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Investigation of dothistroma needle blight development on *Pinus radiata*

A thesis presented in the partial fulfilment of the
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Abstract

Dothistroma needle blight (DNB), caused by the fungi *Dothistroma septosporum* and *Dothistroma pini*, is an important foliar disease of pine species throughout the world and predictions of the future spread of this disease have been made using climate models. Although DNB infection is prevalent in many forests, attempts to achieve infection under controlled laboratory or glasshouse conditions are notoriously difficult. However, artificial infection is a very important tool for studying different aspects of plant-microbe interactions, such as pathogen life style and roles of virulence factors. *D. septosporum* was thought to have a hemi-biotrophic life style but this was not formally investigated *in planta*. The non-host selective toxin dothistromin produced by this fungus was shown not to be essential for pathogenicity but its role in pathogen virulence was unknown.

The aims of this study were to improve the DNB pathogenicity assay and to use this system to test the hypotheses that *D. septosporum* is a hemi-biotrophic pathogen and that dothistromin plays a role in virulence.

A new sporulation medium (pine needle medium with glucose) was used to obtain sufficient viable *D. septosporum* spores. The critical microclimatic component of leaf wetness was optimised to have a short (4-7 d) high wetness period followed by 'medium' wetness (continual misting), and using these conditions >80% needle infection was routinely achieved on *Pinus radiata* seedlings.

A combination of microscopy, biochemical and molecular studies over a time-course of infection of *P. radiata* by *D. septosporum* confirmed its hemi-biotrophic life style. Restricted mesophyll colonisation, shorter lesions and fewer spores from *P. radiata* needles infected with dothistromin-deficient mutants, compared to those with

wild type *D. septosporum*, suggested that dothistromin has a role in virulence. Interestingly ‘green islands’ in which chlorophyll levels were maintained at higher levels than adjacent chlorotic and necrotic regions, surrounded early-appearing lesions caused by both wild-type and mutant isolates. At a later developmental stage of the lesion the green islands were still present in the mutant but appeared to be masked by the extended dothistromin-containing lesions in the wild type, which lead to the hypothesis that chloroplasts could be a site of action of dothistromin.

The discovery that dothistromin is a virulence factor opens up new insights into the *Dothistroma*-pine interaction. This fundamental finding will be useful for management strategies for this important disease in the future.

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2. Raw data of Figs 4.16 and 4.17 (quantitative gene expression in section 4.2.2.1.2).
3. Digital version of thesis

Abbreviation

| Abbreviation | Meaning |
|---------------------|---------------------------|
| µg | micro gram |
| µl | micro litre |
| µM | micro molar |
| ai | after inoculation |
| bp | base pair |
| d | day |
| DNB | dothistroma needle blight |
| h | hour |
| g | gram |
| GFP | green fluorescent protein |
| kb | kilo base pair |
| L | litre |
| M | molar |
| min | minute |
| ml | milli litre |
| mm | milli metre |
| mM | milli molar |
| MQ | Milli Q water |
| nm | nano metre |
| °C | degree celsius |
| PCR | polymerase chain reaction |
| rpm | revolutions per minute |
| UV | ultra violet |
| V | volt |
| w/v | weight per volume |
| wpi | weeks post inoculation |
| WT | wild type |

Chapter 1: Introduction

1.1. Dothistroma needle blight disease

1.1.1. Incidence of the disease

Dothistroma needle blight (DNB) is an important foliar disease of pine species all over the world (Bradshaw, 2004; Watt et al., 2011c) and has been reported to occur in America, Africa, Europe, Asia and Oceania (Bulman et al., 2008; Watt et al., 2009) as shown in Fig. 1.1. Research reports suggest the incidence of DNB is spreading in Europe such as in Finland (Muller et al., 2009), Estonia (Hanso and Drenkhan, 2008) and Norway (Solheim and Vuorinen, 2011).

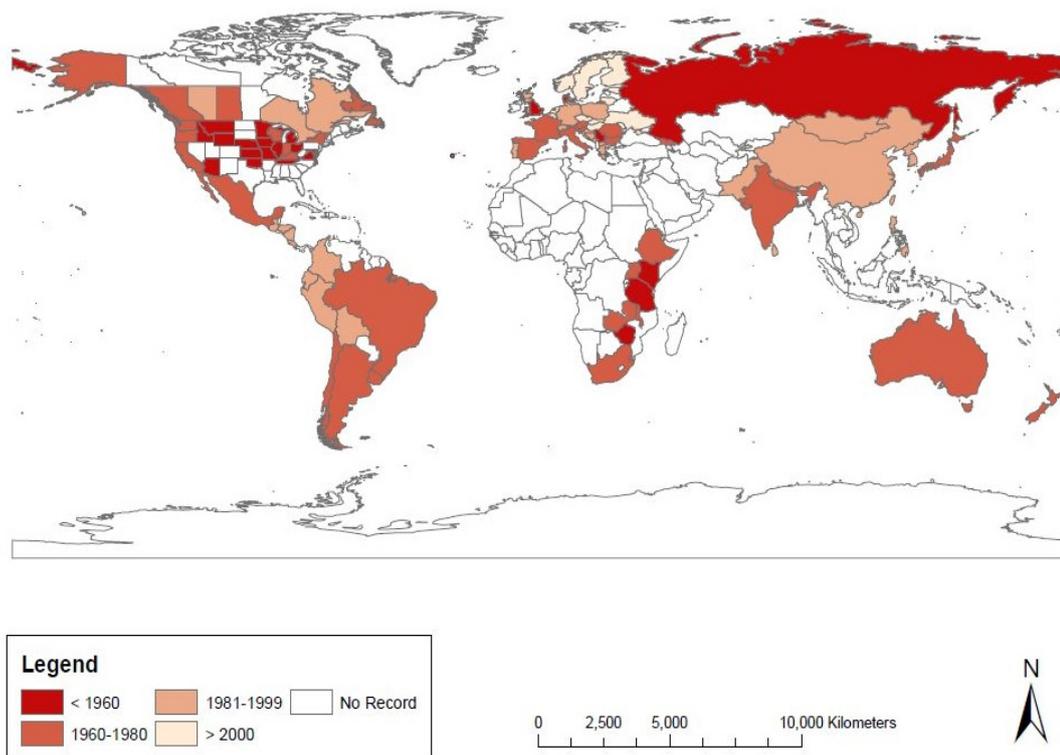


Fig. 1.1. Location of *Dothistroma* spp. on pines throughout the world

World map showing country distribution, colour coded by date of first record (pre-1960, 1960-1980, 1981-2000, 2001-present) (courtesy Bulman et al., 2013)

DNB outbreaks have been observed in various parts of the world since the 1950's (Bulman et al., 2008). In the early 1960's severe outbreaks were noticed in *Pinus*

radiata in East Africa, New Zealand and Chile (Gibson, 1974) and commercial planting of *P. radiata* was subsequently abandoned in East Africa (Gibson, 1974). Outbreaks were also noticed in the late 1990's and early 2000's in British Columbia (Woods et al., 2005) and some areas of Europe (Brown, 2005) with symptoms ranging from premature defoliation to complete death of trees.

Predictions of the future spread of this disease have been made using climate models (Watt et al., 2011b; Watt et al., 2009). Using over seven thousand disease observations throughout Europe, South America and Oceania, it was found that DNB could occur from sub-arctic through temperate, Mediterranean, continental, subtropical to dry tropical regions. The low level of disease detection in some areas was attributed to lower availability of susceptible hosts (Watt et al., 2009).

Although this disease occurs in a wide range of climatic conditions, the pathogen thrives best in areas with high levels of rainfall. Woods (2011) analysed four decades of weather data and linked it with DNB outbreaks in British Columbia. He found that an increase in summer precipitation correlated with the outbreaks of DNB, leading to the hypothesis that climate change has contributed to DNB epidemics.

Symptoms of DNB were first noticed in New Zealand in 1962 and the pathogen, *Dothistroma septosporum* was identified in 1964 (Gilmour, 1967) in the North Island near Tokoroa. By the late 1960's this disease had spread through most of the North Island (Fig. 1.2, left). In the South Island, it was discovered in Nelson in 1966 and spread through this island by the late 1970's (Bulman et al., 2008). Most of New Zealand is suitable or optimal for DNB especially the areas with high rainfall (> 5000 mm/annum) (Watt et al., 2009). Spatial prediction of DNB severity (Watt et al., 2011a) showed a wide range of severity predictions over New Zealand (Fig. 1.2, right),

including high levels in the central North Island region, which is one of the main commercial forest areas in New Zealand.

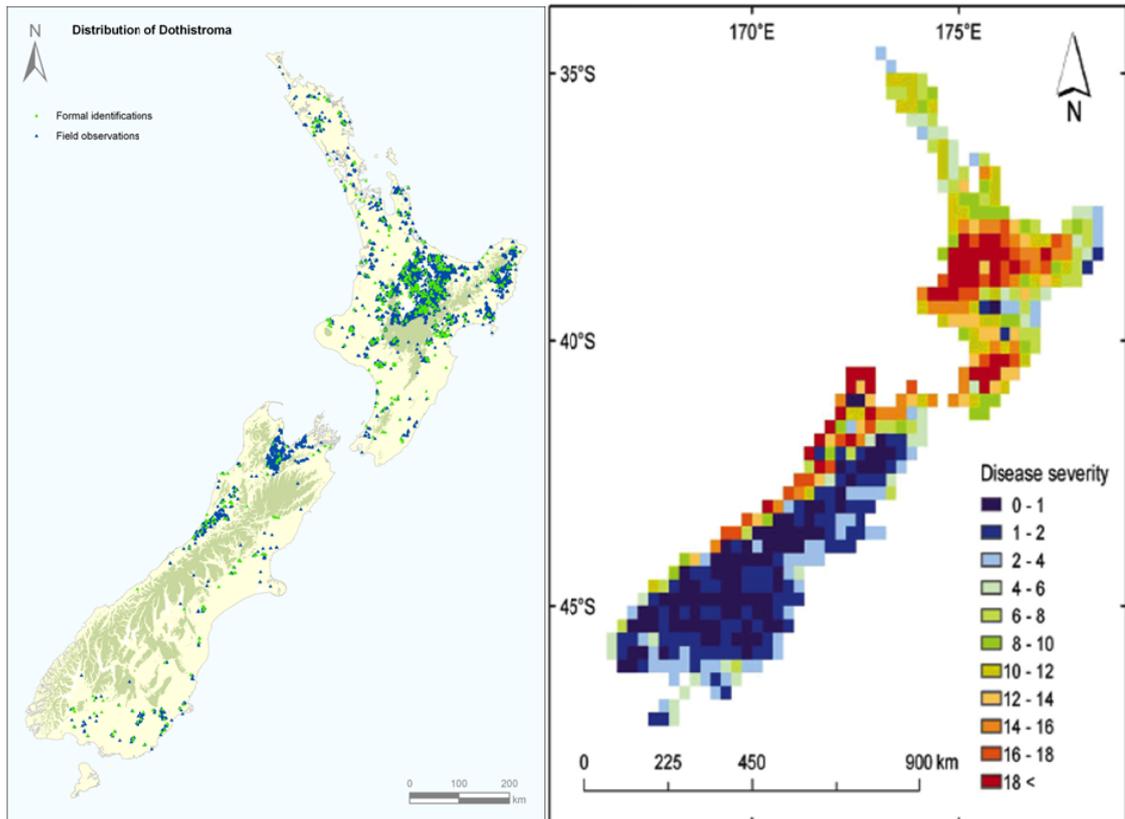


Fig. 1.2. Distribution and prediction of dothistroma needle blight severity in New Zealand.

Map showing distribution of Dothistroma in New Zealand where green and blue indicates formal identifications and field observations respectively (left). Map showing spatial variation of regions suitable for dothistroma needle blight over the whole New Zealand, based on climate modelling, with the potential disease severity in a scale of 0 (no disease) to >18 (severe disease) (right) (courtesy forest health database record 2008; <http://www.nzffa.org.nz/farm-forestry-model/the-essentials/forest-health-pests-and-diseases/> found on 18 May 2014) and Watt et al (2011a)).

Forestry is one of the most important components of the New Zealand economy and contributes \$3.2 billion export revenue each year (www.ffr.co.nz accessed on 16 December, 2013). Economic losses due to DNB disease have been estimated as \$19.8 million/annum when all costs of control and reduced tree growth are included (Watt et al., 2011a).

1.1.2. Dothistroma needle blight symptoms

Dothistroma needle blight symptoms usually first appear on lower needles of pines as yellow spots, these later encircle the needle and widen to a necrotic band (Fig. 1.3). Diseased needles often also show dead tips and a green base (Bradshaw, 2004; Gadgil, 1967; Shain and Franich, 1981). Due to accumulation of dothistromin (Bassett et al., 1970) characteristic red bands are then seen on needles. In later stages, black fruiting bodies appear on the red part of the needle (Fig. 1.3), arranged in rows, parallel to the long axis of the needle.

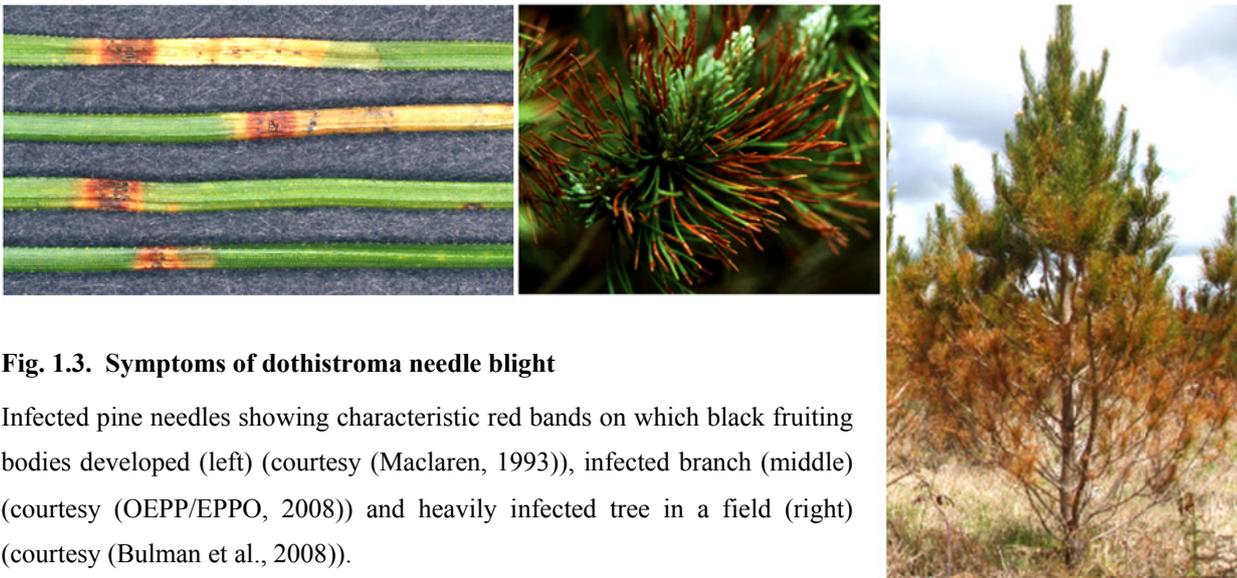


Fig. 1.3. Symptoms of dothistroma needle blight

Infected pine needles showing characteristic red bands on which black fruiting bodies developed (left) (courtesy (Maclaren, 1993)), infected branch (middle) (courtesy (OEPP/EPPO, 2008)) and heavily infected tree in a field (right) (courtesy (Bulman et al., 2008)).

1.1.3. Dothistroma needle blight and its hosts

Over 80 pine species are hosts of *Dothistroma* spp. but they differ in levels of susceptibility (Bulman et al., 2004; Ivory, 1968; Watt et al., 2009). For some species such as *P. radiata*, natural stands do not appear to be infected, but when planted as an exotic outside of its native range shows high susceptibility (Watt et al., 2009). *P. radiata* is the predominant commercial forest species in New Zealand and Chile, where it is planted as an exotic, and therefore its susceptibility to DNB is of serious concern.

In addition to *Pinus* species, other conifers such as *Pseudotsuga menziesii* (Douglas fir) (Mirb.) Franco (Dubin and Walper, 1967), *Larix deciduas* Mill (Bassett, 1969) and some species of *Picea* (Norway spruce) (Watt et al., 2009) have been reported with DNB infections.

1.1.4. Management of dothistroma needle blight

In New Zealand DNB is mainly controlled by spraying copper fungicides. It can also be controlled by avoidance of planting susceptible hosts, or stand management such as pruning and thinning (to reduce inoculum load) (Bradshaw, 2004; Bulman et al., 2013; Pas et al., 1984). The effect of some biological control agents has been evaluated in laboratory conditions but not tested in the forest (McDougal et al., 2011a).

Selection and breeding for host resistance has the potential to manage DNB and has been the topic of much research in *P. radiata* (Carson, 1989; Devey et al., 2004). In New Zealand, variation in resistance was found among the *P. radiata* population and was shown to be polygenic (quantitative) and to have moderately high heritability (Wilcox, 1982). In a later study, four quantitative trait loci (QTL) associated with DNB resistance in *P. radiata* were found (Devey et al., 2004). In Australasia DNB resistance is an important phenotype in breeding programmes and all commercial seed carries a rating for level of Dothistroma Resistance (Ivkovic et al., 2010) but the genetic mechanisms of DNB resistance in pine are still unknown (Bulman et al., 2013). The availability of a reliable laboratory-based pathogenicity assay will help towards selection of resistant cultivars as well as towards research aimed at understanding resistance mechanisms.

1.2. Dothistroma pathogens cause dothistroma needle blight

Two species of *Dothistroma*, *D. septosporum* and *D. pini*, cause dothistroma needle blight (DNB) disease on pine (Barnes et al., 2004). These causal agents belong to the order Capnodiales, within the Dothideomycetes class in the division of Ascomycotina. The teleomorph of *D. septosporum* was previously named *Scirrhia pini* Funk and Parker, and then was renamed as *Mycosphaerella pini* E. Rostrup apud Monk. However the teleomorphic name of *M. pini* is no longer in use under the revised 'one fungus-one name' nomenclature and the sexual stage of *D. pini* has not been identified yet (Barnes et al., 2004; Bradshaw, 2004; OEPP/EPPO, 2008).

The *Dothistroma* pathogens were once divided into different varieties based on conidial length (Thyr and Shaw, 1964). However, this classification based on a single morphological trait was questioned (reviewed in (Bradshaw, 2004)) and it became consolidated to one species. Subsequently, a revised classification was suggested based on DNA sequences of the ribosomal ITS region, β -tubulin, and elongation factor 1- α genes (Barnes et al., 2004) and led to the two separate species (*D. septosporum* and *D. pini*) accepted today. This classification was supported by other molecular studies based on mating type genes (Groenewald et al., 2007) and toxin biosynthetic genes (McDougal et al., 2011b).

The distribution of these two pathogens has been analysed, particularly for *D. septosporum* which is the most intensely studied species. *D. septosporum* is distributed over most parts of the world (Barnes et al., 2004), and was recently reported in Greece for the first time (Tsopelas et al., 2013). Diversity of *D. septosporum* varies from location to location. For example, high levels of *D. septosporum* genetic diversity (based on molecular markers) were found in Estonia, Finland, the Czech Republic, Poland (Drenkhan et al., 2013; Kraj and Kowalski, 2013). The sexual stage has been found in Canada and mainland Europe along with both mating types (Bradshaw, 2004;

Groenewald et al., 2007). However, in the southern hemisphere, particularly in Australia and New Zealand, only one mating type (mating type 2) has been detected. This corresponds with absence of the sexual stage in these countries, and the presence of a clonal population (probably multiplied from a single isolate) of *D. septosporum* in New Zealand (Hirst et al., 1999).

In contrast to *D. septosporum*, *D. pini* is only distributed in the northern hemisphere according to current records. Although it was thought to be limited to north-central USA (Barnes et al., 2004), it was subsequently identified in the Ukraine, south-west Russia (Barnes et al., 2008), France (Ioos et al., 2010), Slovenia (Piskur et al. 2013) and even found to co-occur in one needle with *D. septosporum* in Hungary (Barnes et al., 2011). Although information on the distribution of both *Dothistroma* pathogens is available their centre of origin remains unknown.

1.3. Parasitic phases of plant-fungal interactions

1.3.1. Different life styles in plant-microbe interactions

Depending upon feeding behaviour pathogenic fungi are classified as biotrophic, necrotrophic or hemi-biotrophic (Hammerschmidt, 2006; Newton et al., 2010; Oliver and Ipcho, 2004; Perfect and Green, 2001). Biotrophic fungi grow and reproduce only on living tissues and many of them cannot be cultured extensively in culture. Some biotrophic fungi produce special types of fungal structures called haustoria to collect nutrition from the host cell, secrete limited amounts of lytic enzymes and thus cause little damage to the host. Causal agents of powdery mildews, rusts and downy mildews belong to this group (Dean et al., 2012; Rafiqi et al., 2012).

On the other hand, necrotrophic fungi obtain their food from dead tissue. In most of the cases, necrotrophic fungi release toxins and/or cell wall degrading enzymes to kill

the host cell. *Alternaria brassicicola*, *Fusarium graminearum*, *Fusarium oxysporum*, *Stagonospora nodorum* and *Pyrenophora tritici-repentis* are examples of necrotrophic fungi (Dean et al., 2012; Faris et al., 2010; Ohm et al., 2012).

Hemi-biotrophic microbes have a combination of the above two phases. These types of fungi pass their initial life cycle as biotrophs without producing any symptoms, followed by a necrotrophic phase at the end. Fungi in the genus *Colletotrichum* are well known hemi-biotrophs (Bhadauria et al., 2013; Dean et al., 2012; Perfect and Green, 2001; Vargas et al., 2012).

Besides these morphological and biochemical aspects of plant-fungal interactions, some molecular characterisation of these pathogenic life styles has been conducted in recent years that is described in the following sections.

1.3.2. Molecular aspects of biotrophic plant-fungal interactions

Biotrophic fungal pathogens adopt very complex mechanisms for their survival at the early stages of infection. They secrete a variety of molecules to repress the host defense mechanisms and to obtain food from hosts (Koeck et al., 2011; McDowell, 2013). However, biotrophic fungi can induce a hypersensitive response (HR) by plant cell death in incompatible interactions. This is controlled by specific (gene for gene) resistance genes and involves salicylate-dependent defence pathways (Oliver and Ipcho, 2004). In biotrophic interactions, the gene for gene theory was expanded to a zigzag model described by Jones and Dangl (2006). In this model pathogen associated molecular patterns (PAMPs) are recognised by host pattern recognition receptors (PRRs), leading to PAMP triggered immunity (PTI) that inhibits further colonisation. The pathogen secretes effector molecules to overcome or suppress PTI, resulting in effector triggered susceptibility (ETS). If an effector is recognised by specific host

resistance proteins it results in effector triggered immunity (ETI) which often includes a hyper-sensitive response (HR) (Catanzariti and Jones, 2010; de Wit et al., 2009). The pathogen can overcome ETI by loss or modification of effectors which is an example of the continuous arms race between host and pathogen as shown in Fig. 1.4.

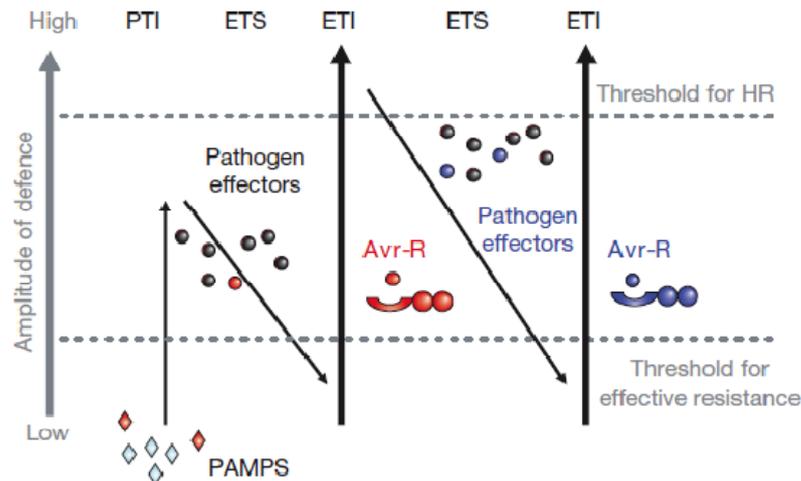


Fig. 1.4. Zigzag model of plant immune system

In the first step of this model, plants detect PAMPS (red diamond) via PRR to trigger PTI. In the next step, the pathogen secretes effectors that interfere with PTI and result in ETS. In the following phase, an effector (red molecule) is recognised by an R-protein activating ETI. In the last step, pathogens that have lost red effector molecules escape recognition but secrete a different molecule (blue) to suppress ETI. When this new effector is recognised by another host R gene again it results in ETI. PAMP- pathogen associated molecular patterns, PTI-PAMP triggered immunity, PRR-pattern recognition receptors, ETI-effector triggered immunity, ETS- effector triggered susceptibility, R- resistant. (Adapted from Jones and Dangl (2006)).

From the characterised fungal effectors of biotrophs, it is known that these molecules are secreted through the endoplasmic pathway into the apoplastic space between the fungus and host cells. Apoplastic effectors function in the extracellular space; whilst others are secreted into host cells (cytoplasmic effectors) and target host cell components (Koeck et al., 2011; Rafiqi et al., 2012).

Every effector has a specific role and its presence varies from pathogen to pathogen. Some effectors of the biotrophic pathogen *Cladosporium fulvum*, causing leaf

mould of tomato, are well studied. It has many types of race-specific Avr proteins and five extracellular proteins (Ecps). Functions of all these effectors are not known yet. However, Avr2 acts as a cysteine protease inhibitor (Rooney, 2005; Shabab et al., 2008) and Avr4 protects fungi against plant chitinase (Burg et al., 2003). Though the effector Ecp6 has the same function as Avr4, due to its involvement in scavenging of fungal chitin, it prevents recognition by PAMP receptors (Bolton et al., 2008).

1.3.3. Molecular aspects of necrotrophic plant fungal interactions

Many necrotrophic fungi use host selective toxins (HSTs) as their main weapons. These fungi induce defence by jasmonate and ethylene dependent defence pathways in their host (Hammond-Kosack and Parker, 2003) and host resistance is generally controlled by quantitative resistance genes (Oliver and Ipcho, 2004). Fungi under this category do not follow the gene for gene theory, instead some follow an inverse gene for gene model as described by Friesen et al. (2007). This model suggests that a HST interacts with a susceptibility gene to result in disease.

With the discovery of effectors, it was found that some necrotrophic HSTs share some common properties on the basis of size and secretion with biotrophic effectors (Tan et al., 2010). Stergiopoulos et al. (2013) reviewed some necrotrophic effectors and their functions that have been characterised in *Dothideomycete* fungi (Table 1.1). Of these the best characterised effector is PtrToxA in *P. tritici-repentis* (Ciuffetti et al., 2010). It is a secreted protein, small in size, causes necrosis in wheat (Tan et al., 2010) and is essential for pathogenicity (Manning et al., 2008). A similar effector is also found in *S. nodorum*, known as SnToxA, having the same activity and being required for *S. nodorum* blotch in wheat (Friesen et al., 2007). Examples with functions of more HSTs/effectors are cited in section 1.4.2.

Table 1.1. Biological functions of some secondary metabolite and proteinaceous host selective toxins from *Dothideomycete* spp.

| Toxin | Organisms | Mode of action | Plant target |
|----------|-------------------------------------|--|---|
| PtrToxA | <i>Pyrenophora tritici-repentis</i> | <i>Tsn1</i> -mediated induction of PCD | Chloroplast ToxABP1 |
| PtrToxB | | Induction of PCD | Probably chloroplast |
| SnToxA | <i>Stagonospora nodorum</i> | <i>Tsn1</i> -mediated PCD induction | Chloroplast ToxABP1 |
| SnTox1 | | <i>Snn1</i> -mediated PCD induction | Probably chloroplast |
| SnTox2 | | <i>Snn2</i> -mediated PCD induction | Probably chloroplast |
| SnTox3 | | <i>Snn3</i> -mediated PCD induction | Unknown |
| SnTox4 | | <i>Snn4</i> -mediated PCD induction | Probably chloroplast |
| Victorin | <i>Cochliobolus victoriae</i> | PCD induction | LOV1:CC-NB-LRR disease resistant protein |
| PM-toxin | <i>Mycosphaerella zeae-maydis</i> | Mitochondrial activity disruption | Mitochondrial protein |
| T-toxin | <i>Cochliobolous heterostrophus</i> | Mitochondrial activity disruption | Mitochondrial protein |

PCD = Programmed cell death; Source: Stergiopoulos et al.(2013)

1.3.4. Molecular aspects of hemi-biotrophic plant fungal interactions

Hemi-biotrophic pathogens have both biotrophic and necrotrophic phases in their life cycle. Understanding of the transition from biotrophic to necrotrophic life style is very important. Recently a transcription analysis was conducted on well-characterised hemi-biotrophic pathogens in the genus *Colletotrichum* (Gan et al., 2013; O'Connell et al., 2012) to monitor gene expression during both biotrophic and necrotrophic phases *in planta*. Gan et al. (2013) found up-regulation of many small secreted proteins (SSP) and secondary metabolite (SM) genes during the biotrophic stage of infection caused by *C. orbiculare*. In contrast, up-regulation of cell wall degrading enzymes (CWDE) and proteases was noticed during the necrotrophic phase (Fig. 1.5).

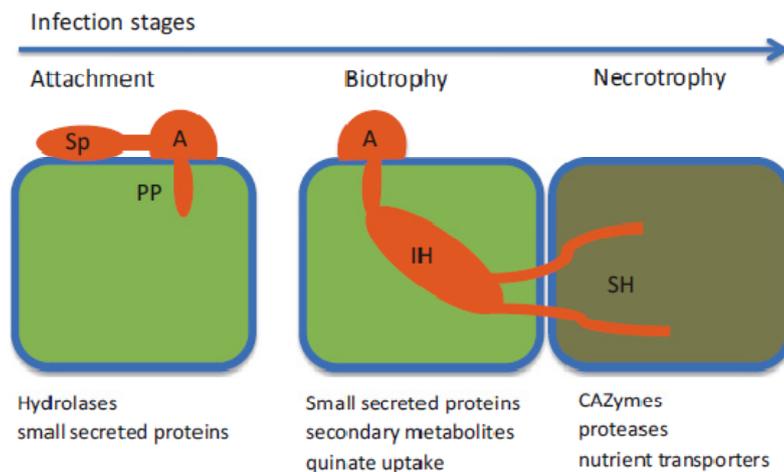


Fig. 1.5. Plant-microbe interaction of *Colletotrichum* fungi along with gene expression

A spore (Sp) germinates and forms an appressorium (A) that attaches to the host cell wall and develops a penetration peg (PP). Hydrolases and small secreted protein (SSP) genes facilitate penetration and manipulation of host cell respectively. During the biotrophic phase, intracellular hyphae (IH) secrete small secreted protein and secondary metabolite (SM) gene products. In the last stage at necrotrophy, secondary hyphae (SH) produce carbohydrate active enzymes (CAZymes), proteases and nutrient transporters. Adapted from (McDowell, 2013).

Although many molecular changes happen during transition between biotrophic to necrotrophic stage, what triggers this transition is not well understood (Koeck et al., 2011; Newton et al., 2010; Rafiqi et al., 2012). A gene named *CLTA1* (encodes a GAL4- like transcriptional activator, in *Colletotrichum* species) was reported to play a reprogramming role in hemi-biotrophs in switching from biotrophic to necrotrophic phase in common bean (Dufresne et al., 2000). More recently an effector ‘CtNUDIX’ (Colletotrichum truncatum Nucleoside Diphosphate linked to some other moiety X) has been characterised in *Colletotrichum truncatum* which was also expressed during transition from biotrophy to necrotrophy (Bhadauria et al., 2013).

Overall, effector molecules that function in both biotrophic and necrotrophic stages are the main basis of molecular interactions between micro-organisms and their hosts. Although these types of molecules were discovered some years back, most are

still not characterised fully. Effectors of *D. septosporum* have not been characterised yet, although homologues of *C. fulvum* *Avr4*, *Ecp2* and *Ecp6* genes have been identified in the *D. septosporum* genome (de Wit et al., 2012). Characterisation of the roles of these effectors requires the pathogen life style to be clearly identified in the host. The recently sequenced *D. septosporum* genome (de Wit et al., 2012) will help to characterise genes that are responsible for biotrophic and necrotrophic behaviour.

1.3.5. Fungi having a latent phase *in planta* and their quantification

In contrast to the above mentioned distinct bi-phasic fungal life style, for many diseases, a latent phase can occur where the pathogen is present in the host but growth is absent or very slow and it does not express any disease symptoms for a certain period. For example, *Sphaeropsis sapinea* in red pine (Stanosz et al., 2001), *Biscogniauxia mediterranea* in oak species (Luchi et al., 2005), *Colletotrichum acutatum* in strawberry (Debode et al., 2009), *Mycosphaerella graminicola* in cultivated wheat (Keon et al., 2007), and *Cyclaneusma minus* in *P. radiata* (Bulman et al., 2008). The majority of members of the genus *Mycosphaerella* are slow growing and most have a latent phase in their host (Goodwin and Kema, 2009). *Dothistroma* spp. are members of the *Mycosphaerella* genus (although the name *Mycosphaerella* is not in regular use for these species due to the introduction of one name one fungus nomenclature); as they are slow growing it is possible they have a latent phase *in planta*.

Latent infections of fungal pathogens can be detected by the use of real time PCR technology in many plants. For instance, latent infections of *C. acutatum*, *M. graminicola*, *B. mediterranea* could be detected in strawberry (Debode et al., 2009), wheat (Keon et al., 2007) and oak (Luchi et al., 2005) respectively using real time PCR.

No research has been conducted on the quantification of *D. septosporum* to determine if it has a latent phase *in planta*.

1.3.6. Identification of life styles of plant-microbe interactions

Identification of biotrophic and necrotrophic phases can be conducted by histopathological studies. Viable and dead host cells are identified with the help of staining and microscopy. Shen et al. (2001) differentiated the biotrophic and necrotrophic phases of *Colletotrichum destructivum* on tobacco by using Neutral red (0.01%) as an indicator of viability. Due to plasmolytic activity viable cells accumulate red colour, whilst host cells killed by the presence of fungal hyphae do not turn red. Double staining methods can also be used to differentiate viable and dead cells (Jones and Senft, 1985). In this study the double stain was a mixture of Fluorescein Diacetate (FD) and Propidium Iodide (PI). In the staining method, viable cells become green (under blue light) due to accumulation of FD and dead cells are bright red due to accumulation of PI. These stains have not previously been tested to identify the trophic phase of DNB.

1.3.7. Use of green fluorescence protein in fungal biology

Green fluorescence protein (GFP), a bioluminescent protein, was isolated from jellyfish (*Aequorea victoria*) in 1992 (Prasher et al., 1992), and is an important tool for visualising fungal growth *in planta*. It consists of a 27 kDa protein having 238 amino acid residues. This protein fluoresces under UV or blue light in the presence of oxygen. It absorbs light at maxima of 395 and 475 nm and emits at maximum of 508 nm. It has been used as a reporter and marker (for example, in gene regulation studies) in many prokaryotes and eukaryotes (Lorang et al., 2001).

In conventional microscopy, the host tissue needs to be cleared and stained to monitor host-fungal interactions. One main advantage of using *gfp*-labelled fungi is that

these steps are not required, reducing time and cost. In addition, with the non-destructive sampling it is possible to monitor host-fungal interactions from initial to final stages (Maor et al., 1998).

In filamentous fungi, GFP expression requires a *gfp* gene variant that can be translated efficiently, and a fungal promoter. Lorang et al. (2001) reported that *sgfp* driven by the *ToxA* gene promoter from *P. tritici-repentis* is widely used in many fungal genera. More recently hemi-biotrophic features of *Colletotrichum graminicola* were studied in maize using *gfp*-labelled fungi (Vargas et al., 2012). In DNB, several *gfp*-labelled *D. septosporum* have been tested in culture and on pine needles (Schwelm et al., 2009); however, no studies have been conducted using these isolates to determine the life style of *D. septosporum*.

1.4. Microbial toxins and plant disease

1.4.1. Introduction and classification of toxins

Microbial toxins are biochemical products produced by microbes that can interfere with the normal metabolic functions of a host. In general, microbial toxins are poisonous and kill or damage the cells of the host even in low concentrations (Agrios, 2005). Some fungal toxins are pathogenicity factors. For example *Cercospora kikuchii* produces cercosporin which is required for soybean disease, as shown by the failure of cercosporin-deficient mutants to produce disease (Upchurch et al., 1991). In contrast, some toxins are not essential for pathogenicity; for example, disruption of the cerato-ulmin gene in *Ophiostoma ulmi* does not affect its ability to cause Dutch elm disease (Bowden et al., 1994; Bowden et al., 1996). Depending upon the specificity of their targets, microbial toxins are classified as host selective toxins (HSTs) or non-host selective toxins (NHSTs).

1.4.2. Host-selective toxins and their roles

Host-selective toxins (HSTs) are microbial products from pathogens that are toxic only on the host of that pathogen with little or no toxicity on non-host species (Agrios, 2005; Friesen et al., 2007; Friesen et al., 2008; Wolpert et al., 2002). Many of the fungi belonging to the Pleosporales order of the class Dothideomycetes produce HSTs (Friesen et al., 2008) and various different mechanisms of action have been determined as previously mentioned in Table 1.1. Some HSTs are the effectors of necrotrophic pathogens where disease occurs in the host when the host has a specific susceptibility gene and localised cell death is triggered, such as the SnTox effectors in the wheat pathogen *S. nodorum* (Francki, 2013; Friesen et al., 2012; Friesen and Faris, 2010; Friesen et al., 2008). Other examples include: HC-toxin (released by *Cochliobolus carbonum* causing leaf spot of maize) that acts in the nucleus to inhibit histone deacetylases; AAL-toxin (released by *Alternaria alternata* causing canker of tomato) that inhibits ceramide synthase and leads to programmed cell death (PCD) (Spassieva et al., 2002); T-toxin (released by *C. heterostrophus* causing leaf blight in maize) that is thought to cause pore formation leading to loss of ion gradients across the mitochondrial membrane and cell death (Turgeon and Baker, 2007); victorin (released by *C. victoriae* causing blight in oat) that leads to cleavage of RUBISCO and subsequent PCD (Markham and Hille, 2001); and Ptr ToxA, Ptr ToxB (released by *P. tritici-ripentis* causing tan spot of wheat) that leads to a light dependent accumulation of reactive oxygen species (ROS) (Manning et al., 2009; Pandelova et al., 2012). Ptr ToxC is another toxin released by this fungus (*P. tritici-ripentis*) whose function is not characterised yet (Faris et al., 2013).

1.4.3. Non-host selective toxins and their roles

In contrast to host-selective toxins, non-host selective toxins (NHSTs) have wider effects on many hosts. Examples of NHSTs include tabtoxin, phaseolotoxin, tagetitoxin, tentoxin, zearalenone, cercosporin, elsinochrome, rubellin and sirodesmin PL that cause various biochemical changes in host species (Agrios, 2005; Meiss et al., 2008; Souza et al., 2012; Stergiopoulos et al., 2013).

Sometimes NHSTs play a role in virulence. Sirodesmin PL, a NHST produced by *Leptosphaeria maculans* which causes black leg disease of canola, is a virulence factor. A cluster of 18 genes are co-regulated during the synthesis of this toxin and disruption of the non-ribosomal peptide synthetase gene (*SirP*) blocks its biosynthesis. The knockout mutant of *SirP* showed less antifungal and antibacterial activity *in vitro* compared to wild type and also produced fewer lesions *in planta* (Elliott et al., 2007). However zearalenone, a NHST produced by *F. graminearum*, is not required for root infection in barley (Lysoe et al., 2006), and production of toxin by the pathogen is not correlated with pathogen virulence (Acosta et al., 2010). Dothistromin is another example of a non-host selective toxin. It is toxic to a wide range of cell types; for example it cause lysis of human blood red corpuscles, beet root tissue, inhibits growth of *Trigonella foenum-graecum* (a medicinal herb) and is toxic to *Artemia salina* (brine shrimp) (Stoessl et al., 1990). Like sirodesmin (Elliott et al., 2007) and cercosporin (Choquer et al., 2007), dothistromin might have a virulence impact on DNB which has not been identified yet.

1.5. Dothistromin toxin

Dothistromin was first isolated from culture broths of *D. septosporum* (Bassett et al., 1970). It is a mixture of two closely related chemical compounds, dothistromin

(C₁₈H₁₂O₉) and deoxydothistromin (C₁₈H₁₂O₈), of which dothistromin is more abundant at about 80-90% (Gallagher and Hodges, 1972). It is a polyketide derived mycotoxin and its difuranoanthraquinone structure was determined by mass spectrometry and nuclear magnetic resonance (NMR) (Bassett et al., 1970). Dothistromin is chemically similar to the aflatoxin precursor versicolorin B (Fig. 1.6) with a common furobenzofuran moiety (Bradshaw, 2004; Gallagher and Hodges, 1972).

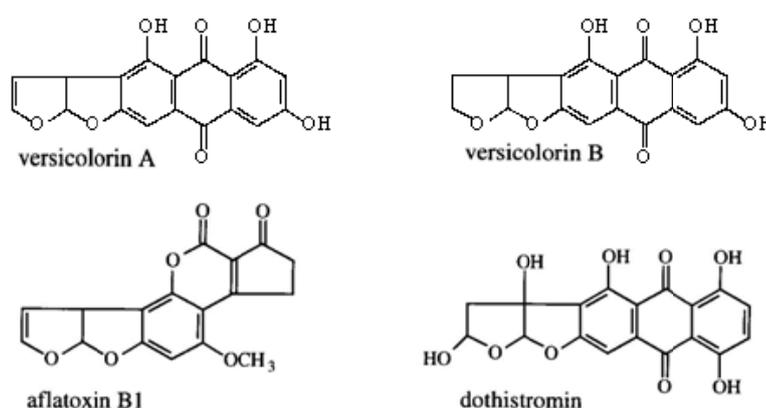


Fig. 1.6. Similarity of chemical structures of dothistromin and aflatoxin precursors versicolorin A and versicolorin B

1.5.1. Biosynthesis of dothistromin *in vitro* and *in planta*

Dothistromin is synthesised *in vitro* where it appears as dark red-brown pigment around the fungal colony in agar plates. However, the exact colour of dothistromin in plates varies depending upon the type of media used for culturing (Bradshaw and Zhang, 2006). Dothistromin is also produced in liquid cultures (Bradshaw et al., 2000; Bradshaw and Zhang, 2006). This toxin is not only produced by *D. pini* and *D. septosporum*, but also by some species of *Cercospora* (e.g. *C. ferruginea*, *C. fusa*, *C. microsora*, *C. rosicola*, *C. rubi*, *C. smilacis*), *Mycosphaerella* (e.g. *M. laricina*) and *Passalora* (e.g. *P. arachidicola*) (Assante, 1985; Assante et al., 1977; Stoessl, 1984). Dothistromin production by species other than *Dothistroma* spp. has only been detected in culture but not tested *in planta*.

Synthesis of dothistromin by *Dothistroma* species is highly variable in culture. In a collection of more than 25 *Dothistroma* isolates from eight different countries, Bradshaw et al. (2000) found dothistromin levels ranging from <1 µg/mg dry weight of mycelium to >100 µg/mg. Variations were also noticed even in a population of *D. septosporum* from New Zealand, where all isolates tested so far appear to have been derived from a single clone (Hirst et al., 1999). The highest dothistromin production in New Zealand was >70.0 µg/ml compared to its lowest production <1.0 µg/ml. However, whether these isolates show similar variations of dothistromin production *in planta* during DNB pathogenesis has not been tested. Slow growth rate, morphological instability or attenuation in culture (Gallagher, 1971) has been predicted to be the cause of these types of variations.

Levels of dothistromin production also depend upon culturing conditions. Bradshaw et al. (2000) showed that dothistromin production in shake flask-grown cultures was much higher than in static tubes. Poor aeration was suggested to be the cause of low levels of dothistromin in tubes. Culture media also play an important role in biosynthesis of dothistromin. Higher levels of dothistromin were produced in glucose-rich than glucose-poor conditions, but lower levels in medium with ammonium instead of nitrate as nitrogen source (Bradshaw et al., 2002). In more complex media higher levels of dothistromin production were supported by a rich yeast-extract/malt extract medium with glucose (dothistroma medium, DM) compared to potato dextrose broth (Schwelm et al., 2008) whilst a minimal medium with glucose and pine needle extract (PMMG) supported even lower levels (Chettri et al., 2013). The basis for these differences is not known. From this work it is clear that production of dothistromin varies between isolates, and is affected by growth conditions at least in culture.

As part of a study of dothistromin biosynthesis in culture, the timing of dothistromin production was monitored (Schwelm et al., 2008). Unexpectedly, it was found that dothistromin biosynthesis occurs at an early growth stage (4-6 dpi) by *D. septosporum* and declines when growth reaches the exponential phase (Fig. 1.7). This early biosynthesis was supported by expression studies of dothistromin genes that were shown to be highly expressed during the same early time points. Early expression of dothistromin genes was confirmed visually in culture plates using a *D. septosporum* transformant containing a *gfp* gene regulated by the *DsDotA* promoter. The results showed *gfp* expression around the margins of the colony, which suggested that the *DsDotA* gene is expressed mainly in the younger growing hyphae (Fig. 1.8) (Schwelm et al., 2008). This finding is unusual compared to secondary metabolite (SM) production in most other filamentous fungi. For example in *Aspergillus flavus* and *A. parasiticus* SMs such as aflatoxin are mainly produced during late exponential and stationary phase (Cleveland and Bhatnagar, 1990; Trail et al., 1995). So far, this unusual feature of dothistromin biosynthesis occurring during a period of exponential fungal growth has not been tested *in planta*. However dothistromin is generally known to accumulate in lesions that occur at a late stage of the disease cycle *in planta*.

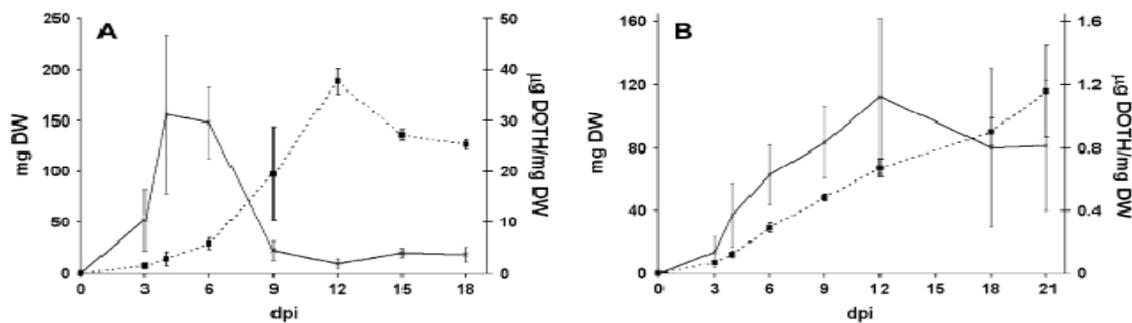


Fig. 1.7. Growth and dothistromin biosynthesis in liquid media by *Dothistroma septosporum*

Results are from growth in dothistroma broth (A) and potato dextrose broth (B). Fungal growth (mg DW) and dothistromin toxin production (µg/mg DW mycelium) are indicated by dotted and normal lines respectively. Values are mean ± standard deviation of 3 independent samples. dpi: days post inoculation. Figure reproduced from (Schwelm et al., 2008).

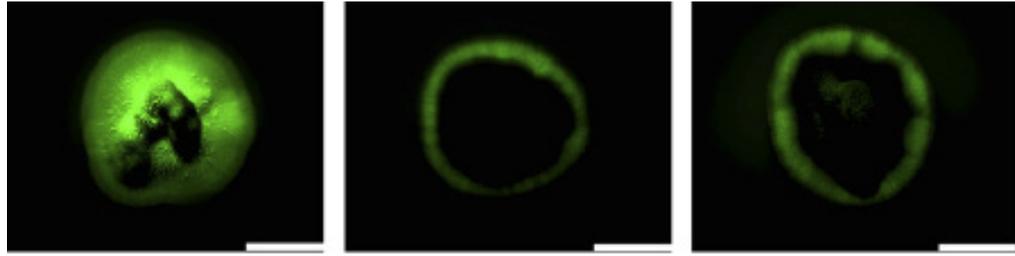


Fig. 1.8. *gfp* expression of *Dothistroma septosporum*

Twelve day old culture of *D. septosporum* transformant with constitutive *gfp* expression (*PgpdA::egfp*; left) shows fluorescence all over the colony, whilst those with regulated *gfp* expression, (*PDsDotA::egfp*; middle and right) show fluorescence only at the margin where young hyphae are actively growing. Size bar = 5 mm. Figure reproduced from (Schwelm et al., 2008).

Although dothistromin is found in diseased needles *in planta* (Bassett, 1972), its production is not well characterised. Gadgil (1967) speculated that dothistromin biosynthesis *in planta* by *D. septosporum* produces the characteristic red band symptoms on pine needles during DNB. *In planta* dothistromin assays have mainly been conducted on *P. radiata*. In naturally infected *P. radiata* a typical red-band lesion on a DNB symptomatic needle contains 1-10 µg of dothistromin (Shain and Franich, 1981).

Red bands can be induced artificially by injecting 10-100 ng of dothistromin (Shain and Franich, 1981). In this experiment lesions with red bands appeared within hours and high light intensity was necessary (Shain and Franich, 1981; Stoessl et al., 1990). While injected dothistromin produces DNB lesion-like symptoms, degradation of the injected toxin was also observed in artificially inoculated needles by 24 hours after its injection (Franich et al., 1986). Research was conducted to determine if there is a positive correlation between *in planta* dothistromin concentrations and DNB lesion length but no correlation was found (Debnam and Narayan, 1994). However, no studies were conducted to determine how dothistromin level change *in planta* over the time course of DNB.

Dothistromin induces host responses in pine needles. It was reported that due to injection of dothistromin, *P. radiata* needles accumulate benzoic acid as a type of phytoalexin, which is very toxic to needle mesophyll cells as well as fungi (Franich et al., 1986). This accumulation depends on age of tissue and possibly on season, being higher in new emerging needles than in one year old needles. Cells adjacent to the induced lesion were most active in accumulating benzoic acid. A linear relationship was noticed between lesion length and benzoic acid accumulation in an experiment in which dothistromin was artificially injected. In addition to benzoic acid accumulation, increased lignification was also found in pine needle tissue of artificially injected lesions as a host response; this was found at the edges of DNB lesions that separate the live part of the needle from the necrotic region. Furthermore, a correlation was noticed between dothistromin concentration and ethylene production in artificially induced lesions as a result of host response (Shain and Franich, 1981). Host defenses were also investigated by treating *P. radiata* cell suspensions with purified cell wall fractions from *D. septosporum* (previously called *D. pini*) (Hotter, 1997). A rapid transient oxidative burst, accumulation of phenolics and induction of phenylpropanoid biosynthesis were detected in *P. radiata* cells within 20-40 minutes after the treatment.

1.5.2. Genetics of dothistromin biosynthesis

The availability of the *D. septosporum* genome sequence facilitated understanding of the genetics of dothistromin biosynthesis. It was reported that chromosome 12 (1.3 Mb in size) carries dothistromin genes at six different loci in an unusual fragmented arrangement (de Wit et al., 2012). More than 20 genes are predicted to be required for dothistromin biosynthesis (Fig. 1.9) (Chettri et al., 2013). Of these, 13 are named as core genes based on their similarities to aflatoxin genes and are listed in

Table 1.2 with their predicted functions. Out of these core genes, functional characterisation has been carried out on *PksA* (Bradshaw et al., 2006), *VbsA* (Zhang et al., 2007), *HexA*, *AdhA* (Chettri et al., 2013), *Ver1* (previously called *dotA*) (Bradshaw et al., 2002) by gene replacement, which showed that these genes are required for dothistromin production. Gene expression studies with the whole set of genes confirmed that they are co-regulated; mutants lacking the pathway regulator gene *DsAflR* gene, an ortholog of the aflatoxin pathway regulator *aflR*, showed down regulation of all dothistromin core genes (Chettri et al., 2013).

Table 1.2. Characteristics of 'core' dothistromin genes of *Dothistroma septosporum*

| <i>Ds</i> core genes | AF ortholog <i>Ap</i> | ST ortholog <i>An</i> | Predicted function |
|----------------------|-----------------------|-----------------------|---------------------------------|
| <i>HexA</i> * | <i>aflA</i> | <i>stcJ</i> | Fatty acid synthase |
| <i>HexB</i> | <i>aflB</i> | <i>StcK</i> | Fatty acid synthase |
| <i>PksA</i> * | <i>aflC</i> | <i>StcA</i> | Polyketide synthase |
| <i>HypC</i> | <i>aflZ</i> | <i>StcM</i> | Anthrone oxidase |
| <i>Nor1</i> | <i>aflD</i> | <i>StcE</i> | NAD(P) reductase |
| <i>AvnA</i> | <i>aflG</i> | <i>StcF</i> | P450 monooxygenase |
| <i>AdhA</i> * | <i>aflH</i> | <i>StcG</i> | Alcohol dehydrogenase |
| <i>CypX</i> * | <i>aflV</i> | <i>StcB</i> | P450 monooxygenase |
| <i>AvfA</i> | <i>aflI</i> | <i>StcO</i> | NAD(P) reductase |
| <i>MoxY</i> | <i>aflW</i> | <i>StcW</i> | Flavin-binding monooxygenase |
| <i>Est1</i> | (<i>AflJ</i>) | (<i>StcI</i>) | Esterase (alpha/beta hydrolase) |
| <i>VbsA</i> * | <i>aflK</i> | <i>StcN</i> | VerB synthase (cyclase) |
| <i>VerB</i> | <i>aflL</i> | <i>stcL</i> | Desaturase (P450 monooxygenase) |
| <i>Ver1</i> * | <i>aflM</i> | <i>stcU</i> | NAD(P) reductase |

Ds- *Dothistroma septosporum*, AF- aflatoxin, ST- sterigmatocystin, *Ap*- *Aspergillus parasiticus*, *An*- *Aspergillus nidulans*, ‘*’ – indicates the genes that are functionally characterised by gene knock out. Table adapted from (Chettri et al., 2013)

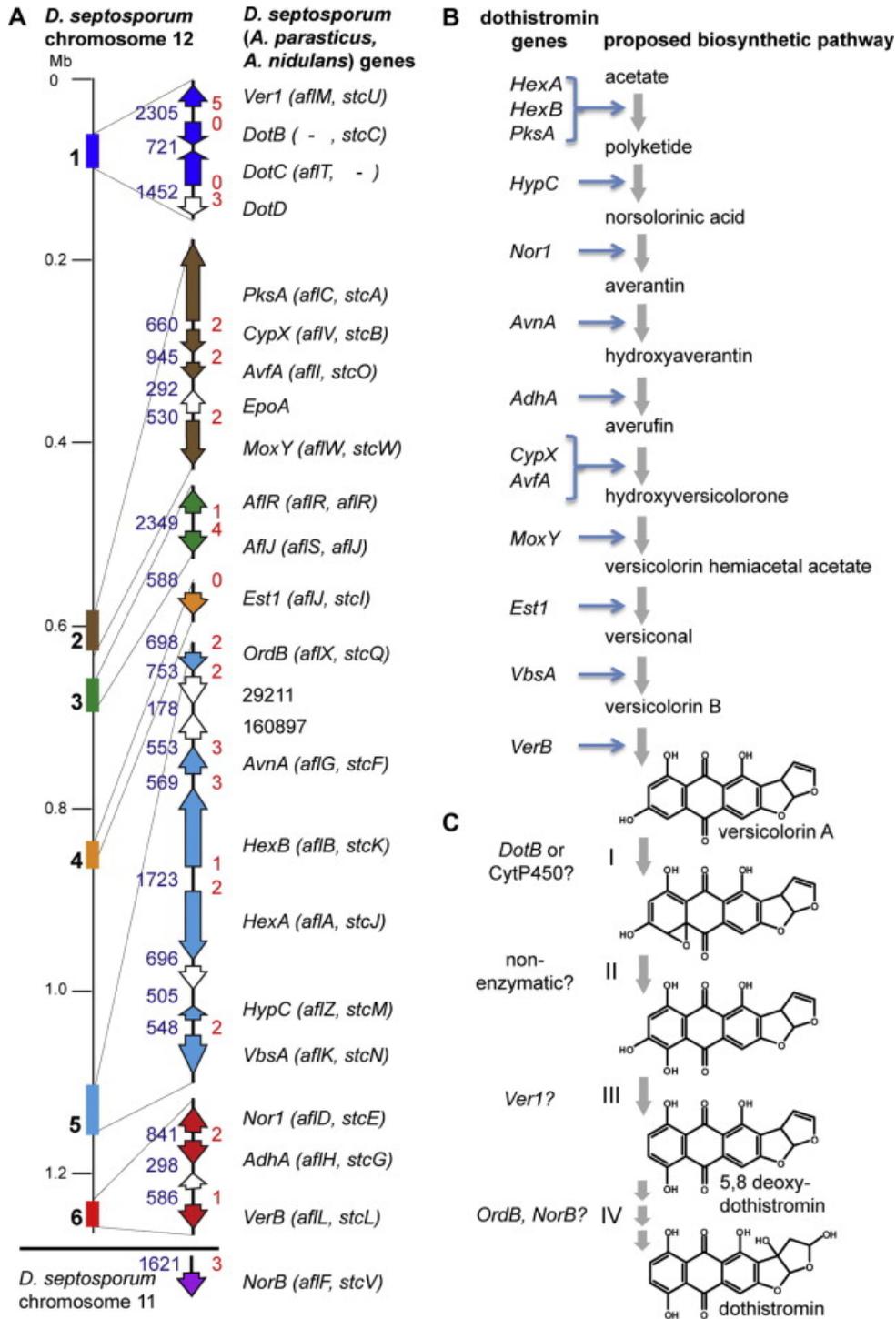


Fig. 19. Dothistromin (DOTH) fragmented gene cluster and proposed dothistromin biosynthetic pathway

(A) Chromosome 12 showing 6 loci with predicted dothistromin genes, along with a fragment of chromosome 11 containing *NorB*. Arrows indicate genes and direction of transcription; predicted DOTH genes are shaded; (B) Proposed DOTH biosynthetic pathway to versicoloin (A), as for aflatoxin; (C) Possible DOTH pathway from versicolorin A to DOTH. Figure adapted from (Chettri et al., 2013).

The genetics of dothistromin biosynthesis was compared with that of aflatoxin (AF) and sterigmatocystin (ST) biosynthesis. AF biosynthesis genes are well characterised in *A. parasiticus* and *A. flavus* and have approximately 25 genes within a 60-70 kb region (Yu et al., 2004). The ST gene cluster of *A. nidulans* contains a similar set of genes in a 60 kb region (Brown et al., 1996). Generally, secondary metabolite genes are clustered and co-regulated in filamentous fungi (Brown et al., 1996; Keller and Hohn, 1997; Martin and Liras, 1989; Yu et al., 2004), in contrast to the situation for dothistromin biosynthesis genes in *D. septosporum* (Fig 1.7A). When dothistromin genes of *D. septosporum* were compared with those of the peanut leaf pathogen *Passalora arachidicola* (Zhang et al., 2010), gene sequence similarity and synteny of gene organisation was noticed among these two pathogens. Gene expression studies of *P. arachidicola* genes also showed similar expression timings as *D. septosporum* which lead to the hypothesis that this toxin might have the same role *in planta* in these two patho-systems. On the other hand, a very similar fragmented arrangement of dothistromin genes was also found in another closely related organism *C. fulvum* (pathogen of tomato), where no biosynthesis of dothistromin was found due to mutations in key genes (de Wit et al., 2012).

Although dothistromin biosynthesis in *D. septosporum* is assumed to follow the same enzymatic pathway as aflatoxin as far as versicolorin A, the steps for conversion of versicolorin A to dothistromin are not known. A biosynthetic pathway was proposed by Chettri et al (2013), as shown in Fig. 1.9C. It was subsequently found that *D. septosporum* mutants deficient in *DotB* and *OrdB*, but not mutants lacking *NorB*, are unable to make dothistromin (Chettri et al. unpublished), providing some support for the proposed pathway.

1.5.3. Mode of action of dothistromin

The mode of action of dothistromin was compared with another non-host selective, and closely related, mycotoxin, cercosporin (Youngman and Elstner, 1984). Both toxins were reported to break down photosynthetic pigments and to result in lipid peroxidation by oxygen activation (Macri and Vianello, 1979; Shain and Franich, 1981) but their mechanisms were found to be different. In the presence of light, cercosporin was converted into an electronically active state that produced singlet oxygen ($^1\text{O}_2$), superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Dothistromin did not produce $^1\text{O}_2$, but instead produced only O_2^- and H_2O_2 , indicative of reductive rather than light-induced production of oxygen radicals by dothistromin. Thus it was predicted by Youngman and Elstner (1984) that the light requirement for the mode of action of dothistromin might be because of reduction of dothistromin by elements of the photosynthetic electron transport chain *in planta*.

An alternative explanation for the light requirement is that dothistromin might act on cellular components which only function in the presence of light. To investigate this hypothesis, Jones et al. (1995) used an immunolabelling assay to locate dothistromin binding sites in *P. radiata* cells. Their results suggested that dothistromin was located within small protein storage vesicles inside embryonic cells. In subsequent research, these authors identified a 40 kDa peptide in mature embryo extracts which bound to dothistromin. Later on their findings were also tested by the use of an anti-idiotypic antibody (MAb 10C12) which mimics dothistromin and was found in protein containing vesicles in embryo cells (Jones et al., 1998). In addition to pine embryo, dothistromin binding sites were also found associated with the chloroplasts of mature pine needles (unpublished work by Jones reported in Jones et al. (1995)), providing some support for the hypothesis.

1.5.4. Role of dothistromin in dothistroma needle blight pathogenesis

Dothistromin was initially thought to be a pathogenicity factor of dothistroma needle blight. Reasons behind this hypothesis were firstly due to an epidemiological study conducted by Gadgil (1967) who noticed the characteristic red bands on pine needle and disintegration of mesophyll cells in this red band. This author suggested that a diffusible toxin or enzyme, secreted from the fungal pathogen, was responsible for disease symptoms seen in regions adjacent to those where fungal hyphae were found. Secondly the biochemical study of Bassett et al. (1970) identified dothistromin as a metabolite in infected needles. Thirdly, the results of phytotoxicity assessments where dothistromin was shown to be toxic to pine mature seed embryo and meristematic leaf callus tissue (Jones et al., 1995). Lastly, due to another phytotoxicity assessment *in planta*, in which the injection of purified dothistromin into pine needles mimicked DNB lesions (Franich et al., 1986; Shain and Franich, 1981). However, no genetic studies were conducted to test this hypothesis due to the lack of naturally occurring dothistromin-deficient mutants of *D. septosporum*.

The hypothesis that dothistromin is a pathogenicity factor was subsequently tested with dothistromin-deficient mutants of *D. septosporum* prepared in the laboratory by use of molecular tools. The *DsPksA* dothistromin gene was knocked out from wild type *D. septosporum* and pathogenicity assays were conducted with the toxin deficient mutant (Schwelm et al., 2009). This dothistromin-deficient mutant was able to infect *P. radiata* seedlings by producing DNB lesions; the percent of symptomatic needles was not significantly different from those obtained by infection with the wild-type isolate, suggesting that dothistromin is not required for DNB pathogenicity. In addition to this finding, it was hypothesised that dothistromin might have a role in competition with other needle dwelling microorganisms as was demonstrated *in vitro* with a few potential

competitors (Schwelm et al., 2009). However, it was not tested whether dothistromin is a virulence factor of DNB *in planta*, having more subtle effects on other aspects of the disease.

1.6. Hypotheses, aims and objectives

Two hypotheses were tested in this study:

Hypothesis 1

D. septosporum is a hemi-biotrophic pathogen, with a latent or biotrophic phase at early stage followed by a necrotrophic phase. The onset of the necrotrophic phase of *D. septosporum* coincides with rapid fungal biomass growth, host cell death and increased dothistromin production.

Hypothesis 2

Dothistromin is a virulence factor of *D. septosporum* during dothistroma needle blight disease.

Aims and Objectives

To test the hypotheses the following aims and objectives were undertaken in this study:

Aim 1: Pathogenicity assay optimisation

Objective 1: To optimise pathogenicity testing of *D. septosporum* to pine seedlings in a controlled environment. This will enable disease synchronisation in pine seedlings that will facilitate the following studies of disease development.

Aim 2: A comprehensive study of the life style of *D. septosporum*

Objective 2: To characterise the parasitic phases of *D. septosporum*. This will enable identification of latent/biotrophic and necrotrophic phases of *D. septosporum* as part of its life style.

Aim 3: Investigation of the role of dothistromin

Objective 3:

To compare dothistromin deficient *D. septosporum* infected *P. radiata* seedlings with those infected with WT *D. septosporum* at various time points during dothistroma needle blight infection.

Chapter 2: Materials and Methods

2.1. Biological materials

2.2.1. Fungal isolates

D. septosporum isolates used in this study are listed in Table 2.1.

Table 2.1. *Dothistroma septosporum* isolates used in experiments

| Isolate/Lab reference | Characteristics or transformed isolate/construct | Plasmid | <i>gfp</i> expression /Doth | Source/ reference |
|-------------------------------|---|---------|-----------------------------|-------------------------|
| NZE10 | Wild type (CBS128990), single spore isolate from pine needle, collected by Ben Doherty from West Coast of South Island, New Zealand | NA | NA/ Doth+ | (Barron, 2006) |
| NZE30 | Wild type, single spore isolate from pine needle, collected by Rebecca Ganley and Margaret Dick from Rotorua, New Zealand | NA | NA/ Doth+ | This study, 2009 |
| <i>PToxA::sgfp</i> / FJT20 | <i>gfp</i> -labelled on wild type NZE7 <i>D. septosporum</i> / NZE7/ <i>PToxA::sgfp</i> , <i>hph</i> | pCT74 | Constitutive/ Doth+ | (Schwelm, 2007) |
| <i>PDotA*:egfp</i> / FJT24 | Contains a <i>gfp</i> gene regulated by <i>DsDotA</i> promoter/ NZE7/ <i>PDotA:egfp</i> , <i>hph</i> | pR242 | Regulated/ Doth+ | (Schwelm, 2007) |
| $\Delta PksA$ / FJT3 | <i>DsPksA</i> replacement mutant NZE7/ $\Delta DsPksA::hph$ | pR226 | NA/ Doth- | (Bradshaw et al., 2006) |
| $\Delta VbsA$ / FJT12 | <i>DsVbsA</i> replacement mutant NZE10/ $\Delta DsVbsA::hph$ | pR254 | NA/ Doth- | (Zhang et al., 2007) |
| $\Delta PksA::gfp$ / FJT30 | <i>DsPksA</i> replacement mutant labelled with <i>gfp</i> / FJT3/ <i>PgpdA:egfp::hph</i> | pN82 | Constitutive/ Doth- | (Schwelm, 2007) |
| $\Delta HexA$ / FJT94 | <i>DsHexA</i> replacement mutant NZE10/ $\Delta DsHexA::hph$ | pR256 | NA/ Doth- | Zhang '08 (un-pub.) |

*' *DotA* is now called *Ver1*; *hph* = Hygromycin resistance gene;

Doth+ = Dothistromin producing; Doth- = Dothistromin non- producing; NA = Not applicable

2.2.1.1. Isolation and confirmation of *Dothistroma septosporum*

A new isolate of *D. septosporum*, isolate NZE30, was isolated from an infected needle of *P. radiata* using the method of (Barnes et al., 2004). After single spore purification and growth DNA was extracted (Bradshaw et al., 2008). The DNA concentration was

measured by Nano Drop ND-1000 (NanoDrop technologies, USA) and PCR was conducted using ITS primers (ITS1 and ITS4) and methods as described by (Bradshaw et al., 2008). PCR products were purified with a QIAGEN (Limburg, Netherlands) column purification kit and sequenced using the ITS1 primer to confirm the isolate's identity as *D. septosporum*.

2.1.2.2. Maintenance of fungal isolates

Fungal isolates were sub-cultured by fungal spore transfers every six months on potato dextrose agar (PDA) (Appendix 1: A1.1) and incubated at 22°C for 7-10 d until the colony diameter reached approximately at 2-3 cm and then stored at 4°C. For long term preservation, actively growing edges of fungal cultures were plugged and stored in 20% glycerol at - 80°C. All incubation of *D. septosporum* was at 22°C unless otherwise stated.

2.2. Plant material

Six to eighteen month-old *P. radiata* seedlings (seed lot number 518, 519, 520, 521) grown from seeds were used for inoculation. These seedlings were used for pathogenicity optimisation, fungal life style studies and to determine the role of dothistromin.

P. radiata clones were used in pathogenicity assays in a blind test to differentiate resistant and susceptible pines. Resistant clones 6/1, 6/2 and susceptible clones 3/1, 3/2 (from control-pollinated *P. radiata*) were used for these studies. These clones were rooted cuttings, less than 6 months old, derived from seedlings <1 year old grown from seeds from families showing dothistroma resistance ratings of 32 (R; resistant clones 6/1, 6/2) and 18 (S; susceptible clones 3/1, 3/2) in forest trials. Of these, only susceptible clones were used for the gene expression studies reported in this thesis.

All seedlings and clones were potted with standard potting mix and supplied by Scion, Rotorua, New Zealand.

2.3. Pathogenicity assay

2.3.1. Sporulation of *Dothistroma septosporum* (*in vitro* and *in planta*)

From a wide range of media initially screened for their ability to support sporulation of *D. septosporum* (isolate NZE10) *in vitro*, four were selected: (1) pine needle minimal medium with glucose (PMMG) (McDougal et al., 2011a), a minimal salts medium (Carsolio et al., 1994) made with water in which pine (*P. radiata*) needles (10%, w/v) had been soaked for 24-48 h at room temperature; (2) pine needle agar (PNA) containing 0.5% (w/v) freeze-dried and ground pine needles in 2% (w/v) water agar; (3) water agar (WA) (2% agar, w/v); (4) dothistroma sporulation medium (DSM) (Bradshaw et al., 2006) containing malt extract, 2% (w/v), yeast extract, 0.5% (w/v) and bacteriological agar, 1.5% (w/v) (Appendix 1: A1.1). All media were adjusted to pH 6.2. Plates containing the different media were inoculated by spreading 5×10^4 *D. septosporum* spores over the surface. Spores were harvested and counted 7 d and 12 d after inoculation by taking five 5 mm diameter plugs (one from the centre of the plate and other four from the corners of a square around the centre sample) from each plate (based on Manandhar (1998)), vortexing (five pooled plugs) for 1 min in 4 ml sterile water and counting spores using a cytometer and phase contrast microscope (Zeiss Axiophot Compound Light Microscope). Four replicate plates were used for each medium and combined results from three independent experiments are presented.

For *in planta* sporulation, ten needles showing DNB symptoms were selected at random from each replicate (taken from different representative positions of each pine seedling from top to bottom) for counting numbers of fruiting bodies and spores from a single DNB lesion. Fruiting bodies were counted under a binocular microscope

(Leica MZ10F, fitted with Leica DFC450C digital camera) then incubated to facilitate fruiting body maturation and spore release as follows.

Needles were cut into small pieces each containing one lesion with additional one cm length on both sides. These pieces were incubated in a moist and humid Petri dish (containing moist blotting paper) at 22° C for 72 h. To prevent spore loss from lesions during this incubation a gap was maintained between lesions and moistened blotting paper by raising one end of each needle piece on a sterile glass slide platform placed on top of the blotting paper. After incubation, one end of needle piece was cut very near to swollen fruiting bodies and soaked in 15 µl sterile water in a micro centrifuge tube. To allow fruiting bodies to release spores the tube was shaken (@100 rpm) for 48 h by mechanical shaker. After incubation in water, one minute centrifuge (@ 10000 g) was carried out to spin down all spores to the bottom of the tube. Finally at the time of removing needle out from tube, the needle was tapped gently on top of the water to obtain maximum yield of spores. These harvested spores were then counted by haemocytometer.

2.3.2. Spore germination, surface growth and penetration

For *in vitro* spore germination assays, 2×10^5 spores in 2 ml were spread on a plate containing 0.5% (w/v) water agar and incubated for 48 h at 22°C. Using a cytometer grid to facilitate assessment, 100 spores were scored at random (diagonally in a focal view) for each replicate and considered germinated if a visible germ tube was observed.

For assessment of spore germination and fungal penetration *in planta*, five needles were randomly collected from pine seedlings and cut longitudinally for infiltration of clearing solutions. Needles were soaked in solution A (acetic acid: ethanol; 1: 3) overnight (16 h) followed by solution B (acetic acid: ethanol: glycerol; 1: 5: 3) for 3 h with slow shaking. Needle sections were subsequently stained with trypan

blue (0.01% in lacto phenol) overnight (based on Mehrabi et al. (2006)). One thousand randomly selected spores or stomata were examined microscopically (Zeiss Axiophot, Germany) for spore germination or fungal penetration respectively from each of four replicates. Surface growth of hyphae was also monitored on cleared needles in the same way.

2.3.3. Pathogenicity assay chamber

The assay chamber (Fig. 2.1) design was based on that used by Schwelm et al. (2009). Inoculated plants were incubated in a 90 L plastic container (39.5 cm × 69 cm × 45 cm) containing ~ 20 L of distilled water in which a water fogger was immersed. Two 36-V home-built water foggers were used instead of the 24-V fogger used previously (Schwelm et al., 2009). Platforms (inverted tip boxes) were placed to keep plant pots above the water level and the chamber was enclosed in a frame covered with plastic wrap to maintain high humidity, leaving a 30 cm × 20 cm gap at the top for air flow and to minimise the growth of contaminating fungi. All experiments were conducted in a temperature controlled glass house under natural lighting conditions throughout the year (summer, autumn, winter, spring); light intensities are outlined in Table A4.2.



Fig. 2.1. Pathogenicity assay chamber

Inoculated pine seedlings in pathogenicity assay chamber (left) where seedlings were kept on a platform; misters in action (middle) that were placed at the bottom of pathogenicity assay chambers; assay chambers in continuous misting covered by plastic wrap around a rectangular frame (right).

Total incident light energy was quantified at morning time (one time point in a day, ten days in a season) using a 'PAR' (photo synthetically active radiation) sensor (SKU 420; Skye Instrument Ltd, Llandrindod Wells, UK) and using Skye Instrument software (<http://www.skyeinstruments.com/Light.htm>).

2.3.4. Plant inoculation techniques

Three plant inoculation techniques were tested: spores were either sprayed on needles using a multipurpose hand sprayer (delivering up to 24 ml per plant to wet the needles thoroughly), painted on with a camel-hair brush, or attached by wrapping a wet cotton cheesecloth (soaked in fungal spore suspension) around the seedlings. In all cases spores from 7 d PMMG plates were used at 3×10^6 spores/ml. Control plants were spray-inoculated with water only and experiments were conducted with at least three replications. After inoculation, pine seedlings were kept in the pathogenicity assay chamber described above with the water fogger system permanently on.

2.3.5. Adhesion tests

Four compounds were tested for their potential to improve spore adhesion based on the studies of Rawlings et al. (2007). The following were added (% w/v) to the water used to prepare the spore inocula: gelatin (1%), mucin (0.5%), tween 20 (0.05%) or polyethylene glycol 8000 (1%). An *in vitro* test was carried out to determine spore adhesion to a glass surface (cytometer grid) using 10 μ l of 1×10^6 spores/ml in solutions of potential adhesives or water control. After drying for one hour, spores were counted, the cytometer washed by agitation under water for 10 min at 90 rpm then spores counted again. Ten independent counts were made for each adhesive and percent adhesion calculated. Spore germination was also assessed in the adhesive solutions, as outlined above. An *in planta* adhesive test was conducted in a similar way with four

replications by spraying seedlings with spores suspended in gelatin (1%), mucin (0.5%), tween 20 (0.05%) or water as above.

2.3.6. Microclimate optimisation

Experiments to optimise DNB infections were conducted in a temperature controlled (20°C) glasshouse. Because of the importance of leaf wetness in DNB, a series of experiments were carried out to optimise this parameter and two are reported here that involved changing the moisture level over the period of the experiment. In the first experiment, all seedlings were placed in the assay chamber with continual fogging but differed in the amount of covering to give wetness conditions that were either low (no cover), medium (plastic cover over the whole chamber with ventilation as described for the pathogenicity assay chamber) or high (individual plants enclosed within a plastic sleeve and within the covered assay chamber). The conditions were changed two weeks post-inoculation (wpi) to give four combinations: low+medium, low+high, high+medium and high+high. In the subsequent follow-up experiment, seedlings were maintained under high wetness conditions (i.e. with plastic sleeve) for different periods of time after inoculation (0, 4, 7, 14, 70 d) before changing to medium wetness conditions inside the pathogenicity assay chamber. Following this, the most effective condition (4 d high wetness) was tested twice more. For each of these experiments, 3×10^6 spores/ml were used for inoculum and four replicate seedlings were used for each condition.

2.3.7. Dothistroma needle blight infection and scoring

Disease symptoms were generally recorded ten weeks post-inoculation (wpi) using the assessment system of Schwelm et al. (2009). Needles were categorised as un-infected (green), chlorotic (with no evidence of infection/faded green) or infected. Infected needles typically had red bands and stromata, and confirmation was made using

microscopy or PCR when required. Needles with symptoms were calculated as the percentage of all non-chlorotic needles showing DNB symptoms ('infected'). Red bands per needle were calculated as the total number of red DNB bands/total DNB infected needles. Chlorotic needles were not considered in this calculation as it was unknown why they were showing chlorosis.

2.3.8. Pathogenicity assays for role of dothistromin

Four pathogenicity experiments were conducted using dothistromin deficient mutants and compared with wild type *D. septosporum* infection *in planta*. During these experiments *in vitro* spore germination was carried out at 4 days post inoculation, fungal growth on the needle surface was quantified at weeks 2, 4, 6 and 8; and other parameters were compared as shown in Table 2.2.

Table 2.2. Summary of experiments conducted to determine the role of dothistromin *in planta*

| Expt | Isolates of <i>Dothistroma septosporum</i> | Lesions measured at wpi ^a | Time/Season | Seed lot no. | Sporulation checked at wpi ^a | Purpose of experiment |
|------|---|--------------------------------------|--------------------------------|-------------------|---|--|
| 1 | $\Delta PksA:gfp$ $\Delta HexA$ WT(NZE30) | 8, 10, 12 | June-August/ Winter | 521 | 12 | To compare spore germination, penetration, fungal growth, mesophyll colonisation, lesion development and spore production between mutant and wild type <i>D. septosporum</i> |
| 2 | $\Delta PksA:gfp$ WT(NZE10) | 6, 8, 10 | February-April/ Autumn | 520 and 521 | 10 | |
| 3 | $\Delta HexA$ WT(NZE10) | 6, 8, 10 | August- November/ Spring | 520 | 10 | |
| 4 | $\Delta PksA$ $\Delta VbsA$ WT(NZE10) | - | August- November/ Spring | 520 | - | To monitor lesion development only at late stage of DNB between mutant and wild type <i>D. septosporum</i> |

^aweeks post inoculation. The timings differ due to differences in the rate of disease progression.

2.4. Microscopy

2.4.1. Light microscopy

For sample preparation for light microscopy, fresh needles were sliced into one mm transverse sections and fixed (3% glutaraldehyde, 2% formaldehyde in 1 M phosphate buffer, pH 7.2) with vacuum infiltration at 4°C for 24 h. Samples were then washed with 1 M phosphate buffer (3 times in 30 min), post fixed with osmium tetroxide (1% OsO₄ in phosphate buffer) for 1 h and dehydrated with an acetone series (diluted in milli-Q water) of 25%, 50%, 75%, 95%, 2 × 100%; 15 min for each and 1 h for last 100% step. Then the samples were embedded in resin (Procure 812, ProSciTech Pty. Ltd; Thruringowa, Queensland, Australia), trimmed transversely into 700 nm sections using a glass knife in a microtome (Ultracut R microtome, Leica Microsystems, Wetzlar, Germany) (Spiers and Hopcroft, 1993) and stained with aqueous toluidine blue (0.05%) according to Christensen et al. (2002). Light microscopy was conducted using a compound microscope (Zeiss Axiophot, Germany) equipped with a DFC320 digital camera (Leica Microsystems, Wetzlar, Germany).

Light microscopic studies were conducted on infected pine needles at stages 1, 2, 3 and 4 to monitor cell morphology and integrity during DNB disease progression. For this experiment needles from an un-inoculated seedling were used as a control (section 4.2.1.2). To study the role of dothistromin (section 5.2.5) needles infected with a dothistromin deficient mutant at stages 3 and 4 were used, with WT infected needles as a control.

2.4.2. Confocal microscopy

For confocal imaging fresh or processed needle samples were mounted in water. A confocal microscope (Leica SP5 DM6000B, Leica Microsystems, Wetzlar, Germany) was used for imaging trypan blue stained and *gfp*-labelled WT *D. septosporum* on pine

needles. It was also used to identify live cells, semi-quantitative gene expression of *D. septosporum* and chlorophyll loss during DNB. The focal depth and optical size varied according to the purpose of imaging. All images were processed in Leica LAS AF lite software (Leica Microsystems).

For trypan blue fluorescent imaging, pine needle samples of stages 1, 2 and 4 were processed as described in section 2.3.2 (*in planta*). In confocal microscopy, trypan blue fluorescence was excited at 561.0 nm and emission detected at the range of 622.3 nm – 758.8 nm. For imaging of *gfp*-labelled wild type *D. septosporum* infections, fresh infected whole needles (stages 1 and 4) were mounted in water and excitation/emission wavelengths of 405 nm and 498.0 – 571.0 nm used respectively. To identify live cells, samples were prepared as in section 2.5.1 and fluorescence of neutral red determined by excitation/emission at 561.0 nm and 575.7 – 688.7 nm respectively. To measure chlorophyll loss during infection of DNB, fresh needles that were un-inoculated or at DNB stages 3 and 4 were collected. Thin hand cut cross sections (about 0.5 mm) were prepared and mounted in water. *gfp*-labelled *D. septosporum* was determined as above, and chlorophyll auto-fluorescence by excitation and emission wavelengths of 561.0 nm and 634.4 – 765.4 nm respectively.

For semi-quantitative gene expression, *gfp* fluorescence was excited at 488 nm and detected in the emission range of 498.0 – 571.0 nm as an expressed *DotA* gene. To visualise auto-fluorescence from fungal structures (without *gfp*), another laser was activated that excited at 561.0 nm and detected in the emission range of 616.0 – 777.0 nm.

2.4.3. Transmission electron microscopy

Transmission electron microscopy was conducted on stage 3 and 4 infected needle samples to monitor details of cell structures and phenolic compounds. Needle samples

were fixed and embedded as for light microscopy samples (section 2.4.1). The area of interest was trimmed down to 0.5 mm × 0.5 mm and cut into 100 nm sections using a diamond knife and ultra-microtome (Ultracut R microtome, Leica Microsystems). Sections were placed on copper grids and stained (4 min) with saturated uranyl acetate in 50% ethanol in water then washed in 50% ethanol followed by a distilled water wash. Later on samples were stained by lead citrate (0.4%) for 4 min followed by a final distilled water wash (Venable and Coggesha, 1965). Samples were viewed under a transmission electron microscope (Philips CM10, USA) fitted with SIS Morada digital camera (Olympus).

2.4.4. Scanning electron microscopy

Needle samples were sliced and fixed as for light microscopy samples (section 2.4.1), then dehydrated using an ethanol series (25%, 50%, 75%, 95%, 2 × 100%; 15 min for each and 1 h for last 100% step) followed by critical point (CP) drying using liquid carbon-dioxide (as the CP fluid). Dried samples were then mounted on to aluminium specimen support stubs, sputter coated with gold as described by Eaton et al. (2010) for visualisation under scanning electron microscopy (FEI Quanta 200 SEM, Hillsboro, USA).

2.5. Histo-chemical studies

2.5.1. Cell viability

To monitor cell viability using neutral red, un-inoculated, stage 1 and 3 whole infected needle samples were sliced into small pieces (2 mm) and washed for five minutes with several changes of tap water. Then samples were vacuum infiltrated with aqueous neutral red (0.01%) for 24 h then fixed in 10% buffered formalin (Appendix 1: A1.2)

for 48 h (Boyer, 1963). After fixation needle pieces were dehydrated using an ethanol series (50%, 60%, 75%, 80%, 95%, 2 × 100%; 1 h for each step). The ethanol (Et) was gradually replaced with histoclear (H) solution (a histological clearing agent; product no #HS – 200; www.nationaldiagnostic.com) (as 25% Et + 75% H, 50% Et + 50% H, 75% Et + 25% H, 95% Et + 5% H, 2 × 100% H; 30 m for each step and 1 h for last step) based on Preuss and Britt (2003). The histoclear was gradually replaced by paraplast chips (Tyco Healthcare, Mansfield, USA) and incubated at 60°C for 3 days with two changes in a day. Then needle pieces were embedded in wax using a Leica EG 1160 wax embedder (Leica Microsystems, Wetzlar, Germany) and trimmed cross sectionally using a microtome (Leica RM 2145, Leica Microsystems, Wetzlar, Germany) having 8 µm thickness. Confocal microscopy (section 2.4.2) was used to visualise live cells that accumulate neutral red (as a red colour) due to plasmolysis (O'Connell et al., 1993).

2.5.2. Reactive oxygen species (ROS) staining

Attempts were made to detect superoxide and hydrogen peroxide in infected needle samples using Nitroblue Tetrazolium (NBT) and 3,3, diaminobenzidine (DAB) using the protocols of (Adam et al., 1989) and Thordal-Christensen et al. (1997) respectively. Due to increased lipid peroxidation when oxygen radicals accumulate in the cells, NBT is taken up by cells and seen by its blue colour (Adam et al., 1989). In the presence of peroxidase activity when DAB is up taken by living plant tissue it forms a reddish-brown colour (Thordal-Christensen et al., 1997).

2.5.3. Lignification test

To visualise lignified areas, phloroglucinol-HCl was used to stain hand cut pine needle cross sections as mentioned in Pomar et al. (2002).

2.6. Molecular and biochemical studies

2.6.1. PCR Diagnostics

2.6.1.1. DNA extraction

A CTAB DNA extraction method was used based on Stewart and Via (1993). Samples (fungal or pine) were ground with liquid nitrogen in a 2 ml micro centrifuge tube for DNA extraction. To this was added 750 µl pre-heated (65°C) DNA extraction buffer containing 2% CTAB. The samples were vortexed for 90 min and incubated in a water bath for 30 min at 65°C with occasional mixing during this period. Then an equal volume of freshly prepared phenol:chlorophorm:IAA (25:24:1) was added and briefly vortexed. The sample was then centrifuged for 5 min at 13,000 g and the supernatant transferred to a new tube and followed by another chlorophorm:IAA (24:1) wash. Then two-thirds volume of isopropanol was added to the extracted supernatant, mixed gently and incubated at room temperature for 5 min at -20° C for 30 min. Then the tube was centrifuged for 5 min at 13,000 g to obtain a DNA pellet. After discarding supernatant the DNA pellet was washed with 70% alcohol and dried for 30 min at room temperature followed by elution in 20 µl elution buffers (Buffer AE, www.qiagen.com). In addition 2 µl RNase (10 mg/ml) was added to the eluted solution and was kept at 55°C for 30 min.

2.6.1.2. Dothistroma needle blight confirmation by PCR

PCR was conducted on DNB infected samples for the confirmation of disease by using dothistroma specific primers (DPS ITS spec fwd1 and DPS ITS spec rev2) (Appendix 1: Table A1.1) PCR reactions were performed using a FIREPol® DNA Polymerase kit (Solis BioDyne, Tartu, Estonia), according to the manufacturer's instructions. Each 25 µl PCR reaction contained 10 × Buffer BD (2.5 µl), 1.5 mM MgCl₂, 0.4 µM of each

primer, 1.25 U of FIREPol[®] DNA Polymerase, 1 µl of DNA (approx 50 ng) and PCR-grade water (up to 25 µl total volume). The cycling conditions consisted of an initial denaturation step of 95°C for 4 min, then 30 cycles of 94°C (1 min), 58°C (1 min) and 72°C (1 min), and a final extension step of 72°C (6 min). Gels were run on 2.5% agarose in TBE (Appendix 1: A1.2) and PCR products were visualised after staining with ethidium bromide (Appendix 1: A1.2). DNB infected samples were compared with the samples from un-inoculated pine seedlings for confirmation of *D. septosporum* on pine needles.

DNB lesions caused by dothistromin deficient mutants ($\Delta PksA:gfp$, $\Delta PksA$, $\Delta HexA$ and $\Delta VbsA$) were confirmed in the same way as above using dothistroma specific primers, followed by amplification using hph Fwd2 and hph Rev2 primers (Appendix 1: Table A1.1) to confirm the presence of the dothistromin-deficient gene knockout construct.

2.6.2. *Dothistroma septosporum* biomass and dothistromin toxin quantification

2.6.2.1. Needle sampling

Due to challenges associated with obtaining reliable data especially at early stages of infection, a series of five experiments was carried out with successive modifications (Table 2.3). Out of five experiments, two were conducted using whole needle samples and three were conducted using DNB lesions (early and late). All experiments were conducted with three replicates (i.e. one seedling per replicate) in a glass house condition as outlined in section 2.3.

Table 2.3. Summary of needle sampling, sampling time and other related information for *Dothistroma septosporum* biomass and dothistromin toxin quantification during dothistroma needle blight

| Experiment | Needle sampling | | | Time/ Season | Type of pine/seed lot no. | Age of pine (years) | Fungal isolate used |
|------------|------------------------------|---------------------|--|-----------------------|---------------------------------|---------------------------|---------------------------|
| | Whole needles (number) | Lesions (number) | Interval | | | | |
| 1 | 5 | - | weekly | June-July/ Winter | Seedling/ 519 | 1.4 | NZE30 |
| 2 | 100 | - | weekly | Feb.-April/ Autumn | Seedling/ 520 and 521 | 2.0 | NZE10 |
| 3 | 20 ^a | 20 ^b | fortnightly | Nov.-Jan./ Summer | Seedling/ 527 | 1.0 | NZE30 |
| 4 | 100 ^a | 80 ^c | at non- symptomatic, early & late DNB stage | Feb.-April/ Autumn | Seedling/ 520 and 521 | 2.0 | NZE10 |
| 5 | 100 ^a | 120 ^d | at non- symptomatic, early & late DNB stage | Jan.-April/ Autumn | Clones/ 3-1 and 3-2 | 1.0 | NZE10 |

^aNon-symptomatic needles, ^blesions from 2 seedling/replicate, ^clesions from 14 seedling/replicate, ^dlesions from 8 clones/replicate

2.6.1.2. Quantification of *Dothistroma septosporum* biomass

D. septosporum biomass was quantified using the qPCR protocol of Chettri et al. (2012) and a LightCycler480 instrument (Roche Applied Sciences, Penzberg, Germany). Quantification of biomass from genomic DNA was based on amplification of the *D. septosporum* polyketide synthase (*PksA*) gene as a target and *P. radiata* cinnamyl alcohol dehydrogenase (*CAD*) gene as a reference. The reaction was performed using a LightCycler® 480 Probes Master kit (Roche Applied Science, Penzberg, Germany) where each 10 µl of reaction volume contained: 5 µl of 2 × Probe master mix (5' and 3' end of *PksA* probe was labelled with 6-carboxyfluorescein (FAM) and quencher dye BHQ-1 respectively; 5' and 3' end of *CAD* probe was labelled with HEX (5') and BHQ-

1 (3') respectively), 1 μ l 10 \times primer probe mix, 2 μ l PCR grade water and 2 μ l DNA template (known and standard). The 10 \times primer probe mix consisted of dothistroma fungal *PksA* probe 88, pine *CAD* probe 945, each at 2 μ M along with dothistroma specific primers *PksA64*, *PksA164* and pine specific primers *CAD918*, *CAD1019* (Appendix 1: Table A1.1) each at 4 μ M. PCR was conducted with pre-incubation at 95°C for 10 min followed by 55 cycles of: denaturation at 95°C for 10 s, annealing at 58°C for 15 s, extension at 72°C for 20 s followed by cooling at 40°C for 10 s.

Relative quantification of *D. septosporum*/biomass was determined by comparison to standard curves (Appendix 2: Fig. A2.1) prepared using LightCycler[®]480 software version 1.5.0 using five-fold dilution series of both fungal (200, 40, 8, 1.6, 0.32, 0.064, 0.0128, 0.00256 ng) and pine (1000, 200, 40, 8, 1.6, 0.32 ng) genomic DNA. The amount of *D. septosporum* biomass per dry weight of needle or lesion was estimated by absolute quantification of fungal DNA (Appendix 2: Table A2.1) using fungal a standard curve (of fungal DNA) as shown in Appendix 2: Fig. A2.1.

2.6.2.3. Dothistromin toxin quantification

Dothistromin toxin was extracted from the by products of DNA extraction from infected needles using the method of Chettri et al. (2012). After pipetting out the DNA-containing supernatant, 1 ml ethyl acetate (acidified with 1% formic acid) was added to the cell debris and phenol chloroform layer. Then these tubes were covered with tinfoil and placed on a shaker for 72 h at room temperature to allow dothistromin toxin to dissolve in this organic solvent. After centrifuging for five minutes @ 8000 g, ethyl acetate layers were transferred to a new tube. From this extracted solution, 200 μ l solution that contained dothistromin toxin were evaporated under dark conditions in a fume hood for 48 h, resuspended in 200 μ l of acetonitrile (acidified with 1% formic

acid) and then filtered through a Minisart RC4 0.2 μm syringe filter (Sartorius Stedim Biotech, GmbH 37070 Goettingen, Germany) for quantification.

Dothistromin toxin was quantified by high performance liquid chromatography (HPLC). This equipment consisted of a Dionex UltiMate 3000 pump, Dionex ASI-100 automated auto sampler and Dionex UVD340U UV detector (Dionex, California, USA). Separations were performed using an analytical C18 column (Phenomenex, USA; 4.6 mm ID, length 150 mm, particle size 5 μm) preceded by a Phenomenex security guard cartridge system (Phenomenex, California, USA; C18 ODS, octadecyl, dimension 4 mm L \times 3 mm ID). During quantification, gradient elution (5% acetonitrile during first 5 min, followed by 75% in next 30 min, then raised to 100% at 38 min, dropping back to 5% between 42 and 45 min with an equilibration in next 15 min) was performed at a flow rate of 1 ml/min using two solvents (solvent A, 1% formic acid in MQ water; solvent B, 1% formic acid in 100% acetonitrile). Dothistromin toxin, 50 μl of standard or extracted sample, was injected into the column and the column temperature was kept at 40°C. Dothistromin UV spectra were measured by a Dionex UVD340U diode array detector, and fluorescence response (excitation and emission wavelengths of 470, 545 nm respectively) was collected by a Dionex RF2000 Fluorometer (Dionex, California, USA). Dothistromin was quantified by fluorescence peak area at 23.1 ± 0.05 min. Dionex Chromeleon software (www.dionex.com) was used to record and process data. Quantification of unknown dothistromin toxin was calculated using a standard curve generated with a 10-fold dilution-series (0.1 to 0.0000001 ng/ μl) of purified dothistromin (Appendix 2: Fig. A2.2) as shown in Appendix 2: Table A2.2. To estimate the amount of dothistromin toxin produced by *D. septosporum* per unit of biomass the amount of dothistromin toxin per needle was divided by the amount of *D. septosporum*

biomass per needle (calculated using an absolute quantitative PCR assay), as shown in Appendix 2: A2.3.

2.6.3. Semi-quantitative gene expression

D. septosporum isolate FJT24 contains a *gfp* gene regulated by the *DsDotA* promoter (Schwelm, 2007). This construct was used to monitor the expression of *DsDotA* in *planta* over the time course of DNB infection. Two seedlings were infected as previously described (section 2.3) by this *D. septosporum* isolate. Ten needles were collected randomly from the two infected seedlings weekly (from week 1 till week 8) and each needle surface was monitored at five points under 100 × magnifications by a florescent microscope (Olympus BX51). Semi-quantitative gene expression was recorded based on eye estimation of the *gfp* fluorescence received from the fungal isolate on the needle surface. When mycelia or spores were seen by auto-fluorescence (uv spectra excitation and emission ranges 330 – 385 nm and 420 nm respectively) covering the whole field of microscopic view it was considered as 100 percent. This microscopic view was monitored by another filter (excitation and emission ranges 460 - 490 nm and 510 - 550 nm respectively) that detects *gfp* fluorescence. The *gfp* fluorescence was estimated by eye as the percentage of auto-fluorescing (visible) hyphae that showed *gfp* fluorescence. Semi-quantitative gene expression of *DsDotA* (now called *Ver1*) in *planta* was calculated as:

Gene expression *DsDotA* = (% of field of view with *gfp* fluorescence × 100) / (% of field of view with auto-fluorescence of uv spectra)

A *D. septosporum* isolate with a constitutively expressed *gfp* gene (NZE7/*PToxA:sgfp*, *hph*) was used as a control in this experiment. Photos were taken with a confocal microscope (Leica SP5 DM6000B, Leica, Microsystems, Wetzlar,

Germany) at stage 1 (spore germination), stage 2 (fungal growth) and stage 4 (late lesions).

2.6.4. Gene expression studies

To obtain samples for gene expression analysis by high-throughput RNA sequencing (RNAseq) at early stages (1 and 2) of DNB disease, the extent of fungal surface growth was determined by light microscopy (section 2.3.2) on two randomly selected needles from each tree (five trees from each replicate assay chamber) at weekly intervals since inoculation. Based on these results, one tree from each susceptible clone series from each replicate was selected which had even fungal growth all over the needle surface. Whole needles were collected for RNA extraction at this stage as the positions of future lesions could not be predicted. At weeks 8 and 12 after inoculation, early and late lesions were checked using a binocular microscope (Leica MZ10F) and termed mid and late stage samples respectively. In these cases approximately 120 lesions were cut out of needles taken from up to five tree clones within each replicate incubation box; this was done to maximise the amount of fungal RNA and minimise plant RNA within these samples.

RNA was extracted from the infected needle samples using a SpectrumTM Plant Total RNA kit (Sigma-Aldrich, St. Louise, USA; Catalog no STRN50-1KT; www.sigma-aldrich.com) by Yanan Guo (Massey University). Five µl of total RNA was submitted to the Massey genome centre for quality check using a Bioanalyser (Illumina). Samples having RNA integration numbers (RIN) above 7 were considered suitable for library preparation for sequencing. For RNAseq analysis two biological replicates were selected for sequencing from non-symptomatic tissue, early lesions and late lesions. A library was made for each individual sample with a unique 6 bp sequence tag and all libraries were combined for sequencing. To ensure the correct proportions of

samples in the mix (based on their different proportions of fungal to plant reads), and correct proportions of sequence tags, Illumina MiSeq 150 base PE (3 consecutive flow cell runs) was carried out first, followed by Illumina HiSeq100 base PE (1 lane) followed by 100 base SE (8 lanes). Library preparation and sequencing was carried out by New Zealand Genomics Ltd. and bioinformatic analysis was performed by Murray Cox (Massey University). Only two replicates were used in this experiment due to the high cost of RNA sequencing by high-throughput Illumina sequencing.

2.6.5. Chlorophyll quantification

For chlorophyll quantification, ten DNB symptomatic needles were sampled from each biological replicate in experiments with wild type and dothistromin deficient *D. septosporum* with un-inoculated pine needles used as a control. Different symptomatic areas were excised carefully from fresh needles using a scalpel blade as follows; 1 = green island, 2 = DNB necrotic region, 3 = chlorotic region, 4 = 'normal' green region. Chlorophyll was extracted from these areas by incubating 1 mg tissue in 20 µl ethanol (96%) overnight at 4°C in the dark. The chlorophyll in the supernatant was quantified by using 2 µl of the extract in the program 'uvvis' in Nanodrop®ND-1000 (NanoDrop technologies, USA). Wave lengths 665 nm and 649 nm were used for quantification of chlorophyll a and b respectively and calculations were performed according to Winterma and Demots (1965) as follows,

$$\text{Chlorophyll a } (\mu\text{g}/\mu\text{l}) = 13.70 \times A_{665\text{nm}} - 5.76 \times A_{649\text{nm}}$$

$$\text{Chlorophyll b } (\mu\text{g}/\mu\text{l}) = 25.80 \times A_{649\text{nm}} - 7.60 \times A_{665\text{nm}}$$

$$\text{Chlorophyll a + b } (\mu\text{g}/\mu\text{l}) = 6.10 \times A_{665\text{nm}} + 20.04 \times A_{649\text{nm}}$$

2.7. Statistical analysis

2.7.1. ANOVA

Data were analysed (analysis of variance, ANOVA) using Minitab 15 statistical software (Minitab Inc. State College, Pennsylvania). Statistical significance based on a null hypothesis of no difference between treatments was calculated as least significant differences (LSD) at a 95% confidence level.

2.7.2. Scattered plot analysis

A Levene's test for equality of variance was used to compare variance between these groups using QI Macros in Excel.

2.7.3. Student t-test

Student t-test was conducted to compare the events between dothistromin containing and dothistromin deficient mutants. Tests were conducted to consider the null hypothesis of no differences between WT and mutant at $P < 0.05$.

Chapter 3: Pathogenicity assay optimisation

3.1. Introduction

Although DNB infection is prevalent in many forests, attempts to achieve infection under controlled laboratory or glasshouse conditions have met with mixed success. Whilst successful DNB infection has been reported (Gadgil, 1974; Gadgil and Holden, 1976; Muir and Cobb, 2005; Parker, 1972) DNB pathogenicity assays under controlled conditions are unreliable and difficult to replicate, with disease levels sometimes as low as 10% of needles showing symptoms (Barron, 2006; Schwelm et al., 2009). Reproducible and high levels of DNB infection, would greatly facilitate research into this disease, ranging from screening pines for resistance to functional studies of candidate virulence genes identified in the *D. septosporum* genome (de Wit et al., 2012).

Microclimatic conditions such as temperature, light and leaf wetness play an important role in DNB infection and optimisation of these is critical for development of pathogenicity assays under controlled conditions. A wide range of temperatures support DNB disease development, ranging from 12-24°C in controlled conditions (Gadgil, 1974; Parker, 1972), but with an optimum at 20°C (Muir and Cobb, 2005). Although light has no effect on early stages of needle infection, it has an impact on later development of symptoms including toxin production (Gadgil and Holden, 1976). Leaf wetness is a critical factor for DNB infection. A long dry period after inoculation lead to less infection, whilst increased leaf wetness gave higher disease levels (Gadgil, 1974; Gadgil and Holden, 1976).

In addition to microclimatic components the inoculum and inoculation methods are important factors in DNB infection. *Dothistroma* sporulation medium (DSM) is used for

culturing *D. septosporum* (Bradshaw et al., 2000) but does not reliably produce sufficient asexual spores for inoculation. Many authors have instead used natural inoculum collected from diseased needles in the forest (Muir and Cobb, 2005) but this is not a feasible option for studies which require testing of specific laboratory isolates of the pathogen, such as dothistromin-deficient mutants. Therefore, improved sporulation conditions are required.

Inoculation methods are important for the success of pathogenicity assays but little has been published on optimisation for DNB. In a pathogenicity test of black leaf streak disease of banana, a camel hair brush was used to ensure sufficient coverage of *Mycosphaerella fijiensis* spores on banana leaves (Donzelli and Churchill, 2007). In contrast *D. septosporum* spores are generally applied by spraying (Bulman et al., 2004) although entry to the needle is reportedly more frequent in the abaxial rather than the adaxial surface (Muir and Cobb, 2005). Other studies advocate the use of adhesive compounds to assist spore adherence onto the host. For example, Natrosol, mucin and gelatine were effective adhesives for spores of *Glomus etunicatum*, *A. niger*, *Colletotrichum gloeosporioides* and *M. fijiensis* (Hung et al., 1991; Mahuku and Goodwin, 1998; Peraza-Echeverria et al., 2008). The use of adhesives was not previously reported for pathogenicity assays with *D. septosporum*.

Host factors such as age and genetic background are other important considerations for pathogenicity assays. *P. radiata* shows ontogenic resistance to DNB (Gadgil and Holden, 1976; Gibson, 1972) and indeed rooted cuttings from 5-7 year old trees showed greater resistance to DNB than <1 year old seedlings (Schwelm et al., 2009). However, cuttings from younger trees have not previously been tested as hosts under controlled conditions.

The aims of this research were, firstly, to develop and optimise a reliable pathogenicity assay for DNB that could provide consistently high levels of needle infection in both seedlings and clones. Secondly, to test the sensitivity and reproducibility of this pathogenicity assay to distinguish between hosts from families with differing levels of DNB resistance.

3.2. Results

3.2.1. Sporulation

Four media, PMMG, PNA, WA and DSM (section 2.3.1 and Appendix 1: A1.1), were tested for their ability to support reliable sporulation and spore quality was assessed (section 2.3.2) by germination tests. Among these four growth media, PMMG consistently supported the highest level of sporulation (Table 3.1), with a significant improvement over other media ($P < 0.05\%$), including dothistroma sporulation medium (DSM). In separate studies (data not shown), it was found that neither pine soaked water medium (PMMG without salts) nor MM medium (MM with MQ water) alone induced the high levels of sporulation seen in PMMG. A synergistic effect of pine needle soaked water and MM contributed to the high level of sporulation obtained with PMMG medium.

In germination tests, 7 d and 12 d old spores from PMMG cultures showed $82.3 \pm 8.0\%$ and $83.3 \pm 3.1\%$ mean spore germination, respectively. However, germination of spores from 17 d PMMG cultures was only $7.0 \pm 10.4\%$ (Appendix 3: Table A3.1). An increasing proportion of darker, ‘shrunken’ spores were noticed consistently in phase contrast microscopy of older cultures on both PMMG and DSM media; these spores were never seen germinating (Appendix 3: Fig A3.1).

Table 3.1. Sporulation of *Dothistroma septosporum* on different media

| Growth medium ^a | Numbers of spores × 10 ⁴ /mL ^b | |
|----------------------------|--|---------------|
| | 7 dpi | 12 dpi |
| PMMG | 158.8a ± 13.5 | 315.9a ± 26.7 |
| DSM | 77.9b ± 10.7 | 174.8b ± 25.9 |
| PNA | 24.2c ± 2.4 | 28.2c ± 4.6 |
| WA | 3.0d ± 0.8 | 3.8d ± 0.9 |

^aPMMG (pine needle minimal media with glucose); PNA (pine needle agar), WA (water agar); DSM (dothistroma sporulation medium)

^bValues are mean ± SD of spore numbers at different days post-inoculation (dpi); combined results from three independent experiments.

Figures in a column followed by a different small letter differed significantly (P<0.05) as calculated from least significant difference.

3.2.2. Inoculation

Different inoculation techniques and adhesives were tested (sections 2.3.4 and 2.3.5 respectively) to determine their effects on the level of DNB infection. Of the three inoculation techniques trialled, spray inoculation was most effective, with needle infection levels of 28.5 ± 14.1%, compared to 2.6 ± 1.8% for brush and 7.6 ± 6.3% for cloth inoculation methods (LSD = 9.9, at 0.05%) (Table 3.2). Spraying was also the fastest of these methods.

Table 3.2. Effect of inoculation techniques on dothistroma needle blight

| Spore inoculation method | Needles with symptoms ^a (%) | Red bands/needle ^a | Total needles counted |
|--------------------------|--|-------------------------------|-----------------------|
| Spray | 28.5 a ± 14.1 | 1.5 a ± 0.2 | 478 |
| Brush | 2.6 b ± 1.8 | 1.0 b ± 0.0 | 321 |
| Cloth | 7.6 b ± 6.3 | 1.1 b ± 0.2 | 375 |
| Negative control | 0.0 b ± 0.0 | 0.0 c ± 0.0 | 605 |

Figures in a column followed by a common letter did not differ significantly (P<0.05) as calculated from least significant difference.

^aMeans ± SD are from three replicate seedlings per treatment

The negative control trees were sprayed with water.

A trial of four potential adhesive compounds was carried out *in vitro* to determine the best candidates for *in planta* screening, in anticipation that improved adhesion of spores to the plant would lead to more reliable and higher levels of infection. When

tested for spore adhesion to a glass surface, none of the adhesives performed significantly better than the water control ($89 \pm 12\%$ spores adhered; $n = 4$) (Appendix 3: Table A3.2). Spore germination assessments with the various candidate adhesive compounds *in vitro* gave inconsistent results, but it was observed that mucin induced clumping of spores. Mucin was associated with high DNB levels in a preliminary *in planta* trial; hence a replicated *in planta* trial was carried out to test the effect of spraying pine seedlings with spores suspended in 0.5% mucin. A moderate level of DNB infection ($35.9 \pm 8.5\%$) was obtained but this was not a significant improvement over infection levels obtained with the water control ($29.2 \pm 8.4\%$; $LSD = 10.4$) (Table 3.3). It was concluded that the candidate adhesives tested do not consistently improve infection levels compared with water alone.

Table 3.3. Effect of adhesives on dothistroma needle blight incidence and red bands

| Treatments | Disease incidence ^a (%) | Red band/ needle ^a | Total needles counted |
|-----------------|------------------------------------|-------------------------------|-----------------------|
| Gelatin (1%) | 10.7 c \pm 3.7 | 1.2 a \pm 0.2 | 697 |
| Mucin (0.5%) | 35.9 a \pm 12.0 | 1.4 a \pm 0.1 | 858 |
| Tween20 (0.05%) | 17.9 b \pm 3.3 | 1.1 b \pm 0.1 | 596 |
| Water (Control) | 29.2 a \pm 8.4 | 1.2 a \pm 0.3 | 652 |
| Un-inoculated | 0.0 d \pm 0.0 | 0.0 b \pm 0.0 | 565 |

Figures in a column followed by a common letter did not differ significantly ($P < 0.05$) as calculated from least significant difference

^aMeans \pm SD are from four replicated seedlings per treatment

3.2.3. Needle Wetness

Many preliminary experiments were conducted to optimise conditions (section 2.3.6) for pathogenicity assays. These involved varying several parameters (such as adhesive, plant height, leaf wetness, light) to determine which had most effect. Some examples are shown in Appendix 3: Fig. A3.2. These experiments led to the hypothesis that high leaf wetness is critical for disease development in controlled conditions. It was further

postulated that high needle wetness would be particularly important during the early stages of infection when fungal spores germinate and hyphae grow over the needle surface prior to penetration, hence a step-wise investigation was carried out to optimise this. In the first step, the effect of level of needle wetness was determined and in the second step the duration of high wetness conditions was evaluated.

As shown in Table 3.4, high needle wetness during the first two weeks post-inoculation (wpi), followed by medium needle wetness conditions (section 2.3.6) significantly favoured high DNB infection (60.0%) compared to low needle wetness conditions followed by medium (13.3%) for the same time frame. It was also found that treatments having high needle wetness at any stage (early or late) showed no significant difference in percent needles with symptoms, although variability between replicates was high in these experiments as is often found in DNB assays.

Table 3.4. Effect of needle wetness on dothistroma needle blight (DNB) infection

| Needle wetness ^a | | Needles with symptoms ^b (%) | Red bands/ needle ^b | Total needles |
|-----------------------------|--------|--|--------------------------------|---------------|
| 0-2 wpi | >2 wpi | | | |
| Low | Medium | 13.3b ± 10.1 | 1.2c ± 0.2 | 497 |
| Low | High | 39.0ab ± 24.5 | 1.5c ± 0.1 | 480 |
| High | Medium | 60.0a ± 16.8 | 2.2a ± 0.2 | 443 |
| High | High | 52.2a ± 25.7 | 1.8b ± 0.1 | 447 |

^aNeedle wetness conditions: Low- No covering of seedlings, Medium- Seedlings under plastic covered pathogenicity assay chamber, High- individual seedlings enclosed within a plastic sleeve and within the covered pathogenicity assay chamber; wpi- weeks post inoculation.

^bValues are mean ± SD are from four replicate seedlings per treatment

Figures in a column followed by a common small letter did not differ significantly ($P < 0.05$) as calculated from least significant difference.

To determine the optimal length of high wetness during the early infection stage, the experiment was repeated, but with variable high wetness periods. Spore germination and needle penetration were also monitored. More than 60% spore germination was obtained when seedlings were kept in early-stage high wetness conditions for any period of time (from 4 to 70 d), whilst those kept under the ‘medium

wetness' conditions (zero high wetness) only showed 25% spore germination when measured at the same time-point (Table 3.5). Likewise needle penetration was very low, or delayed, in the treatment lacking a high-wetness period. Thus an early high-wetness period is conducive for efficient spore germination and penetration. Amongst the samples that did have an early high-wetness period, penetration was significantly higher in those with 4 day high wetness than those with longer high-wetness periods, when measured at 7 and 14 dpi (Table 3.5). Finally, the percentage of needles showing DNB symptoms were significantly higher for the seedlings kept in high wetness conditions for 4 or 7 days and less variability was seen between replicate trees under these conditions (Table 3.5). Disease progression was also monitored in this experiment. It was also noticed that percent symptomatic needles started to increase in 4 or 7 d high wetness conditions after 5 wpi. In contrast, needles kept in high wet condition for the whole duration did not increase DNB symptoms (Fig. 3.1).

Table 3.5. Effect of initial high wetness on spore germination, penetration and dothistroma needle blight symptoms

| High wetness period (d) | Spore germination (%) ^c 4 dpi | Penetration (%) at ^c | | | Needles with symptoms ^c (%) | Red bands/ Needle ^c | Total needles |
|-------------------------|--|---------------------------------|-------------|-------------|--|--------------------------------|---------------|
| | | 4 dpi | 7 dpi | 14 dpi | | | |
| 0 | 25.0b ± 4.6 | 0.0 ± 0.0 | 0.0c ± 0.0 | 0.0b ± 0.0 | 23.2c ± 17.1 | 1.4b ± 0.2 | 163 |
| 4 | 65.5a ± 7.5 | 0.3 ± 0.4 | 1.8a ± 1.0 | 17.5a ± 9.3 | 83.6a ± 5.6 | 2.3a ± 0.2 | 201 |
| 7 | 65.0a ± 2.3 | 0.1 ± 0.1 | 0.8b ± 0.8 | 2.6b ± 1.0 | 77.9a ± 5.9 | 2.2a ± 0.4 | 180 |
| 14 | 62.2a ± 3.0 | 0.2 ± 0.3 | 0.5b ± 0.1 | 0.9b ± 1.3 | 54.5b ± 14.1 | 1.6b ± 0.2 | 260 |
| 70 ^a | 63.2a ± 5.6 | 0.0 ± 0.0 | 0.4bc ± 0.2 | 0.2b ± 0.2 | 4.3d ± 3.8 | 1.1c ± 0.1 | 178 |
| Control ^b | 0.0c ± 0.0 | 0.0 ± 0.0 | 0.0c ± 0.0 | 0.0b ± 0.0 | 0.6d ± 0.7 | 0.8c ± 0.5 | 278 |

^a70 days = full length of trial

^bControl plants were inoculated with water and kept under high wetness for 4 d

^cValues are mean ± SD are from four replicate seedlings per treatment

Symptoms were evaluated at 10 wpi (week post inoculation), spore germination and penetration calculated at 4, 7, 14 days post inoculation (dpi)

Five needles were collected from each replication for spore germination and penetration studies

Figures in a column followed by a common small letter did not differ significantly (P<0.05) as calculated from least significant difference.

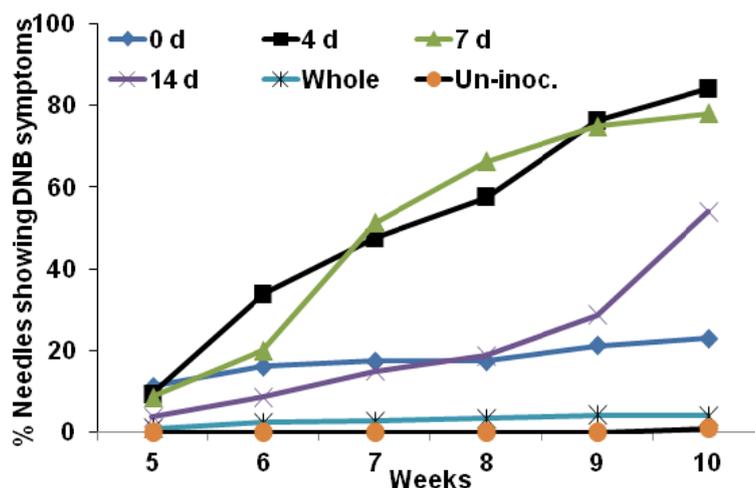


Fig. 3.1. Dothistroma needle blight (DNB) progression

Percent needles showing DNB symptoms from appearance (week 5) to week 10. Initial 4 and 7 d high wetness condition showed higher DNB symptoms compared to high wetness for 14 d or for the whole incubation period. LSD values for weeks 5, 6, 7, 8, 9, 10 are 5.7, 12.3, 11.4, 12.5, 6.9 10.3 respectively at $P < 0.05$. $n=4$

Based on the results of treatments showing highest DNB symptoms (83.6%) during optimisation of wetness conditions, pathogenicity assays were repeated twice with 4 dpi high-wetness followed by medium wetness. In these two independent trials, disease levels with >95% symptomatic needles ($97.6 \pm 1.1\%$ and $96.7 \pm 5.8\%$) were obtained (Appendix 3: Table A3.3 and Fig. A3.3).

3.2.4. Host Genotype

Using the optimised inoculation method, a DNB pathogenicity challenge was performed using young clones (cuttings) taken from seedlings belonging to families showing either moderate resistance (MR) or high susceptibility (HS) to DNB in the forest (Table 3.6). A high level of DNB infection was achieved with clones (3/1, 3/2) from the HS family (>77% of needles showing symptoms) along with long lesions (Fig. 3.2) and high numbers of fruiting bodies. In contrast clones (6/1, 6/2) from the MR family showed less than 12% symptomatic needles (Table 3.6) with shorter lesions and lower numbers of fruiting bodies. Disease progression curves also showed the late

appearance of lesions in MR hosts with no rapid increase of percent symptomatic needles after a lag period (Appendix 3: Fig. A3.4).

Table 3.6. Dothistroma needle blight events on *Pinus radiata* clones

| Pine clone ^a | Needles with symptoms ^b (%) | Red bands/needle ^b | Lesion length ^b (mm) | No. Fruiting body/lesion ^b | Total needles |
|-------------------------|--|-------------------------------|---------------------------------|---------------------------------------|---------------|
| HS (3/1) | 77.8b ± 8.1 | 1.2 ± 0.1 | 4.4a ± 0.2 | 11.2b ± 2.0 | 397 |
| HS (3/2) | 87.9a ± 4.0 | 1.4 ± 0.3 | 3.9a ± 0.8 | 17.4a ± 6.0 | 581 |
| MR (6/1) | 11.3c ± 3.1 | 1.3 ± 0.2 | 2.6b ± 0.5 | 6.7c ± 5.0 | 514 |
| MR (6/2) | 9.8c ± 4.1 | 1.2 ± 0.3 | 2.7b ± 0.5 | 5.1c ± 5.1 | 396 |
| Control | 0.0d ± 0.0 | 0.0 ± 0.0 | 0.0c ± 0.0 | 0.0d ± 0.0 | 267 |

^aClones 3/1, 3/2 are from highly susceptible (HS) and 6/1, 6/2 from moderately resistant (MR) families.

^bValues are mean ± SD; n=4

Figure in a column followed by a common letters did not differ significantly (P<0.05) as calculated from least significant difference.

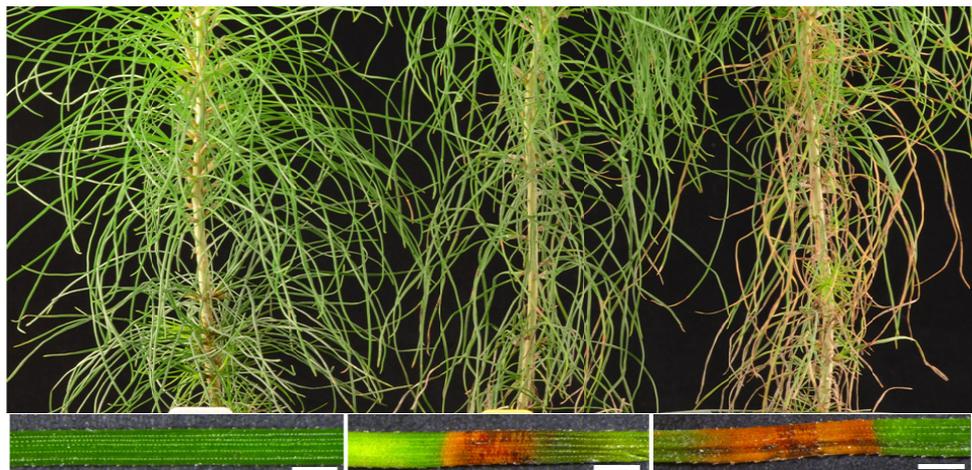


Fig. 3.2. Dothistroma needle blight (DNB) symptoms on pine clones

Un-inoculated pine clones having no DNB symptoms (left), moderately resistant clones showing some needles with DNB symptoms at bottom (middle), highly susceptible clones having DNB symptoms all over the seedlings (right). Representative needles are shown enlarged under each type of seedlings where shorter lesion is shown from moderately resistant clone (middle) compared to highly susceptible clone (right). Size bar = 1 mm.

In a repeat of this experiment only 3/2 and 6/2 clones were available, but these showed similar trends having 82.9% and 6.1% needles with symptoms respectively (Appendix 3: Table A3.4).

It was expected that clonal pine hosts will show less variation during disease development than seedlings that are genetically diverse. In this case clones would be preferable for future molecular, biochemical and physiological aspects of *Dothistroma*-pine interactions. Therefore, the percent symptomatic needles of pine seedlings and pine clones over the time course of infection were illustrated in a scatter plot (Fig. 3.3). Although the scatter plot suggests the clones showed lower variance than the seedlings this was not significant according to a Levene's test as shown in Appendix 3: Table 3.5.

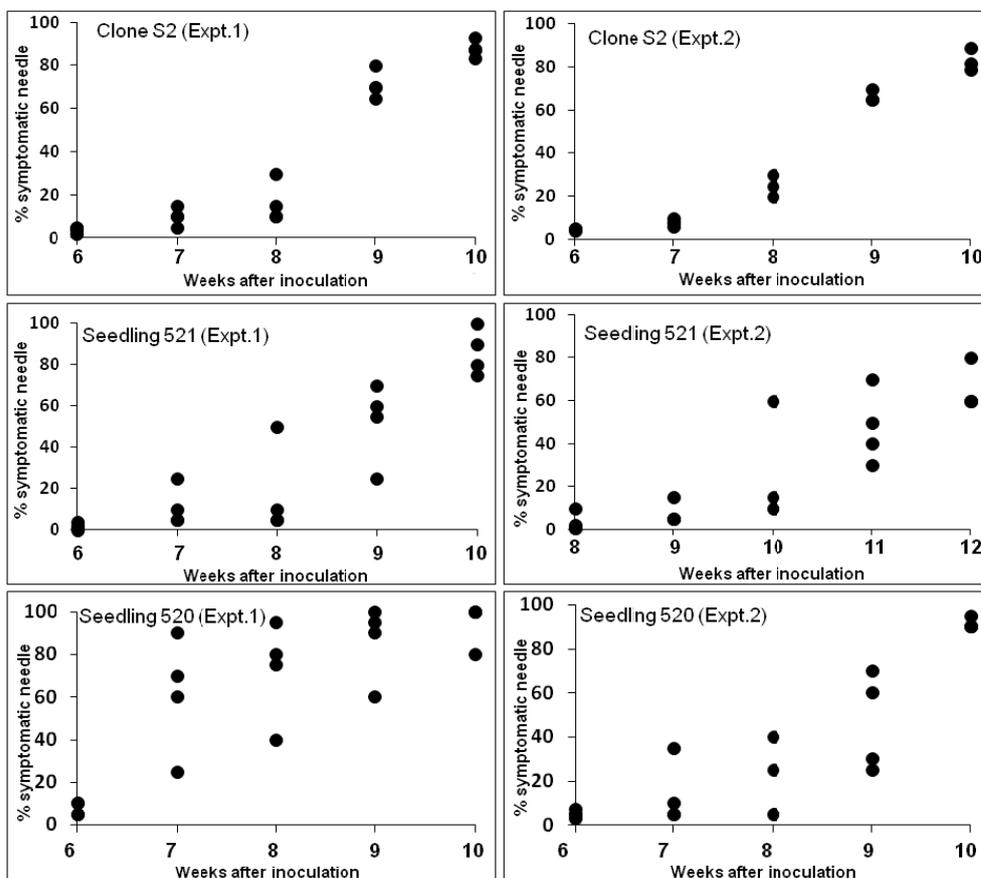


Fig. 3.3. Scatter plots showing percent of needles showing dothistroma needle blight symptoms in *Pinus radiata* clones or seedlings

Each point shows results for one tree, n = at least 3, at each week

In summary, it can be stated that the optimised pathogenicity assay provides the ability to distinguish field resistance and susceptibility in pine clonal populations when testing under controlled glasshouse conditions. However variability in dothistroma

needle blight infection levels is seen between replicate clones as well as between replicate seedlings.

3.3. Discussion

This study was undertaken to achieve a reliable and high rate of DNB infection in *P. radiata* seedlings. To meet this purpose suitable sporulation media were evaluated and different aspects of *in planta* pathogenicity assays were optimised.

Culture media play an important role in nutrition and production of fungal spores. Sometimes addition of host tissue makes the media more productive. For example, carnation leaf pieces induce sporulation of *Fusarium* species in water agar (Fisher et al., 1982), mulberry leaves help sporulation of *Colletotrichum dematium* in potato sucrose agar (Yoshida and Shirata, 2000) and banana and palm petioles favour the sporulation of their endophytes in corn meal agar (Photita et al., 2001) or malt extract (Guo et al., 1998). Likewise, addition of pine needle extract in PMMG enhanced sporulation levels in *D. septosporum* in this study. Similarly, sporulation of another pine pathogen (*Sphaeropsis sapinea*) was induced by addition of sterile pine needles to water agar (Basilio et al., 2007). However the results suggest it is important to check spore morphology and viability to ensure that optimum spore germination will be achieved on the chosen medium; for inoculum routine harvesting of spores after 7 - 12 days incubation on PMMG is advised.

Attenuation of pathogen virulence over time due to serial sub-culturing is often suggested as a possible factor limiting successful disease development and for this reason many authors have preferred to use fresh field inoculum for DNB infections (Ellingbo, 1968; Muir and Cobb, 2005). During the current study and other unpublished studies in this laboratory *D. septosporum* cultures revived from more than ten years of

storage at -80⁰C, followed by three subsequent sub-cultures, did not show any loss of virulence and resulted in high DNB infection.

In pathogenicity assays a spray inoculation technique was found best for DNB infection. It is widely used in other successful pathogenicity assays of pine species (Aitken, 1993; Gadgil, 1974; Muir and Cobb, 2005). Its success is probably due to very tiny droplets of spore suspension that can cover most of the needle surface area evenly, providing a uniform inoculum. Compounds trialled as adhesives did not reliably improve either spore adhesion or overall disease scores. Adhesive is not required in *M. fijiensis* pathogenicity assays (Donzelli and Churchill, 2007) but the surfactant tween 20 is used for another closely related species, *M. graminicola* (Keon et al., 2007).

It is well established that high humidity is required for spore germination of DNB fungi (Gibson et al., 1964; Gilmour, 1981; Ivory, 1972; Muir and Cobb, 2005; Peterson, 1969). These authors found 50-90% *D. septosporum* spore germination on *P. radiata* needles with high humidity (more than 85% RH) and high needle wetness obtained with a water sprinkler. High humidity was used for other pathogenicity assays of pine (Aitken, 1993), wheat (Keon et al., 2007) and banana (Donzelli and Churchill, 2007). However, experiences with DNB during this work showed that even 100% RH, used throughout these experiments, is not sufficient for good spore germination (and consequent needle infection), as shown by the low levels of germination on trees without a 'high wetness' period (Table 3.5). The significant increase in spore germination seen under high wetness conditions suggests an extremely high needle wetness threshold for this critical stage of the disease cycle. In previous studies (Schwelm et al., 2009) it was found that reverse osmosis (RO) or distilled water is preferable to tap water to avoid mineral deposition on needles; this requirement will differ depending on local water quality.

Needle wetness also plays a role in fungal penetration into needles, although in this study most of the penetration occurred after the high wetness period had been downgraded to 'medium wetness' (leaf surface wetness was higher in 'high wetness' compared to 'low wetness' conditions) (Table 3.5). Moisture and humidity levels fluctuate in the forest environment (Peterson, 1976), and accordingly higher fungal penetration was found in variable rather than constant misting regimes (Muir and Cobb, 2005; Peterson and Walla, 1978). In general, it is reported that fungal penetration is avoided in the presence of film water (Bald, 1952). The current study shows that although fungal penetration was significantly higher in 4 d high wetness compared to 7 d high wetness, no significant difference in numbers of diseased needles was found between those treatments after 10 weeks. This suggests that more fungal penetration happened after 14 dpi in the 7 d high wetness condition, i.e. after wetness had been downgraded to 'medium'. Alternatively it is possible that penetration of at least 2.6% of stomatal pores is sufficient to have >70% needles with symptoms.

DNB symptom development also depends on needle wetness. Gadgil (1977) obtained very few DNB symptoms when seedlings were kept in dry conditions immediately after inoculation. In another experiment when comparing the duration of misting, only 16-48% DNB symptoms were noticed when misting was turned on for 16 hour per day, whilst higher disease levels (78-84%) were obtained with continuous misting (Muir and Cobb, 2005). This prolonged wetness condition was also found necessary for disease development of some other species of *Mycosphaerella* (Carisse et al., 2000; Chungu et al., 2001; Goos and Tschirch, 1963). In this experiment, prolonged wetness conditions were also used with very high wetness at the beginning. It appears that a short period (4-7 d) of very high needle wetness is preferable to a longer period to establish better penetration, and that the positive effects of the short early wetness

period are seen during later stages of the disease cycle as shown by higher levels of disease incidence.

Other microclimatic factors such as temperature and light, that are also important components for DNB development, were not tested for optimisation in this study. As it was already known that DNB can occur in a wide range of temperatures (Gadgil, 1974; Parker, 1972), experiments were conducted in a suitable temperature (20°C) in a temperature controlled glass house. However, as all experiments were conducted in natural lighting conditions, the received light energy varied from season to season (i.e. experiment to experiment). Light is required for DNB symptom development (Gadgil and Holden, 1976) and therefore some effect of light intensity on symptom levels was expected although no clear correlation could be discerned over the course of this study (Appendix 4: Table A4.2). Within each experiment, as all treatments received the same amount of light, the comparisons that were done were therefore not expected to be affected by light levels.

High DNB symptom levels (80-90% needle infection) on clones showed that young clonal material is suitable for efficient pathogenicity assessments. Significantly more DNB symptoms occurred on clones from families showing field susceptibility compared to those with field resistance. These results imply that this glasshouse-based artificial inoculation method is suitable for screening young *P. radiata* clones for field resistance and may provide a time- and cost-effective method of resistance screening (De Souza et al., 1990). A reliable pathogenicity assay will be a useful tool for the scientific community and for pine resistance breeding programs.

In summary, high and reliable levels of DNB symptoms were obtained by spraying *P. radiata* with 7-12 d old *D. septosporum* spores (grown in PMMG) at the rate of 3×10^6 spores/ml and keeping them in a high wetness condition by covering for 4

days after inoculation, followed by continual misting in an enclosed but ventilated plastic chamber. A clear distinction between DNB field resistance and susceptibility could be determined using this assay

Chapter 4: Life style of *Dothistroma septosporum* in *planta*

4.1. Introduction

D. septosporum clearly has a necrotrophic phase in its life cycle (Bradshaw, 2004; Gadgil, 1967), at which stage dothistromin is seen in lesions. It is predicted to have a hemi-biotrophic life style *in planta* (de Wit et al., 2012) as the symptoms develop a few weeks after inoculation. This fungus belongs to the Dothideomycetes, a diverse class of fungi (Stergiopoulos et al., 2013) that includes those with biotrophic or latent components of their life cycle, as well as necrotrophs. However, the closest relative of *D. septosporum* characterised so far, the tomato pathogen *C. fulvum*, is a biotroph, and these two species share many similarities at the genome level (de Wit et al., 2012).

Another closely related species is *M. graminicola* that has a symptomless period (Fig. 4.1A) before onset of leaf blotch of wheat (Keon et al., 2007). Its growth on wheat during this symptomless period is minimal (until 8 days after inoculation), hence it can be considered as a 'latent' or biotrophic phase. Later on at nine days after inoculation, host cell death was noticed with rapid increase of fungal biomass (Fig. 4.1B), which is the necrotrophic phase (Keon et al., 2007). This hemi-biotrophic life style is seen in many species of fungi in the genus *Mycosphaerella*, but has never been formally shown for *D. septosporum*. This chapter will describe the hemi-biotrophic life style of *D. septosporum*.

Some Dothideomycete fungi secrete secondary metabolites or toxins during their infection cycle (Stergiopoulos et al., 2013). *D. septosporum* releases dothistromin during plant infection and also when grown in culture. Dothistromin is synthesised during the exponential growth phase in culture (Schwelm et al., 2008), which is unusual as most secondary metabolites are produced mainly after fungal growth has slowed

down in the stationary phase (Cleveland and Bhatnagar, 1990; Trail et al., 1995). *In planta*, on the other hand, dothistromin appears to accumulate in red bands at a late stage of infection. Therefore, a contradiction arises on the timing of dothistromin production in culture and *in planta*, leading to the question of whether dothistromin is produced from the beginning of the life cycle *in planta* or is only produced in high amounts at the late stage. Determining when dothistromin is produced during various stages of DNB infection is important to understand the life style of *D. septosporum*.

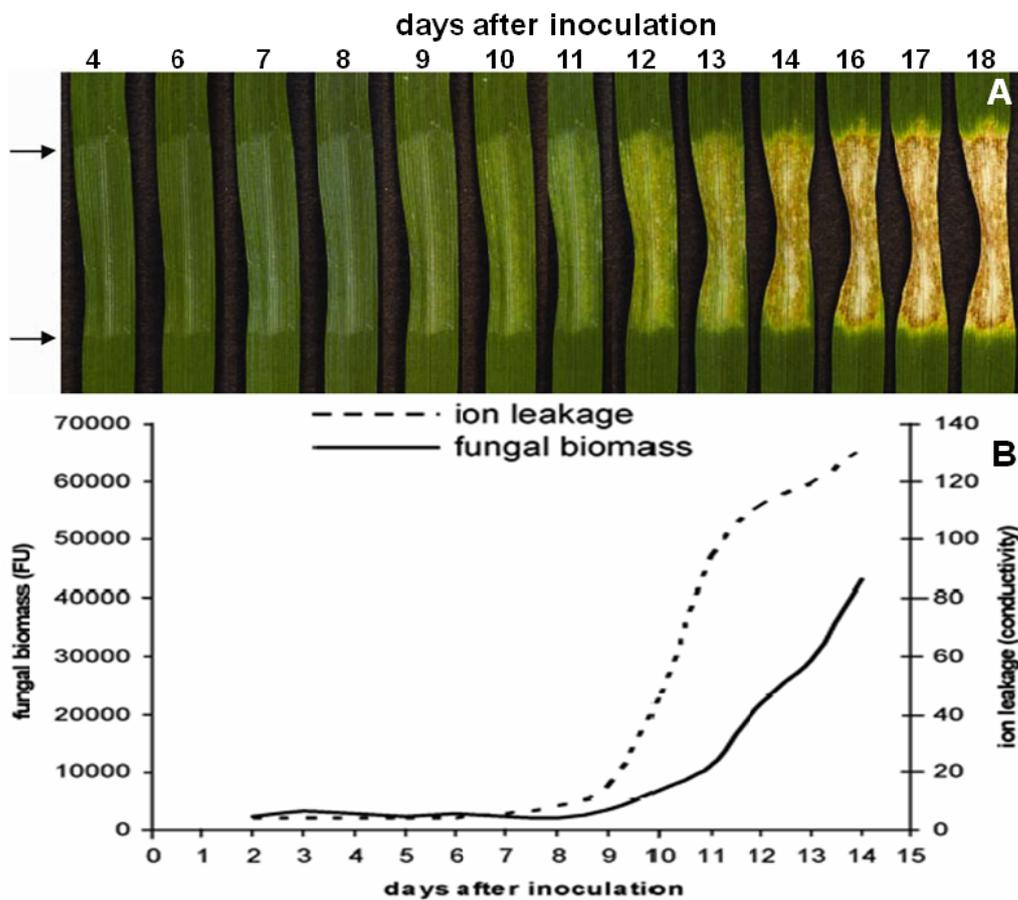


Fig. 4.1. Time course of *Mycosphaerella graminicola* (septoria leaf blotch) on wheat

(A) Time course symptom development on wheat, where visible symptoms started to appear at 9 days after inoculation (dai) followed by leaf necrosis and development of asexual sporulation structures; (B) Ion leakage (indicating host cell death) and fungal biomass increase during the time course, where there is very minimal fungal biomass until 9 dai followed by a rapid increase in later time points.

Knowledge of the life style of *D. septosporum* is helpful in understanding the disease process and the molecular basis of virulence or pathogenicity by this pathogen. For example effector molecules (e.g. Avr4) released by the biotrophic pathogen *C. fulvum* trigger a hypersensitive resistance response (HR) in tomato following gene for gene resistance (de Wit et al., 2009). However, in the inverse gene for gene theory necrotrophic effectors trigger HR which enhances susceptibility, such as with *S. nodorum* on wheat (Friesen et al., 2007). If an effector molecule of *D. septosporum* triggered a HR response in pine this could either inhibit the pathogen (as in the case of a biotroph) or help it (as in the case of a necrotroph). In addition, effectors could potentially be used to screen for resistance (or susceptibility) in pines. Therefore, understanding of the life style will provide deeper knowledge on *Dothistroma*-pine interactions that will facilitate forest management practices in future.

In this study, microscopic, histological and molecular tools were used to describe the hemi-biotrophic life style of *D. septosporum*. In microscopy, the infection process was monitored over a time course and histological studies were carried out to find the time point when host cells start to die. In addition, real time PCR was used to quantify fungal growth over time. Combining those three observations it was possible to separate biotrophic or latent and necrotrophic phases of *D. septosporum* interactions in pine needles. Biochemical studies were also carried out at the same time points to monitor the changes in dothistromin levels over time. Host *P. radiata* seedlings were used and were inoculated with wild type (WT) and *gfp*-labelled WT *D. septosporum* to study the host microbe interaction in glass house conditions. In addition, some gene expression studies were conducted to determine more details of the *D. septosporum* life style over the time course of DNB *in planta*.

4.2. Results

4.2.1. Microscopy, molecular and biochemical studies of *Dothistroma septosporum* life style

4.2.1.1. Overview of *Dothistroma septosporum* life cycle *in planta*

The life cycle of *D. septosporum in planta* was usually completed within ten to twelve weeks after inoculation (ai) under temperature controlled glasshouse conditions using either wild type (WT) (NZE10 and NZE30) or *gfp*-labelled WT *D. septosporum* isolate FJT20. For clarity of descriptions, the life cycle can be divided into four stages as shown in Table 4.1. However, the time periods varied between and within experiments such that the timing of stages of the life cycle overlapped. An overview of the life cycle is illustrated in Figs. 4.2 (macroscopic observations) and 4.3 (microscopic observations). The individual stages are discussed in more detail below, along with further microscopic observations that indicate key features.

Table 4.1. Macroscopic and microscopic overview of the *Dothistroma septosporum* life cycle

| Stages | Events | Weeks after inoculation ^a | Images of life cycle stages | |
|--------|--|--------------------------------------|-----------------------------|--------------------------|
| | | | Macroscopic ^b | Microscopic ^c |
| 1 | Epiphytic: spore germination, surface growth | 1 - 4 | Fig. 4.2B | Figs. 4.3A and 4.3B |
| 2 | Penetration, growth inside epistomatal chambers | 2 - 6 | Fig. 4.2B | Figs. 4.3C and 4.3D |
| 3 | Mesophyll colonisation and early lesion development | 3 - 8 | Fig. 4.2C | Fig. 4.3E |
| 4 | Late lesion development and fruiting body maturation | 6 - 12 | Fig. 4.2D | Fig. 4.3F |

^ausual range of life cycle, ^bobservation on needle surface, ^cobservation by scanning electron microscope

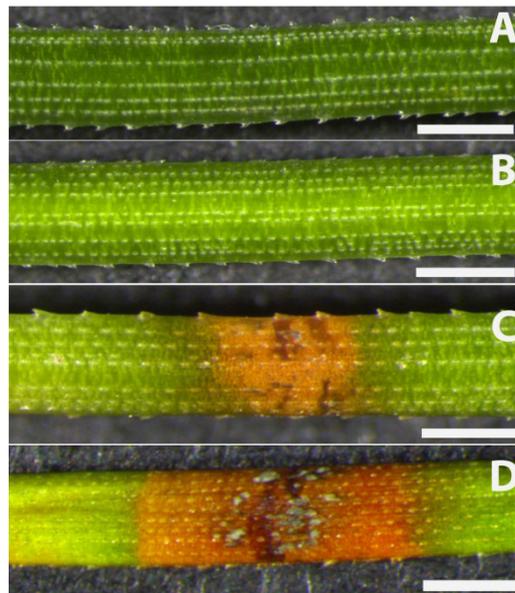


Fig. 4.2. Macroscopic overview of *Dothistroma septosporum* life cycle

(A) Un-inoculated needle; (B) Stages 1-2: Spore germination, surface growth, penetration, fungal growth inside stomata. No symptoms are visible during these times; (C) Stage 3: Mesophyll colonisation and early lesion development. Needle is showing early dothistroma needle blight (DNB) lesion; (D) Stage 4: Late lesion and fruiting body development. Needle shows an extended lesion with fruiting bodies (stromata) bearing conidiospores that have erupted through the epidermis. Size bar = 1 mm.

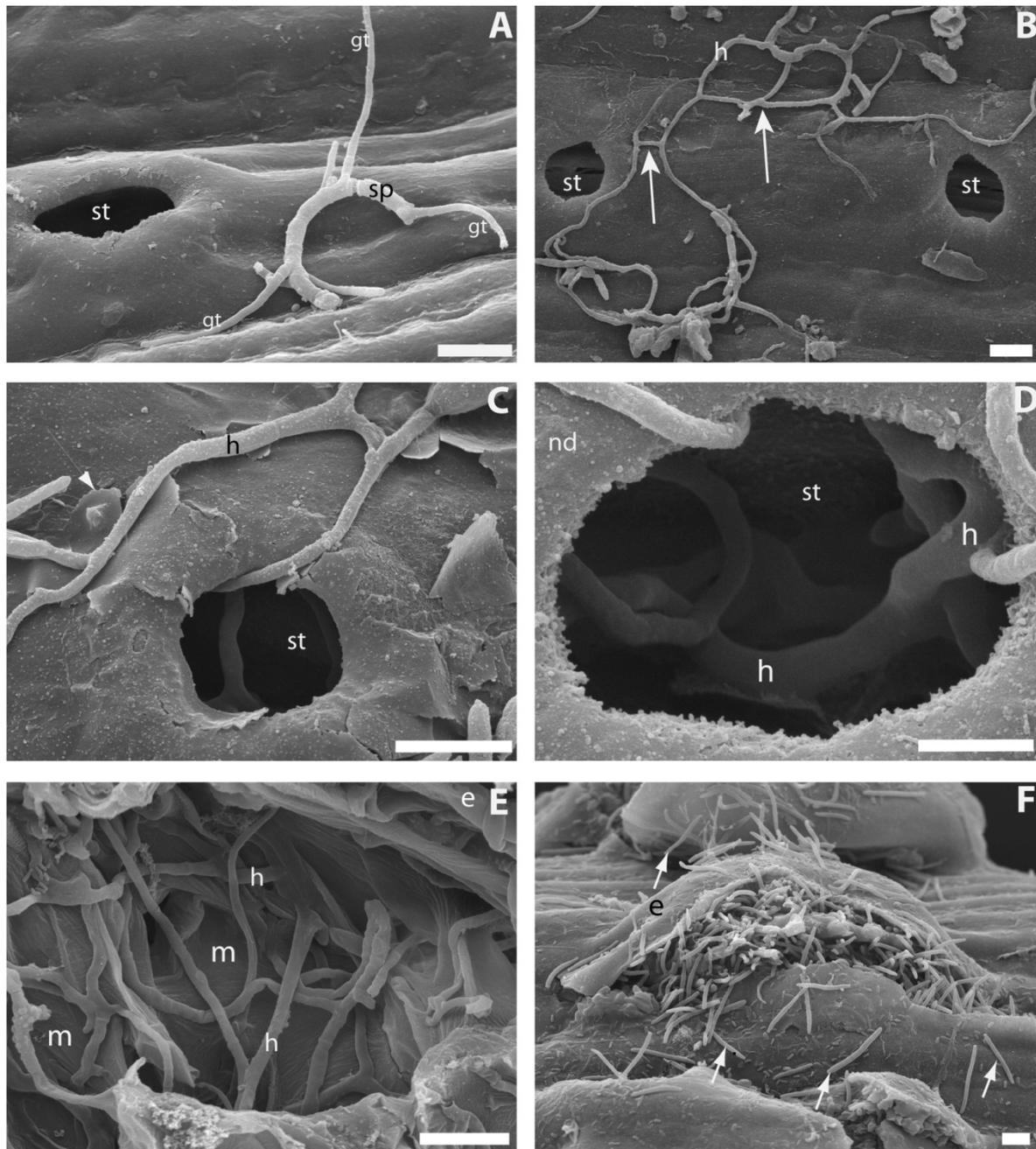


Fig. 4.3. Microscopic (SEM) overview of the *Dothistroma septosporum* life cycle

(A) Stage 1. Spore germination: germinating spore (sp) having several germ tubes (gt) on needle surface near a stomatal pore (st) at week 1 ai (after inoculation); (B) Stage 1. Epiphytic fungal network on pine needle surface at 3 weeks ai. Hyphae (h) appear to grow randomly without targeting to stomatal pores (st) and hyphal anastomosis was also seen (arrows); (C) Stage 2. Penetration: penetration of hyphae into a stomatal pore; (D) Stage 2. Fungal hyphae growing inside epi-stomatal chamber of stomata (st) after penetration from needle (nd) surface; (E) Stage 3. Mesophyll colonisation: part of cross section of a pine needle where fungal hyphae colonised mesophyll cells (m) under the epidermal (e) layer; (F) Stage 4. An erupted fruiting body with mass of spores (arrows). Size bar = 10 μm except D which is 5 μm .

Stage 1: Spore germination and epiphytic surface growth.

Spores of *D. septosporum* started to germinate by week 1 ai. Germ tubes were seen growing from both central and terminal segments of *D. septosporum* spores, often with multiple germ tubes per spore (Figs. 4.3A, 4.4A, Appendix 4: Figs. A4.1A and A4.1B). During weeks 2-4 ai an extensive network of hyphae grew over the needle surface. There was no evidence for directional growth towards stomatal pores. (Figs. 4.3B, 4.4B and 4.4C, Appendix 4: Figs. A4.1C and A4.1D). In some cases anastomosis was seen during this stage on the needle surface (Fig. 4.3B). Hyphal growth direction and anastomosis were not quantified.

Stage 2: Penetration and growth inside epi-stomatal chamber.

From week 2 ai, hyphae started to penetrate into stomata (Fig. 4.3C). Chemotropism or thigmotropism is probably not involved in this process as germ tubes and hyphae sometimes were seen to cross stomata without penetration (Figs. 4.4D and 4.4E). No direct penetration of the epidermis by the fungus was noticed on the needle surface. After penetration fungal hyphae grew inside the epi-stomatal chamber (Figs. 4.3D, Appendix 4: Fig. A4.1D) from inoculation through to this time point no visible DNB symptoms were noticed on infected needles (Fig. 4.2B).

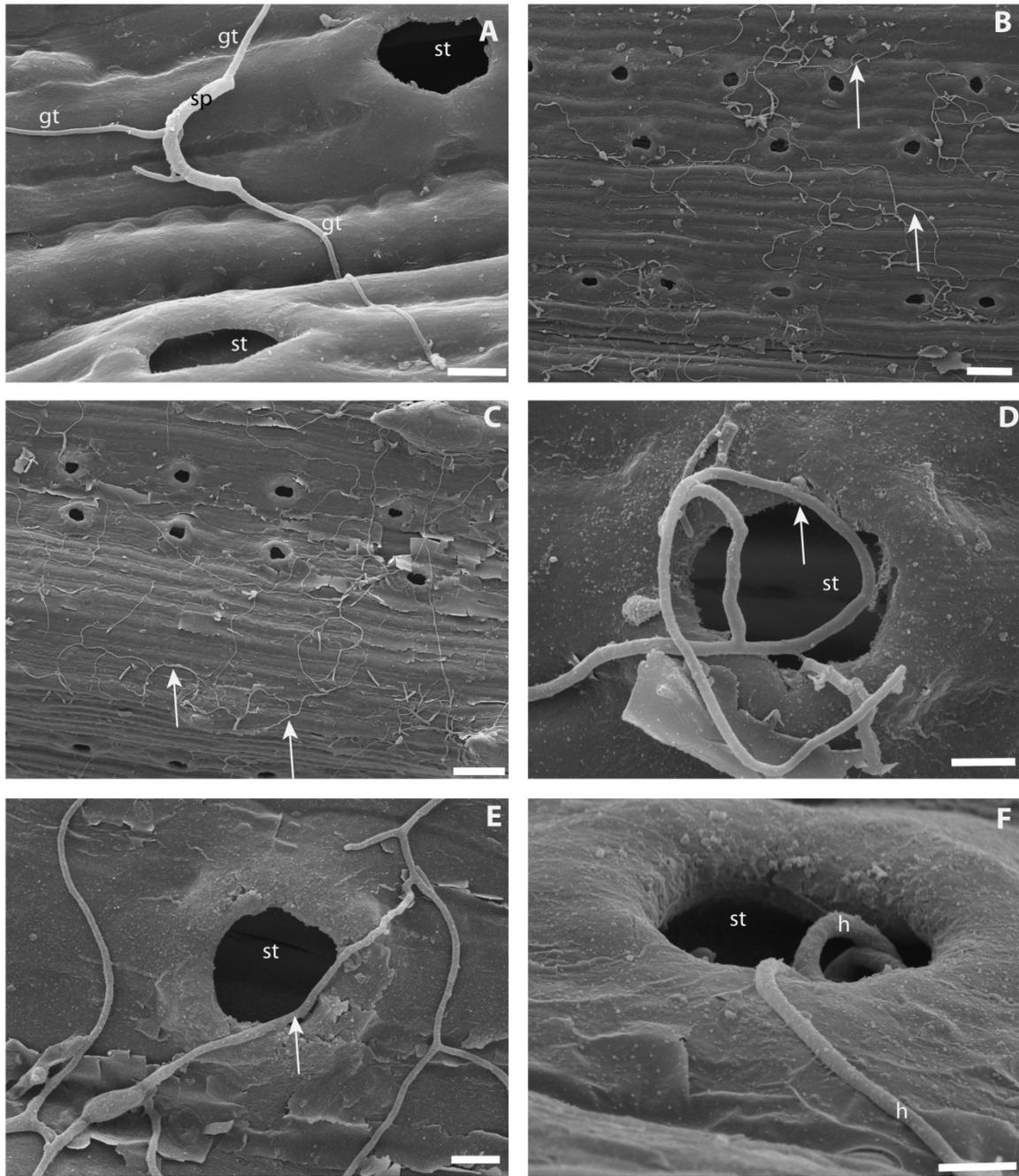


Fig. 4.4. Stages 1 and 2 of *Dothistroma septosporum* life cycle

(A) A germinating spore (sp) with four germ tubes (gt). The direction of growth appears random rather than targeting towards stomata (st); (B-C) Surface growth on needle surface where hyphae (arrow) are growing all over the needle; (D-E) Hyphae (arrow) appearing to avoid penetration; (F) Penetration of hypha (h) into a stomata. Size bars B, C = 40 μm ; A, E = 10 μm ; D, F = 5 μm .

Stage 3: Mesophyll colonisation, early lesion development.

Colonisation of the pine needle mesophyll tissue started to occur from week 3 - 5 ai (Figs. 4.3E and 4.5). Hyphae were found in the intercellular spaces of mesophyll cells and were sometimes seen to attach closely to mesophyll cells (Figs. 4.5A and 4.5B). No evidence of intracellular colonisation was seen during this period. Hyphal width appeared to be the same all over the mesophyll colonised area. Early DNB symptoms (early lesion) sometimes started to appear from this time point (Fig. 4.2C). Early lesions often appeared as water soaked lesions with a small brown (~1mm) necrotic area on the needle surface which usually extended to girdle the whole needle.

Stage 4: Late lesion development and fruiting body maturation.

From week 6 - 12 ai (Fig. 4.2D, at late DNB stage), masses of *D. septosporum* spores were released from fruiting bodies (stromata) that ruptured through the epidermal layer in a longitudinal line (Figs. 4.6, Appendix 4: Figs. A4.2A and A4.2B). Spores were not seen to erupt out from stomatal pores during this period. Over the course of this study it was determined that a single lesion can contain between 13 and 31 fruiting bodies, which in the laboratory, released up to 1.0×10^4 spores. Spores from needle lesions looked uniform in shape and size. Spores were not seen to germinate on the needle surface around these lesion areas during the course of these studies (Figs. 4.3F, 4.6B-F).

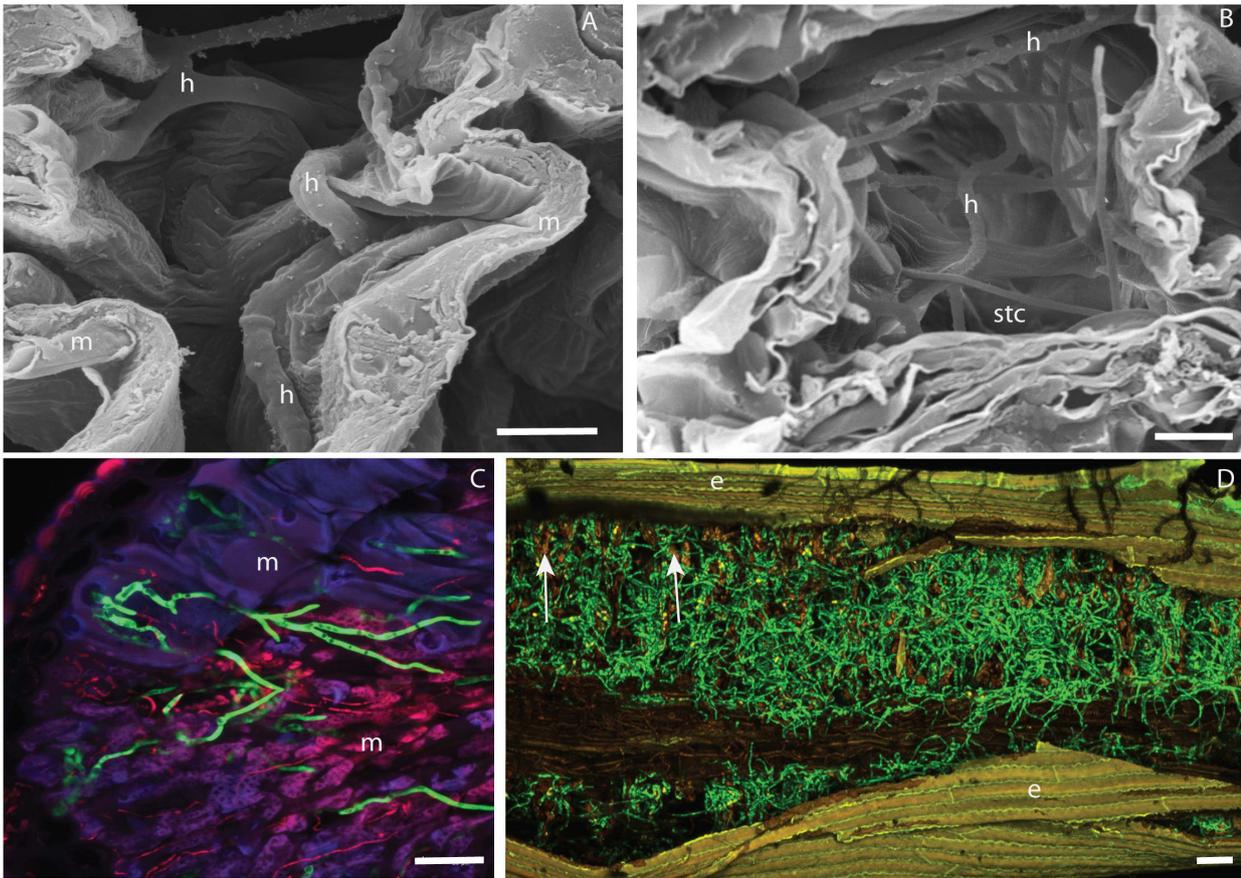


Fig. 4.5. Stage 3 (mesophyll colonisation) of *Dothistroma septosporum* life cycle

(A) Scanning electron micrograph (SEM) is showing fungal hyphae (h) in very close contact to mesophyll (m) cells; (B) SEM showing fungal hyphae (h) inside a sub-stomatal chamber (stc) in a transverse section of pine needle; (C) Confocal image showing cross section of a hand cut pine needle colonised by *gfp*-labelled WT *D. septosporum* (green). Fungal penetration can be seen from the stomatal pore (left) through mesophyll tissue towards the centre of the needle (right). Blue and red colours are auto fluorescence from mesophyll (m) cells; (D) Confocal image showing hand cut longitudinal section of a pine needle, where the spaces between the mesophyll cells (orange coloured, indicated by white arrows) are extensively colonised by *gfp*-labelled WT *D. septosporum* (green). 'e' indicates epidermis. Size bar A = 5 µm; B = 10 µm; C, D = 20 µm.

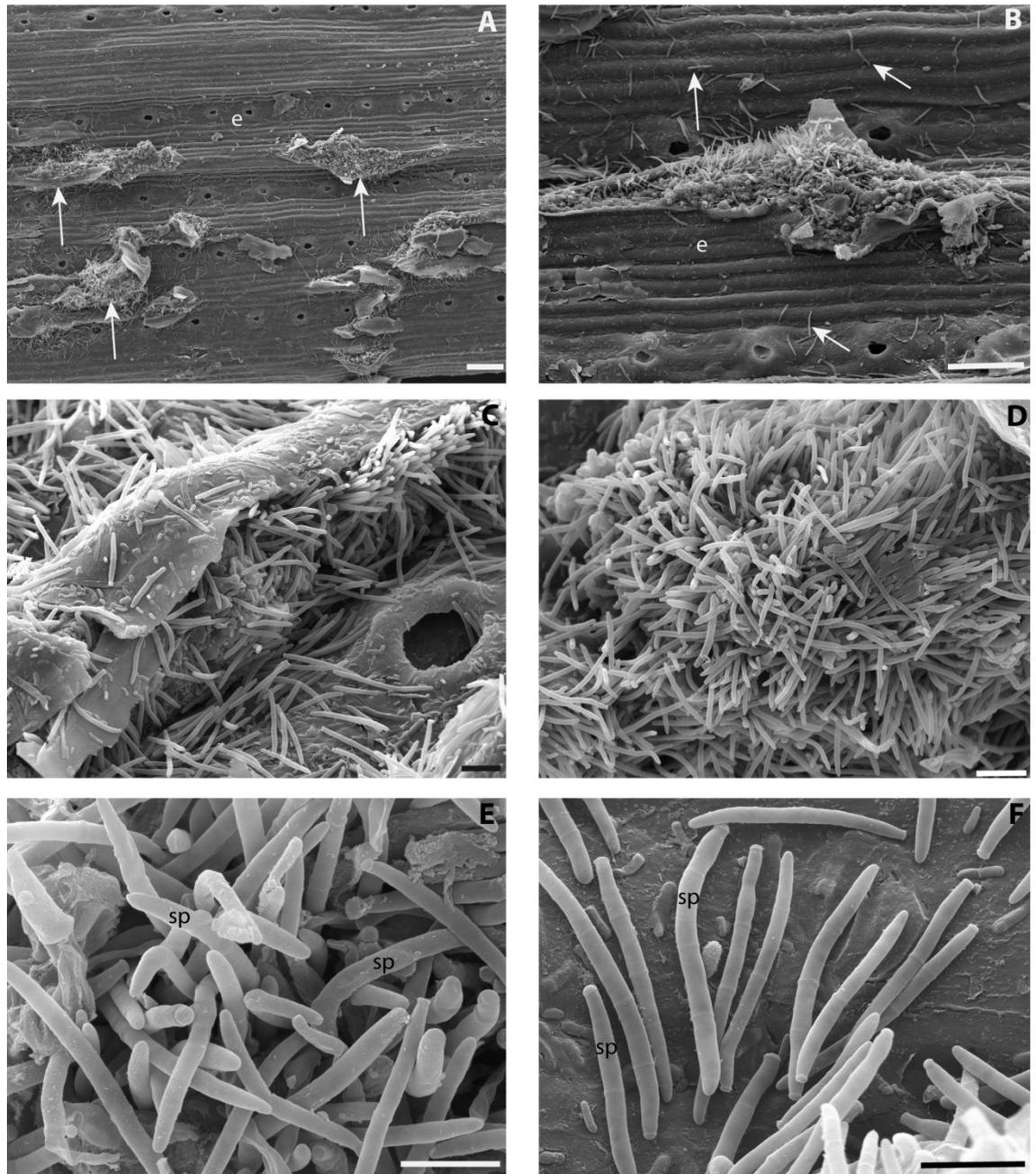


Fig. 4.6. Stage 4 (maturation of fruiting bodies) of *Dothistroma septosporum* life cycle

(A) Masses of spores from fruiting bodies (arrows) are seen erupting through the epidermis along longitudinal lines (e) of pine needle; (B) Close view of an erupted fruiting body showing tearing of the epidermal (e) tissues with some released spores (arrows); (C-D) Mass of spores is seen from erupted fruiting body; (E) Close view of many spores (sp) from an erupted fruiting body; (F) Close view of released spores from fruiting body at late stage on needle surface, showing uniform spore size and shape. Size bar A = 100 μm , B = 50 μm , C, D, F = 10 μm and E = 5 μm .

4.2.1.2. Host cell disintegration during stages 3 and 4 of dothistroma needle blight

The effect of *D. septosporum* invasion on the pine cell morphology, structure and integrity was studied using light microscopy (section 2.4.1) on cross sections of pine needles during progression of DNB.

In un-inoculated needles, although intact epidermal, hypodermal, mesophyll and endodermal cells were seen (Figs. 4.7A and 4.7B), some empty spaces were found among the mesophyll cells and under the stomatal chambers (sub-stomatal chamber). More of these spaces were generally found in older needles (older than two years) as shown in Figs. 4.7A and 4.7B than in young needles such as at six months of age (Appendix 4: Fig. A4.3). In needles inoculated with *D. septosporum*, a similar morphology to un-inoculated needles was seen in stages 1 and 2 (germination, surface growth, penetration and fungal growth inside stomata) with no evidence of host cell death or disintegration (Fig. 4.7C-F). This was consistent with macroscopic observations in which no DNB symptoms were found on infected needles during stages 1 and 2 (Fig. 4.2B) and also consistent with a biotrophic or latent stage for the pathogen.

When early lesions were evident on needles at stage 3, and the mesophyll region was colonised by *D. septosporum* within those early lesions, massive collapse of mesophyll cells was observed, although endodermal cells remained intact (Figs. 4.7G and 4.7H). In stage 4 (late lesions), more extensive fungal growth was seen inside the pine needle that spread towards the endodermal region and endodermal cells had collapsed. During colonisation, fungal hyphae were found between the intercellular spaces of mesophyll cells with very close contact (Figs. 4.7I and 4.7J). This life cycle study was also conducted by inoculating *gfp*-labelled WT *D. septosporum* on pine needles. After confirming the stages by fluorescent microscopy needle samples were prepared for light microscopy and fungal hyphae were identified by diameter (1-2 μm).

Events in stages 2 and 4 (Figs. 4.7F and 4.7J respectively) were studied using TEM (section 2.4.3) to monitor fungal and plant cellular structures in more detail. In stage 2, when fungal hyphae were present in the epi-stomatal chamber some appeared closely attached to stomatal guard cells (Fig 4.8A). Guard cells (Figs. 4.8B and 4.8C), hypodermal and mesophyll cells (with cytoplasmic cell organelles) (Fig. 4.8D) all appeared intact during this time. However, in stage 4, (week 10 ai) by which time fungal hyphae had invaded the mesophyll region of the pine needle, guard cells (Fig. 4.8E) and mesophyll cells showed disintegration and lack of cytoplasmic organelles (Figs. 4.8F and 4.8G). In addition to the disintegrated mesophyll cells, deformed endodermal cells were also found (Fig. 4.8H) in the presence of fungal hyphae.

Other than the cell structures, differences were also noticed in the deposition of electron dense materials between these two stages. Compared to stage 2 (week 4 ai; Fig. 4.8D), more electron dense materials were noticed at stage 4 (week 10 ai) in host guard cells (Fig. 4.8E) and mesophyll cells (Figs. 4.8F and 4.8G). The nature of these materials is unknown but it is possible they were phenolic compounds released as part of the plant defense. A considerable amount of electron dense material was also noticed around the fungal hyphae at this time point.

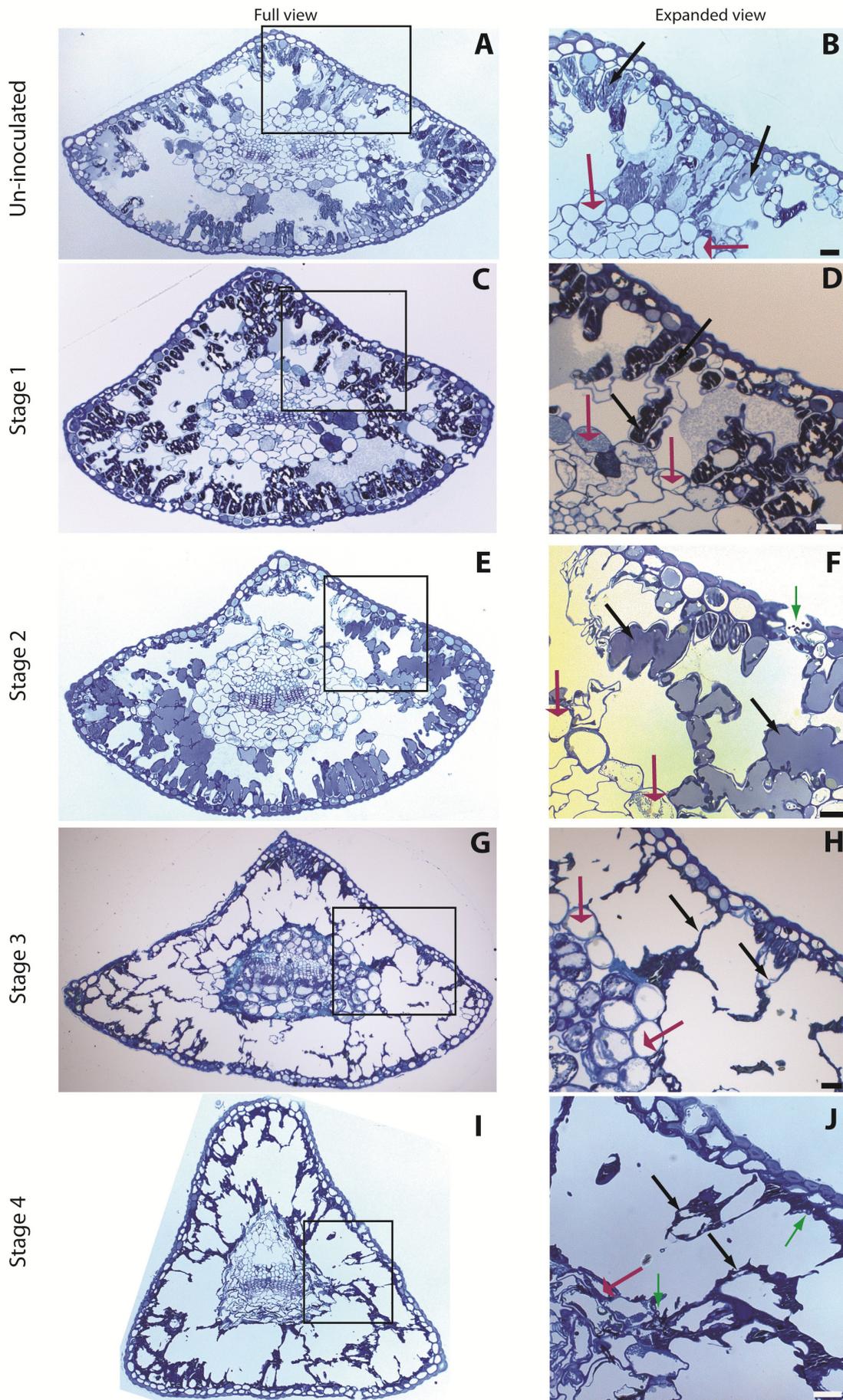
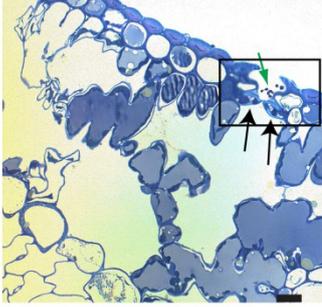


Fig. 4.7. Light microscopic observations on pine needle cross sections during dothistroma needle blight progression

(A, C, E, G, I) (left) are full needle cross section views and (B, D, F, H, J) (right) are expanded views of the parts indicated by rectangles in the respective whole needles. In all sections mesophyll and endodermal cells are indicated with black and purple arrows respectively and the presence of hyphae is indicated with green arrows where applicable. (A-B) Un-inoculated needle showing intact mesophyll cells, endodermal cells and apoplastic spaces between cells; (C-D) stage 1 (germination and surface growth) and (E-F) stage 2 (penetration and fungal growth inside stomata) showing intact cells. During stage 2 fungi were seen in epi-stomatal chambers (green arrow in F); (G-H) stage 3 (early lesions) needle showing disintegrated mesophyll cells and intact endodermal cells but few hyphae; (I-J) stage 4 (late lesions) needle showing disintegrated mesophyll and endodermal cells, and considerably more fungal hyphae than in stage 3. Times of stages 1, 2, 3 and 4 were approximately 1, 4, 7 and 10 weeks after inoculation respectively. Size bar = 20 μm .

Stage 2 (epi-stomatal penetration)



Stage 4 (late lesions)

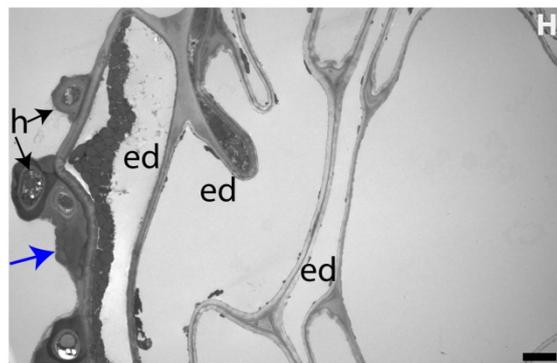
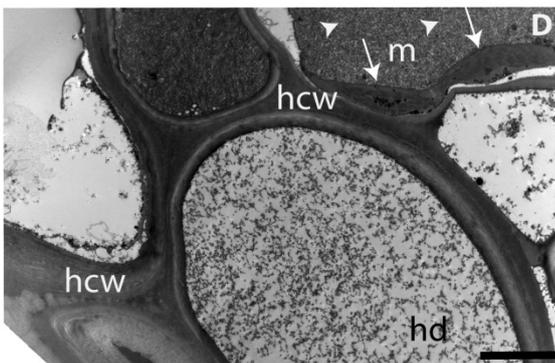
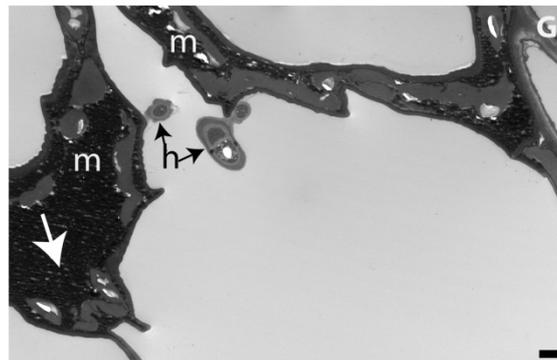
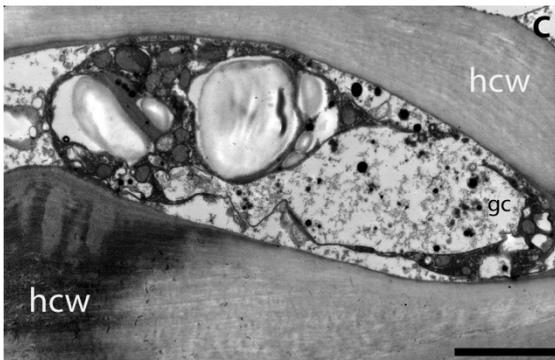
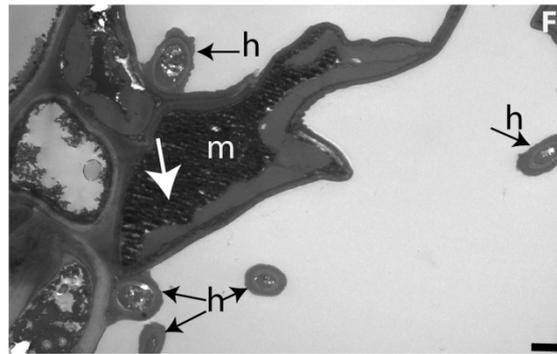
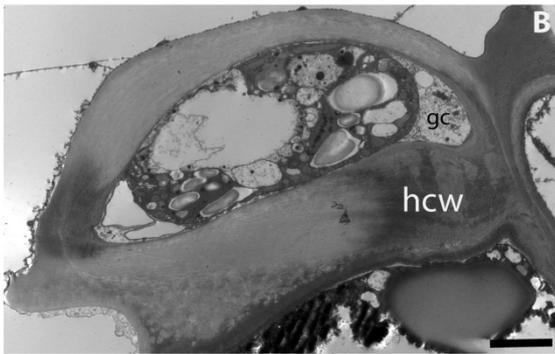
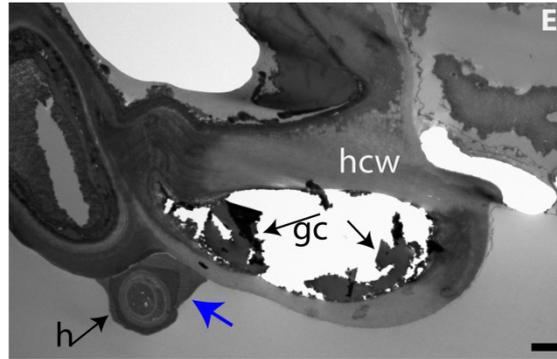
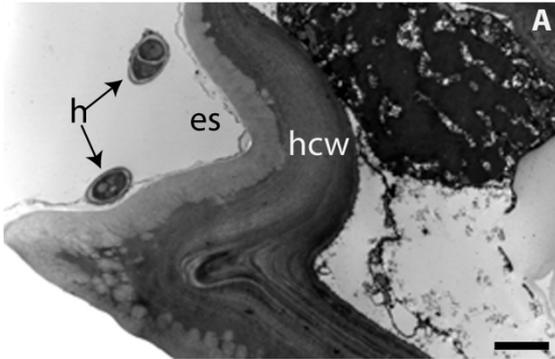
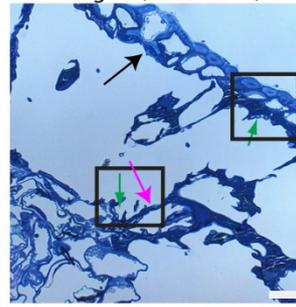


Fig. 4.8. Ultra-structure of dothistroma needle blight infection on *Pinus radiata* needle at stage 2 and 4

(A-D) and (E-H) are TEM views of stages 2 and 4 (weeks 4 and 10 ai respectively) that were prepared from respective light microscopy cross sections shown at the top. (A) Two fungal hyphae (h) (arrows) are seen in the epi-stomatal (es) chamber; (B-C) Intact guard cells (gc) (indicated by black arrows shown on the top), from the same epi-stomatal chamber as shown in (A), with cell contents surrounded by a thick host cell wall (hcw); (D) Intact cells such as hypodermal (hd), and mesophyll (m) cells that clearly contained cell organelles (arrow) were found with light electron dense material (white arrow head); (E) Disintegrated guard cells (gc) (indicated by black arrow shown on the top) appear to have no cell content, with a fungal hypha (h) nearby (arrow). High electron dense materials (black arrow) are seen inside the disintegrated guard cell and surrounding the fungal hypha. A high amount of this electron dense material was noticed at the point where the hypha attached to the guard cell (blue arrow); (F-G) Disintegrated mesophyll (m) cells showing similar characteristics as the disintegrated guard cells in (E) with intracellular deposition of high electron dense materials (white arrow) and fungal hyphae (black arrows) surrounded by electron dense material nearby; (H) Disintegrated endodermal cells (ed) with fungal hyphae nearby (arrow) surrounded by high electron dense material (blue arrow), especially where the hypha attached to the endodermal cell (blue arrow). Size bar = 2 μ m.

These light microscopy and TEM studies revealed that epidermal, hypodermal, mesophyll, and endodermal cells remained intact during fungal epiphytic growth and penetration stages (stages 1 and 2). The progression of infection after this period results in destruction of mesophyll and ultimately the endodermal cells.

4.2.1.3. Histo-chemical analysis of cell death and host response during dothistroma needle blight

The viability of host cells was monitored using neutral red (a vital stain) and confocal microscopy during the DNB infection process (sections 2.4.2 and 2.5.1). Neutral red was retained in vacuoles of living mesophyll cells in an un-inoculated needle (Fig. 4.9A) and in inoculated needles at stage 2 (penetration) (Fig. 4.9B). However, at stage 3 (early lesions), no accumulation of neutral red was seen in mesophyll cells (Fig 4.9C) indicating loss of viability. These results support earlier observations that mesophyll cells remain alive during stages 1 and 2 (germination, surface growth and penetration) and die during stage 3 (mesophyll colonisation/early lesions).

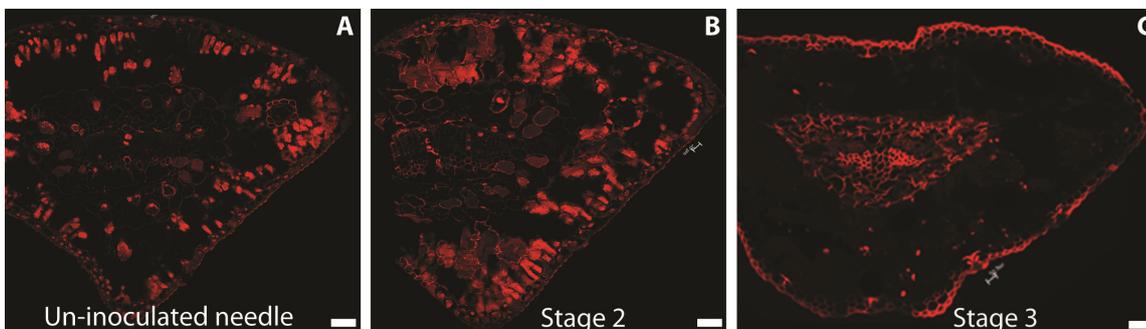


Fig. 4.9. Neutral red stain indicates loss of mesophyll cell viability in stage 3 of dothistroma needle blight

(A) and (B) confocal images of cross sections of un-inoculated and stage 2 (penetration) infected needles respectively show accumulation of neutral red in living cells; (C) At stage 3 (early lesions) loss of neutral red in the mesophyll layer corresponds to cell disintegration in this region. Neutral red emission band width was 575.7 - 688.7 nm during imaging in confocal microscopy. Size bar = 50 µm.

Attempts were made to stain for phenolics (lignins) over the time course of DNB as these compounds are known to accumulate in response to pathogen attack (Franich et

al., 1986; Keon et al., 2007). Due to the high natural phenolic content of pine mesophyll cells, the results were inconclusive. However, in the vascular tissues of pine needles it was possible to identify lignins by histo-chemical staining with phloroglucinol-HCl. Xylem vessels inside the endodermis appeared to be lignified at all stages but with thicker lignification layers at a late stage of infection. However the presence of the red coloured dothistromin toxin interfered with clear interpretation of this lignin deposition (Appendix 4: Fig. A4.4).

Host responses were monitored using diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) to detect reactive oxygen species (hydrogen peroxide and superoxide respectively) that are usually elevated in response to pathogen attack. No staining of host cells appeared at stage 2 (penetration time), indicating no activation of a host defence response (Appendix 4: Fig. A4.5). These stains were also used at stages 3 and 4, but due to production of dark colours (results not shown) in the host (possibly presence of phenolics or dothistromin) the results were inconclusive.

4.2.1.4. Chlorophyll loss during dothistroma needle blight infection

Confocal microscopy was used to monitor mesophyll layer colonisation using a *gfp*-labelled WT *D. septosporum* isolate (section 2.4.2). Bright red auto-fluorescence due to chlorophyll (Anand and Chanway, 2013) meant it was also possible to monitor changes in chlorophyll. Based on auto-fluorescence levels chlorophyll was abundant in the mesophyll cells of an un-inoculated needle cross section (Fig. 4.10B) but greatly reduced in both early (stage 3) and late (stage 4) lesions (Figs. 4.10F and 4.10J respectively). These results suggest that chlorophyll levels reduced over time due to *D. septosporum* colonisation.

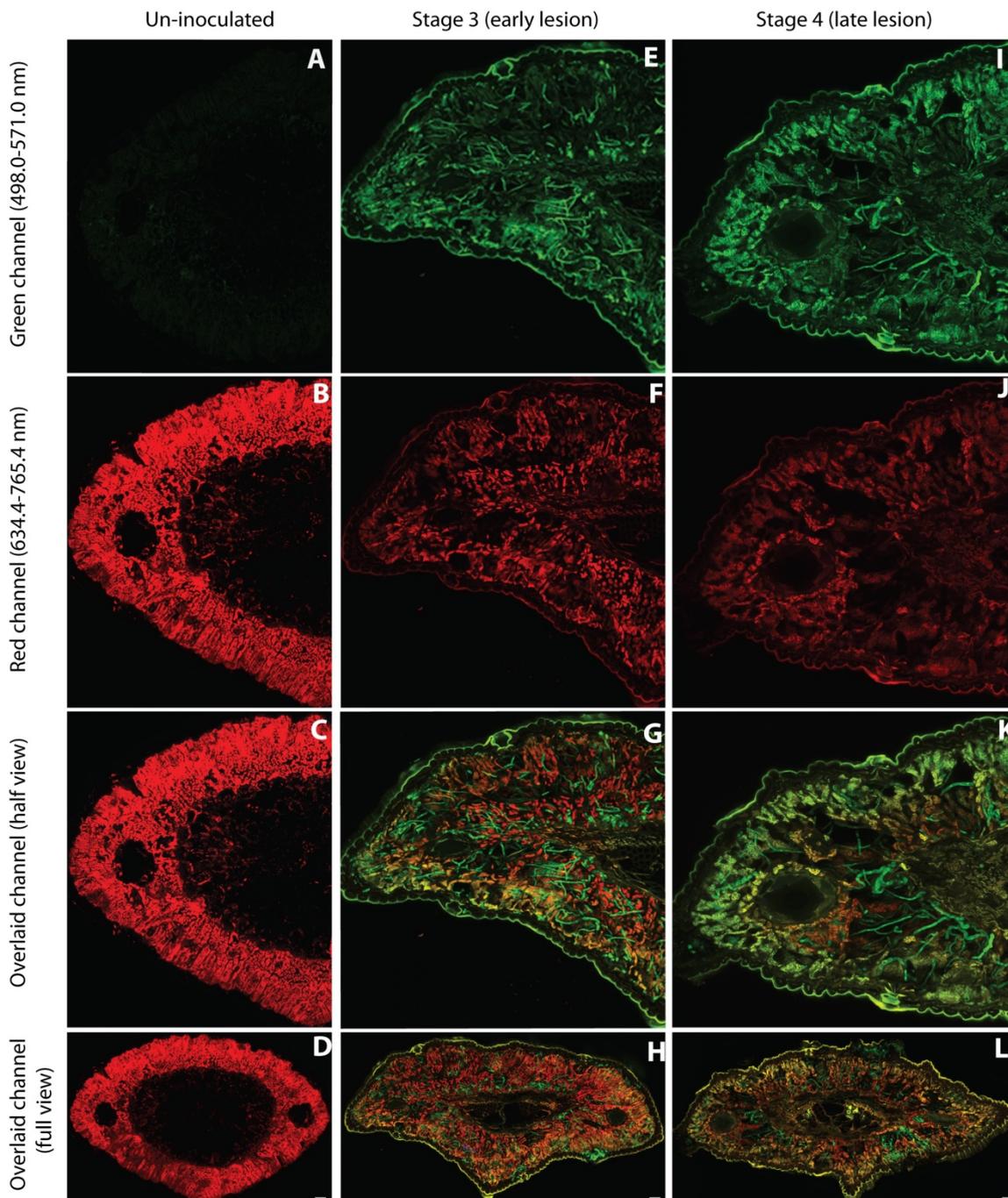


Fig 4.10. Mesophyll colonisation and chlorophyll auto-fluorescence in *Pinus radiata* needles by *gfp*-labelled *Dothistroma septosporum*

(A-D), (E-H), (I-L) are confocal images of cross section of un-inoculated, stage 3 and stage 4 DNB infected pine needle samples respectively, with green (top row) red (second row) and overlaid (bottom two rows) channels as indicated. Mesophyll colonisation by *D. septosporum-gfp* hyphae (green) can be seen at both stages of infection. Chlorophyll auto-fluorescence (red) is reduced at stage 3 (H, F), and further reduced at stage 4 (L, J) compared to un-inoculated needle sections (D, B). Note the overall disintegration of needle cross sections by their shapes in stages 3 and 4 (H, L) compared to the un-inoculated section (D). Size bar = 50µm.

In summary, microscopic studies showed that *D. septosporum* spent the first part of its life cycle without causing visible damage to cells of its host *P. radiata*, living first as an epiphyte then colonising the epi-stomatal chamber. This was followed by mesophyll colonisation and gradual mesophyll cell disintegration, followed by endodermal collapse and eruption of a fruiting body which completed the life cycle.

4.2.1.5. Growth and toxin production by *Dothistroma septosporum* during dothistroma needle blight infection

Molecular and biochemical tools were used to determine the growth of *D. septosporum* *in planta* and the timing of dothistromin biosynthesis over the time course of infection. To assess the fungal growth from genomic DNA, relative quantitative real-time PCR (qPCR) using a single copy gene in both the fungus (*PksA*) and the plant (*CAD*) was used as a measure of fungal biomass (based on relative quantification) with respect to needle biomass (section 2.6.2). To quantify dothistromin, HPLC was used on organic extracts from infected needles or lesions and expressed as amount of toxin per unit of pine tissue. From these values an estimation of the amount of dothistromin per unit of fungal biomass (based on absolute quantification) was calculated.

Because not all areas of a needle, or even all needles, that are sprayed with *D. septosporum* spores develop lesions, it is not possible to clearly define regions of successful infection at early stages. Furthermore, DNB symptoms can develop at different rates on different needles. Consequently reliable, synchronised, localised infection cannot be obtained easily for the *D. septosporum*-pine patho-system as for other patho-systems such as *M. graminicola*-wheat (Keon et al., 2007) and it was necessary to develop methods to achieve a reproducible semi-synchronous infection.

Thus two approaches were taken for analysis of *D. septosporum* biomass and dothistromin toxin over the time course of infection. Firstly, two experiments were

conducted based on whole needle samples taken at weekly intervals. Subsequently, three experiments were carried out using only specific areas of DNB infected needles.

In the first experiment, the life cycle of *D. septosporum* was completed by week 7 and 100% needle infection was obtained. After a lag period, *D. septosporum* biomass appeared to increase rapidly two weeks after the first appearance of lesions with an apparent rapid, but not significant, drop at week 7 (Fig. 4.11A). In the same samples, dothistromin toxin per needle (Fig. 4.11B) and dothistromin toxin per *D. septosporum* biomass (Fig. 4.11C) appeared to increase one week after lesions first appeared. However, statistical analysis did not show any significant differences between these time points for any of the parameters, probably due to high variation among the duplicate samples.

In an attempt to minimise variation between replicates, experiment 2 involved using larger samples (100 needles from 14 seedlings, instead of 5 needles from one seedling) for each of three replicates at each time point. Although the life cycle was not completed until 10 weeks ai, similar trends and a high level of variability between replicates were seen as in experiment 1 (Figs. 4.11D-F). Interestingly, dothistromin toxin per needle was significantly higher at late stage (week 10) compared to earlier time points (Fig. 4.11E). Although dothistromin levels in experiment 2 were significantly higher at week 10 compared to earlier weeks, this amount was less than in late stages of experiment 1; this highlights the variability of dothistromin production.

From these two experiments, it appeared that *D. septosporum* biomass and dothistromin toxin increased at a late stage of infection (particularly stage 4). However their amounts varied considerably and it was not possible to discern details of when biomass and dothistromin synthesis started to increase. This variation was partly due to random selection of whole needles from an infection that was not synchronised, as

shown by overlaps in duration of stages of infection (Fig 4.11). It was also partly because the number of lesions per needle varied at late stages. From this context, further quantification was carried out using DNB lesions instead of whole needles when possible.

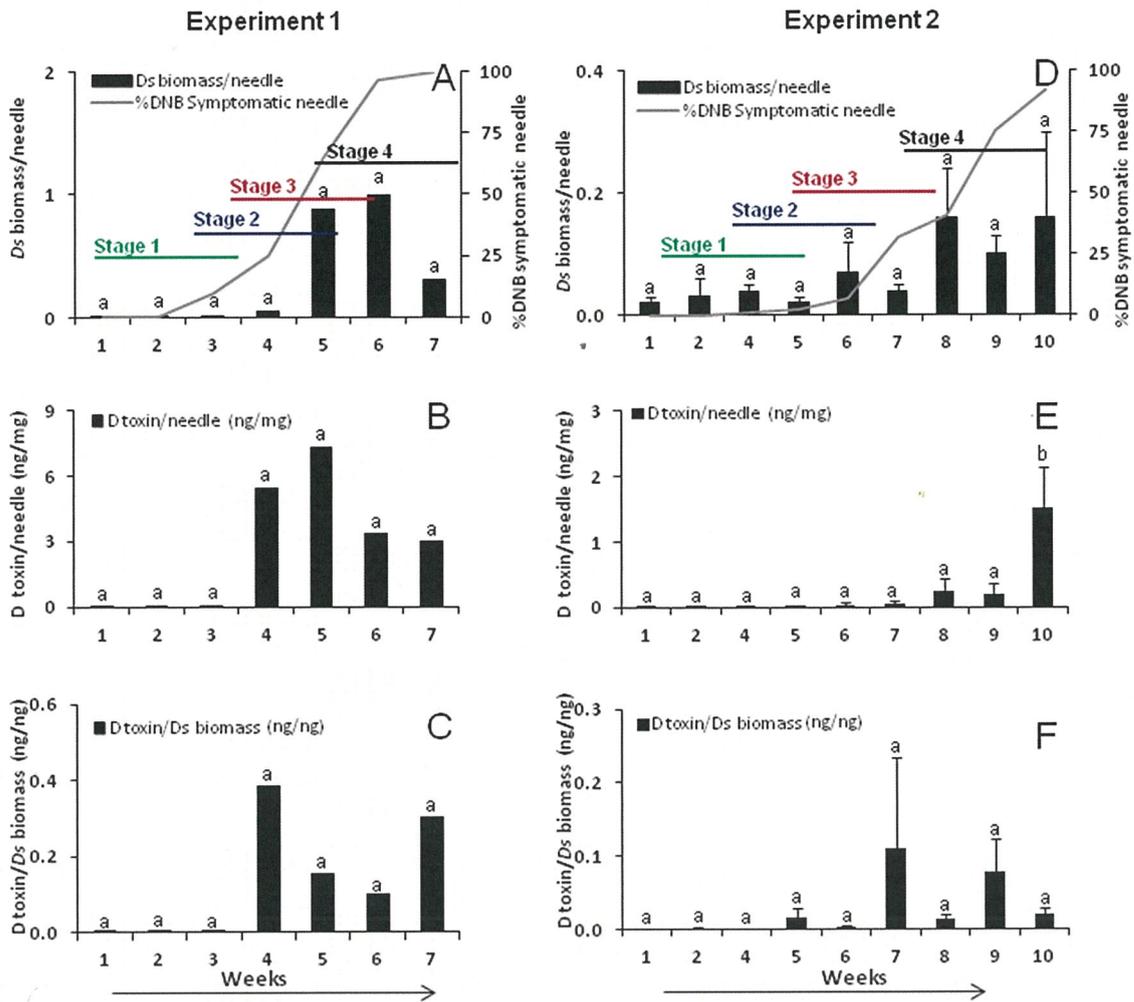


Fig. 4.11. Quantification of *Dothistroma septosporum* biomass and dothistromin toxin from whole needles

(A-C) and (D-F) were from experiments 1 and 2 respectively. Values with common letters in each chart did not differ significantly ($P < 0.05$) as calculated from least significant difference. Values are mean and standard deviation from two biological replicates in experiment 1 and three biological replicates in experiment 2 and two technical replicates per treatment. Approximate durations of stages 1 - 4 indicated in (A) and (D) were assessed visually (with microscopy at early stages). In (A) and (D) fungal biomass was calculated based on relative quantification (ng fungal biomass per ng of pine tissue). In (C) and (F) the rate of toxin production was calculated using an absolute biomass measurement (Appendix 4: Figs A4.6A and A4.6B respectively). *Ds* biomass and D toxin refer to *D. septosporum* biomass and dothistromin toxin respectively.

In the third experiment, samples were taken from whole needles at early (asymptomatic) stages but from lesions (cut out of needles) at later stages in an attempt to reduce variability between samples and to get a more synchronised infection. Samples were collected every two weeks with twenty DNB lesions collected at each later time point. In this experiment *D. septosporum* biomass increased at week 3 ai when DNB lesions first appeared (Fig. 4.12A). However there was considerable overlap in stages of infection, high variation and no significant differences between *D. septosporum* biomass over the time period. Dothistromin toxin production per needle increased significantly two weeks after DNB lesion appearance (Fig. 4.12B) but dothistromin production per *D. septosporum* biomass showed no significant differences (Fig. 4.12C) even though it appeared to be produced at a higher level at the end (week 9).

Based on the results of experiment 3, and taking needle availability into account, more extensive needle sampling was carried out in experiment 4. Therefore, whole needles of weeks 1 and 3 ai (stage 1 and 2) were mixed (non-symptomatic stage), with 100 needles per replicate; 80 early lesions (stage 3) per replicate were collected in weeks 5 and 6 and 80 late lesions (stage 4) at the end of the incubation period. The *D. septosporum* biomass increased significantly in late DNB lesions (Fig. 4.12D) compared to non-symptomatic needles and early lesions. Similarly, a significant increase of dothistromin toxin/lesion was also noticed in late lesions (Fig. 4.12E) but this increase was not significant when expressed as dothistromin toxin per *D. septosporum* biomass (Fig. 4.12F).

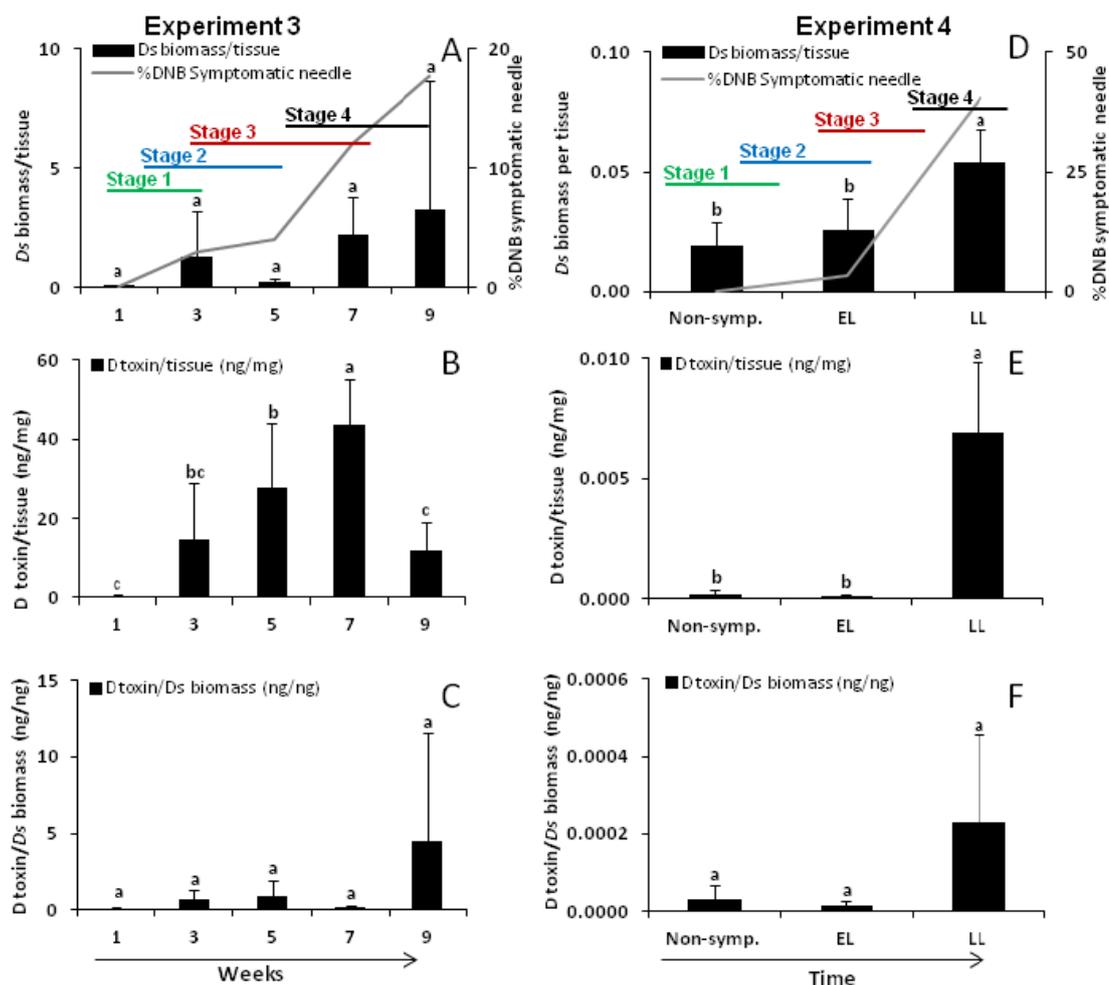


Fig. 4.12. Quantification of *Dothistroma septosporum* biomass and dothistromin toxin from dothistroma needle blight lesions

(A-C) and (D-F) are from experiments 3 and 4 respectively as described in the text. Values with common letters within each chart did not differ significantly ($P < 0.05$) as calculated from least significant difference. Values are mean and standard deviation from three biological replicates and two technical replicates per treatment. Approximate durations of stages 1 - 4 are indicated. The week 1 samples of experiment 3 and 'non-symp' of experiment 4 were from whole needles, as lesions were not present but fungal growth and penetration was confirmed microscopically. In (A) and (D) fungal biomass was calculated based on relative quantification (ng fungal biomass per ng of pine tissue). In (C) and (F) the rate of toxin production was calculated using an absolute biomass measurement (Appendix 4: Figs A4.6C and A4.6D respectively). *Ds* biomass and D toxin refer to *D. septosporum* biomass and dothistromin toxin respectively. 'non-symp', 'EL' and 'LL' refer to non-symptomatic needles, early lesions and late lesions respectively.

Experiment 5 was a repeat of experiment 4 but with more DNB lesions per biological replicate (120 instead of 80). An important additional modification was brought by choice of host. Instead of using host seedlings with a mixed genetic background (from one seed lot) genetically identical *P. radiata* host trees (clones, obtained by cuttings from a *Dothistroma* susceptible seedling) were used. It was hoped there would be less variability in DNB symptom development between clonal plants.

Like in experiment 4, a significant increase of *D. septosporum* biomass was seen in late DNB lesions compared to non-symptomatic needles (Fig. 4.13A). In addition, dothistromin toxin/lesion and dothistromin toxin/*D. septosporum* biomass increased significantly in the late stage compared to the very low levels seen in non-symptomatic and early lesion stages (Figs. 4.13B and 4.13C).

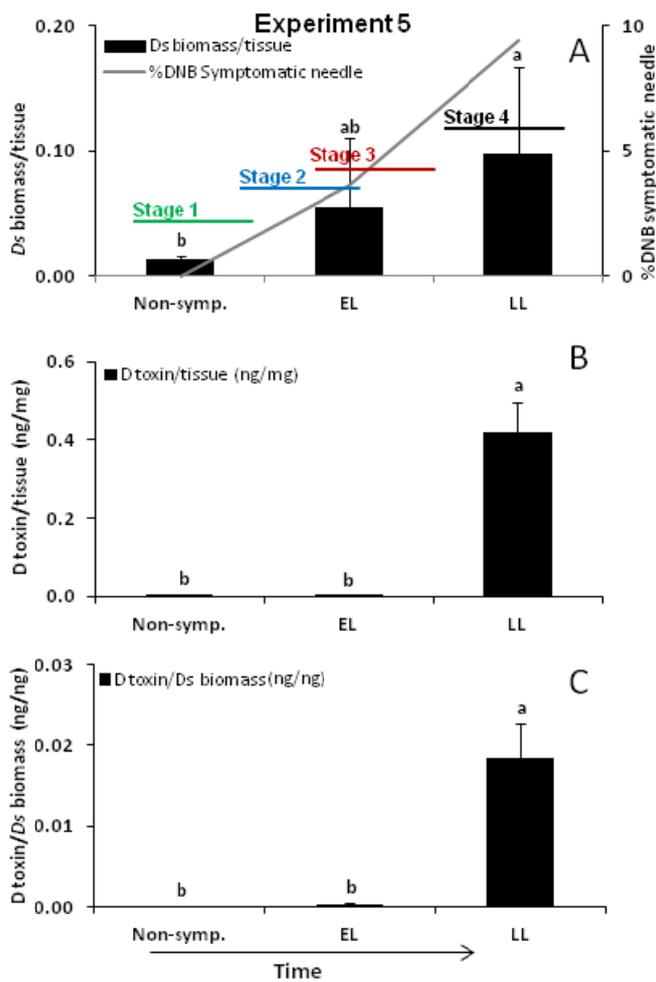


Fig. 4.13. Quantification of *Dothistroma septosporum* biomass and dothistromin toxin from lesions of susceptible clonal plants

Values with common letters did not differ significantly ($P < 0.05$) as calculated from least significant difference. Values are mean and standard deviation from three biological replicates and two technical replicates per treatment. Whole needles were used in case of non-symptomatic needles and confirmed microscopically for fungal growth and penetration. In (A) fungal biomass was calculated based on relative quantification (ng fungal biomass per ng of pine tissue). In (C) the rate of toxin production was calculated using an absolute biomass measurement (Appendix 4: Fig A4.6E). For abbreviations see legend to Fig. 4.12.

In conclusion, sampling from large numbers of whole needles during non-symptomatic stages, and from lesions during stages 3 and 4, provided a consistent way to quantify *D. septosporum* biomass and dothistromin toxin in a semi-synchronised infection of DNB, although there was considerable variability both within and between experiments. Looking at all the experiments together, the results suggest that *D. septosporum* biomass increased over the time course of infection, with the main increase occurring between early and late lesion formation (stages 3 and 4). They also suggest a negligible amount of dothistromin production occurred at the non-symptomatic stage, with a very pronounced increase between stages 3 and 4.

4.2.2. Gene expression studies and the life style of *Dothistroma septosporum*

Biochemical studies in section 4.2.1.5 showed that dothistromin levels increased at late stages of infection. To know more about its life style, expression of dothistromin genes, and the expression of other types of genes related to particular life styles in other fungi, were studied.

4.2.2.1. Expression of dothistromin genes *in planta*

Expression of dothistromin genes *in planta* was studied in two ways; firstly in a semi-quantitative way using a *gfp* reporter gene with a dothistromin gene promoter (section 2.6.3), and secondly in a quantitative way (section 2.6.4) by RNAseq analysis of key dothistromin genes.

4.2.2.1.1 Semi-quantitative gene expression

In this study, a *gfp* (green fluorescent protein) regulated by a dothistromin gene promoter (*DotA*, now called *Ver1*) was transformed into *D. septosporum* to monitor the expression of the dothistromin gene *in planta* over the DNB infection period. A semi-quantitative approach was taken to monitor gene expression by *gfp* fluorescence using a fluorescence microscope, where constitutive *PToxA:gfp D. septosporum* was used as a control.

In week 1, *PDotA:gfp* spores on the needle surface all expressed *gfp* in a similar way to the constitutive *PToxA:gfp* expression seen in spores used for inoculation (Figs. 4.14A, 4.14B (week 1) and 4.15A, 4.15E). However some *PDotA:gfp* germ tubes did not express *gfp* and by stage 2, low levels of *PDotA:gfp* expression were seen in hyphae on the needle surface (Figs. 4.14A (weeks 2-5), 4.15B, 4.15C) compared to constitutive *PToxA:gfp*, (Figs. 4.14B (weeks 2-5), 4.15F, 4.15G) despite extensive hyphal growth by both isolates (Figs. 4.14A and 4.14B). *PDotA:gfp* expression was higher again at stage 4 (Figs. 4.14A (weeks 7-8) and 4.15D) when asexual spores started to erupt out from

fruiting bodies, although it was still less than constitutive *PToxA:gfp* (Figs. 4.14B (weeks 7-8) and 4.15H).

This study suggests that dothistromin is produced towards the end of the infection period and is prevalent during sporulation. However, in this study, gene expression was monitored only on the needle surface, therefore further work, including quantitative dothistromin gene expression studies, needed to be carried out to determine the time of dothistromin gene expression *in planta*.

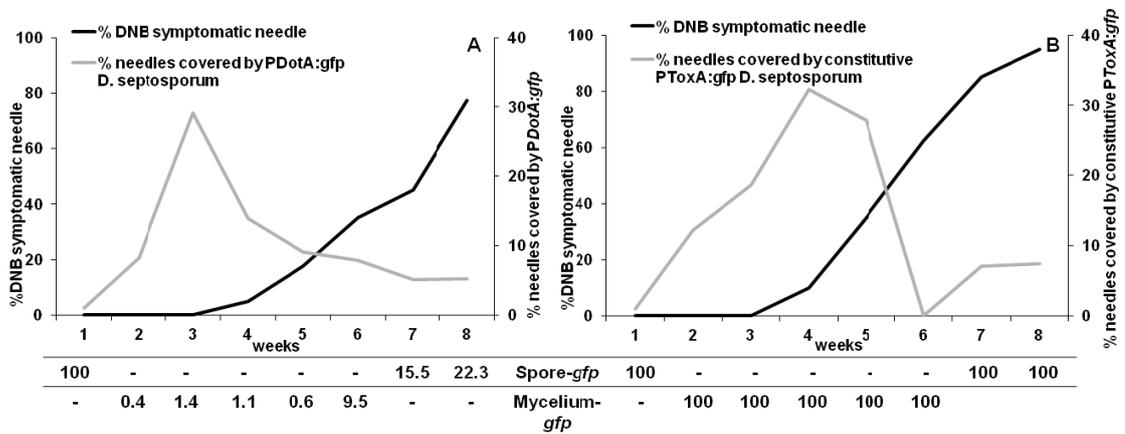


Fig. 4.14. Semi-quantitative *PDotA:gfp* gene expression during dothistroma needle blight

(A) *PDotA:gfp* (isolate FJT24) gene expression and (B) Constitutive *PToxA:gfp* (isolate FJT20) expression (control) over time. Values under the chart indicate the percentage of *Dothistroma septosporum* hyphae that expressed *gfp*.

D. septosporum
P*DotA*:*gfp* Constitutive P*ToxA*:*gfp*

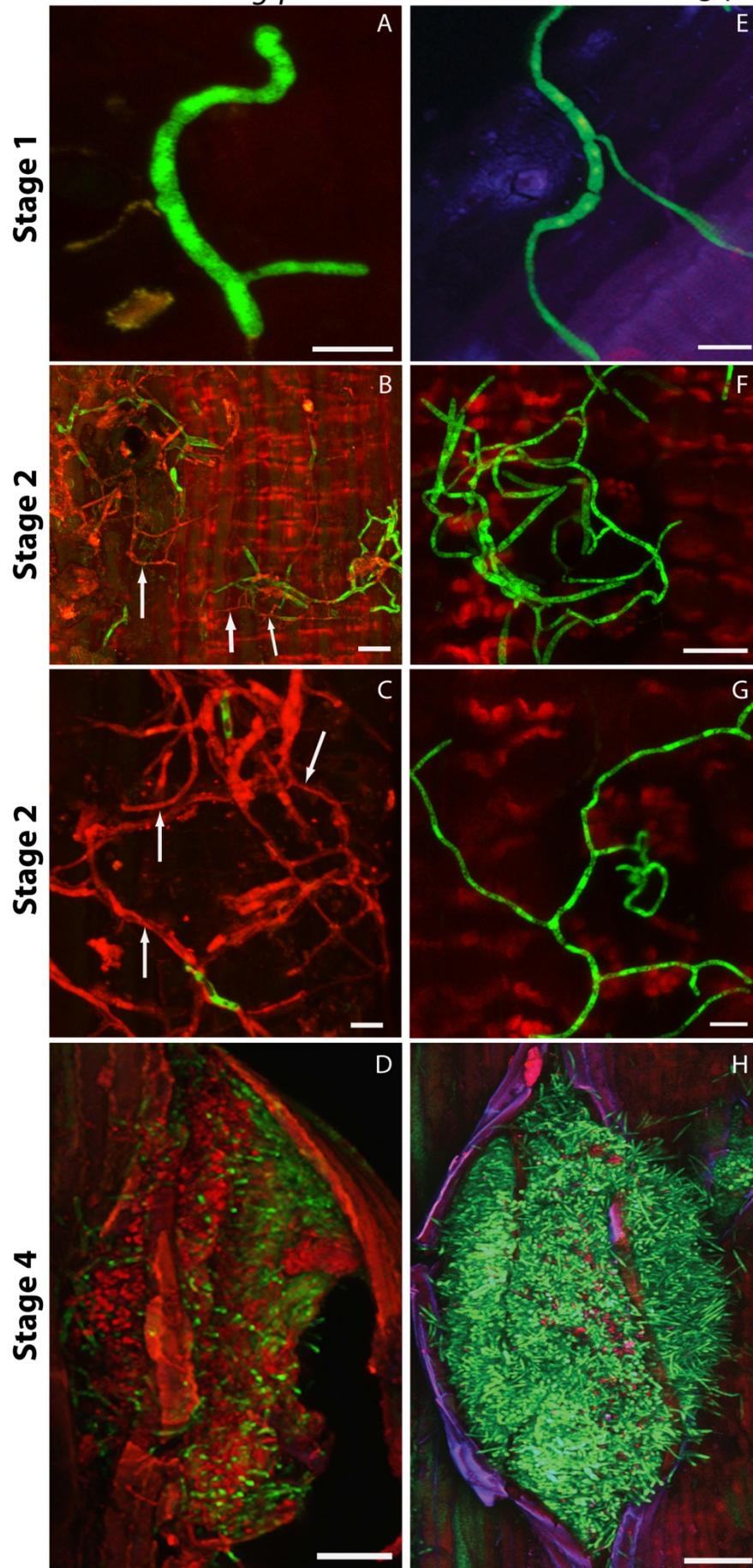


Fig. 4.15. Dothistromin regulated gene *PDotA:gfp* expression during dothistroma needle blight

(A) *PDotA:gfp* was expressed in inoculated spores on the needle surface during stage 1; (B-C) *PDotA:gfp* expression in some parts of the needle surface network (green), but not in other parts (white arrows) during stage 2; (D) Some spores expressed *PDotA:gfp* from a fruiting body at late stage; (E – H) constitutive *PToxA:gfp* genes expressed evenly at the same time points. Size bar A, C, E, G = 10 μm ; B, F = 20 μm ; D, H = 50 μm .

4.2.2.1.2. Quantitative gene expression

Gene expression studies using RNAseq analysis were conducted at non-symptomatic, early and late lesion appearing times of DNB. Expression studies were conducted on key dothistromin genes such as *AflR*, *PksA*, *HexA*, *VbsA* and *Ver1* (previously called *DotA*) (section 2.6.4).

In quantitative gene expression studies, overall it was noticed that expression of early pathway genes (e.g. *PksA*, *HexA*) and regulatory gene (*AflR*) was lower compared to that of late pathway genes (e.g. *VbsA* and *Ver1*). However all of the biosynthetic genes (*PksA*, *HexA*, *VbsA*, *Ver1*) showed similar trends, with lowest expression in non-symptomatic tissue, and highest expression in early lesions that was slightly reduced in late stage lesions. In contrast expression of *AflR* continued to increase over time, but the expression levels were low (Fig. 4.16).

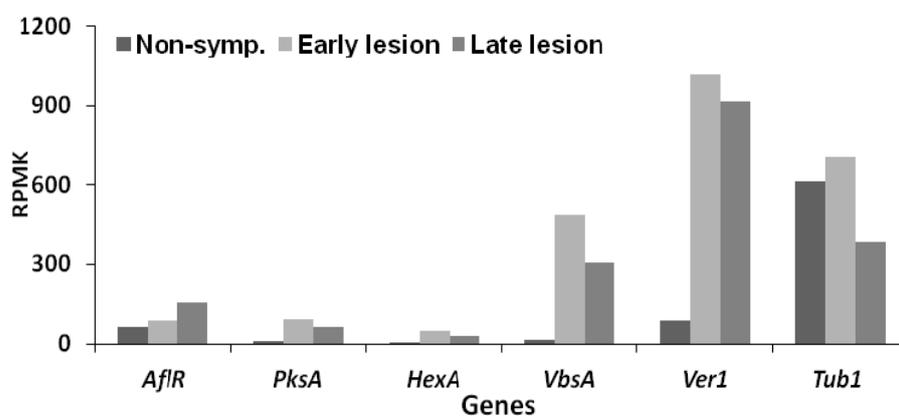


Fig. 4.16. Dothistromin gene expression in planta

Expression of dothistromin genes *AflR*, *PksA*, *HexA*, *VbsA* and *Ver1* and the housekeeping beta-tubulin (*Tub1*) gene during non-symptomatic, early and late lesion stages. Expression values are reads per million kilo bases (RPMK) and were determined from an RNAseq experiment with two biological replicates.

Combining earlier biochemical studies (section 4.2.1.5), these results suggested that although dothistromin was not produced at high levels during the early lesion stage, dothistromin genes were expressed which suggests that the biosynthetic enzymes were

made at this time. Low expression levels of dothistromin genes at non-symptomatic stages support the earlier findings of very low amounts of dothistromin at the non-symptomatic stage of infection.

4.2.2.2. Expression of other genes associated with life style

The genome of *D. septosporum* was analysed to determine if genes associated with a particular phase of plant-fungal interaction in other fungi/micro-organisms (biotrophic, necrotrophic or hemi-biotrophic) are present. Genes related to hemi-biotrophic cell death of *Phytophthora* spp. (e.g. *SNE1*, *PiNPP1.1*, *PsojNIP*), *Magnaporthe oryzae* (*BAS1*, *BAS2*, *BAS3*, *BAS4*, *AvrPiz-t*) and *C. trunctum* (*CtNUDIX*) were not found in the *D. septosporum* genome (Appendix 4: Table A4.1). For other genes that were found in the *D. septosporum* genome, RNAseq expression data from asymptomatic, early and late lesion stages of DNB was analysed to study their expression patterns (section 2.6.4). Genes similar to the *Colletotrichum* biotrophy-necrotrophy switch genes *CLNRI*, *CLTAI* showed very low levels of expression in *D. septosporum in planta*. Similarly there was low expression of the gene for invertase which is associated with biotrophy (sucrose utilisation) in many fungi (Fig. 4.17; Appendix 4: Table A4.1). Among other genes studied, *M. graminicola* *MgXYL1* and *M. fijiensis* *MfGas1* showed 62% and 74% identity with *D. septosporum* genes *Ds137959* and *Ds74988* respectively. *MgXYL1* encodes a xylanase enzyme that is highly expressed during early lesion formation in wheat (Goodwin et al., 2011) and a similar pattern of expression was seen for the putative *D. septosporum* *MgXYL1* ortholog (Fig. 4.17). *MfGas1* is a putative pathogenicity factor in banana and is expressed at highest levels during the early stage (speck) of sigatoka disease (Kantun-Moreno et al., 2013). The putative *D. septosporum* *MfGas1* ortholog had the highest expression in the non-symptomatic or biotrophic stage

(Fig. 4.17). Another *D. septosporum* gene with similarity (but only 32% amino acid identity) to *MfGas2* (*Ds69223*) showed a similar expression trend and was highly expressed (Fig. 4.17).

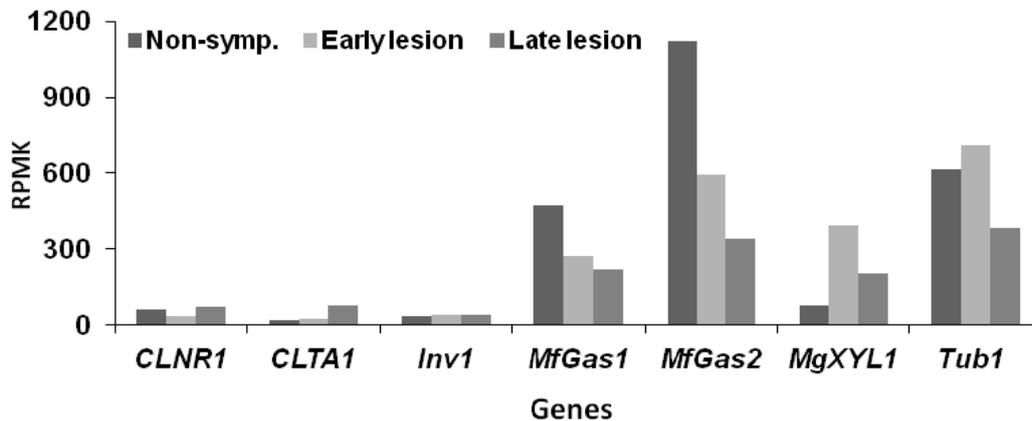


Fig. 4.17. Expression of genes associated with life style in planta

Expression of putative *Dothistroma septosporum* orthologs of *CLNR1*, *CLTA1*, *Inv1*, *MfGas1*, *MfGas2* and *MgXYL1* genes, along with the beta-tubulin housekeeping gene (*Tub1*), during non-symptomatic, early and late lesion stages. Expression was determined by RNAseq from a duplicated experiment based on reads per million kilo bases (RPMK) values.

From these studies, it can be concluded that *D. septosporum* appears to have orthologs of some 'pathogenic life style'-associated genes from closely related species of *D. septosporum* (*MgXYL1*, *MfGas1*) that share similar expression profiles in planta. In contrast the few putative orthologs from more distantly related species (*CLTA1*, *CLNR1*) showed very little expression in *D. septosporum*. These results support a similar hemi-biotrophic life style in *D. septosporum* as for *M. graminicola* and *M. fijiensis* and suggest the *MgXYL1*, *MfGas1* and *MfGas2* orthologs are worthy of further investigation in *D. septosporum*.

4.3. Discussion

The results of this study showed that *D. septosporum* completes its life cycle as a hemibiotrophic fungus. It had a latent or biotrophic phase in stages 1 and 2 (surface growth and penetration), during which time no mesophyll disintegration or host cell death occurred and very minimal fungal biomass and dothistromin were produced. The necrotrophic phase started from the time when early lesions appeared (stage 3) when the mesophyll started to disintegrate and die, followed by rapid increase of fungal biomass and dothistromin biosynthesis (at stage 4). In the necrotrophic phase, lack of spore germination in necrotic lesion areas suggested that viable cells are essential to support initial germination and the latent or biotrophic phase of this fungus.

Organisms that can live and multiply (grow and reproduce) only on living hosts are named as biotrophs. On the other hand, organisms that show infection without having any symptom expression are often called latent pathogens (Agrios, 2005). In the *M. graminicola*-wheat interaction fungal growth was undetectable during the symptomless period and on this basis the authors considered this period a latent phase of the infection (Keon et al., 2007). It is difficult to determine whether *D. septosporum* is actually a latent or biotrophic pathogen at stages 1 and 2. In glass house experiments, it generally took 5-6 weeks for symptoms to appear. In the forest situation this could be even slower so whether it could be considered as a latent pathogen is unknown. However, microscopic studies in this study and others (Gadgil, 1967; Muir and Cobb, 2005) showed that this fungus has an epiphytic growth phase after spore germination. After epiphytic growth and penetration, fungal hyphae were seen to increase in the epistomatal chamber (Figs. 4.3D, Appendix 4: Fig. A4.1D) before the appearance of DNB symptoms. Based on this evidence of growth, the initial DNB phase will be termed 'biotrophic' rather than 'latent' in the rest of this thesis.

Hemi-biotrophy is also seen in close relatives of *D. septosporum* within the Dothideomycetes (Ohm et al., 2012). These fungi include *M. graminicola* (Keon et al., 2007), *M. fijiensis* (Torres et al., 2012), *Cercospora arachidicola* (Guchu and Cole, 1994), *L. maculans* (Elliott et al., 2007). The duration of the biotrophic phase differs between these fungi but most are shorter than the 5-6 weeks for *D. septosporum* found under controlled conditions in the current study. In the case of *M. graminicola* and *M. fijiensis* this phase is about 9 or 28 days in their respective wheat and banana hosts (Keon et al., 2007; Torres et al., 2012). It is predicted that *M. graminicola* produces mycotoxins (Orton et al., 2011) and *M. fijiensis* is reported to produce both HSTs and NHSTs (Cruz-Cruz et al., 2011). Another hemi-biotrophic fungus *L. maculans* has a biotrophic stage of 10 days in *Brassica napus* and produces the non-host selective mycotoxin sirodesmin PL (Elliott et al., 2007). In contrast to hemi-biotrophic fungi, necrotrophic fungi usually take a very short time to produce symptoms in their hosts. For instance, *P. tritici-repentis*, *S. nodorum*, *A. alternata* and *C. heterostrophus* all take only 2-3 days to produce lesions in their respective hosts (Friesen et al., 2007; Pandelova et al., 2012; Rai, 2012; Spassieva et al., 2002). These fungi produce HSTs during their necrotic phase.

To answer the biological question of when dothistromin is produced *in planta*, gene expression studies were carried out in addition to direct measurements of dothistromin levels in plant tissue (section 4.2.2). These experiments showed that minimal levels of dothistromin are produced at the non-symptomatic stage which supports the findings of Schwelm et al. (2008), that dothistromin gene expression commences during an early stage of growth *in vitro*. Highest expression of dothistromin genes occurred in early lesions (stage 3), when both fungal biomass and toxin levels were very minimal suggesting that the fungus may recognise the host environment and

prepare itself to produce dothistromin. This phenomenon was also noticed in the *F. graminearum*-wheat interaction (Mudge et al., 2006), when the *Tri5* gene responsible for producing the mycotoxin DON was expressed ahead of DON production during crown rot infection. Expression of dothistromin genes was slightly reduced at the late stage of DNB, but because the biomass was much higher at this time the amount of dothistromin made during fruiting body formation was high (Figs. 4.14A and 4.15D). The regulation of dothistromin production *in planta* appears to be complex. At each stage in the life cycle, even during the late lesion stage, some compartments of the mycelium, or some spores, appeared to express dothistromin genes whilst others did not, based on *gfp* expression with a dothistromin gene promoter (Figs. 4.15A-D). Why the expression is so variable is unknown. The age of fungal cells and hyphal differentiation might contribute to variable dothistromin gene expression. When gene expression studies were conducted (*in vitro*) on PDA and DM media using a *PDotA:gfp* strain of *D. septosporum*, it was noticed that young hyphae showed dothistromin gene *DotA* expression in contrast to no expression in older hyphae (Fig. 1.8) (Schwelm et al., 2008). Likewise, using *gfp* fluorescence in *Aspergillus niger* it was noticed that expression of *glaA* (glucoamylase) varies cell to cell due to hyphal differentiation (Vinck et al., 2005).

Levels of dothistromin produced *in planta* varied a lot between experiments. For example, experiments 1 and 2 both had >90% needle infection rates but mean dothistromin levels per needle and per biomass were about 4-5 fold higher in experiment 1 (Fig. 4.11 and Appendix 4: Table A4.2). This might have been due to higher fungal biomass content in experiment 1 compared to experiment 2 and a shorter life cycle of *D. septosporum* by three weeks, possibly due to use of a different *D. septosporum* isolate (Table 2.3) in the two experiments. Alternatively the apparent

differences in dothistromin levels may have been sampling artefacts due to taking whole needle samples at random, which may have differed in lesion number and size.

Unexpectedly, dothistromin levels varied even more so in the experiments in which lesions were cut from the needles (in experiments 3, 4 and 5). In experiment 3, dothistromin levels were much higher than experiment 4 (Figs. 4.12 and Appendix 4: Table A4.2). It might be due to storing of experiment 4 lesions for a long time (about 7 months at -80°C) that might have resulted in dothistromin degradation. Although dothistromin quantification for experiments 3 and 5 was carried out from fresh lesions, dothistromin per needle and per biomass was >100 and >200 fold higher respectively in experiment 3 compared to experiment 5. In addition to higher fungal biomass (>200 times) and, use of a different isolate in experiment 3, another possibility might be the different season. Experiment 3 was conducted in summer time when long duration of days (>14.7 h) meant more sunlight energy (about 213 W/m^2) (Appendix 4: Table A4.2) compared to experiment 5 that was conducted in Autumn ($\sim 58.9 \text{ W/ m}^2$). It is well established that light positively affects symptom development (Shain and Franich, 1981) but dothistromin biosynthesis itself does not appear to be affected by light (Chettri, unpublished). Also sampling artefacts might have contributed to the differences seen as fewer lesions were sampled in experiment 3 compared to experiment 5. Therefore based on these variables (fungal biomass, isolate, season and sampling methods), it was difficult to determine the main cause of variability in dothistromin production levels. However the rate of degradation of dothistromin over time needs to be determined by quantifying dothistromin both in ethyl acetate and from plant samples over time.

Time course fungal biomass estimations were carried out based on fungal *PksA* and pine *CAD* genes as mentioned earlier. This type of relative quantification was based

on a method developed for another fungal forest pathogen (*Phaeocryptopus gaeumannii*) of Douglas-fir during Swiss needle cast disease (Winton et al., 2002), where the quantification was carried out successfully by real time PCR using both fungal and host primers and probes. Chettri et al. (2012) successfully quantified and compared fungal biomass of *veA* mutant and WT *D. septosporum* using the *PksA* and *CAD* genes. Although it was carried out in a similar way, the fungal biomass quantification in the current study may not be completely accurate, as overestimation of fungal biomass could be possible at the necrotic phase due to degradation of host cell DNA.

Some hemi-biotrophic pathogens have strategies to overcome host responses. Reactive oxygen species (ROS) production is the earliest host response. As a result, the resistance response is induced, cell walls become thickened, and eventually programmed cell death can occur (Heller and Tudzynski, 2011). Avoidance of host responses was noticed by lack of elevated ROS levels in wheat in the early biotrophic phase of the hemi-biotrophic pathogen *M. graminicola* (Keon et al., 2007; Shetty et al., 2007). The same phenomenon was also noticed in the *D. septosporum*-pine interaction (Fig. Appendix 4: Fig. A4.5) using both DAB and NBT stains (H_2O_2 and superoxide producing stain). These results suggest that *D. septosporum* grew over the needle without triggering rapid host responses. At the late stage of infection it was not possible to detect ROS due to build up of dothistromin. Avoidance of ROS production occurs in some plant-microbe interactions due to ROS scavenging mechanisms of fungi. These mechanisms include the activation of superoxide dismutase, catalases, peroxidases (Lev et al., 2005; Molina and Kahmann, 2007). Analysis of expression of these types of genes over the time course of infection will provide more insight into how the pathogen copes with ROS production *in planta* during dothistroma needle blight infection.

The infection process and fungal structures were monitored during this life style study. Although some direct penetration and intracellular growth was reported (Gadgil, 1967), none of those events were found in this study. Non-directional hyphal growth towards stomata was noticed as in earlier artificial inoculation studies (Gadgil, 1967; Gadgil, 1974; Gadgil, 1977) as opposed to more directed growth seen in field conditions (Muir and Cobb, 2005). Formation of fungal infection structures and invasion of *D. septosporum* into pine needles did not show any similarities with obligate biotrophic fungi. Specialised hyphae called haustorium found in truly biotrophic fungi (Horbach et al., 2011) were not seen in *D. septosporum*. Some hemi-biotrophic fungi, such as *C. graminicola*, maintain irregular, globular hyphae during their biotrophic phase (Vargas et al., 2012) for nutritional purposes. Examination of *D. septosporum* hyphae using scanning electron microscopy and confocal microscopy with a *gfp*-labelled WT isolate revealed no morphological changes between biotrophic and necrotrophic stages. At the end of the life cycle no spores came out of the needles through stomatal pores as seen in the biotrophic pathogen *C. fulvum* (de Wit et al., 2012).

A qualitative experiment was carried out to monitor chlorophyll loss by chlorophyll auto-fluorescence during DNB infection at lesion formation time (stage 3 and 4). In hemi-biotrophism of *M. graminicola*, little or no loss of chlorophyll was found in the symptomless phase but loss was prominent in its necrotrophic stage (Scholes and Rolfe, 2009). Similar results were also found in *C. orbiculare*, in which no loss of fluorescence was noticed until late biotrophic or early necrotrophic phase on tobacco (*Nicotiana benthamiana*) (Scholes and Rolfe, 2009). However, in both cases this loss or damage varied a lot within the infected parts of the host (Scholes and Rolfe, 2009; Tung et al., 2013). In future, detailed studies should be carried out to compare

chlorophyll levels at all stages of DNB with large numbers of samples to obtain reliable conclusions.

To describe the hemi-biotrophic life style of *D. septosporum* in this study, fungal biomass was found to increase after mesophyll disintegration and death had commenced. In other fungi with hemi-biotrophic life styles like *M. graminicola* (Keon et al., 2007), *M. fijiensis* (Kantun-Moreno et al., 2013) and *F. graminearum* (Stephens et al., 2008) fungal biomass was also found to increase significantly in the necrotrophic phase. In the case of *M. graminicola* and *M. fijiensis*, cell membrane integrity and electrolyte leakage also increased in the necrotrophic phase in their hosts wheat (Keon et al., 2007) and banana (Kantun-Moreno et al., 2013) respectively that positively correlated with fungal biomass increase. Kantun-Moreno *et al.* (2013) also found that the increase of *M. fijiensis* fungal biomass and electrolyte leakage was inversely correlated to the expression of the *MfGas1* and *MfGas2* genes. These genes belong to the glycosyl phosphatidylinositol protein (GPI) family in the β 1-3 glucansyltransferase (*Gas*) or glycoside hydrolase ‘GH72’ group of carbohydrate degrading enzyme (CAZymes) that collectively are needed for pathogenesis, morphogenesis and cell wall biogenesis of fungi (Kantun-Moreno et al., 2013). In *D. septosporum*, expression putative orthologs of *MfGas1* and *MfGas2* was high during the symptomless period and gradually dropped down later in the disease cycle. During the asymptomatic phase, the CAZymes encoded by these genes might be expressed at high levels to help in cell wall remodeling to establish *D. septosporum* infection as it starts to invade the apoplast, with their expression becoming gradually less at early and late lesion stages. Seven GH72 genes are reported in the *D. septosporum* genome (de Wit et al., 2012) and the functions of these need to be evaluated to determine their roles in the pathogen life style.

Different CAZymes have different functions in plant-microbe interactions. For instance *MgXYLI* (ortholog of *Ds13759*), is a cell wall degrading enzyme that belongs to another group of glycosidase hydrolases ‘GH11’. These enzymes display endo-xylanase activity and break down the hemi-cellulose components of plant cells (Kema et al., 2008). Increased expression of this gene at the early lesion stage of DNB suggests that *D. septosporum* may be active to degrade hemi-celluloses of pine needle at this time point. Only two GH11 genes are reported in the *D. septosporum* genome and functional studies of these *in planta* are required. The gene expression studies carried out in this experiment were from interim transcriptome read counts where variations in total read numbers between the different stages were prominent (Appendix 4: Table A4.3); more sequencing is required for a reliable statistical interpretation.

Plants produce phenolic compounds in response to many types of stresses, such as pathogen attack (Agrios, 2005), drought (Grossoni et al., 1998) or UV radiation (Laakso et al., 2000). In this ultra-structural study (section 4.2.1.2), phenolic compounds seemed to appear in pine needles as a high electron dense material at the end of the DNB cycle. This material was noticed around hyphae as well as needle cells, especially at attachment points of hyphae to needle tissue (Figs. 4.8E and 4.8H). Similar kinds of high electron dense material termed tannins (which are phenolic compounds) were also noticed in ultra-structural studies of radiata pine (Jarvis and Leung, 2002) and other pine species (Laakso et al., 2000). The presence of phenolics was a limiting factor in other histological studies (such as ROS staining) as they produced a dark colour that masked other expected colours. A similar kind of limitation (due to presence of phenolics) was also reported in the interaction of *Paenibacillus polymyxa* with lodge pole pine (Anand and Chanway, 2013).

In conclusion, *D. septosporum* follows a hemi-biotrophic life style during DNB, having a biotrophic phase followed by a necrotrophic phase. The necrotrophic phase is characterised by production of a non-host selective toxin, which is produced at variable levels in the host. The role of this toxin in disease was unknown, and is addressed in the next chapter.

Chapter 5: Role of dothistromin during dothistroma needle blight

5.1. Introduction

In planta biochemical studies outlined in chapter four showed that dothistromin is synthesised by *D. septosporum* at a high rate during lesion formation in *P. radiata* needles, but is present at low levels during early stages of infection. Although this non-host selective mycotoxin was initially suggested to be a pathogenicity factor, this was an assumption based mainly on its toxicity and its presence in diseased needles (Bassett et al., 1970; Franich et al., 1986; Gadgil, 1967; Jones et al., 1995; Shain and Franich, 1981). This assumption was not supported by a study carried out by Schwelm et al. (2009) with dothistromin-deficient mutants which showed that dothistromin is not required for dothistroma needle blight (reviewed in section 1.5.4).

Consequently, a series of questions arise regarding the role of this toxin in the disease cycle. Does it influence spore germination, surface growth or stomatal tropism on the plant host? Does it have a role in penetration or at the later stages of the disease process when the levels of dothistromin are higher? Other non-host specific toxins have been shown to affect host colonisation (e.g. cercosporin in *Cercospora zea-maydis*, sirodesmin PL in *L. maculans*) (Elliott et al., 2007; Shim and Dunkle, 2003) and symptom appearance (e.g. deoxynivalenol in *F. graminearum*, cercosporin in *C. zea-maydis*, trichothecene in *Gibberella zea*) (Desjardins et al., 1996; Horevaj and Bluhm, 2012; Shim and Dunkle, 2003). Secondary metabolite production has also been associated with sporulation in many systems, possibly due to shared regulatory pathways (Adams and Yu, 1998; Hicks et al., 1997; Shim and Woloshuk, 2001) and a dothistromin-deficient mutant was previously shown to have a 3-fold reduction in sporulation compared to the wild-type (Bradshaw and Zhang, 2006). On the basis of this

work with other non-host specific toxins it is hypothesised that dothistromin has a role in virulence in DNB.

To test this hypothesis, pine seedlings were inoculated with dothistromin-deficient mutants of *D. septosporum* (section 2.3.8), which had deletions of essential dothistromin genes (Table 2.1). Disease events were compared with those obtained with wild type (WT) dothistromin-producing *D. septosporum* isolates at various time points in the disease cycle. Genes chosen for mutation for these experiments function at different steps in the dothistromin biosynthetic pathway and are at different loci on chromosome 12. The *PksA* gene is in locus 2 and encodes a polyketide synthase which functions near the beginning of biosynthetic pathway (Fig. 1.9) (Chettri et al., 2013). Disruption of this gene did not produce dothistromin, or pathway intermediates such as versicolorin A or norsolorinic acid when tested in culture, and had significantly less sporulation than the WT (Bradshaw et al., 2006). The *HexA* gene is in locus 4 and is predicted to encode a fatty acid synthetase enzyme that functions at the beginning of the dothistromin biosynthetic pathway (Fig. 1.9). Functional characterisation showed that disruption of this gene also resulted in undetectable amounts of dothistromin in culture (Chettri et al., 2013). One more gene used in this study is *VbsA*, which is also located in locus 4 (Fig. 1.9), but functions at a later stage of the dothistromin biosynthetic pathway. Functional characterisation also showed that in the absence of this gene, *D. septosporum* produces negligible dothistromin (Zhang et al., 2007).

Studying the role of dothistromin *in planta* using dothistromin-deficient mutants will help to understand whether dothistromin is a virulence factor that affects the severity of the disease. Knowledge of the molecular basis of virulence of *D. septosporum* will facilitate efforts to develop resistant pine (Ivkovic et al., 2010; Pas et al., 1984).

5.2. Results

5.2.1. Spore germination and surface growth of dothistromin-deficient mutants at stage 1

Spore germination and surface growth (section 2.3.2) of two independent dothistromin-deficient mutants ($\Delta PksA:gfp$ and $\Delta HexA$) and WT *D. septosporum* was compared to determine if dothistromin is required for this stage of the life cycle on the needle surface.

No significant differences were found in spore germination between dothistromin-deficient mutants and WT *D. septosporum* either *in vitro* or *in planta* (Table 5.1). In all cases spore germination was more than 80% and dothistromin-deficient mutants appeared to germinate in a similar way to the WT isolate (Fig. 5.1A, 5.1B).

Table 5.1. Spore germination, surface growth and penetration of dothistromin-deficient mutants ($\Delta PksA:gfp$ and $\Delta HexA$) and wild type (WT) *Dothistroma septosporum*

| <i>D. septosporum</i> | Spore germination (%) | | Surface growth ^c (%) (weeks after inoculation) | | | | Fungal penetration ^d (%) (weeks after inoculation) | | |
|-----------------------|------------------------------|-------------------------------|--|--------|--------|--------|--|--------|--------|
| | <i>In vitro</i> ^a | <i>In planta</i> ^b | Week 2 | Week 4 | Week 6 | Week 8 | Week 2 | Week 4 | Week 6 |
| $\Delta PksA:gfp$ | 81 ± 1 | 82 ± 3 | 5 ± 0 | 16 ± 1 | 22 ± 3 | 18 ± 3 | 0 ± 0 | 10 ± 3 | 13 ± 1 |
| $\Delta HexA$ | 81 ± 2 | 84 ± 2 | 5 ± 0 | 15 ± 2 | 23 ± 4 | 10 ± 0 | 0 ± 0 | 15 ± 1 | 17 ± 0 |
| WT | 83 ± 2 | 84 ± 1 | 7 ± 3 | 15 ± 2 | 18 ± 4 | 13 ± 4 | 0 ± 0 | 14 ± 3 | 17 ± 2 |

^aat 4 day after inoculation, ^bat week 2 after inoculation, ^cpercentage of needle surface covered by fungal hyphae, ^dpercentage of stomata where fungal penetration was seen.

Values are mean and standard deviation from three biological replicates per treatment. No significant differences were noticed between dothistromin-deficient mutants ($\Delta PksA:gfp$ and $\Delta HexA$) and WT values for any of the parameters at any time point ($P > 0.05$).

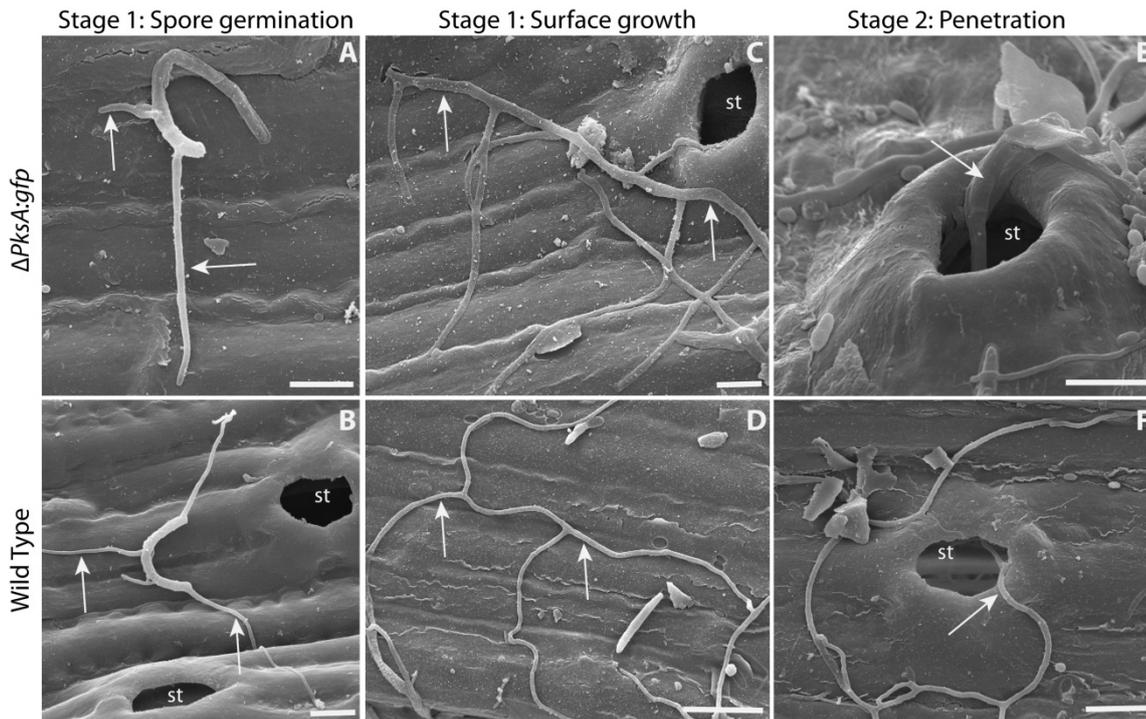


Fig. 5.1. Spore germination, surface growth and penetration of dothistromin-deficient mutant ($\Delta PksA:gfp$) and wild type (WT) *Dothistroma septosporum* at stages 1 and 2

(A – B) Spore germination of dothistromin deficient mutant and WT *D. septosporum* respectively showing germ tubes (arrows) on needle surface 4 days after inoculation (dai); (C – D) Fungal growth (arrows) on the needle surface; overall similar growth patterns were seen in the mutant and WT; (E – F) Fungal penetration (arrow) via stomatal pores (st) by WT and mutant at week 4 ai; Size bar A, F = 5 μm , B, C, D, E = 10 μm .

No significant difference in surface hyphal growth (as assessed by percent needle coverage) was seen between dothistromin-deficient mutants and WT at any of the sampled time-points (Table 5.1; Fig 5.1C, 5.1D). The surface growth increased until week 6 ai then appeared to decrease (presumably by falling off the needle or by degradation) by week 8 ai.

Spore germination and surface growth quantification was repeated using the same dothistromin-deficient mutants ($\Delta PksA:gfp$ and $\Delta HexA$) in two separate experiments. Likewise, there was no significant difference in spore germination compared to WT under any conditions (Appendix 5: Table A5.1 and A5.2). Fungal growth by the $\Delta PksA:gfp$ mutant was significantly higher than WT at weeks 2 and 4 ai;

however this was not a consistent effect as later time points and the $\Delta HexA$ mutant showed no significant difference from WT (Appendix 5: Table A5.1 and A5.2). In these replicate experiments, maximum surface growth was seen at the earlier time point of 4 weeks ai, highlighting the variability of this patho-system.

Overall these results suggest that dothistromin-deficient mutants are not impaired in spore germination or needle surface growth.

5.2.2. Needle penetration of dothistromin-deficient mutants at stage 2

Penetration of *D. septosporum* into stomatal pores on pine needles was quantified (section 2.3.2) to determine if dothistromin is required for this stage of the disease cycle. This quantification was carried out using the same samples as described in section 5.2.1.

The dothistromin deficient-mutants entered pine needles through stomatal pores in the same way as the WT (Fig. 5.1E, 5.1F). No significant differences between WT and mutants were seen with regard to the extent of penetration, as measured by the percentage of stomata penetrated at weeks 4 or 6 ai (Table 5.1). When this experiment was repeated the $\Delta HexA$ mutant behaved as the wild-type, but again the $\Delta PksA:gfp$ mutant showed a slightly different result to the WT in this experiment, with lower penetration at weeks 4 and 6 (Appendix 5: Table A5.1 and A5.2).

Overall there appeared to be no or little effect of dothistromin on these early stage events. Only in one of the experiments did the $\Delta PksA:gfp$ mutant show greater surface growth levels at some time-points and lower penetration levels than the WT.

5.2.3. Mesophyll colonisation of dothistromin-deficient mutant at stages 3 and 4 and 4

Mesophyll colonisation was compared between *gfp*-labelled dothistromin-deficient mutant ($\Delta PksA:gfp$) and *gfp*-labelled WT (NZE10:*gfp*) *D. septosporum* infected needles to determine if lack of dothistromin affects mesophyll colonisation at stages 3 and 4. SEM and confocal imaging suggested the mutant was able to colonise mesophyll cells (Fig. 5.2A and 5.2B) but showed a more restricted colonisation pattern than the WT. Whilst the WT had colonised the entire cross-section of the needle by week 6 ai (Fig. 5.2D), even after 14 weeks the mutant had only colonised one region of the needle (Fig. 5.2C). These images are representative of many (more than 30) consistent observations. These results suggest that dothistromin is required for normal mesophyll colonisation.

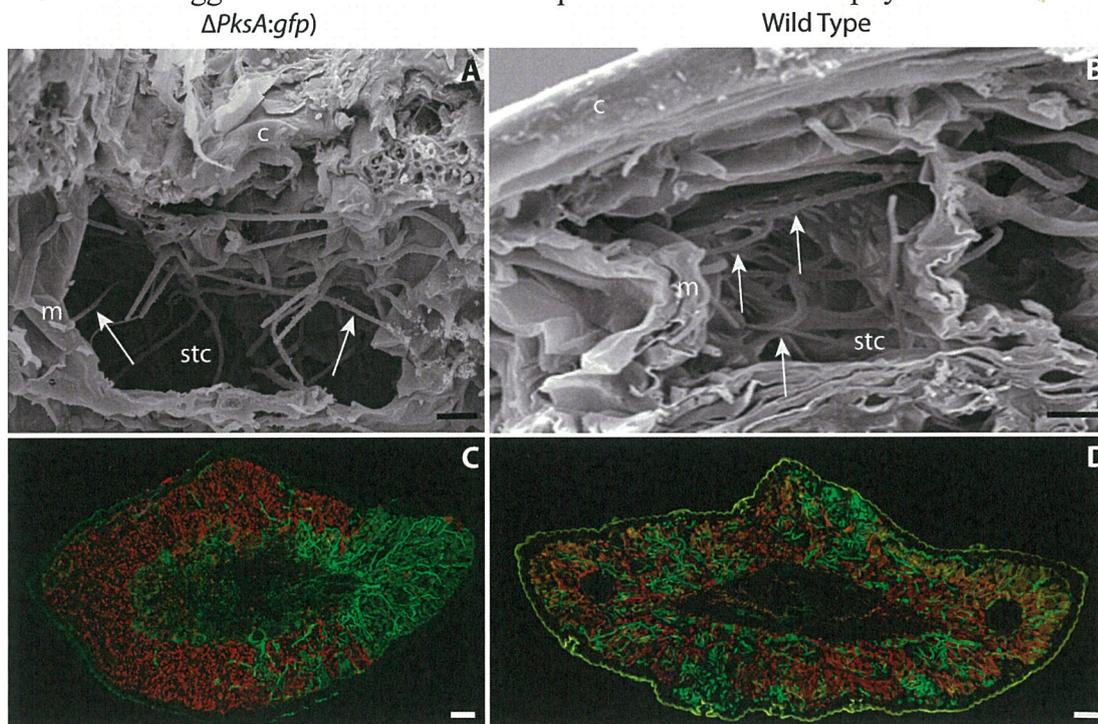


Fig. 5.2. Comparison of mesophyll colonisation between dothistromin-deficient mutant ($\Delta PksA:gfp$) and wild type (WT:*gfp*) *Dothistroma septosporum* infected needles

(A – B) Scanning electron micrograph showing successful mesophyll (m) colonisation in sub-stomatal chambers (stc) and similar extent of hyphal growth by mutant and WT isolates respectively; (C) Confocal image of dothistromin-deficient mutant that colonised about the half the area of a pine needle cross section at 14 weeks ai; (D) Confocal image of WT *D. septosporum* that colonised the entire pine needle cross section within 6 weeks ai. Size bar A - B = 10 μ m, C - D = 50 μ m.

5.2.4. Effect of dothistromin on lesion development at stages 3 and 4

The timing of first appearance of lesions varied from experiment to experiment. In one experiment DNB lesions started to appear on WT inoculated seedlings at week 5 ai whilst on those inoculated with mutants ($\Delta PksA:gfp$ or $\Delta HexA$) lesions first appeared on both dothistromin-deficient mutants at week 6 ai. However, when the experiment was repeated no differences in timing of first symptom appearance were seen.

The percentages of needles showing DNB symptoms were not significantly different between plants inoculated with WT or either mutant at weeks 8 and 10 ai, although the WT had significantly more symptomatic needles at week 12 ai (Fig. 5.3A). The mean numbers of lesions per needle were also compared and no significant differences were seen between WT and mutant-infected seedlings at any time points (week 8, 10 and 12 ai) as shown in Fig. 5.3B. Repeated experiments conducted separately using the same dothistromin-deficient mutants showed no significant differences in either percent of needles with DNB symptoms or lesion numbers per needle throughout the life cycle (Appendix 5: Fig A5.1A and A5.1B).

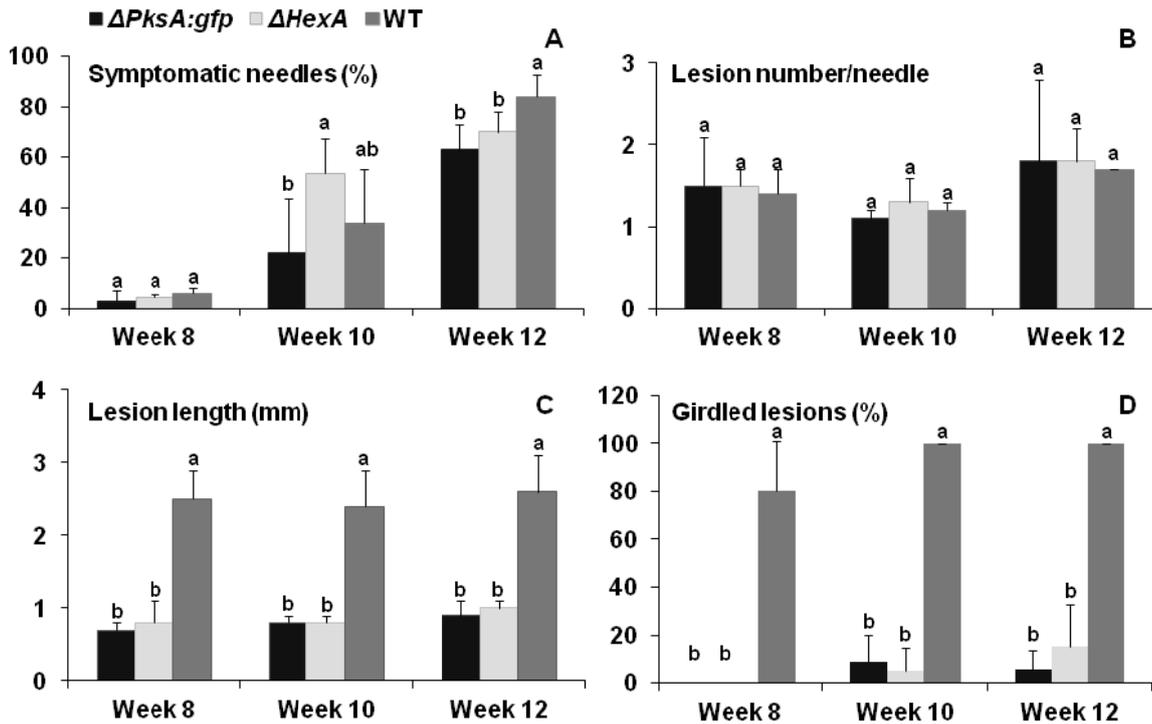


Fig. 5.3. Comparison of dothistroma needle blight (DNB) events caused by dothistromin-deficient mutants ($\Delta PksA:gfp$ and $\Delta HexA$) and wild type (WT) *Dothistroma septosporum* at stages 3 and 4

(A- D) show, respectively, DNB symptomatic needles (%), lesion number per symptomatic needle, lesion length and percent of necrotic regions of lesions that girdled the needle.

Values are mean and standard deviation from four biological replicates per treatment.

Small common letters in each time point indicate values did not differ significantly between mutants and WT (P<0.05).

Next, lesion development was assessed in terms of lesion length and area of coverage, where DNB lesions were considered as brownish/necrotic areas. The length of mature (late stages) lesions varied significantly between plants infected with either of the dothistromin-deficient mutants compared to WT infected needles. In every time point, lesions were two or three times longer in WT infected needles compared to those with the mutants (Fig. 5.3C and 5.4D, 5.4E, 5.4F).

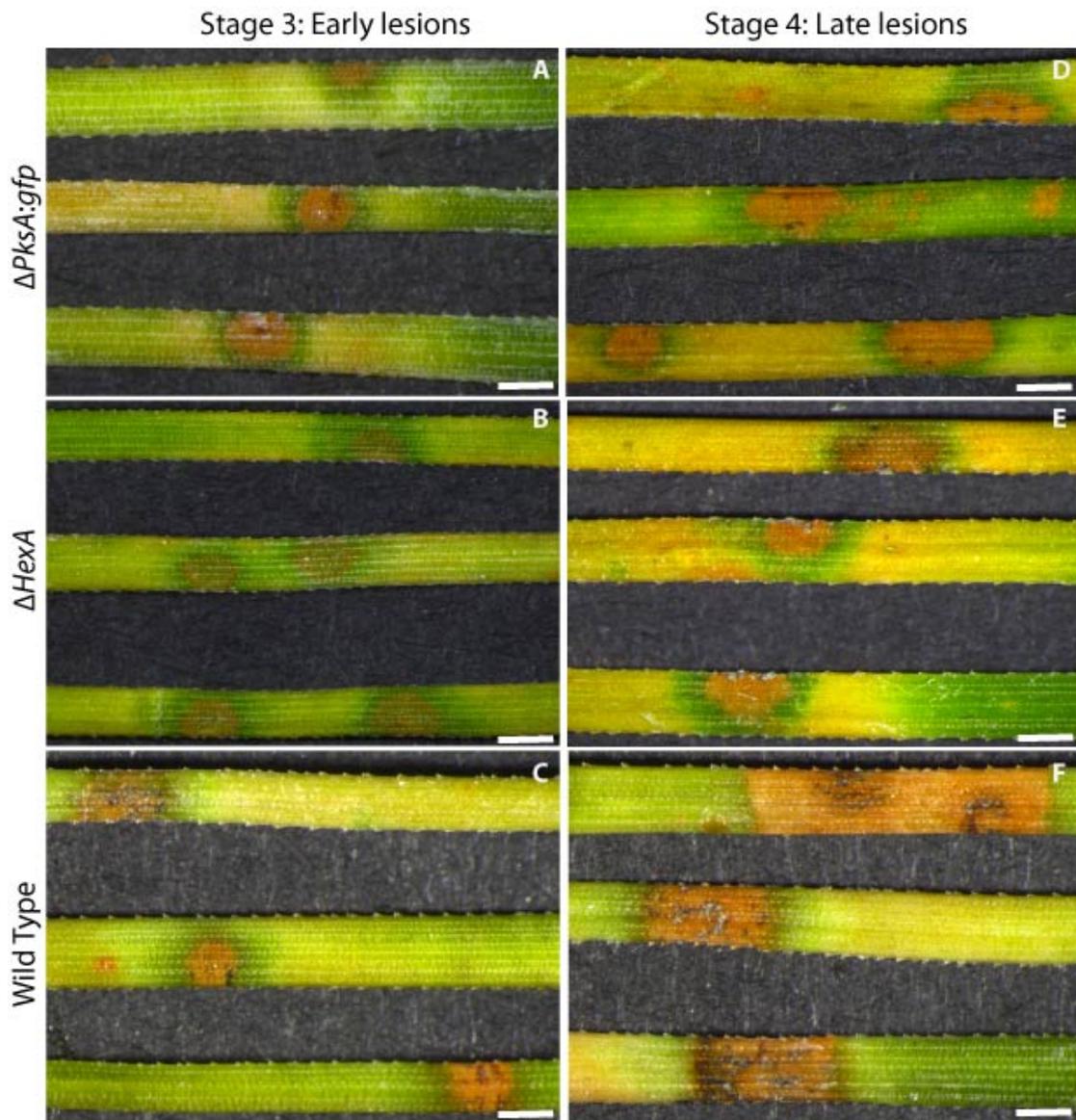


Fig. 5.4. Comparison of dothistroma needle blight (DNB) lesions on needles infected with dothistromin-deficient mutants ($\Delta PksA:gfp$, $\Delta HexA$) and wild type (WT) *Dothistroma septosporum*

(A, B, C) Early DNB symptoms showing similarly small and un-girdled necrotic lesions in both dothistromin-deficient $\Delta PksA:gfp$ and $\Delta HexA$, and WT-infected needles respectively, with green islands around the lesions; (D, E) With the mutants, late lesions are slightly larger than the early lesions in (A) and (B) and do not girdle the needle, but the green islands are still evident; (F) WT lesions are longer than in (C) and girdle the whole needle. At this stage the green islands cannot be clearly distinguished and may be masked or absent. Size bar = 1 mm.

Differences between the extents of lesion development around the needles were noticed between WT and mutants. Early DNB lesions on plants infected with WT *D.*

septosporum are discrete necrotic regions (Fig 5.4C) and these girdle the needle as they mature; in other words the brownish/necrotic area extends around the needle so it can be seen from both sides (Fig. 5.4F). In contrast, most lesions on needles infected with either the $\Delta PksA:gfp$ or $\Delta HexA$ mutant failed to girdle the needle even at a late stage of infection (10-12 weeks ai) (Fig 5.4A, 5.4B, 5.4D, 5.4E). These differences were quantified and shown to be significant (Fig. 5.3D). The experiments were repeated with the same set of mutants, and visual observations made with two additional dothistromin-deficient mutants: $\Delta VbsA$ and $\Delta PksA$ (lacking the *gfp* marker). Similar results were obtained and are shown in Appendix 5 (Fig. A5.1 and A5.2).

During these experiments, dark green regions, similar to green islands reported in the literature (Davis et al., 1994), were noticed around early lesions with both WT and mutants, and persisted in late lesions caused by dothistromin-deficient mutants. In WT infected needles the dark green region was not so evident possibly due to expansion of the lesion over this region (Fig. 5.4).

In summary, results from studies with several independent dothistromin-deficient mutants suggest that dothistromin is required for expansion of small early lesions into large lesions that girdle the needle. Furthermore regions resembling green islands were seen around early lesions irrespective of whether the pathogen made dothistromin or not.

5.2.5. Cell damage by dothistromin-deficient mutants

To determine the effect of dothistromin on cell damage during DNB infection, light microscopy observations were made on needle cross sections of stage 3 and 4 (early and late DNB lesions) caused by dothistromin-deficient mutant $\Delta HexA$ and WT *D. septosporum*.

In this study, most mesophyll cells collapsed during WT infection at stage 3 (early lesion) (Fig. 5.5A) as also shown in Chapter 4. However in needles infected with the mutant, some mesophyll cells were disintegrated around the infection site whereas other mesophyll cells were still intact at the equivalent time point (Fig. 5.5B). In stage 4, severe mesophyll and endodermal collapse was noticed in the WT infection throughout the cross-section (Fig. 5.5C). In contrast in the mutant infection, collapsed mesophyll tissue and limited endodermal cell disintegration was mainly at sites of fruiting body formation and intact mesophyll cells were noted in the same cross-sections (Fig. 5.5D), in accordance with the lack of girdling of the needle by mutant lesions noted earlier.

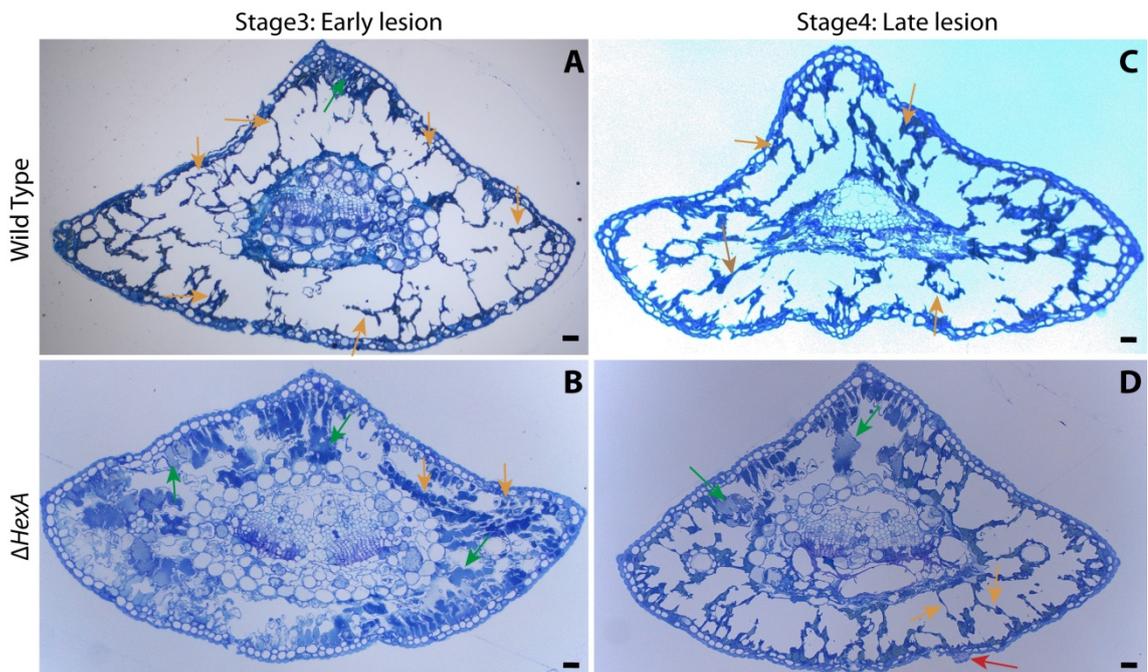


Fig. 5.5. Cross sections through dothistroma needle blight lesions infected with dothistromin-deficient mutant $\Delta HexA$ and wild type (WT) *Dothistroma septosporum* at stages 3 and 4

(A) The WT pathogen resulted in mesophyll collapse over the entire needle section in early lesions; (B) Slight mesophyll disintegration was observed around the infection site of $\Delta HexA$ at the same stage as (A); (C) Extensive mesophyll colonisation with collapsing endodermis is found in WT infected needles in late lesions; (D) Mesophyll colonisation became slightly extended with the mutant, with slightly damaged endodermis at the same stage as (C). In all sections green arrows show intact mesophyll, yellow arrows disintegrated mesophyll and pink arrows *D. septosporum* fruiting bodies. Size bar = 50 μm

The extent of cell damage, and its relationship with fungal colonisation, was evaluated further by SEM of transverse sections of lesions from $\Delta HexA$ mutant and WT infected needles at stage 4. In WT infected needles, fungal hyphae were seen through most of the lesion area, and damaged mesophyll and endodermal cells were seen throughout (Fig. 5.6A). However damaged mesophyll and endodermal cells were also seen in an area where fungal hyphae were not evident. In contrast, the fungal network caused by the $\Delta HexA$ mutant appeared to be confined to one area and had not grown through the whole section. Mesophyll cells, other than those in colonised areas, appeared intact with normal cell contents. In addition, endodermal cells and vascular tissue appeared mostly intact in non-colonised areas (Fig. 5.6B).

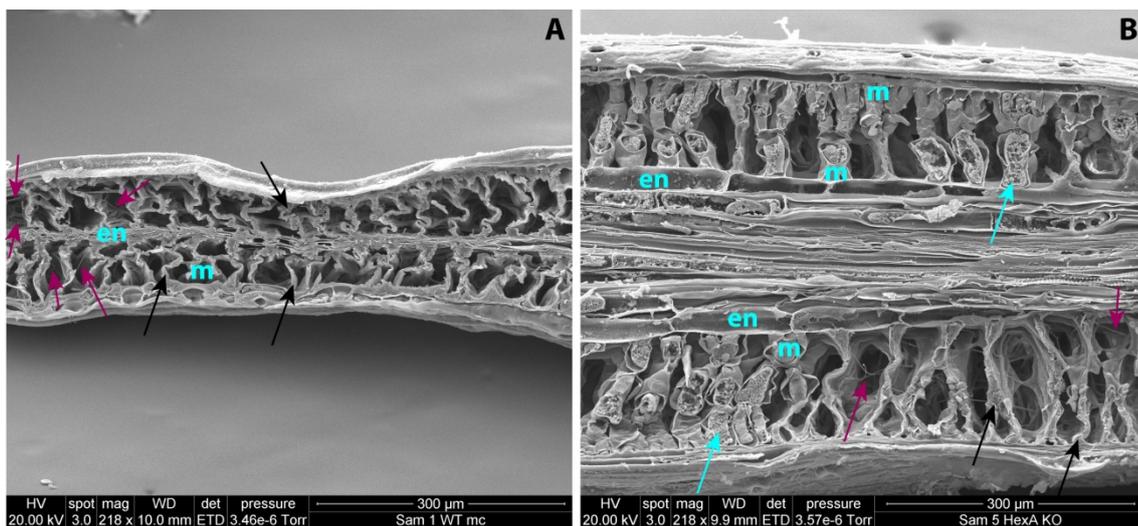


Fig. 5.6. Scanning electron microscopy of transverse sections of late-stage dothistroma needle blight lesions from dothistromin-deficient mutant ($\Delta HexA$) and wild type (WT) infected needles

(A) In the WT infection, both mesophyll (m) and endodermal (en) cells showed severe cell damage and tissue collapse (black arrows) and hyphae (pink arrows) were seen in both upper and lower mesophyll areas; (B) Collapsed mesophyll cells and slightly disintegrated endodermal cells were seen in regions where $\Delta HexA$ hyphae were also present (in lower right of mesophyll region). Intact mesophyll cells (green arrows) were seen in un-colonised mesophyll regions (lower left and upper layers). Both images were taken in same magnification.

These results suggest that the ability of the pathogen to make dothistromin is associated with extensive cell damage during lesion formation in DNB.

5.2.6. Fruiting body formation and sporulation by dothistromin-deficient mutants

The numbers of fruiting bodies formed in late stage lesions, and spores released from single lesions, were quantified to determine if dothistromin affects these parameters.

Significantly lower numbers of fruiting bodies per lesion were found in needles infected with dothistromin-deficient mutants compared to the WT. In addition, significantly fewer spores were released from lesions of mutants compared to WT (Table 5.2). Similar results were obtained when the experiments were repeated using same mutants (Appendix 5: Table A5.3)

Table 5.2. Comparison of fruiting bodies and spores per lesion between needles infected with dothistromin-deficient mutants and wild type (WT) *Dothistroma septosporum*

| <i>D. septosporum</i> | Fruiting bodies/lesion | Spores/lesion |
|-----------------------|------------------------|-------------------|
| $\Delta PksA:gfp$ | 4 b \pm 2 | 84 b \pm 36 |
| $\Delta HexA$ | 3 b \pm 1 | 248 b \pm 120 |
| WT | 17 a \pm 4 | 6678 a \pm 1732 |

Values are mean and standard deviation from 3 lesions from each of four replicate trees. Values in a column followed by a small letter differ significantly ($P < 0.05$).

Scanning electron microscopy was conducted on infected needles to monitor fruiting body eruption through the cuticle. The results showed huge eruption of WT fruiting bodies with masses of spores whilst less pronounced cuticle eruption with fewer spores were seen in needles infected with dothistromin-deficient mutants $\Delta PksA:gfp$, $\Delta HexA$ or $\Delta VbsA$ (Fig. 5.7). PCR amplification was used to confirm that lesions contained spores of the expected *D. septosporum* isolates (Appendix 5: Fig. A5.3).

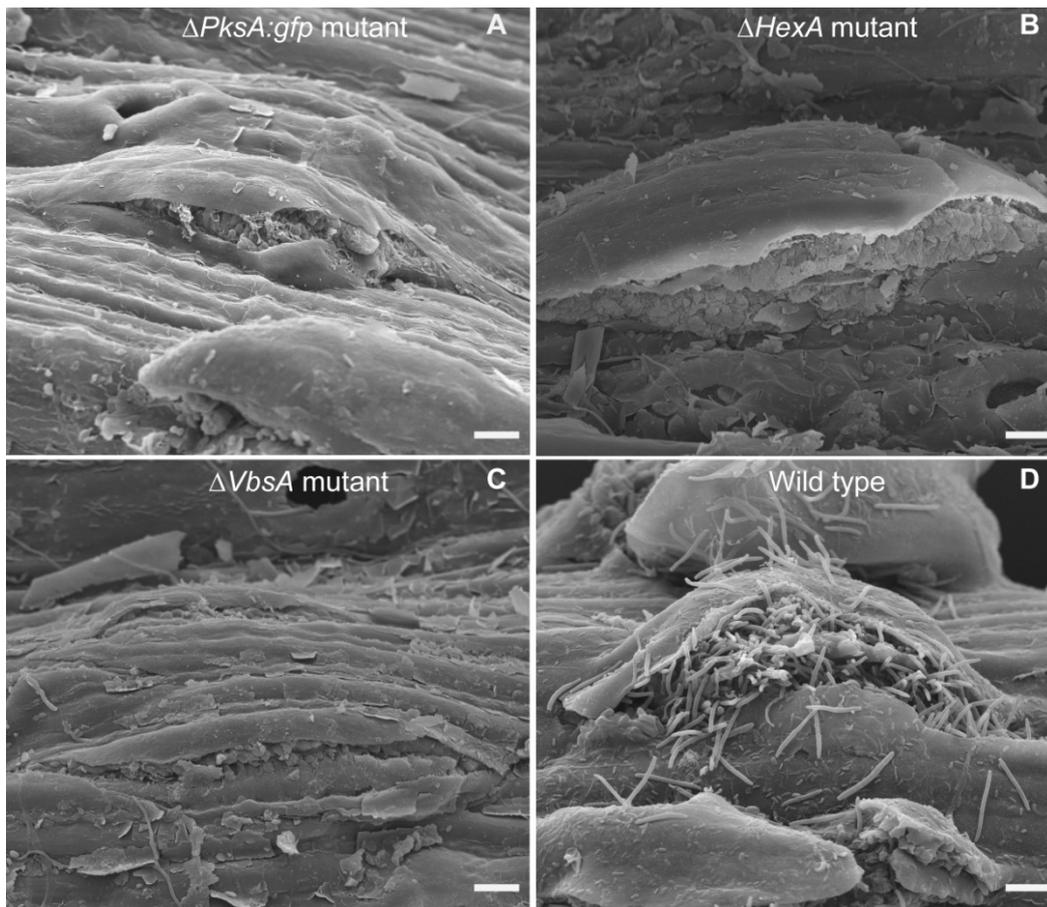


Fig. 5.7. Comparison of fruiting body eruptions from needles infected with dothistromin-deficient mutants and wild type *Dothistroma septosporum*

Scanning electron micrographs of the surface of *Pinus radiata* needles showing erupting and sporulating fruiting bodies that are less pronounced in infections with dothistromin-deficient mutants $\Delta PksA:gfp$, $\Delta HexA$ or $\Delta VbsA$ (A-C) compared to those of the wild type (D). Size bars = 10 μm .

These results suggest that dothistromin is required for normal fruiting body development and sporulation in *D. septosporum*.

5.2.7. Chlorophyll quantification in needles with dothistroma needle blight lesions

Chlorophyll in different areas of DNB infected needles was quantified (section 2.6.5) to see if there was any difference in the green island areas (Fig. 5.4) between dothistromin-deficient mutant and WT infected needles. Total chlorophyll (chlorophyll a and b) was

quantified from (1) the green island area around the lesion, (2) the necrotic portion of DNB lesion, (3) the chlorotic area, and (4) the normal needle green area (Fig. 5.8A, 5.8B).

Although green islands were seen surrounding DNB lesions, the chlorophyll content was lower than normal green areas elsewhere in the needle. The same was found for both mutant and WT lesions. The different areas (1-4 of Fig 5.8) were compared between dothistromin-deficient mutant and WT infected needles. No significant differences in chlorophyll content were found between the mutant and WT lesions in (1) green island, (3) chlorotic or (4) green regions. However, the necrotic regions (2) in the dothistromin mutant lesions had significantly higher chlorophyll levels than the WT lesions (Fig. 5.8C).

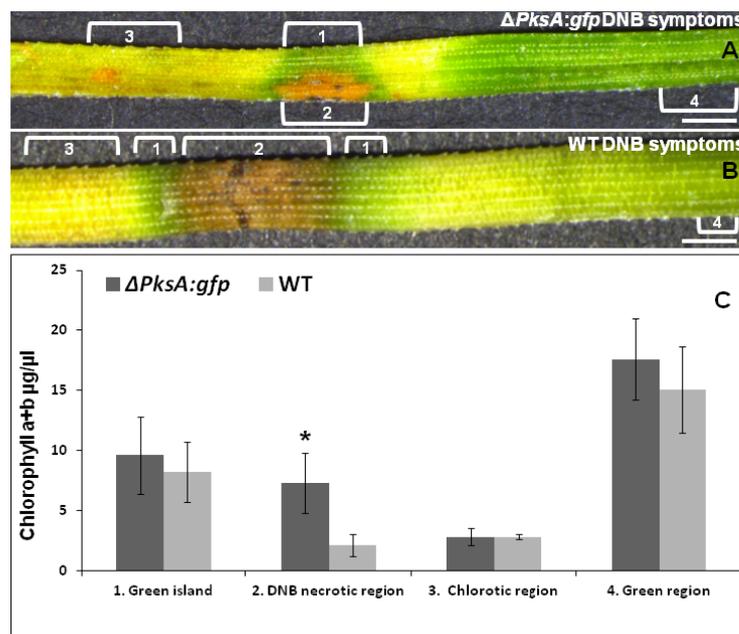


Fig 5.8. Comparison of chlorophyll content between distinct regions of needles infected with dothistromin mutant and wild type (WT) *Dothistroma septosporum*

(A, B) Different areas of $\Delta PksA:gfp$ and WT DNB infected needle, where 1 = green island, 2 = necrotic region, 3 = chlorotic region, 4 = green region; (C) chlorophyll content of marked areas shown in (A) and (B). ‘*’ indicates significant difference between $\Delta PksA:gfp$ and WT values at $P < 0.05$. Values are mean and standard deviations from 10 lesions from each of four replicate trees. Size bar = 1mm.

These results suggest that one consequence of the presence of dothistromin in DNB lesions might be a reduction in chlorophyll content.

5.3. Discussion

Overall, there was clear evidence to support the hypothesis that dothistromin is a virulence factor in pine needle blight. Mutants blocked in dothistromin biosynthesis by gene replacement of either of the early-pathway genes *PksA* or *HexA* led to decrease mesophyll colonisation, smaller lesions and lower numbers of spores, but showed no differences in germination, epiphytic growth or penetration compared to the wild type isolate. In some cases the dothistromin-deficient *PksA* mutant showed higher surface growth and lower penetration, and the wild type showed higher percent lesions at week 12. Given the high levels of variability that can occur in this patho-system, as seen in earlier experiments, it is possible that this was just a natural fluctuation in timing of the disease process. However an effect of dothistromin on the rates of penetration and lesion formation cannot be ruled out completely. Also it is possible that because only a small amount of dothistromin biosynthesis occurs at spore germination until early lesion appearing time in WT isolates (shown in chapter 4), lack of dothistromin in the mutants does not affect these early stages of the disease process.

An equivalent frequency of penetration events with dothistromin mutants or wild-type isolates helps to explain why the overall percentages of needles showing disease were similar, as shown previously (Schwelm et al., 2009) and confirmed in this study. Other non-host selective toxins such as sirodesmin, cercosporin, elsinochrome and trichothecenes have been shown to be virulence factors but collectively their effects on host plants are varied and complex. In contrast to our observations of an equivalent

number of smaller disease lesions in dothistromin mutants, loss of non-host selective toxins such as cercosporin or elsinochrome lead to fewer lesions compared to WT isolates (Choquer et al., 2005; Liao and Chung, 2008). Similarly when sirodesmin-deficient (*SirP*) mutants of *L. maculans* were injected into canola cotyledons they produced fewer lesions but the lesion sizes were the same as those obtained with WT isolates, suggesting it is not required for necrosis (Elliott et al., 2007). Instead sirodesmin appears to have a role in colonisation of stems that normally occurs during a second phase of the disease cycle (Elliott et al., 2007). The trichothecene deoxynivalenol (DON) is another non-host selective toxin, which is required by the pathogen *F. graminearum* to colonise wheat, but not required for it to colonise barley (Maier et al., 2006). In the case of dothistromin-deficient mutants it is possible that different phenotypes could be obtained with different pine host species. At least in *P. radiata*, dothistromin appears to have a role in mesophyll colonisation, which is correlated with lesion expansion, rather than initial colonisation of stomatal chambers and early lesion formation.

Most penetration events in DNB do not result in lesions. An average of about 17% (of stomatal) penetration events per needle was counted yet the maximum number of lesions per needle was about 7. This type of non-correlation between penetration and symptom development was also noticed by Muir and Cobb (2005) while monitoring DNB infection in field conditions. The factors that affect whether any particular penetration eventually becomes a lesion are unknown and might be due to termination of fungal penetration in the sub-stomatal vesicle under the guard cell as found by Muir and Cobb (2005). Also it is difficult to analyse the fungal growth *in planta* to observe events immediately prior to lesion initiation.

Small DNB lesions appeared during infection of pine with the dothistromin-deficient *D. septosporum* mutant and these lesions did not expand much during the life cycle. It is possible that host defense responses restrict further colonisation by this fungus. A host response was noticed in wheat when it was inoculated with a trichothecene-deficient mutant of *F. graminearum*. In the absence of this mycotoxin the host developed thicker cell walls in the rachis node that blocked colonisation by the fungus (Jansen et al., 2005). In another experiment it was found that some host (wheat) defense genes are more highly expressed at late stages of infection in leaves infected with WT rather than with trichothecene-deficient mutants (Desmond et al., 2008). This event is consistent with a model for trichothecenes that these toxins stimulate programmed cell death (PCD) in plants, which facilitates fungal growth. This type of PCD was also observed in Arabidopsis leaves infiltrated with Type A trichothecenes such as T-2 toxin (Nishiuchi et al., 2006) and fumonisin (Asai et al., 2000) and in the latter case was shown to require signaling pathways normally associated with host defense.

In earlier studies, host responses were noticed on *P. radiata* after artificial injection of dothistromin. For example, ethylene production increased with increasing concentration of dothistromin on *P. radiata* (Shain and Franich, 1981). Benzoic acid is also produced in pine needles due to artificial injection of dothistromin and proposed as a phytoalexin of *P. radiata* during DNB (Franich et al., 1986). Expression of host defense genes that are involved in jasmonate, salicylate and ethylene defence pathways from both WT and dothistromin-deficient mutant inoculated needles might help to know more details about the effect of the host response on symptom development and on the formation of the early small DNB lesions *in planta*.

About 25 to 80 fold reduction in sporulation in dothistromin-deficient mutants represents a significant effect of dothistromin on virulence. Dispersal is mainly by rain-splash of conidiospores (Gibson, 1972; Peterson, 1973) and any reduction in spore numbers would severely impair the pathogen's ability to disperse to new needles on the same tree, or to new trees in the area. Indeed one of the main silvicultural control measures used in New Zealand to control DNB is pruning which has the effect of reducing inoculum load in the forest (Van Der Pas et al., 1984).

The effect of dothistromin on sporulation is most likely due to a combination of factors. In culture whilst the growth rate of the dothistromin-deficient *PksA* mutant was indistinguishable from wild-type its sporulation was reduced to less than 50% of wild-type levels (Bradshaw et al., 2006). A similar effect on sporulation was noted for mutants of *A. nidulans* blocked in biosynthesis of sterigmatocystin (a relative of dothistromin) (Wieser et al., 1997). Although the mechanism is unknown, the regulatory networks that control secondary metabolite biosynthesis and sporulation are highly interconnected (Brodhagen and Keller, 2006) hence a direct effect of these toxins on sporulation (or the regulation of sporulation) is possible. In addition to this it seems likely that the lower sporulation seen in dothistromin-deficient mutants was due to the smaller pool of available nutrients associated with the smaller lesion size (fewer dead plant cells) and correspondingly lower numbers of fruiting bodies per lesion.

Green islands, in which green needle colour was retained adjacent to regions colonised with fungal mycelium, were evident in all early lesions. The green islands persisted in the small lesions produced by dothistromin-deficient mutants, but were probably smothered or lost in mature wild-type lesions that expanded due to the presence of dothistromin. Green islands are typically associated with biotrophic pathogens and are regions in which photosynthetic activity is maintained. However

green islands are also seen in plants infected with necrotrophic and hemi-biotrophic pathogens and it has been proposed that their formation is due to cytokinins that delay senescence and enable chlorophyll retention (Behr et al., 2010; Walters et al., 2008).

Presence of this green island during DNB was also reported during the selection of resistant pine against *D. pini* (Carson et al., 1983). The loss of green islands in needles infected with WT *D. septosporum* and the lowering of chlorophyll levels in these regions in mature wild-type lesions, lead to the suggestion that dothistromin might target chloroplasts and/or chlorophyll. Although potential dothistromin-binding sites were identified within vesicles in pine embryos (Jones et al., 1995) studies with an anti-idiotypic antibody led to speculations that dothistromin targets chloroplast components in plants (Jones, unpublished, from Jones et al. (1995)). As with dothistromin, toxin made in the closely related species *M. fijiensis* is proposed to target the chloroplast of banana leaf (Harelimana et al., 1997).

Does dothistromin have a role in protecting the pathogen's niche from competition? Although scanning electron micrographs of the needle surface showed very few other epiphytic fungi (Figure 5.1) it is known that pine needles can host a varied collection of endophytes in the forest (Ganley and Newcombe, 2006). The low levels of dothistromin during the epiphytic/penetration stages suggest that its primary role is not to help *D. septosporum* competing with other epiphytic microorganisms. However the late stages of infection when dothistromin is produced in abundance coincide with extensive host cell death. It is likely that other needle-dwelling organisms take advantage of these nutrients and indeed some endophytic fungi are known to convert to a saprophytic life style and increase their biomass substantially in necrotic tissue (Deckert et al., 2001). Because dothistromin is a broad-spectrum toxin with antibiotic activity against needle endophytes such as *Lophodermium* spp. (Schwelm et

al., 2009), a competitive advantage within necrotic lesions is feasible. Similar effects were noticed with other non-host selective toxins such as zearalenones, which are effective against bacteria, fungi and viruses (Rouxel et al., 1988).

Dothistromin-deficient mutants were able to complete the disease cycle and cause lesions with evidence of mesophyll and some endodermal cell collapse. The cause of this dothistromin-independent plant cell death is not known. It is possible there are proteinaceous effector molecules that act in an inverse gene-for-gene manner to initiate a localised plant cell death response, as for ToxA of *S. nodorum* on susceptible wheat carrying a 'susceptibility' gene (Friesen et al., 2009). Alternatively one of the other secondary metabolites made by *D. septosporum* might cause plant cell death at an early stage of infection. There are only 11 predicted secondary metabolite key genes in the *D. septosporum* genome, some of which are expressed *in planta* (Ozturk and Bradshaw, unpublished results). Another possibility is that carbohydrate active enzymes or other fungal enzymes cause cell death either directly by enzymatic degradation, or indirectly by triggering a PAMP response. Functional analysis of other genes that are highly expressed in the genome will help to answer the question of what causes this dothistromin-independent cell death.

Although this study demonstrated a role in virulence for dothistromin, further work is needed to determine the mode or mechanism of action of dothistromin. For better understanding of how this toxin works *in planta*, experiments could be conducted based on microscopy, biochemical and molecular studies of needles infected with WT compared to dothistromin-deficient mutant *D. septosporum*.

Microscopy using immunolabelling is the way to localise dothistromin in cells and cell organelles during DNB. During this localisation focus needs to be made on the modification of plasma membrane, chloroplasts and mitochondria (based on (Jones et

al., 1998; Kang and Buchenauer, 1999)). Plasma membranes can be damaged slightly or severely due to action of toxin as found for cercosporin (Daub et al., 2005) or AAL-toxin (Tsuge et al., 2013) and invagination of cell membranes can be monitored by TEM. Also it is possible to locate sites of membrane leakage using pyroantimonate fixative (Park et al., 1987). This cytochemical was used to visualise sodium and magnesium ions near the leakage site (cell wall microfibrils) of plasma membranes. In addition lipid peroxidation can be visualised by the generation of hydrogen peroxide using another cytochemical $CeCl_3$ (Park and Ikeda, 2008). Mitochondrial modification can be monitored by swelling of mitochondria, disappearance of matrix components, destruction of cristae, and formation of a bulbous outer membrane (Park and Ikeda, 2008). Chloroplast modification can be monitored for damage of chloroplast membrane, ion leakage (as plasma membrane damage) and disruption of grana and lamellae (Park and Ikeda, 2008).

In biochemical studies, similar events as mentioned in microscopy can be quantified during stages of DNB infection. These include quantification of electrolyte leakage (Keon et al., 2007) and reactive oxygen species (Yang and Chung, 2012).

During the same stages of microscopy and biochemical studies gene expression studies can be carried out to get more information about effects on host. These include expression of pathogenesis related (PR), ethylene biosynthesis, jasmonic acid biosynthesis, PCD, oxidative stress, defense and chloroplast related genes at different time points of DNB.

In conclusion, dothistromin is required for full expression of typical dothistroma needle blight infection on *P. radiata* and further studies are needed to determine its mode of action.

Chapter 6: Conclusions and future directions

The first aim of these studies was to optimise methods for achieving artificial inoculation of the DNB pathogen in controlled conditions. Development of this tool then enabled hypotheses about the life style of *D. septosporum* and the role of dothistromin to be tested.

The DNB pathogenicity assay was improved by introducing a new sporulation medium, pine needle minimal medium with glucose (PMMG), and by optimising microclimatic conditions. The critical environmental parameter for achieving high levels of DNB (>80% of needles with disease symptoms) was leaf wetness. Use of individual covers for 4-7 days post inoculation, followed by incubation in covered chambers containing water foggers for the remainder of the infection cycle, enabled reliably high rates of disease incidence. Using this assay, it was possible to distinguish between trees from families with different levels of field resistance, using clones taken as cuttings from young trees (<1 yr). This optimised DNB pathogenicity assay will benefit the forest industry by enabling screening of resistant and susceptible genotypes at an early stage (about 1 year of seedling age) which will save time and cost compared to testing in the forest.

Based on this optimised pathogenicity system a comprehensive study was conducted on the life style of *D. septosporum in planta*. The hypothesis that *D. septosporum* is a hemi-biotrophic pathogen was supported. Molecular, biochemical and microscopic studies were conducted over the time course of DNB infection to determine its parasitic phases. *D. septosporum* hyphae showed extensive epiphytic growth on the needle surface and growth in stomatal chambers during the non-symptomatic phase. The fungal biomass of *D. septosporum* and levels of dothistromin toxin increased

rapidly after the first appearance of DNB lesions about four to six weeks after inoculation. The plant mesophyll remained intact before the onset of DNB symptoms; however mesophyll collapsed massively when the first lesions appeared. The life style of *D. septosporum* is very similar to those of the closely related hemi-biotrophic fungi *M. graminicola* and *M. fijiensis*. Gene expression studies showed similarities to some genes related to fungal life style in these species. Combining these results, it was concluded that there is a biotrophic phase prior to a necrotrophic phase and *D. septosporum* can be called a hemi-biotrophic pathogen.

Knowledge of this hemi-biotrophic life style will be helpful for a deeper understanding of *Dothistroma*-pine interactions but further work needs to be done. Gene expression studies were carried out for only a few genes in this study. The expression and functions of other genes that might be important in the disease process, such as those encoding hydrolases, secondary metabolites, effectors, carbohydrate active enzymes, proteases and small secreted proteins should be studied in the future. Hydrolases help fungi to penetrate into host cells whilst many effectors such as small secreted proteins manipulate the host cells during early stages of infection (McDowel, 2013; Rafiqi et al. 2012). Many hydrolases and small secreted proteins are present in the *D. septosporum* genome and knowledge of their expression *in planta* over the time course of DNB will help to know more about establishment of this fungal infection. Production and analysis of mutants deficient in effectors that show high levels of *in planta* expression will help to understand the role of these effectors in DNB. Likewise secondary metabolite (SM) genes and their expression *in planta* vary among fungi with different life styles. Some of these SM genes are highly expressed in hemi biotrophic fungi (such as *Colletotrichum* spp., *Magnaporthe oryzae*) (McDowell, 2013; Soanes et al. 2012) at early stages. In contrast, well-characterised SM genes found predominantly

in saprophytes (encoding aflatoxin, sterigmatocystin) are expressed at late stages during necrosis (Moebius and Hertweck, 2009). The complement of SM genes contained in the *D. septosporum* genome needs to be functionally characterized and their *in planta* expression levels determined. Numbers of carbohydrate-degrading enzymes (CAZymes) can also be correlated with pathogen life style. For example, hemi-biotrophic and necrotrophic fungi have more CAZymes than biotrophic fungi (Zhao et al. 2013). Expression of these genes will also help to understand the life style of *D. septosporum* in more detail.

The hypothesis that dothistromin is a virulence factor in dothistroma needle blight infection was supported in this study. To investigate the role of dothistromin, pathogenicity assays were conducted on seedlings of *P. radiata* by inoculating them with two independent dothistromin-deficient mutants under glass house conditions. Similar patterns of DNB infection were noticed with the mutants based on spore germination, penetration and numbers of lesions on pine needles when compared with wild type (WT) controls. However, with the mutants mesophyll colonisation was restricted and lesions smaller (in both length and area) compared to the WT. In addition, restricted cell damage, fewer fruiting bodies and spores were produced in mutants at the end of the DNB cycle. Together these results show that dothistromin is a virulence factor of DNB.

Intriguingly, intense green regions (green islands) initially formed around DNB lesions on needles infected with both mutants and WT. Later in WT infections the green regions were no longer visible due to dothistromin build up and tissue necrosis. A quantitative experiment also supported this loss of chlorophyll. The association with green islands, along with previous observations that light is required for toxicity of dothistromin to plant tissue, leads to the hypothesis that chloroplasts could be a site of

action of dothistromin. How chloroplasts are affected during DNB infection, and the detailed mechanism of action of dothistromin, needs to be investigated using microscopy, biochemical studies and genetic studies as outlined in the discussion of chapter 5.

Whilst testing this hypothesis, some limitations were considered which should be addressed in further investigations. This test was done only in *P. radiata* species and with one clonal *D. septosporum* strain. The role of dothistromin should be tested on other species of pine. In addition, virulence levels of wild type *D. septosporum* isolates that produce different levels of dothistromin should be compared on a common host genotype. These studies would add more insights into the role of dothistromin *in planta*.

Some other questions were unanswered during this study. Host cell disintegration was found in both wild type and dothistromin-deficient mutants, causing needle blight infection. What causes this cell death in the absence of dothistromin is unknown and a possible hypothesis in response to this issue has been described in chapter 5. Using vital staining will help to monitor the extent of cell death at various stages. Functional studies of genes that are highly expressed in early lesions formed by both WT and mutant *D. septosporum in planta* will also help to determine the cause.

Other questions such as 'what affects the virulence of *D. septosporum*?' are unanswered. To answer this question host responses need to be considered along with fungal characterisation, and very little is known about host responses against *D. septosporum*. Gene expression studies of both host (e.g., putative PCD-causing genes) and pathogen (e.g. putative virulence genes) during their interaction will help to answer this question.

In conclusion, *D. septosporum* is a hemi-biotrophic pathogen and the non-host selective toxin dothistromin released by this fungus is needed for the full expression of

DNB. The further research outlined above can be prioritised based on these findings as follows. Firstly, *D. septosporum* known to produce different levels of dothistromin can be challenged against one pine genotype to determine if dothistromin levels are correlated with different virulence levels, followed by a challenge to several pine species to screen suitable genotypes for silviculture in the forest. Secondly, the mode of dothistromin can be studied using microscopy, molecular and biochemical tools. Thirdly, gene expression studies mentioned earlier can be conducted to know the deeper in-sight of the life style of *D. septosporum*. These studies will help us to understand the basis of virulence of *D. septosporum*.

Appendices

Appendix 1: Media, buffers, primers and probes

All media were prepared with Milli-Q water and sterilised by autoclaving at 121°C for 15 minutes.

A1.1. Media

A. Pine Needle Minimal Media with glucose (PMMG)

| | |
|--|--------|
| Minimal Mineral (MM) | (g/l) |
| Magnesium sulphate (MgSO ₄ .7H ₂ O) (Merk, Darmstadt, Germany) | 0.2g |
| Dipotassium hydrogen orthophosphate (K ₂ HPO ₄) (BDH, Poole, England) | 0.9g |
| Potassium chloride, (KCl) (Sigma Chemical Co, Louis, Germany) | 0.2g |
| Ammonium nitrate (NH ₄ NO ₃) (Sigma–Aldrich, Steinheim, Germany) | 1.0g |
| Iron sulphate (FeSO ₄ .7H ₂ O) (APS Chem. Ltd. NSW, Australia) | 0.002g |
| Zinc sulphate (ZnSO ₄ .7H ₂ O) (BDH, Poole, England) | 0.002g |
| Manganese chloride (MnCl ₂ .7H ₂ O) (BDH, Poole, England) | 0.002g |
| Asparagine (Sigma Life Science, St. Louis, USA) | 2.0g |
| Glucose (APS Chem Ltd. NSW, Australia) | 3.0g |
| Agar (NEOGEN corporation, Michigan, USA) | 20.0g |

Water used to make this medium was prepared by soaking fresh pine needles (10% [w/v]) in 1 l Milli Q water for 24-48 hours at room temperature based on Carsolio et al. (1994) and adjusted to pH 6.2

B. Dothistroma Sporulation Medium (DSM) (g/l)

| | |
|--|-------|
| Malt extract (Oxoid, Hants, England) | 20.0g |
| Yeast extract (Life Technologies, UK) | 5.0g |
| Bacteriological agar (Acumedia, Michigan, USA) | 15.0g |

in 1 l MQ water and adjusted to pH 6.2

C. Pine Needle Agar (PNA)

5g ground pine needles in 2% Water Agar (Acumedia, Michigan, USA) and adjusted to pH 6.2 (using NaOH)

D. Water Agar (WA)

2% Water Agar (Acumedia, Michigan, USA) and adjusted to pH 6.2

E. Potato Dextrose Agar (PDA) (Merck, Darmstadt, Germany)

Potato dextrose agar 39g/L

A1.2. Buffer and solutions

All solutions were prepared with Milli-Q water and sterilised by autoclaving at 121⁰C for 15 minutes.

A. Buffered formalin (10%) (Grizzle et al., 2008)

| | |
|--|--------|
| Tap water | 900 ml |
| Formaldehyde (37%) | 100 ml |
| Sodium phosphate, dibasic, anhydrous (Na ₂ HPO ₄) | 6.5 g |
| Sodium phosphate, monobasic, monohydrate (NaH ₂ PO ₄) | 4.0 g |

B. CTAB DNA extraction buffer

| | |
|-------------------------------------|----------------|
| CTAB - | (2%) w/v |
| NaCl - | 1.42 M |
| EDTA - | 20 mM, |
| Tris HCl - | 100 mM pH 8.0, |
| PVP 40 (Polyvinylpyrrolidone) | 2% w/v |
| (Sigma Chemical Co., St. Louis, MO) | |

C. 1×TBE buffer

89 mM tris-HCl, 2.5 mM Na₂EDTA and 89 mM Boric acid (pH 8.3)

D. Ethidium Bromide

Agarose gel was stained in Milli-Q water with a final concentration of 1 µg/ml ethidium bromide.

A1.3. Primers and probes

Table A1.1. Primers and probes used for *Dothistroma septosporum* identification and biomass quantification

| Target species | Primer/probe name | Lab ref. | Primer/probe sequence (5' to 3') | Size (bp) | Ref. |
|---|-------------------|----------|----------------------------------|-----------|------------------------|
| <i>D. septosporum</i> | <i>PksA64</i> | 934 | CTGTCTTCCTCGACCTGTT | 102 | (Chettri et al., 2012) |
| | <i>PksA164</i> | 871 | AAGCACACCTGGAAAGAATGA | | |
| | Probe 88 | - | 6FAM-CCATCGATCCCAGCACCGCT-BHQ1 | | |
| <i>Pinus radiata</i> | <i>CAD918</i> | 935 | CAGCAAGAGGATTTGGACCTA | 101 | (Chettri et al., 2012) |
| | <i>CAD1019</i> | 936 | TTCAATACCCACATCTGATCAAC | | |
| | Probe 945 | - | HEX-TGTGAACCATGACGGCACCC-BHQ1 | | |
| <i>D. septosporum</i> | DPS ITS spec fwd1 | 377 | CTGAGTGAGGGCGAAAG | 409 | - |
| | DPS ITS spec rev2 | 376 | CTCTTCAGCGAAATATATG | | - |
| <i>D. septosporum</i> deficient mutant specific | hph Fwd2 | 175 | ATTCATATGCGCGATTGCTGAT | 536 | - |
| | hph Rev2 | 176 | TATCGGCGAGTACTTCTACACA | | - |

Appendix 2: Calculations of *Dothistroma septosporum* biomass and dothistromin toxin

Standard curves preparation using Real-time PCR

Standard curves of both fungal *PksA* (target) and pine *CAD* (reference) genes were prepared using five-fold dilutions of fungal and pine genomic DNA from starting concentrations of 200 ng and 1000 ng respectively. Each dilution was replicated at least six times for reliable amplification (Figs. A2.1A and A2.1C). Fungal and plant standard curves (Figs. A2.1B and A2.1D) showed an efficiency of 2.026 and 2.017 with a regression value $y = -3.261x + 30.25$ and $y = -3.283 + 36.65$ respectively; where y = crossing point (cp), x = log concentration.

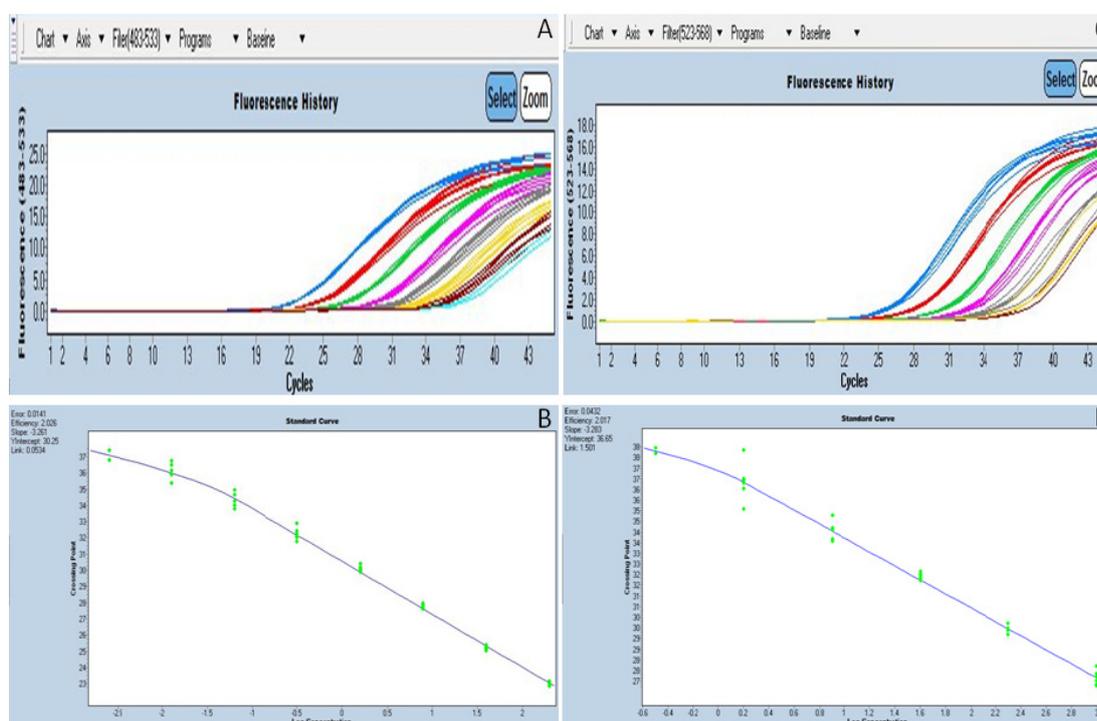


Fig. A2.1. Amplifications and standard curves of *PksA* and *CAD* gene

(A) Amplification of *PksA* genes with 200, 40, 8, 1.6, 0.32, 0.064, 0.0128, 0.00256 ng fungal DNA (8 replicates); (B) Standard curve for *PksA* gene prepared from data shown in (A); (C) Amplification of *CAD* genes with 1000, 200, 40, 8, 1.6, 0.32 ng plant DNA (6 replicates); (D) Standard curve of *CAD* genes prepared from data shown in (C).

Estimation of unknown *Dothistroma septosporum* biomass/needle by real time PCR

Using the standard curve based on the *D. septosporum PksA* gene (Fig. A2.1B) absolute quantification of unknown *D. septosporum* biomass was calculated as shown in the following example (based on Gachon et al. 2004) is was calculated so that the rate of dothistromin production (per fungal biomass) could be calculated (Table A2.2).

In this example, ‘sample Cp’ is the Cp value for unknown *D. septosporum* DNA, and ‘calibrator Cp’ is a single reference Cp used as the basis of relative fold increase in qPCR studies. Example of absolute *D. septosporum* biomass quantification per needle based on data from Table A2.1.

Table A2.1. Calculation of unknown DNA from pine needle

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|-----------|---------------|---|----------------------|------------|--------|----------------------------|---------------|-------------------------------|
| Sample Cp | Calibrator Cp | Cp of calibrator at the time of standard curve generation | Normalised sample Cp | log DNA ng | DNA ng | Eluted total fungal DNA ng | Needle wt. mg | Total fungal DNA/needle ng/mg |
| 27.25 | 31.07 | 30.09 | 26.39 | 1.184 | 15.28 | 168.08 | 41.00 | 4.10 |

Steps:

1. Cp values of unknown *D. septosporum* sample and calibrator were 27.25 and 31.07 respectively (from 1 biological replicate and 2 technical replicates) that obtained from a qPCR run (column 1 and 2 respectively),
2. This calibration Cp value was 30.09 at the time of standard curve preparation (column 3),
3. The sample Cp value was normalised by adjusting the Cp value based on the Cp value of the calibrator in this assay [the unity method, based on based on Gachon and Saindrenan (2004)]; calculated as $27.25 \times 30.09/31.07 = 26.39$ (column 4),

4. The amount of fungal DNA in the qPCR sample was determined from the standard curve based on *PksA* normalised Cp of 26.39. i.e. $y = -3.261x + 30.25$, where y = normalised Cp value 26.39, $x = \log \text{DNA}$. Therefore, $\log \text{DNA}$ obtained was 1.184 ng, amount of unknown DNA obtained, $\text{anti log } 1.184 = 15.28 \text{ ng}$ (column 5 and 6),
5. As the volume of sample used in the qPCR reaction was 2 μl , and DNA pellet was eluted in 22 μl of MQ water, therefore total fungal DNA obtained from the pine needle sample was $15.28 \times 22/2 = 168.08 \text{ ng}$ (column 7),
6. This amount (ng/ μl) DNA was obtained from 41 mg (dry wt.) of pine tissue. Therefore the total fungal DNA per mg of needle tissue was $168.08/41.00 = 4.1 \text{ ng/mg}$ (column 8 and 9).

Standard curve preparation for estimation of dothistromin by HPLC

Dothistromin standards curve was prepared using 10 fold dilution of pure dothistromin starting concentration from 0.1 ng/ μ l to 0.0000001 ng/ μ l. Three technical replications of these 7 dilutions were used to prepare this standard curve which had an efficiency value of 0.9996 with an regression equation $y = 0.8176x + 3.7675$; where $y = \log$ area, $x = \log$ dothistromin concentration (ng/ μ l).

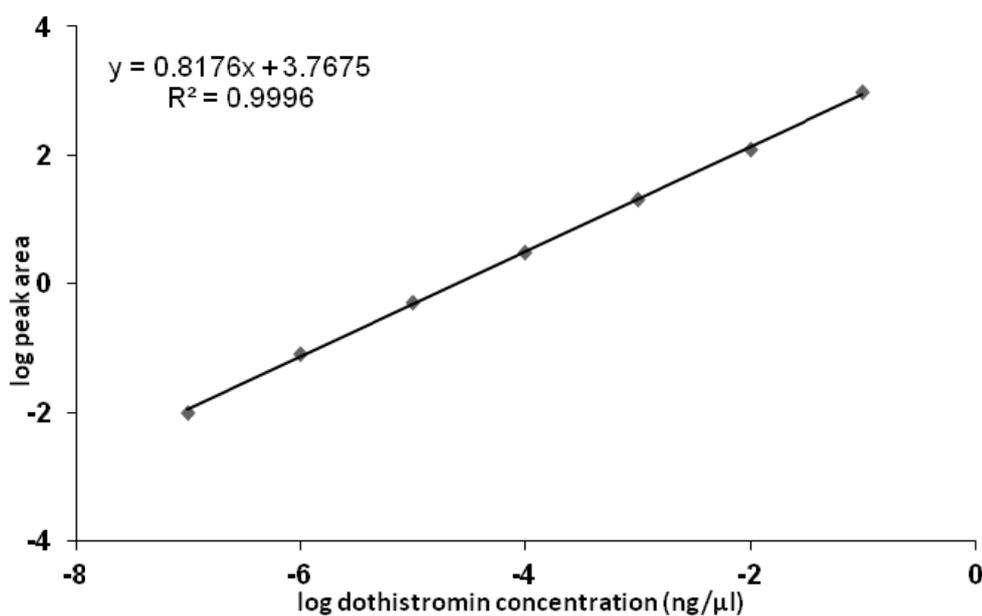


Fig. A2.2. Standard curve of dothistromin

Standard curve of dothistromin quantification prepared by plotting log peak area (y axis) and log dothistromin concentration (x axis) of the diluted dothistromin.

Estimation of unknown dothistromin toxin/needle by HPLC

Example of dothistromin toxin per needle calculation based on data from Table A2.2

Table A2.2. Calculation of unknown dothistromin from needle

| 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|-------------|-----------------|--|--|-------------------------|--------------------|-----------------------------|
| Sample area | Sample log area | Log dothistromin concentration (ng/ μ l) | Dothistromin concentration (ng/ μ l) | Total Dothistromin (ng) | Needle dry wt (mg) | Dothistromin/needle (ng/mg) |
| 152.73 | 2.184 | -1.9367 | 0.01157 | 11.57 | 41.0 | 0.28 |

Steps

1. Firstly area of dothistromin in unknown sample '152.73' was collected from HPLC run (column 1),
2. This area figure was then transformed; $\log_{10}(152.73) = 2.184$ (column 2),
3. Log area figure was inserted to the regression equation ' $y = 0.8176x + 3.7675$ ' of standard curve (Fig. A2.2) to calculate log dothistromin concentration = $(2.184 - 3.7675)/0.8176$; -1.9367 ng/ μ l (column 3),
4. Therefore, dothistromin concentration was $\text{antilog}_{10}(-1.9367) = 0.01157$ ng/ μ l; (column 4),
5. This amount of dothistromin was extracted in 1 ml ethyl acetate solution; total amount of dothistromin was $(0.01157 \text{ ng}/\mu\text{l} \times 1000 \mu\text{l}) = 11.57$ ng (column 5),
6. The amount of dothistromin in column 5 was extracted from 41 mg of needle tissue (column 6). Therefore, dothistromin/needle was $11.57/41 = 0.28$ ng/mg (column 7),

A2.3. Calculation of unknown dothistromin toxin/*D. septosporum* biomass

Steps:

1. We know dothistromin toxin/needle was 0.28 ng/mg dry wt needle tissue (from Table A2.2) and *D. septosporum* biomass/ needle (dry wt needle tissue) was 4.1 ng/mg (from Table A2.1)
2. Therefore, dothistromin toxin/*D. septosporum* biomass = $0.28/4.1$ ng/ng; 0.068 ng/ng

Appendix 3: Pathogenicity assay optimisation

Table A3.1. Spore germination of *Dothistroma septosporum* on PMMG at 7, 12 and 17 day

| Time points | % Germination of spores ^a |
|-------------|--------------------------------------|
| 7 day | 82.3 a ± 8.0 |
| 12 day | 83.3 a ± 3.1 |
| 17 day | 7.0 b ± 10.4 |

Figures in a column followed by a common small letter did not differ significantly ($P < 0.05$) as calculated from least significant difference.

^aMeans ± SD are from three replicates per treatment

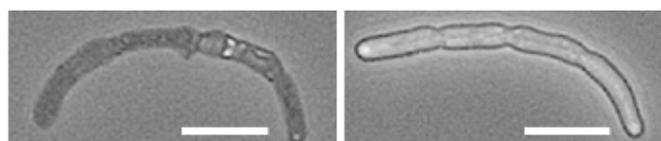


Fig. A3.1. Types of *Dothistroma septosporum* spores seen during sporulation tests

Examples are shown of darker spore that was found in old cultures and failed to germinate (left) and normal healthy spore (right). Size bar = 10µm.

Table A3.2. Percent adhered *Dothistroma septosporum* spores (*in vitro* glass surface) after wash, and spore germination using adhesives

| Treatments | Adhered spores ^a (%) | Spore germination ^a (%) | |
|-----------------|------------------------------------|------------------------------------|----------------|
| | | Experiment 1 | Experiment 2 |
| Gelatin (1%) | 5.6 c ± 5.0 | 68.9 d ± 3.8 | 57.2 a ± 15.4 |
| Mucin (0.5%) | 49.2 b ± 34.3 | 81.0 c ± 5.7 | 30.2 bc ± 12.4 |
| Tween20 (0.05%) | 14.8 c ± 18.6 | 96.2 a ± 6.6 | 34.8 b ± 15.6 |
| PEG (1%) | 95.9 a ± 5.7 | 67.6 d ± 6.8 | 18.3 c ± 8.9 |
| Water (Control) | 88.6 a ± 12.3 | 89.1 b ± 3.1 | 41.3 ab ± 20.1 |
| | n=4 | n=4 | n=4 |

Figures in a column followed by a common letter did not differ significantly ($P < 0.05$) as calculated from least significant difference;

^aMeans ± SD are presented

Example of preliminary pathogenicity assays

Seven experiments (some replicated and some non-replicated) were conducted to test adhesives *in planta*. Due to limited availability of seedlings and space, these tests were conducted at different times, with seedlings having different height but in the same conditions. Comparing the disease incidence within each adhesive, smaller seedlings showed much higher infection in most of the adhesives compared to taller seedlings (Appendix 3: Fig A3.2). It was observed that the misting system used to obtain high leaf wetness was not so effective with tall plants. This led to the hypothesis that high leaf wetness is critical for disease development in controlled conditions

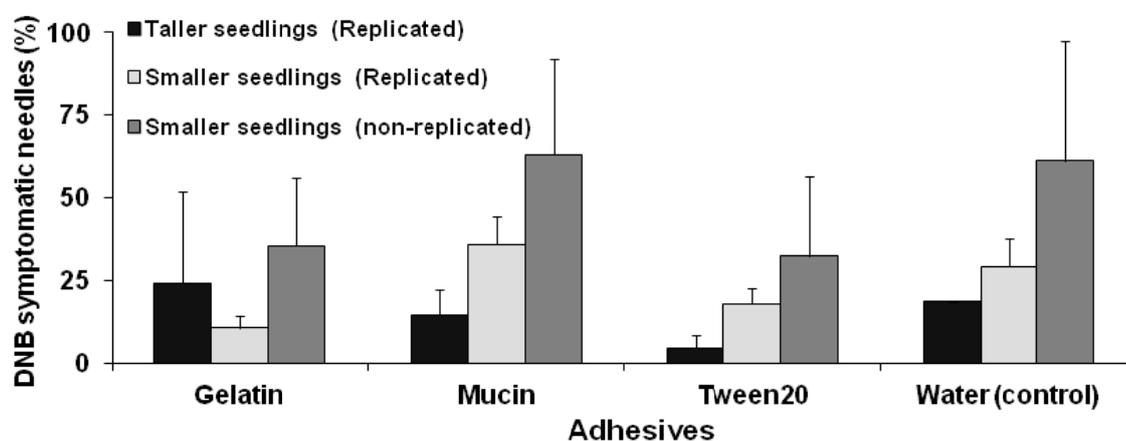


Fig. A3.2. Dothistroma needle blight incidence between taller and smaller seedlings

Higher disease incidence was noticed in smaller seedlings except in gelatin treatment. Replicated experiments (n=2) had at least 3 replicates and non-replicated experiments conducted in 3 different times. Mean and standard deviations are presented.

Table A3.3. Dothistroma needle blight infection with optimised wetness conditions

| Experiments | Treatments | Needles with symptoms ^a | Red bands/needle ^a | Total needles |
|-------------|-----------------------|------------------------------------|-------------------------------|---------------|
| Expt.1 | Inoculated | 97.6 a ± 1.1 | 2.2 a ± 0.4 | 416 |
| | Control (water spray) | 0.0 b ± 0.0 | 0.0 b ± 0.0 | 445 |
| Expt. 2 | Inoculated | 96.7 a ± 5.8 | 2.1 a ± 0.2 | 452 |
| | Control (water spray) | 0.0 b ± 0.0 | 0.0 b ± 0.0 | 530 |

Figures in a column followed by a common letter did not differ significantly ($P < 0.05$) as calculated from t-test; ^aMeans ± SD are from three replicated seedlings per treatment; Experiments were conducted at 4 days post inoculation high-wetness followed by medium wetness.



Fig. A3.3. Optimised dothistroma needle blight

Dothistroma needle blight symptoms at week10 (left) based on optimised wetness condition and no symptoms on water (control) sprayed seedlings (right).

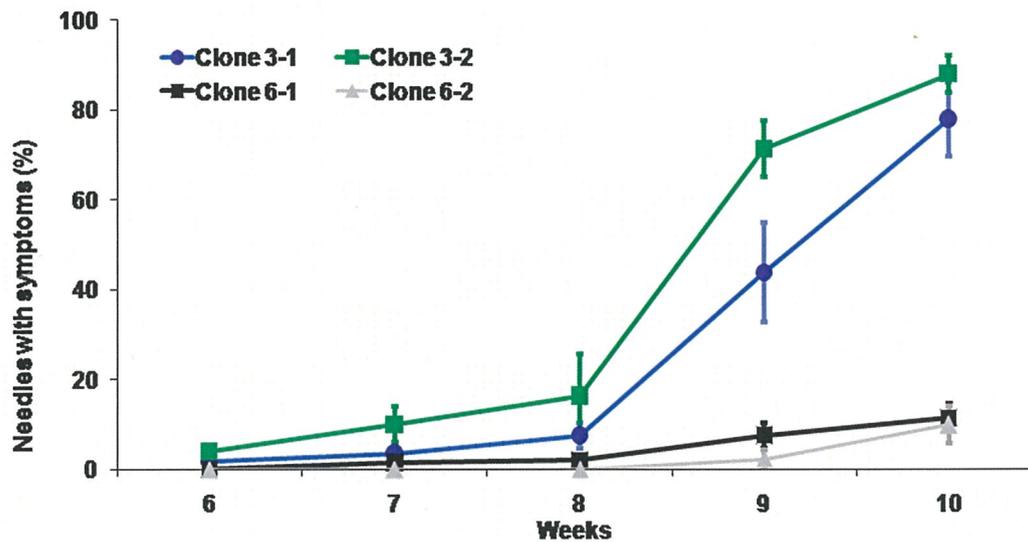


Fig. A3.4. Dothistroma needle blight progression on pine clones

Moderately resistant clones (6-1 and 6-2) show late appearance and lower levels of disease symptoms compared to highly susceptible clones (3-1 and 3-2).

Table A3.4. Dothistroma needle blight events on *Pinus radiata* clones

| Pine clone ^a | Needles with symptoms ^b (%) | Red bands/needle ^b | Lesion length ^b (mm) | No. Fruiting body/lesion ^b | Total needles |
|-------------------------|--|-------------------------------|---------------------------------|---------------------------------------|---------------|
| 3/2 | 82.9a ± 4.6 | 2.3a ± 0.6 | 5.2a ± 1.2 | 14.2a ± 4.7 | 507 |
| 6/2 | 6.1b ± 1.4 | 2.2a ± 0.8 | 2.6b ± 0.6 | 4.3b ± 2.3 | 621 |
| Control | 0.0c ± 0.0 | 0.0b ± 0.0 | 0.0c ± 0.0 | 0.0c ± 0.0 | 517 |

^aClones 3/2 and 6/2 are from highly susceptible (HS) and moderately resistant (MR) families respectively

^bValues are mean ± SD; n=4

Figure in a column followed by a common letters did not differ significantly (P<0.05) as calculated from least significant difference

Table A3.5. Comparison of variance between seedlings and clones

| | | Clone | | | |
|-----------|---------------|------------------------|--------------|-------------------------|--------------|
| | | Mid stage ^a | | Late stage ^b | |
| | | S2 (Expt.1) | S2 (Expt. 2) | S2 (Expt.1) | S2 (Expt. 2) |
| Seedlings | 521 (Expt. 1) | NS (0.58) | NS (0.48) | NS (0.07) | NS (0.16) |
| | 521 (Expt. 2) | NS (0.55) | NS (0.47) | NS (0.66) | NS (0.79) |
| | 520 (Expt. 1) | NS (0.37) | NS (0.29) | NS (0.66) | NS (0.79) |
| | 520 (Expt. 2) | NS (0.21) | NS (0.08) | NS (0.55) | NS (0.41) |

Contingency table showing probability (*p*) values obtained using Levene's test for variance, based on a null hypothesis of no difference in variance in percent dothistroma needle blight (DNB) symptomatic needles between pair-wise combinations of clones and seedlings at a mid (left) or late (right) stage of DNB.

^aMid stage = 8 wai (weeks after inoculation) except 521 (expt. 2), where it is 10 wai; ^bLate stage = 10 wpi except 521 (expt. 2), where it is 12 wai

S2 = clone 3/2; NS = not significant

Appendix 4: Life style of *Dothistroma septosporum*

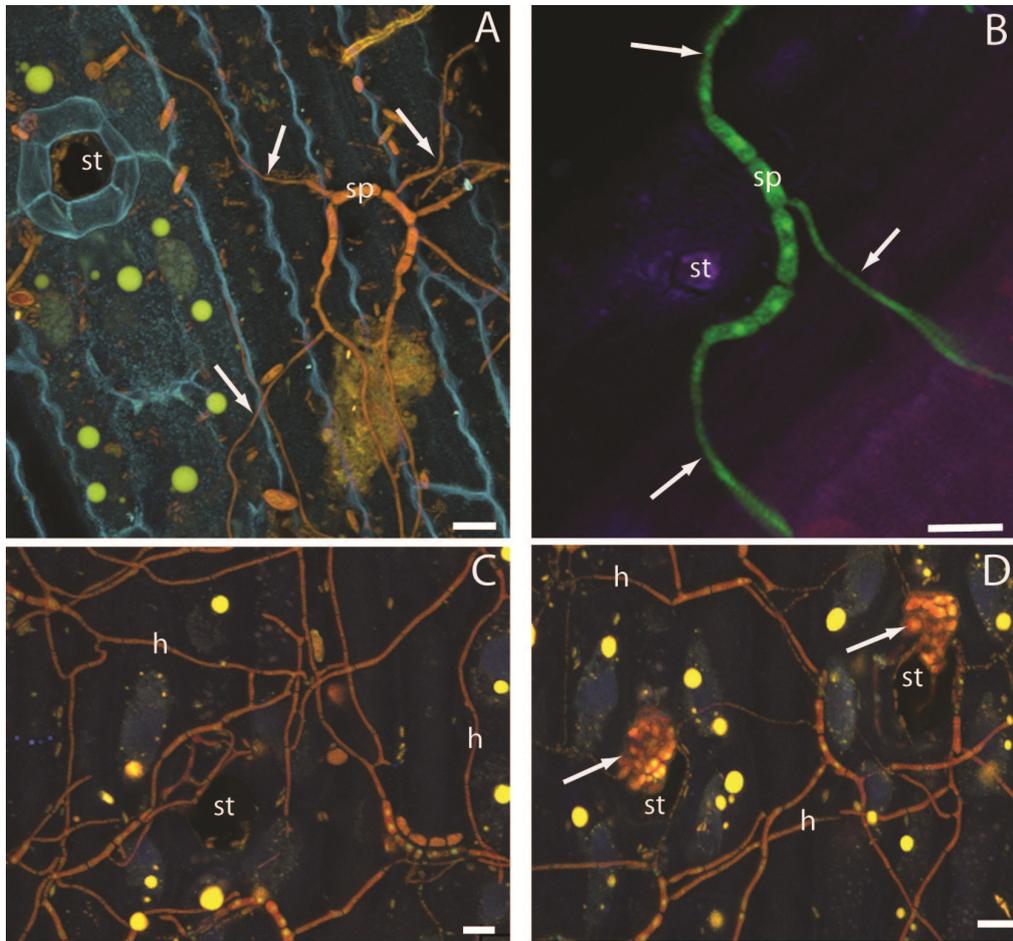


Fig. A4.1. Spore germination and fungal growth on pine needle surface

(A-B) Confocal images of germinated spore (s) having several germ tubes (arrows) near a stomata (st); (C) Hyphal (h) network after germination; (D) Hyphal (h) network after germination. Fungal growth (arrows) was also seen inside of stomata. (A), (C-D) are stained with Trypan blue and (B) is labelled with *gfp*. Size bar = 10 μm.

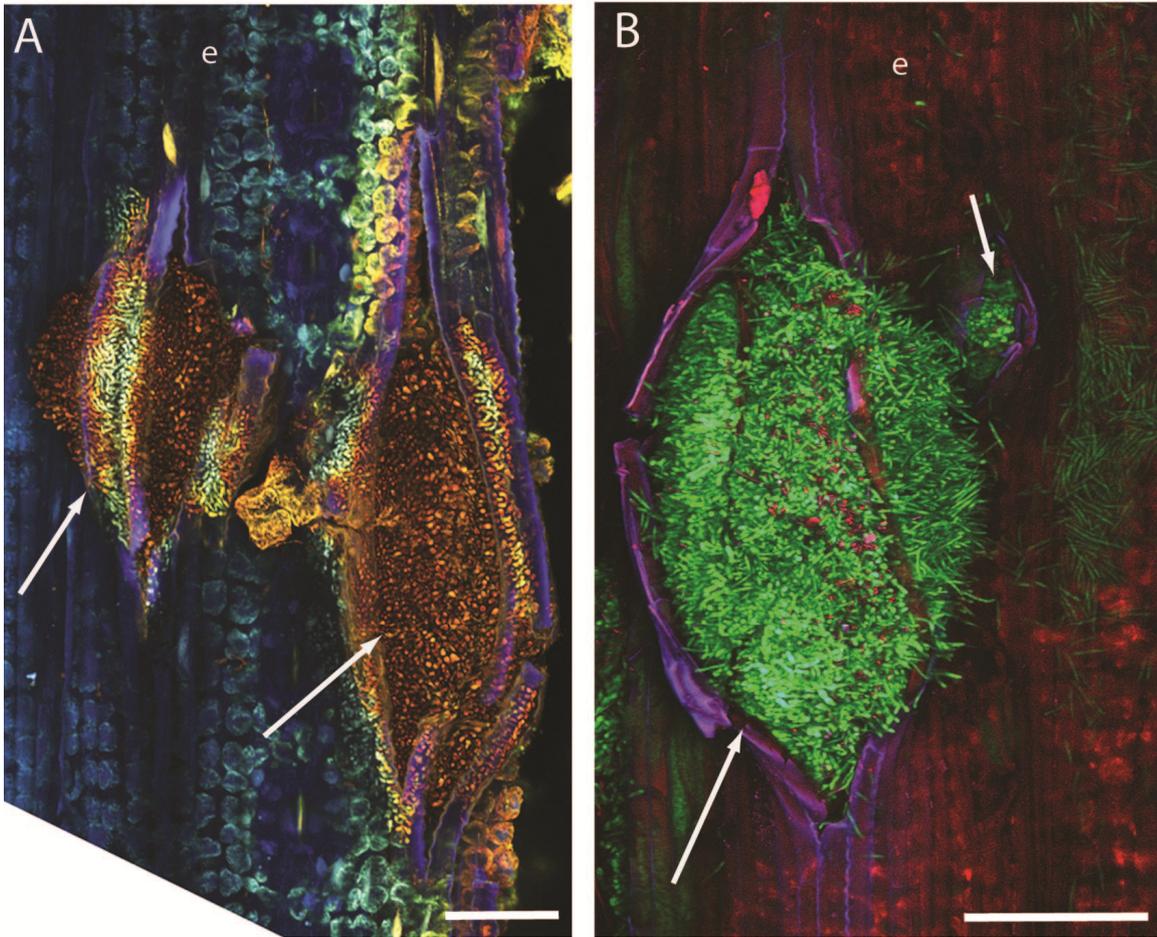


Fig. A4.2. Fruiting body of *Dothistroma septosporum*

(A-B) Confocal images of two fruiting bodies (arrows) are seen that erupted through the epidermis (e) with masses of spores; (A) stained by Trypan blue and (B) labelled with *gfp*. Size bar = 50 μ m.

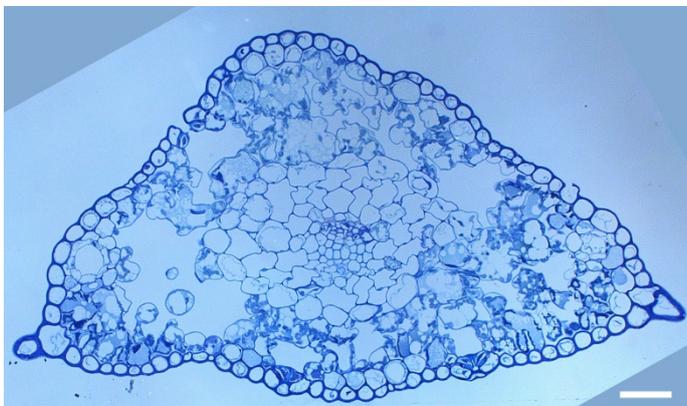


Fig. A4.3. Young pine needle

Light microscopy cross section of young pine needle (6 months old) shows less empty spaces around mesophyll cells than mature needle (2 years old) shown in Fig. 4.7. Size bar = 50 μ m

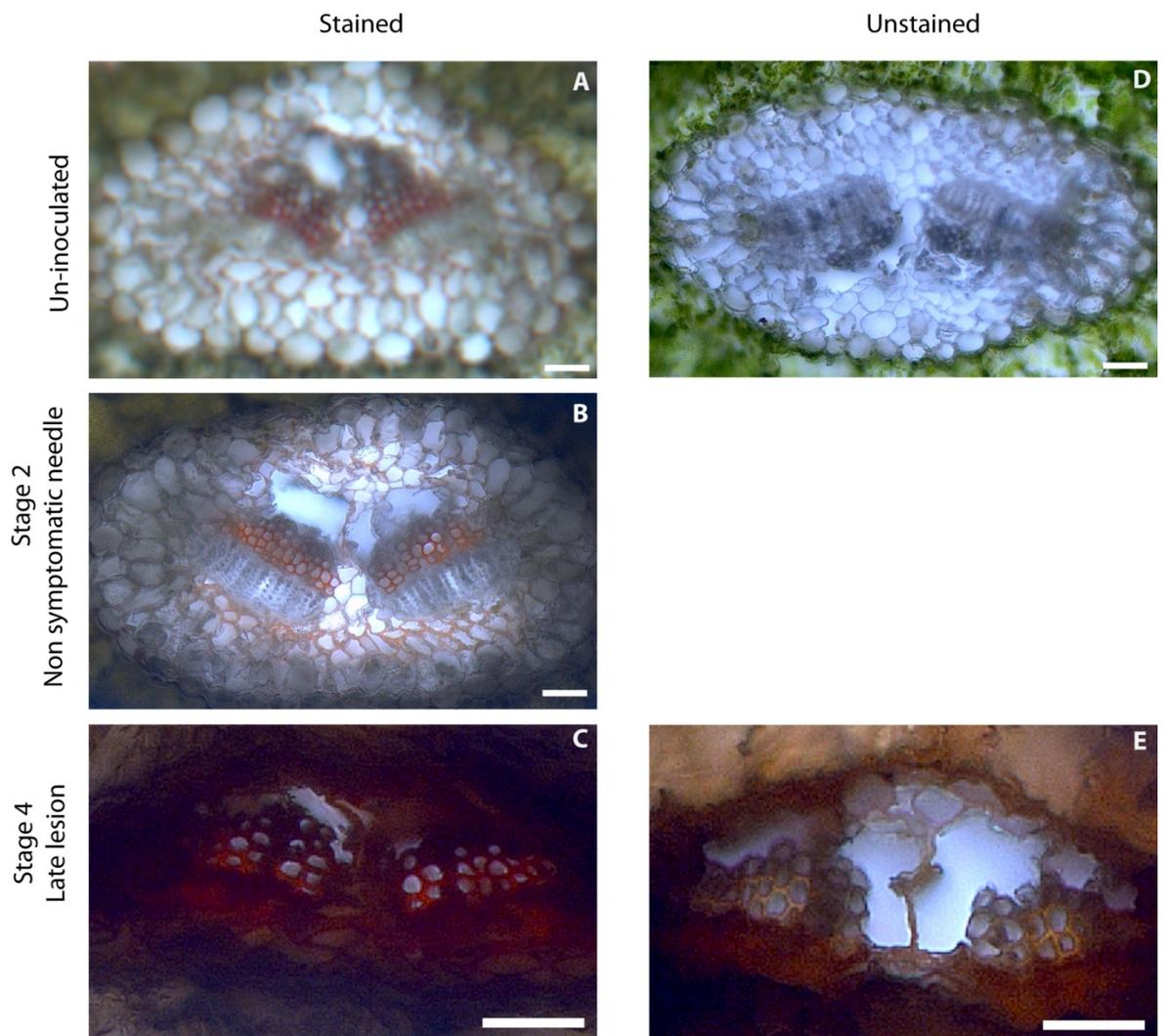


Fig. A4.4. Light microscopic view of lignification on pine needle during dothistroma needle blight (A-C) Pine needle cross sections showing red colour as an indication of lignifications in xylem vessels of endodermis; where vessels of late dothistroma needle blight (DNB) lesion (C) were thicker and more intensely stained compared to un-inoculated (A) and non-symptomatic (B) needle cross sections; (D-E) No red colour was found in un-inoculated needle cross section and late DNB cross section that were not stained with phloroglucinol-HCl, although brown colour associated with dothistromin accumulation is seen in (E). Size bar = 50 μ m

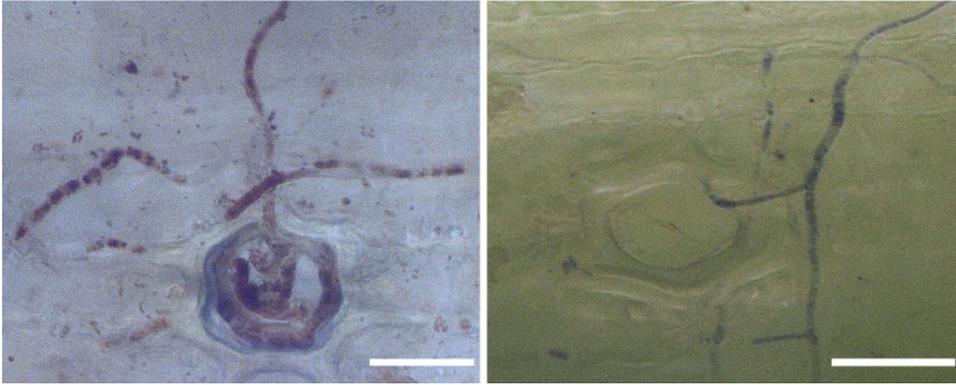


Fig. A4.5. Reactive oxygen species staining during dothistroma needle blight

Light microscopic view of the surface of *Dothistroma septosporum* infected needle showing no host response using diaminobenzidine (DAB) (left) and nitroblue tetrazolium (NBT) (right) staining. Only fungal hyphae were stained as brown (on needle surface as well as around the stomatal pore) (left) and blue (right) respectively at stage 2 (time of penetration). Size bar = 25 μm

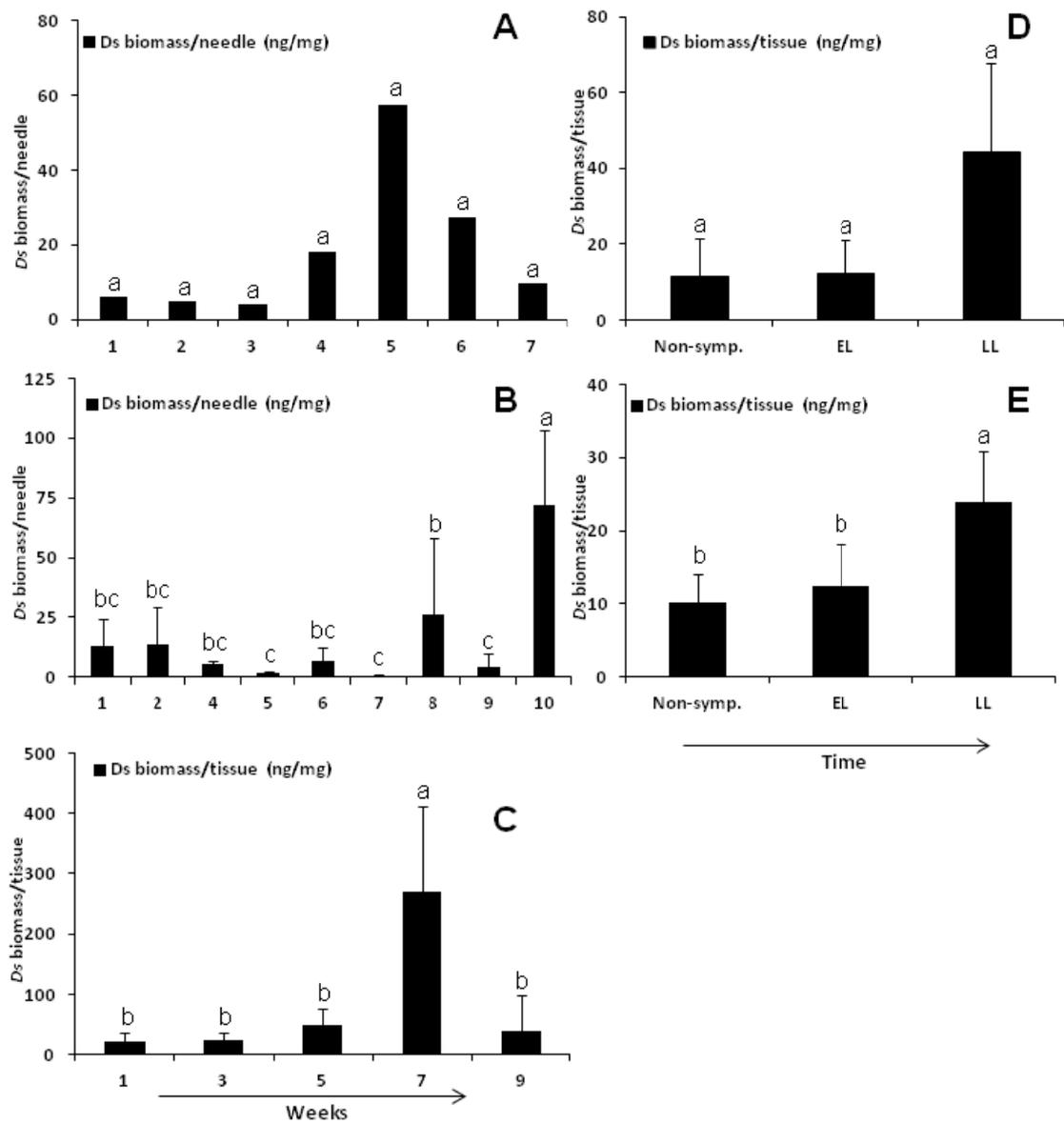


Fig. A4.6. Absolute biomass quantification

(A, B, C, D and E) were calculated *Dothistroma septosporum* biomass (*Ds* biomass) from experiments 1, 2, 3, 4 and 5 respectively. Values with common letters in each chart did not differ significantly ($P < 0.05$) as calculated from least significant difference. Values are mean and standard deviation from two biological replicates in experiment 1 and three biological replicates in experiment 2, 3, 4 and 5, and two technical replicates per treatment. *Ds* biomass refers to *D. septosporum* biomass. 'non-symp', 'EL' and 'LL' refer to non-symptomatic needles, early lesions and late lesions respectively.

Table A4.1. Genes associated with fungal life style

| Pathogen | Gene/ product | Function | GenBank ID | <i>Dothistroma septosporum</i> protein ID | References | % aa ^a ID | E-value | Remarks |
|--|------------------|---|-------------------|--|------------------------------|--|-----------|------------------------------|
| <i>Phytophthora infestans</i> | <i>SNE1</i> | Suppresses host PCD at early biotrophic phase | DQ888318 | Not found | (Kelley et al., 2010) | - | - | Hemi- biotroph |
| | <i>PiNPPI.1</i> | Induces host cell death at necrotrophic phase | AY961431 | Not found | (Kanneganti et al., 2006) | - | - | |
| <i>P. sojae</i> | PsojNIP | Induces host cell death at necrotrophic phase | AY961431 | Not found | (Qutob et al., 2002) | - | - | |
| <i>Mycosphaerella graminicola</i> | <i>MgXYL1</i> | Co-related with necrosis during biotrophy to necrotrophy transition phase | <i>Mg</i> 60105 | <i>Ds</i> 137959 | (Goodwin et al., 2011) | 62 | 2.15E-76 | Hemi- biotroph |
| <i>Mycosphaerella. fijiensis</i> | <i>MfGas1</i> | Pathogenicity factor (slightly increased at early necrotrophic phase) | <i>Mf</i> 87294 | <i>Ds</i> 74988 | (Kantun-Moreno et al., 2013) | 74 | 0E0 | Hemi- biotroph |
| | <i>MfGas2</i> | Pathogenicity factor (expressed high at early necrotrophic phase and gradually decreases) | <i>Mf</i> 38594 | <i>Ds</i> 69223 | (Kantun-Moreno et al., 2013) | 32 | 4.25E-65 | |
| <i>Magnaporthe oryzae</i> | <i>BAS1</i> | Biotrophy associated secreted protein | MGG_04795.6 | Not found | (Mosquera et al., 2009) | - | - | Hemi- biotroph |
| | <i>BAS2</i> | Biotrophy associated secreted protein | MGG_09693.6 | Not found | (Mosquera et al., 2009) | - | - | |
| | <i>BAS3</i> | Biotrophy associated secreted protein | MGG_11610.6 | Not found | (Mosquera et al., 2009) | - | - | |
| | <i>BAS4</i> | Biotrophy associated secreted protein | MGG_10914.6 | Not found | (Mosquera et al., 2009) | - | - | |
| | <i>AvrPiz-t</i> | Suppresses BAX-induced cell death | AEX97148 | Not found | (Li et al., 2009) | - | - | |
| <i>Colletotrichum lindemuthianum</i> | <i>CLTA1</i> | Reprograms metabolism during transition from biotrophy to necrotrophy | AF190427.1 | <i>Ds</i> 68895 | (Dufresne et al., 2000) | 35 | 5.61E-116 | Hemi- biotroph |
| | <i>CLNR1</i> | Major nitrogen regulatory protein that regulates biotrophy necrotrophy switching genes | AY168017 | <i>Ds</i> 41021 | (Pellier et al., 2003) | 41 | 1.81E103 | |
| <i>Colletotrichum truncatum</i> | <i>CtNUDIX</i> | Help to switch from biotrophy to necrotrophy | HO663661 | Not found | (Bhadoria et al., 2013) | - | - | |
| <i>Rhynchosporium secalis</i> | <i>RsecNip1</i> | Virulence factor/toxin or elicitor | AY507845 | Not found | (Schurch et al., 2004) | - | - | Necro troph |
| Ascomycetes and Basidiomycetes | Invertase | Positively correlated with biotrophy | CAZy ID 242231 | <i>Ds</i> 75094 or <i>Ds</i> 66287 | (Parrent et al., 2009) | Not aligned with any specific fungi | | Biotroph and endophyte |

aa = amino acid

Table A4.2. Summary results of *Dothistroma septosporum* biomass and dothistromin toxin quantification along with environmental parameters

| Expt. | Season | Day length ^a (hr) | Light energy ^b (W/m ²) | Considering highest value at stage 4 of dothistroma needle blight (DNB) ^c | | | |
|-------|--------|---------------------------------|--|--|---------------------------|---------------------------------------|--------------------------------|
| | | | | <i>Ds</i> biomass/ needle (ng/mg) | D toxin/needle (ng/mg) | D toxin/ <i>Ds</i> biomass (ng/ng) | DNB symptomatic needles (%) |
| 1 | Winter | 9.24 | 50.1 (1.2- 78.6) | 1.000 | 7.314 | 0.3036±0.0209 | 100 |
| 2 | Autumn | 12.28 | 58.9 (0.8-125.0) | 0.157±0.14 | 1.502± 0.634 | 0.0778±0.0445 | 92± 9 |
| 3 | Summer | 14.74 | 213.0 (5.8-267.5) | 3.269±5.37 | 43.745±11.452 | 4.4991±7.1136 | 30± 5 |
| 4 | Autumn | 12.28 | 58.9 (0.8-125.0) | 0.054±0.01 | 0.007± 0.003 ^d | 0.0002±0.0002 ^d | 41±27 |
| 5 | Autumn | 12.74 | 58.9 (0.8-125.0) | 0.015±0.01 | 0.418± 0.078 | 0.0184±0.0043 | 25± 5 |

^aCalculated from 'www.timeanddate.com'; mean values are presented

^bMean and range (in parenthesis) of received light energy are presented

^cMean ± standard deviation value were presented from 3 replicated trial except in experiment 1 where it was a duplicated experiment

^dSample was kept in storage for 7 months (at -80°C) and degradation of dothistromin probably occurred.

Ds = *Dothistroma septosporum*; D toxin = Dothistromin toxin

Table A4.3. Interim transcriptome read counts for the time-course of dothistroma needle blight

| | | Non-symptomatic | Early lesion | Late lesion | Fungal mycelium |
|---------------------|-------------|-----------------|--------------|-------------|-----------------|
| Mapped fungal reads | Replicate 1 | 47,361 | 585,826 | 1,410,868 | 32,112,201 |
| | Replicate 2 | 220,278 | 285,842 | 472,785 | 28,420,997 |

The sequence reads were from a mixture of plant and fungal reads. There was a problem with ribosomal RNA contamination in some samples, which meant the read numbers were low and there are large differences between the replicates. More sequencing will be done for the samples with the lowest read counts.

Appendix 5: Role of dothistromin

Table A5.1. Spore germination, surface growth and penetration of dothistromin-deficient mutant ($\Delta PksA:gfp$) and wild type (WT) *Dothistroma septosporum*

| <i>D. septosporum</i> | Spore germination (%) | | Surface growth ^c (%) (weeks after inoculation) | | | | Fungal penetration ^d (%) (weeks after inoculation) | | |
|-----------------------|------------------------------|-------------------------------|--|-----------|----------|---------|--|----------|----------|
| | <i>In vitro</i> ^a | <i>In planta</i> ^b | Week 2 | Week 4 | Week 6 | Week 8 | Week 2 | Week 4 | Week 6 |
| $\Delta PksA:gfp$ | 72 a ± 2 | 79 a ± 3 | 26 a ± 6 | 48 a ± 14 | 7 a ± 5 | 6 a ± 3 | 0 a ± 0 | 5 b ± 2 | 12 b ± 3 |
| WT | 76 a ± 3 | 82 a ± 2 | 13 b ± 3 | 30 b ± 6 | 11 a ± 5 | 6 a ± 2 | 0 a ± 0 | 14 a ± 1 | 19 a ± 2 |

^aat 4 day after inoculation, ^bat week 2 after inoculation, ^cpercentage of needle surface covered by fungal hyphae,

^dpercentage of stomata where fungal penetration was seen.

Figures in a column followed by a small letter differ significantly between dothistromin-deficient mutant and WT values ($P < 0.05$), values are mean and standard deviation from three biological replicates per treatment

Table A5.2. Spore germination, fungal surface growth and penetration of dothistromin-deficient mutant ($\Delta HexA$) and wild type (WT) *Dothistroma septosporum*

| <i>D. septosporum</i> | Spore germination (%) | | Surface growth ^c (%) (weeks after inoculation) | | | | Fungal penetration ^d (%) (weeks after inoculation) | | |
|-----------------------|------------------------------|-------------------------------|--|--------|--------|--------|--|--------|--------|
| | <i>In vitro</i> ^a | <i>In planta</i> ^b | Week 2 | Week 4 | Week 6 | Week 8 | Week 2 | Week 4 | Week 6 |
| $\Delta HexA$ | 79 ± 3 | 81 ± 1 | 5 ± 0 | 18 ± 4 | 18 ± 4 | 8 ± 4 | 0 ± 0 | 11 ± 1 | 13 ± 1 |
| WT | 73 ± 3 | 80 ± 3 | 7 ± 3 | 20 ± 0 | 13 ± 4 | 8 ± 4 | 0 ± 0 | 12 ± 0 | 14 ± 2 |

^aat 4 day after inoculation, ^bat week 2 after inoculation, ^cpercentage of needle surface covered by fungal hyphae

^dpercentage of stomata where fungal penetration was seen.

No significant differences were found between dothistromin-deficient mutant $\Delta HexA$ and WT values ($P > 0.05$), values are mean and standard deviation from three biological replicates per treatment

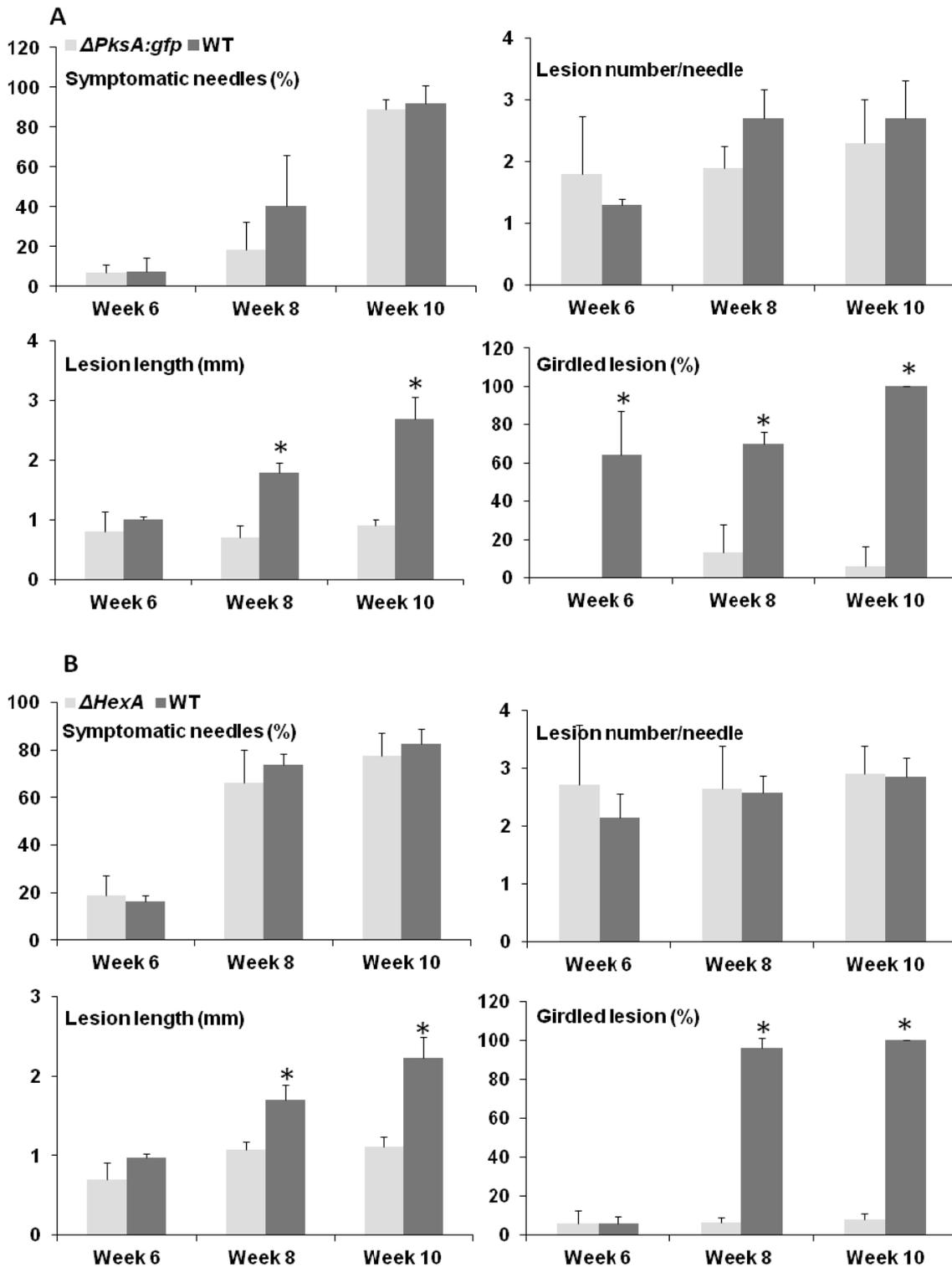


Fig. A5.1. Comparison of dothistroma needle blight (DNB) events caused by dothistromin-deficient mutants and wild type (WT) *Dothistroma septosporum*

(A) and (B) are the experiments carried out with $\Delta PksA:gfp$ and $\Delta HexA$ mutants respectively. In each experiment DNB events were compared with WT infected needles at weeks 6, 8 and 10. ‘*’ indicates the significant difference between dothistromin-deficient mutant and WT at $P < 0.05$. Values are mean and standard deviation from four biological replicates per treatment.

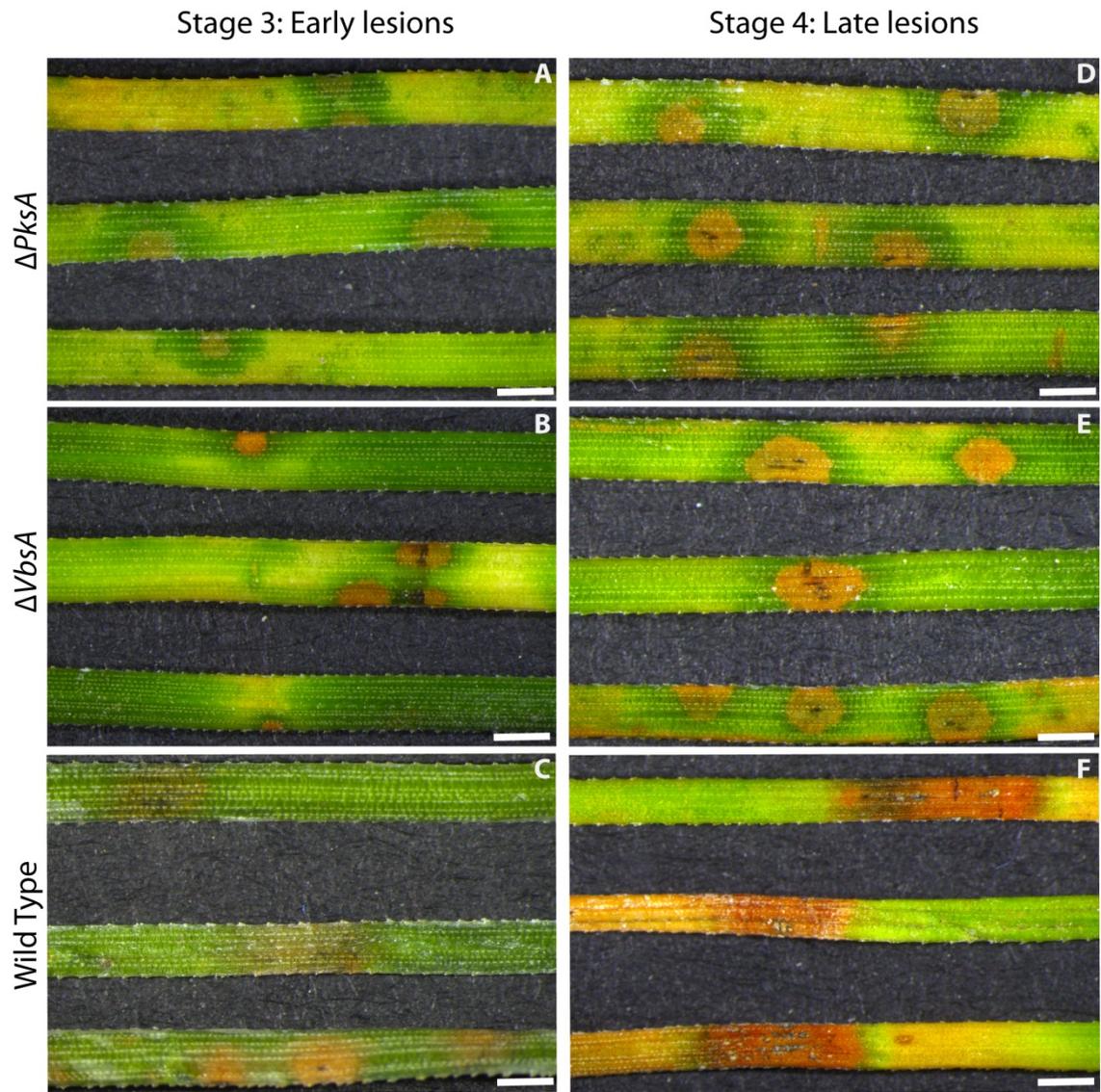


Fig. A5.2. Comparison of dothistroma needle blight needle (DNB) lesions between dothistromin-deficient mutant and wild type (WT) *Dothistroma septosporum* at stages 3 and 4
 (A – B) Early DNB symptoms showing small and un-girdle necrotic lesion in both dothistromin $\Delta PksA$ and $\Delta VbsA$ infected needles with green island around the lesions; (C) WT infected needles showing small early stage lesions similar to those with the dothistromin-deficient mutants; (D – E) $\Delta PksA$ and $\Delta VbsA$ late lesions are slightly longer than the early lesions in (A) and (B) but the green island are still evident; (F) WT lesions are longer than in C and girdle the whole needle. Size bar = 1 mm.

Table A5.3. Comparison of fruiting body and spores per lesion between dothistromin-deficient mutants and wild type *Dothistroma septosporum* infected needle lesions

| <i>D. septosporum</i> | No fruiting bodies/lesion | Spores/ lesion |
|-----------------------|---------------------------|-------------------|
| $\Delta PksA:gfp$ | 9 b \pm 6 | 337 b \pm 140 |
| WT | 23 a \pm 9 | 2123 a \pm 760 |
| $\Delta HexA$ | 4 b \pm 1 | 254 b \pm 73 |
| WT | 17 a \pm 1 | 5777 a \pm 1105 |

Figures in a column followed by a small letter differ significantly ($P < 0.05$), values are mean and standard deviation from three biological replicates per treatment. The $\Delta PksA:gfp$ and $\Delta HexA$ results are from two different experiments, each with WT control.

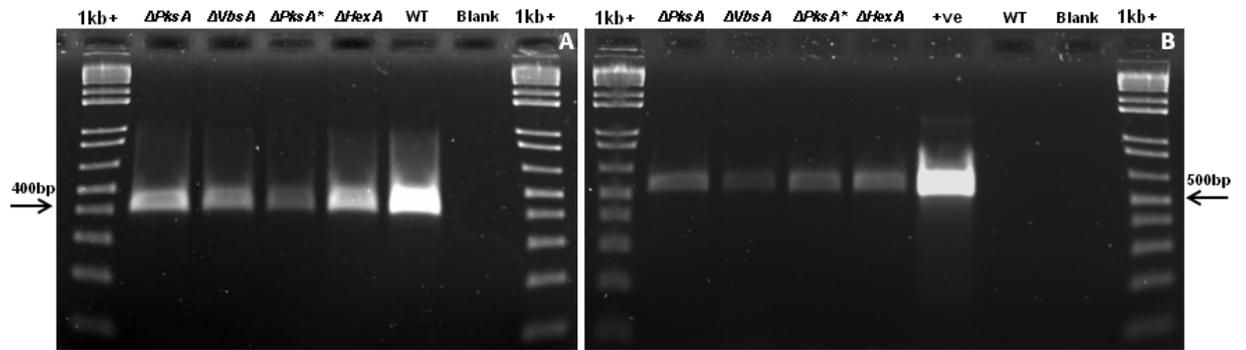


Fig. A5.3. PCR diagnostics of DNB lesions caused by dothistromin-deficient *Dothistroma septosporum*
 (A) All tested DNB lesions by dothistromin-deficient mutants ($\Delta PksA$, $\Delta VbsA$, $\Delta PksA:gfp$, $\Delta HexA$) showed same sized fragment as wild type (positive control, NZE10) with dothistroma-specific primers which confirmed that DNB lesions contained *D. septosporum*; (B) Amplicons of mutants showed the same sized fragment as positive control ($\Delta AfIR$) using dothistromin-deficient mutant specific primers confirmed that these lesions were from dothistromin-deficient mutant constructs. ' $\Delta PksA^*$ ' indicates $\Delta PksA:gfp$

Identification of dothistromin-deficient mutants ($\Delta PksA$, $\Delta VbsA$, $\Delta PksA:gfp$, $\Delta HexA$, where *PksA*, *VbsA* or *HexA* genes were knocked out) forming lesions in inoculated seedlings was confirmed by extracting DNA from lesions followed by PCR with dothistroma-specific ITS primers (Fig. A5.3A) and primers specific to dothistromin mutant strains (Fig. A5.3B).

Appendix 6: Publication and conference presentation

Full scientific papers:

Published:

1. **M. S. Kabir**, R. J. Ganley and R. E. Bradshaw. 2014. Dothistromin toxin is a virulence factor in dothistroma needle blight of pines. *Plant Pathology*. Doi 10.1111/ppa.12229.
2. **M. S. Kabir**, R. J. Ganley and R. E. Bradshaw. 2013. An improved artificial pathogenicity assay for dothistroma needle blight on *Pinus radiata*. *Australasian Plant Pathology* 42(4): 503-510.
3. de Wit, P.J.G.M., van der Burgt, A., Okmen, B., Stergiopoulos, I., Abd-elsalam, K.A., Aerts, A.L., Bahkali, A.H., Beenen, H., Chettri, P., Cox, M.P., Datema, E., de Vries, R.P., Dhillon, B., Ganley, A.R., Griffiths, S.A., Guo, Y., Hamelin, R.C., Henrissat, B., **Kabir, M.S.**, Jashni, M.K., Kema, G., Klaubauf, S., Lapidus, A., Lévasseur, A., Lindquist, E., Mehrabi, R., Ohm, R.A., Owen, T.J., Salamov, A., Schwelm, A., Schijlen, E., Sun, H., van den Burg, H.A., van Ham, RCHJ., Zhang, S., Goodwin, S.B., Grigoriev, I.V., Collemare, J. and Bradshaw, R.E. 2012. The genomes of the fungal plant pathogens *Cladosporium fulvum* and *Dothistroma septosporum* reveal adaptation to different hosts and lifestyles but also signatures of common ancestry, *PLoS Genetics* 8(11): e1003088.

News Letter/Seminar/Workshop/Symposium proceedings:

1. **M. S. Kabir**, R. Ganley and R. Bradshaw. 2013. An improved artificial pathogenicity assay for dothistroma needle blight on *Pinus radiata*. Forest health news. Scion on-line news letter, News letter no 234.
2. **M. S. Kabir**, R. Ganley and R. Bradshaw. 2013. Dothistroma needle blight pathogenicity, life style of *Dothistroma septosporum* and role of dothistromin during dothistroma needle blight on *Pinus radiata*. Paper presented at the IUFRO (Foliage, shoot and stem diseases) meeting held on 20-25 May, 2013 in Czech Republic.
3. **M. S. Kabir**, R. Ganley and R. Bradshaw. 2012. Role of dothistromin during dothistroma needle blight infection on *Pinus radiata*. Paper presented in New Zealand Microbiological Society (NZMS) Conference, held on 26-29 November 2012, at University of Otago, Dunedin, New Zealand.
4. **M. S. Kabir** and R. E. Bradshaw. 2011. *In planta gfp* labelled dothistromin gene expression. Poster presented in New Zealand Microbiological Society (NZMS) Conference, held on 23-25 November 2011, at Convention Centre, Palmerston North, New Zealand.
5. **M. S. Kabir** and R. E. Bradshaw. 2010. Production of *Dothistroma septosporum* spore *in-vitro* and its effectiveness *in planta*. Poster presented in on fungal and yeast cell biology symposium held on 29-30 November 2010, at Massey University, Albany, Auckland, New Zealand.
6. **M. S. Kabir** and R. E. Bradshaw. 2010. Investigation of needle blight disease development on pine. Paper presented on IMBS student symposium held on 8 December, 2010 at Massey University, Palmerston North, New Zealand.

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