught on one of the plastic divisions that had suddenly shifted. On this occasion this caused the misting system to stay on for a period of 20 minutes, spraying the trees with continuous a supply of water. This occurred thirty four days after inoculation of the trees.

The use of a potable water supply meant chlorinated water was being used to mist the trees. This may have had an adverse effect on *D. pini* growth and will be discussed in Section 4.4. The water supply also caused miner al deposits to build up on the surface of the needles (Figs 4.5, 4.6).

Fig 4.5: Pine needle with mineral deposits on the tip. Deposits had been cleared away from a section seen on the right for contrast.

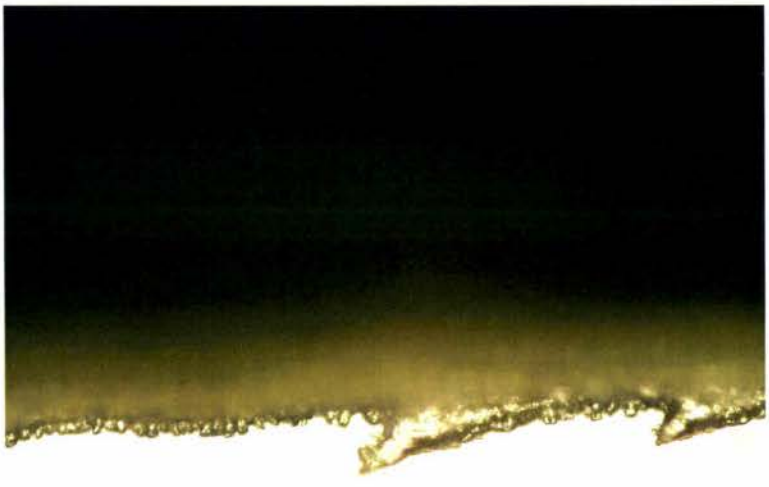


Fig 4.6: Bright field microscopy image of the edge of pine needle showing crystalisation of mineral deposits on the needle surface. 200X magnification.

4.3.2.4 Temperature

Temperature should have been controlled by the GMO suite monitoring system over the length of the pathogenicity test but on examination of data recorded by a data logger at the end of the test this was found not to be the case. Change over of the control systems in the GMO suite had led to a spike in temperature up to 28°C for a period of 5 minutes 10 days after inoculation of the *P. radiata* seedlings. The two weeks following this spike temperature was within +/ - 3°C except for one spike up to 25°C (+ 5°C). Due to improper setup of the data logger information was not collected during the main time period of the pathogenicity test. The last week and a half of pathogenicity tests showed 11 major temperature spikes up to ~ 33°C. The cause of these spikes was later identified as a leak in the air conditioner's compressor. It is not clear when this fault developed. These problems mean temperature was not controlled thought out this experiment and may have adversely affected *D. pini* growth.

4.3.3 Confirmation of *D. pini* strains identity

4.3.3.1 Background

ITS sequencing was chosen to confirm the identity of the different strains used in the pathogenicity test because of its previously proven usefulness in characterising *D. pini* (Hirst, Richardson et al. 1999).

4.3.3.2 Results

The ITS sequences obtained from NZE5 (wild type), 8A1 (ectopic), 34C1 (Δ*dotA*), and 34Y1 (Δ*dotA*) were used to search against nucleotide sequences on the NCBI nucleotide database. NZE5, 8A1, 34Y1 all had *Mycosphaerella pini* as their top scores. Based on this information and pigment production of the fungal strains in media it was decided that the fungal samples were indeed *D. pini*.

4.3.4 *D. pini* spore density and germination on inoculated *P. radiata* seedlings

4.3.4.1 Background

D. pini spore viability had already been investigated when inoculated onto media (Section 4.3.1) but it was necessary for the accuracy of future results, based on observations, to calculate the viability of *D. pini* spores inoculated onto the seedlings as well as their density.

4.3.4.2 Results

Based on the samples taken, the different treatments received similar quantities of inoculum with the 8A1 treatment being slightly higher than the others (Day 0, Fig 4.7). The spore density steadily decreased over the following two weeks. A steady base line value was not observed. The fact only small regions of the needles were analysed introduces variation into the observed spore density, explaining the short term increase in spore density (Fig 4.7). Clustering of spores may also have contributed to this variation.

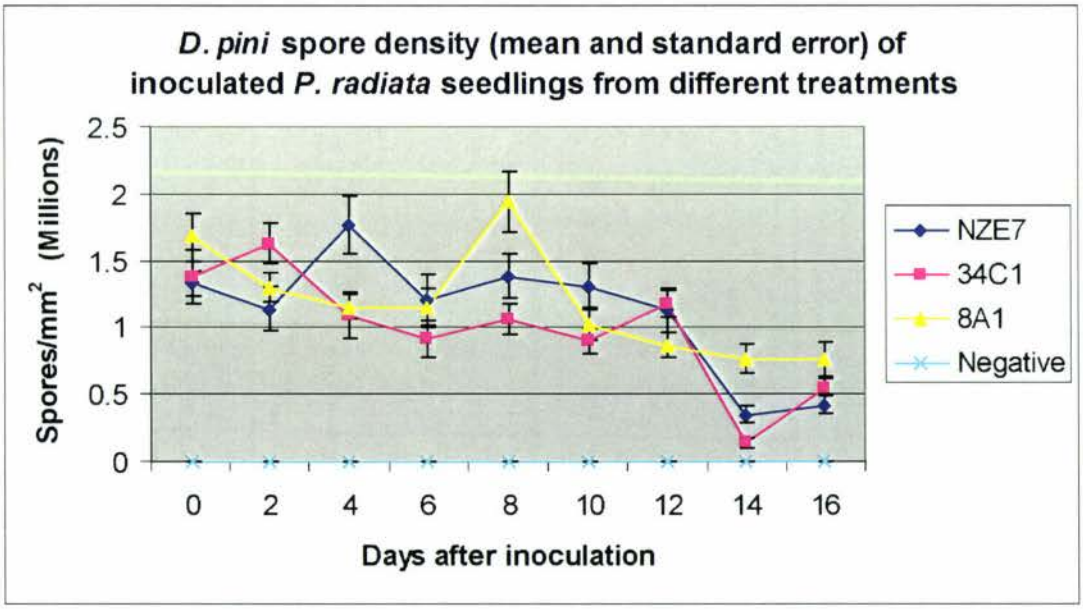


Fig 4.7: Mean *D. pini* spore density within all treatments of *P. radiata* seedlings over the first sixteen days following inoculation.

D. pini spore germination levels were at 5-8 % at the time of inoculation (Fig 4.8). The percentage of germination within the different treatments increased in a linear manner before levelling off at between 40-50 % after eight days (Fig 4.8).There was no significant difference in the spore germination percentage past the eight day time point (10 day P=0.375, F=0.990, with df= 2, 117; 12 day P=0.062, F=2.852, with df= 2, 111; 14 day P=0.337, F=1.101, with df=2,95; 16 day P= 0.625, F=0.473, with df= 2,112). A small decrease in spore germination percentage was observed after 10 days (Fig 4.8).

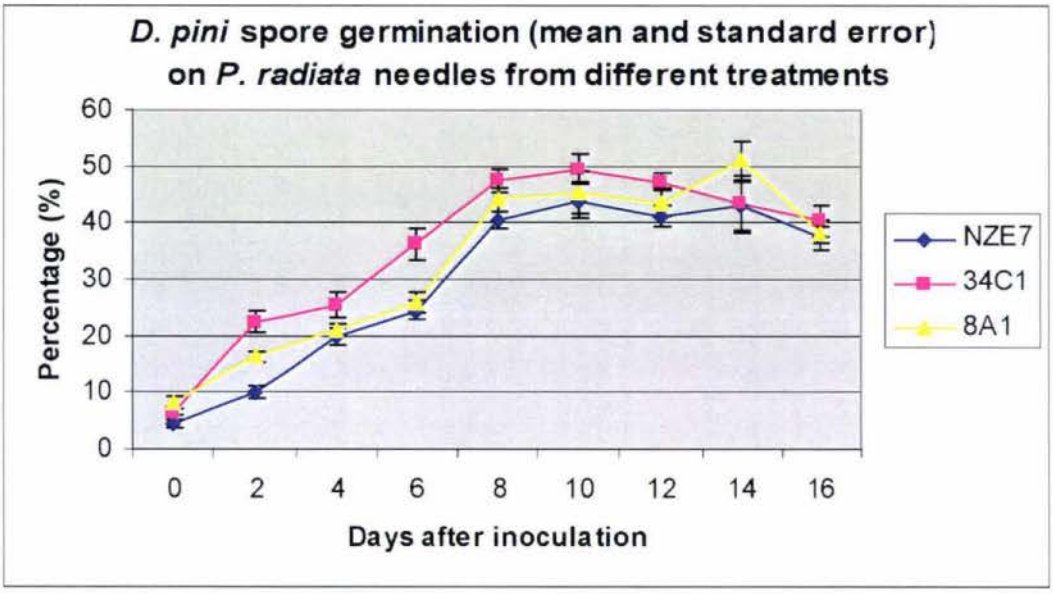


Fig 4.8: Mean *D. pini* spore germination within treatment of *P. radiata* seedlings over the first sixteen days following inoculation.

4.3.5 Monitoring of epiphytic growth by fluorescent microscopy

Inoculation of the *P. radiata* trees generally appeared uniform (Fig 4.9) but clusters of spores were also observed, especially on trees in the NZE7X treatment which had been inoculated with a higher concentration of spores (Fig 4.10). Low levels of germination were visible on day 0 and some of the germ tubes in the 8A1 treatment were quite well developed (Fig 4.11). However samples taken from the 8A1 treatment on days after Day 0 did not show any evidence of spores with well developed germ tubes and showed low levels of germ tube extension similar to the other treatments. Spore germination increased over the first eight days (Day 6, Fig 4.12) but germ tube

length was not as long as observed in the 8A1 treatment (Fig 4.11). A possible stomatal penetration event was observed on day 12 in the NZE7 treatment but the lack of contrast made it impossible to confirm (Fig 4.13). For all treatments generally reorientation of germ tubes towards stomata was not observed (Fig 4.13, 4.14). However some germ tubes did appear to grow to wards stomata (Fig 4.18). Germ tube development after day 12 was generally non-existent with germ tubes not extending or showing any further growth except for the occasional sample that showed more significant growth (Fig 4.17). The NZE7X treatment, which received a higher number of spores than the other treatments, showed clusters of spores where stomatal penetration had every chance of occurring (Fig 4.15). Day 21 images showed the first stages of the mineralization caused by the misting system (Section 4.3.2.2)(Fig 4.16). This problem continued to get worse from day 21 onwards (Fig 4.19). No changes in growth were observed from the observations mentioned above. Spores were still present at the end of the test but their appearance was the same as those observed early in the test so were assumed dead or dormant (Fig 4.20).

NB: Representative photos were taken of each treatment every two days for the first two weeks. From the two-week point onwards photos were taken of representative sections of needle from each tree in the pathogenicity test twice weekly. Only photos that convey something of note have been included in Figures 4.9 – 4.20.

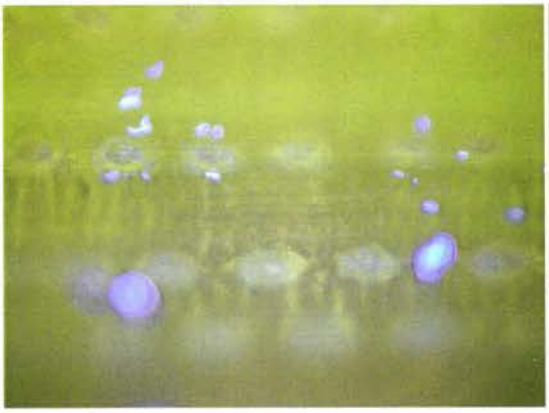


Fig 4.9: Needle taken from NZE7 treatment, 60/5 seedling, Day 0. 200X

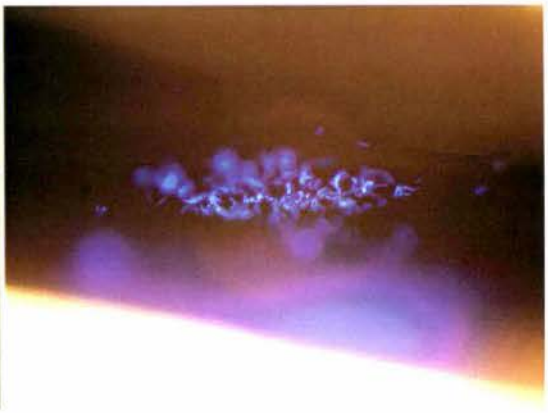


Fig 4.10: Needle taken from NZE7X treatment, 60/5 seedling, Day 0. 200X

Fig 4.11: Needle taken from 8A1 treatment, 13/11 seedling, Day 0. 200X

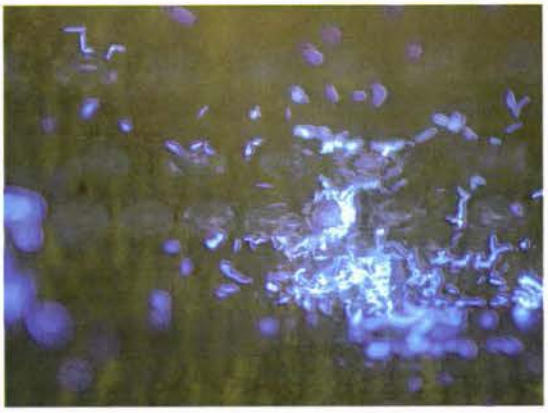


Fig 4.12: Needle taken from NZE7 treatment, 60/5 seedling, Day 6. 200X

Fig 4.13: Needle taken from NZE7 treatment, 40/8 seedling, Day 12. 200X

Fig 4.14: Needle taken from 8A1 treatment, 40/8 seedling, Day 12. 200X

Fig 4.15: Needle taken from NZE7X treatment, 40/8 seedling, Day 21. 200X

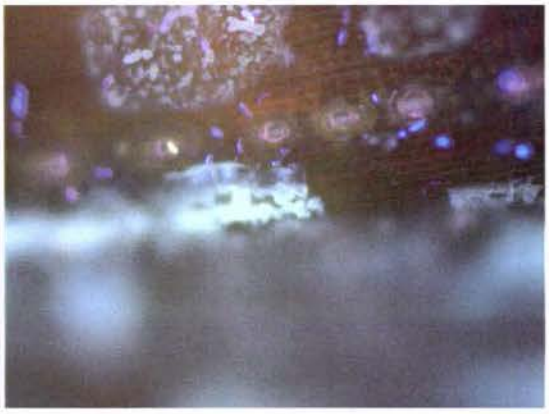


Fig 4.16: Needle taken from 34C1 treatment, 13/11 seedling, Day 21. 200X

Fig 4.17: Needle taken from 34C1 treatment, 60/5 seedling, Day 26. 200X

Fig 4.18: Needle taken from 8A1 treatment, 13/11 seedling, Day 31. 200X

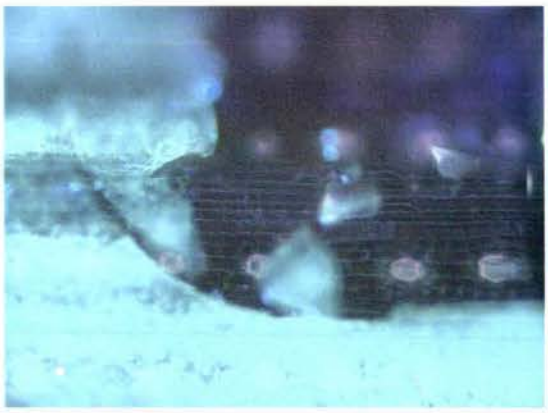


Fig 4.19: Needle taken from 34C1 treatment, 18/19 seedling, Day 48. 200X

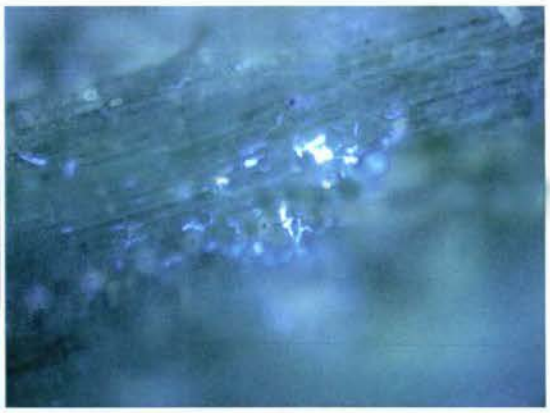


Fig 4.20: Needle taken from 34C1 treatment, 18/19 seedling, Day 83. 200X

4.3.6 Visual observations of trees from different treatments

4.3.6.1 Background

D. pini infection of *P. radiata* causes several characteristic symptoms visible by eye. These include necrotic lesions containing a dark red band that can later develop dark fruiting bodies that rupture the epidermis of the pine needle . Visual monitoring of the trees was used to screen for these and any other physical changes in the trees.

4.3.6.2 Results

Three main types of damage were observed to develop on the trees during the course of the pathogenicity test. The first, and most common, was necrotic lesions. Red bands were not observed in any of the examined lesions. Lesions ranged from small (Fig 4.21), less than 1 mm, to large (Fig 4.22), 3 -4 mm. Several lesions were often on the same needle (Fig 4.23) and sometimes joined to form a larger lesion. Lesions were occasionally in the same position on different needles within a fascicle (group of 3-4 needles) (Fig 4.24). The second kind of damage was loss of pigment from the needle tissue (chlorosis) (Fig 4.25) and the third was tip browning (Fig 4.26).

Damage to the trees was first observed after nineteen days when detailed inspection of the trees began. The types of damage observed on each tree persisted to the end of the pathogenicity test showing a small increase in severity over the time of the test. The damage was present at similar levels throughout all of the treatments including the negative control. The bottom third of the trees were the most commonly seen with damage and the top third the least. All types of damage were observed in the top, middle and bottom thirds of the trees. For more information regarding the type and position on the tree of the damage see appendix. Also located in the appendix are counts of the different types of damage obtained at the end of the pathogenicity test when all the needles from each tree were counted and inspected for damage.

Fig 4.21: Small lesion (< 1 mm) on the base of a *P. radiata* fascicle (Group of 3-4 needles) taken from pathogenicity test.

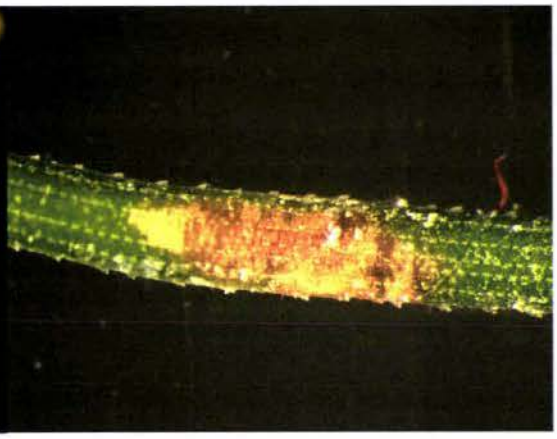


Fig 4.22: Large lesion (3 mm) on a *P. radiata* needle taken from pathogenicity test.

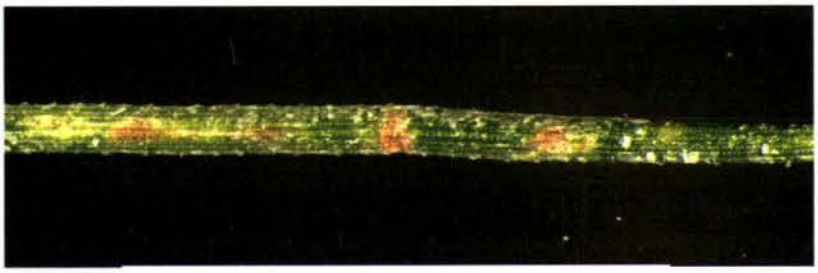


Fig 4.23: Multiple lesions (< 1 mm) on a *P. radiata* needle taken from pathogenicity test.

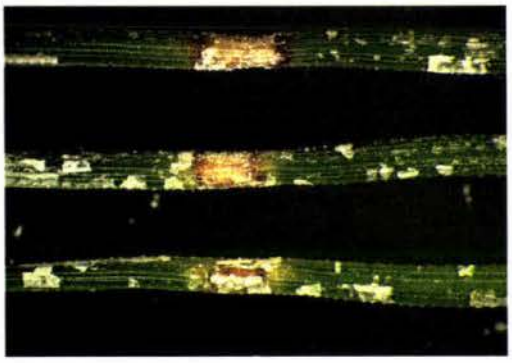


Fig 4.24: Lesions in the same position on different *P. radiata* needles within the same fascicle needle taken from pathogenicity test.

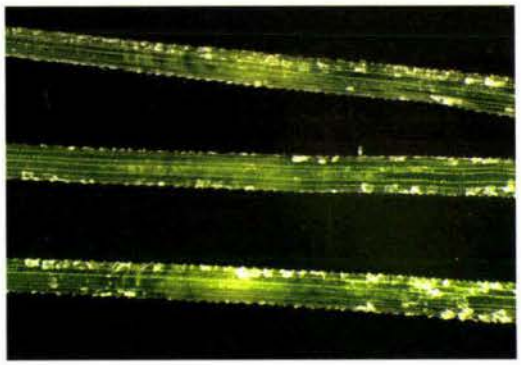


Fig 4.25: Chlorosis in the same position on different *P. radiata* needles within the same fascicle needle taken from pathogenicity test.

Fig 4.26: Tip browning of a *P. radiata* needle taken from pathogenicity test.

4.3.7 Percentage of damaged foliage

4.3.7.1 Background

At the end of the pathogenicity test all the needles were stripped from the trees and counted. The needles were also inspected for *D. pini* disease symptoms and any damage. Counts were made of the number and types of damage observed. It was also noted in what combination the damage symptoms appeared in on single needles.

4.3.7.2 Results

It was observed that all three of the damage types (lesions, chlorosis and tip browning) were capable of appearing on the same needle but this occurrence was not common (Fig 4.29 -4.33). Significant differences were seen between the total damage of the different *D. pini* treatments ($P < 0.001$, chi-square value = 91.87 with 1 df) but no significant difference was seen between the different ramet groups ($P = 0.154$, chi-square value = 2.03 with 1 df) (Fig 4.27, 4.28). Lesions were the most common form of damage with often multiple lesions appearing on a single needle. Needle exhibiting multiple lesions were put into three groups, 'one lesion', 'two lesions' or '3 or greater lesions' (Fig 4.29 – 4.33). Figures 4.29 through 4.33 are Ven diagrams showing the frequency of different types of damage observed on individual needles. Where more than one type of damage was observed on an individual needle (eg, two lesions and chlorosis) this was marked in the area where the 'two lesions' and 'chlorosis' regions of the diagram overlap.

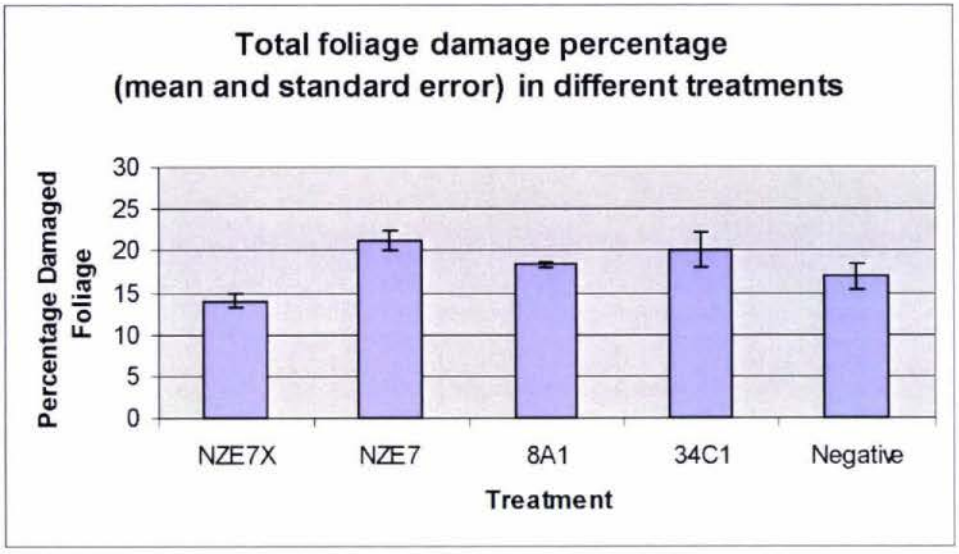


Fig 4.27: Mean percentages of total damaged foliage (Lesions, chlorosis and/or tip browning) in the different treatments after collecting and analysing all pine needles from the different treatments (except 40/8), at the end of the pathogenicity test.

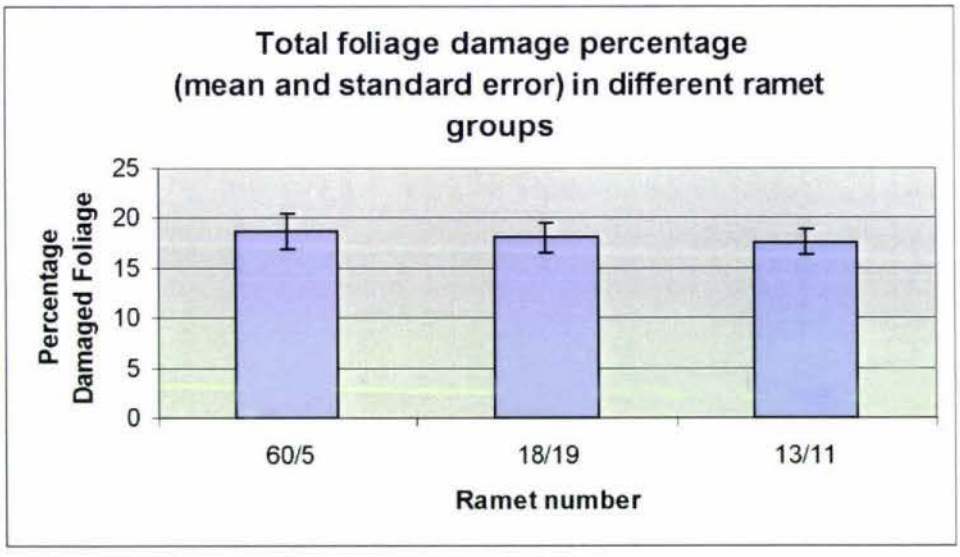
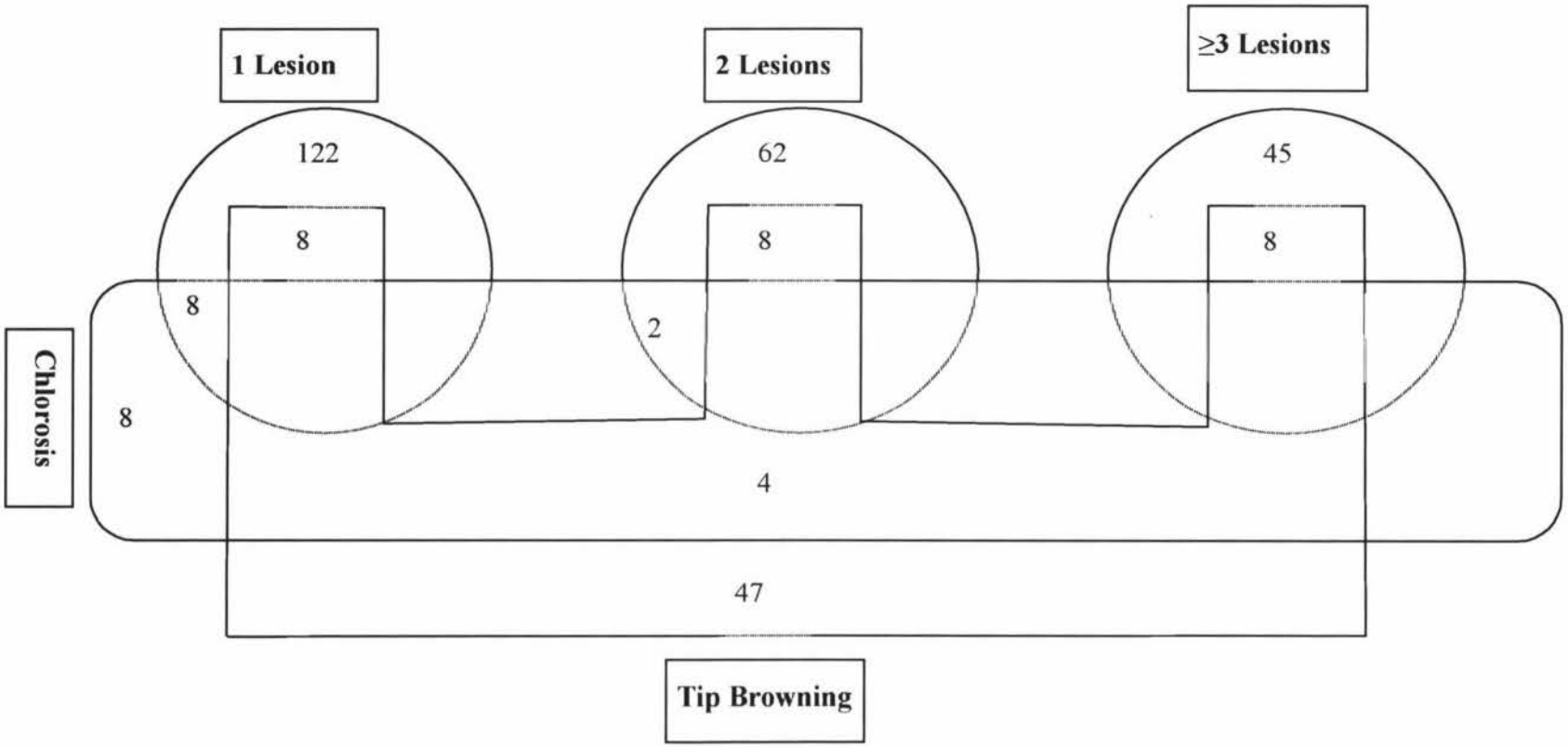
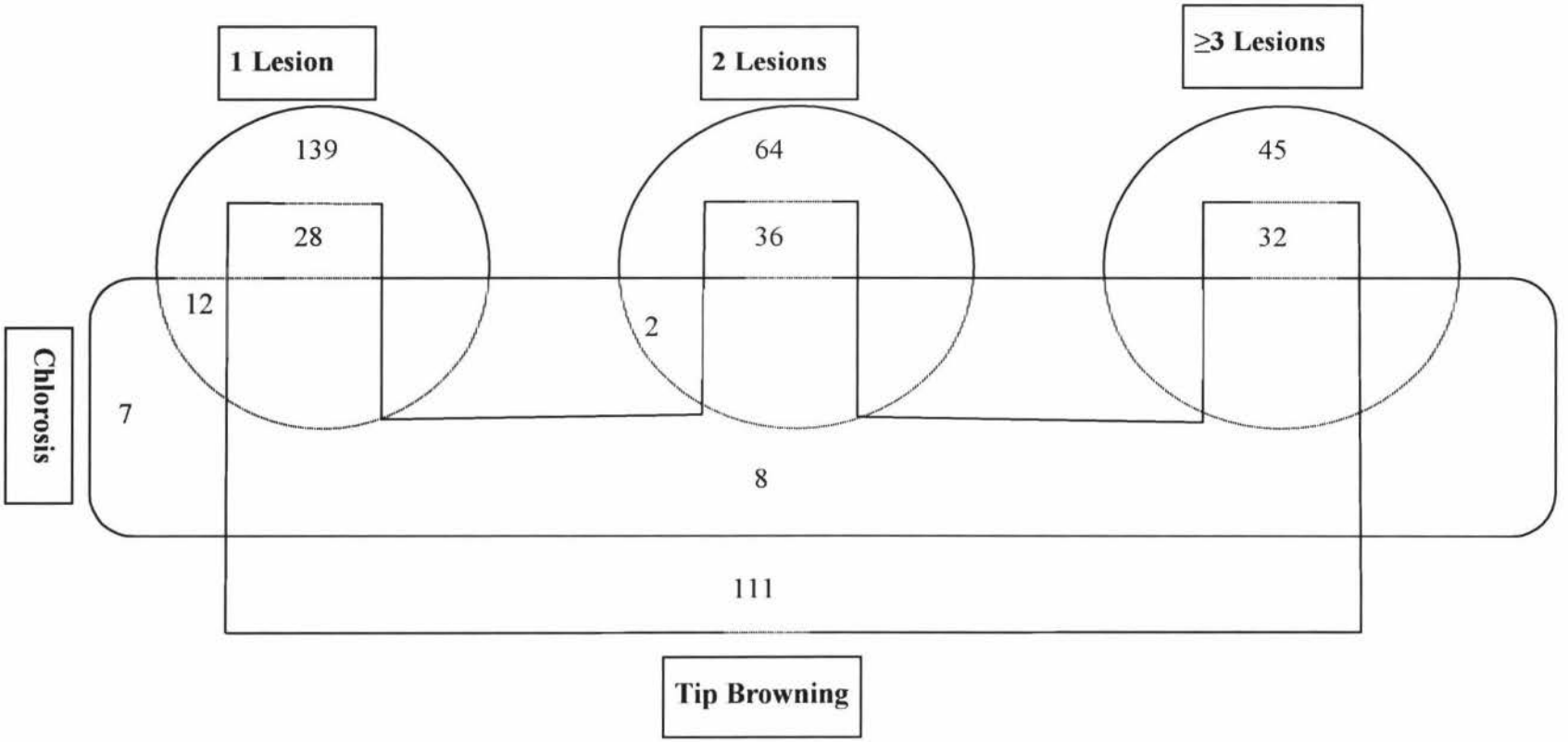


Fig 4.28: Mean percentages of total damaged foliage (Lesions, chlorosis and or tip browning) in the different ramet groups after collecting and analysing all pine needles from the different treatments, except 40/8, at the end of the pathogenicity test.



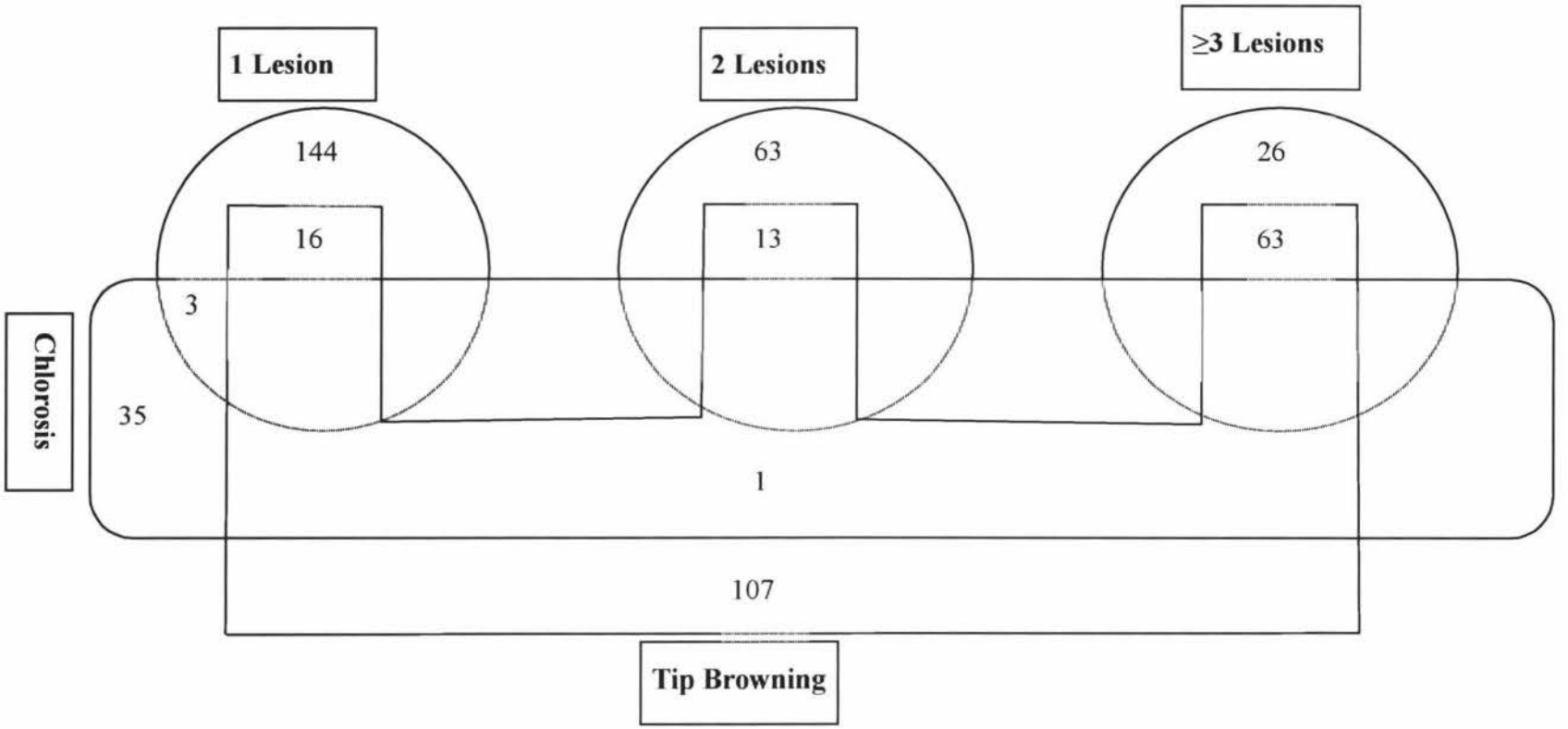
NZE7X
Total Pine Needles= 2596
Total Damaged Needles = 322

Fig 4.29: Diagram showing observed combinations and quantities of damage to treatment NZE7X.



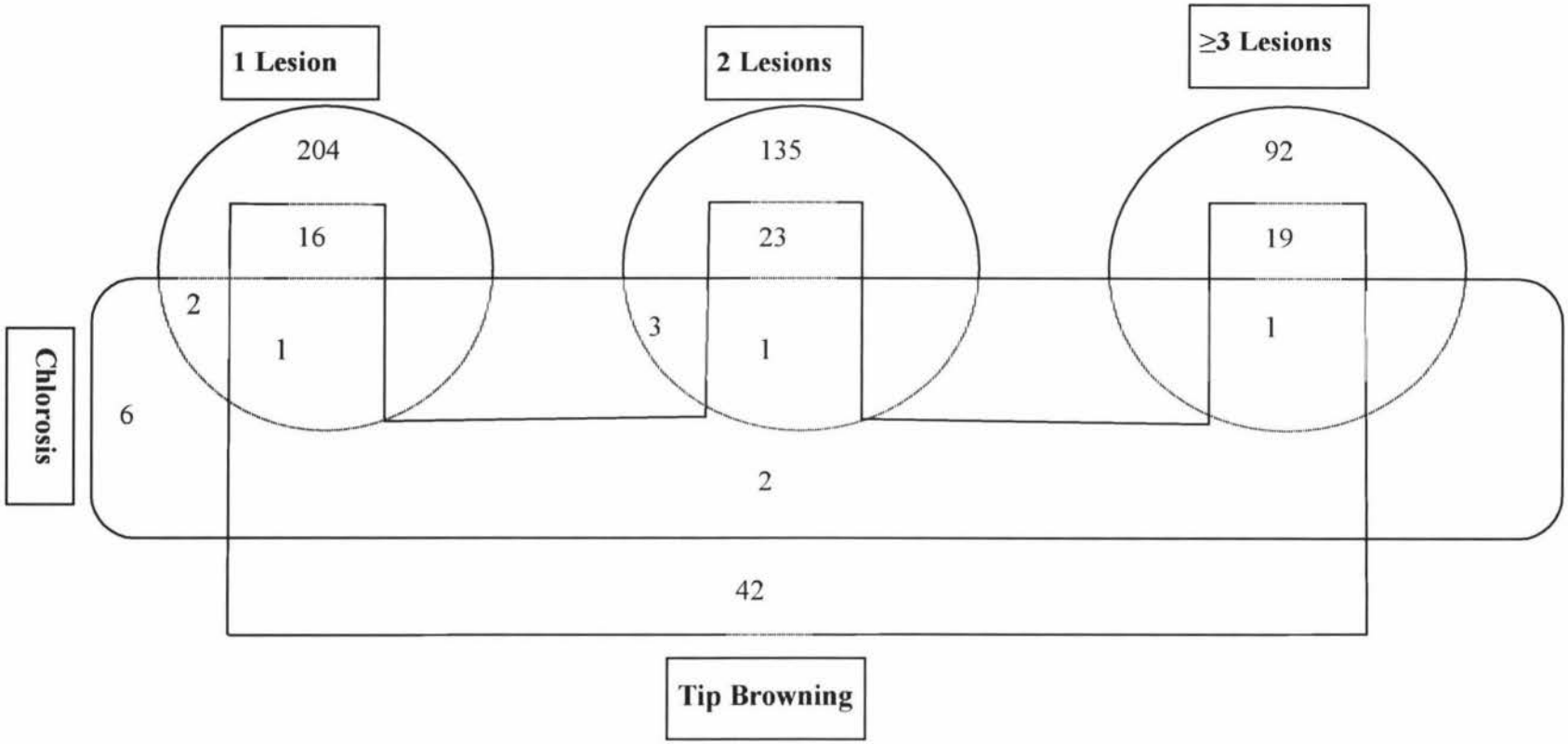
NZE7
Total Pine Needles = 2481
Total Damaged Needles = 482

Fig 4.30: Diagram showing observed combinations and quantities of damage to treatment NZE7.



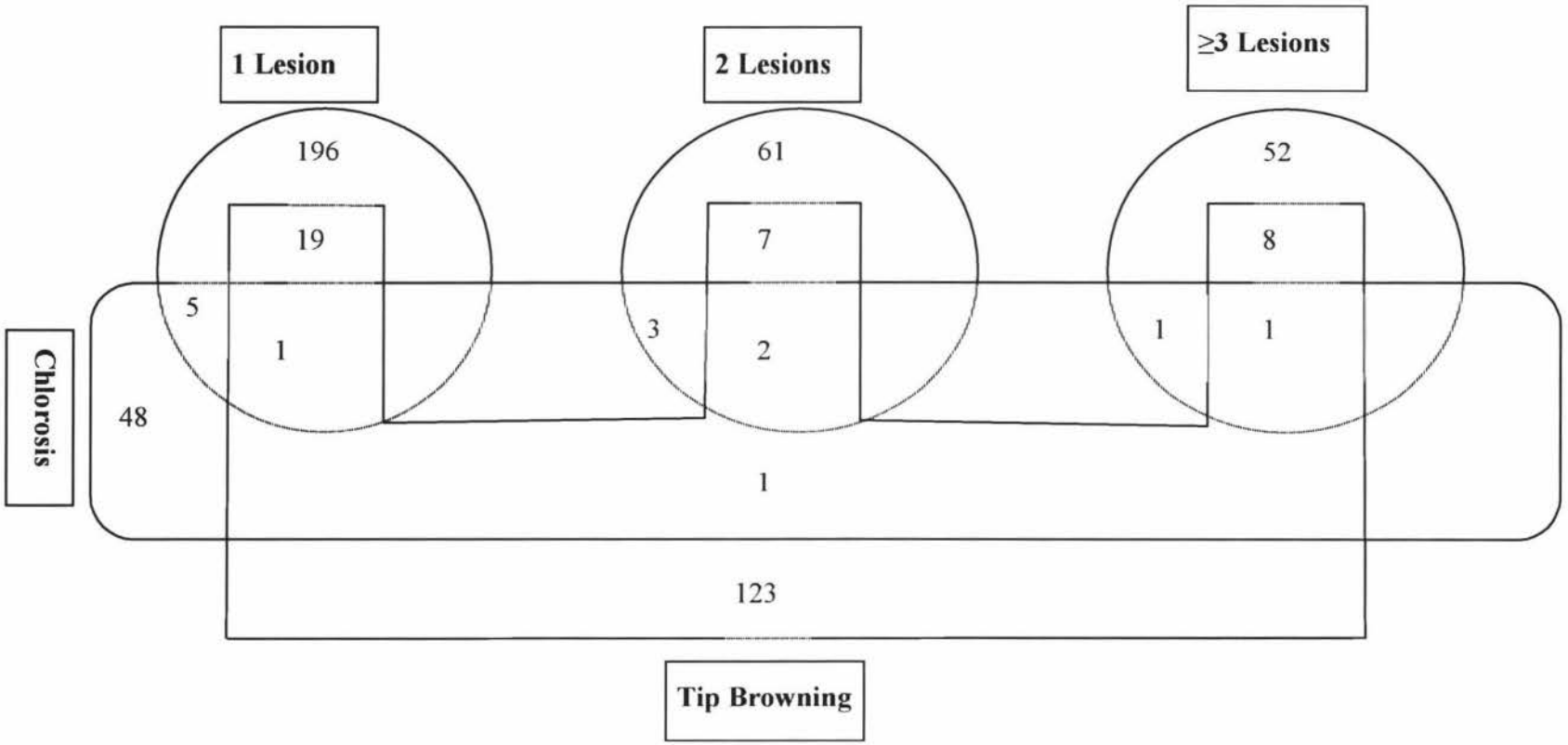
8A1
Total Pine Needles = 2273
Total Damaged Needles = 471

Fig 4.31: Diagram showing observed combinations and quantities of damage to treatment 8A1.



34C1
Total Pine Needles = 2678
Total Damaged Needles = 547

Fig 4.32: Diagram showing observed combinations and quantities of damage to treatment 34C1.



Negative
Total Pine Needles = 3267
Total Damaged Needles = 528

Fig 4.33: Diagram showing observed combinations and quantities of damage to treatment Negative.

4.4 Discussion

There was no significant difference in spore viabilitywhen the *dotA* knockout *D. pini* and wildtype or ectopic strains were inoculated onto pine needles or AMM media . It is not clear why NZE7 had a lower spore viability th an the other *D. pini* strains when grown on PDA, but it is possible the low viability was due to something during spore collection rather than the strain itself. As expected spore germination occurred a lot faster on PDA and AMM media compared to the needle surface. Studies of *in vitro* spore germination have yielded germination percentages as high as 100% (Sheridan and Yen 1970) but more time points were needed in the media spore germination trial done in this study to determine the maximum percentage of germination. The fact that spore germination percentages on AMM were higher than those on PDA suggests carbohydrate is an important factor in germination. Spore germination on the needle surface took a lot longer than has been observed in other studies taking eight days to reach its maximum. The original study that investigated *D. pini* infection in detail of *P. radiata* showed about 70% germination after 48 hours (Gadgil 1967), but other studies have shown levels of spore germination similar to the ones seen in this study (Gadgil 1976). Washing of the spore suspension to remove dothistromin and other media components was not mentioned in either of these studies, but may have contributed the slow and low germination levels seen in this study. It would be interesting to investigate this by setting up an inoculation trial of *P. radiata* trees using washed versus unwashed spores but time did not allow this. The fact no significant difference was observed in spore viability on the needle surface rules out spore viability as a complicating factor in future pathogenicity trials.

The environmental conditions within the pathogenicity trial may also have adversely affected *D. pini* germination and growth. As mentioned several temperature spikes were observed during the pathogenicity test. The first during the early stages of the test was 28°C, which has been shown to inhibit germination by 90% (Sheridan Yen 1970). This may have been another possible contributing factor to the low level of germination observed during the test. Disease symptoms have been shown to occur over a temperature range of between 5 and 26°C (Gilmour and Crockett 1972) but

information about the effects of temperature above 26°C on mycelial growth could not be found. However, it can be stated that the temperature spikes observed at the early stages of the test up to 28°C and the spikes up to 33°C were not conducive to disease development.

The problems introduced with the misting system mentioned in Section 4.3.2.2, also contributed to the problems faced with environmental conditions. The use of chlorinated water for misting and watering was identified as a potential problem during the set up of the environmental conditions but it was decided to use chlorinated water because no unchlorinated water supply was readily available and obtaining one would have been difficult and expensive. While the effect of chlorinated water on *D. pini* growth has not been investigated it may have been a factor in the inhibition of *D. pini* growth. Mineral precipitation on the surface of the needle also was a problem caused by the misting system. The composition of the mineral deposits was unknown but they dramatically changed the topography of the needles surface possibly altering the mycelial growth direction and adhesion to the needle surface. The chemical composition of the needle surface would also have been altered, possibly affecting *D. pini* growth. The increased osmotic potential of the needle surface caused by the deposits would also have limited the amount of free water available. To eliminate this problem in the future a deionisation filter and carbon filter could be installed in line with the misting system to remove minerals and chlorine from the water.

The problems encountered with the misting sensor also meant leaf wetness may not have been continuous throughout the course of the pathogenicity test. These problems have been identified and fixed (Section 4.3.2.2) and should not present any problems for future work. With these changes, the misting system is capable of supplying the continuous leaf wetness important for *D. pini* disease development.

The lighting system supplied light intensities on both sunny and cloudy days that were consistent with achieving infection percentages of 40-50% of foliage on the right side which received the lowest light intensity (Gadgil 1976). The light intensity levels in the middle and left were consistant with obtaining 80-90% infected foliage (Gadgil 1976). The lighting was important for maintaining a high light intensity on cloudy days and for maintaining a day period similar to that seen in summer.

Visual assessment of the *P. radiata* trees throughout the pathogenicity test failed to identify any Dothistroma disease symptoms but several kinds of needle damage were observed. For the first four weeks of the pathogenicity test metal tweezers were used to collect needle samples from the trees. It was thought that sampling with the tweezers had inadvertently damaged other needles on the same tree. From the four week point on, gloved hands were used to collect needles. The occurrence of some of the lesions observed, like the ones seen in Fig 4.22, where the lesion was on the same section of different needles in close proximity to each other suggests that the tweezers were responsible for some of the damage observed. However the appearance of chlorosis despite no obvious physical damage (Fig 4.23) shows that the tweezers were not responsible for all the damage observed on the trees. The development of more damage, including lesions, that was observed despite the change in sampling methods also supports this idea. Adverse environmental factors may also have contributed to the damage observed including temperature spikes and mineral precipitation on needle surfaces. The significant differences in needle damage percentage between the different treatments could also be a function of environmental factors ie. microclimates in different parts of the test rack containing the treatments, given that the negative control also sustained a lot of damage. A repeat of the experiment with the treaments located in different areas of the rack could address this. The fact no significant difference was observed between the ramet groups supports this idea as their random layout would have negated the microclimate effect. Future experiments could possible include ramet groups with higher levels of resistance to *D. pini* to identify whether or not the damage observed is the result of inoculation with *D. pini* or environmental factors.

Microscopic examination of epiphytic *D. pini* growth showed low levels of *D. pini* growth throughout the pathogenicity test supporting the absence of disease symptoms observed by visual inspection.

In summary environmental conditions within the pathogenicity test were not ideal for *D. pini* infection of *P. radiata* and probably have inhibited disease progression resulting in the absence of Dothistroma disease symptoms. However several problems have been identified and solutions either implemented or proposed. I am confident

that once these remaining proposed solutions are implemented an environment encouraging *D. pini* infection will be achieved.

CHAPTER 5: TRANSFORMATION OF *D. PINI* WITH GREEN FLUORESCENT PROTEIN (GFP)

5.1 Introduction

The green fluorescent protein (GFP) was originally isolated from the bioluminescent jellyfish, *Aequorea victoria*. It is a very good biomarker due to the fact it only requires oxygen to fluoresce and rarely causes adverse effects when expressed in different organisms (Chalfie, Tu et al. 1994).

GFP has been increasingly used as a biomarker and reporter in different phytopathogens including species of *Fusarium*, *Colletotrichum*, *Aspergillus*, *Cercospora*, *Alternaria* (Fernandez-Abalos, Fox et al. 1998; Lorang, Tuori et al. 2001; Tavoularis, Scazzocchio et al. 2001; Chung, Ehrenshaft et al. 2002; Horowitz, Freeman et al. 2002; Chen, Hsiang et al. 2003; Isshiki, Ohtani et al. 2003; Oren, Ezrati et al. 2003). Advances in the understanding of GFP's mode of action and the fact that it is becoming more widely used has led to the development of enhanced GFPs that offer beneficial characteristics(Sullivan and Kay 1999). SGFP is one of these and has been successfully used in a number of filamentous fungi (Spellig, Bottin et al. 1996; Maor, Puyesky et al. 1998; Dumas, Centis et al. 1999; Robinson and Sharon 1999; Frietag, Cuifetti et al. 2001; Sexton and Howlett 2001; Horowitz, Freeman et al. 2002). SGFP contains a Ser65-Thr mutation and was also originally designed with modifications of the codon usage to adapt it to animal cells (Hass, Park et al. 1996). GFPs containing the Ser65-Thr mutation have been shown to have increased fluorescence and solubility as well as decreased photobleaching (Cubitt, Heim et al. 1995; Crameri, Whitehorn et al. 1996; Siemering, Golbik et al. 1996). The same mutation causes a 'red shift' in the excitation maxima from 395 and 470 nm to 488 nm. While this makes screening using UV light more difficult (special filters are required) it makes the GFP ideal for use with fluorescent microscopy. The modifications to codon usage in the SGFP gene were also shown to improve expression in plant cells and removed the cryptic splicing points resulting in more functional protein being translated (Chiu, Niwa et al. 1996). SGFP has been very useful in the study of filamentous fungi and provides benefits over the wild -type GFP

(Fernandez-Abalos, Fox et al. 1998). However there is evidence that wild-type GFP can out perform SGFP in *Cercospora nicotianae* (Chung, Ehrenshaft et al. 2002) so it can not be said that SGFP performs better than GFP in all fungi.

To the best of my knowledge GFP has not been used to investigate foliar infection of the *Pinus* species.

A construct was available (pFAT-3gfp) that contained a mutagenised green fluorescent protein gene *gfp* under the control of a constitutive promoter (*Aspergillus nidulans gpd)*. This gene is sourced from the plasmid pGreen Lantern formerly produced by Life Technologies. The gene contains a serine-to-threonine substitution at amino acid 65 (S65T) mutation as well as more than 80 other mutations to create a pattern of codon usage orientated towards mammalian cells making it very similar to *sgfp*. It was decided that two constructs would be made incorporating this *gfp* into vectors with hygromycin B and phleomycin resistance with the aim of transforming both the wild type and *dotA* knockout *D. pini*.

Another *sgfp* construct containing hygromycin B resistance was sourced towards the end of this study that utilised a powerful *ToxA* promoter from *Pyrenophora tritici-repentis* for gfp expression. This construct has been shown to be effective in a wide range of filamentous fungi.

5.2 Materials and Methods

5.2.1 Plasmids

Plasmid	Relevant Characteristics	Source or Reference
pFAT-3gfp	Agrobacterium vector, Spec^R, hygB^R, GFP, An p-gpd (14.822 kb)	(Fitzgerald, Mudge et al. 2003)
pBC-hygro	Chloramphenicol^R, Hygromycin B^R, *lacZ* (6.8 kb)	(Silar 1995)
pBC-phleo	Chloramphenicol^R, Phleomycin^R, *lacZ* (6.1 kb)	(Silar 1995)
pBCH-gfp	pBC-hyg containing fragment from pFAT-3gfp (gfp gene, An p-gpd) (9.1 kb)	This study
pBCP-gfp	pBC-phleo containing fragment from pFAT-3gfp (gfp gene, An p-gpd) (8.4 kb)	This study
pCT74	SGFP, Tox A promoter, hygB ^{R}, Amp^R (~6 kb)	(Lorang, Tuori et al. 2001)
pAN7-1	Hygromycin B^R, Ampicillin^R (6.60 kb)	(Punt and van den Hondel 1992)

Table 5.1: Table of plasmids used in this study

5.2.2 *D. pini* strains

The *D. pini* strains that were used for transformation are listed below with the *gfp* vectors listed beside. More detailed information about *D. pini* strains is available in Table 2.2

NZE7 (wild type): pBCH-gfp, pCT74

NZE5 (wild type): pBCH-gfp

34C1 (*dotA* knockout): pBCP-gfp

5.2.3 Media

5.2.3.1 Osmotically stabilised Dothistroma media (DMSuc)

Malt Extract (Oxoid) 50 g/l, Nutrient agar (Oxoid) 28 g/l, 0.8 M Sucrose (BDH) 273.9 g/l. Made up to final volume with milli Q water.

5.2.3.2 Dothistroma media top agar (with antibiotic additions)

Malt Extract (Oxoid) 50 g/l, Nutrient agar (Oxoid) 11.2 g/l, (0.8 M) Sucrose (BDH) 273.9 g/l. Made up to final volume with milli-Q water. Hygromycin B = 350 µg/ml to give final concentration of 70 µg/ml when 5 ml is added as an overlay to 20 ml of DMSuc. Phleomycin = 30 µg/ml to give a final concentration of 6 µg/ml when 5 ml is added as an overlay to 20 ml of DMSuc.

5.2.4 Buffers and solutions

5.2.4.1 OM buffer

$MgSO_4.7H_2O$ (Merck) 1.6 M, Na_2HPO_4 (BDH) 10 mM, use 100 mM NaH_2PO_4 (BDH) to buffer to pH 5.8

NB: A 1.4 M $MgSO_4.7H_2O$ (Merck) variant of this buffer was also used.

5.2.4.2 Polyethylene glycol (PEG) 6000

40% (w/v) Polyethylene glycol 6000 (BDH) in STC buffer (section 5.2.4.5)

5.2.4.3 ST buffer

Sorbitol (BDH) 1.0 M, Tris-HCl (Invitrogen) 100 mM (pH 8.0).

5.2.4.4 STC buffer

Sorbitol (BDH) 1.2 M, Tris-HCl (Invitrogen) 50 mM (pH8.0), $CaCl_2$ (Merck) 50 mM

5.2.5 Procedures

5.2.5.1 Restriction digestion protocol

Three and a half µg of plasmid pFAT-3gfp (Table 5.1) was digested with 30 units of *NotI* endonuclease (Invitrogen) in a total reaction volume of 100 µl containing a 1X final concentration of H buffer (Invitrogen). The digest was then incubated at 37°C overnight. *NotI* was then inactivated by heating to 65°C for 15 min.

One and a half µg of both pBC-hygro and pBC-phleo (Table 5.1) were digested with 30 units of *NotI* endonuclease (Invitrogen) in a total reaction volume of 50 µl

containing a 1X final concentration of H buffer (Invitrogen). The digest was then incubated at 37°C overnight. *NotI* was then inactivated by heating to 65°C for 15 min.

5.2.5.2 Phosphatase treatment

40 µl of the *NotI* digest was heated in a 65°C water bath for 15 mins. The digest was then cooled to 37°C before the addition of 0.2 µl of a 1 unit/µl calf thymus alkaline phosphatase (Roche). The reaction was then incubated at 37°C for 2 hours. Phenol/chloroform extraction and ethanol precipitation (Section 5.2.5.3) were carried out to remove traces of the alkaline phosphatase enzyme prior to ligation.

5.2.5.3 Phenol/chloroform extraction and ethanol precipitation method

One volume of phenol was added to the sample, which was then vortexed before centrifugation at 13,000 rpm in a micro centrifuge. The aqueous phase at the top of the tube was collected and transferred to a fresh micro centrifuge tube. A half volume of phenol and a half volume of chloroform was then added before vortexing the sample and centrifugation as mentioned above. The aqueous top phase was then collected and transferred to a clean micro centrifuge tube. One volume of chloroform was then added before repeating the vortexing and centrifugation. The top aqueous phase was then collected and again transferred to a clean micro centrifuge tube before adding two volumes of 95% ethanol and putting in a -20°C freezer for 20 minutes. The sample was then centrifuged at 13,000 rpm in a micro centrifuge for 1 min before putting the tube back in the -20°C freezer for a further 5 minutes. The ethanol was then carefully decanted off and the pellet was left to air dry. The dry pellet was then re-suspended in 20 µl TE buffer (Section 2.3.8). (NB: No ethanol wash was carried out because no salts were used to precipitate the DNA and washing the pellet may have further reduced the amount of DNA recovered.)

5.2.5.4 Ligation

The ligation protocol used was based on the one described in the pGEM-T Vector System I' kit (Promega). All enzymes and reagents were sourced from this kit also. The changes to this protocol were the use of the pBC plasmid vectors (Table 5.1) instead of the pGEM-T plasmid and that a restriction fragment containing *gfp* was used instead of a PCR product. A 1:1 ratio of vector to insert was used. The reaction

volume was increased from 10 µl to 40 µl and the amount of T4 DNA ligase buffer was also increased to maintain a final concentration of 1X. The units of T4 DNA ligase remained unchanged. Salts from the ligation mix needed to be removed to prevent arcing when attempting transformation of *E. coli* by electroporation. This was done by pipetting the ligation mix onto a cellulose nitrate membrane (47 mm diameter, pore size = 0.45 µm, Micro Filtration Systems). The membrane was floated on a beaker filled with milli-Q water. The ligated sample was left for 20 mins before being collected with a pipette and being put in a clean micro centrifuge tube.

5.2.5.5 Construction of *gfp* vectors

Plasmid pFAT-3gfp (Table 5.2.1) was digested with *NotI* (Section 5.2.5.1). The digest was run on a gel and the fragment containing the *gfp* gene, constitutive *gpd* promoter from *A. nidulans* and the *G. cingulate gpd* terminator was excised and purified using a 'Qiaquick gel extraction kit' (Qiagen). The pBC-hygro (Section 5.2.1) (hygromycin resistance) and pBC-phleo (Section 5.2.1) (phleomycin resistance) plasmids were also digested with *NotI*. The cut pBC vectors were dephosphorylated using calf thymus alkaline phosphatase (Roche) and a phenol/chloroform extraction was then done to remove the phosphatase enzyme. The *gfp* fragment and two pBC plasmids were then ligated in two separate reactions using a modified method from the pGEM-T Vector System I' kit (Promega) (Section 5.2.5.4). The resulting ligation reaction was then transformed into XL-1 cells (Table 2.1) by electroporation (Section 2.5.2) and was screened for transformants using blue/white selection (Section 2.5.2). Incorporation of the GFP gene was confirmed by digestion with*NotI* followed by electrophoresis on a 1.5 % agarose gel.

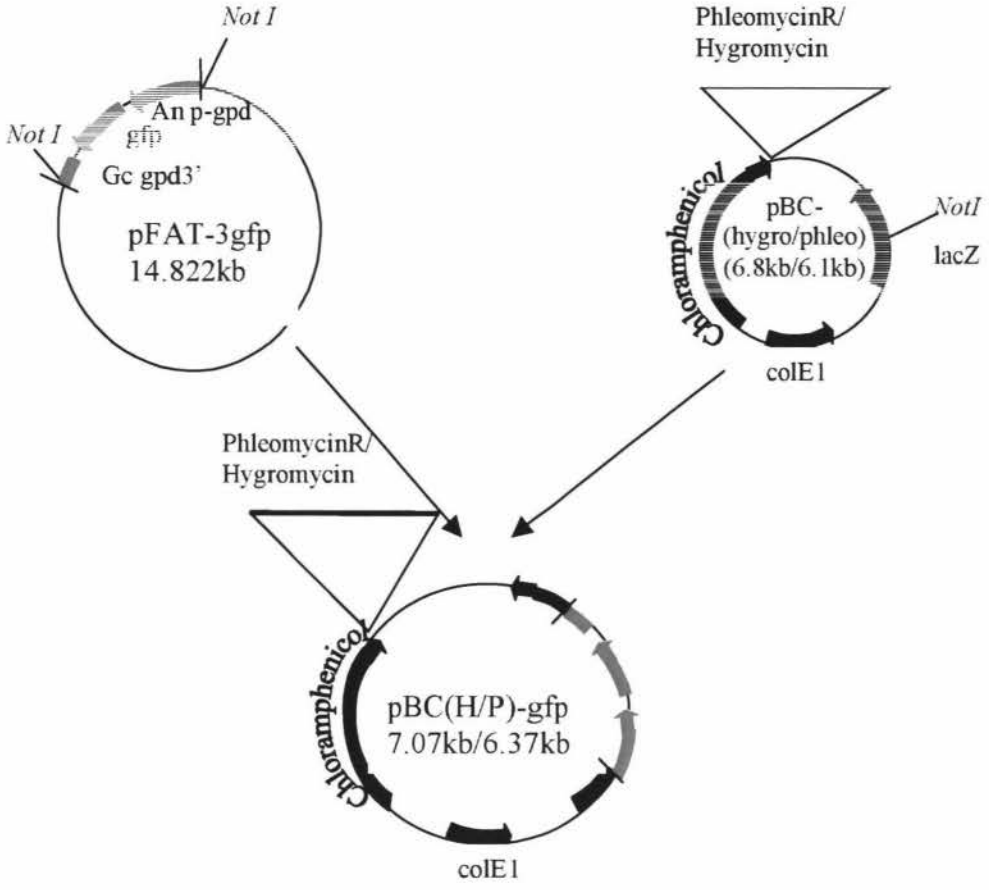


Fig 5.1: Outline of the cloning strategy used to develop gfp constructs with hygromycin B and phleomycin resistance.

5.2.5.6 Generation of competent *D. pini* protoplasts

100 µl of freshly grown *D. pini* mycelium ground up in sterile milli Q water was spread onto cellophane covered DM media. The cellophane plate was incubated in the dark for 6 days at 22°C. After 6 days the cellophane containing a light layer of growth was striped from the media and placed mycelia side down in a sterile petri dish containing 10 ml of sterile 20 mg/ml Glucanex (beta-glucanase, Chemcolour Industry, NZ) dissolved in OM buffer (section 5.2.4.1). Up to 4 cellophanes were placed in a single petri dish. The petri dish was sealed with parafilm and left shaking at 37°C overnight. Protoplast formation was confirmed by placing 20 µl of the incubated suspension onto a microscope slide and examining at 400X magnification under a phase contrast microscope. 5 ml of the incubated protoplast suspension was put in a 30 ml corex tube, overlaid with 1 ml ST buffer (Section 5.2.4.3) and centrifuged at 4°C for 5 mins at 3000 rpm in a SS -34 rotor. The protoplasts forming a white band at

the interface of the two solutions, were removed and washed twice in 5 ml STC buffer (Section 5.2.4.4) being pelleted in between washes by centrifugation as above. If the protoplasts failed to float 20 ml of additional STC was added to the 30 ml corex tube containing ST and STC buffer and the whole sample was mixed and centrifuged at 6000 rpm for 20 mins to pellet the protoplasts. The pellet was then washed twice with 10 ml STC buffer being pelleted each time by centrifugation at 6000 rpm for 10 mins. The pellet was finally resuspended in 200-600 µl of STC buffer and stored at 4°C for no longer than 4 hours before use. The concentration of the protoplast solution was determined by counting with a haemocytometer.

5.2.5.7 Transformation of *D. pini* protoplasts

For each transformation between 5 x 10^6 and 1x10^7 protoplasts in 150 µl of STC buffer (Section 5.2.4.4) were mixed with 5 µg of DNA and incubated at 22°C for 20 minutes. In three steps 250, 250 and 850 µl of 40% polyethylene glycol (PEG) 6000 solution in STC buffer were carefully mixed with DNA/protoplast mixture and the final suspension was incubated for a further 20 minutes at 22°C. The suspension was then diluted in 5 ml of STC buffer and the protoplasts were collected by centrifuging for 10 minutes at 4°C, 3000 rpm using an SS-34 rotor. The pellet was then re-suspended in 500 µl of STC buffer.

NB: Due to plasmid pCT74 arriving towards the end of this study there was not enough time to generate large quantities, but a transformation of NZE7 was carried out using 2.4 µg.

Twon hundred µl, 100 µl and a 10-fold and 100-fold dilution of the re-suspended protoplasts were carefully spread onto 20 ml DM Sucrose. Antibiotics were added 24 hours later by overlaying the DMSuc with 5 ml of antibiotic containing DM top agar.

5.3 Results

5.3.1 Generating GFP constructs for transformation of *D. pini*

5.3.1.1 Background

Hygromycin B and phleomycin constructs were needed for use as selectable markers for the transformation of *D. pini*. Hygromycin B had been used as a selectable marker for the generation of the *D. pini dotA* knockout mutant so could not be used again. Phleomycin was chosen as a selectable marker for the transformation of the *dotA* knockout mutant with GFP.

5.3.1.2 Results

Transformation of *E. coli* XL-1 cells with the initial attempt to construct pBCH-gfp and pBCP-gfp were unsuccessful. Two white colonies (WC1 and WC2) were obtained from the pBCH-gfp transformation but neither possessed the gfp insert as shown by the absence of a 2.27 kb *NotI* fragment (Fig 5.2).

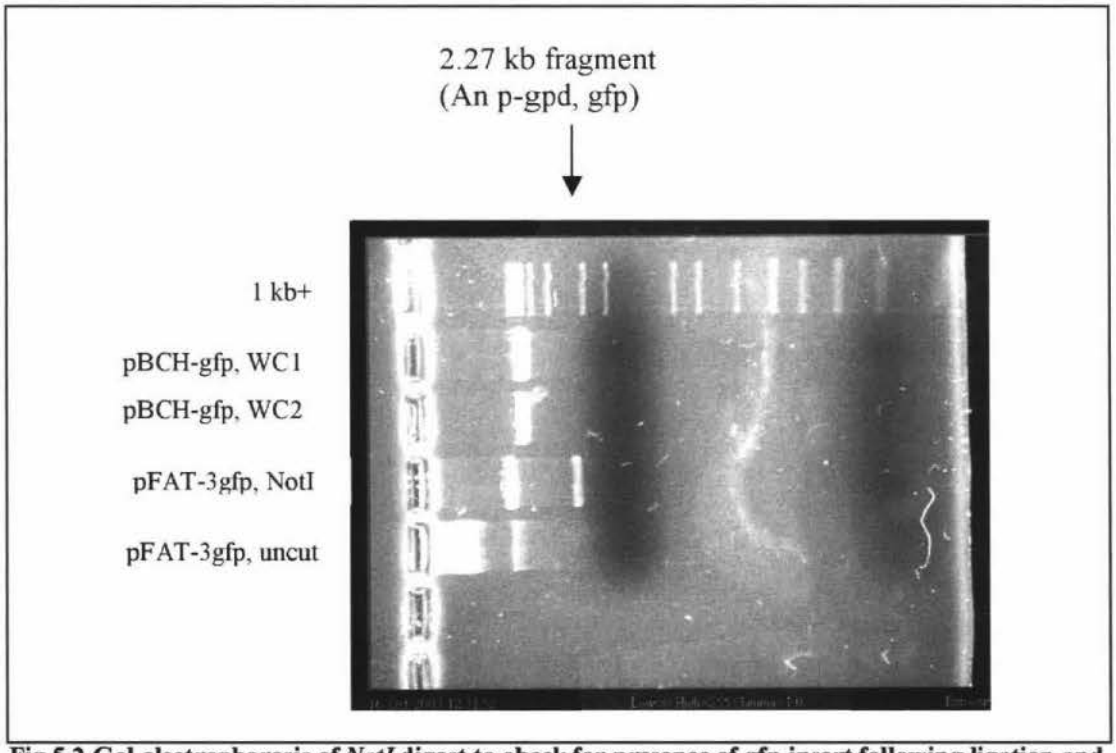


Fig 5.2 Gel electrophoresis of *NotI* digest to check for presence of gfp insert following ligation and transformation of pBCH-gfp. pFAT-3gfp, *NotI* is the positive control.

The second attempt involved the dephosphorylation of *NotI* digested pBC vectors in order to prevent self-ligation and promote uptake of the gfp insert. However a considerable amount of DNA was lost during the phenol/chloroform extraction to remove the calf thymus alkaline phosphatase so the ligation mix contained a low DNA concentration. The consequence of this was that a large volume of ligation mix was needed for electroporation of *E. coli* XL-1 cells. The high salt content resulting from adding 12 µl (30 ng DNA) to 40 µl of *E. coli* XL-1 cells caused an arc during electroporation and subsequently no growth occurred following inoculation onto LB media.

To reduce the amount of salt, the samples were dialysed (Section 5.2.5.4). Of the 28 µl applied to the membrane only 5 µl was recovered. There was not sufficient volume remaining to check the DNA concentration of the dialysed samples so it was assumed still to be 2.5 ng/µl. Transformation was carried out using the remaining 5 µl of dialysed sample.

Three white colonies were obtained from each of the *E. coli* transformations. After single cell isolation these were inoculated into LB broths and plasmid DNA extracted. Plasmids from all three of the pBCH-gfp white colonies (pBCH-gfp WC1-WC3) as well as one phleomycin plasmid (pBCP-gfp WC2) incorporated a DNA fragment of the expected size (Fig 5.3). pBCP-gfp WC1 failed to grow in LB broth containing ampicillin and WC3 showed unexpected additional banding so was not used in further experiments.

E. coli strains containing the plasmids pBCH-gfp (WC1) and pBCP-gfp (WC2) were purified before being grown in LB broth and large quantities of purified plasmid isolated (Section 2.5.3). These plasmid preparations were then used in attempts to transform *D. pini*.

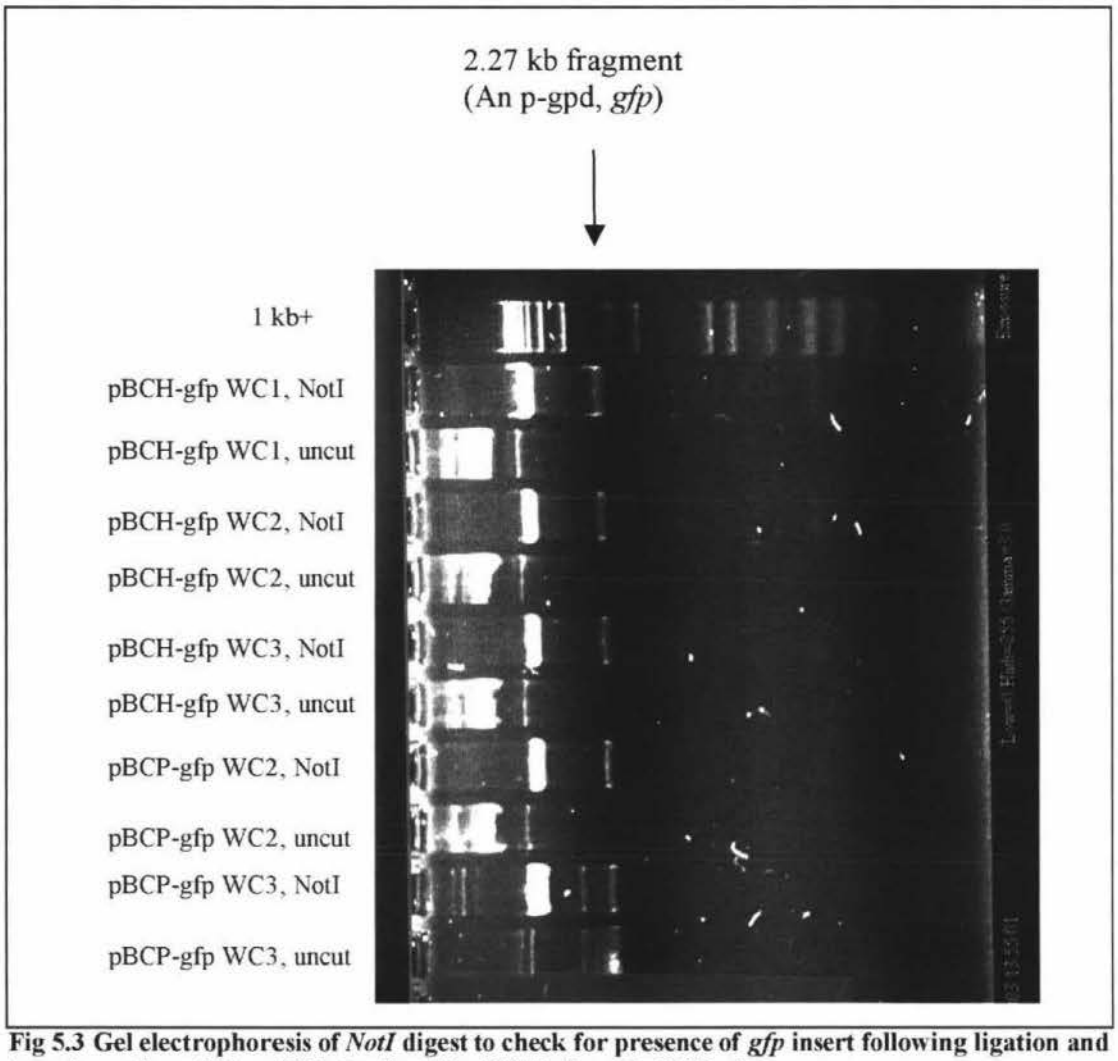


Fig 5.3 Gel electrophoresis of *NotI* digest to check for presence of *gfp* insert following ligation and transformation of *E. coli* XL-1 cells with pBCH-gfp and pBCP-gfp.

5.3.2 Transformation of *D. pini* with GFP constructs

The first stage in transformation of *D. pini* is to obtain protoplasts. However problems were faced trying to obtain sufficient numbers of *D. pini* protoplasts using Glucanex enzyme (Section 5.2.5.6). An alternative enzyme Yatalase (chitinase, chitobiase and β-1, 3-glucanase, Takara Bio Inc, Japan) used at 15 mg/ml in OM buffer (Section 5.2.4.1) was trialled but no improvement was observed. Glucanex digestion yielded protoplasts in the range of 5.4×10^7 to 2.5×10^8 protoplasts/ml (mean $=1.6 \times 10^8$). Yatalase yielded protoplasts in the range of 9.0×10^6 to 8.5×10^7 protoplasts/ml (mean $= 4.4 \times 10^7$).

It was soon revealed that the protoplasts were not floating as well as expected during the flotation step used to purify protoplasts away from mycelial debris. A lot of

protoplasts still remained in the bottom phase when overlaid with ST buffer (Section 5.2.5.6). In an attempt to increase the swelling (and therefore possibly the floating) of the protoplasts, the $MgSO_4$ concentration of the OM buffer was reduced from 1.6 M to 1.4 M. This change had no effect.

Centrifuging the whole sample did yield sufficient number of protoplasts to attempt transformation but mycelial fragments were still present in the harvested suspension. Once sufficient numbers of protoplasts were obtained there was only enough time remaining for one transformation attempt.

D. pini 34C1 (*dotA* knockout mutant) protoplasts did not regenerate resulting in unsuccessful transformation with pBCP-gfp. NZE7 (wildtype) protoplasts were heavily contaminated with fungi as well as bacteria making it impossible to determine protoplast regeneration rate or any successful transformation events with pBCH-gfp or pCT74.

NZE5 (wild type) protoplasts had a low regeneration rate of 0.004 %. No pBCH-gfp transformants were observed with NZE5 protoplasts.

5.4 Discussion

The fact *D. pini* protoplasts did not float very well was an unexpected problem because the transformation protocol has successfully been used previously with NZE5 (Bradshaw, Bhatnagar et al. 2002). The fact that the problem also affected the newly isolated wild type NZE7 suggests that the problem isn't the result of a change in *D. pini* growth characteristics brought on by *in vitro* growth. Other people working on *D. pini* transformation in the laboratory had the same problems. Unfortunately time was not available to investigate this further. One possibility is that the fault lies with the buffers used because the trial done with Yatalase did not improve floating, suggesting the enzyme digestion is not responsible. During protoplast generation some of the corex centrifuge tubes stuck in the rubber sleeves used to adapt the tubes to the rotor. Additional handling was required to remove the tubes and despite the utmost care being taken it is likely that this was the cause of the contamination seen with the

NZE7 protoplasts. If protoplast generation continues to be a problem an alternative method of transformation involving *Agrobacterium tumefaciens* may prove useful. This technique has successfully been used to transform mycelium from a number of different filamentous fungi (de Groot, Bundock et al. 1998; Abuodeh, Orbach et al. 2000; Mullins, Chen et al. 2001; Zwiers and Waard 2001; Amey, Athey-Pollard et al. 2002; Fitzgerald, Mudge et al. 2003). Time constraints also meant attempts to transform the wild type and *dotA* knockout forms of *D. pini* had to be cut short. This was disappointing due to the successful development of GFP constructs capable of transforming both the mutant and wild type *D. pini*. The SGFP plasmid, pCT74, also presents another option to transform *D. pini* with *gfp* and will easily be converted to phleomycin resistance due to its amenable restriction sites. The pCT74 plasmid arrived towards the end of this study, only allowing time for one transformation attempt. I am confident that the constructs developed will be useful for the transformation of *D. pini* with *gfp*. Once GFP expression is achieved informative images of endophytic growth will be possible using confocal microscopy.

CHAPTER 6: GENERAL DISCUSSION

6.1 Introduction

D. pini needle blight has been a significant disease in the southern hemisphere for several decades but outbreaks in the northern hemisphere involving regions of pine in their native environment are making *D. pini* a pathogen of widening global interest. No information regarding why *D. pini* has spread into these new environments has been obtained with possible explanations being climate change or new more virulent strains of *D. pini*. This widening concern and the economic impact of *D. pini* disease is justification for the further investigation of *D. pini* infection of pine trees.

Molecular techniques have developed rapidly in recent history, which has provided new tools with which to investigate the role dothistromin plays during *D. pini* infection of pine trees. One such technique has been applied to knockout the *dotA* biosynthetic gene involved in dothistromin synthesis. By knocking out the gene it is possible to observe the fitness of *D. pini* during its natural infectious life-cycle. This should provide a more definitive explanation about the function of dothistromin.

Early studies indicated that the toxin, dothistromin is a key factor for *D. pini* infection of trees and for disease development. The recent discovery of similarity between the dothistromin and aflatoxin biosynthetic pathways provides additional interest for the further investigation into the function of this toxin. No biological role has been assigned to aflatoxin production despite the large metabolic input needed to produce it. Investigation into the biological role of dothistromin may provide insight into the role of aflatoxin or may provide an evolutionary explanation for the existence of aflatoxin. Unfortunately infection of *P. radiata* with *D. pini* was not observed during this study so no further information can be added to the debate on the role of dothistromin. It is pertinent to remember the lessons learnt through the study of the soy bean pathogen *Cercospora kikuchii* and the dutch elm disease pathogen *Ophiostoma ulmi*. Both are fungal pathogens utilizing toxins thought to be significantly involved in the infection process. Knockout of Cercosporin toxin production from *C. kikuchii* resulted in a non-virulent fungus while knockout of the

cerato-ulmin toxin of *O. ulmi* had no measurable effect on virulence. This highlights the need to confirm dothistromin's role utilizing the dothistromin deficient *dotA* knockout mutant. The suggested role of dothistromin in the infection process of *D. pini* has direct relevance to New Zealand's exotic forests as the strain present in New Zealand produces relatively low amounts of dothistromin compared to overseas strains. Genetic diversity of *D. pini* is low in New Zealand, which presents the opportunity to develop more effective control measures. However if another *D. pini* strain is introduced that produces higher levels of dothistromin the damage caused to New Zealand forestry could be more severe and current control methods could become inadequate if indeed dothistromin is a virulence factor. If an increase in the genetic diversity of *D. pini* also occurs then design of future control measures may be more difficult. The low genetic diversity of the *D. pini* population in New Zealand presents the opportunity that newly designed control methods based on a thorough understanding of the infection process and plant-fungal interaction could significantly reduce the amount of *D. pini* disease in New Zealand.

6.2 Microscopy

In order to investigate the infection process of *D. pini* it is necessary to have techniques to observe the different stages of infection. While the infection process of *D. pini* has been studied in detail previously (Gadgil 1967) this work had been done some time ago and it was deemed desirable to investigate different microscopy techniques and apply new technology to see if a more efficient method could be identified while still supplying detailed information about the infection process. Normal light microscopy proved time consuming and ineffective compared to fluorescent techniques trialled for visualisation of epiphytic growth. Attempts to visualise endophytic *D. pini* growth were unsuccessful. Gluteraldehyde staining combined with confocal laser scanning microscopy showed potential but was later proven to be inconsistent. The early success of this technique suggests further testing and development of this method may yield an effective tool for the visualisation of invasive growth of needle blight pathogens. To my knowledge, fluorescent microscopy is not commonly used to diagnose disease of pine. The work done in this study highlights the sensitivity of fluorescent techniques. Calcofluor white was an especially useful fluorescent stain for the visualisation of epiphytic *D. pini* growth.

Epi-illumination microscopy also provided benefits over bright field microscopy in certain situations. Adaptation and development of some of the techniques used in this study may result in more sensitive and earlier detection of disease in forest samples. This may prove especially beneficial if the spread of a disease from a specific location needs to be monitored.

The techniques outlined in (Gadgil 1967) are still the most effective for the visualisation of endophytic *D. pini* growth. It maybe more practical to split the experiments comparing the *dotA* knockout to the wild type *D. pini* done in this study into two separate areas. The first looking at the level of *D. pini* disease of *P. radiata* based on macroscopic examination only, and then once clear results are obtained in this area the second phase would be to look at the infection process in more detail. Visualisation of sections as outlined in (Gadgil 1967) could then be done along with other microscopy techniques, such as the fluorescent imaging techniques used in this study, to further examine the effect of knocking out dothistromin biosynthesis on *D. pini* infection.

6.3 Green Fluorescent Protein

Introduction of a biomarker, such as GFP, into *D. pini* could obviate the need to section plant material to visualise endophytic growth. GFP has been used successfully in a wide range of phytopathogens. Constructs for the introduction of GFP into the *dotA* knockout and wild type *D. pini* were successfully developed but transformation was not achieved. Successful transformation of *D. pini* with GFP will further broaden the range of organisms where GFP has been used and when combined with confocal microscopy will provide a powerful tool for the visualisation of *in situ* endophytic *D. pini* growth. The *in situ* nature of visualisation techniques incorporating GFP or similar reporter genes opens up new possibilities for study as well as providing benefits over destructive sampling. If characterisation of mutants expressing GFP show little difference to the wild type, the lack of sample processing will reduce the chance of introducing experimental artefacts. This means a higher level of confidence is possible in the results obtained, resulting in biologically more accurate information. The *in situ* nature of the technique also presents the possibility of time series experiments, which could investigate such things as tropisms towards stomata and

response to stimuli. Also the effects of fungicides, new and old, as well as new control measures could be observed in real time.

Necrotic lesions did autofluorese in the gfp channel of the confocal microscopy but the confocal technician was confident GFP expression would still be visible within these regions. While GFP visualisation within these necrotic lesions should be possible it maybe more effective to use a different coloured chromophore such as dsRed (Greer III and Szalay 2002) to make distinction between plant and fungal material. A wide range of chromophores are currently present so combinations of these could also be used to investigate gene expression as well as acting as a biomarker. It maybe possible to visualise at what stage of infection dothistromin expression is switched on as well as different stress response genes in the plant tissue.

6.4 Pathogenicity Test

The use of a *dotA* knockout mutant meant any pathogenicity testing system needed to be contained under PC2 conditions. The limited available space meant the test had to be on a small scale. Conditions were developed that are favourable for *D. pini* infection but the conditions during the pathogenicity test showed large variations caused by several problems. Specifically: temperature fluctuations, a period of no misting as well as a period of over-misting, and mineral precipitation on the surface of needles. These variations were the most likely explanation no definitive disease symptoms were observed during the pathogenicity test. The inclusion of the fresh wild type isolate NZE7 supports the idea that the conditions were not right for infection rather than the *D. pini* being attenuated as a result of being in culture too long. This first trial identified problems and potential causes for the lack of infection, which makes me confident that a second trial will be more successful.

The demonstration of a successful pathogenicity testing system will provide a platform on which to test all future genetically modified *D. pini* strains. The PC2 classification of the construct used means future work requiring PC2 conditions can also be done using this facility. These might include experiments investigating possible biological control agents. Introduction of resistance genes into trees could

also be investigated along with future genetically modified strains of fungi to further investigate the plant fungi interaction.

Smaller scale pathogenicity trials may be possible incorporating plant cell cultures or detached fasicles in tubes. Single needles stored in petri dishes, to maintain humidity, could also be used. However, the most biologically accurate system would be tests involving the whole plant.

The small scale of the system and ideal conditions for *D. pini* infection, which will provide fast results, could make the system useful for other applications. Screening for *D. pini* resistance in *P. radiata* would be faster and the use of young trees would reduce the amount of space needed. New possible control measures could be trialled under different environmental conditions and results obtained quickly. Also other diseases with the same important environmental factors could be studied. Soil conditions are one area that has received little investigation concerning its effect on *D. pini* infection and the potted seedlings used in the pathogenicity testing system would make this possible.

6.5 Conclusions

Progress has been made in developing a system to quickly obtain detailed information about the infection process of *D. pini*. New visualisation methods have been identified and an environment has been developed to expedite the infection process in which genetically modified dothistromin deficient strains of *D. pini* can also be used.

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Appendix I
Visual observations of Inoculated Trees over the Time Course of the Pathogenicity Test.

Key: B = Vertical bottom third of tree, M = Vertical middle third of tree, T = Vertical top third of tree.

NZE7X (60/5)							
	23/1	**25/1**	**27/1**	**29/1**	**31/1**	**2/2**	**7/2**
Lesions							
Chlorosis							
Tip browning							
	11/2	**16/2**	**19/2**	**24/2**	**27/2**	**1/3**	**5/3**
Lesions	B,M	B,M	B,M	B,M	B,M	B,M	B
Chlorosis							
Tip browning							
	8/3	**12/3**	**15/3**	**18/3**	**22/3**	**25/3**	**29/3**
Lesions	B	B,M	B	B	B,M,T	B,M	B,M
Chlorosis			M	M			
Tip browning				B			
	1/4	**5/4**	**8/4**	**13/4**	**15/4**	**19/4**	
Lesions	B,M	B,M	B,M	B,M	B,M	B,M	
Chlorosis							
Tip browning				B			
NZE7X (40/8)							
	23/1	25/1	27/1	29/1	31/1	2/2	7/2
Lesions							
Chlorosis							
Tip browning							
	11/2	16/2	19/2	24/2	27/2	1/3	5/3
Lesions			B	B	B	B	B
Chlorosis	B	B		B,M	B	B	B
Tip browning	B	B,M	B	B	B	B	B
	8/3	12/3	15/3	18/3	22/3	25/3	29/3
Lesions	B	B	B	B	B	B	
Chlorosis	B	B,M	B	B	B	B	B
Tip browning	B	B	B	B	B	B	B
	1/4	5/4	8/4	13/4	15/4	19/4	
Lesions	B,M	B	B	B	B	B,M	
Chlorosis	B	B	B	B,M	B	B	
Tip browning	B	B	B	B	B	B	

NZE7X (18/19)							
	23/1	**25/1**	**27/1**	**29/1**	**31/1**	**2/2**	**7/2**
Lesions							
Chlorosis							
Tip browning							
	11/2	**16/2**	**19/2**	**24/2**	**27/2**	**1/3**	**5/3**
Lesions	B	M	B,M				
Chlorosis	B	B	B	B	B	B	B
Tip browning							
	8/3	**12/3**	**15/3**	**18/3**	**22/3**	**25/3**	**29/3**
Lesions	B	B	B		B	B	B
Chlorosis	B		B	B	B	B	B
Tip browning							
	1/4	**5/4**	**8/4**	**13/4**	**15/4**	**19/4**	
Lesions	B	B	B	B	B	B	
Chlorosis	B	B	B	B	B	B	
Tip browning							
NZE7X (13/11)							
	23/1	**25/1**	**27/1**	**29/1**	**31/1**	**2/2**	**7/2**
Lesions							
Chlorosis							
Tip browning							
	11/2	**16/2**	**19/2**	**24/2**	**27/2**	**1/3**	**5/3**
Lesions	M	B,M	B,M	B	B	B	B
Chlorosis							
Tip browning							
	8/3	**12/3**	**15/3**	**18/3**	**22/3**	**25/3**	**29/3**
Lesions	B	B	B	B	B	B	M
Chlorosis							
Tip browning							
	1/4	**5/4**	**8/4**	**13/4**	**15/4**	**19/4**	
Lesions	B	B	B,M,T	B	B,M	B,M	
Chlorosis		B					
Tip browning							

NZE7 (60/5)							
	23/1	**25/1**	**27/1**	**29/1**	**31/1**	**2/2**	**7/2**
Lesions							
Chlorosis							
Tip browning							
	11/2	**16/2**	**19/2**	**24/2**	**27/2**	**1/3**	**5/3**
Lesions	B	B	B	B	B	B	B
Chlorosis		B	M		B		
Tip browning			B				
	8/3	**12/3**	**15/3**	**18/3**	**22/3**	**25/3**	**29/3**
Lesions	B	B	B	B,M	B,M	B	B
Chlorosis							
Tip browning					B	B	B
	1/4	**5/4**	**8/4**	**13/4**	**15/4**	**19/4**	
Lesions	B	B,M	B	B,M	B,M	B	
Chlorosis					B		
Tip browning	B	B		B			
NZE7 (40/8)							
	23/1	**25/1**	**27/1**	**29/1**	**31/1**	**2/2**	**7/2**
Lesions							
Chlorosis							
Tip browning							
	11/2	**16/2**	**19/2**	**24/2**	**27/2**	**1/3**	**5/3**
Lesions	B	B,M	B	B	B	B,M	B
Chlorosis	B				B,M	B,T	B
Tip browning	B	B	B	B	B	B	
	8/3	**12/3**	**15/3**	**18/3**	**22/3**	**25/3**	**29/3**
Lesions	B	B	T	B	B	B	B
Chlorosis	B		B	B	B	B	
Tip browning			B	B	B	B	B
	1/4	**5/4**	**8/4**	**13/4**	**15/4**	**19/4**	
Lesions	B	B	B	B,M	B,M	B	
Chlorosis	M	B,M	B	B	B	B	
Tip browning		B	B	B	B	B	

NZE7 (18/19)							
	23/1	**25/1**	**27/1**	**29/1**	**31/1**	**2/2**	**7/2**
Lesions							
Chlorosis							
Tip browning							
	11/2	**16/2**	**19/2**	**24/2**	**27/2**	**1/3**	**5/3**
Lesions	B	B,M	B	B	B,M	B	B
Chlorosis	B		B	B		B	B
Tip browning							
	8/3	**12/3**	**15/3**	**18/3**	**22/3**	**25/3**	**29/3**
Lesions	B	B	B	B	B	B	B
Chlorosis	B	B,T	B	B,T	B	B	B
Tip browning				B	B		
	1/4	**5/4**	**8/4**	**13/4**	**15/4**	**19/4**	
Lesions	B	B	B	B	B	B	
Chlorosis	B	B	B	B	B	B	
Tip browning							
NZE7 (13/11)							
	23/1	**25/1**	**27/1**	**29/1**	**31/1**	**2/2**	**7/2**
Lesions							
Chlorosis							
Tip browning							
	11/2	**16/2**	**19/2**	**24/2**	**27/2**	**1/3**	**5/3**
Lesions	B,M	B,M	B,M	B,M	B,M	M	B,M
Chlorosis	T						
Tip browning							
	8/3	**12/3**	**15/3**	**18/3**	**22/3**	**25/3**	**29/3**
Lesions	B,M	B,M	B,M	B	B,M	B,M	B,M
Chlorosis			M	B			
Tip browning				B		B	B
	1/4	**5/4**	**8/4**	**13/4**	**15/4**	**19/4**	
Lesions	B,M,T	B,M,T	B,M,T	B,M	B,M	B,M	
Chlorosis							
Tip browning							

8A1 (60/5)							
	23/1	25/1	27/1	29/1	31/1	2/2	7/2
Lesions							
Chlorosis							
Tip browning							
	11/2	16/2	19/2	24/2	27/2	1/3	5/3
Lesions	B,M	B,M		B	B	M	B
Chlorosis							
Tip browning							
	8/3	12/3	15/3	18/3	22/3	25/3	29/3
Lesions	B	B	B	B	B	B,M	B,M,T
Chlorosis					B		
Tip browning				B	B		
	1/4	5/4	8/4	13/4	15/4	19/4	
Lesions	B,M	B	B	B,M	B	B	
Chlorosis							
Tip browning							
8A1 (40/8)							
	23/1	25/1	27/1	29/1	31/1	2/2	7/2
Lesions							
Chlorosis							
Tip browning							
	11/2	16/2	19/2	24/2	27/2	1/3	5/3
Lesions	B,M		B			B,M	B
Chlorosis		B,M	B,M	B	B,M	B,M	B
Tip browning		T					
	8/3	12/3	15/3	18/3	22/3	25/3	29/3
Lesions		B	M	B,M,T	B	B,M	B
Chlorosis	B	B	B	B,M	B	B,M	B
Tip browning	B	B		B,M,T	B,M,T	B,M	
	1/4	5/4	8/4	13/4	15/4	19/4	
Lesions	B,M	B	B,T	B	B,T		
Chlorosis	B,M	B,M	B	B	B	B	
Tip browning	B			B	B	B	

8A1 (18/19)							
	23/1	**25/1**	**27/1**	**29/1**	**31/1**	**2/2**	**7/2**
Lesions							
Chlorosis							
Tip browning							
	11/2	**16/2**	**19/2**	**24/2**	**27/2**	**1/3**	**5/3**
Lesions	B	B,M	B	B,M	B,M	B,M	B
Chlorosis	M		M				B
Tip browning							
	8/3	**12/3**	**15/3**	**18/3**	**22/3**	**25/3**	**29/3**
Lesions	B	B	B	B	B,M	B	B
Chlorosis	B	B	B				B
Tip browning							
	1/4	**5/4**	**8/4**	**13/4**	**15/4**	**19/4**	
Lesions	B	B,M	B	B	B	B,M,T	
Chlorosis	M	B					
Tip browning							
8A1 (13/11)							
	23/1	**25/1**	**27/1**	**29/1**	**31/1**	**2/2**	**7/2**
Lesions							
Chlorosis							
Tip browning							
	11/2	**16/2**	**19/2**	**24/2**	**27/2**	**1/3**	**5/3**
Lesions	B,M	B	B	B	B,M	B,M	
Chlorosis		B,M	M				
Tip browning							
	8/3	**12/3**	**15/3**	**18/3**	**22/3**	**25/3**	**29/3**
Lesions	B,M	M	M	B		M	B
Chlorosis	B,M		B		M		
Tip browning							
	1/4	**5/4**	**8/4**	**13/4**	**15/4**	**19/4**	
Lesions	B,M	B,M	B	B,M	B,M	B	
Chlorosis		M					
Tip browning							

34C1 (60/5)							
	23/1	25/1	27/1	29/1	31/1	2/2	7/2
Lesions							
Chlorosis							
Tip browning							
	11/2	16/2	19/2	24/2	27/2	1/3	5/3
Lesions	B,M,T	B,M,T	B,M	B,M	B,M	B,M	B,M
Chlorosis							
Tip browning							
	8/3	12/3	15/3	18/3	22/3	25/3	29/3
Lesions	B,M	B	B	B,M	B,M	B,M	B,M
Chlorosis		M					
Tip browning							
	1/4	5/4	8/4	13/4	15/4	19/4	
Lesions	B,M	B,M	B,M	B,M	B,M	B,M	
Chlorosis							
Tip browning							
34C1 (40/8)							
	23/1	25/1	27/1	29/1	31/1	2/2	7/2
Lesions							
Chlorosis							
Tip browning							
	11/2	16/2	19/2	24/2	27/2	1/3	5/3
Lesions	B	B	B	B	B	B	B
Chlorosis		B	B	B		B	B
Tip browning	B	B	B	B	B	B	B
	8/3	12/3	15/3	18/3	22/3	25/3	29/3
Lesions	B	B	B,M	B	B	B,M,T	B
Chlorosis	B		B	B	B	B	B
Tip browning	B	B	B	B	B	B	B
	1/4	5/4	8/4	13/4	15/4	19/4	
Lesions	B	B	B	B	B	B	
Chlorosis	B	B	B	B	B	B	
Tip browning	B	B	B	B	B		

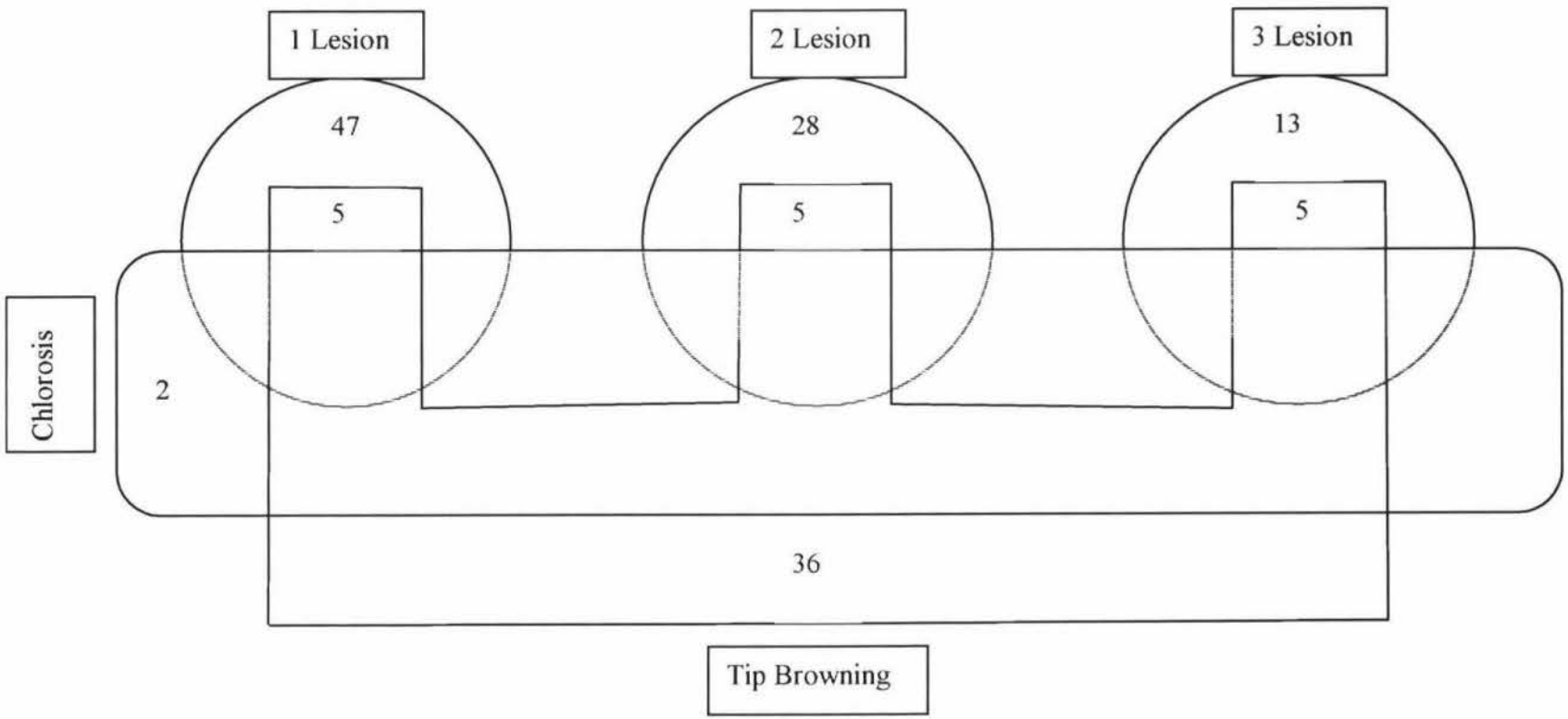
34C1 (18/19)							
	23/1	**25/1**	**27/1**	**29/1**	**31/1**	**2/2**	**7/2**
Lesions							
Chlorosis							
Tip browning							
	11/2	**16/2**	**19/2**	**24/2**	**27/2**	**1/3**	**5/3**
Lesions	B,M	B	B	B	B	B	B
Chlorosis	B,M			B		M	
Tip browning				B			
	8/3	**12/3**	**15/3**	**18/3**	**22/3**	**25/3**	**29/3**
Lesions	B	B	B	B	B	B	B
Chlorosis							B
Tip browning							B
	1/4	**5/4**	**8/4**	**13/4**	**15/4**	**19/4**	
Lesions	B	B	B	B	B	B,M	
Chlorosis		B		B			
Tip browning	B	B		B		B	
34C1 (13/11)							
	23/1	**25/1**	**27/1**	**29/1**	**31/1**	**2/2**	**7/2**
Lesions							
Chlorosis							
Tip browning							
	11/2	**16/2**	**19/2**	**24/2**	**27/2**	**1/3**	**5/3**
Lesions	B,M	B,M,T	B,M	B,M	B,M,T	B,M,T	B,M,T
Chlorosis							
Tip browning							
	8/3	**12/3**	**15/3**	**18/3**	**22/3**	**25/3**	**29/3**
Lesions	B,M,T	B,M,T	B,M,T	B,M,T	B,M,T	B,M,T	B,M,T
Chlorosis							
Tip browning							
	1/4	**5/4**	**8/4**	**13/4**	**15/4**	**19/4**	
Lesions	B,M,T	B,M	B,M,T	B,M	B,M,T	B,M	
Chlorosis							
Tip browning							

NEGATIVE (60/5)							
	23/1	25/1	27/1	29/1	31/1	2/2	7/2
Lesions							
Chlorosis							
Tip browning							
	11/2	16/2	19/2	24/2	27/2	1/3	5/3
Lesions	B	B,M	B	B,M	B	B	B
Chlorosis							
Tip browning	B		B	B	B	B	
	8/3	12/3	15/3	18/3	22/3	25/3	29/3
Lesions	B	B	B	B	B	B,T	B.M
Chlorosis							
Tip browning		B	B	B	B	B	B
	1/4	5/4	8/4	13/4	15/4	19/4	
Lesions	B,M	B	B,M	B,M	B	B	
Chlorosis					B		
Tip browning	B	B		B	B	B	
NEGATIVE (40/8)							
	23/1	25/1	27/1	29/1	31/1	2/2	7/2
Lesions							
Chlorosis							
Tip browning							
	11/2	16/2	19/2	24/2	27/2	1/3	5/3
Lesions	B	B		B	B	B	B
Chlorosis	B	B	B	B	B	B	B
Tip browning	B,M	B	B	B			
	8/3	12/3	15/3	18/3	22/3	25/3	29/3
Lesions	B	B	B	B	B	B,M	B
Chlorosis	B		B	B		B	B
Tip browning	B	B		B			
	1/4	5/4	8/4	13/4	15/4	19/4	
Lesions	B,M	B	B	B,M	B,M	B,M	
Chlorosis	B	B	B	B	B	B	
Tip browning	B		B				

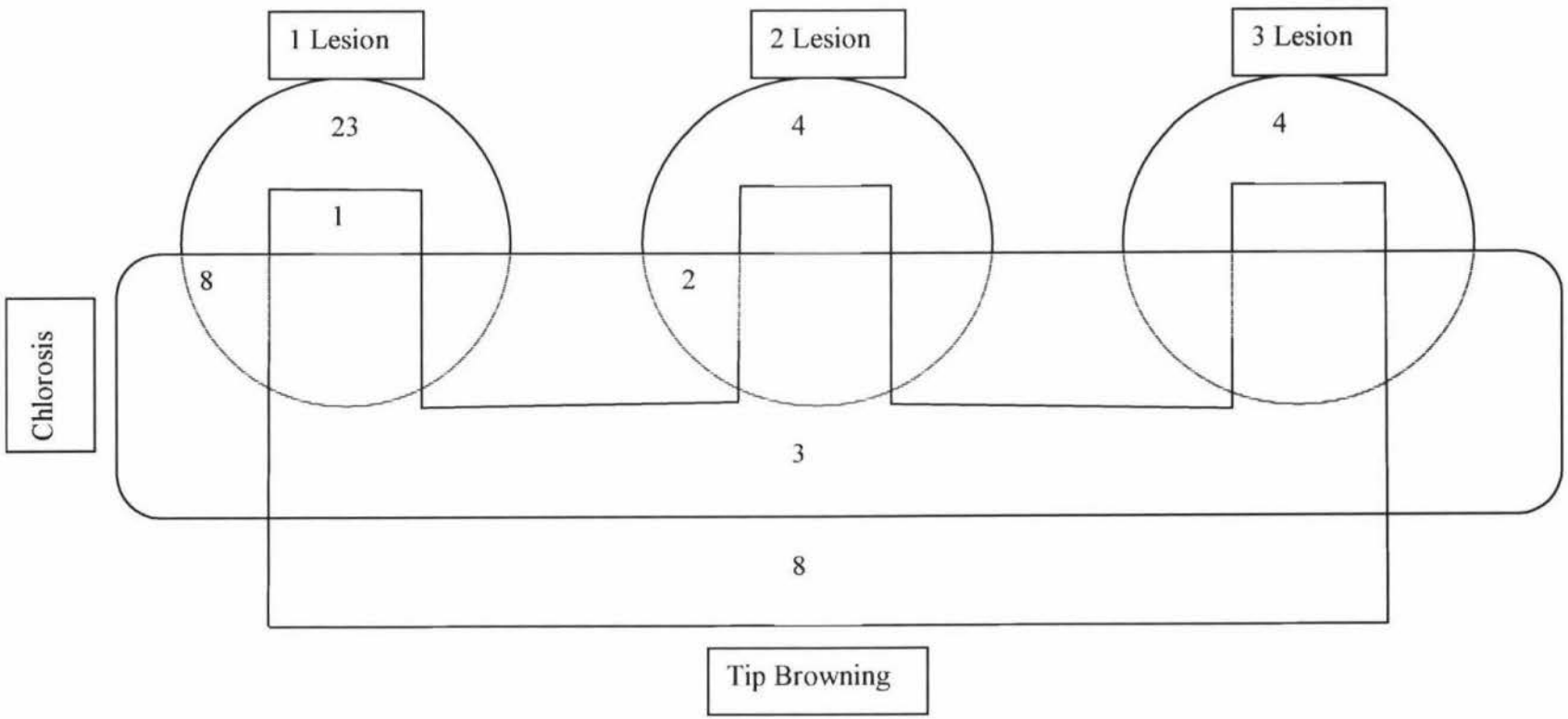
NEGATIVE (18/19)							
	23/1	**25/1**	**27/1**	**29/1**	**31/1**	**2/2**	**7/2**
Lesions							
Chlorosis							
Tip browning							
	11/2	**16/2**	**19/2**	**24/2**	**27/2**	**1/3**	**5/3**
Lesions	B,M	B,M	B,T	B,M	B,M	B,M	B,M
Chlorosis	B	B,M	B	B			
Tip browning							
	8/3	**12/3**	**15/3**	**18/3**	**22/3**	**25/3**	**29/3**
Lesions	B,M	B,M	B	B	B,M	B,M	B,M
Chlorosis		B,M	B	B	B		B
Tip browning							
	1/4	**5/4**	**8/4**	**13/4**	**15/4**	**19/4**	
Lesions	B,M	B	B,T	B	B	B	
Chlorosis	B	B	B	B	B		
Tip browning							
NEGATIVE (13/11)							
	23/1	**25/1**	**27/1**	**29/1**	**31/1**	**2/2**	**7/2**
Lesions							
Chlorosis							
Tip browning							
	11/2	**16/2**	**19/2**	**24/2**	**27/2**	**1/3**	**5/3**
Lesions	B,M	B,M,T	B,M	B,M	B,M	B,M	B,M
Chlorosis					B		
Tip browning							
	8/3	**12/3**	**15/3**	**18/3**	**22/3**	**25/3**	**29/3**
Lesions	B	B,M	M	B,M	B,M	B,M	B,M
Chlorosis							
Tip browning							
	1/4	**5/4**	**8/4**	**13/4**	**15/4**	**19/4**	
Lesions	B,M	B,M	B,M	B,M	B,M	B,M	
Chlorosis							
Tip browning							

Appendix II

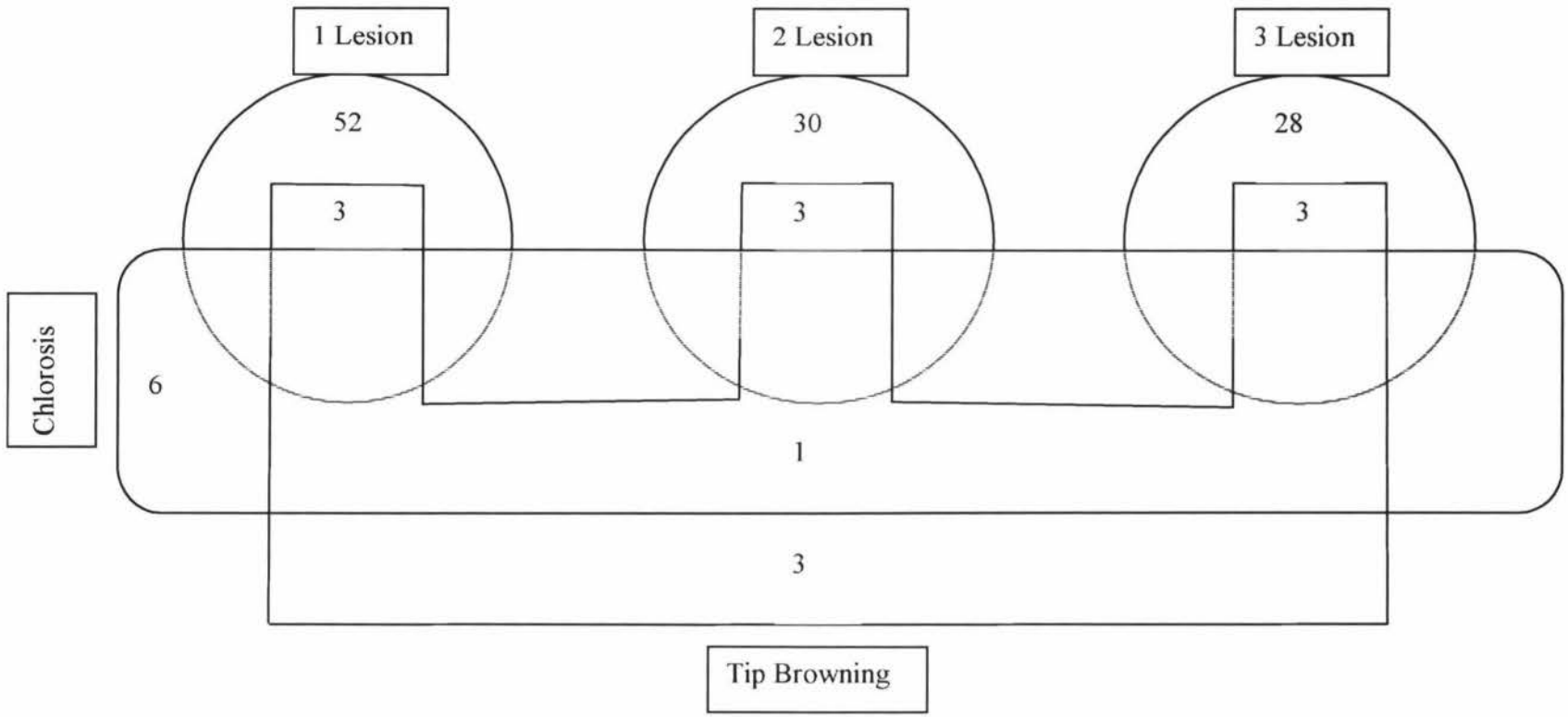
Diagrams of Damage Counts from Pine Needles Collected at the End of the Pathogenicity Test.



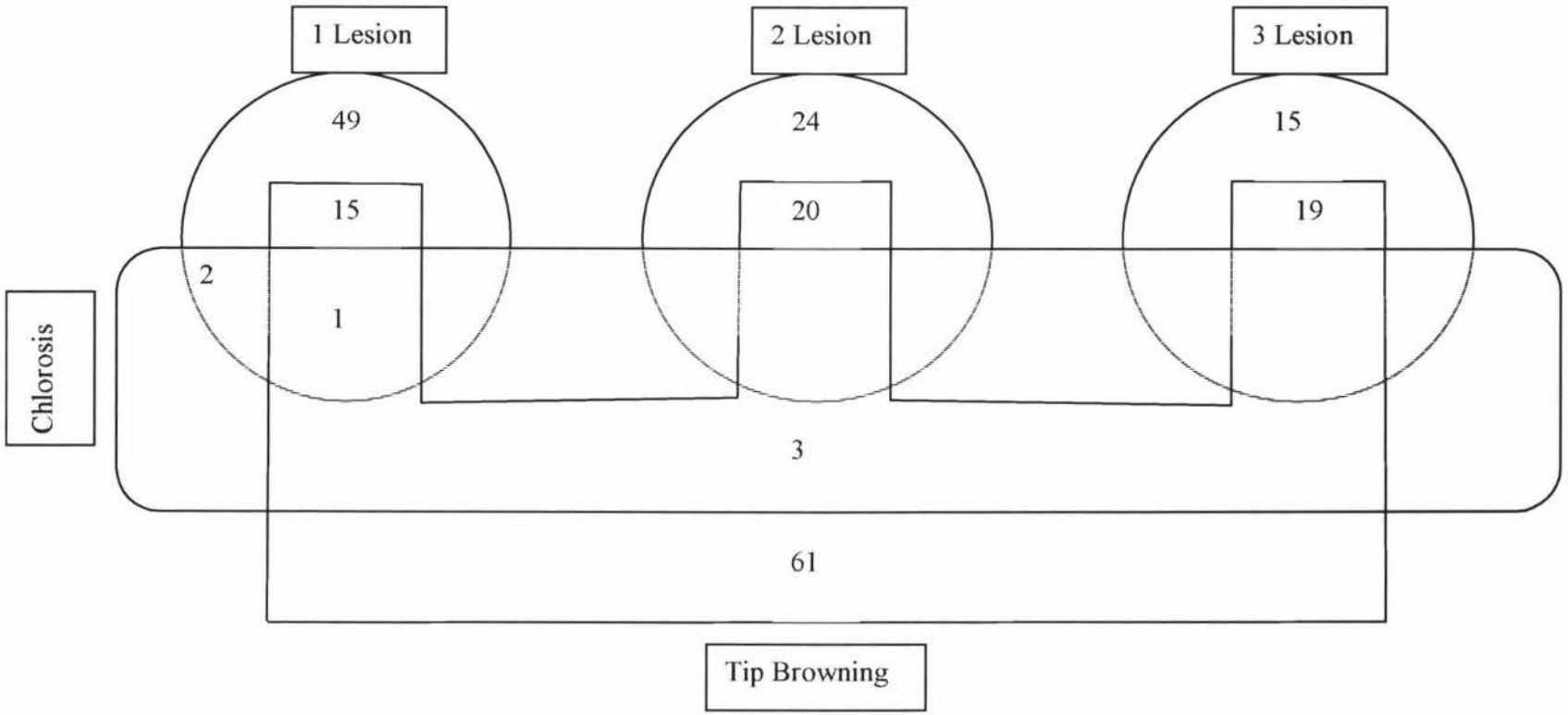
NZE7X (60/5)
Total = 909



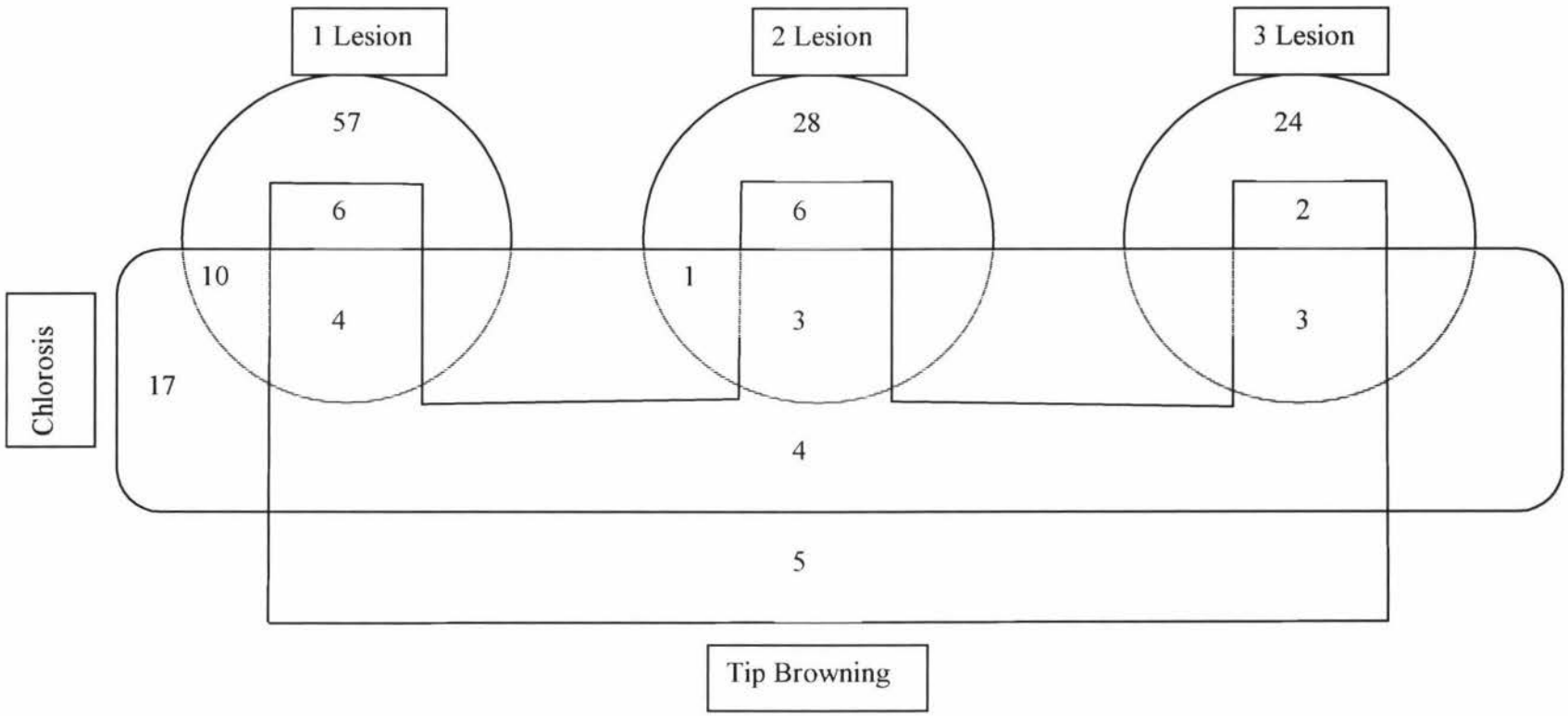
NZE7X (18/19)
Total = 662



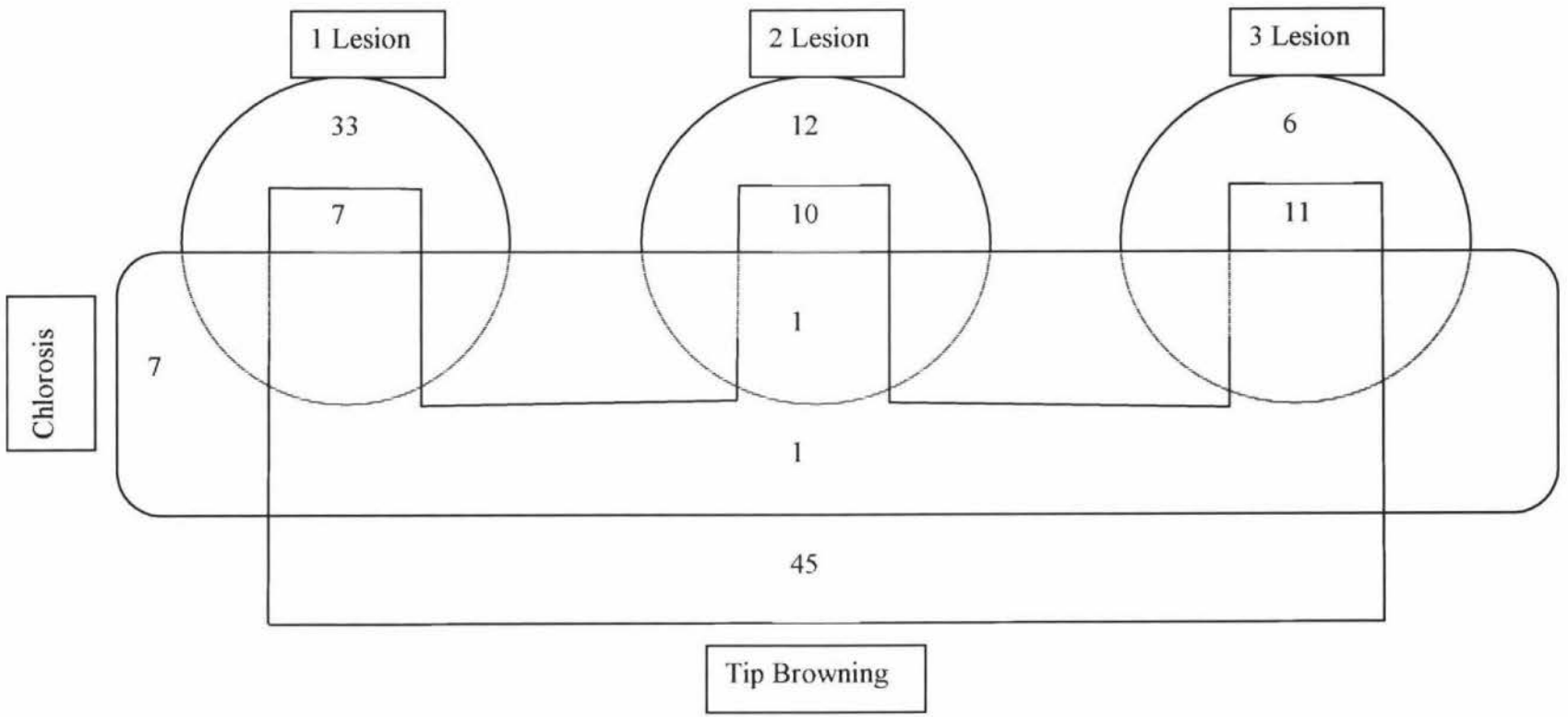
NZE7X (13/11)
Total = 1025



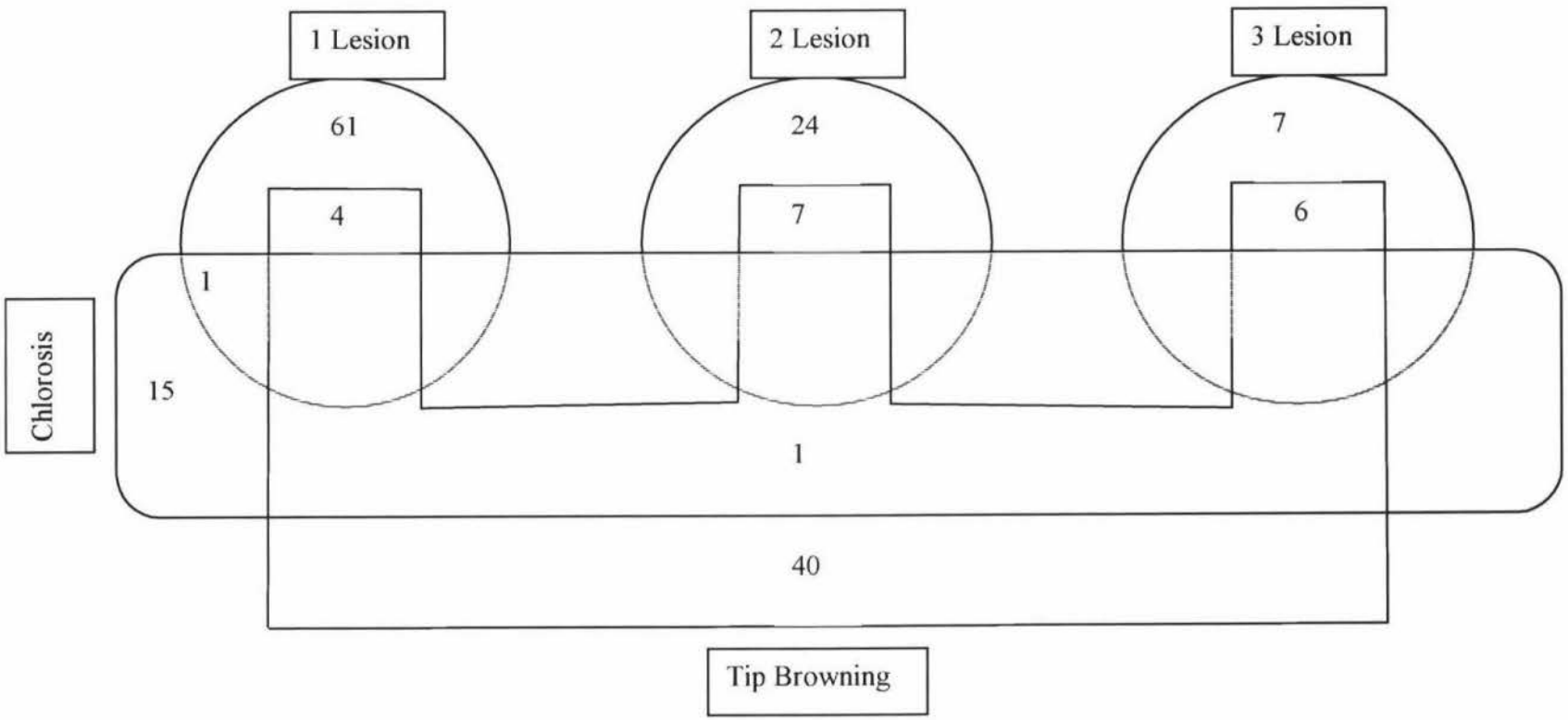
NZE7 (60/5)
Total = 1050



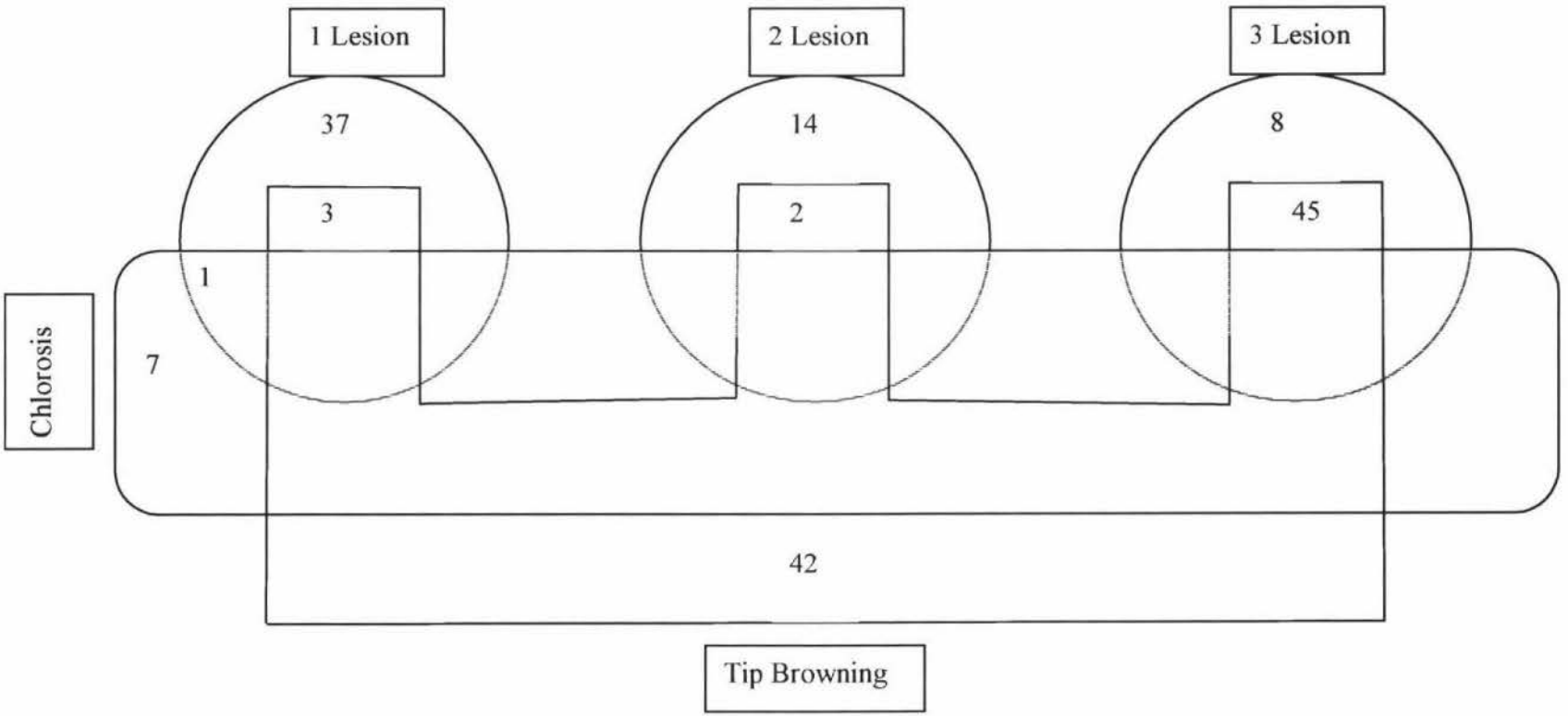
NZE7 (18/19)
Total = 740



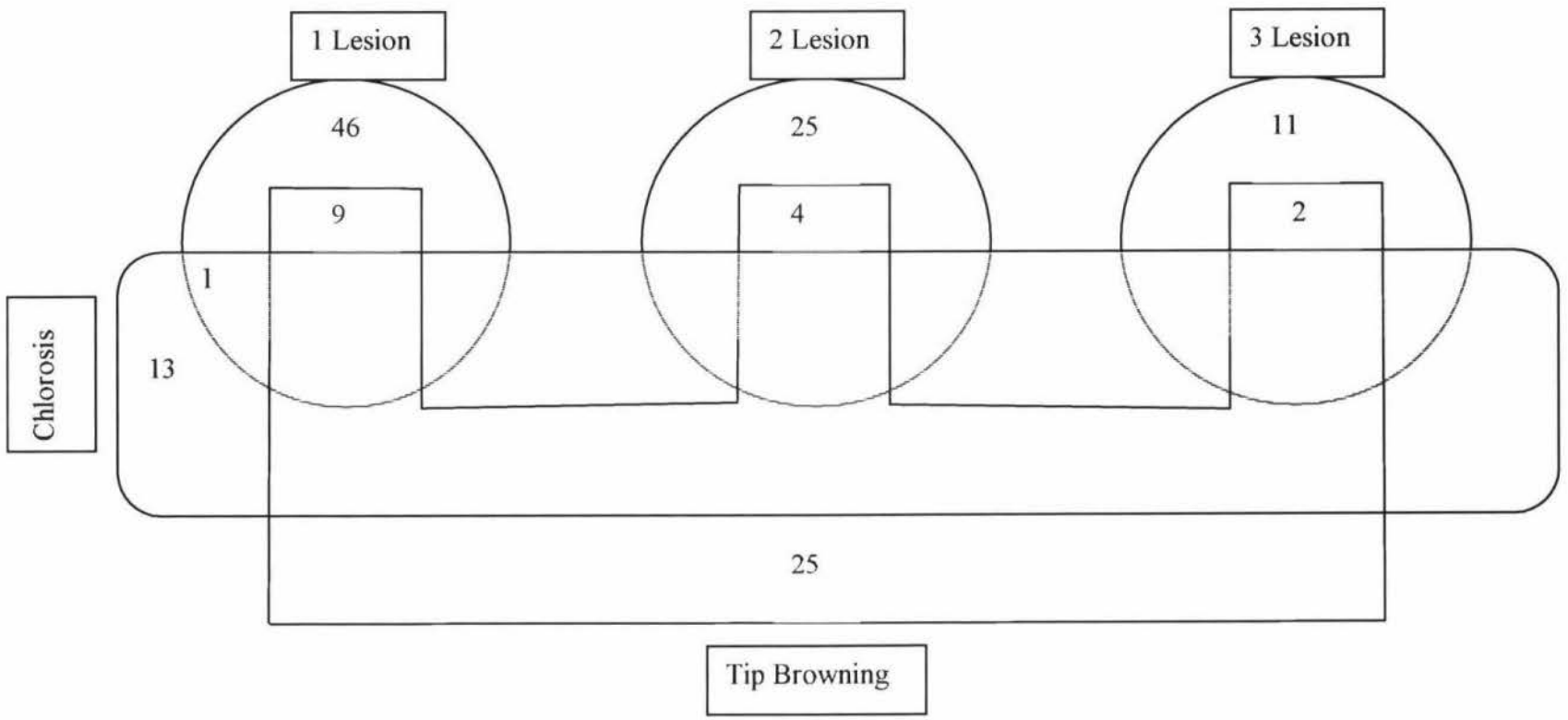
NZE7 (13/11)
Total = 691



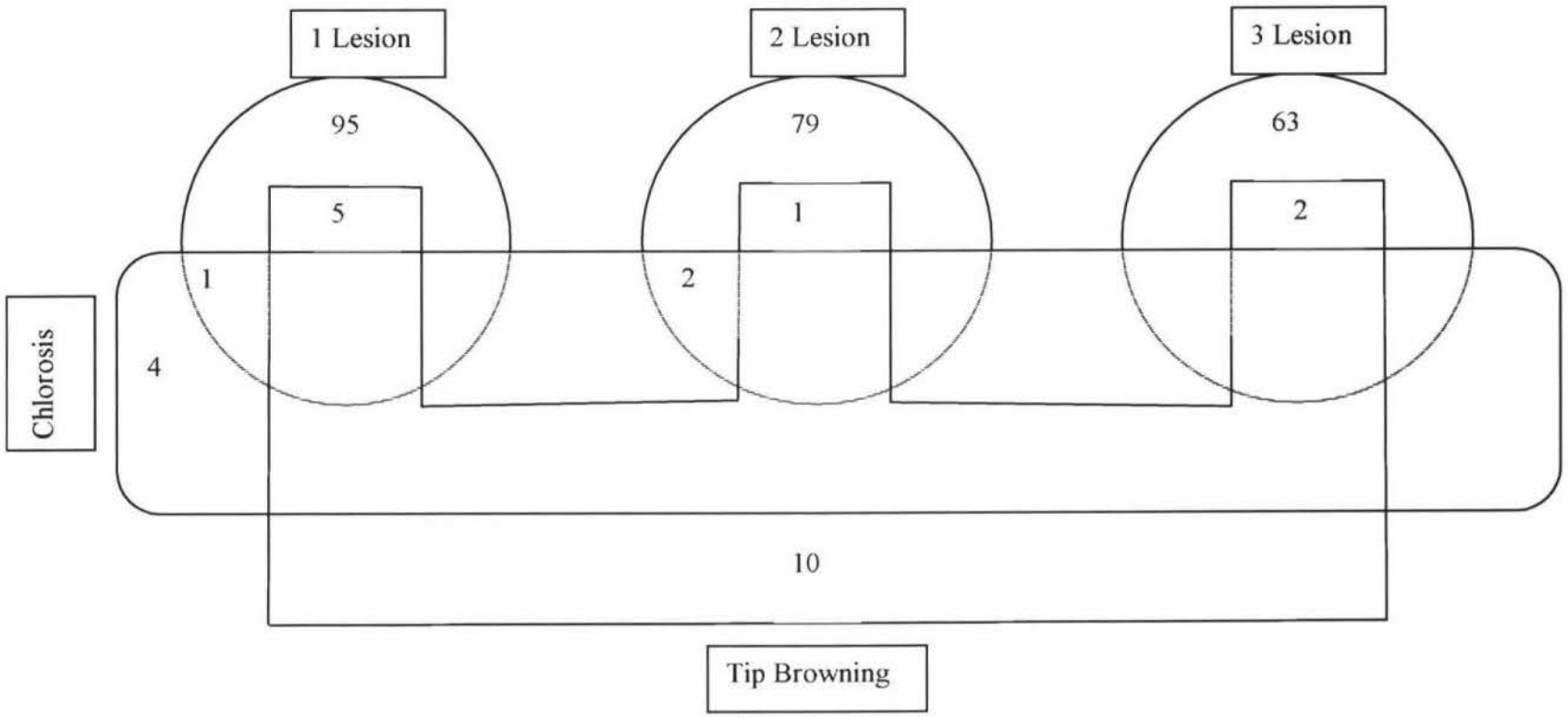
8A1 (60/5)
Total = 911



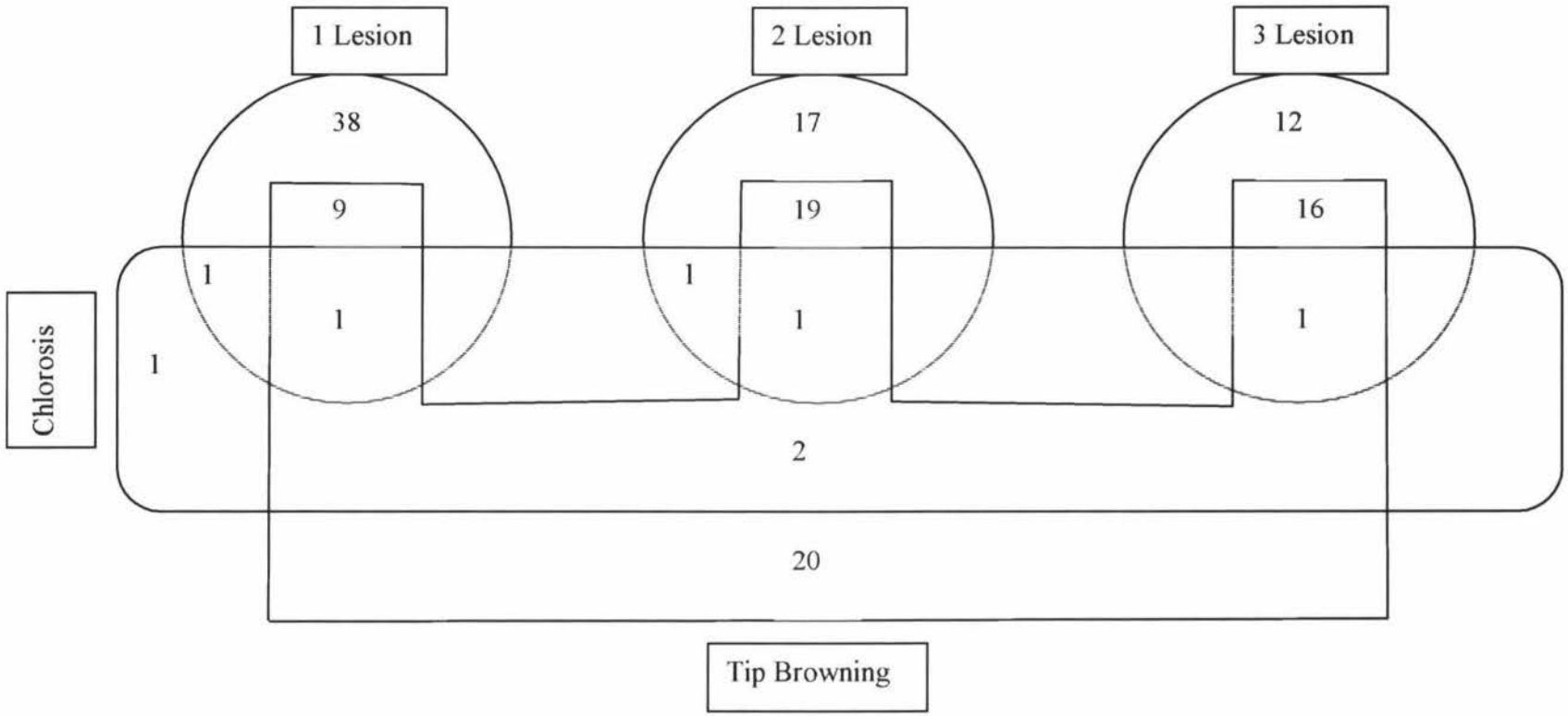
8A1 (18/19)
Total = 602



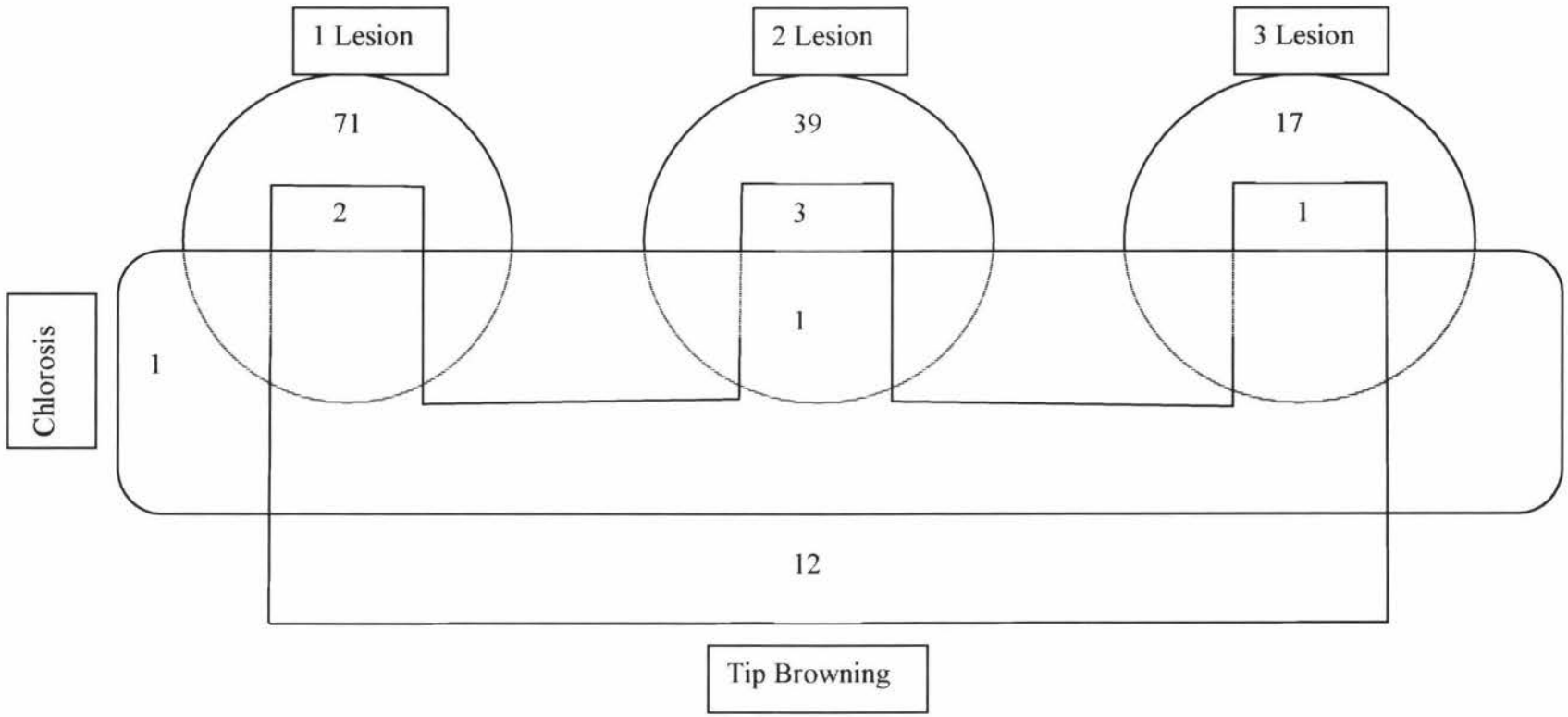
8A1 (13/11)
Total = 760



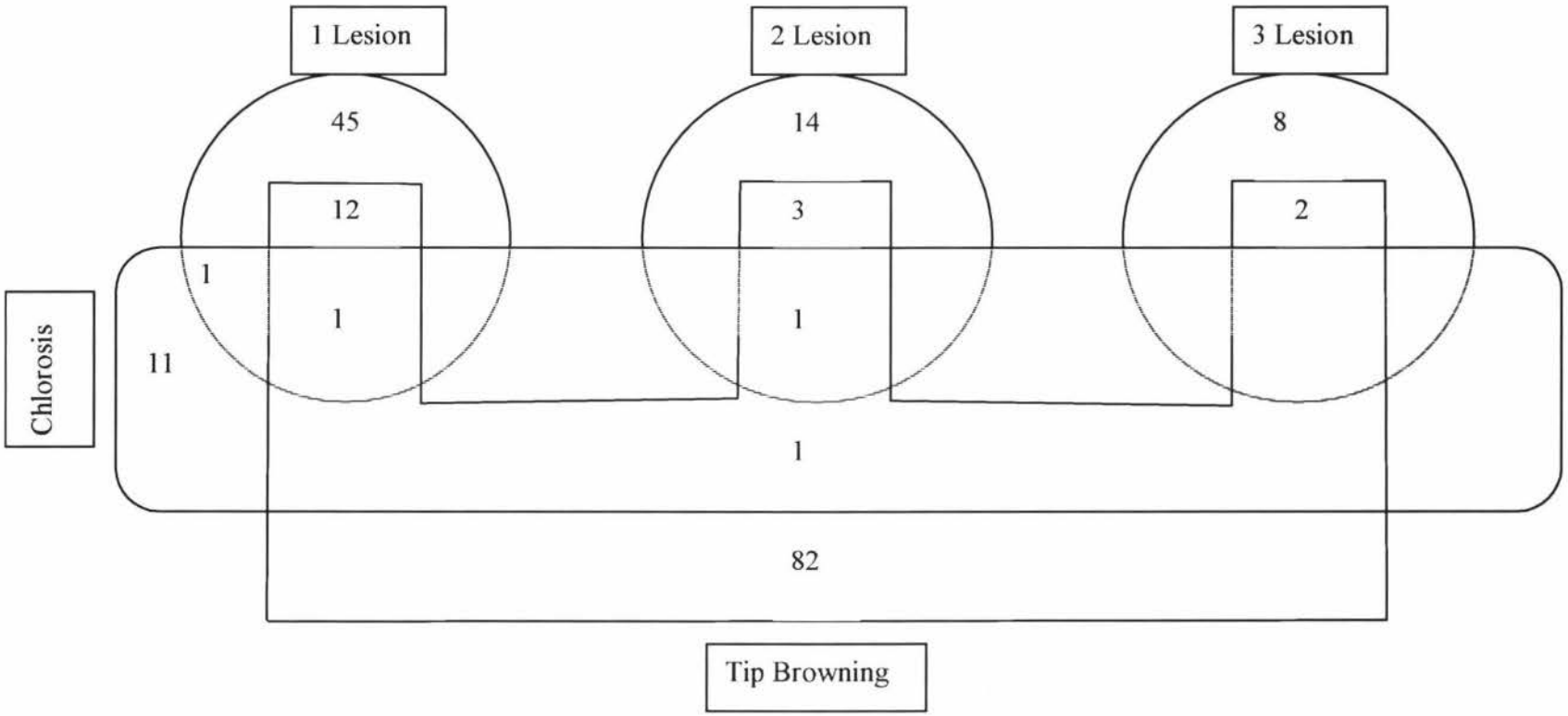
34C1 (60/5)
Total = 1087



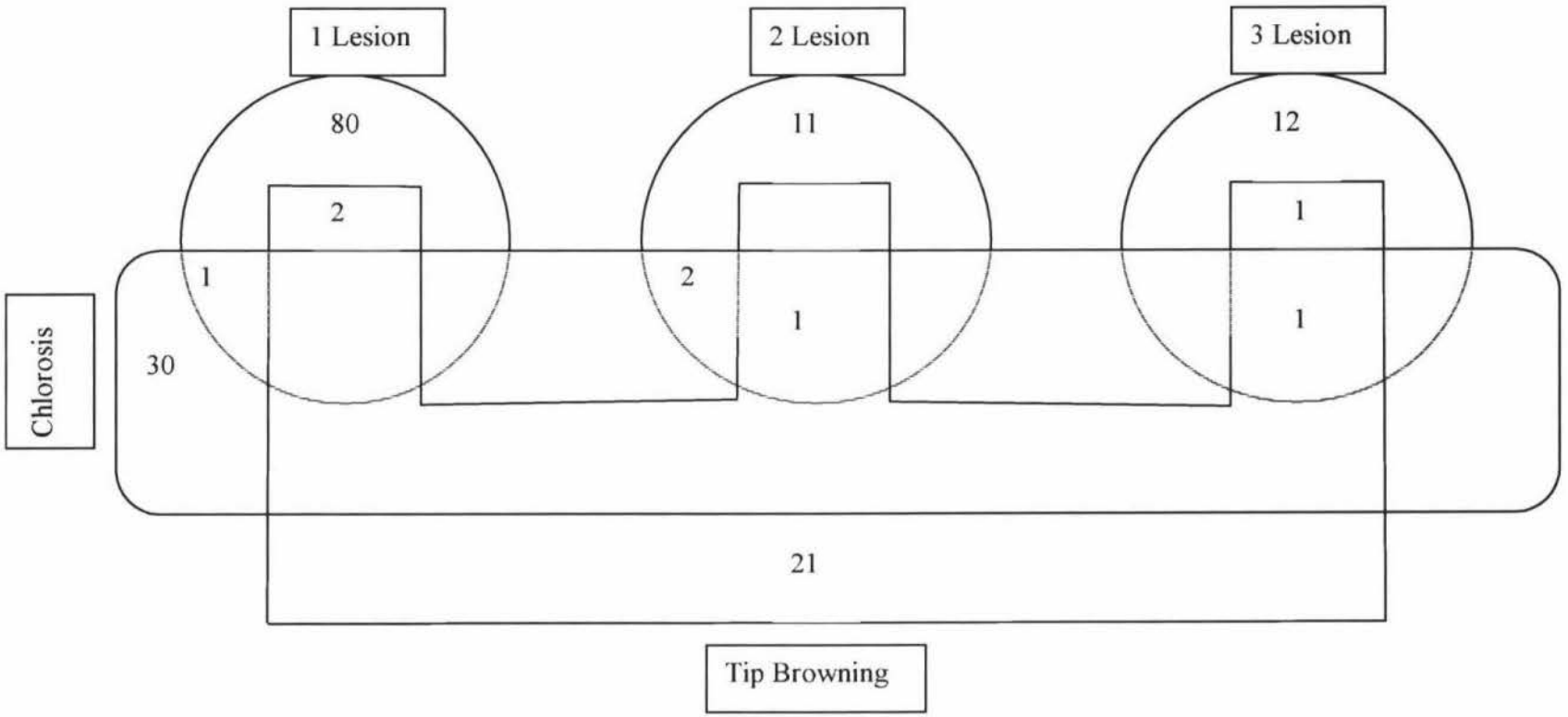
34C1 (18/19)
Total = 796



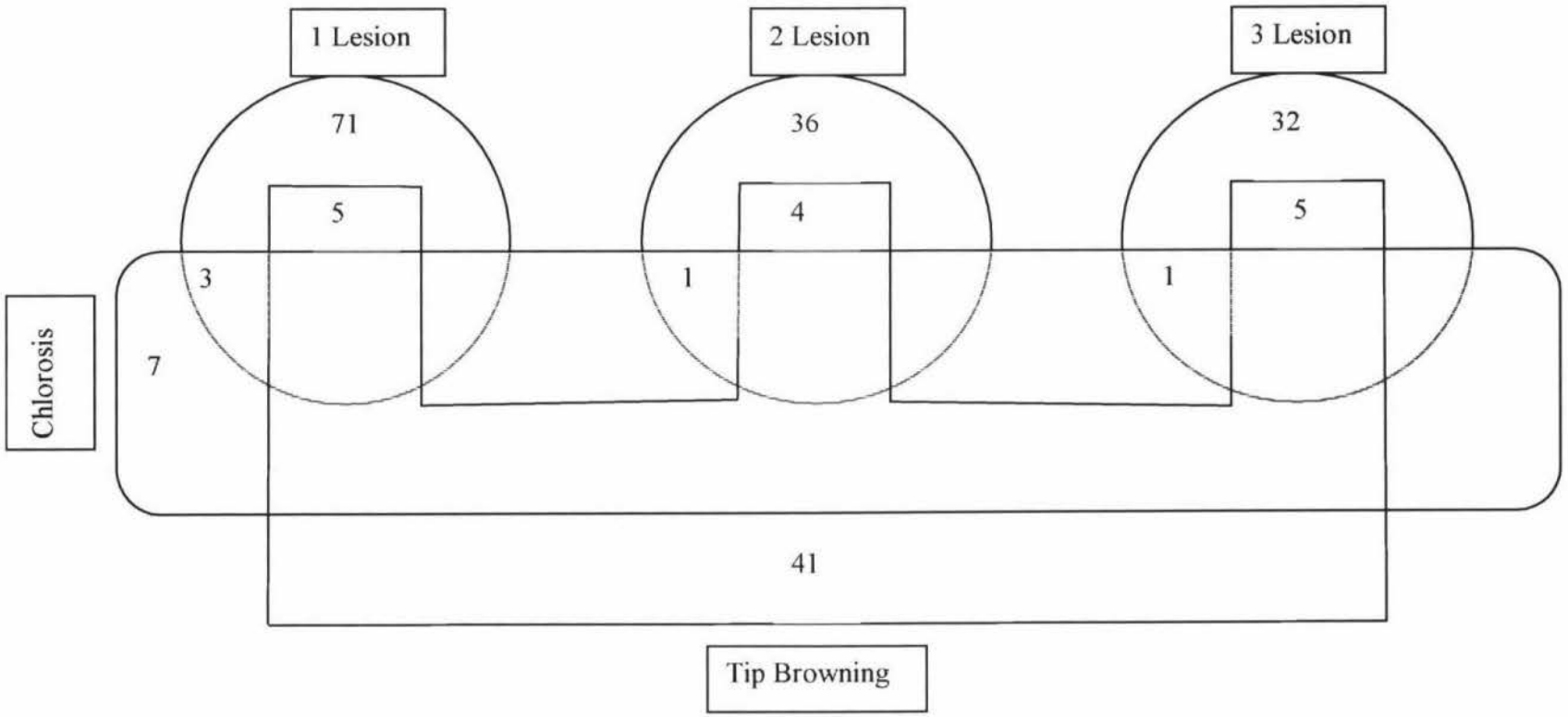
34C1 (13/11)
Total = 795



Negative (60/5)
Total = 1263



Negative (18/19)
Total = 951



Negative (13/11)
Total = 1053

Appendix III

Statistical Analysis

Anova: Single Factor	**Spore germination on AMM compared to PDA**					
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
AMM	15	1053.422	70.22816	104.3953		
PDA	15	803.0541	53.53694	92.65088		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2089.477	1	2089.477	21.20799	8.16E-05	4.195982
Within Groups	2758.647	28	98.5231			
Total	4848.124	29				

Anova: Single Factor	**Spore germination on AMM comparing different *D. pini* strains**					
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
NZE5	3	196.2316	65.41053	73.38023		
34Y1	3	226.01	75.33668	12.31446		
32	3	180.3262	60.10873	10.96513		
8A1	3	198.8341	66.27804	56.69445		
34C1	3	252.0205	84.00684	41.66578		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1071.494	4	267.8736	6.867847	0.006316	3.47805
Within Groups	390.0401	10	39.00401			
Total	1461.534	14				

Anova: Single Factor **Spore germination on PDA comparing different *D. pini* strains**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
NZE5	3	111.5052	37.1684	30.67685
34Y1	3	163.3203	54.44009	3.297707
32	3	170.1224	56.70746	54.66965
8A1	3	171.1718	57.05725	1.890761
34C1	3	186.9345	62.3115	5.747305

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1104.548	4	276.137	14.33997	0.000379	3.47805
Within Groups	192.5645	10	19.25645			
Total	1297.112	14				

Anova: Single Factor **Spore germination on PDA comparing different *D. pini* strains excluding NZE5**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
34Y1	3	163.3203	54.44009	3.297707
32	3	170.1224	56.70746	54.66965
8A1	3	171.1718	57.05725	1.890761
34C1	3	186.9345	62.3115	5.747305

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	99.81334	3	33.27111	2.028559	0.188508	4.06618
Within Groups	131.2108	8	16.40135			
Total	231.0242	11				

Anova: Single Factor

Spore germination on pine needle 10 days after inoculation comparing *D. pini* strains

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
NZE7	40	1748.997	43.72492	361.0742
34C1	40	1984.943	49.62357	240.9405
8A1	40	1813.036	45.3259	526.0995

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	744.3634	2	372.1817	0.989745	0.374762	3.073763
Within Groups	43996.46	117	376.0381			
Total	44740.82	119				

Anova: Single Factor

Spore germination on pine needle 12 days after inoculation comparing *D. pini* strains

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
NZE7	40	1646.887	41.17218	137.8786
34C1	40	1894.227	47.35568	84.14333
8A1	34	1481.301	43.56767	195.3047

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	776.2792	2	388.1396	2.852473	0.06194	3.078057
Within Groups	15103.91	111	136.0712			
Total	15880.19	113				

Anova: Single Factor **Spore germination on pine needle 14 days after inoculation comparing *D. pini* strains**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
NZE7	34	1460.437	42.95403	800.9138
34C1	27	1176.149	43.56107	632.7838
8A1	37	1883.95	50.91757	449.54

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1369.205	2	684.6024	1.101095	0.336712	3.092217
Within Groups	59065.97	95	621.7471			
Total	60435.18	97				

Anova: Single Factor **Spore germination on pine needle 16 days after inoculation comparing *D. pini* strains**

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
NZE7	38	1418.591	37.33134	140.9144
34C1	39	1576.085	40.41245	310.5621
8A1	38	1460.359	38.43049	142.5655

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	188.13	2	94.06502	0.472644	0.62459	3.077309
Within Groups	22290.11	112	199.0189			
Total	22478.24	114				

Chi-Square test comparing damage from different *D. pini* treatments

```
GenStat Release 7.1  (PC/Windows XP)              31 January 2005
16:20:20
Copyright 2003, Lawes Agricultural Trust (Rothamsted Experimental
Station)

                 ------------------------------------------------

                 GenStat Seventh Edition
                 GenStat Procedure Library Release PL15

                 ------------------------------------------------

   1  %CD 'g:/'
   2  "Data taken from unsaved spreadsheet: New Data;1"
   3  DELETE [redefine=yes] Damage_counts
   4  FACTOR [modify=yes;levels=5;reference=1] C2
   5  FACTOR [modify=yes;levels=2;labels=!t('Damaged','Undamaged')]
%tf2_1
   6  TABLE [class=C2,%tf2_1;margins=no] Damage_counts
   7  READ Damage_counts

    Identifier   Minimum      Mean   Maximum    Values   Missing
 Damage_counts     322.0      1330      2739        10         0

   9
  10  "Data taken from unsaved spreadsheet: New Data;2"
  11  DELETE [redefine=yes] Expected
  12  FACTOR [modify=yes;levels=5;reference=1] C4
  13  FACTOR [modify=yes;levels=2;labels=!t('Expected freq (Damaged)',\
  14  'Expected freq (Undamaged)')] %tf2_3
  15  TABLE [class=C4,%tf2_3;margins=no] Expected
  16  READ Expected

    Identifier   Minimum      Mean   Maximum    Values   Missing
      Expected     401.8      1330      2690        10         0

  18
  19  CHISQUARE [PRINT=test,prob; METHOD=maximumlikelihood;
GOODNESSOFFIT=yes] Damage_counts;\
  20    FITTEDVALUES=Expected; DF=1

 Goodness-of-fit test for observed versus expected values

 Likelihood chi-square value is 91.87  with   1 df.

 Probability level (under null hypothesis) p < 0.001
```

Chi-Square test comparing damage between ramet groups

```
                GenStat Seventh Edition
                 GenStat Procedure Library Release PL15

  1  %CD 'g:/'
  2  "Data taken from unsaved spreadsheet: New Data;1"
  3  DELETE [redefine=yes] ramets
  4  FACTOR [modify=yes;levels=3;reference=1] C2
  5  FACTOR [modify=yes;levels=2;labels=!t('damaged','undamaged')]
%tf2_1
  6  TABLE [class=C2,%tf2_1;margins=no] ramets
  7  READ ramets

    Identifier   Minimum      Mean   Maximum    Values   Missing
        ramets     683.0      2618      5220         6         0

  9
 10  "Data taken from unsaved spreadsheet: New Data;2"
 11  DELETE [redefine=yes] expected
 12  FACTOR [modify=yes;levels=3;reference=1] C4
 13  FACTOR [modify=yes;levels=2;labels=!t('damaged','undamaged')]
%tf2_3
 14  TABLE [class=C4,%tf2_3;margins=no] expected
 15  READ expected

    Identifier   Minimum      Mean   Maximum    Values   Missing
      expected     680.7      2618      5246         6         0

 17
 18  CHISQUARE [PRINT=test,prob; METHOD=maximumlikelihood;
GOODNESSOFFIT=yes] ramets; FITTEDVALUES=expected;\
 19   DF=1

 Goodness-of-fit test for observed versus expected values

 Likelihood chi-square value is  2.03  with   1 df.

 Probability level (under null hypothesis) p = 0.154
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