Optimizing *Dothistroma septosporum* Infection Of *Pinus radiata* And The Development Of Red-Band Disease

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Naydene Barron
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Errata (February 2007)

Page 14: Citation
Citation Lubeck et. al., 2002 should be Lorang et. al., 2001.
All reference citations will be accurate upon publication.

Page 34: Word correction (line 7)
'resistance to infection *until* around eight years' should be 'resistance to infection *after* around eight years'.

Page 95: Deletion (paragraph 2, line 3)
'(results not shown)' should be deleted as photograph illustrating results on page 96.

Page 41: Figure 6 legend
The arrows in Figure 6(A) indicate red bands, symptoms of *D. septosporum* infection.

Page 42: Figure 7 legend
The arrows in Figure 7(A) indicate red bands, whilst in Figure 7(B) the arrows indicate naturally occurring necrotic needles.

Page 43: Figure 8 legend
The arrows in Figure 8(A) indicate the natural inoculum suspended over pine seedlings, whilst in Figure 8(B) the arrows indicate necrotic needles on the pine seedling at the end of the trial. The necrotic regions showed signs of *D. septosporum* infection.

Page 64: Significance between treatments
The significant difference between the mean daily radial growth rate of NZE10 in Table 11 was compared with Tukeys HSD test at $p<0.05$. The significant difference in growth rate is between YES media and all other media tested.

Page 64 (Table 11.) and 66 (Figure 13.): Comparison of data
The data presented in Table 11 is the daily growth rate on un-buffered media and this experiment was not conducted at the same time as the daily growth rate on buffered media. Additionally, the pH of the un-buffered media (mentioned page 67) is comparable to the pH of buffered media, but the daily growth rate is considerably higher for the un-buffered media. The difference in growth rate may be due to physiological variability of the fungal isolate, commonly seen, or due to the buffer and not the actual pH affecting growth rate.

Abstract

The filamentous fungus *Dothistroma septosporum* infects pine species throughout the world causing red-band disease, one of the most serious diseases of conifer species. In NZ, a clonally derived asexual strain of *D. septosporum* was identified in 1964, and has spread throughout the country. There are conflicting accounts on the environmental conditions required for infection, which has lead to difficulties in optimizing a laboratory-based system for infection. The pathogen is spread naturally through rain-splashed inoculum of conidiospores from mature stromata that have erupted through the pine needle tissue. Diseased needles become necrotic, often with a red band due to the mycotoxin dothistromin produced by the hyphae. Dothistromin has the chemical structure of a difuranoanthraquinone and shows similarity to the aflatoxin precursor, versicolorin B produced by *Aspergillus parasiticus*. The role of dothistromin in pathogenicity has not yet been determined, although experiments have shown injecting toxin into pine needles results in the characteristic red band lesion.

In this study it was found that fluctuating temperature (16°C/24°C), a 12 h diurnal cycle (white and ultraviolet light), high relative humidity and continuous moisture are conditions conducive to development of red-band disease on inoculated pine trees in an artificial environment. A higher rate of infection was obtained using pine seedlings as opposed to pine cuttings, and using a spore suspension containing a yeast extract. A dothistromin minus mutant was able to infect pine needles, indicating that dothistromin is not a pathogenicity factor, though it may be a virulence factor. The use of GFP-expressing isolates allowed the initial infection process to be monitored with both wild type and mutant isolates. Additionally, a PCR-based diagnostic procedure to confirm infection was developed.

The production of aflatoxin by *Aspergillus* species is regulated by nutritional parameters and extracellular pH, which affect both growth and aflatoxin gene expression. *D. septosporum* similarly has enhanced growth at acidic pH, but it does not appear that pH has a strong influence on physiological processes as toxin biosynthesis and gene expression do not appear to be pH regulated. Different carbon and nitrogen sources also affect the morphology of *D. septosporum*.

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Abbreviations

Abbreviation	**Meaning**
AF	aflatoxin
bp	base pair
cDNA	complementary deoxyribonucleic acid
°C	degree Celsius
DMSO	dimethyl sulphoxide
Dnase	deoxyribonuclease
DOTH	dothistromin
DW	dry weight
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein
G	gram
eGFP	enhanced green fluorescence protein
GFP	green fluorescence protein
sGFP	synthetic green fluorescence protein
GMO	genetic modified organism
ITS	internal transcribed spacer region
kb	kilobase pair
kDa	kilo Dalton
L	litre
M	mole per litre
ml	milliliter
mM	millimole per litre
PCR	polymerase chain reaction
RT-PCR	real time polymerase chain reaction
ST	sterigmatocystin
µl	microlitre
µM	micromole per litre
µg	microgram
UV	ultraviolet

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Introduction

1.1 Dothistroma Needle Blight

Dothistroma needle blight (red-band disease) is a foliage disease of pine trees caused by the filamentous fungus, *Dothistroma septosporum* (Barnes et al., 2004). It is thought that *D. septosporum* originated from South America in high-altitude cloud forest regions (Bradshaw et al., 2000). The fungus has been found to infect both native and exotic pine species throughout the world, resulting in premature defoliation and incremental wood loss in proportion to disease severity. Over the last decade there has been an increase in disease incidence in the Northern hemisphere where the fungus now appears to be colonizing native trees in addition to exotic pines (Woods et al., 2005). Consequently, dothistroma needle blight is now classed as one of the most important diseases of pine, and is of major economic concern to the forest industry in countries such as New Zealand, Australia, Canada, Chile, Europe, Kenya, and the United States of America (Woods et al., 2005).

D. septosporum, the asexual form was first identified in New Zealand *Pinus radiata* pine plantations in 1962, with positive confirmation in 1964 (Gibson, 1972). The sexual form of the fungus, *Mycosphaerella pini* or *Scirrhia pini* has not yet been identified in NZ, although the sexual form was found in Europe and the USA in the early 1920's (Bradshaw et al., 2000). The current asexual form found in NZ is clonally derived with very low genetic diversity, indicating a single strain of *D. septosporum* was introduced into this country (Hirst, 1999).

1.1.1 Environmental Conditions Conductive to Infection

There are conflicting views between scientists, dating back to the earliest studies in the 1970's, on the precise environmental conditions required for *D. septosporum* to infect pine needles. The four most important variables that appear to affect the severity of infection are needle wetness, temperature, humidity and light.

Gadgil (1974 and 1977), inoculated pine seedlings in an artificially controlled environment and showed there was no significant effect of the length of the post-inoculation wetness period on germination and mycelial growth on pine needles. In contrast, there were significant effects of temperature on infection, with stromata appearing two weeks post-inoculation at 24°/16°C (day/night), and four weeks post-inoculation at 20°/12°C (day/night) under continuous wetness conditions with 70-80% relative humidity. At temperatures of 16°/8°C, 12°/4°C (day/night) with either short periods or continuous wetness conditions and lower relative humidity, infection levels were significantly reduced and stromata took seven weeks to appear. Although infection can occur in dry conditions, severity of infection increases with increased length of wetness period, and an optimum temperature between 12°C and 24°C.

Light intensity also has an effect on disease severity, with field observations indicating that there is less infection by *D. septosporum* on shaded foliage than on foliage that is exposed to direct light. Under experimental conditions, the severity of infection decreased linearly with decreasing light intensity (181 W/m^2 – 58 W/m^2). However, stromata appeared post-inoculation within two weeks for all light intensity conditions tested (Gadgil, 1976).

An intensive field study from the early 1990's to the present within an identified *D. septosporum* epidemic area in Northwest British Columbia (BC) Canada, revealed that an increase in precipitation at temperatures over 16°C, correlated with an increase in disease severity (Woods et al., 2005). Over the last two years (2004-2006) in BC, the increase in summer precipitation has led to approximately 70% mortality of log pole pine in certain areas, in conjunction with an extensive increase in disease severity in other areas (Woods, personal communication), as seen in Figure 1. This highlights the importance of environmental conditions that affect extent and severity of disease in the field, whilst also enabling a consensus to be reached on the ideal conditions required to obtain infection in laboratory conditions.

Figure 1. Dothistroma damage of log pole pine forests in British Columbia

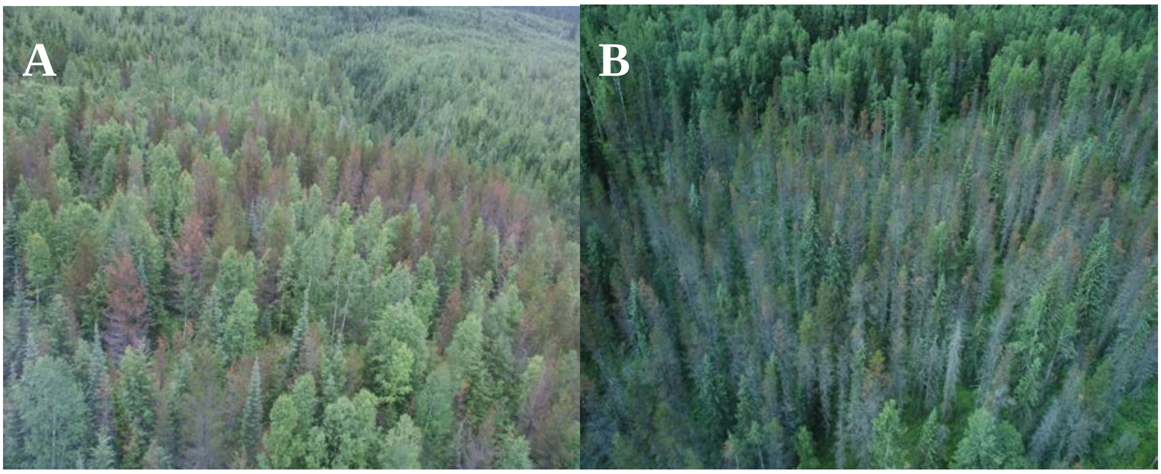


Photo A; extensive Dothistroma damage (Bell Irving, BC, Canada), B; Mortality (Sediesh Creek, BC, Canada), courtesy of Alex Woods.

1.1.2 Mode of Infection

Generally *D. septosporum* is spread over short distances by passive dispersal of conidia, from infected foliage, in water droplets (Ivory, 1972b). How the fungus has spread over long distances is unclear, although research conducted in Kenya showed that conidia were taken up into clouds from infected forests at high altitude (Gibson, 1972). The resilience of conidia to temperature extremes means the spores can remain viable for months until they encounter favorable environmental conditions allowing germination (Gibson, 1972).

Once conidia (typically four celled) are attached to the pine needle, germination takes place within three days with a germ tube arising from each cell (Ivory, 1972b). Generally germ tubes appear first from the terminal cells of the conidia, growing more vigorously than germ tubes arising from the median cells. *In vitro* studies have shown fusion of germ tubes from different conidia in addition to germ tube branching (Gadgil, 1967; Ivory, 1972b). An extensive study by Peterson and Walla (1978) on ponderosa and Austrian pine in Nebraska showed germ tube growth is directed toward the stomatal pore. Two germ tubes from the same conidia or branched germ tubes often grew directly into the stomatal pore. Furthermore, needle topography (abaxial or adaxial side of the needle) did not affect germ tube orientation (Peterson and Walla, 1978). The fungus, once established, forms an appressoria like structure over the stomatal cavity, with an infection peg penetrating the stomata (Peterson and Walla, 1978; Franich, 1983). The stomata of young needles are open pores composed of guard cells that are covered in a microtubular wax that appears to signal appressoria formation. This is in contrast to mature needles where the stomatal opening is occluded with a resinous material that may present a mechanical barrier (Franich, 1983). Stomatal penetration can occur within two days of germination, with the infection peg branching within the pine needle sub-stomatal chamber. Under experimental conditions using macerated mycelium, direct penetration of the epidermis has been observed with hyphae subsequently spreading throughout the mesophyll tissue (Gadgil, 1967).

Once inside the needle tissue, fungal hyphae spread both intra- and inter-cellular within the mesophyll, with lateral spread limited to a few millimeters from point of penetration. The regions of the needle tissue where hyphae are contained, and mesophyll cells adjacent to the hyphae, become necrotic possibly due to the presence of dothistromin (DOTH) toxin produced by the hyphae (Gadgil, 1967) or due to collapsed cells becoming filled with resin (Ivory, 1972b). This area of necrosis produces a red/brown band, a symptom of disease and a key characteristic of red band disease. The lesion area is often contrasted by healthy green tissue or in some cases light green/yellowish tissue may flank the lesion (Gadgil, 1967).

Correlated with the appearance of a lesion is the formation of black stromata within the necrotic region in the hypodermis between needle stomata (Ivory, 1972b). The presence of stromata depends on the environmental conditions such as moisture on the needle surface, and the earliest they have been reported to occur is two weeks post-inoculation (Gadgil, 1974, 1976, 1977). Asexual conidia are produced within the stromata beneath the epidermis, which mature to split the epidermal tissue longitudinally and expose the conidia (Ivory, 1972b; Barnes et al., 2004). The conidia are hyaline, can appear curved or straight, usually one to three septate and produced in a slimy mass (Bradshaw, 2004).

1.1.3 Lifecycle in the Forest Environment

The main infection period in New Zealand is between November and February (late spring-summer). During periods of rain or heavy mist (Gibson, 1972), conidia from erupted stromata collect within the film of water that covers the needle surface. Water droplets falling from needles are broken up upon contact with another surface which allows the conidia to become airborne. Dispersal of conidia is generally within the vicinity of the neighboring tree and considered the most important form of conidia dispersal (Gibson, 1972). Therefore, the severity of infection depends on temperature, needle wetness and the number of viable conidia landing on the needle surface.

Once infection has occurred the length of the pre-reproduction period is variable. The incubation period before stromata are produced may be as short as three weeks in the summer or as long as 16 weeks in autumn; sometimes sporulation may not occur until the following spring (Gibson, 1972). Generally, the shorter the incubation period the more severe infection will be if there is adequate rainfall. In New Zealand it has been established that rainfall above 500 mm spread over 50 rain days between November-February is ideal for severe infection to occur in areas with adequate inoculum. However, conidia can remain viable on dry needles for up to 11 months at 18°C and five months at 30°C (Gibson, 1972).

Brick red bands around the needles, typical of dothistroma needle blight can appear within weeks of infection and are often still visible when the infected needle has died. The needle tissue beyond the band often dies, and the whole needle becomes necrotic (Bradshaw, 2004). Symptoms usually appear on the lower branches of the tree, during late summer but are more obvious during winter. Premature defoliation of dead needles occurs in the spring prior to new needle growth (Franich et al., 1982). Dothistroma does not appear to survive on shed needles on the plantation floor for longer than two months, probably due to microbial competition (Gibson, 1972).

1.1.4 Dothistroma Needle Blight Control

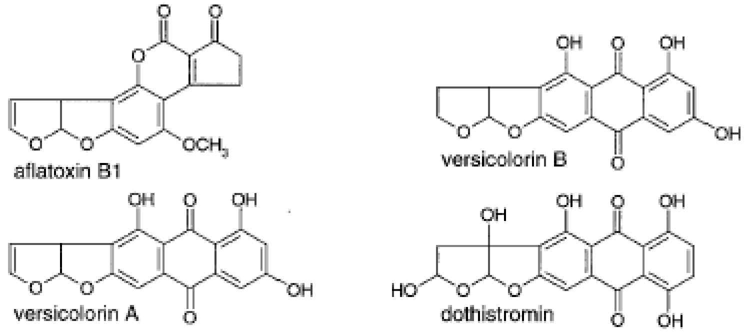
There are three methods used to control Dothistroma needle blight in the commercial pine forests in the Southern hemisphere. These control methods are: use of resistant pine seedlings, silvicultural practices such as pruning and thinning of infected branches, and aerial application of copper fungicide (Bradshaw, 2004). A dothistroma-resistant cultivar of *P. radiata* was developed in 1983 and is available in New Zealand for planting in high incidence areas where needle blight is a problem (Jayawickrama and Carson, 2000). The resistant cultivar has been estimated to reduce incidence of infection by 15% (Dick, 1989). In addition, *P. radiata* becomes more resistant with age, usually around eight years in moderately diseased stands, or around 15 years in heavily diseased stands. However, older pine trees take longer to recover from severe infection, and unfortunately little is known about the mature tree resistance mechanism (Gibson, 1972). The most prominent form of control is still the use of copper fungicides.

Fungicides rose to prominence in 1964 in Kenya, when field trials showed that copper fungicides applied from the air were effective in controlling needle blight. In New Zealand, aerial application of copper oxychloride and cuprous oxide have been effective in controlling dothistroma since the early 1970's (Bradshaw, 2004). All stands within New Zealand are assessed for infection in July/August each year, and stands where infection levels exceed 20% are subjected to aerial application of the copper fungicide at a rate of 5 L/ha (1.66 kg copper oxychloride and 2 L emulsifiable spray oil with sufficient water to make up the volume to 5 L) in November/December when inoculum levels are greatest (Bulman et al., 2004). Copper fungicide is taken up by *D. septosporum* conidia within 60-90 minutes of contact. It prevents germ-tube growth, whilst also inhibiting the production of secondary conidia. The copper spray persists on needles for several months (Franich, 1988) protecting existing foliage from new infection.

1.1.5 Dothistromin Mycotoxin

Dothistromin (DOTH) is a phytotoxin that has been isolated from cultures of *D. septosporum*, and is a difuranoanthraquinone, as determined by mass spectrometry and nuclear magnetic resonance (NMR) (Bear et al., 1972). DOTH is also produced by *Cercospora* species, including the peanut pathogen *C. arachidicola* (Stoessl and Stothers, 1985). In addition, there is structural similarity between DOTH and an aflatoxin precursor, versicolorin B, produced by *Aspergillus parasiticus* and *A. flavus*, with similarity of biosynthetic steps involved in production of aflatoxin (AF) by *A. parasiticus* (Bradshaw et al., 2002). The structural similarity has been confirmed (by ^{13}C NMR), showing the bistetrahydrofurano side chain of DOTH is similar to aflatoxin and sterigmatocystin side chains (Shaw et al., 1978). Furthermore, both DOTH and versicolorin B share a saturated bifuran ring although the arrangement of the hydroxyl groups of the anthraquinone ring is different (Figure 2) (Bradshaw et al., 2002).

Figure 2. Molecular structures of aflatoxin B_1, versicolorin A, versicolorin B and dothistromin



(Bradshaw et al., 2002)

The characteristic red colour of pine needle lesions resulting from dothistroma infection is due to accumulation of DOTH. This has been shown experimentally by inducing artificial lesions through injecting DOTH into pine needles (Shain and Franich, 1981; Franich et al., 1986). In addition, the tissue that separates the live part of the needle from the dothistroma-induced lesion is highly lignified, having four times as much lignin as the rest of the needle tissue (Franich et al., 1986). DOTH is oxidized in needle lesions primarily to CO_2 and oxalic acid, with benzoic acid synthesized by the host in cells adjacent to those killed by the toxin. Light affects the toxicity of DOTH, with greater breakdown of the toxin in the presence of light. DOTH is also reduced in an NADPH-dependent reaction, upon auto-oxidation forming H_2O_2 and O_2^-, and under anaerobic conditions is capable of generating OH radicals (Franich et al., 1986). Shain and Franich (1981) detected an ethylene response in needles injected with DOTH, both in light and dark conditions, albeit a smaller response in darkness, but greater than controls. This suggests the host is capable of a response regardless of light conditions (Shain and Franich, 1981). However, the production of ethylene in dark conditions may have been due to the presence of residual oxygen radicals, which were generated from the NADPH pathway when the needle tissue was previously exposed to light. Perhaps through a photosensitizing process, DOTH may exert toxicity by generating reactive oxygen species (Franich et al., 1986).

Other research has been done to determine possible roles for the mycotoxin DOTH. A study conducted to determine the toxicity of DOTH to *Pinus* tissue found growth of pine embryo and meristematic leaf callus was completely inhibited by 13 nmol DOTH per gram of tissue. Furthermore, an immunoassay confirmed the uptake of DOTH by the pine embryos, they became orange and DOTH disappeared from the solution. Using a dothistromin-mouse albumin conjugate and DOTH-specific antibodies, the experimenters identified DOTH binding in pine embryos to small vesicles and a putative 40-kDa dothistromin binding peptide (Jones et al., 1995). DOTH has also been shown to inhibit the metabolism of the bacterial species *Bacillus megaterium* and *Chlorella pyrenoidosa* (Harvey et al., 1976). Inhibition was dependent on the concentration of DOTH added to the cultures, and it was proposed that bacterial growth in culture recommenced due to DOTH being broken down by light (Franich et al., 1982). However, whether DOTH is absolutely required for infection to occur in pine needles, or simply a virulence factor facilitating infection in respect to red-band disease is yet to be elucidated.

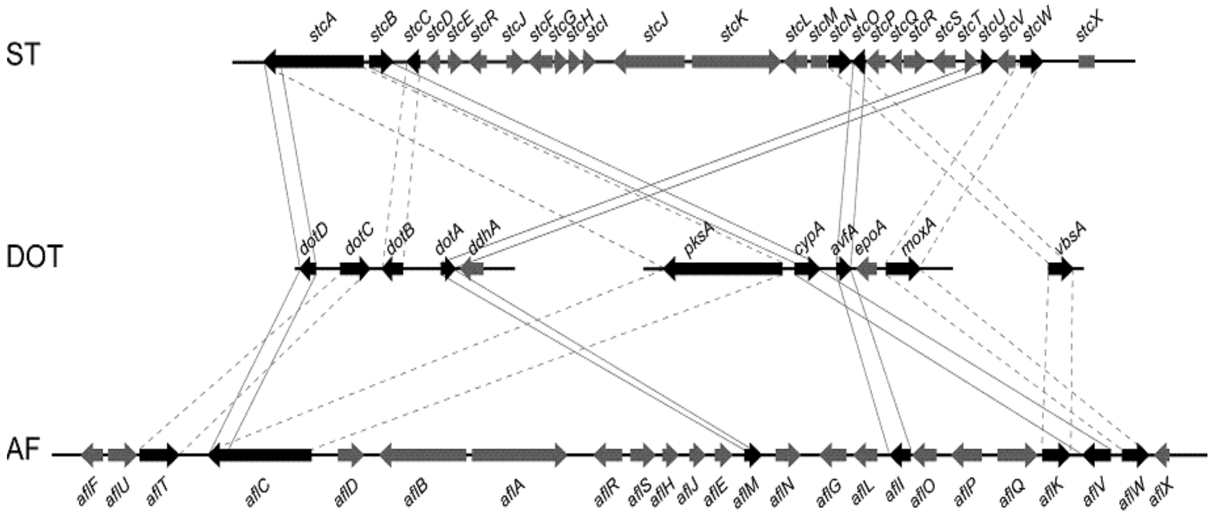
1.1.6 Fungal Gene Clusters

As mentioned above, DOTH biosynthesis appears to be similar to AF biosynthesis, which has been confirmed by using aflatoxin genes as hybridization probes to recover dothistromin genes (Bradshaw et al., 2002). The genes involved in AF biosynthesis are well characterized, due to AF B_1 being a potent natural carcinogen that is of major concern to the food industry. In addition, sterigmatocystin (ST), produced by approximately 20 species of fungi, is an intermediate compound in the AF biosynthetic pathway. Both AF and ST genes are clustered, although the order of genes is different (Klich et al., 2000). The AF gene cluster contains approximately 25 genes within a 60 to 70 kb region (Bradshaw et al., 2002), whilst the ST gene cluster contains 25 genes within a 60 kb region (Brown et al., 1996). Both the AF and ST pathways show conservation in respect of function and regulation (Brown et al., 1996). There are 10 enzymatic steps required for ST, and 12 steps required for AF biosynthesis after generation of the first stable intermediate, norsolorinic acid (Zhang and Keller, 2004). It has been proposed that dothistromin biosynthesis genes are clustered and share biosynthetic steps with AF production, of which several genes have been characterized to date that show homology to AF pathway genes (Bradshaw et al., 2002).

Bradshaw et. at. (2002) found a genomic clone containing part of the putative dothistromin gene cluster, containing four genes showing similarity to AF cluster genes. These genes have predicted functions based on similarities with AF/ST genes, being a ketoreductase (*dotA*), oxidase (*dotB*), major facilitator superfamily transporter (*dotC*) and thioesterase (*dotD*) (Bradshaw et al., 2002). Recently, four other genes have been isolated from the *D.*

septosporum genomic library that show homology to *aflC,, aflV, aflI and aflW* genes from the AF gene cluster of *A. parasiticus*, in addition to the discovery of an epoxide hyrolase (*epoA*) gene which has not been found in the AF/ST gene clusters (Bradshaw et al., 2006). Figure 3 shows the AF and ST gene clusters of *A. parasiticus* and *A. nidulans*, with dark arrows indicating homologous genes found within three genomic regions from *D. septosporum*.

Figure 3. Comparison of putative DOTH gene cluster with AF/ST gene clusters



(Bradshaw and Zhang, 2006)

The *dotA* gene (proposed ketoreductase) encodes for a 263-amino acid sequence which shows 80% identity with the *A. parasiticus* AflM gene product and 79% identity to the ST gene product, StcU from *A. nidulans*. A *dotA* mutant created by gene replacement did not produce DOTH, but produced a bright yellow pigment in media that was confirmed by TLC analysis and mass spectrometry to be versicolorin A. Versicolorin A is also produced by the *aflM* mutant of *A. parasiticus* further suggesting a similar biosynthetic role of DotA to AflM (Bradshaw et al., 2002). The *stcU* gene (homolog *aflM* in *A. parasiticus*) is required for the conversion of versicolorin A to ST (Brown et al., 1996).

The DotB gene product has a predicted oxidase function based on 24% amino acid identity with the StcC gene product from *A. nidulans*, with no homology seen to any *A. parasiticus* AF genes (Bradshaw et al., 2002) Both StcC and DotB show amino acid identity (29% and

24.3% respectively) with a chloroperoxidase (Brown et al., 1996) which in *Caldariomyces fumago* catalyzes a variety of oxygen transfer reactions (Conesa et al., 2001).

A toxin pump activity has been proposed for DotC (585 amino acid protein) based on 31.2% and 30.8% homology to AflT gene (*A. parasiticus*) and ToxA (*Cochliobolus carbonum*) gene products respectively (Bradshaw et al., 2002). In *C. carbonum*, the ToxA gene product is required to export endogenously produced HC-toxin (a cyclic peptide) from the cell, essential for protecting the fungus from the effects of this toxin. A similar gene (*cfp*) has been identified in *Cercospora kikuchii* which produces the toxin cercosporin. Disruption of the *cfp* gene resulted in decreased cercosporin production and loss of virulence (Upchurch et al., 2002).

The *dotD* gene encodes a 322 amino acid polypeptide that has putative thioesterase enzymatic activity with homology to polyketide synthase (*pks*) genes involved in AF/ST biosynthesis (Bradshaw et al., 2002). The Pks gene product involved in the ST pathway has four catalytic domains, of which *dotD* appears to encode only one of these domains, a monofunctional thioesterase (Bradshaw et al., 2002). The thioesterase domain may be involved in either accepting malonyl-CoA or releasing the intermediate product in initial enzymatic steps from the hexanoate starter unit to the first stable intermediate, norsolorinic acid (Yu and Leonard, 1995).

As mentioned early, a cluster of five genes has been found in *D. septosporum* with four genes, *pksA*, *cypA*, *avfA* and *moxA* showing 40-60% amino acid identity to genes involved in AF/ST biosynthesis (Figure 3.) (Bradshaw et al., 2006). The *pksA* gene homolog, *stcA* from *A. nidulans* encodes a polyketide synthase involved in the assembly of norsolorinic acid from hexanoyl CoA and malonate, the first intermediate in the ST pathway (Brown et al., 1996). The essential function of *stcA* in ST production has been confirmed by feeding hexanoic acid to *stcA* mutant strains, in which norsolorinic acid was not produced (Zhang and Keller, 2004). In addition, a gene disruptant of the *A. parasiticus* homolog *aflC* did not produce any AF intermediates, showing a role of *aflC* early on in the AF biosynthetic pathway. The AflC protein contains motifs similar to those found in fatty acid synthases, with the notable difference of no ketoreductase activity. This indicates that AflC is capable of synthesizing norsolorinic acid as long as the starting product is a hexanoate, thus not requiring reduction (Feng and Leonard, 1995). The putative role of the *pksA* gene from *D. septosporum* has been determined by gene replacement, which resulted in a loss of DOTH biosynthesis. The predicted function of PksA in toxin biosynthesis was determined by metabolite feeding experiments with norsolorinic acid and versicolorin A. The *pksA* mutant was able to convert these aflatoxin precursors to DOTH, indicating that PksA is likely involved in some form of condensation reaction in the early stage of toxin biosynthesis.

The *avfA* gene is a putative oxidase with 47% and 43% amino acid identity to both *aflI* and *stcO* (*A. paraciticus* and *A. nidulans* respectively) gene homologs (Bradshaw et al., 2006). A complementation experiment in *A. paraciticus* determined that AvfA is involved in the conversion of averufin to versiconal hemiacetyl acetate (Wen et al., 2005). The *cypA* gene is a putative averufin monooxygenase with 59% amino acid identity to *aflV* and *stcB* genes (Bradshaw et al., 2006). In *A. paraciticus*, the AflV gene product catalyses the reaction from averufin to hydroxyversicolorone (Wen et al., 2005). The AflV protein contains two conserved regions, a heme-binding motif and hydrogen bond region which are typical active sites present in cytochrome P450 enzymes (Yu et al., 1998). The *moxA* gene encodes a 626 amino acid putative hydroxyversicolorone monooxygenase with 55% amino acid identity to the AflW gene product from *A. paraciticus* (Bradshaw et al., 2006). Latest research by Wen et. al. (2005) determined that the moxY (AflW) gene catalyzes two reactions, one from hydroxyversicolorone to versiconal hemiacetal acetate and the other from versicolorone to versiconol acetate (Wen et al., 2005).

1.2 Pathogenicity Assay

The development of a laboratory based pathogenicity assay to monitor infection by *D. septosporum* has several potential benefits for the forestry industry. First, an *in vitro* assay could be used as a prescreening for resistance (Browne and Cooke, 2004a). This would decrease the time and cost involved in screening potential resistant pine cultivars and allow early detection of non-resistant cultivars so they may be excluded from any field trial (Diamond and Cooke, 1999). Secondly, it would allow the infection process to be monitored *in planta* via microscopy. This would allow scientists to monitor behaviour of wild-type and dothistromin mutant strains on the needle surface, thus determining whether the toxin produced by *D. septosporum* has a physiological role in respect to fungal behaviour on the host. Thirdly, in New Zealand there is only a single clonally derived strain of *D. septosporum*, and it has been shown experimentally that some overseas strains produce more toxin (Bradshaw et al., 2000). Therefore introduction of other *D. septosporum* strains would be a major bio-security risk to New Zealand. A pathogenicity assay could be used to determine differences in virulence between strains in New Zealand and overseas.

Detached leaves or seedlings are commonly used in disease assays with fungal pathogens, allowing characterization of susceptible and resistant host-pathogen interactions (Wharton et al., 2003). These types of assay have been used to analyze cherry leaf spot caused by *Blumeriella jaapii* (Wharton et al., 2003), fusarium ear blight of wheat caused by *Fusarium* spp. (Diamond and Cooke, 1999), sclerotina blight of peanut caused by *Sclerotinia minor* (Hollowell et al., 2003), late leaf-spot disease of groundnut caused by *Phaeoisariopsis personata* (Butler et al., 1994) and canker of red pine caused by *Sphaeropsis sapinea* (Blodgett et al., 2003) to name a few. Detached leaf segments of the host are placed on water agar plates (generally adaxial surface upwards), and inoculated with a spore suspension (1 x 10^6 conidia ml^{-1}) or agar plugs taken from the actively growing edge of a fungal colony, placed mycelium side down on the leaf surface (Diamond and Cooke, 1999; Hollowell et al., 2003; Wharton et al., 2003; Browne and Cooke, 2004a). For whole plant inoculations, a spore suspension (1 x 10^6 conidia ml^{-1}) is sprayed with an atomizer to run off (Butler et al., 1994; Wadia and Butler, 1994; Wharton et al., 2003). Incubation conditions conducive to infection obviously vary according to the host-fungal interaction under study. Conditions usually require optimization with respect to temperature, light intensity, humidity and free water if stable conditions are required, or these variables may be manipulated to determine what effects they have on the host-pathogen interaction.

The method used to characterize disease symptoms post inoculation depends on the host and fungus. However this usually involves monitoring the inoculated host tissue for symptoms and signs of infection, such as, formation of lesions and the development of spores. Often the shortest incubation period before disease symptom appearance, and the latent period before sexual/asexual spore production is determined (Browne and Cooke, 2004a). In addition, lesion length (Hollowell et al., 2003), lesion density (Wadia and Butler, 1994), the number of lesions/sporulating lesions (Wharton et al., 2003), stomatal penetrations and percentage of conidial germination (Wadia and Butler, 1994) are often measured. Analysis of symptom development can also be quantified using a disease severity scale, if available (van Jaarsveld et al., 2003; Wharton et al., 2003).

Pathogenicity assays are often developed as a prescreening technique in determining host resistance to the associated fungal pathogen. Browne and Cooke (2004a) have proposed there is a difference in wheat resistance to *Fusarium* species, with Type I being resistance to initial infection, Type II being resistance against the spread of the pathogen within the host and Type III, the ability to degrade or tolerate the mycotoxin deoxynivalenol. These types of resistance may also be applicable to other fungal-host interactions. However there may be variability in the relationship or correlation of the components of resistance shown in a detached leaf assay compared with a whole plant assay (Browne and Cooke, 2004a). Resistance may or may not be under the same genetic control in a detached leaf assay and whole plant field trial, and there is a possibility that susceptibility factors may or may not be detected in a detached leaf assay (Browne and Cooke, 2004a). It has been shown with a wheat cultivar that there is independent segregation of genes controlling head and leaf resistance, therefore traditional field screening for resistance to fungal infection can not be replaced with a detached leaf assay in this instance (Diamond and Cooke, 1999). The expression of plant resistance also depends on the method of inoculation, conditions and timing of inoculation, and stage of plant development. In addition the use of mycelium plugs as inoculum provide a nutrient base for the fungus, therefore this may lead to enhanced lesion development and be a severe test of host resistance (Hollowell et al., 2003). However, many detached leaf assay tests have shown a correlation with field resistance, for example soybean and dry bean resistance to *S. minor* and alfalfa resistance to *S. trifoliorum* (Hollowell et al., 2003). Detached leaf assays are therefore not a substitute for field evaluations but are useful for preliminary screening for resistance.

1.3 Green Fluorescence Protein

The green fluorescence protein (GFP), responsible for bioluminescence in the jellyfish *Aequorea victoria*, was isolated in 1992, and has since been used as a reporter and marker in both prokaryotes and eukaryotes. *Aequorea* GFP is a 27kDa protein consisting of 238 amino acid residues (Lorang et al., 2001). It fluoresces under UV or blue light in the presence of oxygen (Maor et al., 1998). The protein has been fused to cellular and extracellular proteins allowing analysis of gene regulation, protein localization and organelle labeling. Other reporter genes such as GUS require exogenous substrates, co-factors or antibiotics for detection, in addition to destructive sampling (Atkins et al., 2004). GFP is useful as a reporter in living systems, reflecting gene expression and protein localization without the associated problems of using other reporter systems. However, the wild type GFP protein in some applications has a low turnover rate, taking up to two hours for auto-activation of the chromophore responsible for fluorescence. GFP is also subject to incorrect folding at temperatures above 37°C. Additionally, the GFP chromophore is formed exclusively from part of the polypeptide chain (Maor et al., 1998). Another potential problem is the requirement of oxygen for fluorescence, which may not be present in sub-cellular locations or various cell types at equal concentration within the organism (Lorang et al., 2001). To overcome associated problems such as non-fluorescence, insoluble forms, or inefficient translation that occurs in some systems, point mutations have been inserted into the wild type *gfp* gene (Maor et al., 1998). These modified forms of GFP have resulted in faster chromophore formation, increased fluorescence and solubility and decreased photobleaching (Maor et al., 1998; Lorang et al., 2001).

In filamentous fungi, the wild type *gfp* gene is not efficiently translated, and a synthetic version of GFP (sGFP) has been developed that results in faster chromophore formation, and an increase in GFP protein fluorescence. The sGFP protein has a serine-to-threonine substitution at amino acid 65 (Maor et al., 1998). This substitution causes a red shift in excitation maxima from 395 and 475 nm to 488 nm, with light emittance detected at 508 nm, making it ideal for use with fluorescent microscopy. Studies have confirmed that sGFP yields a higher concentration and level of fluorescence than native GFP in filamentous fungi such as *Ustilago maydis*, *Aspergillus nidulans*, *Cochliobolus heterostrophus*, *Colletotrichum gloeosporioides*, *Neurospora crassa* and *Neotyphodium lolii* (Lubeck et al., 2002). An alternative variant to sGFP is enhanced GFP (eGFP) which is a red-shift variant of the wild-type GFP, optimized for brighter fluorescence. This gene contains a double amino acid substitution of Phe-64 to Leu and Ser-65 to Thr. Enhanced GFP has been used successfully with many fungal species to monitor fungal growth on or within plant hosts. Tanaka et. al. (2006) used eGFP *Epichloe festucae* mutants to show the importance of reactive oxygen species in regulating the interaction between the fungal endophyte and ryegrass host (Tanaka et al., 2006). However, further variants of GFP are being developed, now requiring choice of which *gfp* gene to use depending on the organism, promoter driving the *gfp* gene and method of fluorescence detection (Lorang et al., 2001).

The use of GFP as a marker requires a strong constitutive promoter, which usually results in a cytoplasmically located protein that occurs in all fungal morphotypes, such as hyphae, spores, and appressoria (Lorang et al., 2001). However, differences in fluorescence intensity seen within fungal colonies can be due to non-fluorescent hyphae resulting from non-transformed nuclei (Lubeck et al., 2002) in multinucleate protoplasts, or multiple copies of the GFP plasmid (Balint-Kurti et al., 2001). Once GFP transformants are mitotically stable, all cell types expressing the GFP can be easily detected. However, changes in the cytoplasmic conditions of organs or specific cells, such as condensation of the cytoplasm at the two poles of a spore during germination, may contribute to differences in fluorescence intensity (Maor et al., 1998).

1.3.1 The Use of GFP to Follow Infection

The development of GFP expressing strains of *D. septosporum*, both wild type and mutants defective in DOTH biosynthesis would enable monitoring of fungal development, and determination of whether the mutated genes are crucial for infection. In addition, *D. septosporum* transformed with GFP would be a valuable tool allowing visualization of the pathogen-host interaction, infection structures and post-penetration development (Rohel et al., 2001). Host-pathogen interactions have been followed between *Fusarium graminearum* and barley (Skadsen and Hohn, 2004), *Mycosphaerella fijiensis* and banana (Balint-Kurti et al., 2001), *M. graminicola* and wheat (Rohel et al., 2001), *Alternaria citri* and citrus tissue (Isshiki et al., 2003), and *Cochliobolus heterostrophus* and maize (Maor et al., 1998), using GFP transformed fungi.

Microscopy is the common method of detecting GFP transformed fungi on and within inoculated plant tissue. Using epifluorescent microscopy, Maor et. at. (1998) observed hyphae of *C. heterostrophus* colonizing the mesophyll zone under the point of inoculation on maize. Lu et. al. (2004) observed induction of bio-control related genes (fused to GFP) during mycoparasitism of *Pythium ultimum* and *Rhizoctonia solani* by *Trichoderma atroviride* on cucumber seed using confocal laser scanning microscopy. They observed that the bio-control genes were activated by the presence of the host and chitin within 24 hours of *T. atroviride* colonization. Furthermore, they showed that mycoparasitism takes place on the seed surface, with *T. atroviride* hyphal branches growing towards the host and coiling around the host hyphae (Lu et al., 2004). The endophyte *Neotyphodium lolii* has been transformed with GFP, and was visualized in the leaf sheaths of perennial ryegrass. Observations showed the presence of GFP throughout the cytosol of living hyphae, and the lengthwise orientation of hyphae, with infrequent branching within leaf sheath cells (Mikkelsen et al., 2001). The effects of a fungicide, azoxystrobin in impairing *M. graminicola* infection of wheat leaves has been assessed using a GFP transformed strain of the fungus. The growth of *M. graminicola* inside wheat leaves was monitored following treatment with azoxystrobin at various stages of incubation post-inoculation. The results indicated that the fungistatic effect of azoystrobin on *M. graminicola* lasted up to 50% of the time during the incubation phase (Rohel et al., 2001). As a final example of the use of GFP, the mechanism by which *Mycosphaerella* pathogens cause Sigatoka disease of banana has been elucidated. In addition to monitoring the multiple stages of plant infection using fluorescein (FITC), the experimenters determined that the end of the necrotic area was often in advance of fungal hyphae. It has been proposed that several *Mycosphaerella* banana pathogens produce a diffusible phytotoxin, and the lack of hyphae found within the necrotic area, sometimes up to half of this area leads further support for a role of a phytotoxin in Sigatoka disease (Balint-Kurti et al., 2001).

One of the main advantages of using GFP transformed fungi to monitor the host-pathogen relationship is that no clearing of plant tissue is required for observation. Furthermore, GFP allows non-destructive sampling, so the host-fungal interaction from time of inoculation to final stages of disease development is not interrupted (Maor et al., 1998). Therefore, macroscopic symptoms observed due to infection by transgenic fungi are distinguishable from symptoms caused by wild type untransformed fungi (Balint-Kurti et al., 2001). In most instances where GFP has been used to transform fungi, the protein does not seem to interfere with any major physiological pathways (Isshiki et al., 2003), and further suggests that the transformation process does not affect fungal pathogenicity or virulence (Balint-Kurti et al., 2001).

1.4 Ambient pH Regulates Physiological Processes

Research has determined that hydrogen ion concentration of inoculum affects spore attachment to leaf surfaces and subsequent disease severity, whilst also affecting growth and sporulation on media (Schuerger and Mitchell, 1992; Wang et al., 1999). Research with *Aspergillus* spp. has shown growth optima and AF production at acidic pH, with inhibition of AF production at alkaline pH. Furthermore, up to 27 genes have been shown to be regulated by ambient pH, with gene expression induced with increasing pH (Price et al., 2005). Conversely, a limited amount of research has been conducted on *D. septosporum* physiological response to ambient pH. Shaw (1975) showed an increase in DOTH production associated with increasing pH from pH 4.8 to 5.5, 80 h post-inoculation, in liquid media. Therefore, due to the similarity in the biosynthesis of toxins between dothistroma and Aspergillus, it is possible the response of dothistroma to ambient pH may be similar to results found for *Aspergillus* spp.

For most eukaryotic cells, cytoplasmic pH must be maintained within a narrow range for effective cell function and intracellular processes. In fungi internal pH homoeostatis is achieved by a plasma membrane H^+-ATPase pump (Docampo et al., 1996). Research with *Penicillium* showed that weak organic acids or inhibitors of the plasma membrane pump disrupted pH homoeostatis by lowering internal pH which subsequently inhibited fungal growth in culture medium (Zhang et al., 2005). Therefore, the ability of fungi to survive at adverse environmental pH in part depends on the effect of pH on exclusion of protons, and maintenance of a proton gradient (Davis, 2003). Fungi however also maintain pH homoeostatis through pH regulatory pathways, of which many are not well understood. For *Aspergillus* spp. the production of AF acidifies culture medium, and is under the control of the global pH regulator PacC. The *pacC* gene induces expression of alkaline-expressed genes while repressing expression of acid-expressed genes (Price et al., 2005). The ability to alter ambient pH thus ensures the survival of the fungus in conditions that would otherwise be inhibitory for growth.

Fungi are capable of altering external pH by secretion of various compounds into the medium. Often a decrease in pH of the medium during growth is common, as acids are formed from the carbon source (Weiergang et al., 2002). However, the survival of many fungal species in an acidic environment results in a high expenditure of energy on expelling protons from the cytosol (Zhang et al., 2005). Additionally, some fungal plant pathogens have to alter ambient pH in order for specific enzymes associated with early plant infection to be active. For *S. sclerotiorum*, the secretion of oxalic acid acidifies the environment, thus enabling transcription and activity of cell wall-degrading enzymes during pathogensis (Cotton et al., 2003). Dothistroma survives on the acidic surface of pine needles (Ivory, 1967), with infection usually through stomatal openings. However, dothistromin is broken down to oxalic acid, which is non-toxic to pine needles (Franich et al., 1986). Therefore, DOTH production may be in response to ambient pH within the pine needle. Perhaps this may lead to acidification within the internal pine needle environment, creating conditions conducive to transcription and activity of genes and enzymes required for infection. Therefore determining whether Dothistroma is pH responsive in respect to physiological processes may help in understanding the development of red-band disease.

1.5 Summary of Current Research

1.5.1 Hypothesis 1

Wild type and DOTH mutants of D. septosporum *transformed with eGFP will allow visualization of the infection process in planta, and determination of the putative role of DOTH as a pathogenicity or virulence factor.*

1.5.2 Aim 1

To develop a laboratory-based pathogenicity assay to monitor fungal infection of pine fascicles and pine seedlings inoculated with *D. septosporum*.

1.5.3 Objectives

- Optimize environmental conditions (temperature, humidity, moisture and light) conducive to the completion of the infection cycle (stromata on pine needles).
- Determine which form of inoculum, spores, mycelia plugs or macerated mycelium cause the highest incidence of infection.
- Determine which type of host, detached pine fascicles, pine seedlings or cuttings is appropriate to use in a pathogen assay.
- Use eGFP wild type isolates to monitor fungal behaviour on pine needles post-inoculation.
- Determine if the behaviour of *D. septosporum* eGFP DOTH mutants, on pine needles post-inoculation is comparable to that of the wild type isolate, and whether they can cause infection and disease.

1.5.4 Hypothesis 2

Ambient pH will affect D. septosporum *growth, spore production, DOTH biosynthesis and associated DOTH gene expression.*

1.5.5 Aim 2

To determine whether ambient pH has an effect on *D. septosporum* physiological processes such as growth, sporulation, DOTH biosynthesis and gene expression of putative DOTH genes.

1.5.6 Objectives

- Assess daily radial growth rate of *D. septosporum* on a variety of solid media at a predetermined pH.
- Assess growth and DOTH production of *D. septosporum* in liquid media in response to ambient pH.
- Determine level of DOTH gene expression, in relation to growth and toxin production, at different ambient pH values.

2

Materials and Methods

2.1 Biological Strains

2.1.1 Fungal Isolates

In this study the following *D. septosporum* isolates were used: NZE7, NZE10, GFP-38, GFP-19, and GFP-pksA$^-$. NZE7 and NZE10 are single conidia isolates obtained from *D. septosporum* infected *Pinus radiata* needles (West 2004) and Section 2.1.3 respectively). All GFP isolates were developed from NZE7 in this laboratory (Schwelm, unpublished).

2.1.2 Plant Species

Pinus radiata seedlings or cuttings used in the pathogenicity assay were supplied from SCION (Rotorua, NZ). All trees came from the same ramet number, and are genetically identical. Seedlings were raised from seeds, and rooted cuttings were taken from five to seven year old pine trees. All plant material was potted in standard potting mix in small polythene bags and kept in the shade house at Massey University until required. Potted seedlings/cuttings used in the pathogen assays were aged 6-12 months at the time of inoculation. The tops of the seedlings/cuttings were cut off with sterile scissors to ensure uniform height (approximately 15 cm) of plant material within each pathogen trial.

For the detached fascicle assay, fascicles (3-4 needles) were removed from the trunk of *P. radiata* seedlings using a sterile scalpel. Fascicles were surface sterilised in a 1:5 dilution of sodium hyperchlorite (42 g/L) for two minutes, then rinsed in sterile milli-Q water to remove any remaining bleach residue. The bottom of the fascicle was placed in a 0.6 ml tube which contained approximately 0.4 ml of 2% water agar.

2.1.3 Isolation of Fresh Wild-type *D. septosporum*

D. septosporum infected pine needles were placed in a -80°C freezer for 12 h to kill possible contaminate insects or mites. A section of the needle containing mature stroma was cut free from the remaining needle tissue, as close to the stroma as possible. The stroma was rolled across the surface of a DM agar plate (Appendix A2.2) to release the conidia (Barnes et al., 2004). The plate was viewed at 40X magnification under a dissecting microscope and a block of agar containing many conidia, but free from contaminating debris, was excised using a scalpel. The agar block was then lifted and transferred to a new DM plate. The plate was sealed with parafilm, incubated at 22°C and monitored daily for fungal growth. Isolate NZE10 was obtained using this method.

2.2 Growth of Cultures and Culturing Techniques

2.2.1 Fungal Cultures

D. septosporum cultures were grown on DM, DSM or PDA plates (Appendix A2.2-A2.4). Mycelial plugs 4 mm diam. were removed from the leading edge of *D. septosporum* colonies on solid media using a sterile cork borer. Each plug was placed in the centre of a 90 mm petri plate containing approximately 20 ml of medium. The plates were sealed with parafilm and incubated at 22°C in the dark for 7-14 days. Plates were then stored at 4°C.

For DNA extraction, a 1 cm^2 section of mycelium cut with a scalpel from the edge of a *D. septosporum* colony was ground, using a plastic pestle, in 1 ml of sterile milli-Q water. The macerated mycelium (100 µl) was then dispensed onto DM overlain with a cellophane disc, and spread across the surface using a sterile glass spreader. Plates were sealed with parafilm and incubated at 22°C in the dark for 14 days.

For liquid cultures, 125 ml flasks containing 25 ml of low DB (Appendix A2.5) were inoculated with a known concentration of conidia suspension (Section 2.2.3), a cotton plug was placed in the top of each inoculated flask and they were incubated at 22°C, in ambient light with shaking (220 rpm) for the required number of days.

2.2.2 Maintenance of *D. septosporum* Stocks

New single conidia isolates were sub-cultured onto DM (Appendix A2.2) from initial media used during the isolation procedure described in Section 2.1.3. Stock plates where sealed with parafilm and incubated at 22°C until colony diameter reached approximately 2.5-3.0 cm. Plates were then stored in the cold room at 4°C. Sub-culturing was carried out as required and every six months new stock plates were made. In addition, mycelium plugs where taken using a 4 mm cork borer from the outside of an actively growing colony and stored in 20% (w/v) glycerol in the -80°C freezer for each *D. septosporum* isolate.

2.2.3 Obtaining *D. septosporum* Conidia and Quantification (Rack and Butin, 1973)

A 1 cm^2 section, cut from the edge of a *D. septosporum* stock plate, was ground using a plastic pestle in 1 ml of sterile milli-Q water. The suspension (200µl) was dispensed onto DSM (Appendix A2.3) and spread across the surface of the media with a sterile glass spreader. Plates were sealed with parafilm and stored in the dark at 22ºC for 10-16 days before conidia were harvested.

Conidia were harvested from the multiple growing colonies on the DSM plate by first dispensing 3 ml of sterile milli-Q water onto the plate. The conidia were suspended by rubbing the colony surface gently with a sterile glass spreader and harvested by drawing off 3 ml of suspension with a 5 ml pipette. The plate was rinsed once with 1 ml of sterile milli-Q to recover any remaining conidia.

Alternatively, two mycelial plugs taken from the margins of a fungal colony (opposite sides), using a 4 mm cork borer were placed in 200 µl of milli-Q water containing a drop of Tween-20, and vortexed for 1 min.

The concentration of conidia was quantified using a haemocytometer, with the concentration expressed as the number of conidia per ml. If a standardised conidial suspension was required, the appropriate dilution was done using sterile milli-Q water.

2.2.4 Germination Assay

D. septosporum conidia were harvested as described in Section 2.2.3. The spores were standardized to a 2.0 x 10^5 conidia/ml concentration. Two ml of the standardized suspension was dispensed on 0.5% water agar (WA) plates overlain with cellophane, and the plates were incubated at 22°C. At 0 h (initial harvesting time) and every 12 h thereafter, a 20 µl sample of the suspension was taken from the WA plate, and dropped onto a microscope side. The sample was viewed using a fluorescent microscope (Olympus BX51, and Magnafire 2.1.C digital camera software) with 100 conidia scored.

Conidia were scored as germinating if a visible germ tube was seen protruding from any of the conidial cells.

For assessing germination on needle tissue, trees were inoculated as described in Section 2.8.5. At the required time point, six needles of similar size were removed with a sterile scalpel from each tree (needle length was measured). The six needles were aligned on a microscope slide, and the position along the length of the needle where each microscopic observation would be made was marked on the slide with a marker pen.

For determining the rate of germination at different pH values, conidia were harvested as described in Section 2.2.3 with the exception that conidia were harvested with milli-Q water adjusted to the required pH. The concentration of conidia was then standardized and conidia were dispensed onto 0.5% WA plates overlain with cellophane as described above.

2.2.5 McIlvaine Buffered Solid Media

Two volumes of McIlvaine buffer, mixed to the required pH value (Appendix A1.8) were mixed with one volume of a threefold concentration of medium. For the control condition, the medium was unbuffered. The pH prior to autoclaving was measured using a pH probe (Sartorius) and the pH adjusted if necessary with 1.0 M HCl or 1.0 M NaOH. After autoclaving approximately 20 ml of medium was poured into each Petri-dish in a laminar flow cabinet. The pH of a sample of the autoclaved media from each condition was measured as above and recorded.

A 4 mm plug of mycelium taken from the leading edge of a colony was then inoculated onto the plates (1 plug per plate). A cross was drawn on the underside of the plate running through the inoculum plug. Measurements of colony diameter from the underside of the plate were taken from both the *x* and *y* axis along the cross at seven day intervals up to 21 days. The two measurements for each colony were averaged to obtain the colony diameter, with the mean from four replicate plates per condition calculated from this measurement.

2.2.6 McIlvaine Buffered Liquid Media

Liquid media were made and buffered as described in Section 2.2.5. After any necessary adjustment to pH, 25 ml of medium was poured into each 100 ml flask. Flasks were either inoculated with 1 x 10^6 conidia/ml, or a measured weight of mycelia biomass depending on the experiment. A cotton plug was placed in the top of each flask, and flasks placed on a shaker at 160 rpm at 22°C for the required length of time.

2.2.7 Harvesting Mycelium From Liquid Media

A vacuum harvester (BioRad) was used to harvest mycelium from the liquid media pH experiments. A sterile 5 cm disc of Whatman No. 41 paper was used to filter liquid medium from each flask and collect mycelium. The mycelium was scraped from the paper and divided into two portions. Each portion placed in a 1.5 ml sterile tube and weighed. One tube was placed on the freeze drier for 24 h for dry weight determination (Appendix V), and the other tube was snap frozen in liquid nitrogen for 10 min. before storage at -80°C until required for RNA extraction (Section 2.4.1). The filtrate from each flask was collected in a sterile bottle and subsequently poured into 15 ml falcon tubes. The pH of the filtrate from each flask was determined using a pH probe. The filtrate was frozen at -20°C until required for the ELISA assay (Section 2.7).

2.3 DNA Extraction, Quantification and Analysis

2.3.1 DNA Extraction: CTAB Method

DNA was extracted from fungal strains using the CTAB method (Doyle and Doyle, 1987). For all extractions, cultures were grown for 14 days as described in Section 2.2.1.

Mycelium was harvested by scraping from cellophane overlaying DM plates, using the back of a sterile scalpel and was then frozen at -80°C for 24 h. Each sample was freeze dried for 24 h then ground in liquid nitrogen using a mortar and pestle, and the ground sample was placed in a fresh sterile tube.

For each sample, 600 μl of CTAB buffer (Appendix A1.1) and 2 μl of RNAse (Sigma) (20 mg/ml) was added to the tube, mixed thoroughly, and the sample incubated at 37°C for 5-10 min. Samples were heated in a water bath (65°C) for 30-40 min. with occasional inversion. They were removed from the water bath, allowed to cool to 60°C and 600 μl of chloroform (Merck) was added. The tube contents were mixed gently and left to stand for 2 min. to allow separation of the aqueous and organic phases. The aqueous phase was transferred to a sterile tube and 100 μl of both phenol (Sigma) and chloroform was added and mixed. The sample was then centrifuged at 13,000 g in a Biofuge pico (Heraeus Instruments) for 5 min.

The upper aqueous phase was transferred to a sterile tube containing 100 µl of chloroform. After mixing and centrifugation, the more upper phase was transferred to a new tube with 10 µl of 0.3 M Na acetate (Sigma) and two volumes of 95% ethanol (BDH) (~200 µl). This mixture was spun at 13,000 g in a microcentrifuge for 10 min. The upper phase was transferred to a new tube, 600 µl of isopropanol (BDH) was added, mixed gently and the sample left to stand on ice for 5-10 min. Some of the liquid was decanted off without disturbing the precipitated DNA, followed by the addition of 600 µl of 70% ethanol (BDH). Once the DNA settled to the bottom of the tube, the ethanol was again decanted off. This was followed by two more washes with 70% ethanol before the ethanol was decanted off completely, and the sample left to air dry. Once dry, the sample was re-suspended in 50 µl of TE buffer (Appendix A1.4).

2.3.2 Fluorometric Quantification

Extracted DNA was quantified using a 'Hoefer DyNA Quant 200' (Amersham Biosciences) fluorometer. The fluorometer was calibrated by addition of 2 µl (100 ng/ml) calf thymus DNA standard (Amersham Biosciences) to 2 ml of fluorometer working solution (Appendix A1.17) in a cuvette. The value entered into the fluorometer was 120 ng/ml, this made allowance for the difference in AT% between calf thymus DNA (42%), and fungal DNA (50%).

2.3.3 Nano-drop Quantification

Both DNA and RNA samples were quantified using a Nanodrop® ND-1000 Spectrophotometer (Nanodrop Technologies), as described by the manufacturer's instructions.

2.3.4 Agarose Gel Electrophoresis

A DNA sample was mixed with gel loading dye (Appendix A1.19) and loaded onto a 1.2% LE agarose (BMA) gel immersed in 1X TBE buffer (Appendix A1.3). In addition to the genomic DNA, a 1 kb plus ladder (Invitrogen) was loaded onto the gel. For the purpose of DNA quantification, lambda DNA standards of 100, 50, 25, 10 and 7.5 ng (Invitrogen), were also used. The gel was subjected to 80V until the dye moved to ~2 cm from the end of the gel. The gel was then removed from the gel apparatus and placed in ethidium bromide (1 μl/10 ml) staining solution (Appendix A1.18) on a shaking platform for 20 min. After removal from the staining solution, it was rinsed in milli-Q water for 5 min before being visualized and photographed using the Gel Doc (BioRad) and Quantity One 4.4.0 basic software.

2.4 RNA Isolation, Analysis and cDNA Synthesis

2.4.1 RNA Extraction

Mycelium for RNA extraction was freeze dried in liquid nitrogen for 10 min and frozen at -80°C until required. RNA was extracted using the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen) with a single modification to the manufacturer’s protocol. After addition of the lysis solution, the lysate was passed through a 1 ml syringe with an 18 gauge needle six times before centrifugation. The extracted RNA was subjected to DNAse treatment using the TURBO DNA-free™ kit (Ambion) following manufacturers instructions. The RNA was then quantified using a nanodrop (Section 2.3.3). To check that all contaminating DNA had been removed, the RNA was used in a PCR reaction with Universal fungal primers ITS1 and ITS4 (Section 2.5), and the PCR products run on 1.2% agarose gel and visualized as described in Section 2.3.4.

2.4.2 cDNA Synthesis

cDNA was prepared from the isolated RNA (Section 2.4.1) using primed hexamers from the Roche Transcriptor First Strand cDNA Synthesis kit, following manufacturers instructions. The cDNA was subsequently diluted 10-fold and stored at 4°C until required for real-time PCR analysis (Section 2.5.3).

2.5 Polymerase Chain Reaction (PCR)

All PCR reactions were set up on ice, combining all reagents (Invitrogen) used for n+1 PCR reactions in a master mix. A 23 ml aliquot of the master mix was dispensed into 0.2 ml PCR tubes and 2 μl DNA was then added to each tube. Following amplification, tubes were stored at 4°C until the PCR products were visualized by agarose gel electrophoresis (Section 2.3.4).

Table 1. PCR and sequencing primers

Primer	Size (nt)	Tm (°C)	Sequence (5'– 3')	Source or Reference
ITS1	19	65	TCC GTA GGT GAA CCT GCG G	(White et al., 1990)
ITS4	20	58	TCC TCC GCT TAT TGA TAT GC	(White et al., 1990)
ITS5	22	63	GGA AGT AAA AGT CGT AAC AAG G	(White et al., 1990)
pks-F	19	60	CAT TAT GTC GTC CGA GCA A	A. Scwhelm
pks-R	18	59	CGA ACA GAA CTA CCG ACC	A. Scwhelm
dotA-F	19	59	CTG GTG ATG AAT TCG ACC G	A. Scwhelm
dotA-R	18	59	AAG CAC CAC CGT CAA TAC	A. Scwhelm
tub-F	16	60	CCG GCG TGT ACA ATG G	A. Scwhelm
tub-R	16	60	CAT GCG GTC TGG GAA C	A. Scwhelm
NS7	20	52	GAG GCA ATA ACA GGT CTG TG	A. Scwhelm
NS8	20	56	TCC GCA GGT TCA CCT ACG GA	A. Scwhelm
DseptoMat2f	21	58	GTG AGT GAA CGC CGC ACA TGG	M. Groenewald
DotMat2r	21	56	CTG GTC GTG AAG TCC ATC GTC	M. Groenewald

*calculated as Tm= 81.5 + 16.6(log10(Na+))+0.41(%G+C)-600/N; where (Na+)=0.05 M, N= length of oligonucleotide.

2.5.1 rDNA Primers

Universal fungal primers (Table 1) were used to amplify the Internal Transcribed Spacer (ITS) region between the nuclear 18S and 5.8S rDNA genes. Each PCR reaction contained a final concentration of 1X PCR buffer (Invitrogen), 2.0 mM $MgCl_2$ (Invitrogen), 0.1 mM dNTPs (Invitrogen), 2 μM of each primer (Sigma), 0.8 units Taq polymerase (Invitrogen) and 5 ng of genomic DNA in a total reaction volume of 25 μl.

Amplification was carried out in either a Genius thermal cycler (Techne) or a Mastercycler (Eppendorf) with an initial step of 94°C for 2 min., followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min., with a final step of 72°C for 5 min. before holding at 4°C.

2.5.2 Purification of DNA from PCR Products

For all PCR reactions, a sample of the PCR product was run on a 1.2% agarose gel (Section 2.3.4). For PCR reactions that produced multiple bands, the DNA band(s) of interest were excised with a scalpel from the gel. The DNA was then extracted from the gel slice using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Alternatively, DNA was purified directly from the PCR product using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

2.5.3 Real-time PCR

Real-time PCR was performed in the Lightcycler™ (Roche), using 20 µl glass capillaries (Roche), and the ABGene SYBR Green Mastermix Kit. Data was analyzed using the Relative Quantification Method and the Lightcycler™ Master Software, Version 4.0 (Roche). Prior to analyzing cDNA samples, the reaction conditions were optimized for each gene by doing a series of cDNA dilutions and a $MgCl_2$ titration to determine which parameters gave the best melting curve analysis. An example of the melting curve data obtained is in Appendix VII. The 18S RNA gene was used as an endogenous control and active reference so the target gene expression profile could be compared to the constant 18S RNA expression profile. For analysis of unknown samples, a coefficient file was created using the RelQuant Software, (Version 1.1.0) and the standard curve data for each gene and the 18S RNA control imported into the Lightcycler™ Master Software. The coefficient file enabled differences in the amount of total RNA in each reaction to be normalized.

For each mono-fluorescent LightCycler™ run, a 2 µl template of each calibrator (gene of interest and 18S RNA, known concentration) and sample was used. Table 1 shows the primer sequence and annealing temperature for each gene analyzed (grey font). Each 10 µl PCR reaction contained: 1 x concentration of SYBR green mastermix, 10 mM each primer and was made up to 8 µl with PCR grade wate. The PCR amplification parameters consisted of an enzyme activation step (15 min at 95°C) then a hotstart (10 min at 95°) followed by 42 cycles of (a) 10s at 95°C (denaturation), (b) 10s at 59°C (annealing), (c) 16s at 72°C (extension) and 5s at 84°C for acquisition. Following amplification, a melting curve analysis with a temperature range from 65°-95°C at 0.1C°/s was performed. The final step was cooling at 40ºC for 30s.

2.6 Automated Sequencing

DNA sequencing was done by the Allan Wilson Centre Genome Sequencing Service (AWCGS), at Massey University Palmerston North, using a capillary ABI3730 Genetic Analyzer (Applied Biosystems Inc.). A 15 µl sample of purified DNA (approximately 10 ng) and 3.2 pmol of primer were supplied to AWCGS and an electronic nucleotide sequence obtained.

The chromatogram was viewed using FinchTV software, Version 1.3.1, before the nucleotide sequence was entered into the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/) and results compared to the GenBank database.

2.7 Quantification of Dothistromin

The concentration of the mycotoxin dothistromin produced by *D. septosporum* in liquid culture (Section 2.2.6) was determined using a modified competitive enzyme-linked immunosorbent assay (ELISA) described by Jones et. al.(1993). In all instances, mycelia fragments were removed from the liquid culture by filtration (Section 2.2.7) before the ELISA protocol was done.

2.7.1 Preparation of DOTH-MSA Conjugate ELISA Plates

Microtitre wells (96 well plate) were coated with 100 μl of DOTH-MSA conjugate (diluted 1/3000) in 1% phosphate-buffered saline (PBS) (Appendix A1.5). The plates were covered in gladwrap (Glad) and incubated at 37°C for 3 h. The plates were then washed five times with PBST (Appendix A1.6) before 400 μl of BSA (Appendix A1.7) was added to each well. The plates were incubated for another 3 h at 37°C. The BSA mix blocks the remaining protein-binding sites on the micro-well surface. After this step the plates were washed once with PBST (400 μl/well) and stored at 4°C until required (no longer than six weeks).

2.7.2 Preparation of Samples for ELISA

Ten standards were prepared by adding 1 μl of the standard to 1 ml of dilution buffer and broth (Appendix A1.9), an approximate 1000-fold dilution. The dothistromin concentration of the standards was as follows; 1000, 500, 250, 125, 62.5, 31.6, 15.6, 7.8 and 3.9 μg. In addition a BO standard containing no toxin was also used. The standards were made from a 2-fold serial dilution of a 2 mg/ml stock standard using DMSO (BDH). The standards are stored in the fridge until required.

For the test samples, 200 μl of sample dothistromin was added to 200 μl of working buffer (Appendix A1.10). DMSO was added to the standards to compensate for the addition of

DMSO to the test samples. If test samples required diluting, this was done using working buffer and broth (Appendix A1.11). The total volume of each sample was 400 µl.

In another set of micro-titre plates, the pre-incubation plates (not DOTH-MSA plates), 100 µl of labeled peroxidase was placed in each well and 100 µl of test sample from the above were added to the appropriate wells. Each test sample and dilution was replicated three times (ie. three wells per sample) on one plate. Also, 100 µl of each standard (replicated three times per plate) was added to the labeled peroxidase. The plates were then covered with gladwrap and incubated for 1 h at 37°C. Prior to completion of this incubation, the DOTH-MSA plates previously prepared were washed once in PBST by filling all wells then inverting the plate to dispose of the PBST.

2.7.3 DOTH-MSA Plates

After the 1 h incubation period was complete, 100 µl of the test samples and standards were transferred using a multi-pipette to the washed DOTH-MSA plates. The DOTH-MSA plate was then covered in gladwrap and incubated at 37°C for 3 h. The DOTH-MSA plates were washed six times with PBST, ensuring each well on the plate was filled for each wash. The peroxidase labeled monoclonal antibody was detected by adding 200 µl of the substrate to each well. The substrate was made with 100 ml of 0.2 M Na_2HPO_4 (Merck), 40 mg of substrate o-phenylene diamine (Invitrogen), 0.51 g citric acid (BDH) and 40 µl of 30% H_2O_2 (Merck). The plates were covered with tin foil (substrate is sensitive to light) and shaken gently for 30 min. to get a colour reaction. Fifty µl of 4 M H_2SO_4 (BDH) was added to each well to stop the reaction. Before the absorbance in each well of the plate was read using a Anthos Labtec HT2, Version 1.21E ELISA plate reader, the measurement filter was set to 490 nm and the reference filter to 595 nm.

2.8 Pathogen Assay

2.8.1 Assay Chamber

An infection chamber was designed to house inoculated pine material in the GMO suite (Section 2.8.3). The chamber was a 90 L plastic container (39.5 cm x 69 cm x 45 cm) filled with 20 L of RO water, with a 24 V water fogger (Waterwerks, Australasia) placed on the bottom of the container. The water fogger produced a continuous fine mist. The top of the container was covered in glad wrap, allowing a gap 30 cm x 20 cm for air flow. Suspended approximately 40 cm above the infection chamber was a lighting rig consisting of two black light tubes (36 W), and one white light tube (36 W). The lighting rig was on a timer, set on at 0700 h and off at 1900 h.

2.8.2 Ultraviolet Light (UV)

The ability of different types of material to transmit UV light was determined using a spectrometer. The analysis of the suitability of gladwrap (Glad), parafilm (Pechiney), and petri dish lid or petri dish sleeve plastic (Labserv), as a covering material for the assay chamber (Section 2.8.1) (to maintain a humid environment), was determined. A 1 cm x 2 cm piece of each of the above described materials was placed separately in a cuvette which was placed into a spectrometer (Lyco). The wavelength was set to run from 200 nm through to 800 nm, and an electronic readout of absorbance was obtained.

2.8.3 GMO Suite

The infection chamber was placed in the GMO suite in the Institute of Molecular Biosciences (IMBS), Massey University, Palmerston North. The GMO suite has Physical Containment Level 2 (PC2) status, and was therefore capable of housing genetically modified organisms. The containment room was constructed with large glass windows on one side of the bench, with a solid wall opposite the windows, and a tinted glass roof. Environmental conditions, such as temperature (±3°C), humidity, CO_2 levels and to a certain extent light, using shade cloth and auxiliary lighting could be controlled. The temperature was set to 24°C from 0700-1859 h and to 16°C from 1900-0659 h. Relative humidity was set to 80%.

2.8.4 Mycelia as Plant Inoculum

Initially conidia were harvested from the DSM plate as described in Section 2.2.3. Then 1 cm^2 of each remaining colony was cut from the plate, leaving behind as much media as possible. This was placed in a 50 ml falcon tube containing 25 ml of sterile milli-q water and ground with an electric homogenizer until a viscous solution was formed. A haemocytometer was used to determine the number of mycelial fragments and the suspension was diluted to the required concentration.

2.8.5 Pine Seedling and Cutting Inoculation

All plant material was inoculated with a standardized conidia suspension of 1 x 10^6 conidia/ml (or mycelia fragments). Plants were placed in a biological containment cabinet in a PC2 laboratory, and a household trigger action atomizer was used to spray approximately 3 ml of inoculum per plant. This produced a fine mist over the needle surface ensuring a relatively even dispersal of inoculum on the pine needle. The plants were left in the bio-cabinet until the inoculum was dry, then they were relocated to the GMO suite (Section 2.8.3). After 48 h post-inoculation, plants were placed in the assay chamber (Section 2.8.1).

2.8.6 Diseased Pine Needle Analysis

At the conclusion of each infection assay (six weeks post-inoculation), the number of lesions per seedling/cutting was counted. All new growth above the top of the tree (where the stem was cut, Section 2.1.2), was not included in the analysis. All needles were removed from the tree and counted as uninfected, chlorotic or diseased. Needles were classified as healthy if the needle was green and showed no sign of chlorosis. Needles were classified as chlorotic if they had regions of chlorosis which did not show the characteristic red band pattern (symptom of *D. septosporum*), or under the dissecting microscope no stromata were seen. All needles classified as diseased were viewed under a dissecting microscope, and the presence of either a red band or stromata was confirmed. Additionally, for every diseased needle, the number of bands per needle and stromata per needle were recorded. The calculations required to determine disease incidence are shown in Appendix IV.

2.8.7 Diagnostic Procedure Confirming Disease

The following procedure was developed from an adaptation of the DNA extraction protocol used to extract DNA from *Arabidopsis thaliana* (Kasajima et al., 2004). Visibly diseased sections of the pine needles (no less than 2 mg) were excised with a sterile scalpel from surrounding needle tissue. The needle tissue was ground with a plastic pestle in liquid nitrogen in a 1.5 ml tube. Then, 180 µl of TE buffer (Appendix A1.4) and 20 µl of extraction buffer (Appendix A1.14) were added to the ground needle tissue, mixed and left to stand at room temperature for 30 min. Next, 50 µl of isopropanol was added to the mix. The total volume was then placed into a spin column (QIAgen), followed by centrifugation at 12,000 g for 1 min. The contents in the collection tube were discarded and 50 µl of TE buffer was added to the spin column and subsequently centrifuged as above. The extracted DNA was then cleaned using the QIAgin PCR Purification Kit to remove potential inhibitors in preparation for PCR.

PCR analysis using the forward primer DseptoMat2f and reverse primer DotMat2r (Groenewald et. al. unpublished) (Section 2.5), which are both specific for *D. septosporum* and the *Mat1-2* genes, was done using 2 μl of the above purified DNA as template. PCR Enhancer System (Invitrogen) reagents were used in the reaction, as the template, although purified, was still problematic producing inconsistent results when conventional reagents were used. The total reaction volume was 25 μl and contained 1x amplification buffer, 0.4 μM dNTPs, 2.0 mM $MgSO_4$, 0.4 μM of each primer, 0.5x enhancer solution and 0.8 U of Taq (Invitrogen). The cycling conditions consisted of an initial denaturation step 94°C for 5 min, followed by 40 cycles of 94°C (20s), 65°C (20s) and 72°C (40s), followed by a final elongation step at 72°C (7 min). The resulting PCR products were visualised as in Section 2.3.4, with the expected band size being 480 bp.

2.9 Statistical Analysis

All data was entered into Excel (Microsoft Office, 2003), and the data analysis function used for basic statistical calculations and ANOVA analysis. All subsequent graphs were created in Excel. For Tukeys HSD, data from the ANOVA analysis in Excel was entered into an online statistics calculator which performed the analysis (http://graphpad.com/quickcalcs/posttest1.cfm).

3

Red-band Disease Pathogen Assay

3.1 Introduction

Plantation forestry in New Zealand covers approximately 1.7 million ha (Girisha et al., 2003), predominated by *Pinus radiata* D. Don which is susceptible to red-band disease caused by the fungus *Dothistroma septosporum*. The estimated cost of controlling the spread of this fungus and associated loss in wood yield due to disease is approximately $24 million per annum (Bulman et al., 2004). This makes red-band disease one of the most serious diseases of radiata pine in this country, requiring vigilant assessment, treatment and control. However, radiata pine does appear to show natural resistance to infection until around eight years or 15 years maturity in moderately and heavily infected plantations respectively (Gibson, 1972). A pine clone with resistance to dothistroma was developed in 1983 (Carson, 1989), which was estimated to reduce the infection rate by 15% and the associated cost of red-band disease by 56% (Dick, 1989). However, field assessment of potential pine resistance to dothistroma usually occurs three years after planting, and is therefore a lengthy process (Jayawickrama and Carson, 2000). This process may be shortened by the use of a simple assay system which would allow assessment of fungal and host interaction, in addition to red-band disease development, benefiting foresters and scientists.

The development of laboratory-based pathogen assays enables more rapid assessment of host plant resistance than can be achieved in a conventional field trial. However, although this technique is useful as a preliminary screen for resistance, it cannot be substituted for eventual field evaluation (Hollowell et al., 2003). In the 1970-80's a large amount of research was conducted on the environmental conditions required for Dothistroma infection to proceed with pathogen assays conducted under laboratory conditions (Gadgil, 1967; Ivory, 1972b; Gadgil, 1974, 1976; Shain and Franich, 1981; Franich et al., 1986). The results from multiple researchers were in conflict regarding parameters such as light intensity, duration of needle wetness required, and a suitable temperature which enabled germination of conidia and subsequent infection of needle tissue. Muir and Cobb (2005) concluded that a higher level of infection was obtained when seedlings were exposed to a variable temperature and continuous wetness. However, continuous wetness periods do not occur within nature, with the majority of rainfall events being of short duration and usually high evaporation (Butler et al., 1994). Therefore slow growing fungi such as *D. septosporum* must be able to withstand intermittent wetness during the infection cycle, although the duration of free water required on the plant surface varies for different fungal species. It was shown for *Phaeiosariopsis personata*, which causes leaf spot of groundnut that there was an increase in stomatal penetration during intermittent wetness, as opposed to continuous wetness (Wadia and Butler, 1994). It is therefore not uncommon for fungal pathogens to require controlled environmental conditions for successful infection, as is seen with the slow growing fungus *Blumerielle jaapii* which causes cherry leaf spot. Similarly to *D. septosporum*, this fungus infects cherry leaves through the stomata, produces a toxin and, in a laboratory situation, requires light, temperature and relative humidity for successful infection (Wharton et al., 2003). Therefore any system used to house inoculated plant material must be optimized in order to maximize the rate of infection.

Detached plant material is often used in pathogen assay systems, as opposed to whole plants, so that a larger number of replicates can be screened in a small space. Successful pathogen assays using detached plant material have been used for Fusarium wilt of cotton caused by *Fusarium oxysporum* (Wang et al., 1999), ear blight of wheat caused by *Microdochium nivale* (Diamond and Cooke, 1999), and accessing *Sclerotinia minor* aggressiveness and host resistance in peanut (Hollowell et al., 2003). Although there are many instances where the results of detached plant assays correlate with whole plant resistance or disease response to pathogens, it is still possible that using this type of assay does not predict adult plant resistance, as seen with ear glume blotch of wheat caused by *Stagonospora nodorum* (Diamond and Cooke, 1999). Equally, the use of seedling inoculation may not correlate with or represent mature plant traits such as resistance or susceptibility to infection. For certain plants however the use of young seedlings in a pathogen assay was able to predict specific and non-specific resistance to infection as seen with tobacco seedling inoculations (van Jaarsveld et al., 2003). However, detached plant material and/or seedling inoculation may give inconsistent results between replicates, with interpretation of no sign of infection difficult to assign to host resistance. Conversely, a high level of infection may be due to high inoculum load or perfect environmental conditions conducive to disease development (Hollowell et al., 2003), which is not valid in respect to natural habitat conditions, and may mask partial resistance of plants to certain pathogens. Additionally, certain parts of plants are known to respond differently to pathogens and the use of detached plant material or seedlings may be a severe test of host resistance (Hollowell et al., 2003). Therefore, for individual pathogen assay systems using detached plant material or seedlings, field trials need to be conducted to validate results obtained with laboratory based assay systems.

Dothistroma is a slow growing fungus, and it is widely noted there is considerable variability between isolates of the fungus with respect to growth, and production of spores and toxin (Barnes et al., 2004; Bradshaw and Zhang, 2006). Despite the morphological instability of the fungus, asexual conidia can be obtained (Rack and Butin, 1973). In most pathogen assays the primary form of inoculum is conidia that are often sprayed on host plant tissue using an atomizer until run-off (Van der Zwet and Stroo, 1985; Browne and Cooke, 2004). Often the spore harvesting procedure produces a crude suspension that contains both condia and mycelial fragments. Possibly there may be different modes of infection between conidia and mycelial fragments (Ivory, 1972b), or a reduction in infection as seen when wheat was inoculated with *M. nivale* spore suspension free from mycelial fragments (Browne and Cooke, 2004). However, whether infection is attributed to conidia or mycelia fragments may be of little importance for some pathogen assays, and in the case of dothistroma the development of an assay that works would be of advantage regardless of the type of inoculum needed to cause infection. Another potential problem seen for other slow growing fungal species, such as *B. jaapii* and *E. maculatum* was the loss of pathogenicity after continuous subculturing (Van der Zwet and Stroo, 1985; Wharton et al., 2003). This may also apply to the viability of laboratory cultured dothistroma spores, and potential to cause infection. Therefore, it would be beneficial to validate the potential use of a pathogen assay system with inoculum from naturally infected plant material and compare levels of infection obtained using both fresh inoculum and laboratory derived inoculum (Muir and Cobb, 2005).

Another potential issue when attributing high levels of infection in artificial conditions to the pathogenicity of the fungal isolate is the role nutrients within the spore suspension may play in enhancing the potential to cause infection. It is known for example that the pathogenicity of *F. oxysporum* towards cotton can be affected by the composition of the media the inoculum was derived from. Wang et. al. (1999) showed there was a significant difference in the level of infection obtained for inocula suspended in the media it was isolated from, as opposed to being suspended in un-inoculated media or distilled water. Prior to establishment within host tissue, most fungi rely on nutrients stored within the spore (Solomon et al., 2004) or nutrient resources available from the host tissue. Sometimes, as seen with infection of corn kernels by *A. flavus*, reliance on stored carbon can affect growth and toxin production (Mellon et al., 2005). For *Sclerotinia minor* inoculation of peanut plants, the use of agar plugs as inoculum enhanced lesion development due to the presence of nutrients within the agar plug (Hollowell et al., 2003). For fungi in general, the presence of water and a carbon source is normally sufficient to trigger germination (Zuber et al., 2003), the initial process required for most pathogenic fungi to infect their hosts. Therefore, when developing an assay system, how the inoculum is prepared and the substrate used to suspend the inoculum in may be an important variable to consider when analysing infection rates obtained.

The aim of the following experiments were to develop a reliable laboratory based pathogenicity assay that would enable monitoring of pine needle infection and development of red-band disease caused by *D. septosporum*. Previously in this laboratory, the development of a reliable pathogen assay was unsuccessful. This was attributed to attenuation of the laboratory cultured *D. septosporum* isolates, and unsuitable environmental conditions for the pathogenicity assay (West, 2004). Therefore, in developing a reliable pathogenicity assay, three groups of variables considered important for successful infection were investigated: environmental conditions, host factors and pathogen factors. Each variable were investigated to determine which set of conditions is most appropriate to obtain consistent infection.

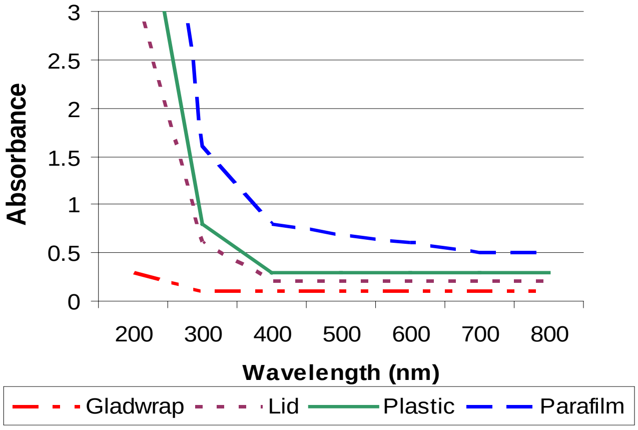
3.2 Results

3.3 Environmental Variables

3.3.1 Ultraviolet Light

Gadgil and Holden (1976) determined light is an important variable in whether infection proceeds, with higher levels of infection obtained when light intensity was at least 185 W/m^2 (400-700 nm) compared with a lower light intensity. Black light tubes produce UV-B (280-315 nm) and UV-A (315-400 nm) light within the solar spectrum, and were used in the developed pathogen assay, in conjunction with visible light to resemble natural sunlight conditions (Section 2.8.2). The assay chamber had to be covered to ensure the mist generated from the water fogger was maintained around inoculated plant material (Section 2.8.5). Therefore, the cover had to allow transmittance of UV-B and UV-A light rays. Possible usable materials were assessed for ability to transmit UV light. The four materials selected for spectrometric analysis were, glad-wrap, petridish plastic, petridish plastic sleeve and parafilm (Section 2.8.2). Figure 4 shows the wavelength each sample recorded the lowest absorbance.

Figure 4 Absorption of light vs wavelength for each sample



Glad-wrap was chosen as the most suitable material, as it had the lowest absorbance around 308 nm, therefore allowing transmittance of UV-B and UV-A light.

3.3.2 Temperature and Humidity

A data logger (Tinytag) was placed inside the assay chamber to determine whether the temperature and relative humidity (RH) within the chamber were similar to the parameters set for the GMO suite (Section 2.8.3), where the chambers were housed. Table 2. shows the temperature and humidity readings taken from downloaded data from the Tinytag logger, for all pathogen assays which will be discussed in the following sections. Appendix III shows a graphical view of the temperature and humidity data retrieved for the pathogen assay in Section 3.5.4 as an example. As seen in Table 2, the mean RH within the assay chamber was below the constant 80% RH set for the GMO suite, except for trials three, five and seven. The minimum temperature was within ±3°C of the set 16°C (12 h per day). However the maximum temperature fluctuated considerably from the set 24°C for 12 h per day (±8.6°C) for some trials. The average temperature over all trials was within a range conducive to infection (Gadgil, 1974).

Table 2. Temperature and humidity readings for all pathogen assays

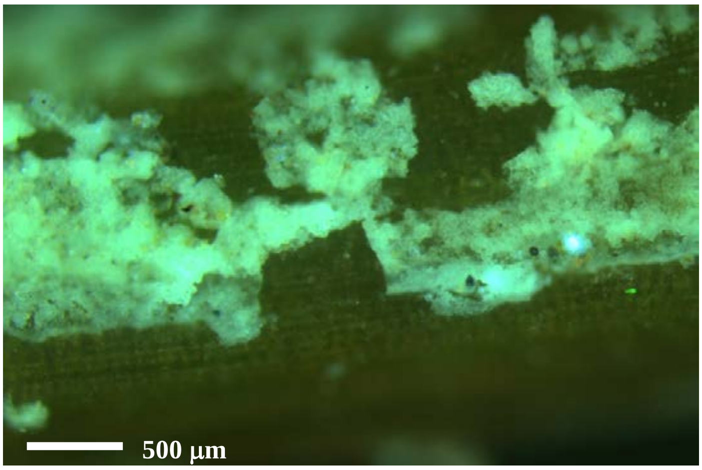
Trial[a]	**Relative humidity (%)**	**Min temperature °C**	**Max temperature °C**	**Average temperature °C**
1	25.6	16.8	28.7	22.0
2	55.8	16.0	28.3	21.7
3	80.9	14.2	32.6	19.4
4	64.3	13.1	27.6	18.2
5	88.6	13.1	27.6	18.1
6	45.8	13.7	27.6	18.2
7	88.6	13.1	27.6	18.1

[a] 1, mycelium vs conidia; 2, seedlings vs cuttings; 3, milli-q, pH 3, rainwater; 4, DSM, pH 5, PNA; 5, milli-q, milli-q + Y, seed broth, lowDB; 6, double inoculum; 7, pks$^-$.
Note: The relative humidity percentage represents the average from data recorded per pathogen trial.

3.3.3 Water Source

Mineral deposits on pine needles watered with laboratory tap water were discovered by a previous researcher in this laboratory (West, 2004). As the buildup of mineral deposits increases over a period of time, it may be inhibitory to dothistroma infection, perhaps by blocking access to pine needle stomata. Figure 5 is an image of mineral deposits on the surface of a pine needle taken from an assay chamber which contained laboratory tap water. Generally the buildup of minerals on the needle surface is clearly visible to the naked eye and does not require microscopic analysis.

Figure 5 Mineral deposits on pine needle



Pine needle viewed at 100x magnification

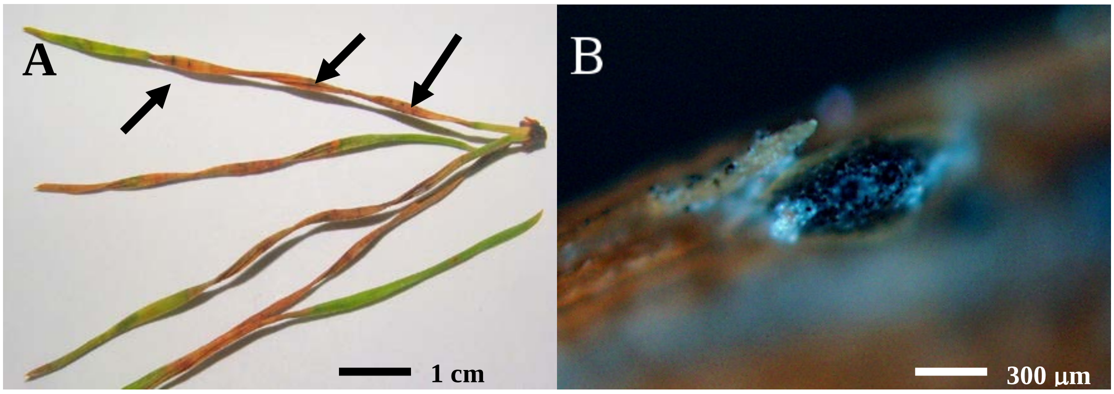
The use of RO water (distilled water) instead of tap water reduced the buildup of mineral deposits on the surface of pine needles (visual observation).

3.3.4 Scoring Infection

The procedure outlined in Section 2.8.6 for scoring disease was followed for all subsequent pathogen assays, to ensure all data collections were consistent between assays conducted at different times. Chlorotic symptoms of disease, such as areas of brown/tan colouration within regions of the pine needle maybe due to other organisms or tissue damage not necessarily associated with *D. septosporum*. Therefore, more specific signs of *D. septosporum* infection (red bands and fruiting bodies) were used to evaluate the success of the pathogen assay.

All needles with red-bands were viewed at 40x magnification to determine the presence or absence of fruiting bodies. Under normal white light of the dissecting microscope or in natural daylight, the bands display a red colouration that is distinct from normal chlorotic lesions often seen on needles, appearing tan or brown. The red colouration is due to the red pigmented mycotoxin, dothistromin. In Figure 6, photograph A shows multiple red bands on single needles (arrow heads), while photograph B shows a mature fruiting body erupting through the epidermal layer of the needle tissue, both signs characteristic of dothistroma disease.

Figure 6 Signs and symptoms of *D. septosporum* disease



Photograph B taken at 100x magnification

The fruiting body is oval in shape, and splits the epidermal layer longitudinally. Sometimes fruiting bodies appeared in areas that did not show the characteristic red band. However on several occasions, red crystals were visible around the outside and within the fruiting body. It is probable these were dothistromin crystals.

Figure 7 shows a typical comparison between an infected seedling (A) and uninfected seedling (B) within the pathogen assay system. The majority of infection usually occurred on the lower half of the seedling, with the young needles becoming necrotic while still attached to the seedling. The red bands are prominent and distinguishable along the length of the pine needle (arrow heads). In instances where a chlorotic region within a green needle is present, and this region is not prominently reddish in colouration (characteristic red-band), the needle is classified as chlorotic and subsequently classed as non-infected. In addition, a small number of the lower needles on the pine seedling become necrotic due to natural reasons (arrow photograph B). These needles are very distinct from infected needles as they do not display the banding pattern and are generally of a dark brown colouration, as opposed to the tan colouration of chlorotic needles or red colouration of infected needles.

Figure 7 Infected and uninfected pine seedlings

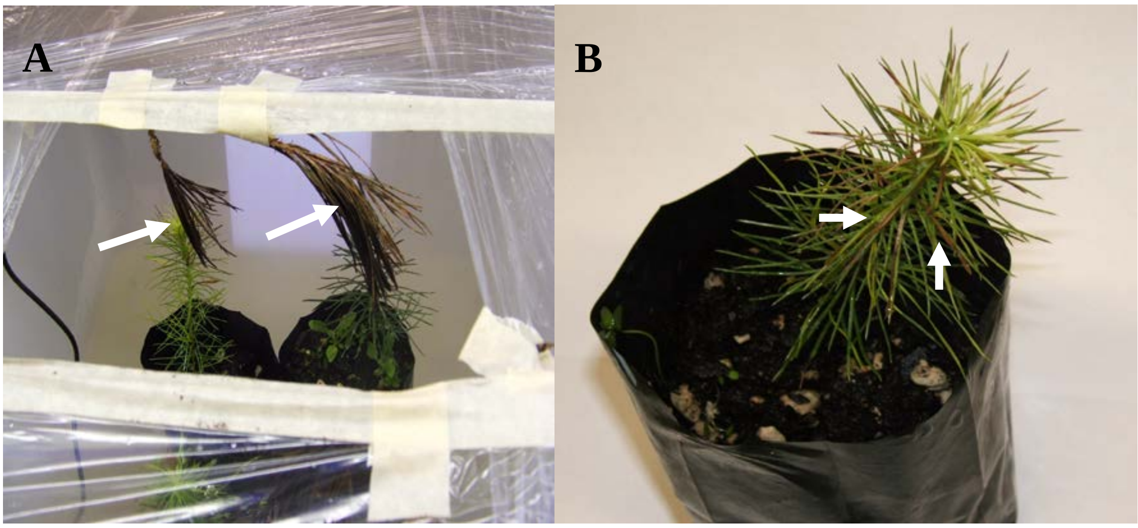
A = Dothistroma infected seedling, B = Uninfected control seedling

3.3.5 Natural Inoculum Validates Developed Pathogen Assay System

Once an adequate assay chamber had been developed which met all required environmental conditions for infection to occur, ie. light, temperature and humidity, the next step was to use natural inoculum in order to test the system. This was a preliminary test prior to the set up of the pathogen assays with cultured inoculum.

The assay chamber was set up as described in Section 2.8.1, with infected needles (of the same batch from which NZE10 had been isolated), suspended over pine seedlings. Six weeks later the needles on the top third of the pine seedling showed signs of dothistroma disease, ie. red bands and fruiting bodies. During the experiment the fascicles containing the natural inoculum became very wet due to continuous misting and subsequently drooped down onto the top of the seedlings. Consequently, many needles also became very necrotic, appearing to be swollen and water logged. However, the fruiting bodies within the red bands on diseased needles were very visible. Figure 8 shows the assay chamber set up and also a pine seedling six weeks post inoculation.

Figure 8 Natural inoculum pathogen assay



A = Natural inoculum suspended over pine seedlings, B = Infected pine seedling

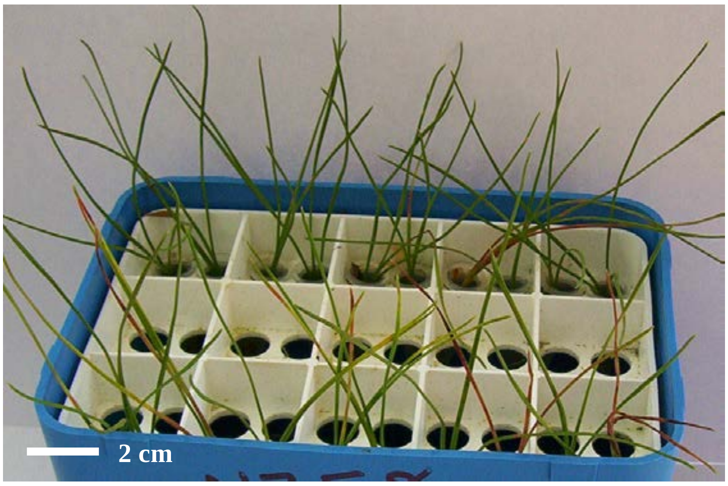
In Figure 8 the arrows in photo A indicate the source of natural inoculum, whilst the arrows in photo B indicate necrotic needles which upon closer observation showed signs of dothistroma disease.

3.4 Host Factors

3.4.1 Use of Detached Plant Material in Pathogen Assay

Preliminary pathogen assays using detached *P. radiata* fascicles were set up in the assay chamber and found to be unsuitable. The fascicles were prone to severe infection and contamination from other micro-organisms, particularly other fungal species. The contamination occurred regardless of whether the plant material had been surface sterilized in sodium hydrochloride prior to inoculation. The water agar plug in which the bottoms of fascicles were placed (Section 2.1.2.) facilitated contamination from other micro-organisms by providing a food or inoculum source. The contamination lead to the subsequent death of the pine needles prior to any signs of dothistroma disease. Furthermore, fascicles placed in separate test-tubes normally turned necrotic within two weeks. This could have been due to a higher temperature in the test-tube environment, no circulation of air and mist or a combination of these environmental factors. Figure 9 shows a container used to hold the pine fascicles and subsequent contamination and death of pine needles.

Figure 9 Detached pine fascicles



Due to contamination problems, the decision was made to use pine seedlings or cuttings, rather than detached fascicles in subsequent pathogen assays. This would also eliminate the possibility that detached plant material does not respond to fungi in a similar way as a whole tree system in the field.

3.4.2 Seedlings vs Cuttings

In order to fit the required number of replicates of seedlings and controls within the same assay chamber, young (6-12 months) plants approximately 15 cm in height were used. *P. radiata* seedlings and rooted cuttings (Section 2.1.2) were tested in the assay chamber to determine which type would be more susceptible to infection and beneficial to use in a model system. Previous research not specifically looking at comparison of infection between seedlings and cuttings had shown that seedlings were more severely infected in their system than cuttings (Gadgil, 1976).

With *D. septosporum* isolate NZE10 as inoculum, ANOVA showed that there was a significant ($p<0.01$) difference in percentage of disease incidence on seedlings compared with cuttings (Table 3). The percentage of disease incidence on seedlings was double that obtained with cuttings. The number of bands seen per needle was similar for both conditions; however there was a 3-fold increase in the number of fruiting bodies per infected needle on the seedlings. In addition, the percentage of chlorotic needles was almost 3-fold greater for seedlings compared with cuttings.

Table 3. Difference in disease incidence according to plant type

	Disease[a] Incidence (%)	Bands/ Needle[b]	Fruit bodies/ Needle[b]	Chlorotic Needles (%)	Needles[c] Counted (n)
Seedlings	19.0 ± 3.0	1.4 ± 0.1	0.8 ± 0.1	34.9 ± 13.2	495
Cuttings	9.1 ± 0.8	1.3 ± 0.1	0.3 ± 0.2	13.6 ± 3.9	498

[a] Percentage of needles with red bands (does not include chlorotic needles).
[b] Mean ± SD per infected needle.
[c] Total needles counted from three replicate trees.
Note: All data are average scores from three tree samples per condition with ± being SD.

These results indicate it would be preferable to use pine seedlings rather than rooted cuttings in an assay system. Since these cuttings were taken from five year old pine trees it is possible some form of mature tree resistance was present within the rooted cuttings.

The disease incidence on needles of un-inoculated control seedlings and cuttings was 1.2% and 1.8% (n=412 and 446) respectively, probably due to contact with inoculated seedlings in the assay chamber. On one control seedling, approximately three quarters of the needles were completely necrotic. Samples of these necrotic needles were viewed at 40x magnification and through visual inspection it was concluded they did not display any signs or symptoms of dothistroma.

3.5 Pathogen Factors

3.5.1 Type of Inoculum

Previous research found infection occurred through stomatal penetration by germ tubes or by direct penetration of the needle tissue by hyphae (Gadgil, 1967). The morphological instability of *D. septosporum* means it is sometimes difficult to obtain conidia from cultured isolates. Therefore this experiment was conducted to determine which form of inoculum, either NZE10 mycelium fragments (Section 2.8.4) or conidia (Section 2.2.3) would be more efficient to use in a pathogen assay system (Section 2.8.1).

The results in Table 4. indicate the percent infection was double when conidia were used as inoculum as opposed to mycelia fragments. However, ANOVA determined that the difference in percent infection between inoculum types was not significant. In addition, the number of bands and fruiting bodies seen per infected needle was slightly higher for conidia than for mycelium fragments. However, the number of chlorotic needles was similar for both conditions. It is very likely that both types of inoculum contained a small percentage of the opposite contaminating fungal structure (ie. spore suspension contained mycelium fragments and vice versa) due to the procedures used to prepare the inoculum. However, the results indicate that regardless of inoculum type the assay system and inoculation procedures are conducive to infection of pine material.

Table 4. Differences in disease incidence according to inoculum type

	Disease[a] Incidence (%)	**Bands/ Needle[b]**	**Fruit bodies/ Needle[b]**	**Chlorotic Needles (%)**	**Needles[c] Counted (n)**
Myc	4.1 ± 3.6	0.9 ± 0.8	0.6 ± 0.5	13.3 ± 4.2	460
Conidia	10.6 ± 5.4	1.4 ± 0.2	0.9 ± 0.1	13.0 ± 0.3	586

[a] Percentage of needles with red bands (does not include chlorotic needles).
[b] Mean ± SD per diseased needle.
[c] Total needles counted from three replicate trees.
Note: All data are average scores from three seedling samples per condition with ± being SD.

There were no *D. septosporum* infections on un-inoculated control seedlings, although 6% (n=427) of needles were chlorotic. The difference between inoculum types was not significant, but a slightly higher disease incidence was achieved using conidia opposed to mycelia fragments as inoculum. In nature, spores are the primary source of inoculum, so for all subsequent pathogen assays, spores were used as inoculum.

3.5.2 The Effect of Conidia Suspension on Infection

Optimal environmental conditions are crucial for *D. septosporum* infections to occur (Gadgil, 1974, 1976, 1977). Therefore, the medium in which conidia are suspended may play a role in conidia viability and plant infection on the needle surface. Three types of medium were chosen to harvest and suspend conidia from NZE10 colonies cultured on DSM (Section 2.2.3): Milli-Q water (representing a suspension depleted of minerals and salts), pH 3 water (mimicking acidic conditions favourable for growth) (Chapter 4), and rainwater (representing a condition as close to natural as possible).

A higher disease incidence was obtained with rainwater, than with milli-Q or pH 3 water, but the standard deviation was large and an ANOVA showed no significant difference between the three types of medium in which conidia were suspended. Generally, over all conditions the number of bands and fruiting bodies present per diseased needle were similar, with slight variations in the number of chlorotic needles per condition. These results indicate that perhaps the nutrient store within the conidia itself may be of more importance than the external conditions around the conidia. Alternatively, if there is free water available on the host surface, regardless of mineral and salt composition, the fungus may be able to infect the needles and cause disease.

Table 5. Difference in disease incidence according to conidia suspension media

	Disease[a] Incidence (%)	**Bands/ Needle[b]**	**Fruit bodies/ Needle[b]**	**Chlorotic Needles (%)**	**Needles[c] Counted (n)**
Milli-q	19.0 ± 3.0	1.4 ± 0.2	0.8 ± 0.2	34.9 ± 13.2	495
pH 3	13.1 ± 2.4	1.4 ± 0.2	0.9 ± 0	23.8 ± 0.3	530
Rainwater	25.3 ± 17.5	1.3 ± 0	0.9 ± 0.1	26.4 ± 9.3	367

[a] Percentage of needles with red bands (does not include chlorotic needles).
[b] Mean ± SD per diseased needle.
[c] Total needles counted from three replicate trees.
Note: All data are average scores from three seedling samples per condition with ± being SD.

For the control seedlings, on average 7.5% of needles appeared chlorotic, likely due to natural causes with no symptoms of dothistroma present.

3.5.3 The Effect of External Nutrients on Infection

An adequate disease incidence was obtained for conidia in a relatively depleted external environment (in respect to minerals and salts) as seen in Section 3.5.2. However, in an enhanced external nutrient environment, it may be possible to increase the numbers of infections. Different nutrients would be available due to the type of suspension in which the conidia were harvested, in addition to the presence of DOTH or other fungal metabolites in the seed broth. Additionally, *D. septosporum* is often isolated from diseased needles along with a yeast species which to date has not been identified (Dick, personal communication). The use of a yeast extract (Appendix A1.21) added to culture flasks in this laboratory resulted in a significant increase in DOTH production by *D. septosporum* (Schwelm, unpublished).

NZE10 conidia were harvested from DSM plates in milli-Q water, or milli-Q water plus yeast extract (Appendix A1.21), seed broth (membrane filtered culture broth, after seven days of *D. septosporum* growth), or lowDB (liquid media) (Section 2.2.3). The assay for seedlings inoculated with spores suspended in seed broth and lowDB finished two weeks after the milli-Q and milli-Q plus yeast assay. Lack of conidia to inoculate all seedlings at the same time caused a delay of two weeks. The highest disease incidence was 32.7% for conidia harvested in milli-Q water plus yeast extract, and although the standard deviation was large, ANOVA determined that the treatment differences for all conditions was significant ($p<0.01$), (Table 6). The significant difference between the means was compared using Tukeys HSD test at $p<0.05$, and showed the difference in disease incidence obtained with milli-Q and yeast extract was significant compared to all other conditions. The number of bands and fruiting bodies per needle, in addition to the percent of chlorotic needles was similar for all conditions. No disease symptoms were seen on un-inoculated control seedlings, with 5.9%, 6.9%, 10.6% and 13.8% (milli-q, milli-q + yeast, seed broth and lowDB respectively) on average chlorotic needles recorded per treatment.

Table 6. Disease incidence with addition of external nutrients to inoculum

	Disease[a] Incidence (%)	**Bands/ Needle[b]**	**Fruit bodies/ Needle[b]**	**Chlorotic Needles (%)**	**Needles[c] Counted (n)**
Milli-q	4.5 ± 4.4	0.9 ± 0.8	0.7 ± 0.7	7.3 ± 3.1	533
Milli-q + Y	32.7 ± 10.5	2.2 ± 0.4	1.7 ± 0.1	9.9 ± 6.8	420
Seed broth	9.0 ± 3.7	1.8 ± 0.6	2.5 ± 0.9	7.9 ± 1.7	454
lowDB	3.7 ± 1.5	1.1 ± 0.1	0.9 ± 0.1	11.9 ± 0	590

[a] Percentage of needles with red bands (does not include chlorotic needles).
[b] Mean ± SD per diseased needle.
[c] Total needles counted from three replicate trees.
Note: All data are average scores from three seedling samples per condition with ± being SD.

All replicate seedlings in the yeast treatment showed more disease incidence than seedlings in the other treatments. It is possible that the unexplored association of *D. septosporum* with a yeast species is a key to obtaining more disease. The disease incidence in this assay is the highest seen in all assays conducted in Section 3.2.

3.5.4 The Effect of Conidia Growth Media on Disease Incidence

As mentioned, conidia rely on internal nutrient reserves to enable them to germinate, a critical step in causing infection. Whether conidia harvested from artificial media that are more similar to growth conditions experienced by *D. septosporum* in the forest, would enhance the potential to cause infection was investigated.

Isolate NZE10 conidia were used, and caused most disease at 7.7% for pH 5 media (Table 7). However the standard deviation was large and an ANOVA showed no significant difference between the three types of media from which conidia were harvested from. The number of bands and fruiting bodies per needle, in addition to the percentage of chlorotic needles (Appendix IV) was similar for all three conditions. For un-infected control cuttings, 2.3% of the needles were chlorotic, with no needles showing signs/symptoms of dothistroma disease (Section 2.8.6). The results show that the type of media on which conidia are grown and harvested does not affect the level of infection obtained when inoculated onto pine cuttings.

Table 7. Difference in disease incidence according to media from which conidia were harvested

	Disease[a] Incidence (%)	**Bands/ Needle[b]**	**Fruit bodies/ Needle[b]**	**Chlorotic Needles (%)**	**Needles[c] Counted n**
DSM	6.2 ± 5.6	1.7 ± 1.0	1.2 ± 0.2	9.0 ± 11.0	304
pH 5	7.7 ± 6.2	1.8 ± 0.5	0.9 ± 0.8	12.4 ± 0.5	272
PNA	2.6 ± 0.4	1.0 ± 0	0.4 ± 0.2	12.3 ± 0.4	320

[a] Percentage of needles with red bands (does not include chlorotic needles).
[b] Mean ± SD per diseased needle.
[c] Total needles counted from three replicate trees.
Note: All data are average scores from three cutting samples per condition with ± being SD.

Results in Section 3.4.2, showed a significant difference between disease incidence obtained with seedlings vs cuttings. Disease incidence between the three conditions in this experiment was not significant; it is unlikely there would be a significant difference in disease incidence between conditions if seedlings were used in the assay.

3.5.5 Double Inoculation With Two *D. septosporum* Isolates

It is common for many fungal species to produce toxins to ward off competition from other microorganisms within their niche. Possibly having two isolates within the same niche would facilitate up-regulation of toxin production in response to each other and therefore may lead to a more infections if both isolates were inoculated together on the same site. Multiple isolates or species of fungi (as seen with *Mycosphaerella* spp. on eucalyptus) are often found within the same lesion area. This idea assumes the presence of the mycotoxin dothistromin is important for infection to proceed.

For this experiment the concentration of conidia used to inoculate plant material was the same as in other assays (Section 2.8.5). An ANOVA showed the difference in disease incidence between the treatments with single or multiple isolates of *D. septosporum* was not significant (Table 8).

Table 8. Difference in disease incidence when two Dothistroma isolates are present

	Disease[a] Incidence (%)	**Bands/ Needle[b]**	**Fruit bodies/ Needle[b]**	**Chlorotic Needles (%)**	**Needles[c] Counted n**
GPF-38	8.1 ± 6.9	1.4 ± 0.4	1.3 ± 0.9	21.0 ± 18.2	384
Both[d]	3.4 ± 2.4	1.0 ± 0.1	1.3 ± 0.4	7.6 ± 3.8	559

[a] Percentage of needles with red bands (does not include chlorotic needles).
[b] Mean ± SD per diseased needle.
[c] Total needles counted from three replicate trees.
[d] NZE10 and GFP-38
Note: All data are average scores from three cutting samples per condition with ± being SD.

For control seedlings, on average 23% of the needles were chlorotic with 1.5% of the needles showing signs or symptoms of *D. septosporum* infection (likely due to cross-contamination between controls and inoculated seedlings with the assay chamber).

3.5.6 Does Infection Occur in the Absence of DOTH?

Dothistromin is thought to be important in the development of disease symptoms, such as the red band seen on infected pine needles (Bradshaw et al., 2006). This has been shown through injection of purified toxin into needle tissue (Shain and Franich, 1981). However, whether DOTH is required for infection to occur is unknown. Recently, a *D. septosporum* isolate, with a mutated polyketide synthase gene (*pksA*), defective in production of DOTH was developed in this laboratory (Bradshaw et al., 2006). The *pksA* mutant was inoculated onto pine seedlings to determine whether DOTH is essential for infection to occur.

The disease incidence was low for both the wild type and DOTH mutant (Table 9), and an ANOVA showed the difference in disease incidence was not significant. No bands were seen on infected needles with the *pksA* mutant, and the number of fruiting bodies per diseased needle was higher for the mutant than for the wild type (NZE10). For un-infected control cuttings, 12% of the needles were chlorotic, with no needles showing signs or symptoms of *D. septosporum* infection.

Table 9. Difference in disease incidence caused by a DOTH mutant and wild type *D. septosporum*

	Disease[a] Incidence (%)	**Bands/ Needle[b]**	**Fruit bodies/ Needle[b]**	**Chlorotic Needles (%)**	**Needles[c] Counted (n)**
NZE10	3.7 ± 1.7	1.5 ± 0.6	1.3 ± 0.4	6.4 ± 2.6	492
pksA	2.6 ± 2.1	0	2.7 ± 0.4	4.6 ± 0.8	499

[a] Percentage of needles with red bands (does not include chlorotic needles).
[b] Mean ± SD per diseased needle.
[c] Total needles counted from three replicate trees.
Note: All data are average scores from three seedling samples per condition with ± being SD.

The lack of a red band on diseased needles inoculated with the mutant was expected, with identification of such needles solely due to the presence of fruiting bodies. This experiment needs to be replicated in order to validate these results. However, it does appear that a comparable level of disease can be obtained in the absence of dothistromin biosynthesis. The testing of further *D. septosporum* mutants, defective in DOTH biosynthesis needs to be conducted to confirm that the mycotoxin DOTH is not a required pathogenicity factor.

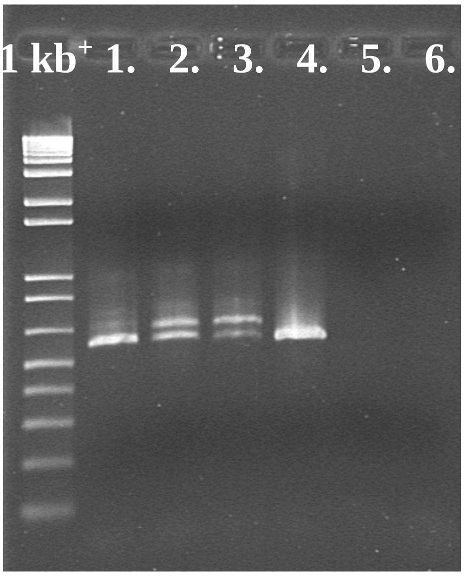
3.6 Diagnostic Procedure to Confirm Red-band Disease

3.6.1 Use of Universal Primers to Confirm Infection

A diagnostic procedure was developed to determine whether the signs and symptoms of disease on needles from the pathogen assay were due to *D. septosporum*. A method for extracting DNA from such needles was developed, with the procedure outlined in Section 2.8.7. Initially it was envisaged that universal ITS primers would be sufficient to amplify the internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S gene from fungal ribosomal DNA using PCR.

PCR products were obtained from infected needles using universal ITS4 and ITS5 primers (Section 2.5), (Figure 10). Lanes 1-3 represent DNA extracted from diseased needles: Lane 1 spore inoculum, lane 2 mycelium inoculum (Section 3.5.1), and lane 3 conidia harvested in rainwater (Section 3.5.2). Lane 4 is *D. septosporum* isolate NZE7 (positive control), lane 5 is DNA extracted from healthy pine needles and lane 6 is the PCR negative control. Multiple bands were obtained with DNA extracted from diseased needle tissue, compared to the single band (approximately 580 bp) obtained for the positive control. Many fungal species share similar sequence identity in respect to the ITS region, and it is likely that the extracted DNA from the pine needle tissue contains extracts of DNA from multiple fungal species which were colonizing the needle tissue (eg. endophytes). Plant and fungi also share similar ITS sequence, but since no band was obtained for the uninfected needle tissue extract, it is unlikely any bands in lanes 1-3 are due to plant DNA.

Figure 10. ITS4 and ITS5 PCR products



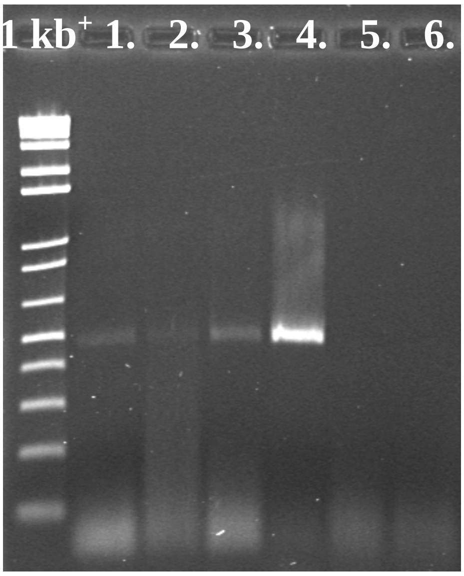
Lanes: 1-3 extracted DNA; 4 NZE7, 5 uninfected needle DNA and lane 6 negative control.

Subsequently the approximate 580 bp band which matched the size of the NZE7 positive control was gel purified directly (Section 2.5.2) and sequenced (Section 2.6). The chromatogram of the sequence data showed many bases were unable to be identified by the sequencer. An alignment with the *D. septosporum* ITS sequence (GenBank Accession AY808280.1) obtained by Barnes et. al. (2004), using the NCBI bl2seq program (http://www.ncbi.nlm.nih.gov) showed 93% identity. Universal primers were not specific in identifying *D. septosporum* within diseased needles.

3.6.2 Specific Mating Type Primers Confirm Presence of *D. septosporum*

Recently the mating type genes, *Mat1-1-1* and *Mat1-2* of *D. septosporum* have been isolated and sequenced. In addition, a set of species and mating type (both genes) specific primers have been designed (Groenewald, unpublished). Dothistroma *Mat1-2*-specific primers, DseptoMat2f and DotMat2r (Section 2.5), were therefore used to confirm the presence of *D. septosporum* within infected needles. When conventional PCR reagents were used (eg. 10 x PCR buffer, and $MgCl_2$) no bands were obtained for DNA extracted from the infected needles. The conventional PCR reagents were then substituted with reagents from the PCR enhancer system (Section 2.8.7), which is useful for problematic and G/C-rich templates, offering higher primer specificity. Figure 11. shows bands consistent with the 480 bp product expected with this primer set. The lanes represent extracted DNA in the same order as described in Section 3.6.1.

Figure 11 Mating type gene PCR



Lanes: 1-3 extracted DNA; 4 NZE7, 5 uninfected needle DNA and lane 6 negative control.

The developed diagnostic procedure has been used to confirm the presence of *D. septosporum* in the pathogen assays listed in Table 10.

Table 10. Presence of *D. septosporum* in diseased needles confirmed by PCR analysis

Pathogen Assay	**Section No.**
Type of inoculum	3.5.1
Spore growth media	3.5.4
Double inoculum	3.5.5

Further optimization of the DNA extraction procedure from infected needle tissue needs to be done. Pine needles are very coarse and stringy, and are not easily ground to a fine powder in liquid nitrogen. Therefore it is difficult to rupture enough cells to obtain sufficient DNA. The procedure at this stage is not robust enough to categorically rule out *D. septosporum* infection if no PCR product is detected, as this could be due to lack of fungal DNA or the presence of PCR inhibitors preventing amplification of DNA.

3.7 Discussion

3.7.1 Environmental Conditions

The results of the current study indicate that infections can be obtained under controlled conditions, with a temperature of 24°C/16° (day/night), relative humidity continuous at 80%, continuous moisture and a diurnal cycle of 12 h consisting of white light and ultraviolet light. These conditions enable *D. septosporum* to complete the full life-cycle from infection structure to production of asexual spores within six weeks.

There are conflicting views between scientists in respect to the environmental conditions, such as needle wetness, humidity, temperature and requirement of light, needed for successful infection of pine material under laboratory conditions. The environmental variable that appears to cause the most discrepancy between researches is temperature. In the current study, preliminary pathogen assays conducted in a variety of conditions where temperature and humidity were not controlled were unsuccessful, including placement of the assay chamber outside under natural light. Therefore it appears that a controlled environment when artificially inoculating pine material is required.

A recent publication by Muir & Cobb (2005) showed disease levels were significantly higher within a controlled but variable temperature environment in conjunction with continuous misting, as opposed to a constant temperature and continuous misting (Muir and Cobb, 2005). In addition, a recent study by Woods et. al. (2005) showed a correlation between increase in dothistroma disease (Northwest British Columbia forests) and increased precipitation and temperature above 16°C (Woods et al., 2005). As the above two researchers have shown, in both an artificial and natural environment, it appears temperature and moisture are two key variables required for successful infection and disease development. The results from the data logger indicate that a variable temperature was maintained, and the use of a temperature controlled room to conduct the assay was therefore of considerable benefit in reducing variability of temperature. The humidity within the assay chamber for half of the assays conducted was lower than the optimal 80% (Table 2). Since infection still occurred in these assays, the fluctuation in humidity may not be as detrimental to the ability of the fungus to cause infection as other variables.

However, the effect of light needs to be further investigated. No infection was obtained in this laboratory when a previous researcher used visible light and sodium lamps (West, 2004). In addition, previous research has noted less dothistroma disease on shaded pine foliage in the field (Gadgil, 1976), in addition to no disease on pine seedlings in the dark (Ivory, 1972b). In the field, pine trees are exposed to varying levels of solar UV radiation, according to both weather conditions and the position of the tree within the forest. Whether a reduction in the disease incidence under low light intensity or in darkness is due to the host or the pathogen needs further investigation. However, although light intensity was not measured in this study, the use of UV light may be an important variable requiring further analysis for laboratory based assays.

The infection chamber created met all environmental variables considered important for infection to occur. The chamber is easily created using simple materials, allowing the duplication of this system within other laboratories. The importance of the observation that de-ionized water reduced the buildup of minerals on the needle surface is unclear. It is possible that mineral deposits may not interfere with potential infection rates, and indeed dothistroma appeared to survive within the mineral deposits on the needle surface as seen with the GFP-isolates (discussed in Chapter 5). However, eliminating any variable that could potentially block the infection process is helpful due to the natural instability of any work or system developed concerning *D. septosporum*.

3.7.2 *P. radiata* and *D. septosporum* Factors

Different species within the family of *Pinus* have different susceptibilities to red-band disease. The most susceptible species planted within New Zealand's pine forests are *P. radiata, P. nigra* and *P. attenuata*. Due to the length of time required to develop an adequate pathogen assay that produced acceptable results, the current work was restricted to *P. radiata*. Considering host factors (Section 3.4), the results from this study determined that the use of detached pine fascicles was unsuitable for the assay developed. However, pine seedlings were found to be more susceptible to *D. septosporum* than pine cuttings, possibly due to cuttings having partial resistance as they were taken from more mature trees (Gadgil, 1976).

Most pathogen assays use spores as inoculum, since spores are the primary source of inoculum in natural conditions. The results in Section 3.5.1 showed no significant difference in disease incidence regardless of whether conidia or mycelia fragments are used as inoculum. In addition, most spore suspension contain mycelia fragments (Browne and Cooke, 2004b). However, mycelia fragments are capable of direct penetration of either the stomata or epidermis (Gadgil, 1967), and in natural conditions the primary inoculum is conidia. But due to the morphological variability of *D. septosporum* producing conidia in culture, the use of mycelia fragments as inoculum is therefore an option for future use in a pathogen assay.

In the assays which considered; the role of spore suspensions, the media on which conidia were grown, the use of two isolates of *D. septosporum*, and one which did not synthesis DOTH, there was no significant difference between any of the variables in each assay in respect to increasing the disease incidence. The only significant increase in disease incidence within an assay was when the role of external nutrients was considered (Section 3.5.3). The addition of the yeast extract to the inoculum suspension resulted in more disease. On occasions *D. septosporum* has been isolated from diseased needles along with a yeast species (Dick, personal communication). During this study, during routine PCR analysis of NZE7 DNA using universal primers, an additional PCR product was obtained and the sequenced product showed 93% identity to a *Rhodotorula* yeast. On occasions, yeast-like colonies fitting the description of this yeast have been seen growing on *D. septosporum* culture plates (personal observation). Whether this is of any significance will require further investigation. However, it is plausible that *D. septosporum* may respond to the presence of yeast by up-regulation of DOTH, which may increase disease incidence if DOTH is a virulence factor. Further analysis on the response of the host to a combination of yeast and *D. septosporum*, in addition to *D. septosporum* response to the phyllophore of microorganisms on the pine needle needs to be conducted.

The majority of diseased needles on the pine seedling/cuttings were on the bottom third of the tree. This is probably due to inoculum being collected within water droplets and subsequently dropping onto the lower needles. Conversely, in the natural inoculum assay (Section 3.3.5), disease was seen predominately on the top third of the tree. This is likely due to a higher level of inoculum being present in this region, as the naturally infected branches drooped onto the top of the seedlings. In all assays, the presence of a red band was seen around three week’s post-inoculation at the earliest (personal observation). As the red band has been attributed to the presence of DOTH (Shain and Franich, 1981), if it is produced in the early stages of infection, it would be expected that signs of needle necrosis would be evident earlier. Alternatively, at the early stages of infection, the level of toxin produced may be insufficient to cause necrosis or the toxin may be broken down on the surface of the needle tissue by light, or possibly diffused by the presence of water. Alternatively, the toxin may not be required for initiation of infection, as the disease incidence for the *pksA* mutant was comparable to that of the wild type (Section 3.5.6). It is also possible *D. septosporum* grows biotrophically within the host tissue after successful penetration, and does not produce DOTH until it has established itself within the host and is ready to form stromata. Alternatively, the red band may be a result of limited breakdown of toxin within the host tissue in addition to a cumulative level of toxin reached before the symptom is evident. Since needles infected with the pksA mutant turned necrotic with no red band, it is likely that needle necrosis is due to a host defense response. Although the assays with the pksA mutant needs to be replicated to validate these results (since low levels of disease were obtained), current results indicate that DOTH may be a virulence factor. Therefore, DOTH is not required for infection nor for formation of stromata, but must confer some ecological benefit to the fungus. The synthesis of DOTH may ensure a higher percentage of penetration events are successful, a higher probability of stromata formation, and the ability of Dothistroma to defend its niche against other pathogens or endophytes.

The current work in this study has shown that *P. radiata* seedlings inoculated with *D. septosporum* conidia, under controlled environmental conditions in the assay chamber developed, resulted in development of dothistroma red-band disease. There do not appear to be any external factors which result in more disease with laboratory cultured stains of *D. septosporum*. However, there was an increase in level disease incidence with the addition of a yeast extract that requires further analysis to determine whether this is due to a response by the host or by the pathogen. Additionally, with the use of the *pksA* mutant, it appears that DOTH may be a virulence factor. Together all these results show that an adequate laboratory system can be set up to achieve infection. This will enable further work to proceed characterizing DOTH mutants, and the screening of resistant pine cultivars to *D. septosporum*.

3.7.3 Developed Diagnostic Procedure

Currently, to my knowledge, no diagnostic procedure has been developed to confirm the presence of *D. septosporum* within needles displaying signs and symptoms of red band disease. The procedure, as outlined in Section 3.6.2, although requiring further optimization in respect to extraction of DNA from the needle tissue, offers potential benefit to scientists. In particular, the procedure in conjunction with the developed mating type gene primers (Groenewald, unpublished) would enable scientists to screen infected pine tissue for the presence of either *D. septosporum* or *D. pini* species. This would help with further characterization of the global distribution of both these species. Additionally, the procedure could be used to undeniably confirm the presence of *D. septosporum* DOTH mutants, within infected pine tissue, further supporting the idea that DOTH is a possible virulence factor not a pathogenicity factor.

4

Does pH affect *D. septosporum* physiological processes?

4.1 Introduction

Essentially all microorganisms have some ability to sense the environment they inhabit. That includes the ability to sense extracellular and intracellular pH and to respond in a manner that favours competitiveness and survival (Davis, 2003; Nozawa et al., 2003). In fungi, ambient pH has been shown to affect most physiological processes, and at a molecular level to regulate expression of genes involved in these processes (Ramon et al., 1999; Cotton et al., 2003; Davis, 2003; Xiao and Sitton, 2004). Disease severity may also be influenced by factors such as the composition of the media the fungi are grown on, inoculum pH, and various other environmental conditions (Wang et al., 1999). Research with *Fusarium oxysporum* showed that an acidic inoculum caused more severe disease symptoms on cotton than neutral inoculum (Wang et al., 1999). Furthermore, for *Candida albicans*, ambient pH affects virulence in addition to being a signal for morphological differentiation, with acidic growth conditions favouring yeast growth and alkaline conditions favouring hyphae growth (Davis, 2003).

Many fungal species have a significantly higher growth rate on media adjusted to an acidic pH. This was shown with *Potebniamyces pyri*, a pathogen of apple tree that causes post-harvest fruit rot. Growth for this fungus was optimum on media adjusted to pH 4, with no growth at pH 7, consistent with the natural environment (pH 4) in which this fungus grows (Xiao and Sitton, 2004). The pH of pine needles, *D. septosporum* natural host, is around pH 4 (Ivory, 1967), and it would also be expected that growth of *D. septosporum* would be preferential at acidic pH. *D. septosporum* is a slow growing fungus with an average daily growth rate of 0.2 mm/day on standard malt based solid media (Bradshaw et al., 2000). It would be beneficial to determine if *D. septosporum* growth rates and conidia production by laboratory isolates could be improved by adjustment of the media pH. However, as research by Xiao and Sitton (2004) showed, not all media that supports growth is suitable for the production of conidia for pathology research.

The production of the secondary metabolite dothistromin by *Dothistroma* spp. must give some beneficial gain to the fungi in their natural niche due to the high energy cost associated with toxin production. It has been proposed that dothistromin is an essential factor for pathogenesis of host tissue and indeed the characteristic symptom of the red-band associate with infection has been reproduced by injection of pure toxin into needle tissue (Shain and Franich, 1981). However, the production of mycotoxins is not necessarily related to virulence, with no correlation found between mycotoxin and virulence for isolates of *Ascochyta pisi, A. fabae* and *Fusarium culmorum* (Weiergang et al., 2002).

Research has determined for other fungal plant pathogens that ambient pH regulates the expression of toxin biosynthetic genes through a conserved signaling cascade mediated by the zinc finger transcription factor PacC (Caracuel et al., 2003a). In *Aspergillus* spp., aflatoxin (AF), penicillin and sterigmatocystin (ST) secondary metabolites are regulated by extracellular pH and some of the toxin biosynthesis genes contain PacC binding motifs and are regulated by the PacC transcription factor (Orejas et al., 1995; MacCabe et al., 1996; Keller et al., 1997). Extensive research conducted on *A. nidulans* has shown that PacC activates alkaline-expressed genes and represses acid-expressed genes and is under pH regulation itself (Flaherty et al., 2003). Additionally, penicillin biosynthesis by *Penicillium chrysogenum* and *A. nidulans* is regulated by nutritional parameters such as nitrogen and carbon sources; however changes in AF/ST production as a result of change in pH, regardless of nitrogen or carbon source has been observed for other *Aspergillus* spp. (Keller et al., 1997). Dothistromin biosynthesis is similar to that of aflatoxin and sterigmatocystin, with similar genes associated with toxin biosynthesis identified (Bradshaw et al., 2006). It is therefore plausible that Dothistroma also has a signaling cascade that will similarly respond to extracellular pH, resulting in a change of toxin biosynthesis and associated toxin gene expression.

The pH of inocula can affect spore attachment to leaf surfaces and subsequent disease severity (Schuerger and Mitchell, 1992; Wang et al., 1999). The initial pathogen-host interaction may involve proteins from either, with pH affecting surface charge of proteins, protein stoichiometry, denaturation of proteins or the availability of micronutrients (Schuerger and Mitchell, 1992). It has been shown that pH affects the regulation of genes involved in proteolytic degradation of the host cell wall as in *S. sclerotiorum* infection (Cotton et al., 2003). Although it has been determined that *D. septosporum* conidia enter through stomatal openings on the pine needle (Gadgil, 1967), there may be a synergistic relationship between pH, germ tube sensing and penetration of the open pore on the needle surface. Additionally, inocula from numerous fungal pathogens are known to secrete glycoproteins or a form of mucilage involved in adhesion to the host surface, with adhesion affected by ambient pH (Wang et al., 1999). In the fungus *F. oxysporum* which causes vascular wilt disease of tomato, PacC is a negative regulator of endopolygalacturonase genes required for virulence. The PacC loss-of-function mutants showed increased virulence at acidic pH due to lack of repression of two acid-expressed endopolygalacturonase genes (Caracuel et al., 2003a). Most, microorganisms have an efficient pH homeostatic mechanism, with extracellular enzymes and permeases functioning over a narrow range of pH, hence they are normally synthesized at pH values which allow them to function efficiently (Caddick et al., 1986).

In order to optimize conditions for a pathogenicity assay to screen *D. septosporum* isolates, a preliminary investigation into the role pH may have in key physiological processes such as growth, spore production and toxin biosynthesis was conducted. Often conventional media have a low buffering capacity, and organic and inorganic acids are used to stabilize pH (Caracuel et al., 2003b). For this investigation, McIlvaine buffer (Dawson et al., 1969), which has been used with other fungal species such as *Sclerotinia sclerotiorum* (Cotton et al., 2003), *Potebniamyces pyri* (Xiao and Sitton, 2004), and *Sphaeropsis pyriputrescens* (Kim et al., 2005) was selected. Ivory (1967) established that citric acid (a component of McIlvaine buffer) did not support growth of *D. septosporum*, and therefore was a suitable constituent along with di-sodium hydrogen phosphate as a buffer for media (Ivory, 1967). However, there are possibly multiple mechanisms within an organism that are pH-responsive (Nozawa et al., 2003), affecting a large array of physiological processes and molecular regulation within the cell.

The aim of the following experiments was to determine at the simplest level of analysis whether pH-responsiveness by *D. septosporum* to ambient pH exists, and whether this can be exploited to optimize conditions favorable for laboratory research. The results of this analysis could therefore lead to further experimentation of pH-responsive mechanisms within this species. If such pH-responsive mechanisms do exist and are shown to be part of the regulatory cues for pathogenesis (Rollins and Dickman, 2001), then blocking the fungus' ability to sense and respond to ambient pH may be another avenue to explore to prevent red-band disease. However, it is plausible that while pH may have an effect on physiological processes in vitro, this is not necessarily applicable *in planta*. Furthermore, favorable conditions for growth will not necessarily correlate with conditions required for optimal toxin biosynthesis and sporulation (Yamanaka, 2003).

4.2 Results

4.2.1 Growth on Different Media

The effect of the type of media on radial growth rates of two Dothistroma isolates, NZE7 and NZE10 was determined. In addition to the media listed in Table 11, the following common media were trialed: Vogel's medium, Czapek dox media, Sporulation media, Cornmeal agar and Nutrient agar (data not included). One week after inoculation it was determined that these media did not enhance the growth rate of *D. septosporum* and therefore would not be used for further growth analysis. For media that appeared to enhance daily growth rate, colony diameter at 21-days was measured as described in Section 2.2.5, and the daily radial growth rate was calculated after subtraction of the initial 4 mm inoculation plug from the measurement.

Table 11. Daily radial growth rates of NZE7 and NZE10

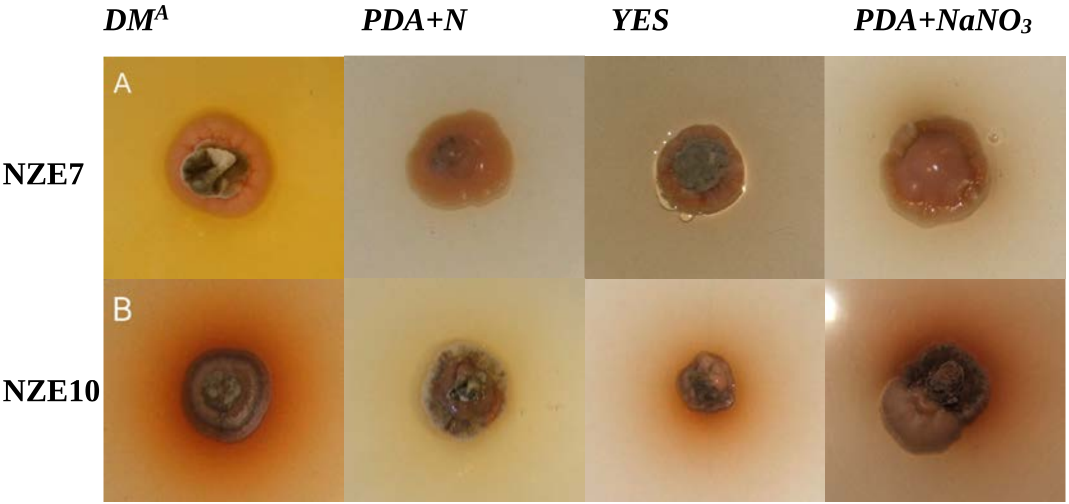
	NZE7 (mm/day)	**NZE10 (mm/day)**
DM[A]	0.62 ± 0.06	0.51 ± 0.03
PDA+N	0.68 ± 0.11	0.52 ± 0.08
YES	0.62 ± 0.10	0.32 ± 0.02
PDA+NaNO$_3$	0.69 ± 0.06	0.50 ± 0.11

[A] DM, dothistroma medium; PDA+N, potato dextrose + niacin agar; YES, yeast extract and sucrose agar; PDA+NaNO$_3$, potato dextrose, sodium nitrate and glucose agar.
Note: Mean calculated from daily growth rate of four colonies per media type, one colony per plate (± SD).

The daily growth rate for NZE7 was similar across all media tested, with non-significant differences in growth as per an ANOVA. However, there was a significant ($p<0.01$) difference in daily growth between media types for isolate NZE10. This would likely be due to the slower rate of growth at 0.32 mm/day for YES media compared to growth of at least 0.50 mm/day for the other media tested.

Visual observations were made of all replicate plates for each medium tested. Under laboratory conditions, the production of dothistromin in culture is seen as of a dark reddish/brown halo around the fungal colony, distinct from the colour of the media. Both isolates produced toxin when inoculated onto YES and PDA+NaNO$_3$ media. Isolate NZE10 (Figure 12. row B) clearly produced toxin when grown on DM and PDA+N, while no toxin was seen for isolate NZE7 (Figure 12. row A) on these media. This observation may not necessarily be significant due to the fact that production of dothistromin in culture for laboratory isolates can be variable and sporadic. One distinct difference observed over all media tested was the difference in colony morphology (Figure 12).

Figure 12 Colony morphology on various media



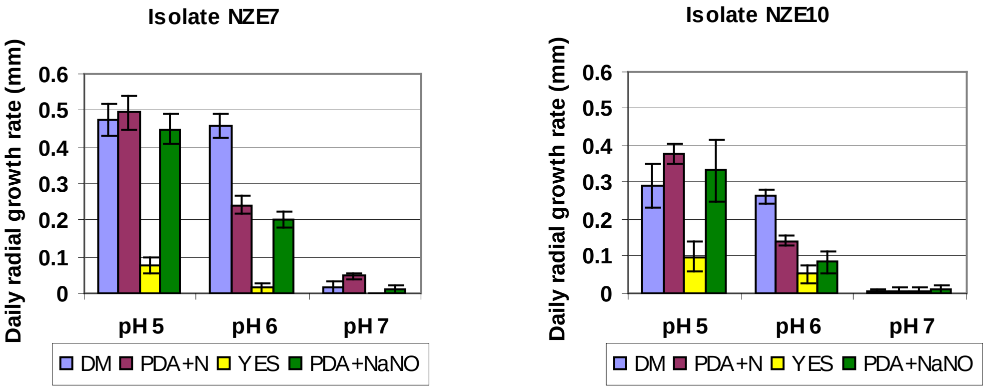
[A] DM, dothistroma media; PDA+N, potato dextrose + niacin agar; YES, yeast extract and sucrose agar; PDA+NaNO$_3$, potato dextrose, sodium nitrate and glucose agar.

On all media the fungus formed circular colonies, with flat growth on the leading edges of the colony and raised growth in the centre of the colony on and around the inoculum plug. Isolate NZE7 colonies on DM were apricot in colour around the circumference, with ridges and little aerial mycelium. The colony was grey/brown, with wrinkled growth and dense aerial mycelium in the centre. On PDA+N and PDA+NaNO$_3$, colonies were apricot in colour, with smooth, near slimy elevated growth with little aerial mycelium present. The growth of NZE7 on YES agar was similar to DM, except for a greater density of aerial mycelium over the whole colony. Isolate NZE10 had a very brown flat colony circumference on DM, lacking aerial mycelium. The centre of the colony was raised and smooth, with aerial mycelium found only around the inoculum plug. For PDA+N and YES agar, there was ridged growth around the circumference of the colony with dense aerial mycelium, which gave the colony the appearance of being white/grey in colour. Again there was raised growth up to and around the inoculum plug with patches of smooth growth and dark grey/brown patches of aerial mycelium dispersed in between. All isolate NZE10 fungal colonies produced sectors on PDA+NaNO$_3$. Interestingly, in Figure 12, the sector that appeared grey/brown and contained aerial mycelium was the sector that produced the greatest amount of toxin, as judged by the pigmentation of the surrounding media. The apricot coloured sector was smooth, lacking aerial mycelium.

4.2.2 Growth and Sporulation on pH Adjusted Media

The four types of media described in Section 4.2.1. were amended to a pH range of 5-7 with McIlvaine Buffer (Section 2.2.5). It was not possible to buffer the media to a more acidic pH due to the media not solidifying, and McIlvaine buffer was unable to maintain alkaline pH beyond pH 7. Growth and sporulation of isolates NZE7 and NZE10 were determined at 21-days. Figure 13 shows the mean daily growth rate across media sampled at the determined pH value.

Figure 13 Daily radial growth rate of *D. septosporum* isolates NZE7 and NZE10 on buffered media



Results are mean of growth rate from four colony counts per media pH

One-way ANOVA showed that the difference in daily growth rate between media within each individual pH condition was significant ($p<0.01$) for both isolates with the exception of NZE10, for which the difference in daily growth rate was not significant at pH 7. Additionally, for both isolates one-way ANOVA showed that the differences in daily growth rate between pH conditions for each medium singularly were significant ($p<0.05$). Comparison of the graphs in Figure 13 shows there is a difference in daily growth rate between the two isolates. Isolate NZE7 has a faster daily growth rate across all pH values and media tested (with the exception of YES agar) than isolate NZE10. On average the growth rate was greatest for both isolates grown on PDA + N buffered to pH 5, with both isolates showing limited growth of media buffered to pH 7.

In addition to daily growth rate, the number of conidia each isolate produced on the four different media was determined for un-buffered media and media adjusted to pH 5 and 6. The pH values of un-buffered media were, pH 5.8 (DM and PDA + N), pH 6.7 (YES) and pH 6.0 (PDA + $NaNO_3$). The numbers of conidia for media adjusted to pH 7 were not determined due to insufficient colony growth. The results shown in Table 12 are the average of the number of conidia per ml from four replicate plates in each condition in which two plugs of mycelia (5 mm diameter) were taken from the opposite sides of the colony margin at 21-days and suspended in 200 μl milli-q water (Section 2.2.3).

Table 12. Number of conidia produced on different media at different pH

	Conidia x 10^5 per ml					
	NZE7			**NZE10**		
	Control	**pH 5**	**pH 6**	**Control**	**pH 5**	**pH 6**
DM	0.66 ±0.19	0.70 ±0.31	1.00 ±0.09	0.38 ±0.13	0.47 ±0.31	1.70 ±1.30
PDA + N	0.41 ±0.50	0.59 ±0.45	0.66 ±0.28	0.58 ±0.24	1.70 ±0.14	2.50 ±0.39
YES	0.75 ±0.40	0.90 ±0.30	0	13.00 ±3.80	2.60 ±2.10	0
PDA + $NaNO_3$	0.56 ±0.13	0.67 ±0.45	1.90 ±1.20	2.60 ±1.10	0.50 ±0.30	0.88 ±0.26

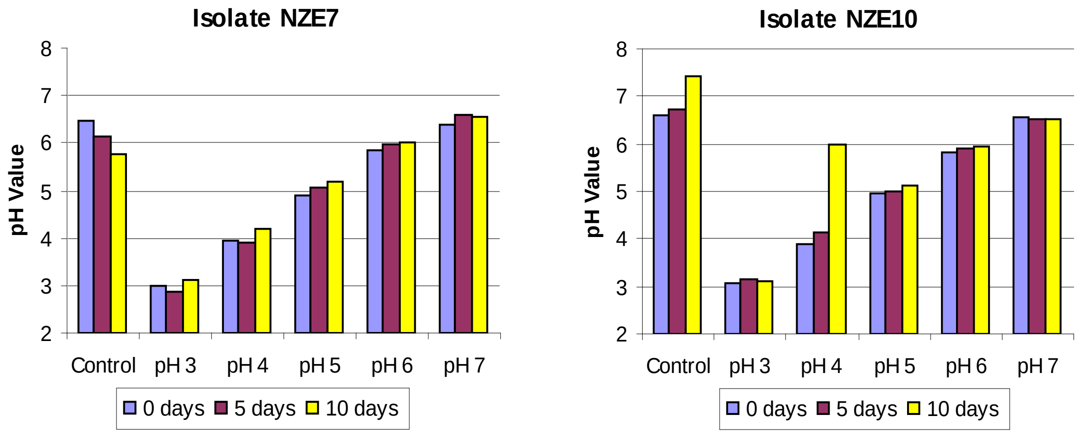
Results are mean of two conidia counts per plate, four plates per media for each pH condition.
-: no conidia were observed.
±: SD

One-way ANOVA showed a significant difference ($p<0.05$) between the number of conidia produced on each medium at pH 5 for NZE7, as well as un-buffered (control) and media adjusted to pH 5 for NZE10. For individual media over all three conditions (ie. un-buffered, pH 5 and pH 6) one-way ANOVA showed that the only significant ($p<0.01$) differences were for isolate NZE10 on PDA+N and YES media. All other results for both isolates were non-significant between conditions. The greatest number of conidia produced for both isolates was on YES media, at pH 5 for NZE7 and un-buffered for NZE10. Isolate NZE10 tended produce more conidia than NZE7 across all media and pH conditions. In summary the composition of the growth media in addition to pH can affect the daily growth rate and the number of conidia produced, with media buffered to pH 5 on average enhancing growth rate with conidia production varying across media and pH depending on the isolate.

4.2.3 Liquid Media Inoculated with *D. septosporum* Conidia

Liquid media buffered with McIlvaine buffer to pH values of 3-7 was inoculated with conidia as described in Section 2.2.6, and growth (Section 2.2.7), toxin production (Section 2.7) and expression of toxin genes (Section 2.5.3) was determined at 5-days and 10-days post-inoculation. The trial was conducted for both NZE7 and NZE10 isolates of *D. septosporum* with triplicate flasks for each condition and time point. The pH of the media for each pH condition (including control) was determined before autoclaving and at three subsequent intervals: after autoclaving but prior to inoculation (0-days), and 5 and 10-days post-inoculation. The following figure shows the mean pH values of the media over three time points.

Figure 14 pH of each liquid media condition at three time points

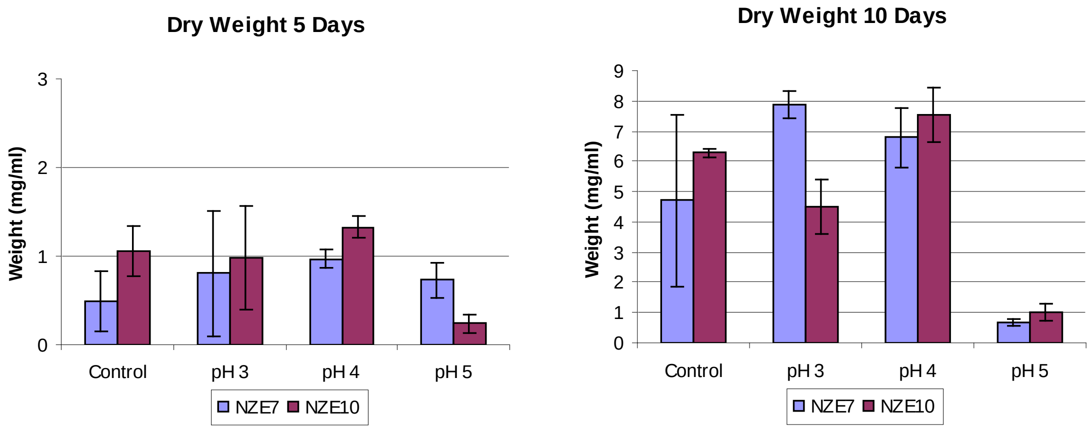


Note: pH determined from pooled media for each time point.

Generally, for both isolates there was very little change in pH over the duration of the experiment. However, NZE7 did show a decrease in the un-buffered control from pH 6.5 at 0-days down to pH 5.8 at 10-days post-inoculation. Conversely, isolate NZE10 showed an increase in pH for the un-buffered control from pH 6.6 at 0-days to pH 7.4 at 10-days post-inoculation. The largest shift in pH was an increase in pH for isolate NZE10 at pH 4.

Mycelial growth was harvested at 5 and 10-days post-inoculation and the dry weight determined as described in Appendix IV. Due to poor growth or no growth at pH 6-7, no dry weight calculations were able to be made for these conditions. One-way ANOVA of the data presented in Figure 15 for NZE7 showed that the difference in growth between pH conditions was non-significant at 5-days but significant ($p<0.01$) at 10-days. The significant difference between the means was compared with Tukeys HSD test at $p<0.05$ (Section 2.9). The only significant difference between the means in respect to growth was between pH 5 and the other three conditions.

Figure 15 Dry weight of mycelia harvested for each growth condition



Results are mean dry weight of three replicate flasks per pH condition at each time point.
Note the differences in y axis scales.

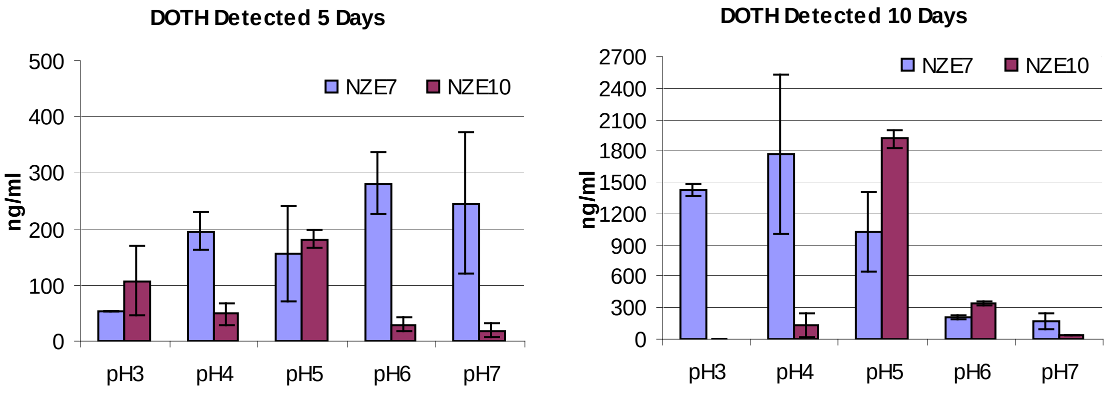
There is overlap between the amounts of dry weight recovered in each pH condition for both time points, for each of the strains, except for the pH 5 condition at 10-days. Results indicate there was no increase of growth from 5-days to 10-days (weight 0.73 and 0.66 mg/ml respectively) for NZE7 at pH 5, but an increase in growth between both time points for NZE10 (weight 0.24 and 1.00 mg/ml respectively). For NZE7, the loss in dry weight recovered at 10-days (pH 5) could possibly be due to the harvesting procedure, larger growth variation between flasks or autolysis of fungal cells. The largest growth increase between 5 and 10-days was 7.05 mg/ml for NZE7 in pH 3, followed by 5.81 mg/ml in pH 4.

For NZE10, one-way ANOVA showed that the growth between pH conditions was significant ($p<0.05$) for both 5 and 10-day time points. The significant difference between the means was compared with Tukeys HSD test at $p<0.05$. The only significant difference (p<0.05) between the means in respect to growth was between pH 5 and control/pH 4 groups at 5-days. However, the difference was significant (p<0.01) for all groups at 10-days with the exception of the control and pH 3/pH 4. The largest growth increase between 5 and 10-days for NZE10 was 6.22 mg/ml at pH 4 followed by 5.22 mg/ml for the un-buffered control.

The variability between isolates in growth is not as pronounced is it may appear by looking at the mean dry weight, considering the overlap in the standard deviation. The only difference in which extremes in weight do not overlap is greater growth for NZE10 at pH 4 (5-days) and NZE7 at pH 5 (5-days). NZE7 growth is greater at pH 3 (10-days), but both isolates follow the same trend of significantly reduced growth at pH 5 for the 10-day time point. In summary both isolates show a significant difference in growth between pH conditions at 10-days, with greatest growth at acidic pH and limited growth at pH 5.

The amount of toxin produced in each flask for each pH condition was determined using an ELISA assay as described in Section 2.7. The results represent the mean of triplicate flask values per condition for each isolate. The calculation of the final result and an example of the standard curve used to generate the results is displayed in Appendix VI. Figure 16 shows the mean toxin level detected for both NZE7 and NZE10 at 5 and 10-days for each pH condition. The control values (un-buffered) at 5-days were 478 and 9366 ng/ml and 10-days 971 and 2530 ng/ml for NZE7 and NZE10 respectively. These values were not placed in the graph as they are considerably higher than those obtained in the buffered pH conditions and distorted the representation of data for the other values. As mentioned, there was minimal growth detected at pH 6-7 for both isolates, but toxin was still detected in the flasks.

Figure 16 Toxin production by isolates NZE7 and NZE10 at 5 and 10 days post-inoculation



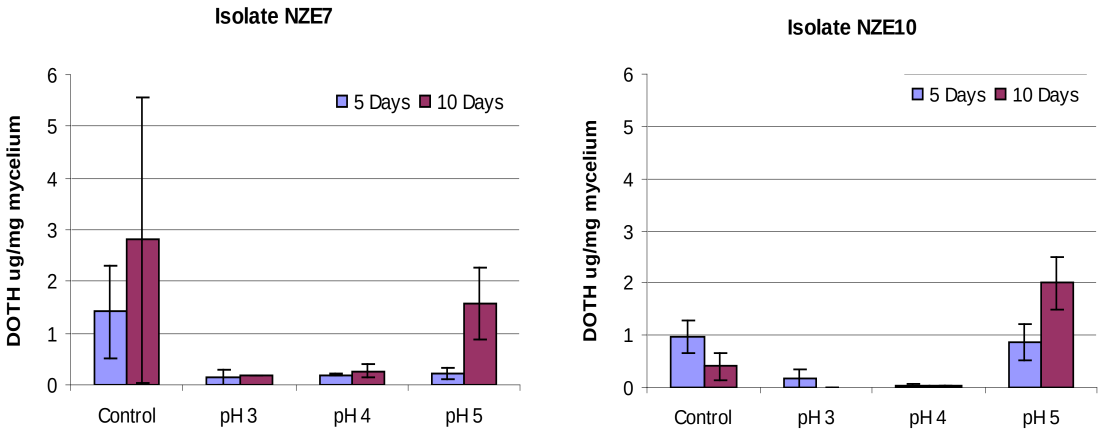
Results are mean toxin value from three replicate flasks per pH condition at each time point.

The difference in the amount of toxin detected between pH conditions (per ml of culture filtrate) is significant ($p<0.01$) at 5 and 10-days for both isolates using a one-way ANOVA. The significant difference between the means for each time point was compared using Tukeys HSD test at $p<0.05$. For NZE7 the only significant difference in toxin detected was between pH 3 and pH 6 at 5-days. For NZE10 at 5-days, there was a significant difference in toxin detected between group pH 5 and pH 4/pH 6-7 in addition to a difference between pH 3 and pH 7. At 10-days for isolate NZE10 the significant difference was between pH 5 and pH 3/pH 7. For both isolates there is considerable overlap between standard deviations at 5-days with the exception of a small amount of toxin detected for NZE7 at pH 3 (52 ng/ml). However the toxin level detected for NZE10 in most pH conditions was lower than what was detected for NZE7. At 10-days, isolate NZE7 produced greater amounts of toxin at more acidic pH as opposed to pH 6-7. Conversely, isolate NZE10 showed increasingly more toxin detected at pH 5 opposed to other pH conditions, and no toxin detected by the ELISA assay at pH 3.

The amount of toxin detected per dry weight of mycelium for each pH condition was calculated to determine a more biologically relevant assessment of the effects of pH on toxin biosynthesis. A one-way ANOVA showed that the results are significant ($p<0.05$) between pH conditions for toxin per dry weight of mycelium for both isolates at 5-days and isolate NZE10 at 10-days (non-significant for NZE7 at 10-days).

Figure 17 shows the amount of toxin produced by dry weight for both isolates over all conditions except pH 6-7 where not enough growth was recovered in order for dry weight to be calculated. Excluding the large variability for the control condition and pH 5 at 10-days, the data represent less than 0.22 µg/mg (toxin per growth). In pH 5 media, there was an increase in toxin produced from 0.22 µg/mg at 5-days to 1.58 µg/mg at 10-days.

Figure 17 DOTH produced per dry weight mycelium

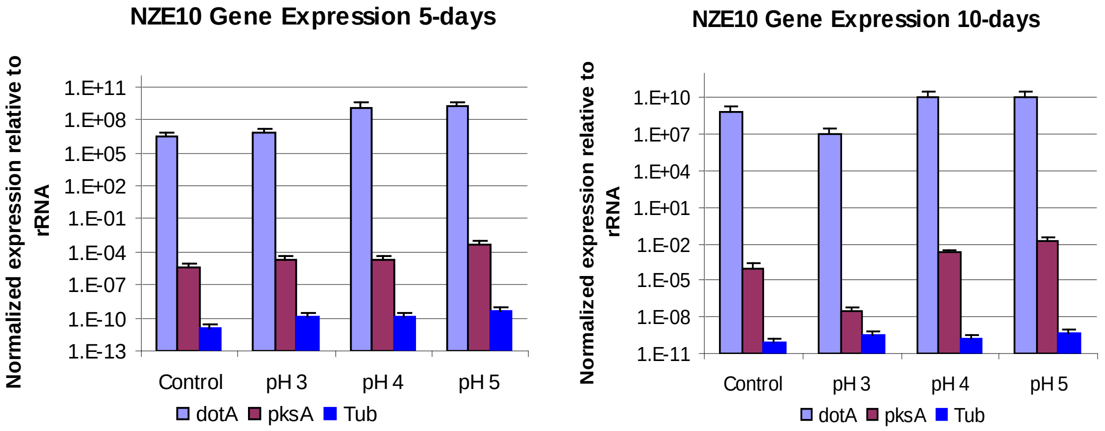


Results are mean of data from three replicate flasks in each pH condition.

For isolate NZE10 the trend in toxin per dry weight was similar to NZE7 despite previous differences across conditions in respect to growth and toxin detected (Figures 15 and 16). A very small amount of toxin was produced per dry weight at pH 4 for both time points (0.04 µg/mg and 0.02 µg/mg respectively), with no calculation of toxin per dry weight for pH 3 at 10-days due to no toxin detected in the ELISA assay. Similarly to NZE7, there was an increase in toxin per dry weight in pH 5 media from 0.85 µg/mg at 5-days to 2.00 µg/mg at 10-days.

The gene expression profile for *pksA*, *dotA* and *tubulin* (control) genes determined by quantitative RT-PCR relative to rRNA is shown in Figure 18. The results are for isolate NZE10 and are the mean of three independent runs, with specificity of the amplified PCR product determined by performing a melting curve analysis (Appendix VII). One-way ANOVA showed that the difference in gene expression across all pH conditions for the genes analyzed was non-significant. Expression of all genes is relatively consistent across pH conditions. No gene expression profile was obtained for pH 6 and 7 media, as there was not enough mycelium available for RNA extraction (Section 2.4).

Figure 18 Normalized ratio of gene expression for putative DOTH genes



Results represent the mean ± SD of three independent RT-PCR runs.
Note: dotA and pksA are dothistromin genes and tub (ß-tubulin) is a control.

In summary, the amount of toxin produced per dry weight of mycelium was similar across media pH with the exception of an increase in toxin per dry weight at pH 5 (10-days) for both isolates, with gene expression relatively uniform across all media pH for NZE10.

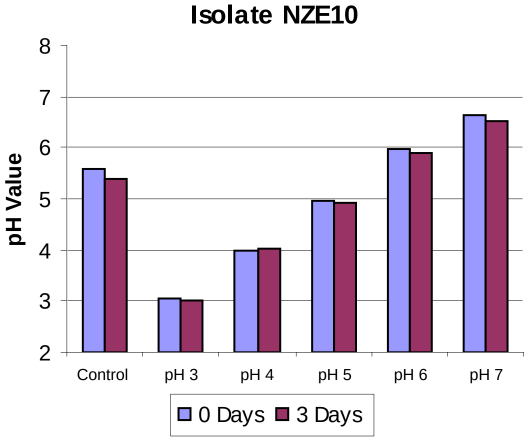
4.2.4 Liquid Media Inoculated with *D. septosporum* Mycelium

This experiment using mycelium to inoculate buffered media at determined pH values was conducted in order to obtain growth data at pH 6-7, which in the previous experiment using spores as inoculum was unsuccessful. In addition, by using mycelium as inoculum, if there is an immediate effect of pH on the fungus’ metabolic processes during the short time period, it may be possible to see an effect on toxin biosynthesis independent of the effect of pH on growth. Price et. al. (2005) used a similar method of inoculating flasks with seed inoculum and looking at secondary growth and toxin production for *Aspergillus parasiticus*. They found that optimal growth conditions at pH 4.5 were also conducive to aflatoxin production, with no growth and toxin produced at pH 8 (Price et al., 2005).

Liquid media buffered with McIlvaine buffer to pH values of 3-7 was prepared as in the previous experiment (Section 4.2.3). The media were inoculated with 7-day old mycelium of NZE10 from a seed flask as described in Section 2.2.6. Growth, toxin production and toxin gene expression were determined at 3-days post-inoculation. There were four replicate flasks per pH condition. The pH of the media was determined before and after autoclaving (0-days) prior to inoculation with mycelium and at 3-days post-inoculation.

Figure 19 shows the pH value of the media for each condition at two time points. As the graph indicates, the pH of the media changed only marginally over the 3-days. The largest change was a drop in pH for the un-buffered control from pH 5.6 to pH 5.4.

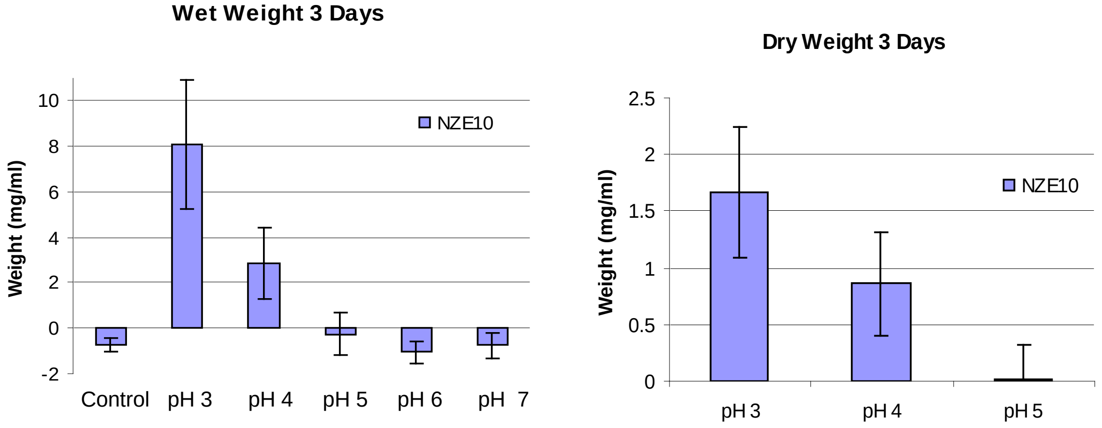
Figure 19 pH reading of each condition at two time points



Note: pH determined from pooled media for each time point.
0 Days = after autoclaving but before inoculation.

Mycelia were harvested 3-days post-inoculation according to the procedure described in Section 2.2.7. For pH conditions 6 and 7, mycelia from four replicate flasks per condition were harvested on the same filter paper to collect enough mycelium for RNA extraction and the average weight per flask determined. Figure 20 shows the mean results for growth (wet weight) and dry weight (DW) of mycelium minus the initial weight of the inoculum, over the 3-day incubation period calculated as described in Appendix V.

Figure 20 Wet and dry weights of NZE10 mycelium harvested at 3-days

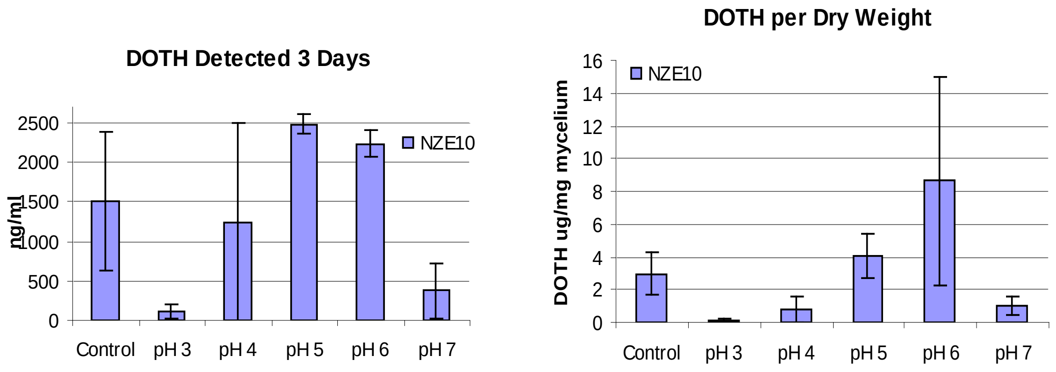


Note: Results are mean of data from four replicate flasks in each pH condition.
Control is un-buffered and inoculated at 0 days.
A negative weight is the difference between the initial inoculums weight and the weight recovered.

One-way ANOVA showed that the difference in wet weight between media pH was significant ($p<0.001$). For the control, pH 6-7 and several of the pH 5 flasks there was a loss of mycelium from the initial weight of the seed flask inoculum (Figure 20). Growth was greatest at acidic pH with loss of growth in the control condition most likely due to the alkaline pH (5.4). One-way ANOVA of the DW shown in Figure 20 showed that the difference in DW between these conditions is significant ($p<0.01$).

Dothistromin levels were determined as before, with the amount of toxin detected and produced per total mg DW (includes initial inoculum) of mycelium for each media pH shown in Figure 21. (calculation Appendix VI).

Figure 21 Toxin detected at 3-days and toxin per dry weight of mycelium



DOTH: mean of data from triplicate samples in the ELISA assay per flask (n=4) per pH condition.

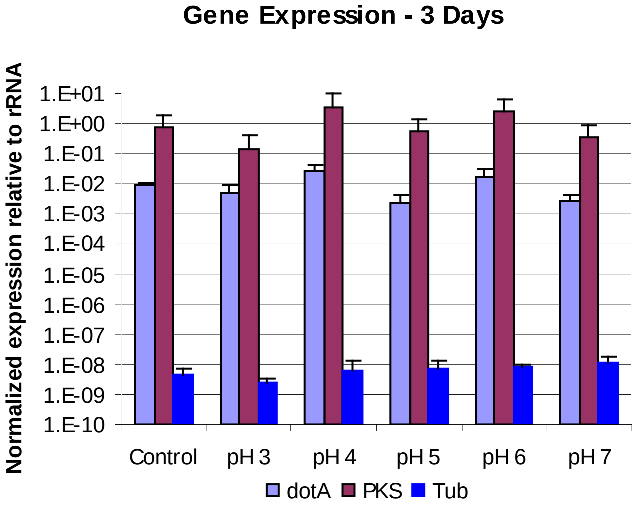
One-way ANOVA showed that difference between the mean toxin (ng/ml) detected for each pH condition was significant ($p<0.001$). The difference between the means was compared using the Tukeys HSD test at $p<0.01$, with a significant difference seen between pH 3 – pH 5-6 and pH 7 – pH 5-6. On average, the most toxin was detected in pH 5-6 media with 2480 and 2239 ng/ml detected respectively. In contrast, only 118 and 380 ng/ml of toxin was detected for both pH 3 and pH 7 conditions respectively with large variability in toxin detected in the control and pH 4 media.

One-way ANOVA showed that the difference between the amount of toxin produced per mg DW is significant at $p<0.01$ (Figure 21). Using Tukeys HSD test, the difference between the pH conditions was significant for pH 6 – pH 3/pH 4/pH 7. The results in Figure 21 show higher levels of dothistromin per mg DW for the control, and pH 5-6 compared to pH 3-4 and 7. Interestingly for the control, and pH 5-6 there was minimal growth, as seen in Figure 20. In contrast, low levels of dothistromin per mg DW correlate with greater growth, as seen for the pH 3-4 conditions.

In order to further determine if there is any effect associated with ambient pH on metabolic processes, the relative expression levels of two putative toxin biosynthesis genes, *pksA* and *dotA* (and ß-*tubulin* control) were determined for each condition using real-time PCR (Section 2.5.3). If there was an association between the reproduction of toxin and pH, then it would be expected that associated gene transcripts would show a similar pattern as toxin production for each pH condition.

The gene expression profiles for *pksA*, *dotA* and ß-*tubulin* (control) relative to rRNA are shown in Figure 22. The results are the mean of three independent runs, with specificity of the amplified PCR product determined by performing a melting curve analysis. ANOVA showed that the difference in gene expression across pH conditions for the *dotA* gene was significant ($p<0.01$), with results for the *pksA* and ß-*tubulin* genes non-significant. The significant difference between the means was compared with Tukeys HSD test at $p<0.05$. The only significant difference was for *dotA* gene expression between the following groups, pH 4 - pH 3/pH 5/pH 7. Comparison of all three genes shows a higher level of expression for the *pks*A gene than for other genes, with a similar pattern of gene expression found for both *dotA* and *pksA* genes. Expression of the ß-*tubulin* control gene appears to be uniform across all pH conditions as would be expected due to no effect of pH on global gene expression.

Figure 22 Expression of DOTH genes in different pH

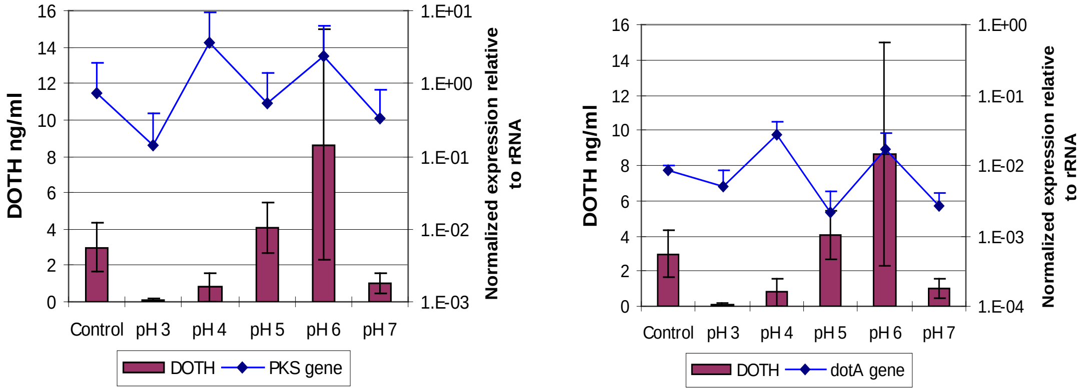


Results represent the mean of three independent RT-PCR runs.

In respect to the toxin genes, both *pksA* and *dotA* show the highest expression at pH 4, followed closely by pH 6. The lowest normalized ratio was 1.44 x 10^{-1} for the *pksA* gene at pH 3 and 2.18 x 10^{-3} for the *dotA* gene at pH 5.

There is considerable variation between fungal species at which point relative to growth toxin biosynthesis commences. To determine whether a trend in putative toxin gene expression exists relative to the amount of toxin produced per dry weight of mycelium, the data for each pH condition from Figures 21 and 22 were contrasted against each other and the results displayed in Figure 23. In interpreting these data, the assumption was made that the fungus is in a dynamic state in respect to physiological processes (ie. toxin levels are not simply an accumulation of toxin produced, but represent production and degradation).

Figure 23 Relative gene expression compared to DOTH per total mycelium dry weight



For both genes there does not appear to be a correlation between high levels of gene expression and high levels of toxin. The highest level of gene expression for both genes coincides with one of the lowest levels of toxin per mg DW as seen at pH 4. Lower levels of gene expression are seen for the pH 3 and 7 conditions for both genes, coinciding with low levels of toxin per mg DW. However, at pH 5 a lower level of gene expression for both genes is seen when there is a relatively median level of toxin per mg DW, and conversely at pH 6 there is a greater level of gene expression which correlates with a higher level of toxin per mg DW. A correlation between *D. septosporum* gene expression and dothistromin has been seen in un-buffered media between three and 15 days (Schwelm unpublished).

4.3 Discussion

4.3.1 Colony Growth and Morphology on Different Media

D. septosporum is a slow growing fungus, and is inherently unstable under laboratory conditions. The morphological appearance of the fungus often changes, with sectors often seen in colonies, and frequent whole colony morphology changes when sub-cultured. These changes often include alterations of mycotoxin production and the ability to produce conidia in culture. The presence of sectors within a colony has been documented for many fungal species, and is often associated with conditions being unfavourable for growth (Xiao and Sitton, 2004) or with attenuation of the fungal colony. Within this experiment there was however also between-isolate variation in morphology, growth rate and levels of toxin produced. This in itself is interesting as *D. septosporum* has been shown to have very low genetic diversity, with the NZ strains proposed to be clonally derived (Hirst, 1999).

The growth analysis determined that *D. septosporum* does have some nutritional preference for which media it will grow on. It is not uncommon for carbon and nitrogen sources to affect physical characteristics of fungi (Daza et al., 2006). However, analysis of the daily growth rate of the four different media used in this experiment did not highlight a single medium that would be more beneficial in increasing daily growth rate.

Although differences in colony morphology for *D. septosporum* have been mentioned in previous research (Hirst, 1999; Barnes et al., 2004b), there are several morphologies apparent on certain media which have not been described before. The slimy apricot/pink appearance of the colony on PDA + N and PDA + $NaNO_3$ is of interest. The colony initially appeared to be contaminated with a yeast or bacterium species or to be producing a type of slimy ooze. However, when sub-cultured the colony reverted back to the common *D. septosporum* colony morphology usually seen. Therefore, it is possible that some differences in colony morphology may be related to carbon or nitrogen source as is seen with other fungal species such as *P. pyri* (Xiao and Sitton, 2004) and *S. pyriputrescens* (Kim et al., 2005). To confirm this theory, further research on colony morphology in response to various carbon and nitrogen sources will need to be conducted.

4.3.2 How Does the pH of Solid Media Affect Growth and Sporulation?

The existence of a pH homeostatic mechanism has been determined for several fungal species which allows the fungus to regulate synthesis of extracellular enzymes and proteins within a pH range that ensures they function effectively (Caddick et al., 1986; Bignell et al., 2005). Therefore, pH as an environmental factor will likely have a role in biological processes such as growth and sporulation. Many fungal species have been documented to have preferential growth within the acidic pH range (Xiao and Sitton, 2004). It would therefore seem likely that *D. septosporum*, whose host tissue is acidic, would preferentially grow at more acidic pH.

The results did indicate that *D. septosporum* growth was greatest at acidic pH as opposed to alkaline pH. Unfortunately, the pH of solid media could not be buffered below pH 5, but higher radial growth rates were obtained at pH 5 than at pH 6-7. It could be postulated that growth would be enhanced at more acidic pH according to the current observations. As seen for the fungal species *S. pyriputrescens* (Kim et al., 2005) and *P. pyri* (Xiao and Sitton, 2004), *D. septosporum* showed a decline in growth at pH 6 with minimal or no growth at pH 7. There may be several reasons as to why growth was stunted at higher pH. Possibly higher pH reduces the ability of the fungus to uptake nutrients or substances essential for growth (O'Callaghan et al., 2006), or maybe the ability of the fungus to acidify the medium, as seen with *Aspergillus tubingensis* (Krishna et al., 2005) was inhibited due to the presence of a buffer in the medium. Additionally, the effect of pH may be dependent on the composition of the medium (Buchanan and Ayres, 1975).

Sporulation is another biological process that can be affected by environmental conditions such as nutrient availability and ambient pH. Both NZE7 and NZE10 produced more spores at pH 5 than pH 6-7. However, the only medium that enhanced the production of spores was YES agar, particularly for isolate NZE10. Interestingly, no spores were recovered from colonies grown at pH 6 on this medium for this isolate. Perhaps this is an indication of a nutritional preference, in addition to inability to acquire nutrients at alkaline pH. Further analysis of nutrient preferences for growth and sporulation, in addition to a buffer system that allows solidification of media below pH 5 would therefore be beneficial for optimizing laboratory conditions.

4.3.3 Inoculation of Conidia Into Liquid Medium at Adjusted pH

Fungi are capable of producing various compounds which help acidify the medium (Krishna et al., 2005) to within a range that is optimal for physiological processes such as growth, sporulation and toxin production. In buffered media inoculated with *D. septosporum* conidia, the pH did not significantly change over the duration of the experiment. This inability to alter pH or acidify the medium may be one reason as to why no growth was seen in the pH 6-7 conditions. For *D. septosporum* high pH is unsuitable for growth, with greatest growth at acidic pH.

For many fungi that produce mycotoxins, the biosynthesis of toxin is normally associated with cell differentiation and/or conidial development. Often the production of toxin occurs at the end of the exponential growth phase (Calvo et al., 2002), and toxins such as aflatoxin have been shown to be non-essential for growth (Ehrlich et al., 2005). However, for many species the interaction between differentiation, toxin biosynthesis and the environment is not clearly understood. When comparing results of the toxin analysis between *D. septosporum* isolates there is a noteworthy difference: (Figure 16), NZE7 produces similar levels of toxin over the pH 3-7 range at five days in contrast to NZE10 producing a lower level of toxin at higher pH 6-7. At 10 days post inoculation there is a considerable difference in toxin detected. NZE7 appears to produce more toxin at acidic pH, whilst NZE10 produces the greatest amount of toxin at pH 5 with low levels detected either side of this pH. The difference in toxin detected between isolates however does not necessarily represent differences in ability to respond to environmental conditions. When the toxin data is related to the biomass of growth for both isolates, the trend appears to be very similar as can be seen in Figure 17. For both isolates the amount of toxin produced is greatest per biomass of growth in pH 5.

Studies with *Aspergillus ochraceus*, *A. nidulans* and *A. parasiticus* have shown that larger amounts of toxin are produced at more acidic pH and lower amounts at higher pH, which correlates with growth (Calvo et al., 2002; O'Callaghan et al., 2006). Research with *A. parasiticus* also concluded that toxin production depends on the composition of the media (Calvo et al., 2002) or that buffering media causes a decrease in aflatoxin and sterigmatocystin production with increasing pH (Keller et al., 1997). Furthermore, it is possible that dothistromin production is not correlated with the end of the exponential growth phase. Unpublished data from this laboratory suggest that the mycotoxin dothistromin, supported by associated toxin gene expression data, is expressed at higher levels at the onset of the exponential growth phase around 2-3 days post inoculation. In this experiment, data only represents sampling from two time points, so it is possible that the isolates were also in different stages of growth in the different media. Also since dothistromin is degraded, possibly the toxin has been oxidized or reduced by light (Franich et al., 1986). A similar situation was found for *A. flavus* with AFB_1 reduced to AFB_1 hemiacetal at five days (Cotty, 1988). A further explanation is the role of phosphate based buffers. It has been proposed by one researcher that increasing phosphate concentration increases toxin production, whilst lower phosphate concentrations increase growth as seen with *Pyrenophora teres* and the phytotoxin aspergillomarasmine A (Weiergang et al., 2002). Again this could partially explain greater growth at acidic pH and high levels of toxin towards alkaline pH.

Due to the growth and toxin data for both *D. septosporum* isolates not conclusively indicating a significant relationship between pH and fungal response, the next obvious step was to assess if pH affected gene expression. The two genes, *pksA* and *dotA* were chosen as they are both well characterized and their predicted roles in the synthesis of dothistromin have been determined (Bradshaw et al., 2002; Bradshaw et al., 2006). The gene expression data as seen in Figure 18 for NZE10 showed there was no significant difference between pH conditions in respect to the expression of these genes. When contrasting the gene expression data against the toxin detected, it would have been plausible to assume there should have been an increase in gene expression for the pH 5 conditions at both time points, considering the toxin levels were highest in this condition, but this was not seen.

There are multiple additional possibilities to explain the pattern of growth, toxin and gene expression seen for *D. septosporum* in response to ambient pH. Some possible explanations are as follows:

- Toxin is produced until the carbon source is exhausted (Shaw, 1975).
- The rate of carbon acquisition may depend on pH (Caddick et al., 1986).
- Lack of growth at pH 6-7 due to autolysis of fungal cells in nutrient limited environment, possibly releasing dothistromin.

- Gene expression not representative of toxin detected, as toxin inactivated at different rates under different pH conditions.

In light of all the above mentioned possibilities, a further experiment was conducted to determine the response of mycelium as inoculum to ambient pH. It was expected that early onset of toxin gene expression would be detected at three days post inoculation, and hoped that sufficient mycelium would be recovered at pH 6 and 7 for further analysis due to the use of seed mycelium.

4.3.4 Analysis of Mycelium as Inoculum in pH Adjusted Liquid Medium

Similarly to the results obtained with spores as inoculum, the pH of the media did not change significantly over the three day period. The dry weight of mycelium recovered is representative of the same trend seen with spores as inoculum. The greatest growth being at acidic pH, with the amount of mycelium recovered at alkaline pH (pH 6-7) less than what the flasks were initially inoculated with (Figure 20). The loss of mycelium in culture over the three day period may be due to autolysis of the fungal cells as mentioned in the previous section.

The level of toxin detected for isolate NZE10 (Figure 21) shows a similar trend to toxin detected when conidia were used as inoculum (Figure 16). The toxin level was low at acidic pH, with an increase at higher pH. In considering the amount of toxin produced per biomass, it appears that toxin produced per biomass at pH 5 and 6 is greatest (Figure 21), with low levels of toxin per biomass at acidic pH, similar to data obtained for both NZE7 and NZE10 (Figure 17). The differences in toxin seen across pH conditions may due to multiple reasons, besides a direct influence of pH on toxin production. As growth is favourable at acidic pH, the crucial onset of toxin expression may have been missed and occurred prior to the three day sampling period. Conversely, the toxin may have been rapidly degraded at acidic pH, hence the lower level detected, and the fungus may still be in the exponential growth phase not requiring toxin production. For the more alkaline conditions (pH 5-6), where toxin level is greatest, *D. septosporum* may have difficulty growing under these conditions and hence has not been able to reach the exponential growth phase. Therefore, there is little mycelium biomass, and the toxin level per biomass will be greater. Possibly the response of producing more toxin at higher pH may be a stress response, due to inability to reach the exponential growth phase. Finally for pH 7, little toxin is detected in this condition for both conidia and mycelium as inoculum experiments (Figure 16 and 21), and it is likely this condition is inhibitory for growth with toxin detected representing what has been released by autolysis of fungal cells, or due to expression of toxin from a few surviving cells.

Therefore the gene expression profile for both *pksA* and *dotA* genes was of considerable interest to determine whether the differences seen in toxin levels could be related to response to ambient pH. The expression profile for *dotA* showed a significant pH effect. Expression tended to be unexpectedly higher at pH 4, compared to the other conditions for both genes. When contrasting gene expression against toxin produced per DW (Figure 23), the higher level of *dotA* expression at pH 4 was not correlated to toxin per DW. As mentioned previously, there is possible degradation of dothistromin at acidic pH, as opposed to higher pH, where higher levels of *dotA* gene expression would correlate with increase in toxin per DW as seen. The degradation rate of dothistromin at different pH values has not been determined as yet. There was a lower level of toxin per dry weight for pH 3, 5 and 7, compared to pH 6, and this also follows the trend in lower gene expression for *dotA* found in these conditions. Although gene expression levels for the *pksA* gene are non-significant, looking at Figure 22, both genes do follow a similar trend in expression over the pH conditions. The *pksA* gene product is required at the beginning of the dothistromin biosynthetic pathway (Bradshaw et al., 2006). Perhaps it may be beneficial to thus access *pksA* gene expression earlier than three days post-inoculation if toxin expression peaks prior to exponential growth.

Results are thus far speculative, based on either earlier or later onset of toxin production in relation to growth. Research with the necrotrophic fungus *S. sclerotiorum*, in a similar experiment as the one conducted here, showed variation of expression in the *pg1-3* genes which encode endoPG isozymes in response to ambient pH. Expression was low at pH 3 and 6, but increased at pH 4 and 5, with decrease in gene expression over time (Cotton et al., 2003). Interestingly, experiments with a North American isolate of *A. flavus* showed that changes in AF production in response to pH was not correlated with changes in *aflR* mRNA levels (AflR being the transcriptional regulator of AF biosynthesis genes) (Ehrlich et al., 2005). Conversely, research with *A. ochraceus*, showed a strong correlation between expression of the *pksA* gene and OTA production at various pH (O'Callaghan et al., 2006). It therefore appears obvious that within and between fungal species there can be considerable differences in physiological response to conditions such as ambient pH in respect to growth, toxin secretion and mRNA expression. It is possible that pH at some level may exert some form of transcriptional regulation over *dotA* gene expression due to the significance of the results found. Possibly this and other toxin biosynthesis genes could be regulated by the PacC transcription factor. Analyses of the toxin genes from the putative dothistromin gene cluster have shown possible PacC binding motifs (Zhang, personal communication). A brief search in our laboratory for a *D. septosporum pacC* homolog, using degenerative PCR, as was done for *F. oxysporum* (Caracuel et al., 2003a), so far has not identified this gene. However, further investigation into the role of nutrients in media, onset of growth stages, onset of toxin biosynthesis, and what toxin biosynthetic genes are correlated with detected toxin production in media, will enhance understanding of the potential role ambient pH may play, if any in regulating physiological processes.

In summary, over a range of pH media (spores or mycelium as inoculum) the expression of dothistromin is higher at pH 5-6 than at pH 3-4, although the growth at pH 5-6 is less than pH 3-4. Additionally, the dot gene expression did not follow the same trend as toxin production, therefore indicating there is no clear evidence for pH control. However, there are multiple complicating factors which may affect growth, toxin production and *dot* gene expression, which the above experiments can not discriminate between. Generally, there are growth differences in different pH media, and it is known that growth-stage affects dothistromin production (Personal communication, (Shaw, 1975)). Additionally, we also know that dothistromin is degraded in culture (Franich et al., 1986), but the ELISA assay used looked at steady-state levels of toxin, not production of toxin. The rates of production and degradation of dothistromin in culture are not known, therefore confounding the possibility of correlating gene expression with toxin levels detected. Further research could look at the rate of degradation of toxin in pH media, in addition to establishing whether there is a difference in the time of onset of toxin production across pH. It would also be beneficial to analyze *dot* gene expression earlier than three days post-inoculation to determine at what time point prior to toxin expression *dot* gene expression becomes up-regulated, if at all. Lastly, the whole experiment needs to be repeated to validate these results, as gene expression at pH 4 seems to be an outlier in the trend seen at other pH values, in addition to the large standard deviations.

5

Green Fluorescent Protein as a Marker to Monitor Infection

5.1 Introduction

Previous research with dothistroma has been useful in determining the environmental conditions conducive to disease, the percentage of penetrating spores and the incidence of disease. However reports of the mode of penetration and formation of penetration structures have been conflicting (Muir and Cobb, 2005). Other research has determined that the mycotoxin dothistromin induces a red-band within needle tissue when injected directly, a symptom of disease (Shain and Franich, 1981). However the putative role of this toxin in causing disease is unknown. For some fungi, secondary metabolites such as this are dispensable for causing disease and only offer some form of selective advantage, perhaps within their host niche or to gain nutrients from the host (Howlett, 2006). Recent advances in the use of green fluorescent protein (GFP) as a reporter and marker gene in fungi, either under the control of a constitutive or inducible promoter, have enabled researchers to follow toxin gene expression patterns and development of fungal pathogens on and in their hosts (Kaufman et al., 2004).

Microscopy is the general form of analysis used to examine behaviour of *D. septosporum* on its natural host. Previous research has employed the use of multiple types of microscopy, ranging from scanning electron, transmitted light, confocal and bright field (Franich, 1983; Jones et al., 1995; Muir and Cobb, 2005), with all methods requiring preparation, fixation and adequate staining. Recently within this laboratory, eGFP-expressing isolates of *D. septosporum* wild type (NZE7) and gene replacement mutants (*dotA* and *pksA*) were developed. The *dotA* and *pksA* mutants have previously been verified to be defective in the biosynthesis of dothistromin (Bradshaw et al., 2002; Bradshaw et al., 2006). The *dotA* mutant accumulates versicolorin intermediates and is involved in a late step of toxin biosynthesis, while the *pksA* mutant is predicted to be involved in a condensation reaction in the early stages of dothistromin biosynthesis (Bradshaw et al., 2006). Therefore, the ability to visualise the mutants behaviour compared to wild type behaviour on pine needles using fluorescent microscopy, may aid in determination of the importance of dothistromin in the disease process.

The role of toxins in disease has been determined for a number of fungal species using visual comparative analysis between toxin mutants and wild type strains. Research with *Leptosphaeria maculans*, mutants deficient in sirodesmin PL production, showed the mutants were capable of causing lesions on canola similar in size to the wild type, with the exception that the mutants colonised canola stem tissue less effectively (Howlett, 2006). Discrepancies in the role of botrydial produced by *Botrytis cinerea* as a virulence factor were determined, as several mutants were still able to infect several different plant types comparative to the wild type while other mutants were not (Howlett, 2006). Additionally, disruption to genes for biosynthesis of secondary metabolites, such as the host-specific toxins produced by *Alternaria alternata* lead to a change in host range and altered the pathogenicity of this fungus (Ito et al., 2004). Furthermore, toxin production by certain fungal species may be induced by signals or substrates derived from the host plant. This is seen with certain *Aspergillus* species which derive acetyl CoA from the fatty acids in maize kernels to facilitate the biosynthesis of sterigmatocystin and aflatoxin (Maggio-Hall et al., 2005). An alternative example is the release of AB-toxin from *A. brassicicola* spores upon germination on brassica plants. The production of AB-toxin does not occur on non-host plants (Oka et al., 2005). Therefore, the ability to monitor GFP *D. septosporum* mutant behaviour on pine material may elucidate differences in germination and penetration behaviour compared to the wild type which may affect the potential to cause disease.

D. septosporum transformed with GFP has not been used in a pathogen assay prior to this research. Therefore the use of these isolates will enhance the understanding of the stages of infection without the need to modify or damage the pathogen-host interaction, therefore allowing accurate and reliable analysis. The use of GFP to follow stages of infection has successfully been used to study the interaction of *Cochliobolus heterostrophus* and maize (Maor et al., 1998), *Trichophyton mentagrophytes* and skin (Kaufman et al., 2004), and *Stagonospora nodorum* and wheat (Solomon et al., 2006). In all of these examples, germination, hyphal growth across host surfaces, and detection of the fungus within the host tissue was observed. Furthermore, GFP did not affect morphology, growth rate, pigmentation or virulence of spores (Maor et al., 1998; Kaufman et al., 2004; Solomon et al., 2006).

The aim of this work was to monitor the mode of infection of pine needles by GFP-expressing *D. septosporum* isolates. It was anticipated that the availability of both wild type and mutant GFP isolates would allow a comparative analysis of differences (if any) in behaviour of the fungal isolates inoculated onto plant material. However at the outset it was realised that there are some limitations of this system. The onset of toxin biosynthesis in most fungal species is correlated with a change in physiological state (Price et al., 2005), and it is not known if dothistromin is correlated with sporulation or germination. Therefore, it is possible that spores of dothistromin-deficient mutants may not germinate on the needle surface. Additionally, it is also possible that the behaviour observed under laboratory conditions may not be representative of the infection process that occurs in a natural environment (Muir and Cobb, 2005). The density of pine needle tissue may also affect the ability to detect a fluorescent signal if the fungus is growing intracellular within the needle, and it has been noted by several researchers that mycelial growth disappears from the needle surface once infection has proceeded (Gadgil, 1967). The length of time the GFP isolates will be able to be detected post-inoculation is unknown. Therefore, at a later stage confocal microscopy may be a more suitable option to look at growth within the needle tissue and perhaps quantification of fungal biomass.

5.2 Results

5.2.1 Conidia Germination

D. septosporum conidia germinate on the needle surface, eventually producing an infection peg which subsequently enters a stomatal opening on the pine needle. Confirmation that the two eGFP expressing isolates (GFP-19 and GFP38) were able to germinate at a comparative rate to the wild-type isolates was required. The time taken for the majority of conidia to germinate may be an indication of the length of time required for germination on the needle surface. Research by Gadgil (1967), showed cultured conidia and conidia derived from stromata inoculated onto needles behaved similarly, with 70% of conidia germinating by 48 h post-inoculation. Cultured conidia were harvested and monitored at 12 h intervals as described in Section 2.2.4. The results are shown in Table 13, with germination determined until 72 h.

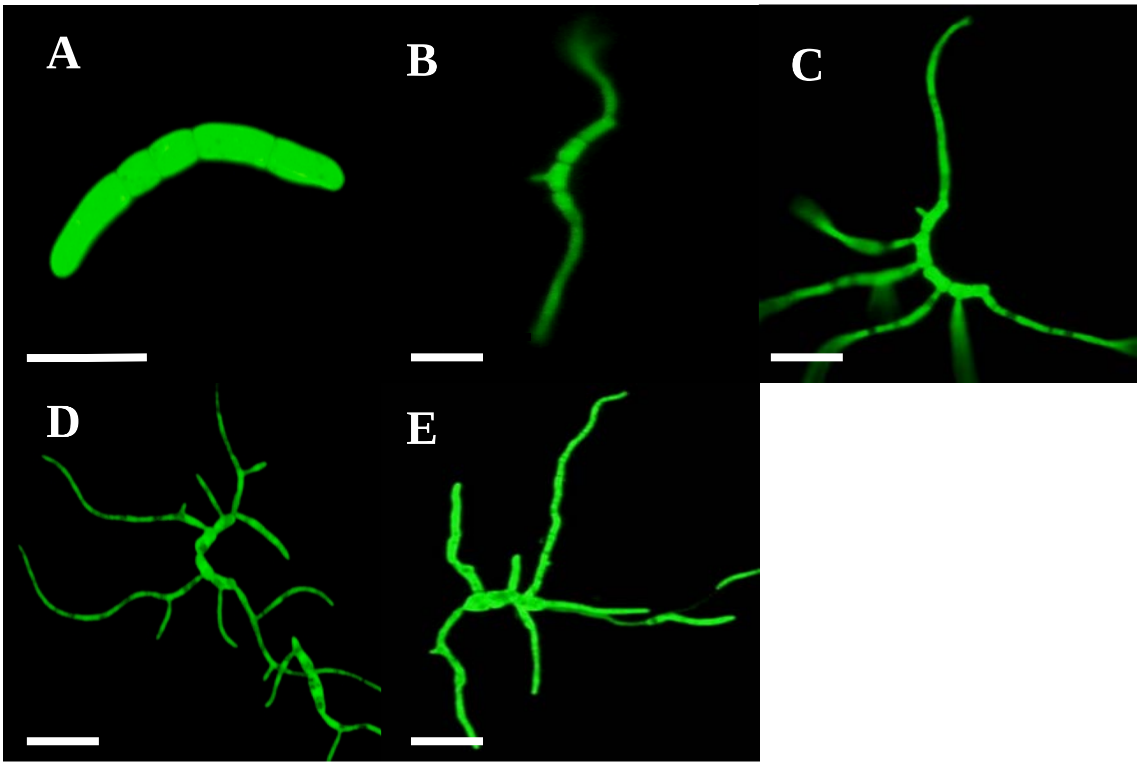
Table 13 Rate of conidia germination for GFP-expressing and wild-type isolates

	Germination		
Time (h)	**GFP-19 (%)**	**GFP-38 (%)**	**NZE7 (%)**
12	15	19	24
24	30	49	53
36	52	73	71
48	65	81	86
60	70	84	91
72	76	73	87

Data are the percentage (of 100 conidia) germinated at each time interval.

For all isolates it was observed that most conidia germinated initially from the terminal cells of the conidia followed by germ tubes from the median cells. Typically most conidia were four celled with one germ tube arising from each cell; however a small number of conidia had multiple germ tubes arising from median cells. At 36 h, over 50% of the spores had germinated for all isolates (Table 13) and indicate a comparable rate of germination between the GFP isolates and NZE7. Figure 24 represents the appearance of germinating spores at different time points for isolate GFP-38.

Figure 24 Progression of germination from 0-60 h for isolate GFP-38



A = 12 hrs, B = 24 hrs, C= 36 hrs, D = 48 hrs and E = 60 hrs
Note: Photographs taken at 400x magnification (Size bars = 4µm).

Visually it was determined that germ tubes from terminal cells were longer (Figure 24 B and C) than germ tubes from median cells up until 48 h (Figure 24 D). From 48 h the germ tubes from both terminal and median cells showed comparable growth, with branching seen from germ tubes protruding from both conidia cell positions.

5.2.2 Effect of pH on Conidia Germination

As results in Chapter 4 indicated that growth is preferential at acidic pH, and naturally conidia are in an acidic environment on the pine needle surface, it was decided to test whether pH would have an effect on the rate of conidia germination. Cultured conidia of GFP-38 were harvested and monitored at 12 h intervals as described in Section 2.2.4, with results shown in Table 14.

Table 14. Percentage of conidia germinated at 12 h intervals at varying pH

	Germination			
	12 hr (%)	**24 hr (%)**	**36 hr (%)**	**48 hr (%)**
pH 3	50	86	95	91
pH 4	27	45	62	65
pH 5	34	41	63	71
pH 6	31	34	45	60
pH 7	15	30	60	47
pH 8	25	25	35	55
pH 9	22	26	30	45
pH 10	24	29	26	30

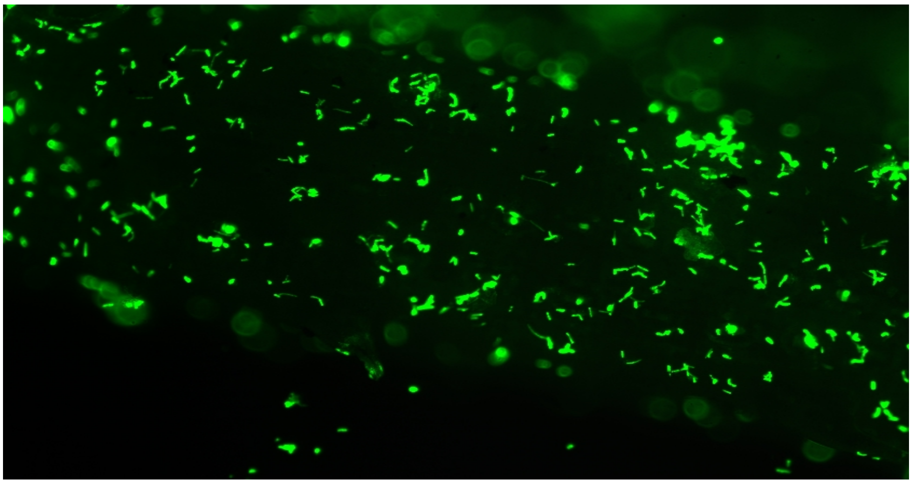
Data are the percentage (of 100 conidia) germinated at each time interval for each pH condition.

The results indicate that spore germination is initiated sooner, with 86% of conidia germinated at pH 3, compared with 45% or less at other pH conditions. In alkaline pH, 50% or less of all conidia germinated by 48 h compared to over 60% or more at lower pH values. At the higher pH values, the rate of conidia germination was slower between time points than at acidic pHs.

5.2.3 Survival and Distribution of GFP Conidia on Pine Needles

The GFP-38 isolate was used to monitor conidia behaviour on the needle surface post-inoculation. Preliminary observation determined that GFP-38 glowed more brightly on the needle surface under UV light than GFP-19, possibly due to having a greater number of copies of the eGFP gene. The GFP-38 isolate was still seen glowing six weeks post-inoculation on pine needles. Seedlings were typically inoculated with a rate of 1 x 10^6 conidia/ml (Section 2.8.5), and Figure 25 shows part of a pine needle directly after inoculation with isolate GFP-38.

Figure 25 *D. septosporum* GFP-38 inoculum on pine needle surface



Microscope photograph at 100x magnification.

As evident in Figure 25, there was a relatively even dispersal of conidia along the length of the needle surface. In the initial stages of the pathogen assay development, there had been a problem with even dispersal of conidia. If inoculated seedlings were placed in the assay chamber with continuous moisture directly after inoculation, over half the inoculum would accumulate at the base of the needle attached to the stem. It is not known if *D. septosporum* conidia excrete an adhesive which allows attachment of the conidia to the needle surface. Possibly as a wet inoculum was sprayed onto the seedlings with continuous moisture present in the chamber, and the angle of the pine needles, the conidia slid down the length of the needle to accumulate at the base. Therefore plant material was kept without continuous moisture for 48 h after inoculation, to facilitate the attachment of inoculum to the needle surface. This ensured a relatively even dispersal of conidia along the length of the needle. After 48 h, continuous moisture was maintained around the plant material (Section 2.8.1).

5.2.4 Germination of *D. septosporum* Conidia on Radiata Pine Needles

It is essential for *D. septosporum* conidia to germinate on the host tissue and produce a penetration peg for infection to proceed. As the time frame in which the majority of conidia germinated in distilled water after harvesting was previously determined for GFP-19 and 38 (Section 5.2.1), an analysis of the number of conidia which germinated on the needle tissue was conducted. Only conidia which glowed under UV light were included in this analysis. It had previously been found that once the conidia land on a susceptible host, germination takes place within three days (Bulman et al., 2004). Table 15 shows the average number of conidia germinating on the needle surface for both GFP-19 and 38 up to three days post-inoculation (Appendix VIII).

Table 15. Germination of *D. septosporum* conidia on the surface of *P. radiata* needles at these times after inoculation

	Conidia/needle[a]		Germinating conidia/needle[b]			
Time	**GFP-38**	**GFP-19**	**GFP-38**	**Mean (%)**	**GFP-19**	**Mean (%)**
0 hours	12192 ± 1711	2185 ± 581	0		0	
24 hours	10547 ± 1380	4186 ± 1264	1056 ± 164	12 ± 4	272 ± 143	7 ± 2
48 hours	14469 ± 1995	4911 ± 1280	2339 ± 327	14 ± 2	338 ± 154	5 ± 5

a Value is mean of conidia/needle (n=6 needles), as calculated in Appendix VIII
b Value is mean of germinating conidia/needle (n=3 needles) as calculated in Appendix VIII
± = SD

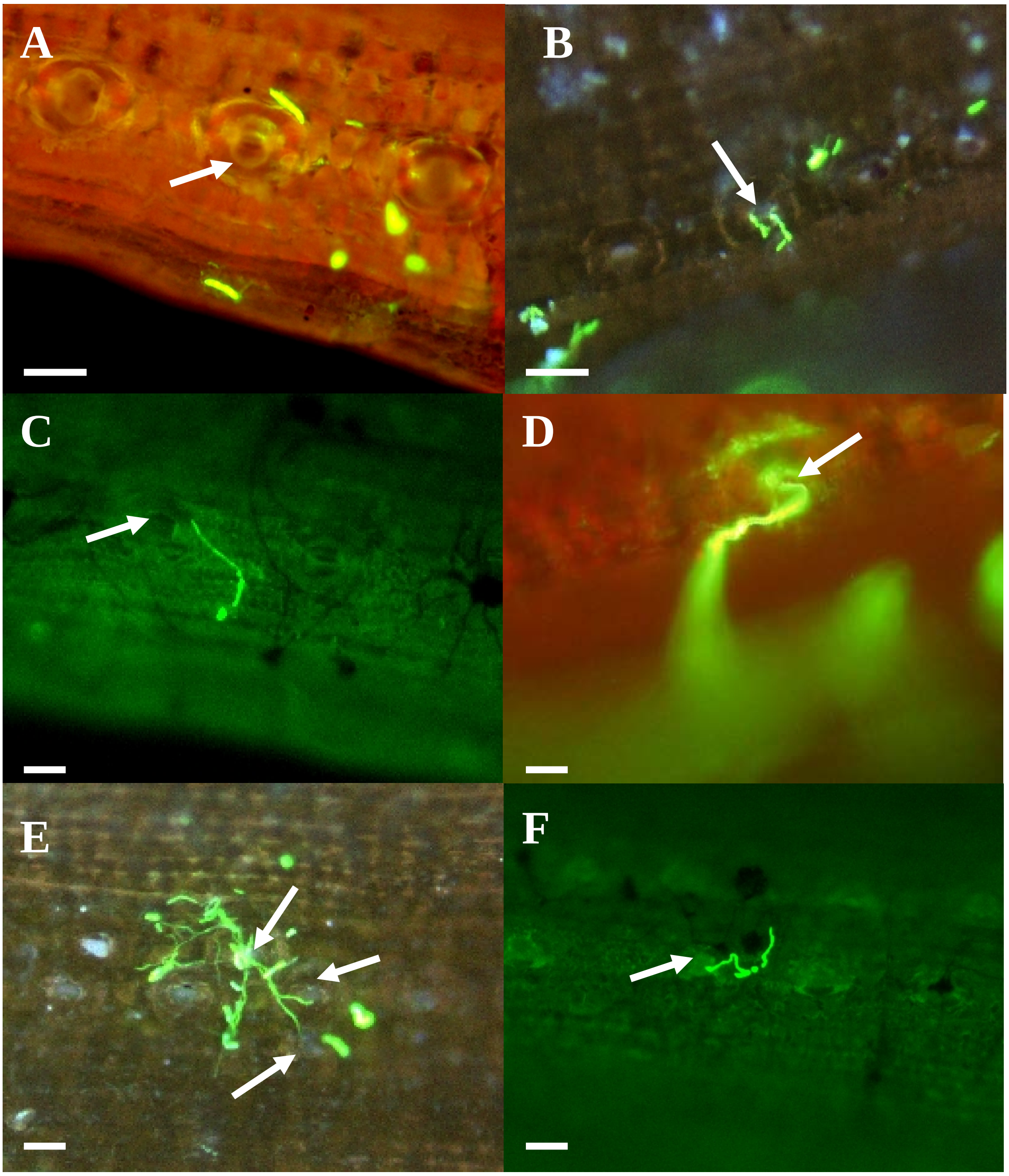
The same inoculum concentration was used for isolates GFP-19 and 38. However between these isolates there was on average at least a two-fold difference in the number of conidia on the needle surface, and at least a three-fold increase in the number of conidia germinating on the needle surface for GFP-38 compared to GFP-19. It is unclear if GFP-19 had subsequently died, whether conidia disappeared upon inoculation onto the plant material, or whether the isolate may not have been detected due to lower GFP expression. The results do indicate that the number of conidia which germinate within three days post-inoculation is very small compared to the inoculum load on the needle surface, and very low compared to germination in distilled water (Section 5.2.1).

5.2.5 Characterizing the Initiation of the Infection Process

The use of eGFP-expressing isolates of *D. septosporum* allows visualization of fungal behaviour on plant material without disrupting the pathogen-host interaction. Quelching of the GFP fluorescent signal and disruption of plant cellular tissue does occur at magnifications above 400x, and prolonged exposure destroys the sample. An additional problem with pine material is the topography of the needle. As the abaxial surface of the needle is curved, this prevents the total area of the needle being viewed in focus under the microscope objective.

Most infection of needle tissue is through penetration of the stomata pores on the pine needle, with appressoria formation reported over the stomata opening (Gadgil, 1967). The needle stomata, under the microscope, are very distinguishable along the length of the pine needle. However, non-directional hyphae growth along with penetration of needle tissue either directly or through stomata by *D. septosporum* has been observed (Muir and Cobb, 2005).

Figure 26 Observations of GFP-38 on inoculated radiata pine needles

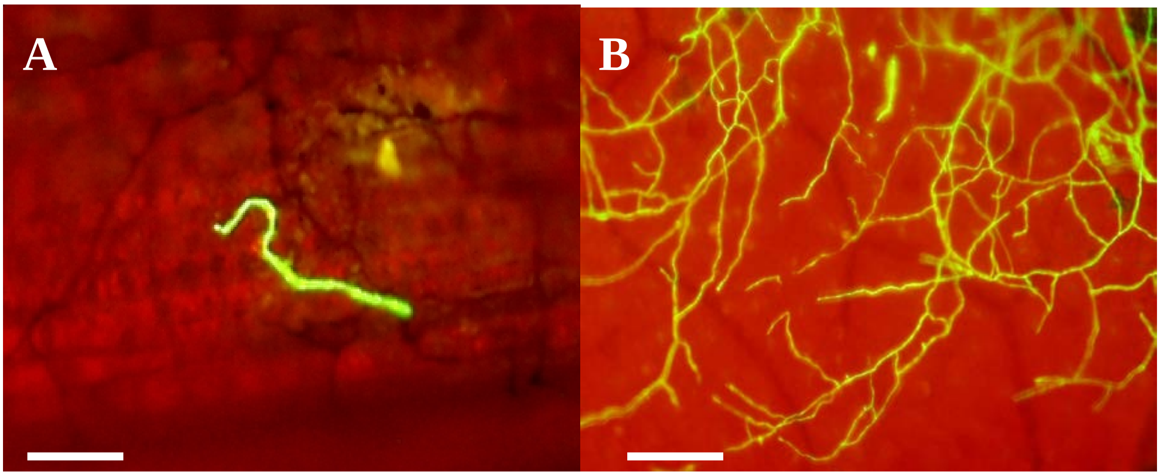


Photograph A & B at 400x magnification, and C-F at 200x magnification.
Arrows indicate stomata pores on *Pinus radiata* needles (Size bars = 10 μm)

Figure 26 A and B show non-germinating conidia next to open stomatal pores, as seen six weeks after inoculation. Figure 26 C shows germinated conidia with hyphal growth directed towards a stomatal pore. Directed growth was very rarely observed, with the majority of hyphae showing no orientated growth toward stomata. Growth was seen traversing the needle tissue both longitudinally and horizontally, and on occasions germ tubes were seen growing directly past or over stomatal openings with no sign of penetration. Previous research has found *D. septosporum* hyphae coiling down into the stomatal pore (Ivory, 1972b), and this was observed only once as seen in Figure 26 D. Penetration of the host was seen on several occasions, albeit less frequently than expected considering the high number of conidia present on the pine needle. It does appear that when the germ tube reaches the lip of the stomatal pore, the germ tube then bends and grows into the open stomatal cavity, as seen in Figures 26 E and F. These observations are representative of behaviour that was repeatedly found on inoculated pine needles over the duration of this study. The presence of GFP-38 intracellular within the needle tissue was not observed, and it is unclear whether the fluorescent signal at this level of penetration would be detectable.

The results shown in Figure 26 suggest that the behaviour of *D. septosporum* on the host tissue is variable and perhaps related to conditions involving the host, environment and physiological state of the fungus. This idea is further support by a preliminary study (results not shown) in which *D. septosporum* conidia was point inoculated onto peanut leaves. In contrast to what was observed on pine material, isolate GFP-38 inoculated onto peanut leaves showed extensive colonization over the surface of the plant material, as seen in Figure 27 six weeks post-inoculation. No penetration or symptoms of infection were seen on the peanut leaves (Figure 27 B).

Figure 27 Isolate GFP-38 germination and growth six weeks post-inoculation on pine needles compared to peanut leaves



Photograph A, GFP-38 on pine needle; Photograph B, GFP-38 on peanut leave (both 100x magnification) Size bar = 6 µm.

It is unknown why no extensive colonization on needle tissue (Figure 27 A) was observed in any of the assays, compared to the rapid growth of GFP-38 on peanut leaves. Possibly, peanut leaves are richer in available nutrients than pine needles, facilitating germination and rapid growth of hyphae.

When viewing needle tissue using fluorescent microscopy, the needle tissue autofluoresces red (as seen in Figure 27 A), as chlorophyll in the needle tissue absorb light. This phenomenon is useful contrasted against the green autofluorescence of the GFP isolates. However, necrotic regions on the needle tissue autofluoresce green, although the fluorescence is lower in intensity than the GFP expressing isolates. Additionally, the stromata of needles infected with GFP-38 showed no green fluorescence. As GFP-38 conidia harvested from cultured plates fluoresce green, it would be expected that the conidia within the stromata would therefore fluoresce. Figure 28 shows lesions autofluorescing green (Photo A), and stromata (arrows Photo B) from infected needles inoculated with both GFP-38 and NZE10 (Section 3.5.5).

Figure 28 Autofluoresence of diseased needle tissue

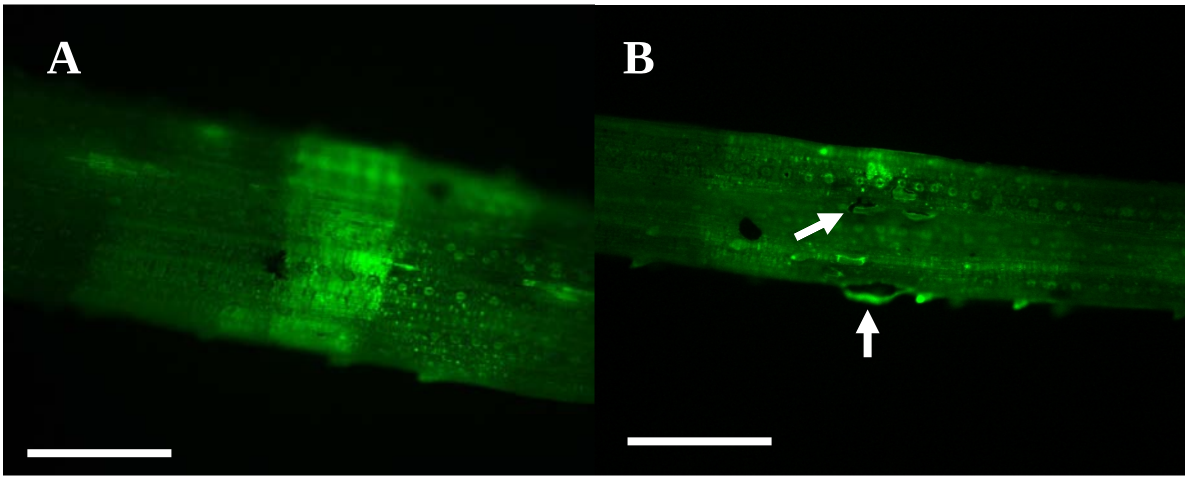


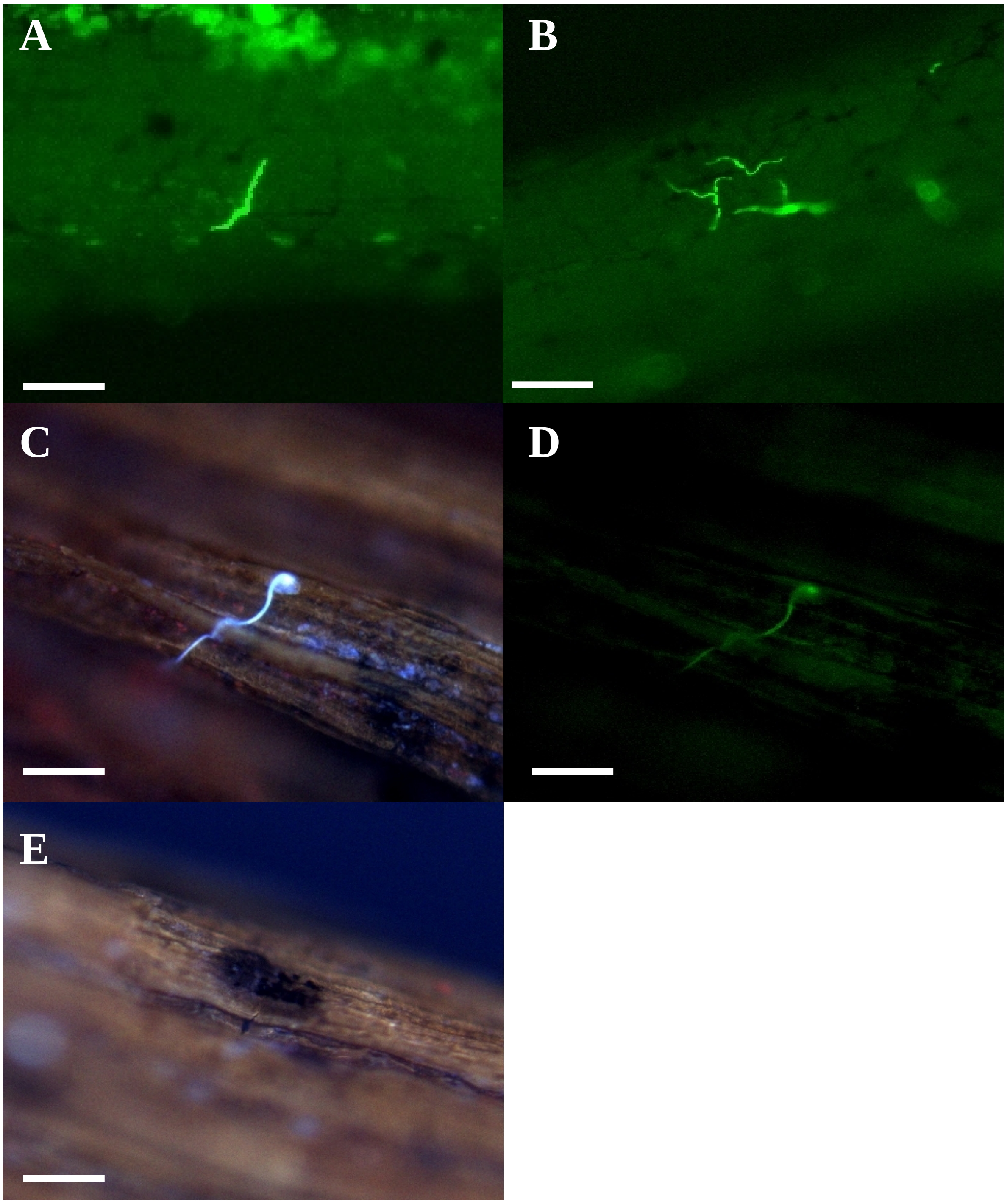
Photo A and B taken at 100x magnification.
Size bar = 0.5 mm

It is not known if the autofluoresence is due to toxin within the lesion, but it is unlikely the fluoresence seen is due to GFP-38, as observation determined damaged regions of a pine needle (no fungal infection) also autofluoresce. It is also possible that all infection observed with NZE10 and GFP-38 was solely due to NZE10, therefore it would be expected no fluorescing stromata be seen. However, as results indicate in Section 5.2.6, no fluorescence of stromata formed by GFP-*pksA* was seen either.

5.2.6 Behaviour of GFP-*pksA* DOTH Mutant on Pine Needles

The role of DOTH in the infection process is unknown as is the behaviour of mutants defective in toxin production and whether they would be any different to wild type isolates in behaviour on the needle surface. As results in Section 5.2.5 showed, the infection process or behaviour of *D. septosporum* on pine needle tissue is not consistent. Two eGFP-expressing mutants, GFP-*pksA* and GFP-*dotA* were available for analysis, but due to time constraints and a low level of sporulation for the GFP-*dotA* isolate, only the GFP-*pksA* isolate on needle tissue was observed. Figure 29 is a series of photographs of GFP-*pksA* at varying times post-inoculation showing the different behaviours observed on pine needles.

Figure 29 Isolate GFP-*pksA* germinating on pine needles



Photograph A-E taken at 100x magnification.
Size bar = 20 µm

The *pksA* mutant germinated on the pine needle surface (Figure 29 A and B), thereby confirming that DOTH is not required for germination of conidia. An appressoria-like structure shown in Figure 29 C and D (same point of focus but two different filters) was seen on a necrotic needle which had been inoculated with the *pksA* mutant (Section 3.5.6). For all other pathogen assays in this study, using wild type or GFP-38 isolates, no such structure has been observed before or since. On the same needle as in C and D (Figure 29), two other appressoria were seen. It did not appear that the appressoria were formed over a pine needle stomatal opening. Additionally, from the *pksA* mutant assay in Section 3.5.6, the presence of stromata was confirmed as shown in Figure 29 E. No fluorescence was observed from any stromata as with similar observations of GFP-38 in Section 5.2.5. It is not understood why the stromata do not fluoresce, and re-isolation of the *pksA* mutant and analysis of the isolated conidia would need to be conducted to further study the lack of fluorescence. However, these results are the first observations of a *D. sseptosporum* isolate deficient in toxin biosynthesis, and its associated behaviour on host tissue. Following germination of conidia on host tissue, the presence of appressoria and completion of the lifecycle from conidia to stromata was observed for the *pksA* mutant. It appears that DOTH may be a virulence factor, perhaps required for enhancing the spread of infection.

5.3 Discussion

5.3.1 Rate of Germination

The rate of germination between GFP expressing and wild type isolates of *D. septosporum* was comparable as seen in Table 13. Germination is the first step in the infection process, and this experiment showed that the introduction of a foreign gene did not prevent germination of the transformed isolates, GFP-38 and GFP-19. The rate of germination for the GFP isolates and wild type was consistent with that previous reported for *D. septosporum* (Gadgil, 1967).

D. septosporum grows best at an acidic pH, and in nature conidia have to germinate within the acidic pine needle environment. As exprected, pH had an effect on the rate of germination with over 80% of the conidia germinating within 24 h at pH 3 compared to less than 45% in all other pH conditions. Therefore it appears the *D. septosporum* is well adapted to survival in an acidic environment.

5.3.2 Germination of Conidia on Pine Needles

The ability to observe *D. septosporum* on pine needles without having to disrupt the pathogen-host interaction, due to the availability of GFP-expressing isolates, is a considerable advancement on the limitations of previous *D. septosporum* research. At the onset of this study, two wild-type isolates were available, GFP-19 and GFP-38, of which GFP-38 contained more copies of the eGFP gene than GFP-19. Preliminary observation showed that the intensity of the GFP-19 fluorescence was considerably lower than that of GFP-38 on the needle surface and GFP-19 did not fluoresce after several days post-inoculation. Differences in the level of fluorescence seen with *L. maculans* transformed with eGFP were attributed to differences in the site of integration of the gene into the genome and the number of copies present (Sexton and Howlett, 2001). Therefore, most of the characterisation of conidia behaviour on the host surface was conducted with GFP-38 since it could be visualised more readily.

Spray inoculation of plant material is a standard procedure used in many pathogen assays. In natural conditions, *D. septosporum* is dispersed via rainsplash, and experimentally a heavy inoculum load is required to obtain moderate levels of disease (Bulman et al., 2004). The use of GFP-38 allowed the effectiveness of spray inoculation of pine trees to be assessed. As the result in Figure 25 indicates, a heavy inoculum load in addition to even dispersal of conidia along the length of the needle was obtained. Daily monitoring of needles post-inoculation showed that continuous moisture straight after inoculation caused the conidia to slide down the needle surface and accumulate at the base of the needle. It is not known whether *D. septosporum* conidia secrete any form of adhesive, and if so at what stage after the conidia has come in contact with the host. The inoculated trees were therefore maintained in a humid environment minus continuous moisture for 48 h post-inoculation. Generally rain droplets would not adhere to any surface (Skadsen and Hohn, 2004), and pine needle topography would also facilitate run off of water droplets, so it is likely some form of adhesive is secreted by *D. septosporum*. Clarification of this will required further experimentation.

Previous research showed that conidia germinate on the needle surface within three days of inoculation (Bulman et al., 2004). As environmental variables have an effect on infection rate, an analysis of the number of conidia germinating on inoculated needles within the developed pathogen assay (Section 2.8.1) was required. The results in Section 5.2.4 indicate that a similar level of inoculum per needle was seen during the 48 h post inoculation, with a small number of conidia germinating by 24 h. There did not appear to be a large increase in the number of conidia germinating between 24 and 48 h. A low percentage of germination on the needle tissue is common, with reports of only 1-16% of germinated conidia seen entering stomatal chambers (Muir and Cobb, 2005). With such a low germination rate, and even lower probability of germinating conidia entering stomatal chambers it is expected that infection rates would be very low. The results of this experiment, highlight the importance of obtaining a high inoculum load on the host tissue, in addition to the importance of correct environmental conditions to maximise the possibility of infection occurring. Further analysis into the number of germinating conidia per needle under different environmental conditions may aid in maximising the chances of obtaining more infections.

5.3.3 Infection Process

The results in Section 5.2.5, with the use of the GFP-expressing isolate, illustrate that *D. septosporum* displays a diverse range of behaviour on the needle surface. The results in this section however highlight the usefulness of GFP as a marker, facilitating ease of monitoring *D. septosporum* on its natural host requiring minimal preparation to do so. However, there are some limitations with this system. First, the pine needle tissue is destroyed under UV light at 400x magnification, often also resulting in quelching of the GFP signal. Second, the topography of the pine needle means only a small area within the field of view is in focus. This often results in only a portion of the germinating conidia being in focus. Additionally, the green autofluoresence of damaged needle tissue may make it difficult to distinguish between the host and pathogen when needle sections are viewed. However, GFP is useful for observing the infection process in general.

Monitoring of GFP-38 on the needle surface showed that the level of conidia germination, in relation to the large number of conidia present, was very low. Additionally, germinating conidia mostly showed no directed growth towards stomatal chambers. Research by Peterson and Wella (1978) concluded the directional growth of hyphae towards stomata is more common in natural conditions, and possibly non-directional growth is an artefact of artificially inoculating needles. Of the hyphae that were seen entering a stomatal chamber, the hyphae appeared to bend at the edge of the chamber and grow down into the stomata. It has been proposed that the hyphae respond to a chemotactic signal from the host which results in hyphal bending and subsequent growth into the chamber (Franich, personal communication). If a chemical signal or some form of recognition signal was not present this could be one reason why some hyphae grew directly across stomatal chambers. However, it has been noted for other fungal pathogens such as *C. fulvum*, that runner hyphae grow non-directionally over the host surface until a main germ tube is produced, which then enters a stomatal chamber (Thomma et al., 2005). Possibly, *D. septosporum* may behaviour in a similar way. Research by Ivory (1972) showed a germ-tube coiled over a stomatal chamber. In this research, coiling of a germ-tube around the parameter and directed down into the stomatal chamber was seen once. Additionally, there have been several reports of hyphae swelling above stomatal openings (Ivory, 1972b; Franich, 1983). Current observations showed hyphae on the edge of and bent into the pine stomata but with no sign of swelling. Other fungal pathogens such as *S. nodorum* and *C. fulvum* also show no signs of hyphal differentiation once they have reached host stomata (Solomon et al., 2006).

The green autofluorescence of chlorotic or necrotic needle tissue is a disadvantage with respect to distinguishing GFP-expressing hyphae intercellularly or within the site of a lesion. For other fungal plant pathogens, such as GFP expressing *L. maculans*, the invading hyphae were clearly seen as intercellular and within the developed lesions which formed 14 days after inoculation on canola (Sexton and Howlett, 2001). Additionally, fluorescent hyphae of *Trichophyton mentagrophytes* were seen at a depth of 9.8 μm in skin explants (Kaufman et al., 2004). Since it is unclear whether the green autofluorescence within lesion areas was due to *D. septosporum* or needle tissue autofluorescence, it would be difficult to determine whether an increase in fluorescence intensity at a region on the pine needle distinct from stomata was host-induced or induced by direct penetration of *D. septosporum* (Solomon et al., 2006). However, by taking transverse sections of needle tissue potentially infected with GFP-expressing *D. septosporum* and examination with confocal scanning laser microscopy, it should be possible to confirm the presence and distribution of *D. septosporum*.

The mature stromata of both GFP-expressing isolates (GFP-38 and GFP-*pksA*) seen on infected needles did not fluoresce under UV light. The stromata seen on these needles appeared to be consistent with the morphology of stromata observed on wild type infected needles. However, conidia from *S. nodorum* (GFP-expressing) did not fluoresce in mature pycnidia as the GFP gene was driven by a promoter involved in glycolysis. It was therefore unlikely that the mature pycnidia were undergoing glycolysis, hence the lack of fluorescence detected (Solomon et al., 2004). It is likely that the *D. septosporum* conidia within the stromata were not metabolically active, and therefore the GFP gene would not be expressed, thus explaining the lack of fluorescence. Additional experimentation is required to confirm that the mature stromata are a result of infection caused by the GFP-expressing isolates. One possibility is to isolate the spores directly from the infected needle tissue, and check that the colonies they produce on agar fluoresce under UV light. However, the use of GFP as a reporter molecule to monitor *D. septosporum* infection of pine needles has led to some interesting observations and to the potential for further experimentation.

5.3.4 Dothistromin as a Virulence Factor

The results in Section 5.2.6 suggest DOTH is a virulence factor, not a pathogenicity factor, as it is not required to cause red-band disease. These are the first observations of a mutant defective in toxin biosynthesis, and associated behaviour on the pine needle surface. As the photographs in Figure 29 indicate, similar behaviour was seen for the mutant as was seen for the wild type (Figure 26). The GFP-*pksA* was capable of germination on the needle surface, in addition to germ tubes having non-directional growth as seen for the wild type. It is unknown if *D. septosporum* conidia produce any form of spore-germinating or inhibiting fluid, often produced by fungi to facilitate adhesion to the host, or as seen for *A. brassicicola* a signal to induce toxin production (Oka et al., 2005). As infection was obtained with the *pksA* mutant, albeit at a low rate, it appears DOTH is not required for morphological differentiation on the host surface. It is possible dothistromin is produced in response to, and toxic to, other microorganisms within its environmental niche. As *D. septosporum* is slow growing, it would need some form of defensive strategy in order to compete. Recently it has been noted in extensively infected forests in British Columbia, that the distinct red-band caused by DOTH is not always present on the needles (Woods, personal communication). Perhaps, this is reflective of *D. septosporum* not having to compete on its host with other microorganisms. Therefore, the toxin may facilitate the successive invasion of host tissue, as the host is defenceless against the presence of toxin, as seen with injected toxin into pine tissue (Franich et al., 1986). Further studies into the flora of microorganisms within the same environment as *D. septosporum*, in addition to differences between microorganisms present on needles that do have the characteristic red-band as opposed to those that do not would facilitate a better understanding of the role of DOTH and disease.

As shown in Figure 29, an appressorium type structure was seen, with no appressoria found on any needles inoculated with the wild type strain of *D. septosporum*. It would seem unlikely that the formation of an appressorium structure by GFP-*pksA* is an artefact of no toxin production, as other research has confirmed wild type strains forming these structures over stomatal pores (Gadgil, 1967, 1974; Peterson and Walla, 1978). However, some appressoria function by providing enzymes required for penetration (Hutchison et al., 2000). Due to the absence of DOTH, which causes cellular breakdown of host tissue, an obvious advantage for *D. septosporum* to penetrate, perhaps the formation of appressoria functioned to facilitate infection. Further pathogen assays using mutants defective in DOTH biosynthesis need to be conducted, in order to replicate and validate the results found in this assay. However, the potential applications of using GFP-expressing *D. septosporum* in pathogen assays are numerous. Of particular interest would be fusions of GFP to the promoters of genes involved in DOTH biosynthesis and monitoring the role of the toxin in the infection process. Combined with confocal microscopy of infected needle tissue, it would be possible to determine at site-specific stages of infection when dothistromin is synthesised and possibly allow quantification of the level of toxin produced within lesion areas. Additionally, fusion of GFP to mutated toxin genes, and the observation of the infection process compared to wild type isolates would confirm dothistromin is a virulence factor, not a pathogenicity factor, and primarily enhances the probability of successful infection.

6

Summary

The results of this research have contributed to the current knowledge of the infection process in Dothistroma red-band disease, the potential role of dothistromin and the response of *D. septopsorum* to ambient pH. In an artificial environment, the conditions conducive to red-band disease are a temperature of 24°C/16°C (day/night), high relative humidity, continuous moisture and a white and UV light diurnal cycle of 12 h. Under these conditions *D. septosporum* completes the disease cycle within six weeks post-inoculation.

Increased disease incidence within an artificial environment is achieved using *P. radiata* seedlings as opposed to cuttings. Cuttings possibly have some form of mature tree resistance to *D. septosporum* infection due to being taken from 5-7 year old pine trees. It was also shown that there is no difference in disease incidence when *D. septosporum* conidia or mycelial fragments are used as the primary source of inoculum. Additionally, the media the fungus is grown on and conidia harvested in do not appear to increase the incidence of disease. However, the addition of an external nutrient such as yeast extract to the conidial suspension resulted in a significantly higher disease incidence. There are several possibilities why this may increase disease. First, *D. septosporum* is often associated naturally with an unknown yeast species (Dick, personal communication), and the presence of yeast may increase the availability of external nutrients, enabling a greater number of conidia to survive, germinate and infect. Second, yeast may induce the production of dothistromin which may be toxic to other microorganisms, enabling the slow growing fungus to compete within the host niche to establish itself.

Previous to this research, it was unclear whether *D. septosporum* was able to infect pine needles and cause disease in the absence of dothistromin. Furthermore, it is not known if dothistromin is a virulence or pathogenicity factor. Using the GFP-*pksA* mutant it was shown that dothistromin is not required for germination on the host surface or for subsequent infection, as disease incidence was comparative to that of the wild type. This eliminates the possibility of dothistromin being a pathogenicity factor, but further research is required to show that dothistromin is a virulence factor, as there may be some role of dothistromin in the infection process. Limited research has been conducted on the host response to dothistromin, and the characteristic red-band seen on diseased pine needles may be due to a host response. Often in British Columbia in areas of forest that have a high incidence of disease, mature stromata are seen on diseased needles but no red-band. If the *Dothistroma* spp. within these needles does not produce toxin, combined with the results from the mutant in this study, this may suggest that dothistromin is not a virulence factor either.

The use of GFP-expressing isolates of *D. septosporum* enabled the infection process to be monitored. The addition of the GFP gene did not disrupt the rate of germination, or the ability of the GFP-expressing isolates to infect and cause disease. The germination rate on the needle surface observed with these isolates was comparable to previous research, with only a small percentage of conidia germ tubes observed entering stomatal pores. For all GFP-expressing isolates used in this study, no consistent growth towards stomatal pores was seen on the needle surface. Often germ tube growth was undirected or germ tubes grew directly over stomatal pores. On some occasions however germ tube growth did appear to be directed towards stomata. Prolific germination of conidia and hyphal colonization of the needle surface was not observed, with many un-germinated conidia still fluorescing at six weeks post-inoculation. For the mutant GFP-expressing isolate, the formation of appressoria on the needle surface was observed. None of the GFP-expressing isolates were observed below the needle surface, and mature stromata did not fluoresce. However, red-bands on the needle did fluoresce, and it is unlikely this was due to the presence of GFP-expressing hyphae as often naturally damaged pine tissue fluoresced green as well. Whether the intensity of the fluorescence seen within the red-band is due to dothistromin is unknown.

Historically, red-band disease is confirmed by the presence of the characteristic signs and symptoms, or re-isolation of the fungus from diseased needles. Due to the morphological instability of this fungus in culture in addition to slow growth, the re-isolation method is often unsuccessful. In this study a molecular based diagnostic procedure was developed, due to the availability of mating type primers specific to the *Dothistroma* spp. and mating type genes. Further optimization of the DNA extraction procedure is required, as often low yields of fungal DNA are recovered from pine tissue in addition to the presence of PCR inhibitors. However, upon further development the method can be used to confirm red-band disease in addition to identification of the infecting *Dothistroma* spp.

The effect of ambient pH on physiological processes such as growth, the production of conidia and dothistromin and the related gene expression of toxin biosynthesis genes was analysed. The only significant effect of ambient pH was more growth at acidic pH, with more toxin detected per biomass at pH 5. There does not appear to be a consistent response by *D. septosporum* to differing ambient pH, as isolates NZE7 and NZE10 showed differences between them in respect to growth, and toxin production. Expression of genes involved in toxin biosynthesis also did not correlate with the levels of toxin detected in the analysis. Therefore, unlike *Aspergillus* spp., *D. septosporum* physiological processes do not appear to be regulated by ambient pH.

There are several areas of future work that have been uncovered by the results of this study. Importantly, UV light should be used in any pathogen assay with *D. septosporum*, as the principle difference between previous work in this laboratory, and the inability to obtain disease, and the current developed assay system was the inclusion of UV light. It should be possible to test dothistroma resistant breeds of pine, based on what is known in respect to environment, host and pathogen factors from this study. Further research into the role of yeast extract on increasing disease incidence, in conjunction with the relationship between yeast organisms and *D. septosporum* on the pine needle, needs to be conducted. A comparison of microorganisms found on diseased needles between forests that show different levels of disease incidence and analysis of *D. septosporum* response to these organisms, would also be informative. The role of dothistromin as a virulence factor needs clarification, in addition to replication of assays using dothistromin mutant isolates of *D. septosporum*. Further analysis into the response of the host to the presence of dothistromin needs to be done to determine whether the red-band is due to a host response. Dothistromin is not required for infection, and *D. septosporum* may grow biotrophically within the needle tissue until biosynthesis of toxin causes the host to respond, triggering *D. septosporum* to complete its lifecycle by production of stromata, enabling survival of the fungus. If confocal microscopy can be used to monitor *D. septosporum* intracellular within the pine tissue, then combined with GFP as a reporter of toxin synthesis, characterization of the role dothistromin has in disease may be able to be determined. Additionally, using confocal microscopy it should be possible to quantify the biomass of the fungi within the pine needle and monitor disease progression and the formation of stromata.

Appendices

Appendix I

Buffers and Solutions

A1.0 Buffers and Solutions

A1.1 Hexadecyl trimethyl-ammonium bromide buffer (CTAB)

CTAB (2%, Sigma) 0.8 g, PVP40 (1%, Sigma) 0.4 g, 5M NaCl (Merck) 11.2 ml, 0.5M EDTA (BDH) 1.6 ml, 1M Tris HCL (pH 8.0 Invitrogen) 4.0 ml, and milli-Q water 22.4 ml.

A1.2 TNE buffer (10X)

Tris (Invitrogen) 12.11 g/l, EDTA (BDH) 3.72 g/l, NaCl (Merck) 116.89 g/l. Dissolve in ~800 ml of milli-Q water, adjust pH to 7.4 using concentrated HCl (BDH), then make volume up to 1 L with milli-Q water.

A1.3 TBE buffer (10X)

Tris (Invitrogen) 108.0 g/l, EDTA (BDH) 9.3 g/l, Boric acid (Univar) 55.0 g/l. Added milli-Q water to make up to 1 L, followed by adjustment of pH to 8.2 using concentrated HCl (BDH).

A1.4 TE buffer (1X)

Tris (Invitrogen) 10 mM, EDTA (BDH) 1 mM pH8.5.

A1.5 Phosphate-buffered saline (PBS 10X)

8% NaCl (BDH), 2.9% Na_2HPO_4 (BDH), 0.2% KH_2PO_4 (BDH), pH 7.4.

A1.6 Phosphate-buffered saline plus 0.1% Tween 20 (PBST)

1 X PBS (Section 1.5) and 0.1% Tween 20 (Difco).

A1.7 BSA

1% skim milk powder (Pams), 1% PBS (Appendix A1.1.5) and 1% thiomersal (BDH)

A1.8 Dilution buffer (1 plate)

PBS (10x) 8 ml, RO water 32 ml, milk powder 0.8g, Tween 20 80µl.

A1.9 Dilution buffer and broth (1 plate)

Dilution buffer (Appendix A1.1.8) 7 ml, lowDB 7 ml.

A1.10 Working buffer (1 plate)

Dilution buffer (Appendix A1.1.8) 10 ml, DMSO 15 µl.

A1.11 Working buffer and broth (1 plate)

Dilution buffer (Appendix A1.1.8) 20 ml, lowDB 20 ml, DMSO 40 µl.

A1.12 Labelled peroxidase (1 plate)

Dilution buffer (Appendix A1.1.8) 198 µl, 10C12 2 µl makes 1/100 dilution. Add 40 µl of 1/100 labelled peroxidase to dilution buffer 12 ml (makes 1/300 dilution).

A1.13 McIlvaine Buffer

0.1 M Citric acid (BDH) 21.01 g/l and 0.2 M $Na_2HPO_4.2H_2O$ (Merck) 35.59 g/l

The following volumes of buffer are combined to obtain 100 ml with the corresponding pH values:

	0.1 M Citric Acid	**0.2 M $Na_2HPO_4.2H_2O$**
pH 3	79.45	20.55
pH 4	61.45	38.55
pH 5	48.50	51.50
pH 6	36.85	63.15
pH 7	17.65	82.35

A1.14 Extraction Buffer

200 mM Tris-HCl (Invitrogen) 31.52 g/l pH 7.5, 250 mM NaCl (Merck) 14.61 g/l and 25 mM EDTA (BDH) 9.3 g/l.

A1.15 Fluorometer DNA standard

100 ng/µl Calf thymus DNA (Amersham Biosciences) in 1X TNE.

A1.16 Hoechst dye stock solution

10 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 the light.

A1.17 Fluorometer working solution

Hoechst H 33258 stock solution 10 µl, 10X TNE 10 ml, milli-Q water 90 ml.

A1.18 Ethidium bromide staining solution

1 mg/ml ethidium bromide in milli-Q water.

A1.19 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)

A1.20 Acid and Base

10M HCl (BDH) 36.46 g/l and 10M NaOH (Scientific Supplies Ltd) 399.00 g/l.

A1.21 Yeast extract

Dissolve 10 g yeast extract (BD) in 80 ml milli-q water. Add 320 ml of 95% EtOH and leave overnight at 4ºC. Pour off EtOH, allow to air dry and re-dissolve yeast in 50 ml of milli-q water. Then freeze at -80ºC before freeze drying.

Appendix II
Media

A2.0 Media

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

A2.1 Aspergillus minimal media (AMM)

$NaNO_3$ (Univar) 6 g/l, $MgSO_4$ $7H_2O$ (Merck) 0.52 g/l, KCI (BDH) 0.52 g/l, KH_2PO_4 (BDH) 1.52 g/l, $FeSO_4$ $7H_2O$ (Univar) trace, $ZnSO_4$ $7H_2O$ (BDH) trace, bacteriological agar (Oxoid) 20 g/l, H_2O to 950 ml and pH 6.5.

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

A2.2 Dothistroma medium (DM)

Malt extract (Oxoid) 5% (w/v), Nutrient agar (Oxoid) 2.8% (w/v)

A2.3 Dothistroma sporulation media (DSM)

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

A2.4 Potato Dextrose Medium (PDA)

Potato dextrose agar (Merck) 39 g/l.

A2.5 Low Dothistroma Broth (low DB)

Malt extract (Oxoid) 2.5% (w/v), Nutrient broth (Oxoid) 2.0% (w/v).

A2.6 Potato Dextrose, Sodium Nitrate and Glucose Medium (PDA + G)

Potato dextrose agar (Merck) 39 g/l, $NaNO_3$ (Univar) 6 g/l, Glucose (Univar) 2.0% (w/v). Sterile glucose from a 40% stock solution was added to the media after autoclaving.

A2.7 Yeast and Sucrose Agar (YES)

Yeast extract (Oxoid) 5 g/l, Peptone (Difco) 5 g/l, Sucrose (BDH) 30g/l, Bacteriological agar (Oxoid) 15 g/l.

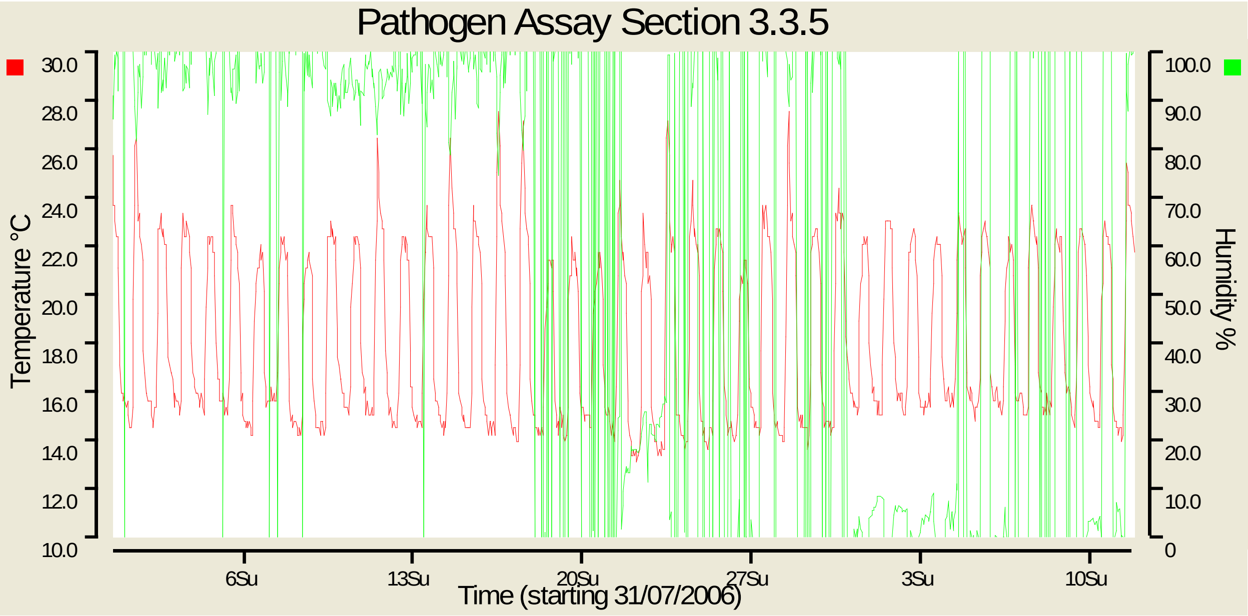
A2.8 Potato Dextrose and Niacin Agar (PDA+N)

Potato dextrose agar (Merck) 39 g/l, Nicotinic acid (Merck) 0.5 g/l, Sucrose (BDH) 30 g/l.

Appendix III
Temperature and Humidity

The following graph is an example of an overlay of temperature and humidity data downloaded from the Tinytag logger using the manufacturer's software (GLM Version 2.8) for the assay to assess the effect of conidia growth medium on disease incidence (Section 3.5.4).

Figure 30. Temperature and humidity data



The data logger was set to take both temperature and humidity readings every hour for the six week duration of this assay. Using the functions in the GLM software, the minimum, maximum and average reading of both temperature and humidity between specified time points can be determined (corresponding results for each assay in Table 2.). Looking at the data in Figure 30, it is apparent that the temperature changed every 12 h, corresponding to the temperature variables set in the GMO suite (Section 2.8.3). The humidity readings show considerable variation, possibly due to moisture buildup on the sensor of the data logger preventing accurate reading, in addition to 80% humidity not being maintained, as was set on the control panel of the GMO suite (Section 2.8.3). However the data logger is a valuable asset for any assay, to assist with determining if environmental parameters are as they should be.

Appendix IV
Disease Incidence

A3.0 Calculations for disease incidence

All data is available in Excel format on the CD-rom at the back of this thesis. There were three replicate seedlings/cuttings per condition per assay. The data presented in Sections 3.3 and 3.4 is the mean of the three replicates for each condition. The calculations are as follows:

1. Disease incidence

$$\frac{100\%}{\text{needles}^{a}} \times \text{diseased needles} = \%$$

(^{a}un-infected + diseased)

2. Bands per needle

$$\frac{\text{total bands}}{\text{total diseased needles}} = \text{bands/needle}$$

3. Fruit bodies per needle

$$\frac{\text{total fruit bodies}}{\text{total diseased needles}} = \text{fruit bodies/needle}$$

4. Chlorotic needles

$$\frac{100\%}{\text{total all needles}^{b}} \times \text{chlorotic needles} = \%$$

(^{b}un-infected + diseased + chlorotic)

Appendix V
Dry Weight Calculation

A5.0 Dry weight calculations

Harvested mycelium from each flask was divided into two approximate equal portions and each placed in a 1.5 ml tube. The wet weight (mg) of each tube was recorded and one tube was placed on the freeze drier while the other was stored at -80°C for subsequent RNA extraction. The dry weight (mg) of the tube after freeze drying was recorded and the calculation shown below was used to determine the approximate dry weight (mg) of the tube placed at -80°C.

1. Calculation to determine dry weight (mg)

$$\frac{\textit{dry weight (mg)}}{\textit{wet weight (mg)}} = \textit{mg dry/mg wet}$$

total mg wet X mg dry/mg wet = total dry weight (mg)

Worked example:
Wet weight: tube 1. 39.3 mg (freeze dryer)
tube 2. 68.4 mg (-80°C)

Dry weight: tube 1. 3 mg

$$\frac{3 \text{ mg}}{39.3 \text{ mg}} = 0.076 \text{ mg dry/mg wet}$$

39.3 mg + 68.4 mg = 107.7 mg wet (total)

107.7 ~~mg wet~~ X 0.076 mg dry/~~mg wet~~ = 8.19 mg dry

DOTH/mycelium (µg/mg) calculations

For each replicate flask in each condition the amount of toxin (µg/25 ml) and mycelium dry weight (mg/25 ml) were divided by 25 ml to obtain DOTH µg/ml and mycelium mg/ml. These numbers were then used in the calculation shown below to find the mean amount of toxin per mycelium for each condition.

2. Calculation to determine DOTH μg/mg mycelium

$$\frac{\text{DOTH } \mu g/ml}{\text{mycelium } mg/ml} = \text{DOTH } \mu g/mg \text{ mycelium}$$

Worked example:

	Replicate	DOTH μg/ml	Mycelium mg/ml	DOTH μg/mg mycelium
5 days	**1**	0.983	0.732	1.342
	2	0.938	1.248	0.752
	3	1.005	1.204	0.835
	Mean	**0.975**	**1.061**	**0.976**

Note that the mean value of 0.976 DOTH μg/mg mycelium used in subsequent analysis was determined by finding the average for all values calculated for the replicate flasks. If you divide the means of each column to obtain the final DOTH μg/mg mycelium the number would be 0.919.

Appendix VI
ELISA Assay

A6.0 Calculations for toxin levels using an ELISA Assay

A set of 10 standards (0-1 µg/µl dothistromin) were run in triplicate on each 96-well ELISA plate. Readings were placed in a table from which the % inhibition was determined, a standard curve generated using the graphical function in Excel, and the linear portion of the curve determined with an R^2 value between 0.9-1 using the functions in Excel. The following data is an example.

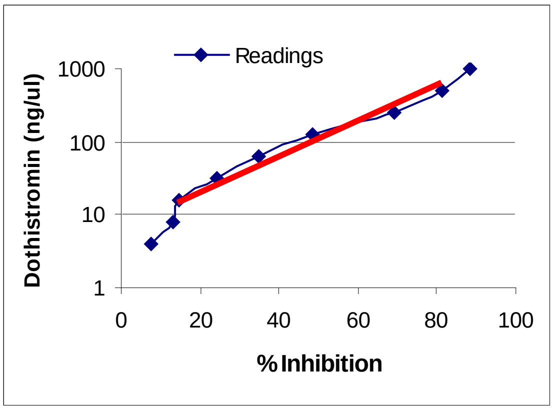
Standard readings

Standards ng/µl	**1**	**Absorbance 2**	**3**	**Average Abs. (B)**	**% Inhibition**
1000	0.201	0.201	0.235	0.212	88.61076
500	0.356	0.354	0.33	0.346	81.40532
250	0.66	0.545	0.532	0.579	68.94332
125	0.983	0.997	0.907	0.962	48.3819
62.5	1.23	1.226	1.199	1.218	34.65044
31.6	1.499	1.381	1.343	1.407	24.49489
15.6	1.771	1.499	1.508	1.592	14.57177
7.8	1.681	1.519	1.665	1.621	13.01625
3.9	1.767	1.741	1.668	1.725	7.455732
0	1.944	1.704	1.945	**Bo** 1.864	-1.8E-05

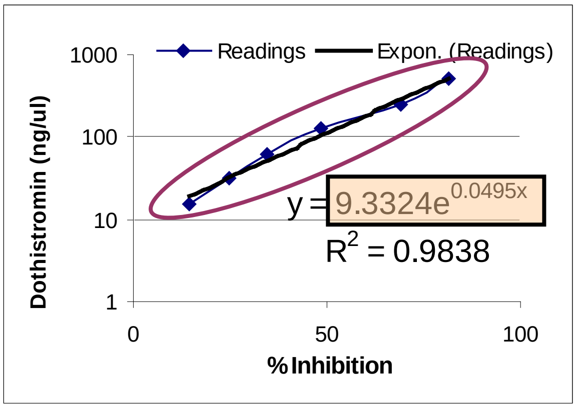
% Inhibition =

$$\frac{B_o - B}{B_o} \times \frac{100}{1}$$

Standard curve



Linear portion of standard curve



The linear portion of the standard curve was determined by selecting a group of data points within the standard curve using Excel that gave an R^2 value as close to 1 as possible. The red line through the standard curve data is representative of the most linear portion of the standard curve. All subsequent readings for the toxin analyses must fall within the corresponding % inhibition range (— = linear portion of standard curve).

The following is an example of ELISA readings taken for a series of samples, and the calculations required to determine the final concentration based on the information above. Every sample analysed is run in triplicate on the ELISA plate, same as the standards.

Sample readings

	Absorbance			Average	%	DOTH (ng/μl)	DOTH (ng/μl)
Samples	**1**	**2**	**3**	**Abs. (B)**	**Inhibition**	**(diluted)**	**(undiluted)**
1	1.549	1.503	1.535	1.529	17.98675	22.73343	2273.343
2	0.866	0.823	0.874	0.854333	54.17485	136.3394	13633.94
3	1.595	1.634	1.434	1.554333	16.62791	21.25461	2125.461
4	0.984	0.921	0.981	0.962	48.39978	102.4404	10244.04

— = 9.3324*(2.71828182845904^(0.0495* % cell))

— Multiply diluted DOTH reading by dilution factor used for the sample (in this example 100x).

Appendix VII
Quantitative RT-PCR

A7.0 Samples

Each LightCycler™ run contained two replicates of the target gene calibrator and reference gene calibrator, known concentration. Two replicates of unknown cDNA (one to determine target gene concentration and the other to determine reference gene concentration) were used for each sample. Each run was done in triplicate, with the mean of these runs shown in Section 4.2.3 and 4.2.4.

A7.1 Melting Curve Analysis

A melting curve analysis was done to determine the specificity of the amplified PCR product. The melting curve analysis allows discrimination between both the target and reference gene, as both genes have a different melting point. Figure 31 is an example of a melting curve obtained for the *pksA* gene (Section 4.2.3).

Figure 31. Melting curve

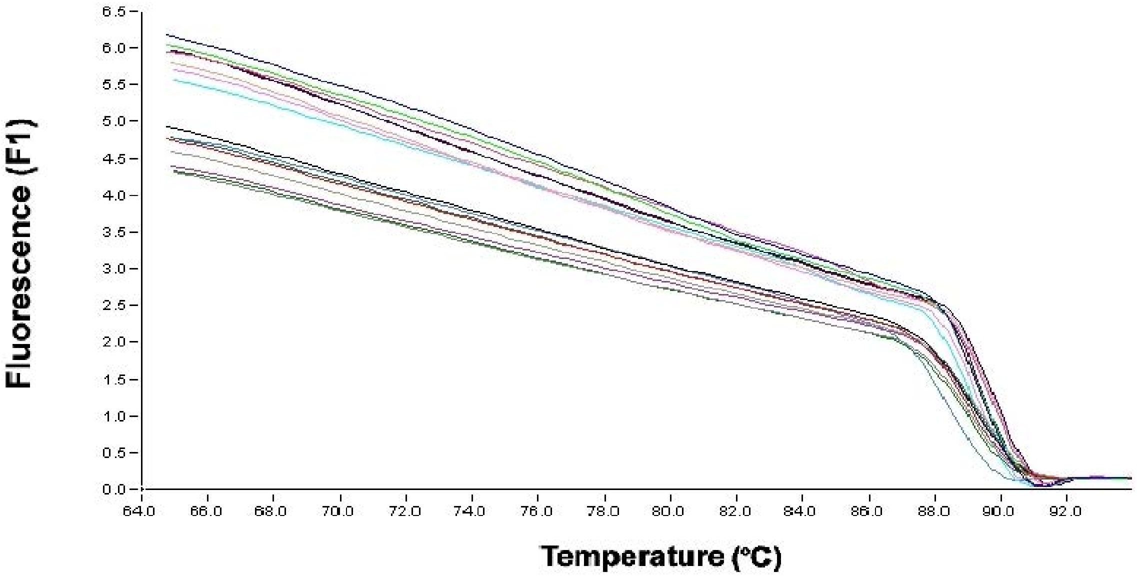


Figure 32 shows an amplification curve obtained for samples using the *pksA* gene primers. The dark blue line which shows no increase in fluorescence is the negative control (no template cDNA).

Figure 32. Amplification curve

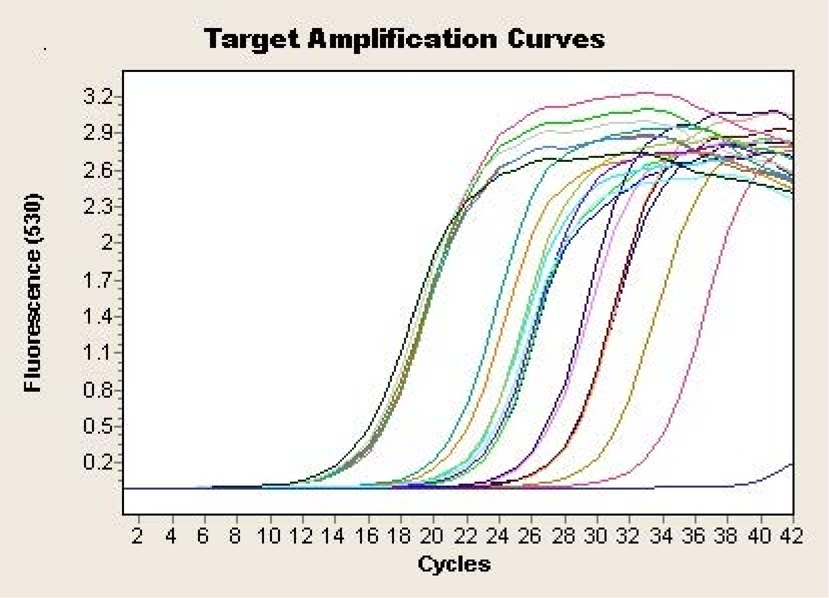
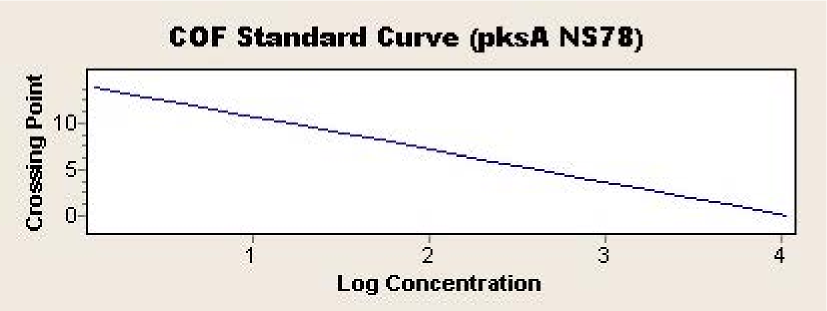


Figure 33 is the standard curve used in which the unknown samples crossing point is contrast on the curve to determine the initial concentration of the target DNA for the *pksA* gene (using Lightcycler™ Master Software). This standard curve is generated from a coefficient file of the crossing points for standards of known concentration run for each gene (both the known gene and reference gene).

Figure 33. Standard curve



Appendix VIII

Conidia Germination on the Needle Surface

The length of each replicate needle used for analysing the number of spores in addition to the number of spores germinating within the defined region was recorded. The area of the needle viewed under the microscope at 100x magnification was determined, using a calibrated eyepiece micrometer, to be **9.86×10^{-3} mm^2** (the average width of a pine needle was determined to be 0.56 mm and used in all subsequent calculations). Five observations per needle were made when counting the number of spores, and three observations per needle when counting the number of germinating spores. The approximate total number of spores and germinating spores per needle was calculated as below. The mean number of spores/germinating spores was then determined from six individual needles per time point.

1. Total area of the needle:

length x width = mm^2

$mm^2 \times 2$ = ***Total area of needle*** (x2 accounts for both sides of the needle)

2. Total spores/germinating spores per needle:

$$\text{Spores counted} \quad \times \quad \frac{\text{Total area needle}}{\text{Area/spore}} \quad = \quad \textbf{Spores/needle}$$

The data used to calculate the number of spores per needle in Table 15 available on the enclosed CD. The data is the mean number of spores per needle (5 counts per needle), from six individual needles taken from two inoculated pine seedlings (3 needles from each) as described in Section 2.2.4.

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