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

A thesis presented in partial fulfilment of
the requirements for the degree of
**Master of Science in Genetics**
at
Massey University,
Palmerston North, New Zealand.

**Naydene Barron**
**2006**

## 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*.

# Acknowledgements

I would like to thank both my supervisors Dr Rosie Bradshaw and Dr Peter Long for their dedication to this research project. Rosie you have been both supportive and understanding during the course of my Masters. You have been an outstanding supervisor who believed in my ability to complete this research, in addition to being approachable and appreciative, and with your dedication and guidance you made the completion of this task so much easier. Peter, thank you for your time and ideas and especially for the humor and making sure my pronunciation of fungal species in addition to data analysis was up to scratch! You have both been inspirational and a pleasure to work with. I have gained so much knowledge from both of you in the last two and half years and enjoyed working on this research project.

To my lab colleagues, both past and present, it has been great to be associated with such a dedicated group of people who helped make this journey possible. Arne, thank you for your technical assistance, sharing of your knowledge and ideas and having a great sense of humor. Shugang thank you for sharing your knowledge and your willingness to listen and provide new information. I wish everyone in the lab the best of luck in completion of their studies and success for the future.

To my partner, thank you for being so supportive, encouraging and understanding over the duration of my studies, in addition to helping me with revision before exams, and making sure I got it right. To my daughter, you have been patient over the last few years, but understand the importance of studying. I hope you have learnt that even though you can't see the forest for the trees, with a lot of hard work and dedication there is always light at the end of the tunnel. To both of you, we have missed out on a lot of time together, but that sacrifice has enabled me to meet my goal, and complete this part of my life, thank you. To mum and dad, thank you for believing in me, your support, financial assistance and helping with Chantel.

Lastly, I would like to thank Rosie for sourcing financial assistance, the CoRE, Bio-protection Centre, NZ Plant Protection Society, C. Alma Baker Trust and Massey University for providing financial assistance for myself and this project.

# Table of Contents

# List of Figures

# List of Tables

# 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 |

# 1

# 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**

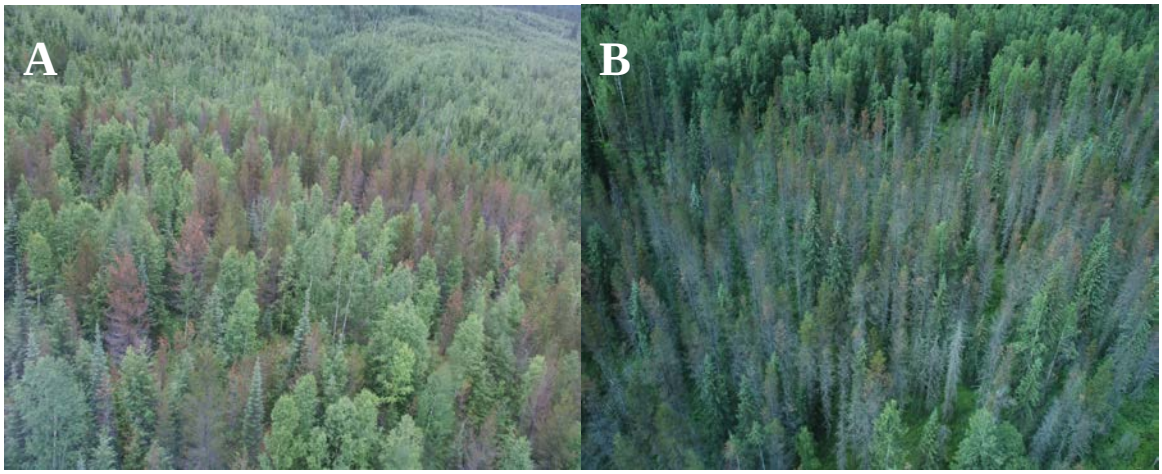



**Photo A; extensive Dothistroma damage (Bell Irving, BC, Canada), B; Mortality (Sediesh Creek, BC, Canada), courtesy of Alex Woods.**

### 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

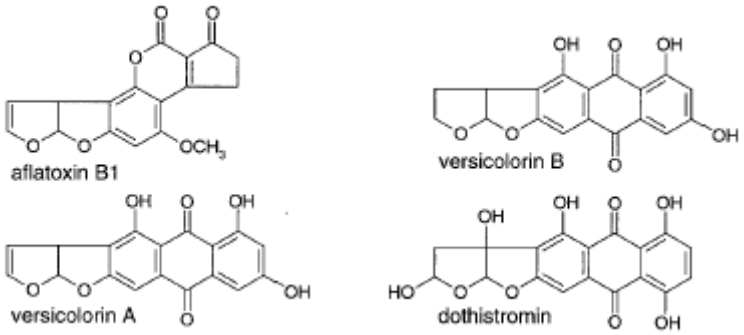
about the mature tree resistance mechanism (Gibson, 1972). The most prominent form of control is still the use of copper fungicides.

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.

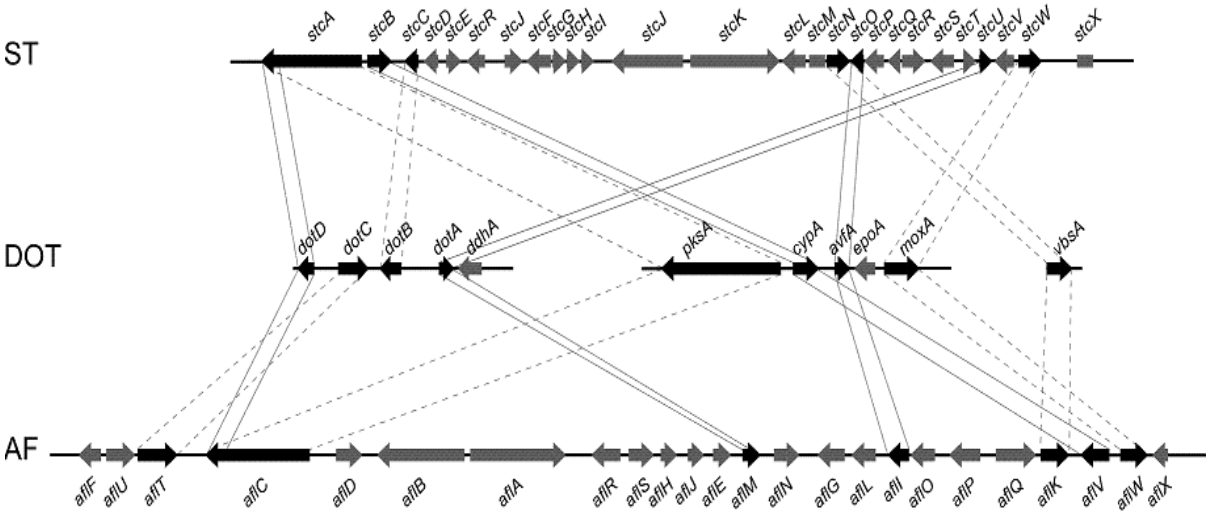
### 1.1.6 Fungal Gene Clusters

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 ($1 \times 10^6$ 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 ($1 \times 10^6$ 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.