Investigations of dothistromin gene expression in *Dothistroma septosporum* and the putative role of dothistromin toxin.

A thesis presented in the partial fulfilment of the requirements

for the degree of

Doctor of Philosophy (PhD)

in

Molecular Biology

at

Massey University, Palmerston North, New Zealand.

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2007

Abstract

Dothistroma septosporum causes pine needle blight, a foliar disease currently causing epidemics in the Northern hemisphere. *D. septosporum* synthesizes dothistromin, a mycotoxin similar in structure to the aflatoxin (AF) precursor versicolorin B. Orthologs of AF genes, required for the biosynthesis of dothistromin, have been identified along with others that are speculated to be involved in the same pathway. The dothistromin genes are located on a mini-chromosome in *Dothistroma septosporum* but, in contrast to AF genes, not in a continuous cluster.

The aim of this study was to increase knowledge of the biological role of dothistromin, which was previously a suspected pathogenicity factor. To identify putative roles of dothistromin, the dothistromin gene expression was investigated and green fluorescence protein (GFP) reporter gene strains of *D. septosporum* were developed.

Expression analyses of dothistromin genes revealed co-regulation. More surprisingly, dothistromin is produced at an early stage of growth and gene expression is highest during exponential growth. This is fundamentally different to the late exponential/stationary phase expression usually seen with secondary metabolites such as AF. Strains with a dothistromin gene (*dotA*) promoter-regulated GFP confirmed early expression of the toxin genes, even in spores and germtubes. Parallel studies with transformants containing a GFP-DotA fusion protein suggest spatial organization of dothistromin biosynthesis in intracellular vesicles. The early expression of dothistromin genes led to the hypotheses that dothistromin is either required in the early stage of the plant/fungi interaction, or for inhibiting the growth of competing fungi.

Constitutive GFP strains helped to determine that dothistromin is not a pathogenicity factor. However, a putative role of dothistromin in competition with other fungi, including pine-colonizing species, was detected, supporting the second hypothesis. It was shown that dothistromin-producing strains appear to have a competitive advantage which is lacking in dothistromin-deficient strains. However, some competitors were not affected and have potential as biocontrol agents.

In summary, this work has led to the discovery of an unusual pattern of regulation of a secondary metabolite, has made substantial progress in identifying the biological role of dothistromin, and has indicated potential for biocontrol of Dothistroma needle blight.

Acknowledgments

First and foremost, I would like to thank my supervisor Rosie Bradshaw. Thank you for your initial trust in giving me the opportunity to do my PhD in New Zealand. Further more I am deeply thankful for your time, efforts, encouragements and patience during my PhD. Thank you for giving me opportunities to present my work at conferences (not only because it meant that I could travel a bit). I couldn't have asked for a better supervisor.

I would like to thank my co-supervisors Peter Long and Margaret Dick for sharing their knowledge and ideas in the plant-pathogenic aspects of this study and providing fungal strains and pine seedlings when available.

I would like to thank former and current members of the Fungaljungle lab; Olivia and Phillip for the warm welcome they gave me when I first arrived, which made it easy for me to settle in; Naydene for useful discussions and technical support, but also for keeping the English in the lab, correcting mine, being fun to have around, and sharing cigarettes (were you trying to kill me?); Shuguang for helpful discussions and even more helpful technical advise; and Hong-Ping Jin for her help and support in the never-ending story of 'maybe getting transformants'. I would also like to thank Justine Baker for carrying out main parts of the competition trials.

Further thanks go to Aiko Tanaka for providing the GFP plasmids, and Sanjay Saikia for useful help in the HPLC assay, for the Indian football shirt, and sharing his PayTV to watch the last European Cup at 5 am. I am also very grateful to Trevor Loo for his HPLC advice, Caroline Young for help and advise in the Northern and Southern experiments, and Brendon Monahan for advice in the RT-PCR. Thanks to Corey Laverty, Max Scott, and Anja Schiemann who logged me on and provided time for me to use the UV microscope. Thank you to Kathryn Stowell for giving me the opportunity to work with the light cycler at any time and to Lili Griffiths for her help and support with light cycler issues (although she didn't by me a bach on the beach). I am very grateful to Jerry Hyams and Isabel Jourdain for making time for me to use their microscope and for advice on

how to use it too. For his advice and support on my work during my PhD, I would also like to thank Barry Scott.

For non-scientific but not less important support, friendship, and company, I would like to thank and hug Dan, Simone, Mirle, Phillip, Andrea, Kelly, Jana, Dorit, Claudia, Rich, Jean-Luc, Jen, Anna, The Massey Handball Club (especially Barbara and Alex for founding it; that was/is fun), and Lutz, for selling his car to come and visit me in Aotearoa (unfortunately my car was never worth a plane ticket).

For putting up with my veganism and making my time enjoyable, I would like to thank my flatmates: Haider, Mofa, Carolina, Amelie, and Mana. I would like to especially thank Yvan and Dorothee for their friendship and Peter, Karine and David for giving me a home when I needed one.

I would also like to thank my family, who, despite the great physical distance, gave me a lot of support. I could not have it done without you.

Finally, I am very grateful to the New Zealand National Centre for Advanced Bio-Protection Technologies CoRE for the scholarship funding that I received throughout my PhD.

Cheers!

Table of contents

Paper:

Schwelm A, Barron NJ, Zhang S and Bradshaw RE, 2007. Early expression of aflatoxin-like dothistromin genes in the forest pathogen *Dothistroma septosporum*. *Mycological Research.* **in press**.

List of Tables:

List of Figures:

Chapter 1: Introduction

1.1 Dothistroma needle blight

Dothistroma needle blight is one of the most serious diseases of pine trees worldwide. Over the last decade incidences of the disease have become more frequent and severe, and this trend has been connected to global climate change. This section gives an overview of the causal fungi, infection conditions and epidemiology regarding Dothistroma needle blight.

1.1.1 *Dothistroma* species

The causal agents of Dothistroma needle blight are two closely related Ascomycetes in the order Dothideales, *Dothistroma septosporum* and *D. pini*. Like many fungi, *Dothistroma* spp. have a tortuous taxonomic history littered with synonyms of both teleomorphic and anamorphic names (Evans 1984; Roux 1984; Sutton 1980). The anamorphic form was first known as *Cytosporina septospora* Dorog. and independently as *D. pini* (Hulbary 1941). Morelet (1968) realized these fungi were the same and made a new combination *D. septospora* (Dorog.) Morelet. The anamorphic state has subsequently been divided into varieties based on the differences in length of conidiospores (reviewed in Bradshaw 2004). However, recent DNA analyses do not support the division of Dothistroma into different varieties based on morphological features. Instead the *Dothistroma* varieties are now divided into two separate species, *D. septosporum* and *D. pini* (Barnes *et al.* 2004). *Dothistroma septosporum* has a worldwide distribution and is the causal agent of disease in exotic pine plantations in the Southern hemisphere. In contrast, the *D. pini* distribution appeared to be restricted to the North Central United States (Barnes et al. 2004), but it has recently been isolated in the Ukraine (Groenewald *et al.* 2007).

The teleomorphic form of *D. septosporum* was first described as *Scirrhia pini* Funk and Parker, then subsequently renamed *Mycosphaerella pini* E. Rostrup apud Monk, although both names are still used (Bradshaw 2004). The sexual form of *D. pini* has so far not been

identified, although a recent study showed that both *D. pini* and *D. septosporum* have two different mating types (Groenewald *et al.* 2007). There are no reports of the sexual form of *D. septosporum* in the Southern hemisphere, and, with the exception of South Africa, only one of the mating types has been isolated there (Groenewald *et al.* 2007).

The origin of *D. septosporum* and *D. pini* is not known (Bradshaw 2004). The majority of isolates subject to research are classified as *D. septosporum* (Bradshaw & Zhang 2006b), and this species is the subject of this study.

1.1.2 The disease: infection process, symptoms and environmental conditions

Dothistroma needle blight is often characterized by distinct brick-red bands (1–3 mm wide) around the needle. These can appear within weeks of infection and sometimes still be seen after the needle has died, therefore the alternative term "red-band disease" is also used. The red colour of the band is due to the fungus producing the mycotoxin dothistromin (Shain & Franich 1981). The early symptoms including yellow bands and tan spots on the live needles. These signs are usually short-lived and the bands rapidly turn red. At later stages small black fruiting bodies form within the red bands on the needle (Figure 1.1A). Adjacent to the red band are areas of yellow necrotic tissue and flanking this region are sometimes areas of dark green tissue containing highly lignified cells (Franich *et al.* 1986). The end of the needle beyond the band dies and eventually the whole needle may develop extensive necrosis (browning) and drop prematurely, leaving a brush-like branch (Figure 1.1B).

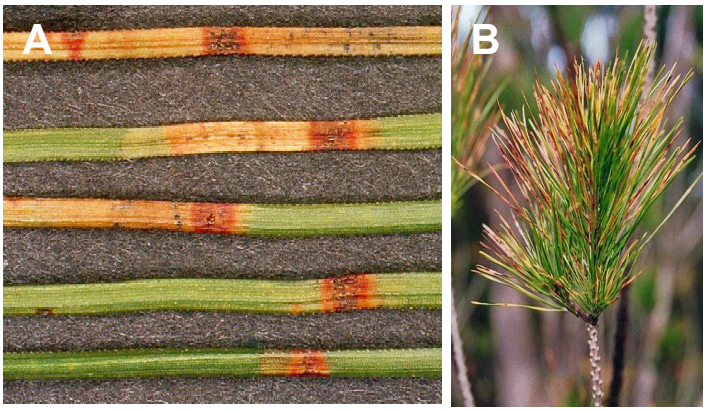

Figure 1.1: Dothistroma infection symptoms. (A) Infected needles often show characteristic red bands, caused by the mycotoxin dothistromin, in which black fruiting bodies develop. Picture from MacLaren (1993) (B) Infected needles drop off prematurely leaving brush like structures at the end of branches. Picture by R.E. Bradshaw.

The main source of inoculum is the asexual conidiospores from the black fruiting bodies (stromata). The first conidiospores are generally released in spring from attached needles infected in the previous year. Conidiospore production can continue in infected stands for up to 7 months of the year, with the main infection period from spring to summer (Gilmour 1981). However, spore production is dependent on sufficient temperature and leaf wetness. The resilience of conidiospores to temperature extremes means they can remain viable for months until they encounter favorable environmental conditions allowing germination (Gibson 1972). Once established, the fungus forms an appressorium-like structure over the stomatal cavity, with an infection peg penetrating the stomata (Franich *et al.* 1983; Peterson & Walla 1978). The stomata of young needles are open pores composed of guard cells that are covered in a microtubular wax that appears to signal appressorium formation. This is in contrast to mature needles where the stomatal opening is occluded with a resinous material that may present a mechanical barrier (Franich *et al.* 1983). Stomatal penetration can occur within two days of germination, with the infection peg branching within the pine needle sub-stomatal chamber. From this, hyphae branch into intra- and intercellular regions of the mesophyll tissue.

Hyphal growth is restricted to within the necrotic tissue, but the extension of necrosis beyond the region directly infected with the fungus suggests that host cells are killed by a toxin or by the host defense response. The host cells collapse after 32–114 days (depending on environmental conditions) and needle symptoms and stromata appear (Ivory 1972; Peterson 1973). The stromata generally mature and produce conidia the year after infection (Butin & Kowalski 1989; Peterson 1982), but in some regions it can take an additional year for the fungus to complete its life cycle (Peterson 1982; Taylor & Schwandt 1998). In diseased needles, the asexual fruiting bodies are the most visible part of the fungus, reaching diameters of 300–600 μm. Initially white and subepidermal, they become brown-black, acervular and erumpent as they develop. Eventually the host epidermis and cuticle are broken, leaving torn flaps around the fruiting bodies. The spores are passively transported in water droplets on to fresh needles where they germinate and infect current year or 1-year-old needles. Dispersal of conidiospores from infected foliage is generally restricted to short distances through water droplets (Ivory 1972). Research conducted in Kenya however showed that conidia were taken up into clouds from infected forests at high altitude, which could lead to dispersal of conidiospores over longer distances (Gibson 1972).

There are conflicting reports about whether the direction of germ tube growth is random or targeted towards stomata. In artificial laboratory conditions germ tube growth appears to be random and in some cases penetration occurs directly through the cuticle (reviewed in Bradshaw 2004; Muir & Cobb 2005). In outdoor conditions Muir and Cobb (2005) observed conidial germ tube growth directed towards and entering the nearest epistomatal opening on *Pinus radiata* needles (Figure1.2), but this was not found on bishop pine plantation trees. The authors suspect that penetration and infection by *M. pini* is highly variable, depending on tree species and age and the environment. Therefore experimental studies on factors affecting penetration and infection by foliage pathogens must be substantiated by analyses of pathogen behavior under outdoor environments of forests or plantations. Relying exclusively on results obtained under "controlled" conditions could result in misleading or incorrect epidemiological concepts. Current knowledge of infection and epidemiology might be based on artefactual or abnormal infection behavior as a result of experimentation conducted in a controlled environment (Muir & Cobb 2005).

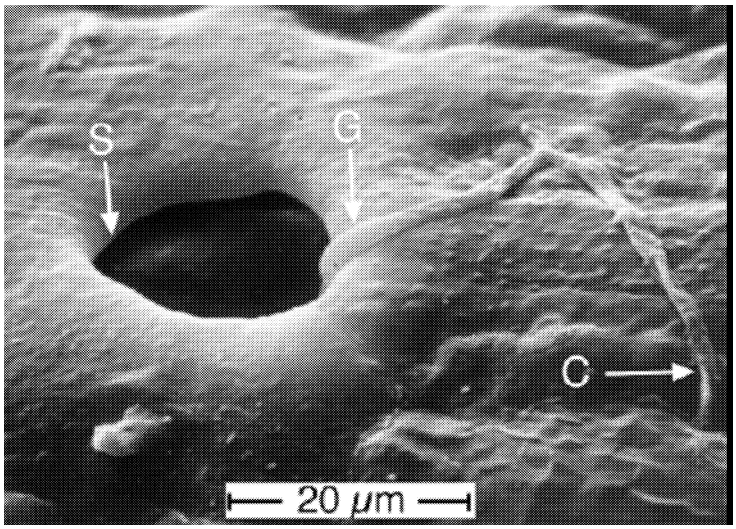

Figure 1.2: Stomatal invasion by *M.pini.* Needle of *P. radiata* plantation tree with *M. pini* germ tube (G) that grew directly from conidium (C) and entered an epistomatal opening (S) (scanning electron micrograph taken from Muir & Cobb (2005)).

In the 1970-80's a large amount of research was conducted on the environmental conditions required for Dothistroma infection to occur, using laboratory based pathogen assays (Franich et al. 1986; Gadgil 1967; Gadgil 1974; Gadgil & Holden 1976; Ivory 1972; Parker 1972; Shain & Franich 1981). Results were inconsistent with respect to the optimal light intensity,

duration of needle wetness and temperature, which enabled germination of conidia and subsequent infection of needle tissue. However, it is clear that a high moisture level is favourable for the disease (Bradshaw 2004; Gadgil 1974; Gadgil 1977; Muir & Cobb 2005). In addition, a recent study showed a correlation between increase of dothistroma disease and increased precipitation and temperatures above 16°C in British Columbia (Woods *et al.* 2005). Therefore it appears that in both an artificial and natural environment, temperature and moisture are two key variables required for successful infection and disease development.

1.1.3 Distribution and impact of Dothistroma needle blight

The fungus *D. septosporum* is able to infect both native and exotic pine species throughout the world. The infection results in premature defoliation and incremental wood loss in proportion to disease severity, and in extreme cases infection leads to the death of the tree. Although pine species appear to be the favorable host for *D. septosporum*, infections of *Larix decidua* (Basset 1969), *Douglas fir* (Gadgil 1968), and *Picea abies* (Lang 1987) have been observed.

While Dothistroma needle blight has been a major problem in exotic pine plantations in the Southern hemisphere for decades (Bassett 1972; Cobb *et al.* 1969; Edwards & Walker 1976; Gibson 1974) there has been a dramatic increase in disease incidence in the Northern hemisphere since the 1990s (Figure 1.3). In the Northern hemisphere the fungus is now colonizing pines in their native environment as well as in plantations (Woods *et al.* 2005). In Southern Germany, Dothistroma infection prior to 1990 was only observed in small local areas and mainly in parks and gardens (Pehl & Butin 1992). At present the disease is more widespread and has been found in the Alpine ecosystem infecting native *P. mugo* at altitudes between 1200 and 1600 m (Maschning & Pehl 1994). In Austria the disease has spread from local infections of *P. mugo* (known since the 1950s) to other native species such as *P. nigra*, *P. sylvestris*, *P. uncinata* and *P. cemba* (Kirsitis & Cech 2006). This disturbing trend has been observed in several Northern hemisphere countries and is summarized in Table 1.1.

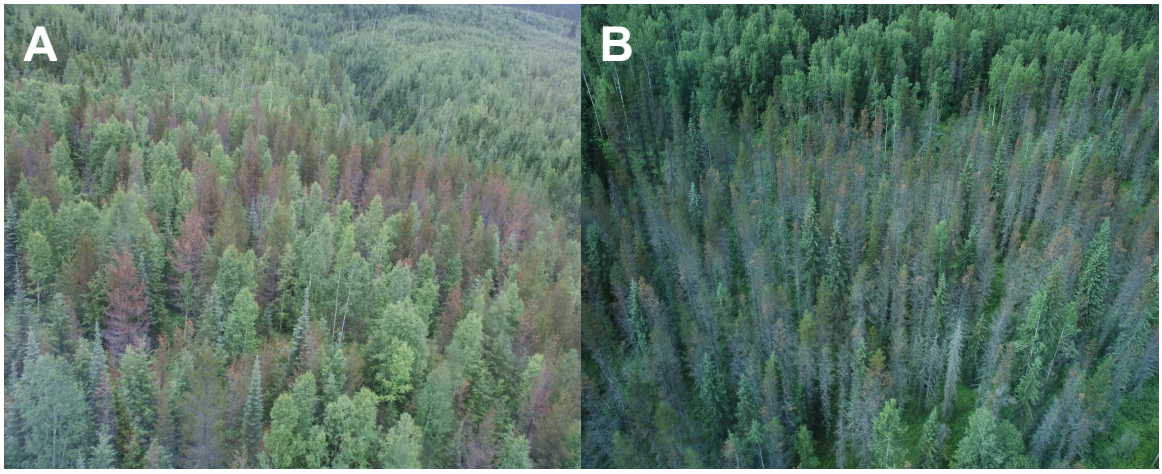

Figure 1.3: Damage caused by Dothistroma in British Columbia, Canada.
(A) Extensive Dothistroma damage (Bell Irving, BC) and (B) Mortality (Sediesh Creek, BC) in lodgepole pine (*Pinus contorta* var. *latifolia*). Pictures are courtesy of Alex Woods.

Consequently, Dothistroma needle blight is now classed as one of the most important diseases of pine, and is of major economic concern to the forest industry in countries such as New Zealand, Australia, Canada, Chile, Europe, Kenya, and the United States of America (Woods *et al.* 2005). *D. septosporum* is also classified as an organism which could be used as a biological weapon or in bioterrorism (Beck 2003). This classification is probably due to the possible ecological and economic consequences of a Dothistroma needle blight outbreak. Of greater concern is the increase of Dothistroma needle blight in the Northern hemisphere being correlated to global climate change, as shown for the recent epidemic in British Columbia, Canada (Woods *et al.* 2005). Different climatic conditions are also suspected to play a role in the spread of the disease in Austria (Kirsitis & Cech 2006). Therefore, global climate change may lead to even more frequent and severe epidemics of Dothistroma needle blight in the future.

Table 1.1: New outbreaks/increased incidence of Dothistroma needle blight recorded in the northern hemisphere since 1994. Table altered from Bradshaw (2004).

Country/ Region	*Pinus* species	Distribution notes	References
Poland	*P. nigra*	First report in Poland	Kowalski and Jankowiak (1998)
Germany	*P. mugo*	First outbreak in native ranges	Maschning & Pehl (1994)
Portugal	*P. pinaster* *P. pinea* *P. radiata*	First report of teleomorph	Fonseca (1998)
Hungary	*P. nigra*	Nationwide epidemics	Koltay (2001)
France	*P. nigra var. laricio*	Considerable foliage damage	Landmann (2000)
Slovakia	*Pinus nigra*	First report in 1996	Zubrick *et al*. (2006)
United Kingdom	*P. nigra var. laricio*	Increased incidence in England First report in Scotland	Evans & Webber (2003) Brown (2005)
Austria	*P. mugo* *P. nigra* *P. sylvestris* *P. uncinata* *P. cemba*	Increased incidence	Kirsitis & Cech (2006)
British Columbia, Canada	*P. contorta* var. *latifolia*	High incidence and mortality	Woods (2003, 2005)
Montana, USA	*P. flexilis* *P. albicaulis*	First report in native ranges	Taylor & Walla (1999)
Vermont, USA	*P. nigra* *P. mugo* *P. pomderosa*	First report in New England	Pfister *et al*. (2000)

In New Zealand, *D. septosporum* was first identified in *Pinus radiata* pine plantations in 1962, with positive confirmation in 1964 (Gibson 1972). The sexual form of the fungus, *Mycosphaerella pini*, has not yet been identified and it appears that there is only one mating type present in New Zealand (Groenewald *et al.* 2007). This supports the results of Hirst (1999) which suggested that the current asexual form found in NZ is clonally derived from a single isolate of *D. septosporum* introduced into this country. As a consequence, the Dothistroma resistant *P. radiata* developed for increased resistance to the New Zealand strain (Jayawickrama & Carson 2000) may not be resistant to other strains around the world.

In 2006 the value of New Zealand forestry export products was estimated as NZ$ 3.1 billion (MAF 2006). The forest sector accounted for 11.2% of the New Zealand export market in 2005 (MAF 2007) and *Pinus radiata* plantations account for approximately 90% of the

commercial forest in New Zealand. This makes Dothistroma needle blight an important factor in the New Zealand economy, being responsible for an estimated economic loss of NZ$ 24 million per year (MAF 2003). With an increasing trend towards monocultures, and of genetically identical trees of selected genotype (clonal forestry), the introduction of potentially more virulent overseas strains of *D. septosporum* into the country should therefore be a major concern for the forest industry and the New Zealand economy. This is a particular concern since the distribution of fungal forest pathogens is facilitated by the worldwide increased movement of people, wood and wood products (Wingfield *et al.* 2001).

1.1.4 Control of Dothistroma needle blight

There are three main methods used to control Dothistroma needle blight in commercial pine forests in the Southern hemisphere. These control methods are: use of resistant pine seedlings, silvicultural practices such as pruning and thinning of infected branches, and aerial application of copper fungicide (Bradshaw 2004). A Dothistroma resistant cultivar of *P. radiata* was developed in 1983 and is available in New Zealand for planting in high incidence areas where needle blight is a problem (Jayawickrama & Carson 2000). The resistant cultivar has been estimated to reduce incidence of infection by 15% (Dick 1989). In addition, *P. radiata* becomes more resistant with age, usually around eight years in moderately diseased stands, or around 15 years in heavily diseased stands. However, older pine trees take longer to recover from severe infection, and unfortunately little is known about the mature tree resistance mechanism (Gibson 1972). The most prominent form of control is still the use of copper fungicides. As it has proven particularly difficult to establish a reliable pathogenicity test (Barron 2006; Devey *et al.* 2004; West 2004), exploring other control options, such as breeding of better Dothistroma resistant pine cultivars, is progressing slowly.

Fungicides were first used in Kenya (1964), when field trials showed that copper fungicides applied from the air were effective in controlling needle blight. In New Zealand, aerial application of copper oxychloride and cuprous oxide have been effective in controlling dothistroma since the early 1970's (Bradshaw 2004). Copper fungicide is taken up by *D. septosporum* conidia within 60-90 minutes of contact. It prevents germ-tube growth, whilst

also inhibiting the production of secondary conidia. The copper spray persists on needles for several months (Franich 1988) protecting existing foliage from new infection.

As *D. septosporum* is usually not spread over long distances, the easiest and probably most effective form of control would be planting non-susceptible hosts in certain areas in addition to avoiding dense planting (Engelbrecht 2006).

1.1 The dothistromin toxin

D. septosporum produces the toxin dothistromin which is visible in the red bands of infected needles. The toxin is similar in structure to a precursor of the potent carcinogen aflatoxin. It has therefore been subject to both plant pathology and non-plant related research.

1.1.1 The toxin

Dothistromin is a toxic metabolite produced by several plant pathogenic fungi. It has been isolated from *D. septosporum* (Bassett *et al.* 1970) and from several species of *Cercospora* (Assante *et al.* 1977; Bradshaw 2004; Stoessl & Stothers 1985).

D. septosporum produces and secretes the polyketide dothistromin in culture as well as *in planta*. The brick red bands frequently seen in infected needles are caused by the accumulation of dothistromin in the needle tissue (Shain & Franich 1981). The secretion of the toxin in culture facilitated purification and chemical characterization (Bassett *et al.* 1970). Purified extracts of "dothistromin" usually consist of dothistromin (80-90%) and the closely related deoxydothistromin (Gallagher & Hodges 1972). Both of them show close structural similarities to versicolorin B, an aflatoxin precursor, produced by *Aspergillus parasiticus* and *A. flavus*. This implied a possible similar biosynthetic pathway for dothistromin and aflatoxin. Subsequently a ^{13}C-NMR study of dothistromin biosynthesis by *D. septosporum* showed a labeling pattern consistent with a bistetrahydrofurano side chain, similar to those found in the biosynthesis of aflatoxin (AF) and sterigmatocystin (ST) (Shaw *et al.* 1978). Furthermore,

both dothistromin and versicolorin B share a saturated bifuran ring although the arrangement of the hydroxyl groups of the anthraquinone ring is different (Figure 1.4).

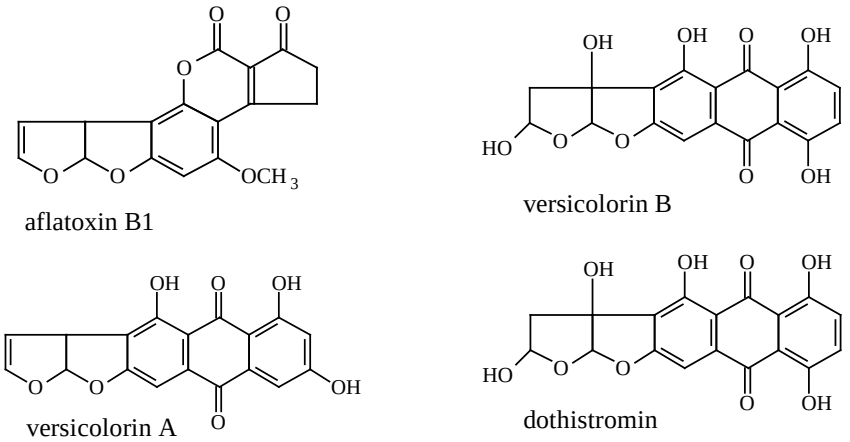

Figure 1.4: Structural similarity of dothistromin and aflatoxin precursors versicolorin A + B**.**

Based on the chemical evidence of the AF intermediates and dothistromin it was predicted that *D. septosporum* contains genes orthologous to some of those involved in aflatoxin and sterigmatocystin synthesis, which were indeed subsequently identified (see Section 1.4.2, Bradshaw & Zhang 2006b).

1.1.2 Occurrence and mode of action of dothistromin

As disease symptoms can be induced through injecting dothistromin into pine needles (Shain & Franich 1981) it is feasible that the dothistromin toxin is either required for pathogenicity (essential for the disease to occur) or virulence (contributes to the severity of the disease). Further early studies of the infection process revealed necrotic needle tissue damage occurring beyond the growth margin of the pathogen, suggesting host tissue death due to a diffusible toxin (Gadgil & Holden 1976). However, Kimura *et al*. (2001) mention, in a review, that those are only circumstantial observations and are far from being accepted as persuasive evidence for a causal role of toxins in pathogenesis. Indeed, in the case of the Dutch elm disease toxin cerato-ulmin, injection of the toxin reproduced disease symptoms on elm seedlings (Takai 1974) but toxin-deficient mutants of *Ophiostoma ulmi* were still as virulent as the wild type

(Bowden *et al.* 1996). In a different pathosystem, treatment of soybean tissue with the purified toxin cercosporin resulted in development of disease symptoms normally caused by the pathogen *C. kikuchii*. Cercosporin-deficient mutants of this fungus were non-pathogenic, confirming that cercosporin is a pathogenicity factor (Upchurch *et al.* 1991).

There are some indications that dothistromin might not be a pathogenicity factor. Although the red bands are a characteristic of Dothistroma needle blight, they are not always seen in *Dothistroma*-infected needles (A. Woods, M. Dick personal communication) and dothistromin content of needle lesions is not correlated with the length of the lesion (Unpublished Project Record 4225, New Zealand Forest Research, Bradshaw & Zhang 2006b). Indeed, 80-90% of the dothistromin is degraded in pine needles to oxalic acid and CO_2 within 24h (Franich *et al.* 1986). Furthermore, isolates of *D. septosporum* and *D. pini* from the same or different countries appear to vary greatly in their ability to produce dothistromin in culture (Bradshaw *et al.* 2000). Whether the production levels seen in culture are correlated with the toxin production *in planta*, or indeed virulence, is not known. In addition, a defence response of *P. radiata* cell suspension cultures can be induced in the absence of dothistromin when challenged with a *D. septosporum* cell wall fraction (Hotter 1997). Therefore it appears that the host response may contribute to the symptoms of the disease. Injection of dothistromin into pine needles is accompanied by a strong host response, such as the accumulation of benzoic acid in the needle. This accumulation occurs at high enough concentrations to inhibit *D. septosporum* growth, but is also toxic to the plant cells (Franich *et al.* 1986).

Plants often use the hypersensitive response as a protection mechanism but some necrotrophic or facultative saprophytic fungi use this defense system to their advantage. Fungal toxins can trigger the programmed cell death (PCD) of their host-plants (Howlett 2006; Markham & Hille 2001). Reactive oxygen species are involved in the signaling pathways of PCD and the ability of dothistromin to activate oxygen has been shown (Youngman & Elstner 1984). Dothistromin requires light and oxygen for its toxicity (Franich *et al.* 1986; Shain & Franich 1981). This might suggest that a photodynamic mechanism of O_2 activation is involved in the phytotoxic response. Dothistromin is also able to act as a reductant, reduced in NADPH-dependent reactions and forming H_2O_2 and $O_2^{\cdot-}$ upon autoxidation. It is feasible that dothistromin might be reductively activated *in vivo* via photosynthetic electron transport, which also would explain the requirement of light in the disease process (Heiser *et al.* 2002;

Youngman & Elstner 1984). However, Shain and Franich (1981) detected an ethylene response in needles injected with dothistromin both in light and dark conditions, albeit with a smaller response in darkness. This suggests the host is capable of a response regardless of light conditions.

Possible toxic effects for dothistromin on a cellular level have been seen. Using pine embryo and meristematic leaf callus it was determined that their growth was completely inhibited by 13 nmol dothistromin/g of tissue. Furthermore, an immunoassay confirmed the uptake of dothistromin by the pine embryos. Using a dothistromin-mouse albumin conjugate and dothistromin-specific antibodies, it was identified that dothistromin binds to a 40-kDa binding peptide in small vesicles in pine embryos (Jones *et al.* 1995). Whether this is the cellular target of dothistromin in the natural system is not known.

Further research has been conducted on dothistromin toxicity due to the structural similarities to aflatoxin. Dothistromin has been shown to be toxic to a broad range of cell types including plant, bacterial, animal and human cells (Elliott *et al.* 1989; Ferguson *et al.* 1986; Harvey *et al.* 1976; Skinnider *et al.* 1989; Stoessl *et al.* 1990). Aflatoxin B_1 is the most potent naturally occurring carcinogen known in addition to being teratogenic and toxic. There is also evidence for dothistromin being a weak mutagen and clastogen, which led to concerns about the risk of forestry workers exposed to dothistromin (reviewed in Bradshaw 2004).

1.3 Fungal secondary metabolites

Secondary metabolites are metabolites that are not essential for growth and reproductive metabolism, unlike intermediates and co-factors such as those that take part in cell-structure synthesis and energy transduction of the organism. Secondary metabolite production in fungi is a complex process coupled with morphological development (Calvo *et al.* 2002). The functions of secondary metabolites for the producing organism are often speculative or unknown but the metabolites are often of tremendous importance to humankind in that they display a broad range of useful antibiotic and pharmaceutical activities as well as less desirable toxic activities (Pelaez 2005; Seo & Yu 2005). Examples of important pharmaceuticals are penicillin, cyclosporin and statins, while aflatoxins and trichothecenes are

potent toxins. Mycotoxins secreted by fungi as they grow in various commodities have great implications for humankind (reviewed in Bennett & Klich 2003; Seo & Yu 2005).

Despite their enormous chemical complexity and diversity, all fungal secondary metabolites arise from a limited number of precursors obtained from primary metabolism. The main classes of fungal secondary metabolites are polyketides (e.g. aflatoxins, dothistromin), non-ribosomal peptides (e.g. penicillin, gliotoxin), alkaloids (e.g. lysergic acid diethylamide (LSD)) and terpenes (e.g. tricothecenes, gibberellin) (Keller *et al.* 2005). Examples are shown in Figure 1.5. The biosynthesis of secondary metabolites of filamentous fungi have been recently summarized by Hoffmeister and Keller (2007). A model which explains the evolution, and reasons for chemical diversity, of secondary metabolites has been described by Firn and Jones (2000; 2003).

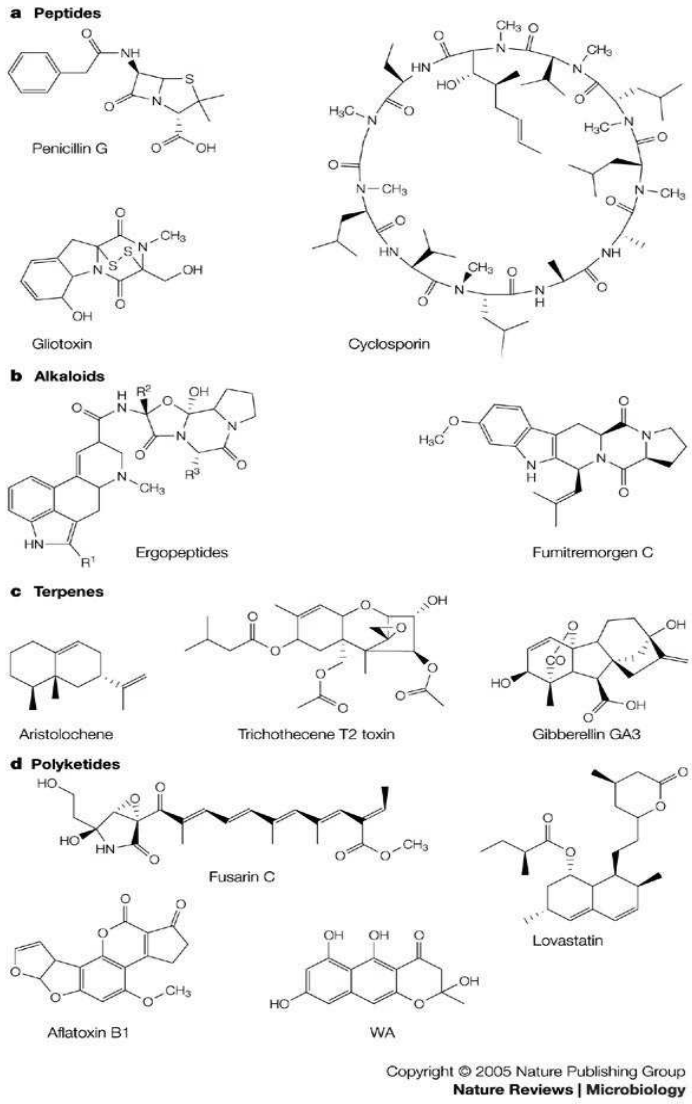

Figure 1.5: The main groups of fungal secondary metabolites.
Examples of secondary metabolites derived from (a) peptides, (b) alkaloids, (c) terpenes and (d) polyketides are shown. Figure taken from Keller *et al.* (2005).

In some cases it has been shown that secondary metabolites play a beneficial role for the fungi. The melanins, which give color to spores, appressoria, sclerotia, sexual bodies, and other developmental structures, can act as plant and animal virulence factors or can be required for general survival, presumably as UV protectants or ROS scavengers (Henson *et al.* 1999; Jacobson 2000). Grass endophytes as *Epichloë* and *Neotyphodium* produce a range of bioactive secondary metabolites, e.g. ergot alkaloids and indole diterpenes, which are known to have anti-insect and anti-mammalian properties. This way the protection of the host assures protection of the environment of the fungi. Interestingly, *Epichloë typhina* also produces the secondary metabolite chokol K, an antifungal volatile, which is also responsible for the attraction of the female *Botanophila* fly that transfers fungal gametes (Schiestl *et al.* 2006).

Some fungal phytotoxins are proven pathogenicity or virulence factors that cause significant disease on agricultural crops (reviewed in Thines *et al.* 2006; Upchurch *et al.* 1991). *Alternaria alternata* pathotypes produce different host-specific toxins and cause disease on different hosts. Three of these classes of toxin (ACT, AF-toxin and AK-toxin) are produced by a core set of three biosynthetic genes, which are located in multiple copies on a conditionally dispensable chromosome. Isolates that lack this dispensable chromosome are non-pathogenic on all hosts (Hatta *et al.* 2002). Conidia of *Cochliobolus carbonum* secrete a toxin (HC-toxin) during appressorium formation on maize leaves. On resistant plants the toxin gets inactivated and the fungus fails to the colonize the host (Weiergang *et al.* 2004). Trichothecenes were also shown to be involved in virulence of *Fusarium* spp. (reviewed in Kimura *et al.* 2001).

The biological role of AF/ST is less clear. Bu'Lock (1965) postulated that AF production was a mechanism for the organism to release excess carbon when the fungus is growing in a carbon-rich environment, but little evidence exists to support or defend this hypothesis and atoxigenic fungi can compete with toxigenic fungi on the same medium (Cotty 1989). A putative benefit for ST production has been shown in *A. nidulans*. ST appears to be important for sporulation, as more conidia accumulate with each progressive conversion of intermediates in the ST biosynthesis pathway (Wilkinson *et al.* 2004). Wether ST production does indeed provide a fitness advantage in nature is not known. AF deficient strains of *A. parasiticus* and *A. flavus* are successfully used as biocontrol agents to compete with toxigenic

strains, but they need to outnumber the toxigenic spores ten- to fifty-fold (Pitt & Hocking 2006). Narasaiah *et al*. (2006) showed that the production of AF in *A. parasiticus* is related to oxidative stress. Their results suggest that formation of AF and its precursors by *A. parasiticus* may occur as a compensatory response to reactive oxygen species accumulation.

1.4 Fungal gene clusters

Although the biological function of many secondary metabolites is still unknown, there is a considerable amount of knowledge about the organisation and regulation of secondary metabolite genes. One remarkable property of secondary metabolites produced by fungi is the frequent clustering of genes involved in their biosynthesis and regulation (for recent reviews see Bok *et al.* 2006; Hoffmeister & Keller 2007; Keller *et al.* 2005; Miller & Linz 2006; Yu & Keller 2005; Zhang *et al.* 2005). Furthermore, they also frequently appear to be located near telomeres (Keller *et al.* 2005). A gene cluster is considered to be the linkage of two or more genes that participate in a common metabolic or developmental pathway (Keller & Hohn 1997). While the clustering of genes was well known for prokaryotic organisms in the form of operons (where genes of related functions are under control of a single promoter and transcribed as a single mRNA), the clustering of fungal genes was not predicted as it had became a dogma that eukaryotic genes involved in functionally related pathways are not linked (Keller *et al.* 2005). However, since the 1990s gene clusters have been frequently identified in fungi. Secondary metabolite gene clusters often include regulatory genes in addition to genes involved in the biosynthesis of the secondary metabolites. Examples of secondary metabolite clusters in fungi include those for AK-toxin (Tanaka *et al.* 1999), ergot alkaloids (Haarmann *et al.* 2005), gibberellins (Tudzynski & Hölter 1998), fumonisin (Proctor *et al.* 2003), β-lactam antibiotics (Liras & Martin 2006), sterigmatocystin and aflatoxins (Brown *et al.* 1996; Keller & Hohn 1997), trichothecenes (Hohn *et al.*, 1993), aflatrem (Zhang *et al.* 2004), sirodesmin (Gardiner *et al.* 2004), gliotoxin (Gardiner & Howlett 2005), lolitrem (Young *et al.* 2005), cercosporin (Chen *et al.* 2007) and ergovaline (Fleetwood *et al.* 2007).

The evolution of fungal gene clusters remains unclear. Most fungal genes for other biosynthetic pathways follow the normal eukaryotic model of dispersal throughout the genome. There is probably some selective pressure that drives and maintains clustering of

secondary metabolite genes, although there are no known internal genomic mechanisms that promote or maintain clustering. In contrast, there are known mechanisms that act to disperse genes, e.g., translocation, inversion, and unequal crossing over. Several hypotheses have been put forward to rationalize clustering of fungal secondary metabolite genes (reviewed in Zhang *et al.* 2005). One is that clustering optimises co-regulation of the constituent genes either by *cis* regulatory elements or by having them all in a similar chromatin environment. In contrast, Walton (2000) argued that genes for "housekeeping" pathways in fungi and secondary metabolite pathways in other eukaryotes are dispersed and that chromatin context is not, in general, important for correct co-regulation. Instead he proposed a model of selfish-gene clusters, which might have been acquired by fungi through horizontal gene transfer (HGT). Because of the discontinuous distribution of secondary metabolite production in fungal taxa, HGT from other species might be the origin of some metabolite gene clusters in fungi (Rosewich & Kistler 2000; Walton 2000). The amino acid identity between some proteins required for the biosynthesis of *β*–lactams, the low G/C content and the lack of introns in *β*–lactam gene clusters in fungi supports the theory of HGT from bacteria to fungi (Liras & Martin 2006; Rosewich & Kistler 2000).

In the case of the AF/ST gene clusters there is no convincing evidence for HGT. The AF gene cluster rather appears to have originated from gene duplication. Arguments against HGT of AF/ST pathway genes include the presence of introns, a similar G/C content inside and outside the cluster (Brown *et al.* 1996) as well as the observation that the genes in the cluster quite closely follow the expected evolutionary lineage of other non-clustered, essential genes in the same species, isolated from widely divergent geographical locations (Ehrlich *et al.* 2003). In addition, many of the cluster genes have obvious similarities to eukaryotic primary metabolic activities (e.g., fatty acid synthases) and could have been obtained from a primary metabolism gene through duplication followed by mutations (Brown *et al.* 1996). The availability of a sexual stage, which facilitates recombination, in *A. nidulans* may account for the differences in organization of the ST and AF gene clusters (Bhatnagar *et al.* 2003).

Cary & Ehrlich (2006) outlined a model for the evolution of AF/ST gene clusters which suggests that blocks of biosynthetic genes were acquired sequentially. The ability to make AF, ST or their precursors is not confined to some *Aspergillus* species but is also found in others such as species of *Penicillium* and *Chaetomium* (Barnes *et al.* 1994; Cole & Cox

1981) or *D. septosporum* (Table 1.2, Bradshaw & Zhang 2006b). HGT is one possible explanation for the presence of genes similar to those of the AF/ST gene cluster in these species (Bhatnagar *et al.* 2003). But phylogenomic analysis of the highly conserved ketosynthase domain of fungal polyketide synthase genes revealed that the origin and diversity of most polyketide genes could be explained by gene duplication, loss or divergence rather than by HGT. There are a few exceptions, however, where polyketide synthases may have been acquired by HGT (Kroken *et al.* 2003).

1.4.1 Aflatoxin and sterigmatocystin gene clusters

Due to the prevalence of the potent toxin AF in foodstuffs an enormous amount of research has been carried out with the aim of understanding, and ultimately controlling, the biosynthesis of AF. In consideration of the structural similarity of dothistromin to AF/ST and the identification of dothistromin genes with high similarities to AF/ST the clustering of the AF/ST genes is of special interest.

The *A. parasiticus* AF gene cluster is approximately 70 kb long and contains 25 genes, including regulatory as well as structural genes, re-named as *afl* genes to reflect their common purpose and genomic location (Yu *et al.* 2004). The involvement of the *afl* gene products in AF biosynthesis has been shown for most of the genes (Ehrlich *et al.* 2005a; Sakuno *et al.* 2005; Wen *et al.* 2005; Yabe & Nakajima 2004). The *A. flavus* AF gene cluster is similar to that of *A. parasiticus*, with the positions and orientations of the genes being conserved between the two species (Yu *et al.* 1995). In *A. nidulans* a 60 kb gene cluster was shown to contain 25 ST genes (Brown *et al.* 1996; McDonald *et al.* 2005). As well as the different order and different orientations of homologous genes in the AF and ST gene clusters (Keller & Hohn 1997), some genes are missing from one of the clusters but present in the other (McDonald *et al.* 2005). For example, the MFS transporter *aflT* is found only in the AF cluster and *stcC* (oxidase) only in the ST cluster.

1.4.2 The dothistromin genes

On the basis of the chemical evidence it was predicted that *D. septosporum* would contain genes orthologous to some of those involved in the biosynthesis of AF and ST, and orthologous genes to the AF/ST genes were indeed identified in *D. septosporum* (Bradshaw & Zhang 2006b). Their high similarities to the aflatoxin and sterigmatocystin genes led also to the expectation that the identified dothistromin genes are part of a big cluster, as seen for the AF/ST genes and many other fungal secondary metabolite genes (Zhang *et al.* 2005). However, recent results show that this is not the case for the dothistromin genes (Bradshaw & Zhang 2006a; Zhang *et al.* 2007). Instead three mini-clusters of eleven putative dothistromin genes have been identified. Other genes that appear to have no connection with dothistromin biosynthesis are located adjacent to, or between, the dothistromin genes in each mini-cluster.

The *dotA* gene was the first identified gene shown to be involved in the biosynthesis of dothistromin. It encodes for a putative versicolorin reductase with 80% amino acid identity to AflM (previously called Ver-1) and was identified in a clone of a *D. septosporum* genomic library by hybridisation with an *A. parasiticus aflM* probe (Bradshaw *et al.* 2002). Targeted gene replacement of *dotA* in *D. septosporum* resulted in a dothistromin negative phenotype, thus implying a requirement for DotA in dothistromin biosynthesis. The *dotA* mutant accumulated versicolorin A (Figure 1.4) but, because dothistromin is structurally more similar to versicolorin B than versicolorin A, it is not known whether versicolorin A is a true precursor or an accumulated side-product in the blocked mutant. However, Henry and Townsend (2005) published a possible biosynthetic pathway for the last stages of dothistromin biosynthesis that suggested how versicolorin A could be a precursor.

Clustered alongside the *dotA* gene are *dotB*, *dotC* and *dotD* that show similarity to AF/ST genes (Table 1.2). Despite this similarity it is not clear if any of these three genes have a functional role in dothistromin biosynthesis. The predicted product of the *dotB* gene contains a peroxidase domain and a putative heme-binding site. Although it shows similarity to the product of the ST gene *stcC,* no equivalent is seen in the AF gene cluster. Furthermore, the role of StcC in *A. nidulans* is unknown since the disruption of *stcC* appears to have no effect on ST production (mention in Zhang *et al.* 2005). The *dotC* gene is predicted to encode a major facilitator superfamily (MFS) transporter. The homologous gene in the AF gene cluster,

aflT, is not required for AF production or secretion (Chang *et al.* 2004). However, in some plant pathogenic fungi, MFS proteins have an important role in toxin secretion and can therefore affect virulence as well as providing self-protection against endogenous toxins (reviewed in Martin *et al.* 2005). The *dotA-D* genes are located on a 32.5 kb contig (mini-cluster 1) which contains no further genes with homologies to genes of the AF/ST cluster (Figure 1.6, Table 1.2).

Screening the *D. septosporum* genomic library with a hybridization probe of the *aflC* (previously called *pksA*) AF gene from *A. parasiticus* led to the identification of a second clone including genes with high similarities to AF/ST genes. This clone contains *pksA*, a gene encoding a polyketide synthase with 55% amino acid identity to AflC. The predicted PksA protein contains the same set of domains found in aflatoxin and sterigmatocystin PKS proteins, including domains for β-ketoacyl synthase, acyltransferase, acyl carrier protein (ACP) or phosphopantotheine attachment site and thioesterase/claisen cyclase, except it has three tandem ACP domains compared to the single ACP found in *A. parasiticus* AflC and the two ACPs found in the *A. nidulans* sterigmatocystin StcA. A *pksA* gene replacement mutant of *D. septosporum* was shown not to produce dothistromin or any known AF precursors. Further feeding studies with norsolorinic acid and versicolorin A showed that this mutant is able to convert those intermediates into dothistromin (Bradshaw *et al.* 2006). This result showed the involvement of the *pksA* gene in the biosynthesis of dothistromin and also supports the suggested biosynthetic pathway of Henry and Townsend (2005) that proposes versicolorin A is a true dothistromin precursor.

Clustered alongside *pksA* in *D. septosporum* are genes named *avfA*, *cypA* and *moxA*, on the basis of their similarity to AF genes that are likely to have functional roles in dothistromin biosynthesis. The *avfA* gene is predicted to encode an oxidase corresponding to *A. parasiticus* AflI (AvfA) and *A. nidulans* StcO. The *aflI* gene was able to complement an atoxigenic mutant of *A. parasiticus* that accumulates averufin (AVR), and therefore is thought to be required for the conversion of AVR to versiconal hemiacetal acetate (VHA). Since the dothistromin biosynthetic pathway is probably very similar to that of aflatoxin at this stage, a similar role for *D. septosporum* AvfA is possible. The conversion of AVR to VHA was subsequently shown to involve a complex metabolic grid with several steps. In *A. parasiticus* an AVR monooxygenase AflV (CypX) and a hydroxyversicolorone monooxygenase AflW

(MoxY) have been confirmed to be involved in this stage of aflatoxin biosynthesis (Wen *et al.* 2005). In *D. septosporum*, possible orthologs of the genes encoding these enzymes lie alongside *avfA*: *cypA* and *moxA*. The *D. septosporum cypA* gene is predicted to encode an AflV ortholog and the *moxA* gene to encode the AflW ortholog (Table 1.2). An equivalent role of these gene products in dothistromin biosynthesis would seem likely. Those genes are located in a 53 kb contig (mini-cluster 3) containing 15 additional genes, with only one, DS31, showing similarities to a AF/ST gene (*stcT*) (Table 1.2, Zhang *et al.* 2007).

S. Zhang (Massey University) identified a *vbsA* gene using degenerate PCR (Schwelm *et al.* 2007). The predicted *vbsA* protein showed high amino acid identities to the versicolorin B synthases AflK and StcN (from *A. parasiticus* and *A. nidulans* respectively). The recent construction of a *D. septosporum* gene replacement strain of *vbsA* showed its involvement in the biosynthesis of dothistromin (Zhang *et al.* 2007). The *vbsA* gene is located on a contig of 14 kb (mini-cluster 2) which includes non-dothistromin genes as well as the partial sequence of a gene designated *hexA*. The predicted amino acid sequence of the partial *hexA* suggests it to be an AflA and StcJ ortholog from the AF and ST clusters and to encode a dothistromin fatty acid synthase (Table 1.2, Zhang *et al.* 2007).

The identified dothistromin genes and the adjacent non-dothistromin genes in the three mini-clusters are summarized in Table 1.2 and the synteny comparison of the dothistromin genes and the AF/ST gene clusters is shown in Figure 1.6.

In the evolutionary model of these clusters proposed by Cary and Ehrlich (2006) it was speculated that the dothistromin cluster diverged earlier than the AF/ST divergence. The basal cluster they proposed as being ancestral to all of these clusters includes homologs of *pksA* and *hexA*, which are now shown to be located in two separate mini-clusters in *D. septosporum*. However the relative positions of these genes to each other in the *D. septosporum* genome are not known and homologs of the other genes speculated to be in the basal cluster (*hexB, aflR, aflJ and nor-1*) have not yet been identified. The hypothesis further suggests that genes encoding modifying enzymes such as the dehydrogenases NorA and AvfA were recruited to the basal cluster (Cary & Ehrlich 2006). It is possible that in *D. septosporum*, the recruited genes were never formed into a close cluster. Alternatively, tight clustering may have been an ancestral feature that degenerated over time in *D. septosporum*.

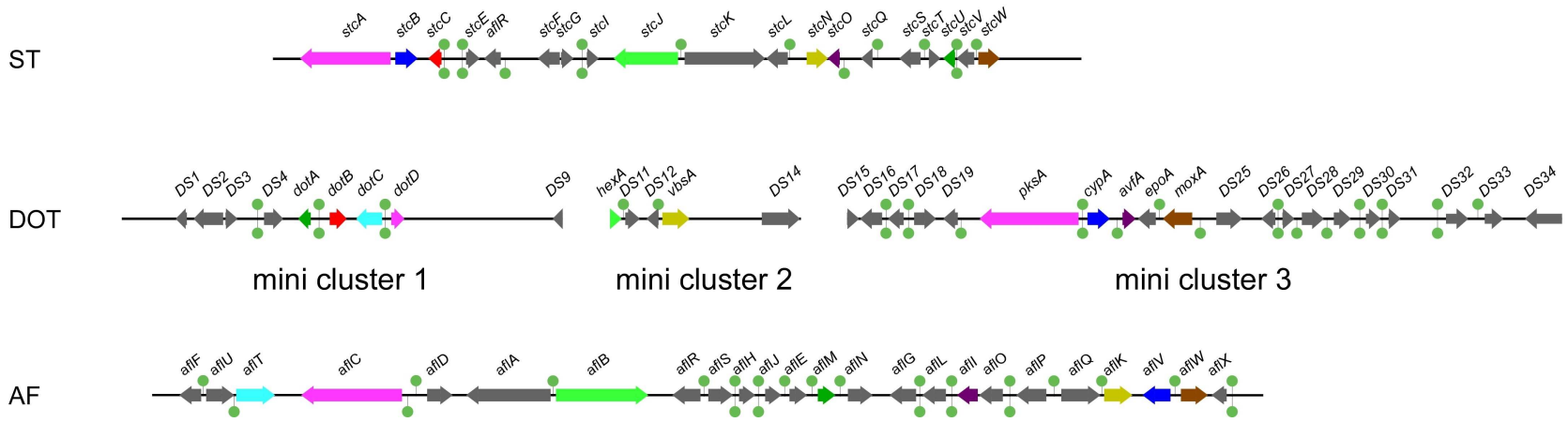

Figure 1.6: Synteny comparison of gene clusters.

The three dothistromin (DOT) mini-clusters are compared with the *A. nidulans* sterigmatocystin (ST, accession number ENU34740) and *A. parasiticus* aflatoxin (AF, accession number AY371490) gene clusters. Genes predicted to have similar functions in the three fungi are displayed using the same colour. Putative AflR binding sites in promoter regions of the genes are indicated with stemmed balls: those above the genes indicate the sequence $TCGN_5CGR$ and those beneath the genes indicate the sequence $TCGN_{11}CGR$. The number of binding sites in the individual promoter regions is not shown. D. septosporum genes labelled DS X do not show any similarity to AF/ST genes. Figure is taken from Zhang (2007).

Table 1.2: Genes identified in the three dothistromin gene mini-clusters. Table altered from Zhang *et al*. (2007). Different background colours indicate mini cluster 1, minicluster 2 and minicluster 3.

Gene	Size[1] (aa)	Putative function (domain)[2]	Highest BLAST match[3]	Genbank accession no.	Species	Indicative E-value	*A. parasiticus* AF cluster		*A. nidulans* ST cluster	
							Probable homolog[4]	% aa identity	Probable homolog[4]	% aa identity
DS1	271	unknown	UG	AACS01000171	*Coprinopsis cinerea*	9e-08	-	-	-	-
DS2	646	chitin synthase	UG	AE017346	*Cryptococcus neoformans*	8e-10	-	-	-	-
DS3[5]	157	unknown	UG	XM_001227301	*Chaetomium globosum*	1.6	-	-	-	-
DS4 *ddhA*[5]	469	UDP-N-acetyl-D-mannosaminuronate dehydrogenase	UG	AP007161	*A. oryzae*	1e-110	-	-	-	-
dotA	**263**	**ketoreductase**	***stcU***	**EAA61594**	***A. nidulans***	**2e-112**	***aflM* (M91369)**	**80.2**	***stcU* (L27825)**	**79.1**
dotB	**414**	**oxidase**	**UG**	**XM_743185**	***A. fumigatus***	**5e-59**	**-**	**-**	***stcC* (U34740)**	**24.0**
dotC	**602**	**toxin pump**	**UG**	**XM_749669**	***A. fumigatus***	**8e-141**	***aflT* (AF268071)**	**31.2**	**-**	**-**
dotD	**322**	**thioesterase**	***pksP***	**XM_001261234**	***Neosartorya fischeri***	**4e-67**	***aflC* (L42766)**	**34.8**	***stcA* (U34740)**	**37.9**
DS9[6]	71	cytoskeleton assembly protein	*SLA2*	AJ884600	*Xanthoria parietina*	8e-16	-	-	-	-
hexA[6]	**113**	**fatty acid synthase**	***hexA***	**AY510454**	***A. nomius***	**1e-20**	***aflA* (AF391094)**	**38.3**	***stcJ* (AN7812)**	**36.0**
DS11	304	unknown	UG	AP007174	*A. oryzae*	3e-33	-	-	-	-
DS12	262	unknown (DUF1772)	UG	AP007159	*A. oryzae*	1e-30	-	-	-	-
vbsA	**643**	**versicolorin B synthase**	***vbs***	**AY510454**	***A.nomius***	**0.0**	***aflK* (AF169016)**	**72.0**	***stcN* (AN7812)**	**69.1**
DS14	702	potassium channel	*toka*	AJ510245	*N. crassa*	2e-116	-	-	-	-
DS15[6]	228	unknown	UG	XM_001217330	*A. terreus*	9e-26	-	-	-	-
DS16	482	unknown	*AocAR*	AB240531	*A. oryzae*	9e-46	-	-	-	-
DS17	274	unknown	UG	XM_387929	*Gibberella zeae*	1e-20	-	-	-	-
DS18	498	unknown (PCI)	UG	AP007167	*A. oryzae*	4e-176	-	-	-	-
DS19	356	unknown (SURF1)	UG	XM_959428	*N. crassa*	2e-98	-	-	-	-
pksA	**2399**	**polyketide synthase**	***pksA***	**AY510452**	***A. flavus***	**0.0**	***aflC* (AAS66004)**	**54.8**	***stcA* (EAA61613)**	**57.0**
cypA	**511**	**averufin monooxygenase**	***stcB***	**XM_676001**	***A. nidulans***	**0.0**	***aflV* (AAS66022)**	**59.3**	***stcB* (EAA61612)**	**59.8**
avfA	**301**	**oxidase**	***aflI***	**AY371490**	***A. parasiticus***	**3e-73**	***aflI* (AAS66010)**	**47.8**	***stcO* (EAA61613)**	**43.7**
epoA	**420**	**epoxide hydrolase**	***EPH2***	**DQ443738**	***A. niger***	**4e-93**	**-**	**-**	**-**	**-**
moxA	**626**	**hydroxyversicolorone monooxygenase**	***moxY***	**AY510454**	***A. nomius***	**0.0**	***aflW* (AAS66023)**	**55.1**	***stcW* (EAA61592)**	**59.0**
DS25	575	amino acid permease	*Agp2*	XM_001267393	*Neosartorya fischeri*	9e-139	-	-	-	-
DS26	345	unknown (YqcI_YcgG)	UG	XM_391678	*G. zeae*	1e-97	-	-	-	-
DS27	196	unknown	UG	XM_391679	*G. zeae*	5e-84	-	-	-	-
DS28	523	MFS multidrug transporter	UG	XM_391680	*G. zeae*	1e-169	-	-	-	-
DS29	300	unknown	UG	XM_741940	*A. fumigatus*	0.75	-	-	-	-
DS30	348	unknown (NmrA)	UG	XM_387985	*G. zeae*	2e-30	-	-	-	-
DS31	231	translation elongation factor	*stcT*	XM_675984	*A. nidulans*	1e-47	-	-	*stcT* (ENU34740)	41.1
DS32	529	methionine permease	UG	XM_001267118	*Neosartorya fischeri*	0.0	-	-	-	-
DS33	458	unknown	UG	XM_677390	*A. nidulans*	7e-75	-	-	-	-
DS34[6]	889	unknown (DUF1785, Piwi, PAZ)	UG	AACS01000213	*Coprinopsis cinerea*	2e-88	-	-	-	-

[1] The size of the peptide was predicted from the putative genes.
[2] Putative function was based on BLAST search at NCBI (restricted to fungal data) and the putative domains were from the Pfam database. The putative domains or Pfam family IDs are in brackets.
[3] Best match from BLAST search at NCBI (restricted to fungal data) (UG: unnamed gene).
[4] Genbank acession numbers are in brackets.
[5] Pseudogenes with stop codons within open reading frames.
[6] Partial genes.

The arrangement of dothistromin genes described here is not the only example of a fragmented secondary metabolite gene cluster in fungi. A fragmented secondary metabolite gene cluster is also known for cephalosporin biosynthesis in *Acremonium chrysogenum*, where two genes are located on chromosome II and four genes on chromosome IV (Liras & Martin 2006). The complete lolitrem gene cluster of the grass endophyte *Neotyphodium lolii* consists of three clusters containing lolitrem genes but no lolitrem-unrelated genes were reported within the cluster (Young *et al.* 2005; Young *et al.* 2006). Instead, AT-rich regions, direct repeats and retrotransposon relics were identified between or adjacent to the mini clusters (Young *et al.* 2006). AT-rich regions containing remnants of retrotransposon sequence were also found in the gliotoxin gene cluster in *A. fumigatus* (Gardiner & Howlett 2005) and in the ergovaline gene cluster of *N. lolii* (Fleetwood *et al.* 2007). The presence of those AT rich regions and retrotransposon relics allows speculation about their role in clustering or dispersing secondary metabolite genes.

A model for the putative biosynthesis pathway for dothistromin is shown in Figure 1.7. The proposed biosynthesis pathway is based on the similarity of the dothistromin genes to AF toxin genes. Identified dothistromin genes are allocated to biosynthetic steps in which the gene products of their AF homologues have been shown to be involved. Included are also the *D. septosporum* genes *norA*, *avnA* and *ver-B* which were recently isolated by degenerate PCR by Z. Feng and J. Baker in this lab. Their partial sequences suggest that the genes code for homologues of the AF genes *aflE*, *aflG* and *aflL* respectively.

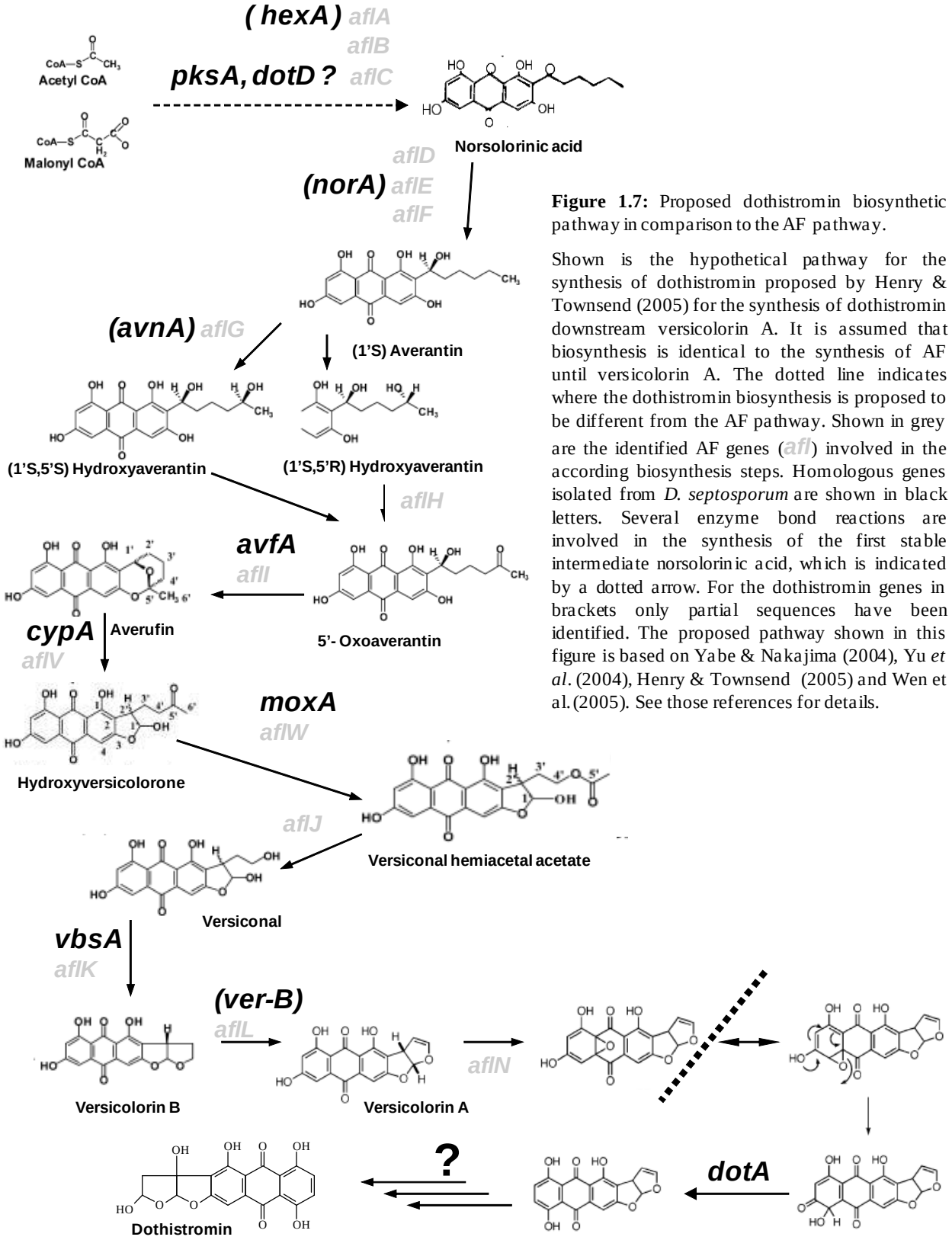

Figure 1.7: Proposed dothistromin biosynthetic pathway in comparison to the AF pathway.

Shown is the hypothetical pathway for the synthesis of dothistromin proposed by Henry & Townsend (2005) for the synthesis of dothistromin downstream versicolorin A. It is assumed that biosynthesis is identical to the synthesis of AF until versicolorin A. The dotted line indicates where the dothistromin biosynthesis is proposed to be different from the AF pathway. Shown in grey are the identified AF genes (*afl*) involved in the according biosynthesis steps. Homologous genes isolated from *D. septosporum* are shown in black letters. Several enzyme bond reactions are involved in the synthesis of the first stable intermediate norsolorinic acid, which is indicated by a dotted arrow. For the dothistromin genes in brackets only partial sequences have been identified. The proposed pathway shown in this figure is based on Yabe & Nakajima (2004), Yu *et al*. (2004), Henry & Townsend (2005) and Wen et al. (2005). See those references for details.

1.5 Regulation of secondary metabolite genes

One theory why secondary metabolite gene clusters evolved suggests that there is a selective benefit if functionally related genes are located in a similar chromatin environment (Zhang *et al.* 2005). In some cases the clustering appears to play a role in the regulation of the secondary metabolite genes. For instances the AF gene *aflD* (*nor-1*) is differently transcribed when it is located outside, as opposed to within, the gene cluster (Chiou *et al.* 2002). In *Aspergillus* spp. the nuclear protein LaeA was identified and disruption of *laeA* resulted in strains of *A. nidulans* (Bok & Keller 2004) and *A. fumigatus* (Bok *et al.* 2005) with reduced secondary metabolite production. LaeA was shown to specifically regulate the expression of secondary metabolite gene clusters and its similarity to methyltransferases involved in histone modification suggests a possible regulation through chromatin modification (Bok *et al.* 2006; Zhang *et al.* 2005).

Secondary metabolite gene clusters often include genes for their own regulation. An example of such pathway specific regulators is AflR, a Zn_2Cys_6 domain regulator protein in the AF/ST gene cluster. AflR binds to a palindromic sequence 5'-TCGN_5CGR-3' and positively regulates the transcription of AF/ST genes. It regulates its own biosynthesis and also influences transcription of some genes outside the cluster (Price *et al.* 2006). AflR interacts with a second regulatory protein in the AF gene cluster, AflJ which appears to be a co-regulator (Chang 2003). Most AF and ST genes contain AflR binding sites (Figure 1.6, Cary & Ehrlich 2006). The dothistromin mini clusters also contain putative AflR binding sites for most genes (Figure 1.6, Bradshaw & Zhang 2006b). A sequence motif (5'-TCGN_{11}CGR-3') that is similar to the AflR sequence is also present in most dothistromin genes (Figure 1.6), but it is not known if either of these sequences function in gene regulation, and no *aflR* homologue has been identified yet in *D. septosporum.*

Environmental influences such as light, pH, carbon and nitrogen source and temperature also affect the production of secondary metabolites. The three Cys_2Zn_2 zinc finger proteins CreA (carbon source), AreA (nitrogen source) and PacC (pH) have all been shown to regulate clustered metabolite genes (Yu & Keller 2005).

In *A. nidulans*, PacC activates alkaline-expressed genes and represses acid-expressed genes and is under pH regulation itself (Denison 2000; Penalva & Arst 2002; Penalva & Arst 2004). The synthesis of penicillin is positively regulated by PacC in *A. nidulans*, while AF, ST and ochratoxin in *Aspergillus* spp. and fumonisin in *F. verticillioides* appear to be negatively regulated (reviewed in Hoffmeister & Keller 2007). Although AF and ST biosynthesis is partly regulated by the pH regulator PacC (Keller *et al.* 1997), the role of pH regulation in AF production is not resolved and often contradictory (Calvo *et al.* 2002), even for different *A. flavus* strains (Ehrlich *et al.* 2005b). Homologues of *pacC* and other genes of the transcriptional regulatory system in fungi have been identified in other fungi and the pH regulatory system appears to be universally present in all major groups of ascomycetes (reviewed in Penalva & Arst 2002; Penalva & Arst 2004). Indeed, upstream of all of the dothistromin genes characterized in *D. septosporum* so far are putative binding sites for the pH regulatory protein PacC (5'-GCCARG-3') (Bradshaw & Zhang 2006b).

Seven of the dothistromin genes also contain a putative binding motif for the nitrogen metabolism regulator AreA (5'-HGATAR-3') (Bradshaw & Zhang 2006b), which is postulated to mediate nitrate repression of aflatoxin production in *A. parasiticus* (Ehrlich & Cotty 2002). Influences of different nitrogen sources on the production of dothistromin by *D. septosporum* have been observed (Bradshaw *et al.* 2002; Ganley 2001).

The production of secondary metabolites is also linked with asexual sporulation. In *Aspergillus* and *Fusarium*, sporulation and mycotoxin production are linked by G protein signaling pathways (Brodhagen & Keller 2006). A G-protein-mediated signaling pathway negatively regulates both sporulation and metabolite biosynthesis such that, in general, derepression of both systems occurs when growth signals are down (Calvo *et al.* 2002; Hicks *et al.* 1997). There is no evidence yet to determine whether a similar pattern of regulation is seen with dothistromin biosynthesis in *D. septosporum*. However, seven of the dothistromin genes also have potential binding sites for the conidiation/developmental regulator AbaA (5'-CATTCY-3') (Bradshaw & Zhang 2006b).

1.6 Reporter genes in plant pathogen studies

One way to investigate regulation of genes of interest is the use of reporter genes that encode easily-assayed proteins. They are used to replace other coding regions whose protein products are difficult to assay and can be introduced into an organism to study temporal and spatial patterns of expression. Alternatively they can be used as marker genes to label whole cells or organisms. Some reporter genes, such as *β*–glucuronidase, *β*–galactosidase, chloramphenicol acetyltransferease or luciferase, require exogenous substrates, co-factors or antibiotics for detection. More recently the green fluorescent gene (*gfp*) has become the gene of choice for labelling fungal cells or for gene expression studies as the GFP protein only relies on oxygen and UV/blue light for its activity (Lorang *et al.* 2001). The GFP is a spontaneously fluorescent polypeptide of 27 kDa, derived from the jellyfish *Aequorea victoria*, which absorbs UV or blue light and emits green light (Tsien 1998). It was isolated in 1992, and has since been used as a reporter and marker in both prokaryotes and eukaryotes (Larrainzar *et al.* 2005; Lorang *et al.* 2001).

1.6.1 Green fluorescence protein in fungi

The wild type GFP protein has a low turnover rate in some applications, taking up to two hours for auto-activation of the chromophore responsible for fluorescence. It is also subject to incorrect folding at temperatures above 37°C. Another potential problem is the requirement of oxygen for fluorescence, which may not be present in sub-cellular locations or various cell types at equal concentration within the organism. Furthermore, in most filamentous fungi the wild type *gfp* gene is not efficiently translated (Lorang *et al.* 2001). For example, the wild type GFP was not expressed in *A. nidulans, Acremonium chrysogenum* or *Sordaria macrospora*, even though the DNA was successfully integrated in the genome (Fernández-Ábalos *et al.* 1998; Pöggeler *et al.* 2003). Some plant species recognize a splice site in the coding sequence of the wild type *gfp* gene, resulting in a partial gene product which is non-fluorescent (Haseloff *et al.* 1997).

Modified forms of *gfp* with optimized codon usage for yeast, plant and mammalian cells, whose protein products show increased fluorescence and solubility compared to the native GFP, have been developed (Larrainzar *et al.* 2005; Lorang *et al.* 2001). Most of the GFP variants include a serine-to-threonine substitution at amino acid 65 (S65T). This substitution causes a red shift in excitation maxima to 488 nm, with light emittance detected at 508 nm, making it ideal for use with fluorescent microscopy. Fernández-Ábalos et al. (1998) showed that the *sgfp* gene, which contains a plant optimised codon usage and a S65T substitution in the protein, was the most effective *gfp* gene in *A. nidulans*. The SGFP was also the first GFP version which was successfully expressed in *Cochliobolus heterostrophus* (Maor *et al.* 1998) and the most common *gfp* gene used for transformation up until 2001 (Lorang *et al.* 2001). Interestingly, Chung *et al*. (2002) reported that the SGFP does not produce sufficient GFP fluorescence in *Cercospora nicotianae* in contrast to the native GFP version. Another GFP variant is the EGFP. The *egfp* gene contains 190 silent base mutations, optimizing the gene to mammalian codon usage and a P64L substitution in addition to the S65T substitution in the protein (Yang *et al.* 1996). EGFP has been used successfully with many fungal species to monitor fungal growth on or within plant hosts. While the *sgfp* and *egfp* genes have been expressed in many Ascomycetes, they appear to require introns in order to obtain GFP expression in most Basidiomycetes (Burns *et al.* 2005).

Besides the choice of the most applicable version of GFP, the use of GFP as a marker requires a strong constitutive promoter, which usually results in a cytoplasmically located protein that occurs in all fungal tissues (Lorang *et al.* 2001).

A gene encoding the red fluorescent protein, DsRed, has been obtained from a reef coral *Discosoma* sp.. This protein resembles GFP, although it tends to form tetramers, and the gene may be used as a reporter in a similar way as the *gfp* gene (Larrainzar *et al.* 2005). Mikkelsen et al. (2003) showed expression of the DsRed protein in *Penicillium paxilli*, *Trichoderma harzianum* and *Trichoderma virens*. Co-expressed DsRed and GFP in dually marked *Trichoderma* transformants, and mixed cultures of two different *P. paxilli* transformants expressing either DsRed or GFP, were distinguishable using conventional fluorescence microscopy. This allows easy detection of mixed cultures or differences in

expression if DsRed or GFP are used as reporter genes in the same strain (Mikkelsen *et al.* 2003).

1.6.2 The use of GFP in plant-fungi interactions

There are numerous examples of the use of GFP as a reporter gene in plant fungal interactions, either with *gfp* genes under control of promoters (constitutive or regulated) or as fusion genes, with the GFP fused to the protein of interest to determine their cellular localisation. Some examples of using GFP transformants, and the numerous possibilities of using GFP strains, are described in the following.

The colonization of host plants by fungi has been frequently investigated using GFP transformants and six examples are outlined here. (1) GFP expressing *C. heterostrophus* colonizing the mesophyll zone under the point of inoculation on maize was observed using epifluorescent microscopy (Maor *et al*. 1998). (2) The endophyte *Neotyphodium lolii* was transformed with GFP, and visualized in the leaf sheaths of perennial ryegrass. Observations showed the presence of GFP throughout the cytosol of living hyphae, and the lengthwise orientation of hyphae, with infrequent branching within leaf sheath cells (Mikkelsen *et al.* 2001). (3) Tanaka *et al*. (2006) showed that fungal ROS production is critical in maintaining a mutualistic fungus–plant interaction between *E. festucae*, and its grass host, *Lolium perenne*. Plants infected with an *E. festucae* NADPH oxidase mutant die. Using GFP strains of mutant and wild type it was shown that this antagonistic interaction with the host is accompanied by a dramatic increase in endophyte biomass within the plant compared with that in the wild type. (4) GFP-transgenic isolates of *C. acutatum* were used to characterize differences between pathogen development on the susceptible host (strawberry) and resistant asymptomic plants (pepper, eggplant and tomato). While extensive branching and penetration through appressoria was observed on strawberries, cell penetration in asymptomatic plants only occurred through invasion of stomatal cells (Horowitz *et al*. 2002). (5) A GFP expressing strain of *Fusarium verticillioides* allowed colonization to be observed at different development stages of maize, when grown in *F. verticillioides*-infested soil (Oren *et al*. 2003). (6) The use of GFP

expressing strains has elucidated the mechanism by which *Mycosphaerella* pathogens cause Sigatoka disease of banana. The study also supports a proposed involvement of a diffusible phytotoxin that several *Mycosphaerella* banana pathogens are thought to produce, as the end of the necrotic area is often in advance of fungal hyphae (Balint-Kurti *et al.* 2001). Further examples where host-pathogen interactions were followed using GFP transformed fungi are *Fusarium graminearum* and barley (Skadsen & Hohn 2004), *M. graminicola* and wheat (Rohel *et al.* 2001a), *Alternaria citri* and citrus tissue (Isshiki *et al.* 2003).

GFP genes fused to promoters of genes of interest are also used in fungi to study gene expression *in vivo*. GFP fused to the *L. maculans* promoter for cyanide hydratase gene *cht*, which encodes a fungal enzyme that catalyses the detoxifcation of cyanide, was used to study the expression of the *cht* gene during the infection of *Brassica napus* and *Brassica juncea* by *L. maculans* (Sexton & Howlett 2001). Similarly a *Mycosphaerella graminicola* strain transformed with GFP control by a carbon source-repressed promoter was used to study carbohydrate uptake during penetration of the fungus in wheat leaves and showed that repression occurs directly after penetration and lasted until sporulation (Rohel *et al.* 2001b).

GFP technology has also facilitated the study of biocontrol agents of pathogenic fungi. Lu *et al*. (2004) used regulated and constitutive expressing GFP strains of *Trichoderma atroviride* during mycoparasitism of *Pythium ultimum* and *Rhizoctonia solani* on cucumber seed. *T. atroviride* with the *gfp* gene under control of either a constitutive pyruvate kinase promoter (induced by cell wall oligosaccharides or digested colloidal chitin) or an inducible promoter (induced by cell wall oligosaccharides or *N*-acetylglucosamine) were used in this study. They observed induction of the regulated reporter genes by the presence of the host and chitin within 24 hours of *T. atroviride* colonization. Furthermore, they showed that mycoparasitism takes place on the seed surface, with *T. atroviride* hyphal branches growing towards the host and coiling around the host hyphae (Lu *et al.* 2004). Neveu *et al*. (2007) transformed the biocontrol agent *Pseudozyma flocculosa* with GFP to study the tripathogenic interaction between pathogenic powdery mildew, host plant and *P. flocculosa*. Transformation of *Clonostachys rosea* with GFP also facilitated study of possible uses of the organism as a biocontrol agent (Lubeck *et al.* 2002). The effects of a fungicide, azoxystrobin in impairing *M. graminicola* infection of wheat leaves has been assessed using a GFP transformed strain of the

fungus. The growth of *M. graminicola* inside wheat leaves was monitored following treatment with azoxystrobin at various stages of incubation post-inoculation to study the fungistatic effect of azoxystrobin on *M. graminicola* (Rohel *et al.* 2001a).

In most instances where GFP has been used to transform fungi, the protein does not seem to interfere with any major physiological pathways (Isshiki *et al.* 2003), and further suggests that the transformation process does not affect fungal morphology, pathogenicity or virulence (Balint-Kurti *et al.* 2001; Horowitz *et al.* 2002; Lubeck *et al.* 2002).

1.6.3 The use of GFP fusion proteins in filamentous fungi

In addition to their use as marker proteins or for studying gene expression, GFP genes can be translationally fused. GFP fusion proteins have been successfully used in filamentous fungi to localize proteins of interest. For example, Fernández-Ábalos *et al*. (1998) could visualize the dynamics of nuclear division in *Aspergillus* using a GAL4::GFP fusion protein. Another example involves the *Aspergillus nidulans* UapC protein, which is a high-affinity uric acid–xanthine transporter. Using a functional UapC–GFP fusion protein it was shown that the protein is removed from the plasma membrane and is concentrated into the vacuolar compartment after addition of ammonium (Valdez-Taubas *et al.* 2004).

Chung et al. (2002) showed that the PDX1 protein in *Cercospora nicotianae* is localized to circular vesicles within the cytoplasm by using a PDX1::GFP fusion protein. PDX1 is involved in the synthesis of the pyridoxine antioxidants and PDX1 is required for growth in the presence of cercosporin and other $^1O_2$-generating photosensitizing compounds. The authors speculated that the vesicles containing the PDX1 protein might present an unknown organelle which is involved in the protection of the fungus from reactive oxygen species, In respect to the synthesis of dothistromin this is interesting as dothistromin can also activate oxygen (Youngman & Elstner 1984) and is produced by several *Cercospora* species (Bradshaw 2004).

1.7 Aims of this study

At the beginning of this study the dothistromin toxin was suspected to be a pathogenicity factor in Dothistroma needle blight. While this assumption seemed reasonable, evidence for this was limited and attempts to test the pathogenicity of *Dothistroma septosporum* wild type and toxin deficient mutants met technical difficulties. Parallel studies carried out by Masterate students in this laboratory aimed to overcome those difficulties and develop a workable pathogenicity testing system. The study described in this thesis was intended to broaden the knowledge about the role of dothistromin for *D. septosporum*. Therefore several approaches were used:

Aim 1: To study expression of the dothistromin genes in culture.

This should reveal if genes are co-regulated as seen for secondary metabolite gene clusters in other fungi. This is of particular interest as the dothistromin genes are more fragmented and not organised in a continuous cluster as seen for AF/ST genes. Therefore it is not necessarily expected that a high degree of co-regulation will be seen. Furthermore, the expression study in culture might give an indication of possible expression pattern *in planta* and allow hypotheses to be developed about the role of dothistromin *in planta*.

Aim 2: To create GFP reporter gene strains of *D. septosporum*, which can be used as tools in the study of the fungal plant interactions.

As no reliable pathogenicity test was available, the development of GFP expressing strains was expected to facilitate the optimisation of the pathogenicity system, as the GFP transformants are easier to observe *in planta*. Once a pathogenicity system is established, both *D. septosporum* wild type and mutants defective in dothistromin biosynthesis will enable monitoring of fungal development, and determination of whether the mutated genes are crucial for infection.

GFP reporter strains of *D. septosporum*, with the GFP expressed under the control of a dothistromin regulatory region, will allow studies of the toxin gene expression *in planta*. The onset of dothistromin gene expressions *in planta* should reveal important information about the role of the toxin in the plant host interaction.

A fusion protein construct, with the GFP fused to a dothistromin biosynthesis protein, may allow the localization of dothistromin biosynthesis within the cell. The localization of the dothistromin synthesis might also reveal clues about a biological role of the toxin or allow the identification of possible control targets.

During this study, it was speculated that dothistromin might play a role in the interaction with other fungi. To test this, the effect of dothistromin producing and dothistromin deficient *D. septosporum* strains in competition with other fungi was also determined.

Chapter 2: Material and Methods

2.1 Biological Material

2.1.1 Fungal isolates

Dothistroma septosporum isolates and other fungal strains used in this study are listed in Table 2.1.

2.1.2 Bacterial strains

Bacterial strains used in this study were *Escherichia coli* XL1-Blue (SupE44 *hsd*R17 *rec*A1 endA1 gyrA46 *thi rel*A1 *lac*$^-$ F'[proAB$^+$ Δ*lac*I^q Δ(*lac*Z) M15 Tn10 (*tet*^{R})]) (Bullock *et al.* 1987) and Top10 (F$^-$ *mcr*A Δ(*mrr-hsd* RMS-*mcr*-BC) 80 (lacZ) ΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697) (Invitrogen).
E. coli strains were used to propagate or maintain plasmids used and constructed in this study.

2.1.3 Plant material

Pinus radiata seedlings and cuttings used in this study were obtained from ENSIS (Rotorua, NZ). Seedlings were raised from seeds, and rooted cuttings were taken from 5-year- to 7-year-old pine trees. All plant material was potted in standard potting mix in small polythene bags and kept in the shade house at Massey University until required. Potted seedlings/cuttings used were aged 6-12 months at the time of inoculation.

Table 2.1: Fungal strains used in this study

Dothistroma septosporum			
Fungal strain /transformant	Transformed plasmid (Reference)	Characteristics	Source/Reference
NZE 5	-	Wild type; Single spore isolate from infected pine needles; needles collected by P. Hirst from Kinleith, New Zealand	(Bradshaw *et al.* 2002; Hirst *et al.* 1999)
NZE 7	-	Wild type; Single spore isolate from infected pine needles; needles collected by Margaret Dick (ENSIS) from Bay of Plenty, New Zealand	(West 2004)
NZE10	-	Wild type; Single spore isolate from infected pine needles; needles collected by Ben Doherty from West Coast of the South Island, New Zealand	(Barron 2006)
FJT2	pR209 (Seconi 2001)	NZE5/Δ*dotA::hph*; Hyg^R	(Bradshaw *et al.* 2002; Seconi 2001)
FJT3	pR226, (Jin 2005)	NZE7/Δ*pksA::hph*; Hyg^R	(Bradshaw *et al.* 2006; Jin 2005)
FJT20	pCT74 (Lorang *et al.* 2001)	NZE7/ P*toxA::sgfp*; hph, Hyg^R	This Study
FJT21	pPN82 (Tanaka *et al.* 2006)	NZE7/P*gpdA*::*egfp*::*TtrpC,hph*; Hyg^R	This Study
FJT22	pPN82 (Tanaka et al. 2006)	NZE7/P*gpdA*::*egfp*::*TtrpC,hph*; Hyg^R	This Study
FJT24	pR242 (this study, Section 4.2.2)	NZE7/ P*dotA::egfp, hph*; Hyg^R	This Study
FJT26	pR242 (this study, Section 4.2.2)	NZE7/ P*dotA::egfp, hph*; Hyg^R	This Study
FJT29	pPN82 (Tanaka et al. 2005) pBC-Phleo (Silar 1995)	FJT2/ P*gpdA*::*egfp*::*TtrpC,hph*; Hyg^R; $Phleo^R$	This Study
FJT30	pPN82 (Tanaka et al. 2005) pBC-Phleo (Silar 1995)	FJT3/ P*gpdA*::*egfp*::*TtrpC,hph*; Hyg^R $Phleo^R$	This Study
FJT31	pR261 (This study; Section 4.2.3) pBC-Phleo (Silar 1995)	FJT2/ P*dotA-dotA*::*egfp*::*TtrpC,hph*; Hyg^R , $Phleo^R$	This Study
FJT32	pR261 (This study; Section 4.2.3) pBC-Phleo (Silar 1995)	FJT2/ P*dotA-dotA*::*egfp*::*TtrpC,hph*; Hyg^R , $Phleo^R$	This Study
FJT34	pPN82 (Tanaka et al. 2006)	NZE7/P*gpdA*::*egfp*::*TtrpC,hph*; Hyg^R	This Study
FJT35	pPN82 (Tanaka et al. 2006)	NZE7/P*gpdA*::*egfp*::*TtrpC,hph*; Hyg^R	This Study
FJT36	pPN82 (Tanaka et al. 2006)	NZE7/P*gpdA*::*egfp*::*TtrpC,hph*; Hyg^R	This Study
FJT37	pPN82 (Tanaka et al. 2006)	NZE7/P*gpdA*::*egfp*::*TtrpC,hph*; Hyg^R	This Study
FJT39	pR237 (This study; Section 2.4.1)	NZE7/P*gpdA*::*dsred*::*TtrpC,hph*; Hyg^R	This Study

Hyg^R = resistance to hygromycin
$Phleo^R$ = resistance to phleomycin

Other fungi used in this study		
Fungal species	Source number, place of origin, collection date	Source
Trichoderma atroviride *Trichoderma viride*	LU132; Pukekohe, Auckland, NZ; 1991 LU761; Lincoln University Arable and Forage Cropping Farm, NZ; 2002	Allison Stewart (Lincoln University, NZ)
Lophodermium conigenum *Lophodermium pinastri* *Cyclaneusma minus* *Strasseria geniculata*	9262/C; NZ NZFS804, NZ NZ NZFS1023; Rotorua, NZ	Margaret Dick (ENSIS, Rotorua, NZ)
Aspergillus nidulans	Wild type NRRL 194 "glasgow"	Lab strain
Glomerella cingulata	ICMP 11061	Peter Farley (Massey University, Palmerston North, NZ)
Eutypa lata *Alternia alternata* *Fusarium graminearum*	E10-10, wild type from grapevines at Hawkes Bay, NZ, 2003 NZ, 2003 NZ, 2002	Peter Long (Massey University, Palmerston North, NZ)
Phoma glomerata	Black 'contaminating' fungus isolated from pine seedlings in *D. septosporum* pathogenicity trials; identity determined by ITS sequence	Naydene Barron (Massey University, Palmerston North, NZ)

2.2 Growth and maintenance of microorganisms

All media used in this study are listed in Appendix A1.

2.2.1 Growth and maintenance of *E. coli* cultures

E. coli cultures were grown overnight at 37°C on LB agar plates or in LB broth with shaking at 220 rpm. For selection of *E. coli* transformants ampicillin, IPTG and X-gal were added. Plate cultures were stored at 4°C. For long term storage, overnight cultures in LB broth were mixed with glycerol to a final concentration of 20% and stored at –80°C.

2.2.2 Growth and maintenance of fungal cultures

2.2.2.1 General growth of *D. septosporum*

Generally *D. septosporum* was grown and sub-cultured on a malt based dothistroma media agar (DM) or potato dextrose agar (PDA) at 22°C. To obtain spores an area of about 0.5 x 0.5 cm was cut from the margin of a growing colony and ground with a sterile pestle in a sterile microcentrifuge tube. The ground mycelia were then diluted in water, spread on dothistroma sporulation media agar (DSM) plates and grown at 22°C for 10-14 days before spores were harvested. For long-term storage, spore suspension or mycelium fragments of *D. septosporum* strains were stored in 17-30% sterile glycerol in water at –80°C.

2.2.2.2 Growth in liquid cultures

In growth experiments conidia were collected from 10-12 days cultures of *D. septosporum* grown on DSM. Approximately 1 x 10^6 conidia/ml were inoculated into 25 ml of dothistroma broth (DB) medium or potato dextrose broth (PDB) in 125 ml conical flasks and incubated at 22°C on an orbital shaker at 180 rpm. At appropriate times mycelium was harvested by vacuum filtration, weighed and divided into two parts. One part was weighed, freeze-dried and re-weighed to calculate total dry weight (DW), while the other was frozen in liquid nitrogen and used for RNA extraction.

Fungal biomass for genomic DNA extraction was obtained by inoculating 20 ml of DB or PDB media with ground mycelia. About 0.5 x 0.5 cm mycelia from the edge of a fungal colony were finely ground with a sterile pestle in 1 ml sterile milli Q water. Each flask was inoculated with 200 µl of the suspension and incubated at 22°C on an orbital shaker at 180 rpm for 5-6 days.

2.2.2.3 Growth of other fungi

Fungal species other than *D. septosporum* were grown and maintained on PDA media at 22°C and stored at 4°C unless otherwise stated.

2.3 DNA isolation, purification and quantification

2.3.1 Isolation of *D. septosporum* genomic DNA

DNA from *D. septosporum* was obtained either directly from plate cultures in a small scale extraction (2.3.1.1) or from a larger volume of mycelium grown in liquid cultures (Section 2.2.2.2) using the CTAB (hexadecyltrimethylammonium bromide) method (2.3.1.2) when required for Southern analyses.

2.3.1.1 Small scale genomic DNA isolation

Jin (2005) developed this method to isolate DNA directly from colonies of *D. septosporum*. The method is based on the method of Al-Sammarrai & and Schmid (2000) but using fresh mycelia instead of freeze-dried mycelia. Areas of mycelia were cut from a plate culture (approximately 0.5 x 0.5 cm) and ground with a sterile pestle in a microcentrifuge tube. Freshly prepared lysis buffer (500 µl) was added and vigorously mixed by pipetting, followed by addition of 2 µl of RNaseA (Sigma) (10mg/ml) and incubation at 37°C for 5 min. 165 µl 5 M NaCl were added, mixed by inversion and microcentrifuged for 10 min at 4°C and maximum speed to precipitate cellular debris. The supernatant was transferred into a new tube and one volume of chloroform was added and mixed by inversion. After another centrifugation as above the aqueous phase was transferred to a new tube. DNA was precipitated as described in Section 2.3.5 or 2.3.6.

2.3.1.2 Large scale genomic DNA isolation (CTAB method)

DNA was extracted from *D. septosporum* using the CTAB method (Doyle & Doyle 1987). Mycelium was harvested by filtering, washed with sterile water and subsequently snap frozen in liquid nitrogen. After freeze-drying for 24 h, the mycelium was ground in liquid nitrogen to a fine powder using a mortar and pestle. Approximately 0.1-0.2 g ground mycelium was transferred into a sterile microcentrifuge tube and re-suspended in 600 µl of CTAB buffer (Appendix A2.2) including 2 µl of RNase (Sigma) (10 mg/ml). The suspension was mixed thoroughly and incubated at 37°C for 5-10 min. The samples were incubated at 65°C for 30- 60 min with occasional inversion and cooled down to 60°C before adding 600 µl chloroform (Merck). The contents of the tube were mixed gently and centrifuged at 13000 rpm in microcentrifuge for 5 min. After centrifugation the upper aqueous phase was transferred into a new microcentrifuge tube and mixed again with 600 µl chloroform, centrifuged at 13.000 rpm and the aqueous phase transferred to a new tube. DNA was precipitated as described in Section 2.3.6 and stored at –20°C.

2.3.2 Plasmid DNA isolation

Plasmid DNA was isolated from *E. coli* overnight cultures using either a QIAGEN plasmid minikit (Qiagen, Hilden, Germany) or a Bio-Rad Quantum Prep® plasmid miniprep kit (Bio-Rad Laboratories) commercial kits according to the manufacturers' instructions.

2.3.3 Isolation of DNA from agarose gel

DNA products from restriction digest or PCRs were isolated using a QIAEX II Gel extraction kit (Qiagen) according to the manufacturer's instruction.

2.3.4 Purification of PCR products

PCR products were purified either by gel purification (Section 2.3.3) or using a QIAquick PCR purification Kit (Qiagen) according to the manufacturer's instructions.

2.3.5 Precipitation of DNA using n-butanol

For electroporation of *E. coli* with DNA ligations, DNA was precicipated with n-butanol to eliminate salt from ligation buffer so as to eliminate electrical arcing in cuvettes. This method was modified from Tillett and Neilan (1999). Generally, after DNA ligation, 1 ml n-butanol was added to the ligation reaction (10 μl), mixed on a vortexer for 30 seconds and then centrifuged for 1-2 min at 13,000 rpm using an Eppendorf centrifuge 5415R. The liquid was discarded by decantation and liquid traces removed by pipetting. The DNA pellet was dried either at room temperature for 5-10 min or in a 37°C room for 2 min before resolved in sterile water.

2.3.6 Precipitation of DNA using isopropanol

To precipitate DNA by isopropanol, the DNA solution was mixed with one volume of cold isopropanol (BDH), mixed by inversion and left to stand on ice for 5-10 min. The sample was centrifuged at maximum speed for 2 min in a microcentrifuge and the liquid decanted off. The pellet was washed three times with 1 ml of 70% ethanol (BDH). The ethanol was decanted off and the sample left to air dry. Once dry, the sample was re-suspended in an appropriate volume of TE buffer or sterile water.

2.4 Agarose gel electrophoresis of DNA

SeaKem® LE Agarose (Cambrex Bio Science, Rockland, ME), at a concentration best suited for the desired separation, was melted in TBE buffer (Appendix A2.1) and set in a gel chamber. Mini or overnight horizontal gels were used to separate DNA fragments by electrophoresis in TBE buffer at 80-100 V or at 20 V for overnight gels. DNA samples were mixed with ¼ volume of gel loading dye (Appendix A2.1) before loading into the wells. The gels were stained in ethidium bromide solution (1 µg/ml in milli-Q water) on a shaking platform for 10-20 min and rinsed in milli-Q water. Bands were visualized on a UV transilluminator and documented using a Gel Doc (BioRad) and the Quantity One 4.4.0 basic software (BioRad). The DNA fragment sizes or concentrations were determined in comparison to known size markers such as the 1 kb plus ladder (Invitrogen), *Hin*dIII digested λ DNA or the Low Mass Ladder (Invitrogen).

2.5 DNA quantification

DNA-samples were quantified using a comparison to the staining intensity of the Low Mass Ladder (Invitrogen) bands on an agarose gel (Section 2.4) for an approximate estimation of DNA quantity.

Accurate quantification of DNA was accomplished by fluorometric quantification of DNA using a 'Hoefer DyNA Quant 200' (Amersham Biosciences) fluorometer according to the manufacturer's instruction. Alternatively DNA was quantified using a Nanodrop® ND-1000 spectrophotometer and software V 3.1.0 (Nano Drop Technologies Inc., Wilmington), as recommended by the manufacturer.

2.6 Ligations

Standard ligations of PCR products were performed using the pGEM-T easy and pGEM-T vectors (Promega, Appendix A3), according to the supplier's manual.

Ligations into other plasmids were performed in a 20 µl reaction containing 2µl of 10x ligation buffer (Roche), 0.5-1 µl T4 DNA ligase (Roche), 20 ng vector and a 2-3 fold molar excess of insert DNA. Ligation mixtures were incubated at 4°C overnight.

PCR products were purified prior to ligation by either PCR column (Section 2.3.3) or gel extraction (Section 2.3.4). Restriction fragments used in ligation reactions were purified using gel extraction. Ligation reactions were purified by either isopropanol or n-butanol precipitation and transformed into suitable *E. coli* strains (Section 2.1.2) for plasmid propagation.

2.7 Restriction endonuclease digestion of DNA

Restriction endonuclease digestion was carried out in the commercial buffer recommended by the manufacturer. A typical digestion reaction of plasmid DNA or PCR products contained an excess of enzyme (1-2 units of restriction enzyme/ µg DNA) and was performed in a water bath set to the recommended temperature for 1-3 h. A small aliquot of the digested DNA was checked by agarose gel electrophoresis.

For Southern analyses restriction endonuclease digestion of genomic DNA (gDNA) was performed overnight. Each reaction contained 3-8 µg gDNA and 10 units of enzyme. The digestion was performed overnight at the temperature recommended by the enzyme manufacturer.

2.8 DNA sequencing

Sequence data were generated from purified PCR fragments or purified plasmids. DNA sequencing was carried out by the DNA analysis Service at the Allan Wilson Centre, IMBS, Massey University using an ABI Prism BigDye^{TM} Terminator cycle sequencing ready reaction kit and an ABI3730 Prism Genetic Analyzer (Applied Biosystems, Foster City, CA) and analysed using the Vector NTI software.

2.9 Transformation of *E. coli*

Competent *E. coli* cells were transformed either by electroporation or by a chemical transformation. Competent *E. coli* were prepared by Jin (2005) or S. Zhang from overnight cultures of *E. coli* strains Top10 or XL-1 blue.

For transformation by electroporation, an aliquot (40 µl) of competent *E. coli* cells was mixed with 2 µl ligated DNA and the mixture was transferred into an ice cold 0.2 cm cuvette and pulsed at 25 µF, 2.5 kV and 200 Ω pulsed using a Gene Pulser (Bio-Rad). The cells were immediately suspended 1 ml of non-selective LB broth, incubated at 37°C for 1 hour and then plated at suitable dilutions onto selective LB Agar plates.

Chemical transformation was achieved by mixing 200 µl of competent *E. coli* cells with 2 µl ligated DNA. The mixture was left on ice for 40 min, subsequently heat shocked at 42°C for 2 min, placed on ice for 2 min and 1 ml non-selective LB broth was added. Following incubation at 37°C for 1 h, *E. coli* cells were plated onto appropriate selective LB agar plates and incubated overnight at 37°C.

For standard ligations (pGEM-T easy, pGEM-T) a blue/white selection was used for initial screening of transformants and positive transformants were confirmed by PCR (Section 2.10), DNA digestion (Section 2.7) or, where appropriate, by sequencing (Section 2.8).

2.10.1 Polymerase chain reaction (PCR) analyses

All PCRs were carried out in an Eppendorf Gradient Mastercycler® t (Eppendorf, Hamburg, Germany). Oligonucleotide primers used in this study are listed in Table 2.2. Primers were synthesized by Invitrogen or Sigma Genosys. Stock concentrations of 200 µM were made and working stocks for PCR use were further diluted to 10 µM. All primer stocks were stored at -20°C.

2.10.1 PCR primers

Table 2.2: Primers used in this study

Primer	sequence (5'-3')	Reference	use	amplified gene
dotA-egfp connect	GACGGTGGTGCTTTCCGAATGGTGAGCAAGGGCGAG	This Study	construction of fusion vector pR260	P*dotA::dotA::egfp*
egfp-dotA connect	CTCGCCCTTGCTCACCATTCGGAAAGCACCACCGTC	This Study	construction of fusion vector pR260	P*dotA::dotA::egfp*
pdotA fusion	GAACAGCCCGGGAGATTTGG	This Study	construction of fusion vector pR260	P*dotA::dotA::egfp*
TtrpC fusion	ATACCCGGGTTACTTGTACAGC	This Study	construction of fusion vector pR260	P*dotA::dotA::egfp*
TUB2	CGAAGGTAGACGACATCTTGAGAC	(Bidlake 1996)	control PCRs gDNA	*tub1*
TUB3	TCCAGGTCAGCTCAACAGTGATC	(Bidlake 1996)	control PCRs gDNA	*tub1*
pdotA-fw	ACGGTCATGATGGGAGATGAGGTGGTGT	this study	pR242 construction, southern probe	P*dotA*
pdotA-rev	GTCTCTTAAGAATCTTCGTCGCTCAGCT	this study	pR242 construction, southern probe	P*dotA*
rt Cyp fwd I	CGATGCTGCGGACGTTGA	This Study	real time RT-PCR	*cypA*
rt Cyp rev I	CGTGTGGTCGAATGTCTG	This Study	real time RT-PCR	*cypA*
rt ddhA fw I	AGTCTTCCAAACTCTCGTCC	This Study	real time RT-PCR	*ddhA*
rt ddhA rev I	ATCCCGCATCCGCTC	This Study	real time RT-PCR	*ddhA*
rt DotA fw I	CTGGTGATGAATTCGACCG	This Study	real time RT-PCR	*dotA* cDNA
rt DotA rev I	AAGCACCACCGTCAATAC	This Study	real time RT-PCR	*dotA*
rt DotB fw I	CCCAGGCTTGAACAGCA	This Study	real time RT-PCR	*dotB*
rt DotB rev I	GATTGTTCGCCACGGAGT	This Study	real time RT-PCR	*dotB* cDNA
rt DotC fw I	GCTTCTTCATCATCGGCG	This Study	real time RT-PCR	*dotC* cDNA
rt DotC rev I	TGGTCCGTTGCCGATAC	This Study	real time RT-PCR	*dotC*
rt DotD fw I	AGCCCTCAACATGGACA	This Study	real time RT-PCR	*dotD*
rt DotD rev I	GTGGAGTAGCCGCCTAT	This Study	real time RT-PCR	*dotD*
rt EpoA Fw I	GCCGGCGAACATGAAAG	This Study	real time RT-PCR	*epoA*
rt EpoA rev I	GACCTCCACTCTCATGTCT	This Study	real time RT-PCR	*epoA*
rt MoxA fw I	AGGATGTTGGTGGAACG	This Study	real time RT-PCR	*moxA* cDNA
rt MoxA rev I	ACCAGAGTTTCCGGCTA	This Study	real time RT-PCR	*moxA*
rt OrdA fw I	ACTACAGCCATCTCGACAT	This Study	real time RT-PCR	*avfA*
rt OrdA rev I	GCCAAACTCACCATAACGC	This Study	real time RT-PCR	*avfA*
rt pksA fw I	CATTATGTCGTCCGAGCAC	This Study	real time RT-PCR	*pksA* cDNA
rt pksA rev I	CGAACAGAACTACCGACC	This Study	real time RT-PCR	*pksA*
rt TUB fw I	CCGGCGTGTACAATGG	This Study	real time RT-PCR	*tub1* cDNA
rt TUB rev I	CATGCGGTCTGGGAAC	This Study	real time RT-PCR	*tub1*
SZDP-6	AAGCCGTAGAACGCGAGTAG	(Zhang *et al.* 2007)	real time RT-PCR	*DS2*

SZDP-124	ACCTGACGAACCAGATCTTG	(Zhang *et al.* 2007)	real time RT-PCR	*DS2*
SZDP-125	TTGTCTGCTGCCATCCCATC	(Zhang *et al.* 2007)	real time RT-PCR	*DS14*
SZDP-114	TGGCAGAGGCGCTGAAGG	(Zhang *et al.* 2007)	real time RT-PCR	*DS14*
SZDP-117	GCCATATGTCATTGCTATGCGT	(Zhang *et al.* 2007)	real time RT-PCR	*DS25*
SZDP-118	TCTTGCGGTCGACACCTTGG	(Zhang *et al.* 2007)	real time RT-PCR	*DS25*
rt VBS fw I	CCGAGCCACAAGAGGG	This Study	real time RT-PCR, Northern probe	*vbsA*
rt VBS rev I	CGGGTGAATGGGCTGA	This Study	real time RT-PCR, Northern probe	*vbsA* cDNA
151R1300comp	CGTCTATGGCCTGAACTGTC	(Monahan 1998)	RT-PCR, Northern probe	*dotD*
151Rconep	CGAACGTCGAAGACATTCAAC	(Monahan 1998)	RT-PCR	*dotD*
152f844comp	AGCATGGCCAACCATGGATAC	(Monahan 1998)	RT-PCR, Northern probe	*dotB*
152fep	GTCACGCTGTAGAAGGACTTG	(Monahan 1998)	RT-PCR, Northern probe	*dotB*
MF4151p4	AGACCAGCAGGCAGATGACAG	(Monahan 1998)	RT-PCR	*dotC*
pMF4152ep2	CTATCATTGTCGCTTCGTAACG	(Monahan 1998)	RT-PCR	*dotC*
RTCexon	GATGCAAGGAAGCAGACCAC	(Monahan 1998)	RT-PCR, Northern probe	*dotA*
RT DotA fw	ATCGGTGATGAATTCGACCG	This Study	RT-PCR; Northern probe	*dotA* cDNA
RT DotC fw	GTGTGACCTGGAGATGGTGC	This Study	RT-PCR	*dotC*
RT DotC rev	TGTGCCGGTTGCGATATCGC	This Study	RT-PCR	*dotC* cDNA
RT DotD rev	GCATAGATGATGTGGGTGCG	This Study	RT-PCR, Northern probe	*dotD*
RTFexon	AACAAAGTCGTCGAGACCATC	(Monahan 1998)	RT-PCR	*dotA*
rt mox dot fw	GTTTGCTTTGAACCCTGACTG	This Study	RT-PCR	*moxA* cDNA
OT R162 A rev4	GCCTTGGATTGGATGGGTACA	(Teddy 2004)	RT-PCR	*epoA*
rt epo dot fw	GACCTCCACCATCTCTGC	This Study	RT-PCR	*epoA* cDNA
mox dot 3' end	ACTACCAGAGTTTCCGGCTATCA	(Teddy 2004)	RT-PCR	*moxA*
cyp dot fwd	GCTGATGAACGCCATCATTGACG	This Study	RT-PCR	*cypA*
cyp dot rev	CTCGAAGAAGTGAGCAACGGC	This Study	RT-PCR	*cypA*
pks CB2	TGAAGAAGTATGTCGCCG	(Seconi 2001)	Northern probe	*pksA*
dmo F2	GTCTTCGATGACGCGGAGG	(Seconi 2001)	Northern probe	*pksA*
JSdkr1C	TCCTGCCCATGGTGCGA	(Seconi 2001)	sequencing	*PdotA*
egfp fwd	AGGAGCGCACCATCTTCT	This Study	sequencing screening	*egfp*
egfp rev	AGAAGATGGTGCGCTCCT	This Study	sequencing screening	*egfp*
pPN81 2003 fw	GTCTGGACCGATGGCTG	This Study	sequencing screening	plasmid
pPN81 2978 rev	TGTCAACTCCGGAGCTGA	This Study	sequencing screening	plasmid
SP6	TTTAGGTGACACTATAGAATAC	Promega	sequencing screening	plasmid
T7	TAATACGACTCACTATAGGGCGA	Promega	sequencing screening	plasmid
rt eGFP fwd I	GCCATGCCCGAAGGCTA	This Study	Southern probe	*egfp*
rt eGFP rev I	CATGCCGAGAGTGATCCC	This Study	Southern probe	*egfp*
153Fep	GTCGACGGACATTATGGGAGATG	(Monahan 1998)	gDNA sectoring	*dotA-dotB*
152Fconep2	TCTTGTCGACGACGGACTTG	(Monahan 1998)	gDNA sectoring	*dotA-dotB*
3' dotB7670fwd	CCGACATGGTGACAGTATACTCCAA	(Jin 2005)	gDNA sectoring	*dotC-dotB*
dotC8888fwd	GTATCCTGCTCGTCAGCAAGACTGA	This lab	gDNA sectoring	*dotC-dotB*
OT R162 A rev1	TTCTCGACAACAATCGTCCATA	(Teddy 2004)	gDNA sectoring	*avfA-epoA*
OT R162 A fwd1	GTCGCCGACAATGCCTCCAA	(Teddy 2004)	gDNA sectoring	*avfA-epoA*
8forwd4	GATTGTCGACGATGGATGTG	(Teddy 2004)	gDNA sectoring	*pksA-DS19*
3'dotD12476fwd	CGAAGCCAACACGACCAATCTCCAA	This lab	gDNA sectoring	*dotD-DS9*
SZDP-60	CAACCAGATCGTGTAGACAG	(Zhang *et al.* 2007)	gDNA sectoring	*dotD-DS9*
SZDP-91	ATCGCTGTGGCAATTGGCAT	(Zhang *et al.* 2007)	gDNA sectoring	*hexA-DS9*
SZDP-65	GTGTGAGTTACAGGCTGAGT	(Zhang *et al.* 2007)	gDNA sectoring	*hexA-DS9*
SZDP-14	GTGTCGGCCAGAACATGCAA	(Zhang *et al.* 2007)	gDNA sectoring	*vbsA-DS14*
SZDP-45	GATTGTCGACGATGGATGTG	(Zhang *et al.* 2007)	gDNA sectoring	*pksA-DS19*
SZDP-23	GGCAGCTTCGTTCTATCCTA	(Zhang *et al.* 2007)	gDNA sectoring	*vbsA-DS14*

Positions where primers connect two exons are underlined

2.10.2 Standard PCR

A standard PCR contained 5 ng genomic DNA, 0.5 U Taq polymerase (Invitrogen), and final concentrations of 1 x Taq polymerase buffer (Invitrogen), 50 μM each dNTP, and 0.2 mM each primer in a volume of 25 μl. The PCR cycling conditions were 94ºC for 2 min followed by 30-35 cycles of 94ºC for 30 s, annealing temperature according to primer for 30 s, 72ºC for 1 min/kb of the expected product, followed by a final step of 72ºC for 4 min.

Proof reading Taq polymerase was used in the PCR's to construct vector pR241 and pR261. The PCR reactions (50 μl) contained 1 unit KOD HotStart DNA polymerase (Novagen, Toyobo), 1 x PCR buffer provided by the manufacturer, 1.5 mM $MgSO_4$, 0.4 mM of each primer, and 50 μM of each dNTP. Thermocycle conditions were as described above, but 1U recombinant Taq polymerase was added after the final step of 72ºC for 4 min and incubated for 2 min at 72ºC in order to create A-overhangs for subsequent ligations.

2.10.3 Colony PCR

For screening of *E. coli* transformants, colonies were picked-up from selective plates and resuspended in a 25 μl PCR reaction and the PCR cycling conditions were as for the standard PCRs but the first step of 94ºC was extended to 4 min.

2.10.4 Real time PCR analyses

Analysis of gene transcription was assessed by relative quantitative RT-PCR using the Faststart DNA Master^{PLUS} SYBR Green I system (Roche Applied Science). Real time PCR reactions were carried out using a LightCycler® 2.0 and software version 3.5. Analyses were performed using the RelQuant software (Roche). The cDNA templates for the PCR reactions were generated as described in Section 2.10.6.

Each reaction was performed in a 10 µl volume, containing 2 µl template and 8 µl proprietary PCR mix. The PCR mixed contained 2 µl Faststart DNA Master^{PLUS} SYBR Green I system and 500 nM each reverse and forward primers in PCR grade water.

Where possible primers were designed to connect two exons (Table 2.2) to avoid any amplification of possible contaminating gDNA.

The PCR program used for all reactions had an initial step of 95°C for 10 min to activate the Faststart Taq DNA Polymerase. Reactions were subsequently subjected to 45 cycles of PCR (10 s of 95°C, 10 s of 59°C, 16 s of 72°C and 5 s at 84°C). The amplified products were detected every cycle during the 84°C step to negate effects of primer dimer accumulation on fluorescence levels.

A melting curve program followed the PCR as follows: the PCR products were heated to 95°C then immediately decreased to 65°C for 15 s and then gradually increased up to 95°C at a rate of 0.2°C/s with constitutive fluorescence acquisition.

2.10.4.1 Creation of standard curves and coefficient files

For real-time RT-PCR analyses a coefficiency corrected calibrator-normalized relative quantification analysis was used as described in the Roche Applied Science Technical Note No. LC 13 (Sagner & Goldstein 2001). Therefore standard curves for each gene of interested were generated. The gene specific cDNA was synthesized as described in Section 2.10.6 using primer pairs which were used in the successive real time PCR reaction for each gene. The PCR product was purified as described in Section 2.3.3 and diluted in a tenfold dilution series over 5 orders of magnitude. Dilutions of the cDNA were used in the light cycler reaction to generate standard curves for each gene and primer pair, with triplicate reactions for each dilution. The data generated by the real time PCR were saved and processed in the RelQuant 1.1.1 software (Roche), according to the manual, to generate coefficient files.

The standard curves were used to create fit coefficients using the RelQuant 1.1.1 software. The software compares the efficiency of the PCR reaction of the target (gene of interest) and reference (control gene, which is constant for all samples) at different concentrations of the template. The coefficient files were used in the subsequent gene

expression analyses to correct for differences in the efficiency of the target and reference reaction. The only classical housekeeping gene known of *D. septosporum* is the β–tubulin gene *tub1*. Initial trials suggested that the expression of *tub1* is not constant as needed for a reference gene. Therefore the 18S rDNA was used as an endogenous reference gene in this study. Although the concentration of 18S rRNA is higher than that of mRNA in the cells it appeared to be an appropriate reference gene in *A. parasiticus* (Chang *et al.* 2004). As the gene expression studies were performed with pure cultures of *D. septosporum*, it was decided to use the universal fungal rRNA primers NS7 and NS8 (White *et al.* 1990). All real time PCR products of target genes were designed to have a similar amplification size as the NS7/NS8 product (~400bp). Primers were designed using the Probe Design Software 2.0 (Roche). Example data are shown in Appendix A5.

2.10.4.2 Gene expression analyses

Expression of the dothistromin genes relative to the 18S rDNA was determined in duplicate for each of three biological replicates. Expression of *tub1* was included in the expression experiments to show that the calculated expression levels did not result from variations in the level of 18S rDNA. Samples were analysed in triplicate from a 10-fold or 100-fold dilution of the original cDNA. Two technical replicates of three independent samples (biological replicates) were used for calibrator-normalized relative quantification analysis. Levels of gene expression in each sample were calculated against the endogenous 18S rRNA gene, as a reference.

In each light cycler run a calibrator for the 18S rDNA and for the target gene were included in duplicate. The calibrators for target and reference gene had the same concentration for each reaction of one experiment. The differences between the target and reference calibrators were used to normalize each reaction by correcting for differences between reactions (as shown below).

$$\text{Normalized Ratio} = \frac{\text{conc. target (sample)}}{\text{conc. reference (sample)}} : \frac{\text{conc. target (calibrator)}}{\text{conc. reference (calibrator)}}$$

2.11 Southern blotting and hybridisation

Southern blotting and hybridisation were performed with standard methods (Sambrook *et al.* 1989), using both radioactive and DIG-dUTP labelling.

2.11.1 Probe labelling

2.11.1.1 Probe labelling with Digoxygenin (DIG)-11-dUTP

DNA probes were labelled with Digoxygenin (DIG)-11-dUTP using a DIG-random priming labelling kit (Roche) according to the manufacturer's instructions. Alternatively DIG labelling of probes was achieved by PCR, performing a standard PCR, but with 200 μM of each dATP, dCTP, and dGTP, 133 μM dTTP and 67 μM (DIG)-11-dUTP in the PCR reaction.

2.11.1.2 Radioactive labelling

Radioactive probes were labelled using the high prime kit (Roche) and [α-^{32}P]dCTP (3,000 Ci/mmol, Amersham) according to the manufacturers' instructions. After labelling, the unincorporated isotope was removed using a Quant G50 Micro Column (Amersham). The labelled probe was denatured in boiled to denature the DNA before it was added to the pre-hybridised blot.

2.11.2 Southern blotting

The DNA blotting method used was based on that of Southern (1975). DNA was prepared using the CTAB method (Section 2.3.1.2) and digested as described in Section 2.6. Separation of gDNA was achieved by gel electrophoresis through a 0.7% TBE agarose gel overnight at 4°C. The was been stained as described in Section 2.4 and photographed alongside a ruler. The gel was depurinated in 250 mM HCl for 15 min to

2.11.4 Signal detection

After washing the radioactive signals were detected by autoradiography on X-ray films (Kodak, Japan) with the required exposure time. X-ray films were developed using a 100Plus^{TM} automatic X-ray processor (All-Pro Imaging Corporation). Alternatively, the membrane was exposed to a BAS Imaging Plate (Fujifilm) and scanned in a FLA-500 Phosphoimager to detect the signal. Resulting images were analysed using the software Image Gauge ver4.4 (Fujifilm).

For chemiluminescent detection of DIG-labelled blots the membrane was first washed in buffer I (Appendix A2.4) for 2 min and then incubated in buffer II (Appendix A2.4) for 30 min, before incubating in the antibody solution (Appendix A2.4) for 30 min. Membranes were then washed twice in buffer I for 15 min and equilibrated in buffer III (Appendix A.2.4) for approximately 5 min. The wet membrane was placed onto acetate paper with the DNA side facing up. CSPD ready-to-use lumigen (Roche) was dispensed over the surface of the membrane and a second acetate sheet was placed over top. The CSPD lumigen was spread evenly and gently over the surface and left for 5 min at 15-25^{o}C. The excess liquid was squeezed out, the edge sealed and the membrane incubated at 37^{o}C for 15 min. Detection of the luminescence signals was then performed as described above.

2.11.5 Removal of signals

Membranes that were radioactively probed and hybridised several times were stripped of their signals by washes in boiling 0.1% SDS.

DIG labelled blots were washed thoroughly in double distilled water, then washed twice for 15 min at 37^{o}C in 0.2M NaOH + 0.1% SDS. Membranes were rinsed in 2 x SSC.

facilitate capillary transfer of high molecular weight DNA onto the membrane. The DNA in the gel was denatured in denaturing solution for 30 min before being washed twice for 20 min each time in neutralisation solution. The gel was then washed in 2x SSC and placed on the blotting apparatus as described in Sambrook et al. (1989). The DNA was transferred to a Hybond-N^+ membrane (Amersham) overnight. After blotting, the DNA was fixed on the membrane by UV irradiation of 120,000 μJoules/cm^2 using a Cex-800 UV-crosslinker (Ultra-Lum Inc.)

2.11.3 Southern hybridisation

2.11.3.1 Radioactive hybridisation

When using radioactive labelled probes, hybridisation was performed in a glass hybridisation tube in a Bachofer hybridisation oven. Membranes were prehybridised for two hours at 68°C with 20-30 ml hybridisation solution (Appendix A2.4). After pre-hybridisation about half of the volume was decanted and the radioactive labelled probe added, followed by overnight hybridisation. Subsequently, membranes were washed 3-4 in 2 x SSC + 0.1% SDS for 20 min each time, until only weak overall radioactivity on the membrane was detected using a Geiger counter.

2.11.3.2 Hybridisation with DIG-labelled probes

If a DIG-dUTP labelled probe was used, hybridisation was performed in a glass hybridisation tube in a Bachofer hybridisation oven overnight at 42°C. The probe was denatured by boiling for 5 min and immediately plunged into ice water. The probe was added to the blot which was pre-hybridised with hybridisation buffer (Appendix A2.4) at 42ºC. After hybridisation, excess probe was washed off the blot with 2 x SSC + 0.1% SDS at room temperature for 10 min, followed by two washes in 0.2-0.5 x SSC + 0.1% SDS at 68°C for 15 min.

2.12 RNA manipulations

2.12.1 RNA extraction

Mycelia for RNA extraction were snap frozen in liquid nitrogen and stored at -80°C until required. RNA was extracted using the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen) with a single modification to the manufacturer's protocol for plant RNA extraction. After addition of the lysis solution, the lysate was passed through a 1 ml syringe with an 18-gauge needle six times. The extracted RNA was quantified using a Nanodrop and subjected to DNase treatment using the TURBO DNA-free™ kit (Ambion) following manufacturers instructions.

RNA was purified after DNase treatment either using the Turbo DNase free kit or by precipitation. In the latter case two volumes of chloroform were added, mixed and centrifuged for 10 min. The RNA in the aqueous phase was precipitated by adding 1/10 volume of 3 M sodium acetate (RNase free) and 2 volumes of 100% ethanol, being stored at –80°C for 10 min and centrifuged at 13,000 rpm for 10 min. The pellet was washed twice with 70 % ethanol, air-dried and re-suspended in RNase free water.

The purity and concentration of RNA were determined using a Nanodrop and the quality of RNA was determined by SDS gel electrophoresis (Section 2.12.2).

To determine whether all contaminating DNA was removed by the DNase treatment, the RNA was used as a template in a PCR reaction with fungal primers TUB2 and TUB3. For the GFP expressing strains FJT24 and FJT26 primers rt-egfp-fwd1 and rt-egfp-rev1 were used. A non-template control and a reaction containing gDNA as positive control, were set up to ensure the reaction was not contaminated with other DNA, and that the PCR worked under the conditions used. PCR products were visualized as described in Section 2.4. If the RNA template reactions did not show a product it was used to generate cDNA (Section 2.12.6).

2.12.2 SDS/Agarose gel electrophoresis of RNA

An aliquot of the RNA was mixed with SDS loading dye and separated through a 1.5% agarose gel containing 0.3% SDS in TBE buffer at 5-8 volts/cm. After electrophoresis, the agarose gel was stained in ethidium bromide for 10-20 min and visualized under UV light. The RNA quality was estimated by the presence and sharpness of the 28s and 18s RNA bands. This method was used as a quality check for RNA used in cDNA synthesis and RT-PCRs.

2.12.3 Formaldehyde gel electrophoresis and Northern blotting

For Northern analyses total RNA (2-5 µg per sample, with equal amount for each sample of the same blot) was separated in a 1.2 % formaldehyde gel. A 150 ml formaldehyde gel was prepared by melting 1.8 g agarose in 110 ml DEPC treated H_2O and adding 15 ml of 10 x MOPS buffer (Appendix A2.5) and 25 ml 37% formaldehyde after cooling down to 60°C. RNA sample (4.7 µl) was mixed with a master mix containing 2 µl 10 x MOPS buffer, 10 µl formamide, 3.2 µl formaldehyde (37%) and 0.08 µl ethidium bromide, then incubated at 65°C for 15 min to denature the RNA. Loading dye was added and samples loaded into the wells. The RNA was separated in 1 x MOPS buffer with a voltage of 1-2 volts/cm. The gel was visualized with a ruler alongside the gel. The blotting apparatus was constructed as for Southern blotting (Section 2.8.1), except that the 20 × SSC buffer was treated with DEPC. After blotting overnight, the apparatus was disassembled and the Hybond-N^+ membrane (Amersham) was exposed to shortwave UV light for 3 min to crosslink the RNA to the membrane, then air-dried in the dark.

2.12.4 Northern hybridisation

Hybridisations were performed using radioactively-labelled probes (Section 2.9.1.2) amplified from cDNA. Northern blots were prehybridised in hybridisation buffer (Appendix A2.5) for two hours at 68°C and hybridised with the labelled probes overnight at 68°C. The membranes were washed at room temperature for 20 min in 1 x SSC + 0.1 % SDS, followed by three washes at 68°C in 0.1 x SSC + 0.1 % SDS. Signals were detected as described in Section 2.9.4.

2.12.5 One-step RT-PCR

Gene expression was determined by RT-PCR using gene specific primers and the SuperScript™ One-step RT-PCR with Platinum *Taq* (Invitrogen) according to the manufacturer's instructions using 100 ng of total RNA per reaction. If possible at least one primer per pair was designed to connect two exons to avoid amplification of any contaminating *D. septosporum* gDNA. Reactions were performed in a 25 µl volume. cDNA synthesis was carried out at 50ºC for 30min and directly followed by PCR. Cycling conditions for PCR were 94ºC for 2 min followed by 25 cycles of 94ºC for 30s, 60ºC for 30s, 72ºC for 1 min/kb of the expected product, and a final step of 72ºC for 4 min.

2.12.6 cDNA synthesis

cDNA was prepared from total RNA (Section 2.4.1) using random hexamer primers from the Roche Transcriptor First Strand cDNA Synthesis kit, following the manufacturers instructions. The cDNA was subsequently diluted 10-100 fold and stored at 4°C until required for real-time PCR analysis (Section 2.8.3).

2.13 Quantification of dothistromin

The production of dothistromin was assessed using a competitive enzyme-linked immunosorbent assay (ELISA).

2.13.1 Competitive enzyme-linked immunosorbent assay (ELISA)

The concentration of the mycotoxin dothistromin produced and secreted by *D. septosporum* in liquid culture (Section 2.2.2.2) was determined using a modified competitive enzyme-linked immunosorbent assay (ELISA) described by Jones *et al*. (1993). Solutions used in the ELISA are listed in Appednix A2.6). The principle of the assay is as follows: The solution to be tested is exposed to an antibody of dothistromin conjugated to a peroxidase (10C12). The antibody part of the conjugate binds the free dothistromin in the solution. The samples are then transferred to a microtitre plate covered with a dothistromin-MSA conjugate. All free dothistromin antibodies in the sample solution then bind on the dothistromin of the dothistromin-MSA coat of the plates. This way the peroxidase of the antibody conjugate is fixed on the wells. The plates are washed and leaving only the fixed peroxidase behind while the dothistromin-antibody-peroxidase conjugate that bound to the dothistromin in the sample solution is washed off. The less dothistromin the original sample contained the more peroxidase is left in the well. The peroxidase can then be detected and quantified in an enzyme reaction using a colour substrate of the peroxidase. This way the concentration of dothistromin in culture can be determined by comparison to standards of known concentration.

In all instances, mycelium fragments were removed from the liquid culture by filtration before the ELISA protocol proceeded.

2.13.1.1 Preparation of DOTH-MSA conjugate ELISA plates

Microtitre plates (96 wells, Maxisorp (Nunc, Denmark)) were coated with 100 µl of Doth-MSA conjugate (diluted 1/3000) in 1% phosphate-buffered saline (PBS, Appendix

A2.6), covered with plastic wrap and incubated at 37°C for 3 hours. The microtitre plates were washed five times with 1% PBS including 1% thiomersal (PBST), then the wells were each filled with 400 µl of 1% skim milk powder (SMP) (Pam's Products LtD, Auckland, NZ) in PBST. To block the remaining protein-binding sites on the micro-well surface the plates were incubated for another 3 hours at 37°C, followed by one wash with PBST (400 µl/well). The plates were then stored at 4°C (maximum storage 4 weeks) until required.

2.13.1.2 Preparation of ELISA standards

Standard solutions were prepared by diluting stock dothistromin solution in DMSO. Working standards were then prepared by diluting 1 µl of the standard solutions in 1 ml of modified dilution buffer. To equilibrate the working standards to the sample solutions, the modified dilution buffer contained equal volumes of 2x PBST (plus 2% SMP) and the medium in which the particular fungal cultures were grown.

2.13.1.3 Preparation of samples and pre-incubation

Ten working standards were prepared by adding 1 µl of the dothistromin stock standard to 1 ml of dilution buffer plus broth (equal volume of dilution buffer (2% SMP and 2x PBST) and media the samples were originally cultured in), to an approximate 1000-fold dilution. The dothistromin concentration of the standards was as follows; 1000, 500, 250, 125, 62.5, 31.6, 15.6, 7.8 and 3.9 µg/ml in DMSO as well a zero standard containing DSMO only. These standards were made from a 2-fold serial dilution of a 2 mg/ml stock using DMSO. The working standards were stored in the fridge until required.

For the test samples, 200 µl sample solution was added to 200 µl of working buffer (dilution buffer with 0.2% DMSO added to compensate for the addition of DMSO in the standards), resulting in a 2-fold dilution. If test samples required diluting, this was done using a diluent consisting of an equal volume of dilution buffer and the appropriate medium, plus 0.2% DMSO.

Pre-incubation was performed in another set of non- DOTH-MSA micro-titre plates. 100 µl of labelled peroxidase (initial 100-fold dilution of 10C12 in dilution buffer, followed by a 300-fold dilution; to a final 1/30000 dilution) was placed in each well. To this 100 µl of the test samples or standard mentioned above were added to the appropriate wells. Usually three replicates of each dilution of the test samples were loaded per plate. The plates were then covered with glad wrap and incubated for 1 hour at 37°C.

2.13.1.4 Incubation of samples on DOTH-MSA plates

After the 1-hour pre-incubation 100 µl sample of each well were transferred to a DOTH-MSA plate (Section 2.13.1.1) using a multi-pipette. Prior to this incubation, the DOTH-MSA plates previously prepared were washed once in PBST by filling all wells then inverting the plate to dispose of the PBST. The DOTH-MSA plate was covered with glad wrap and incubated at 37°C for 3 hours, followed by 6 times washing with 400 µl PBST per well.

2.13.1.5 Detection of the peroxidase labelled antibody

For detection of the peroxidase labelled monoclonal antibody, 200 µl of substrate solution was added to each well. The substrate solution consists of 100 ml of 0.2 M Na_2HPO_4 (Merck) including 40 mg of substrate o-phenylene diamine (Sigma), 0.51 g citric acid (BDH) and 40 µl of 30% H_2O_2. The plate was covered with tin foil (as the substrate is sensitive to light) and shaken gently for 30 min to get a colour reaction. The reaction was stopped by adding 50 µl of 4 M H_2SO_4 to each well.

The absorbance of the plate was read using an Anthos Labtec HT2, Version 1.21E ELISA plate reader. The measurement filter was set to 490 nm and the reference filter set to 595 nm.

Comment on the ELISA

During this study it became obvious that the ELISA assay developed for dothistromin was not completely accurate. Intermediate feeding of the *pksA* replacement mutant FJT3 revealed some possible cross-reaction with dothistromin precursors or related metabolites. Also duplicates of samples showed a high level variation. Therefore it was considered that an HPLC assay would be a more sensitive way to determine the dothistromin concentrations in the future. Primarily experiments were done during this PhD but due to technical difficulties the HPLC assay was not optimised for routine use. However the trends observed in the preliminary HPLC assays mirrored in the ELISA assay results. Results of the HPLC experiments are shown in Appendix A4.

2.14 Transformation of *D. septosporum*

Transformation of *D. septosporum* was achieved using a protoplast based transformation developed by Bidlake (1996), as described by Bradshaw et al. (1997; 2006).

2.14.1 Preparation of *D. septosporum* protoplasts

A mycelial plug from approximately 0.25 cm^2 was ground in 1 ml sterile milli-Q water and about 150 µl was used to inoculate 25 ml DB medium in 125 ml conical flasks. The flasks were incubated at 22°C with shaking in an orbital shaker at 160-180 rpm until sufficient growth was achieved (5-7 days). Mycelia were harvested by centrifugation in a sterile centrifugation tube at 5860 x g for 5 min in a GSA rotor. Mycelia were washed 3 times with sterile water followed by two washes in OM buffer (Appendix A2.3).
Fungal cell walls of the harvested mycelia were digested with filter sterilized Glucanex® 200G (10 mg/ml) (Novozymes, Dittingen, Switzerland) in OM buffer for 12-16h at 30°C and shaking at 80-100 rpm. Protoplasts were harvested by filtering through a sterile miracloth into a sterile flask. The mixture was divided into several 30 ml Corex tubes (approximately 10 ml each) and overlaid with 4 ml ST buffer (Appendix A2.3). Samples were centrifuged at 5860 x g (Rotor SS34) for 5 min at 4°C. The protoplasts formed a white layer at the interface of both solutions. The upper and interphase were transferred

to a new sterile tube and washed 4 times by re-suspending in 5 ml STC buffer (Appendix A2.3) followed by 5 min centrifugation at 4°C. The protoplasts were finally re-suspended in 100 µl STC buffer.

Concentration of the protoplasts was determined using a haemocytometer. The protoplast solution was then diluted to a concentration of 1.5 x 10^8 protoplasts/ml in STC buffer. For long-term storage protoplasts were stored at –80°C in aliquots of 80 µl of 1.5 x 10^8 protoplasts/ml mixed with 20 µl 40% PEG 6000 in STC.

2.14.2 Transformation of *D. septosporum* protoplasts

For protoplast transformation 80 µl of 1.5 x 10^8 protoplasts/ ml were mixed with 20 µl 40% PEG 6000 in STC (Appendix A2.3) and about 5 µg DNA and incubated on ice for 30 min. A further 900 µl of 40% PEG 6000 in STC were added, carefully mixed by pipetting and incubated for further 20 min at room temperature. Aliquots of 100 µl were mixed with 5 ml DM top agar (Appendix A1.3) at 50°C and spread onto a 10 ml DMSuc agar (Appendix A1.3) plate. After incubation overnight at 22°C the plates were overlaid with another 5 ml DMtop agar including the appropriate selection antibiotic.

The selection antibiotics hygromycin B (Roche) or phleomycin (Apollo Scientific Ltd., Stockport, UK) were used at final concentrations of 70 µg/ml and 7µg/ml respectively as previously determined to be efficient for selection (Bidlake 1996).

A negative control for transformation (no DNA) was performed with protoplasts only. This was to determine the viability of the protoplasts on unselective medium (by plating 10^{-1} – 10^{-4} fold dilutions). To confirm the efficiency of the antibiotic selection 100 µl undiluted protoplast suspension were plated on selective medium.

2.14.3 Single plasmid transformation

D. septosporum wild type strain NZE7 was transformed using plasmids containing the *hph* gene as a selection marker. Plasmids used for wild type transformation were the *egfp* containing pPN82 (Tanaka *et al.* 2006) and P*dotA*::*egfp* vector pR242, the sGFP

containing plasmid pCT-74 (Lorang *et al.* 2001), and the $DsRed_{express}$ containing plasmid pR237. For each transformation 5 μg plasmid pAN7-1 (Punt & van den Hondel 1992) was used as a transformation positive control and to determine the transformation frequency.

Fungal colonies that grew up first were transferred to a new selective plate containing 70 g/ml of hygromycin B. The successful transformation and expression of the GFP or DsRed was checked by fluorescence microscopy (Section 2.18). Single spore transformants were purified by plating ground mycelia from the colony margin of selected transformants onto DSM agar plates containing 70 μg/ml hygromycin B for selection. After 12 days at 22°C spores were scratched from the mycelia and streaked out on DM-Hyg plates. After 10-12 days' growth colonies were checked for fluorescence and single colony was transferred onto a fresh DM-Hyg plate, grown to a colony of about 2-3 cm diameter and the whole process was repeated with mycelium taken from the margin of this colony.

2.14.4 Co-transformation

For the transformation of *D. septosporum* strains FJT2 (Bradshaw *et al.* 2002; Seconi 2001) and FJT3 (Bradshaw *et al.* 2006; Jin 2005) a different approach was used. Selection with hygromycin was not possible as FJT3 and FJT2 were created by replacing the *pksA* gene or *dotA* gene (respectively) with the *hph* gene. A co-transformation was used to introduce pPN82 into FJT3 and pPN82 and pR261 into FJT2. The plasmid pBC-Phleo (Silar 1995) which contained the $phleo^R$ gene was co-transformed as a resistance marker (Appendix A3). For transformation 3.5 μg pBC-phleo and 3.5 μg GFP vector pPN82 or pR261 were used. As a transformation control 3.5 μg pBC-phleo were transformed into FJT3 and FJT2.

Selection was performed as described in Section 2.12.3 but using phleomycin at a final concentration 7 μg/ml as previously determined to be efficient for selection (Bidlake 1996).

2.15 Phenotype characterisation of *D. septosporum* transformants

2.15.1 Radial growth rates

Radial growth was measures following transfer of agar plugs (Ø 3 mm) from the growing edge of colonies on fresh PDA or DM media plates. Agar plugs in each experiment were taken from colonies of the same age and from the same media for the colonies which were compared to each other. The plates were incubated at 22ºC for up to 45 days. The diameter were measured regularly along two axes at right angles. The average of these two measurements were taken as the diameter of the colony.

2.15.2 Germination rate

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

2.15.3 Sporulation rate

Two mycelial plugs taken from the margins of a fungal colony (opposite sides), using a 3 mm cork borer were placed in 150 µl of 0.1% Tween-20, and vortexed for 1 min. The concentration of conidia was quantified using a haemocytometer. Values were expressed as number of conidia per mm^2 colony surface.

2.16 Competition assays

Petri dishes (∅ 9 cm) containing approximately 20 ml PDA were each inoculated with one agar plug (approximately 2 mm in diameter) of *D. septosporum* mycelium taken from the growing margin of a colony of both *D. septosporum* wild types NZE7/NZE10 and the dothistromin deficient mutant FJT3. After 7-10 days of growth at 22°C a plug (∅ 2 mm) of a competitor species was placed 1.5 cm from the *D. septosporum* growing edge. Two radial growth measurements were made for each colony: from the inoculation point to the growing margin nearest *D. septosporum* and from the inoculation point to the growing margin furthest from *D. septosporum*. Measurements were made at various time points which differed according to the growth rate of the competitor species.

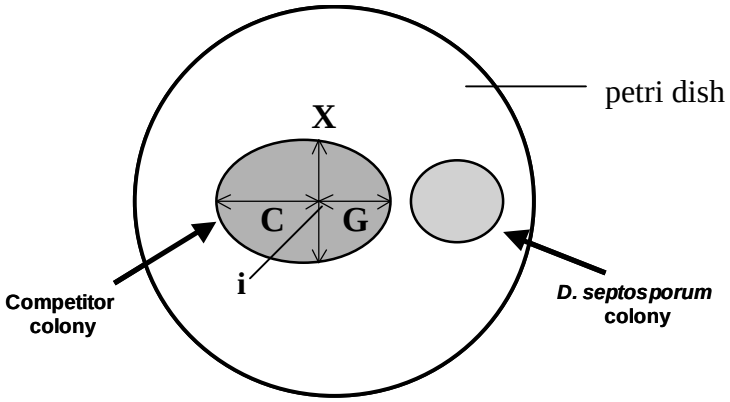

Figure 2.1: Outline of measurements taken for plate competition assays.
Measured were the radii of the competitor colony from the inoculation point (i) towards the *D. septosporum* colony (G) and away from *D. septosporum* (C). X was measured to define the diameter of the competitor colony.

The relative inhibition of growth of each species was calculated according to Wardle and Parkinson (1992), as

$$\mathbf{I = (C-G)/C \times 100}$$

where

I = % inhibition of competitor colony by *D. septosporum* colony

C = growth of competitor colony away from growth zone of *D. septosporum*

G = growth of competitor colony towards growth zone of *D. septosporum*.

2.17 Toxin induction assays

To determine whether competitors induced dothistromin production and/or dothistromin gene expression in *D. septosporum* two approaches were used. Firstly GFP expressed by *D. septosporum* strains FJT24 and FJT26 containing the P*dotA::gfp* gene were analysed in the plate competition assays described in Section 2.16 and GFP expression was monitored by microscopy as described in 2.18. Secondly the potential for dothistromin induction by metabolites secreted by competitors was assessed using "elicitor broths" as described and shown in Appendix A.6

2.18 Microscopy

Colonies on agar media were documented using an Olympus SZX12-RFL3 microscope, with images captured using an Olympus DP70 digital camera and the analySIS software package (Soft Imaging System GmbH). GFP expression was monitored with the excitation and barrier filters set at EX460-90 nm, and EM510-550 nm respectively.

GFP expression of spores and geminating spores was documented using an Olympus IX71 microscope, a Hamamatsu ORCA-AG digital camera and Metamorph 6.3 (Molecular Devices Corp., Downingtown, PA) software. GFP expression was monitored with an EX460-90 nm/EM>510 nm filter set.

2.18.1 Vacuole staining

The red fluorescence stain FM4-64 (Molecular Probes; Invitrogen) was used to stain the vacuoles of *D. septosporum*.

Approximately 0.5 x 0.5 cm mycelial plug from the edge of a growing colony was ground with a sterile pestle in a microcentrifuge tube in 800 µl PDB and incubated on a benchtop shaker for 4 h at 25°C before 5 µl FM4-64 stain (1.64 mM in DMSO) was added. After addition of the stain the mixture was kept in the dark and incubated for another 5 h. Aliquots of 10 µl were put on a microscope slide and FM4-64 fluorescence was detected using the FITC filter settings using an Olympus IX71 microscope, a

Hamamatsu ORCA-AG digital camera and Metamorph 6.3 (Molecular Devices Corp., Downingtown, PA) software.

2.19 Statistical analyses

2.19.1 Single-factor ANOVA

Single factor ANOVA was performed for the growth rate data of *D. septosporum* wild type and transformants, based on the null hypothesis of no difference between growth of all strains, using the Microsoft Excel program. Significant ANOVA data ($P<0.05$) were further analysed by a Tukeys HSD using an online statistic calculator (http://graphpad.com/quickcalcs/posttest1.cfm) to determine pairwise significant differences of each transformant and wild type of *D. septosporum* ($P<0.05$).

2.19.2 Pearson Correlation

Pearson correlation was used to analyze real time PCR data using the online calculator on http://department.obg.cuhk.edu.hk/researchsupport/PearsonCorrelation.asp. The data set analyzed consisted of the means of the gene expression for each time point of each investigated gene. Every single data set was compared to all other data sets and correlation coefficients determined.

2.19.3 T-test

T-tests were used to analyse the data of growth inhibition of competitor species by *D. septosporum*. T-test values were calculated using the unpaired T-test online calculator on www.graphpad/quickcalcs, testing the null hypothesis of no difference in inhibition between competitor challenged with dothistromin producing or dothistromin non-producing strains of *D. septosporum* ($P<0.05$).

Chapter 3: Co-regulation and expression of dothistromin genes in culture

3.1 Introduction

At the beginning of this study two groups of genes that were thought to be involved in dothistromin biosynthesis were known. Those genes were identified on the basis of their similarity to aflatoxin (AF) and sterigmatocystin (ST) genes, from a λ clone library (Bradshaw *et al.* 2002; Bradshaw *et al.* 2006). Using a degenerate PCR approach a further dothistromin gene, *vbsA*, was isolated (Bradshaw & Zhang 2006b; Schwelm *et al.* 2007). One isolated λ clone contains the ketoreductase gene *dotA*, an ortholog of the AF pathway gene *aflM* (*ver-1*) which is adjacent to three other putative dothistromin genes: *dotB*, *dotC* and *dotD* (Table 1.2, Bradshaw *et al.* 2002). The other λ-clone contains the polyketide synthase gene *pks*A, an ortholog of the AF pathway gene *aflC (pksA)* that is clustered with the three putative dothistromin genes *cypA*, *avfA* and *moxA*, orthologs of AF/ST genes. This clone contained also the putative epoxide hydrolase *epoA*, with no homologies to any AF or ST genes (Table 1.2, Bradshaw *et al.* 2006). The *vbsA* gene encodes a homolog of the versicolorin B synthases AflK (AF) and StcN (ST) from *A. parasiticus* and *A. nidulans* respectively. Recent results showed that the *vbsA* is located in a mini-cluster which also contains the AF/ST homolog *hexA* (Figure 1.6, Table 1.2). While *dot*A, *pks*A and *vbsA* have been confirmed to be involved in dothistromin biosynthesis by gene replacement (Bradshaw *et al.* 2002; Bradshaw *et al.* 2006; Zhang *et al.* 2007) the functions of the other genes are predicted based on their amino acid sequences.

In contrast to genes of primary metabolism, secondary metabolite genes tend to be clustered in fungal genomes (Zhang *et al.* 2005). Because of the high similarities between dothistromin genes and the AF/ST genes, it was expected that the identified groups of dothistromin genes form part of a bigger cluster as seen for AF and ST (Zhang *et al.* 2005), but recent results show that this is not the case for the dothistromin genes (Bradshaw & Zhang 2006a; Zhang *et al.* 2007). Instead the identified dothistromin genes

are dispersed with other genes and are grouped in three mini-clusters (Figure 1.6).

In this work three aspects of the dothistromin genes were investigated:

- Although dothistromin genes are not organised in a single non-dispersed cluster it is not known how the genes are distributed in the *D. septosporum* genome. Therefore the chromosomal location was determined by CHEF blot analyses.

- One feature of secondary metabolite gene clusters is co-regulation either by specific or general regulators (Bhatnagar *et al.* 2003; Bok & Keller 2004; Zhang *et al.* 2005). It is of interest to determine if the dothistromin genes are co-regulated despite not being organised in a single non-dispersed cluster.

- As the role of dothistromin in the disease is unknown the study of dothistromin gene expression could also reveal clues to function.

Therefore the co-expression of the putative dothistromin genes and their relation to dothistromin production was determined for two different isolates of *D. septosporum*, NZE5 and NZE7, using RT-PCRs, Northern analyses and real-time RT-PCR.

3.2 Results

3.2.1.1 CHEF blot analyses

A CHEF blot was hybridised with probes from each mini-cluster containing dothistromin genes. The CHEF blots, made by C. A. Young (Massey University) as described in Bradshaw *et al.* (2006).

Probes of the *dotA* and *pksA* gene hybridised with a 1.3 Mb mini-chromosome on a mid-range separation CHEF blot as shown previously (Bradshaw *et al.* 2006, Figure 3.1 A). For a better resolution a low-range separation CHEF blot was hybridised with probes of the *dotA*, *pksA* and *vbsA* gene, each representing one of the three mini-clusters. Probes were generated by PCR (Section 2.10.2) and radioactive labelled (Section 2.11.1.2). Each hybridised with the 1.3 Mb chromosome (Figure 3.1 B). Southern analyses of the *dotA* (Bradshaw *et al.* 2002), *pksA* (Jin 2005) and *vbsA* (Zhang *et al.* 2007) imply all three genes have only a single copy in the *D. septosporum* genome. Therefore the results suggest that all identified dothistromin genes are located on this mini-chromosome.

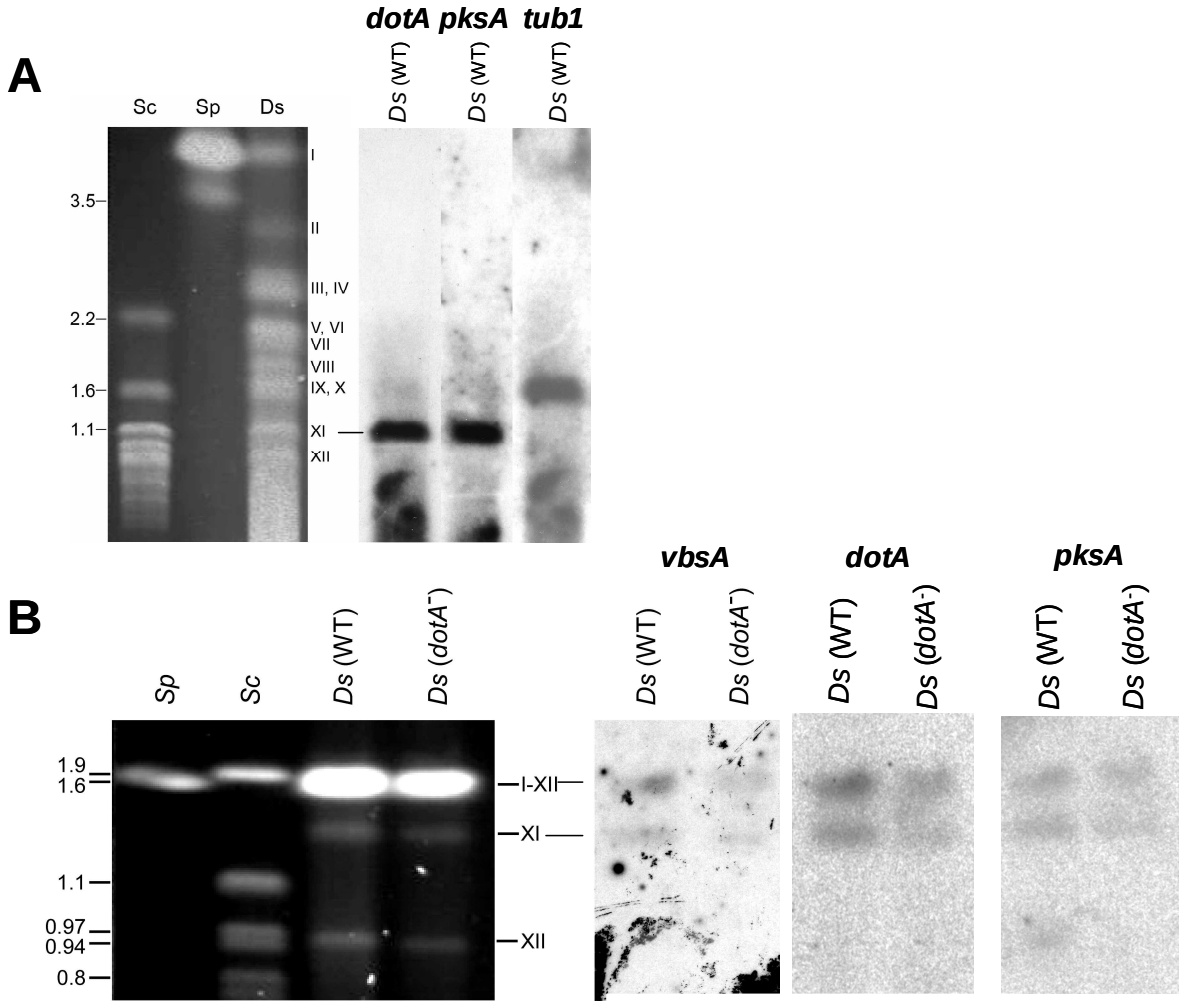

Figure 3.1: CHEF blot analyses.

The CHEF blot membranes, made by C. A. Young, were hybridised with probes for the *vbsA*, *dotA* and *pksA* genes. Separation of chromosomes (left) and hybridization (right). (**A**) The *dotA* and *pksA* gene hybridise with the 1.3 Mb chromosome (XI) from *D. septosporum*. A non-dothistromin gene (X) is shown as comparison. *Schizosaccharomyces pombe* (*Sp*) and *Saccharomyces cerevisiae* (*Sc*) chromosomes were used as size markers, with sizes shown in Mb at the left side of the gel pictures (Figure taken from Bradshaw *et al*. 2006). (**B**) CHEF blot with increased resolution of smaller chromosomes of *D. septosporum* wild type (*Ds* (WT)) and *dotA* mutant (*Ds* (*dotA*$^-$)). For this low range separation 1% Fastlane agarose (FMC) in 0.5 x TBE buffer (89 mM Tris, 89 mM boric acid and 2.5 mM Na_2EDTA, pH 8.2) were used at 150 volts with a switch time of 70 s for 20 h, followed by 80 volts with a switch time of 300s for 48 hours at 14^{o}C. The blot was hybridized with ^{32}P-labeled PCR fragments amplified using primers rt-vbs-fw1 and rt-vbs-rev1 for the *vbsA*, primers RTCexon and RTdotA-fw for *dotA* and primers pksCB2 and dmoF2 for the *pksA* gene (this work). All probes hybridised with the 1.3 Mb chromosome (XI). The top band is probably due to non-specific binding to the accumulated Chromosomes I-X, as no specific hybridisation was seen on other chromosomes in (**A**).

3.2.2 Co-expression of dothistromin genes in culture

The expression of the dothistromin genes was determined in liquid culture to assess if they are co-expressed. Spores of *D. septosporum* NZE5 were used to inoculate liquid media and the dothistromin production and gene expression were determined over a time course (as described in Section 2.2.2.2). RNA was obtained from mycelium pooled from three flasks per time point. Gene expression was determined by one step RT-PCR with, wherever possible, one primer specific for cDNA connecting two exons to prevent amplification of gDNA (Section 2.12.5). This assay was performed for all putative dothistromin genes of the two previously published sequences of mini-clusters 1 and 2 (Figure 1.6, Bradshaw *et al.* 2002; Bradshaw *et al.* 2006). To validate the results obtained by RT-PCR, Northern hybridisation was performed for some of the dothistromin genes.

The dothistromin production occurred early in the growth phase. The highest production of dothistromin (µg DOTH/mg DW) was seen between days 3 and 4, although the amount of dothistromin showed a huge variation between samples from the same day (Figure 3.2). No samples were taken prior to day 3 because the biomass was not sufficient for dry weight and RNA extraction. At day 6, when the growth was still in the early exponential phase, the production of dothistromin decreased significantly and stayed at a lower level for the rest of the growth experiment. The highest concentration of dothistromin in the media (µg DOTH/ml media) was seen at day 9 which is in the mid-exponential growth phase. The concentration of dothistromin in the media decreased thereafter. The expression of the dothistromin genes is low at this growth stage and no new biosynthesis of dothistromin appears to occur. The expression patterns of the dothistromin genes obtained by RT-PCR and Northern analyses mirrored high production of dothistromin at day 3 and day 4 (Figure 3.3), although a delay is seen in the peak of gene expression and peak of toxin concentration in the media. An exception was the *dotC* gene from which bands of similar intensity for each day were obtained by both RT-PCR and Northern analyses, and therefore appeared to be constitutively expressed under these conditions. The results suggest that the dothistromin genes are indeed co-regulated and that their expression is correlated with dothistromin biosynthesis.

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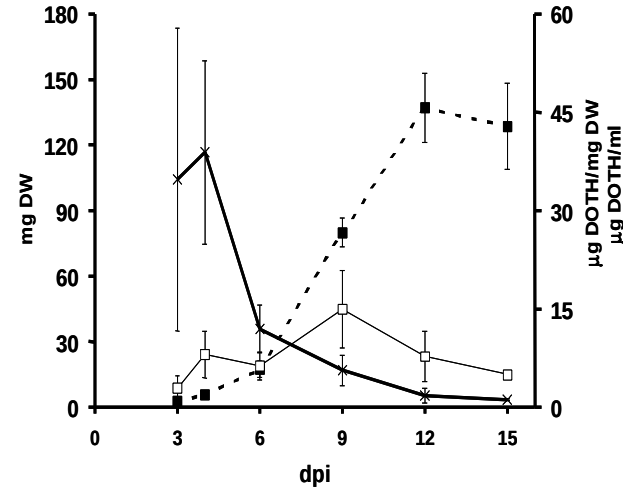
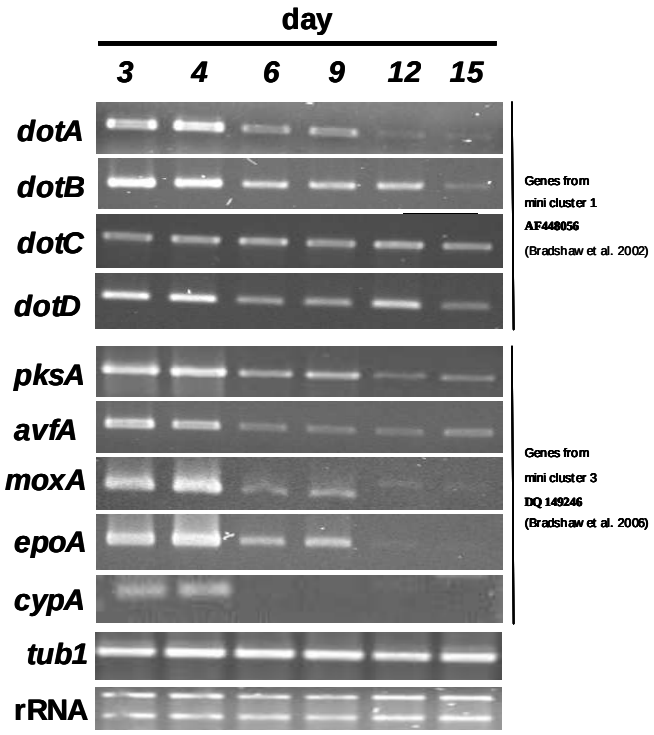


Figure 3.2: Growth and dothistromin production of *D. septosporum* NZE5 in liquid media.

Flasks containing 25 ml DB media were inoculated with approximately 10^6 conidia/ml. Growth (-■-)is shown in mg dry weight (mg DW), toxin production (–x–)in µg dothistromin per mg DW (µg DOTH/mg DW) and dothistromin concentration per ml media (□)µg dothistromin per ml media (µgDOTH/ml) for days after inoculation (dpi). Values are means and standard deviations of three replicates



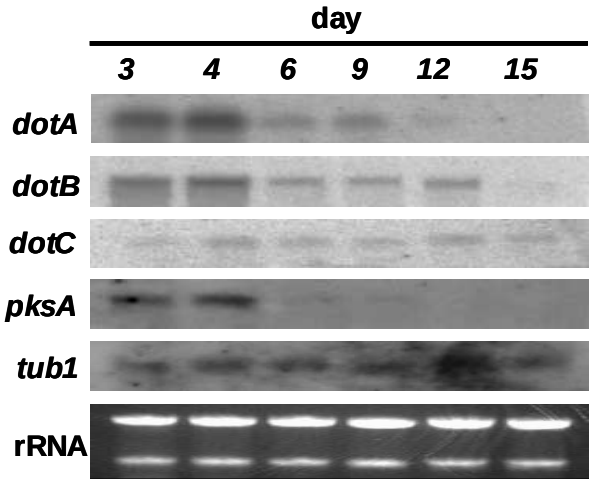


Figure 3.3: RT-PCR and Northern analyses of dothistromin gene expression.

(A) RT-PCR results from mycelium obtained in the experiment of Figure 3.2 are shown. Except for *dotC* and *tub1* all genes show stronger bands at day 3 and 4 compared to later time points. **(B)** Northern analyses confirmed the expression pattern for the selected genes *dotA, dotB, dotC* and *pksA.* The *tub1* gene is included as a constitutive control. The rRNA bands are shown as loading and quality control for the RNA. Primer pairs used for RT-PCR and Northern probes were as follows:

dotA:	RTC exon	RT dotA fw	*dotB:*	152f844comp	152 fep
dotC:	RT dotC fw	RT dotC rev	*dotD:*	RT dotD rev	151R1300comp
pksA:	pks CB2	dmoF2	*avfA:*	OTR162 fwd1	OTR162 rev6
moxA:	RT mox dot fw	mox dot 3' end	*epoA:*	RT epo dot fwd	OTR 162A rev 4
cypA:	cyp dot fw	cyp dot rev	*tub1:*	Tub2	Tub3

For sequences see Table 2.2.

The co-regulation seen in the RT-PCR and Northern results and the unexpected early expression of the dothistromin genes led to the decision to investigate the degree of co-regulation in more detail. RT-PCR and Northern analyses have limitations as statistical analyses are limited and differences in the expression are only detectable if the RT-PCR or Northern hybridisation are not saturated. Therefore the pattern of gene expression for all putative dothistromin genes was analysed by real-time RT-PCR, except for *epoA*. The expression of *epoA* was not determined as melting curve analyses for the *epoA* standards and samples always showed more than one PCR product, despite trying several batches of primers. Southern analyses showed that the *D. septosporum* genome contains only one copy of the *epoA* gene (Jin 2005) and therefore the different melting curves were most likely due to sub-optimal PCR conditions. The time frame of this study did not allow further attempts to optimise the *epoA* real-time PCR assay.

The expression of all other genes was quantified using relative calibrator normalized quantification compared to the levels of 18S rDNA (Section 2.10.4). The expression of the β–tubulin gene *tub1* relative to the 18S rDNA, that was included as a constitutive control, showed no significant change in the level of expression over the time course (ANOVA). Data for each sample were obtained from a duplicated real time RT-PCR with cDNA obtained from RNA from mycelium of three pooled flasks per time point. The results confirmed the highest expression levels of the putative dothistromin genes at day 3 and day 4 and the constitutive expression of *dotC* (Figure 3.4) as seen before by RT-PCR and Northern analyses (Figure 3.3).

Although the dothistromin genes showed highest expression at days 3-4 there was some variation in the expression trends over the whole time course. Therefore the degree of co-regulation of dothistromin gene expression was analysed by pairwise comparison of relative expression levels over time using Pearson correlation, as shown in Table 3.1. Most of the putative dothistromin genes showed high correlations (>0.7) in their pattern of expression levels over time, except for *vbsA*, *dotB* and *dotC* (Table 3.1). The expression of *dotC* showed no significant correlation to the expression of any other genes investigated in this assay. The *dotB* gene only showed significant correlation to the *avfA* gene ($p<0.05$). The *vbsA* gene showed a significant correlation ($p<0.1$) to most of the dothistromin genes except to *dotA* and *avfA*, with the p-value for a significant positive correlation of *dotA* and *vbsA* just exceeding 0.1. Interestingly, in this study, the

expression patterns of genes in mini-cluster 3 have a higher correlation to those of other putative dothistromin genes compared to the genes in clusters 1 and 2; high correlations were seen to five other dothistromin genes. In mini-cluster 1 the *dotD* gene expression was highly correlated to five other putative dothistromin genes. Similar *dotA* gene expression showed high correlation coefficients to the other dothistromin genes. The exception was the gene expression of *dotA* and *vbsA* expression, which were less correlated in this experiment. The expression of the *vbsA* gene in mini-cluster 2 had a lower number of correlated genes. In addition to not showing a strong correlation to the expression of the *dotA* gene, its expression was also not highly correlated to *avfA* of mini-cluster 3 (Table 3.1).

The same cDNA samples were used to determine the expression patterns of recently isolated genes, which are suspected not to be involved in dothistromin biosynthesis but which are neighbouring the putative dothistromin genes (Figure 1.6). Those genes are: *DS2* from mini-cluster 1 which encodes a putative chitin synthase, *DS14* in mini-cluster 2, a putative potassium channel, and *DS25* of mini-cluster 3, a putative amino acid permease. The expression levels of *DS2*, *DS14* and *DS25* and their correlation to the expression of the putative dothistromin were determined to check if non-dothistromin genes in the mini-clusters were co-regulated with the dothistromin genes. *DS14* did not show the same early expression pattern as the dothistromin genes and no correlation to any of the other genes. Likewise the expression of *DS2* did not show the same expression pattern as the dothistromin genes but showed significant correlation to the expression of *tub1* (Table 3.1). Interestingly *DS25* showed significant positive correlation with all co-regulated dothistromin genes except for *avfA* (Table 3.1). The *tub1* gene, which was included as a constitutive expression control, did not show correlation to any of the dothistromin genes.

The expression patterns of the putative dothistromin genes suggest that they are co-regulated. Involved in co-regulation in the AF gene cluster is the regulatory gene *aflR*, which encodes a positive regulator protein for the AF genes (Liu 1998). Putative AflR binding motifs are seen for most of the dothistromin genes (Bradshaw & Zhang 2006b; Zhang *et al*. 2007), as shown in Figure 3.5.

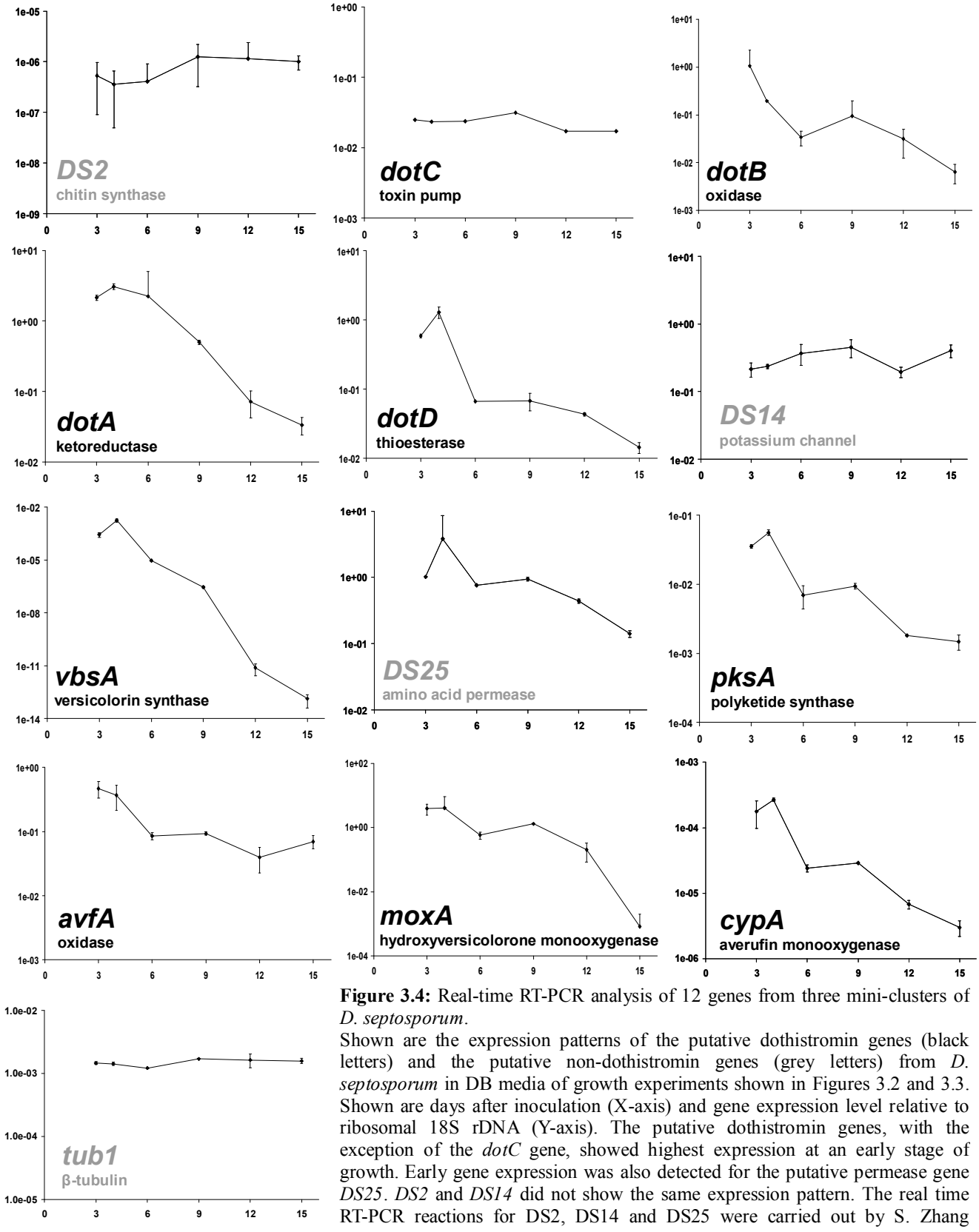


Figure 3.4: Real-time RT-PCR analysis of 12 genes from three mini-clusters of *D. septosporum*.

Shown are the expression patterns of the putative dothistromin genes (black letters) and the putative non-dothistromin genes (grey letters) from *D. septosporum* in DB media of growth experiments shown in Figures 3.2 and 3.3. Shown are days after inoculation (X-axis) and gene expression level relative to ribosomal 18S rDNA (Y-axis). The putative dothistromin genes, with the exception of the *dotC* gene, showed highest expression at an early stage of growth. Early gene expression was also detected for the putative permease gene *DS25*. *DS2* and *DS14* did not show the same expression pattern. The real time RT-PCR reactions for DS2, DS14 and DS25 were carried out by S. Zhang (Massey University). The β-tubulin gene expression (*tub1*) was included as a constitutive control. Each data point is the mean ± standard deviation of two replicate PCR reactions. The error bars do not show equal lengths because of the logarithmic scale of the Y-axis.

In mini-cluster 1 *dotA* and *dotB*, as well as *dotC* and *dotD*, are divergently transcribed. But putative AflR binding sites are located closer towards *dotA* and *dotD* than to *dotB* and *dotC*. The 11 AflR binding sites between the *dotA* and *dotB* coding regions are all within 1489 nucleotides upstream of *dotA* with the first eight within the 642 nucleotides upstream of the *dotA* coding region. The other three binding sites are at least 815 nucleotides upstream of the *dotB* gene. Similarly, all 5 putative AflR binding sites in the intergenic region of *dotC* and *dotD* are within 276 nucleotides upstream of the *dotD* coding region and there is no AflR binding site in the first 1175 nucleotides upstream of *dotC*.

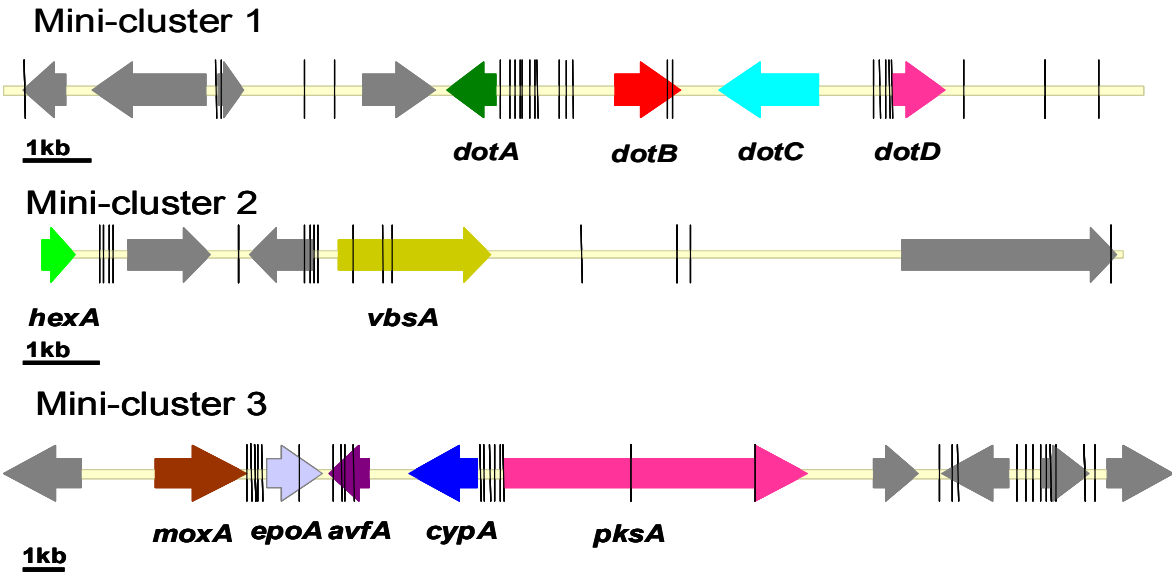


Figure 3.5: Schematic overview of AflR-binding sites in the dothistromin mini clusters.
Shown are the regions of mini-clusters 1-3 (Figure 1.6) which include the putative dothistromin genes. Horizontal arrows show the direction of transcription of identified genes with colored arrows indicating putative dothistromin genes. Vertical lines show putative AflR-binding sites (5'-TCGN_5CGR-3'). Positions of the putative AflR-binding sites are approximately to scale.

The *vbsA* gene contains putative AflR binding sites in both its ORF and upstream region. The *vbsA* gene shows co-regulation with the other dothistromin genes although the upstream AflR binding sites are in the ORF of the neighbouring DS12 gene. In mini-cluster 3, the *pksA* and *cypA* genes show also high correlation in their expression pattern compared with the other putative dothistromin genes. The *pksA* and *cypA* gene share 6 putative AflR binding sites in their intergenic region. Expression of the *avfA* showed high correlation to 5 dothistromin genes, but not to *vbsA*, as seen for the other dothistromin genes. It also was the only gene with a significant correlation to the expression of *dotB* and no significant correlation to the expression of *DS25*. Interestingly

		Mini cluster 1					Mini cluster 2		Mini cluster 3				
	gene	*DS2*	*dotA*	*dotB*	*dotC*	*dotD*	*vbsA*	*DS14*	*pksA*	*cypA*	*avfA*	*moxA*	*DS25*
AflR binding sites		-	**11**	-	-	**5**	**4 (ORF) + 3**	-	**6**	**6**	**4 (ORF)**	-	-
	tub1	**0.892**	-0.754	-0.132	0.033	-0.278	-0.246	0.139	-0.29	-0.293	-0.276	-0.230	-0.260
Mini cluster 3	*DS25*	-0.579	0.760	0.098	0.247	**0.942**	**0.978**	-0.343	**0.899**	**0.879**	0.602	0.797	**6**
	moxA	-0.629	0.784	0.675	0.35	**0.923**	0.786	-0.343	**0.976**	**0.978**	**0.956**	**6**	
	avfA	-0.647	0.731	**0.843**	0.259	0.810	0.615	-0.537	**0.890**	**0.906**	**5**		
	cypA	-0.686	**0.818**	0.545	0.221	**0.981**	**0.888**	-0.536	**0.998**	**6**			
	pksA	-0.68	**0.825**	0.515	0.263	**0.983**	**0.899**	-0.496	**6**				
Mini cluster 2	*DS14*	0.358	-0.349	-0.472	0.387	-0.518	-0.412	-					
	vbsA	-0.587	0.716	0.103	0.066	**0.960**	**4**						
Mini cluster 1	*dotD*	-0.662	0.792	0.377	0.142	**6**							
	dotC	-0.033	0.308	0.248	-								
	dotB	-0.381	0.408	**1**									
	dotA	**-0.936**	**5**										
	DS2	-											

Table 3.1: Pair-wise comparisons of correlation coefficients (Pearson correlation) of gene expression patterns.
The putative dothistromin genes are compared as well as the other *D. septosporum* genes DS2, DS14, DS25 and the β-tubulin gene *tub1*. Shown are the Pearsons product moment correlation coefficients. Statistically significant positive correlations ($p < 0.1$) are shown with a grey background. Values in bold have a p-value of < 0.05. Correlation of *dotA* and *vbsA* is shown in light grey as the p-value just exceeded 0.1. The expression patterns of *dotA* and DS2 were significantly negatively correlated ($p<0.01$) and therefore indicated in white bold letters. Analyses were performed using an online statistics program (http://department.obg.cuhk.edu.hk/researchsupport/PearsonCorrelation.asp). The number in the black box shows the number of significantly correlated dothistromin ($p < 0.1$) genes for each corresponding gene. DS2, DS14, DS25 and *dotB* were assumed to be non-dothistromin genes. The first line shows the number of putative AflR binding sites (5'-TCGN_5CGR-3') upstream of the gene. The AflR binding sites in the intergenic region of *dotA/dotB* and *dotC*/*dotD* are designated to *dotA* and *dotD* respectively (see Section 3.3 and Figure 3.5).

the *avfA* gene does not contain putative AflR binding sites upstream of its ORF. The *moxA* and *DS25* genes were co-regulated with other dothistromin genes despite no putative AflR binding sites in their upstream regions.

In summary, it was shown that the dothistromin genes are co-regulated. The genes in mini-cluster 3 show a higher degree of correlation of expression than the genes in clusters 1 and 2. In addition the highest expression of the genes was seen at early growth stages (Figure 3.3 and 3.4). This was an unexpected finding as secondary metabolites are usually synthesized during late exponential or stationary growth in culture. Therefore further investigations of the onset of the dothistromin gene expression were performed.

3.2.3 Confirmation of early dothistromin gene expression

To confirm the unexpected early onset of expression of the dothistromin genes the gene expression study was repeated with the isolate *D. septosporum* isolate NZE7. All isolates from New Zealand appear to be clonal (Hirst *et al.* 1999) but the NZE5 isolate used in the growth experiments described in Section 3.2.2 had been in culture for over 3 years and therefore it was possible the expression pattern observed could be an adjustment to this. Moreover, NZE5 showed poor sporulation and dothistromin production were low at the time of the experiment. Therefore the newer isolate NZE7 was used instead. Furthermore, in this experiment the RNA samples for each culture day were not pooled prior to cDNA production. The data given here are the mean and standard deviation of three independent samples (biological replicates), with the real time RT-PCR data from each sample obtained from a duplicated PCR (technical replicates). Additionally the expression level of the genes was determined for the inoculum (time zero). Expression relative to the 18S rDNA of the dothistromin genes *dotA*, *dotC*, *pksA* and *vbsA*, as well as for *tub1* was determined for the initial inoculum and during growth in liquid culture. The genes *dotA*, *pksA* and *vbsA* were chosen as representatives for each mini-cluster and *dotC* because of its putative role in toxin secretion. Expression of *tub1* was included to show that the calculated expression levels were not the result of variations in the level of 18S rDNA.

In this experiment, data obtained showed in general high standard deviations. This indicates high variability between the biological replicates. The synthesis of dothistromin

by *D. septosporum* NZE7 occurred at an early stage of growth and declined when growth reached the end of the exponential phase, as seen before for isolate NZE5 (Figure 3.6A). Therefore the expression pattern observed earlier for NZE5 does not appear to be an artefact due to culturing. In DB media the maximum production of dothistromin (per mg DW) occurred at the onset of exponential growth between days 4-6 and the concentration of dothistromin in the media (per ml) peaked at day 6 then slightly declined after (Figure 3.6 A).

Expression levels of the dothistromin genes *dotA*, *pksA* and *vbsA* in DB media mirror the toxin production. The highest levels of expression of these genes occurred in the early growth stage, with maximum expression at days 3-4, before their expression levels declined (Figure 3.7A). Determination of transcript levels before day three was not possible due to insufficient biomass, but they were determined for the inoculum (day 0), where expression for all investigated genes was detected. The similar patterns of expression of the dothistromin biosynthesis genes *dotA*, *pksA* and *vbsA* again suggest co-regulation. Pair-wise comparisons yielded correlation coefficients greater than 0.95 ($p<0.01$) for each pair. Unlike in the experiment in Section 3.2.2 the expression of *dotA* and *vbsA* was also highly correlated. This supports a putative function for *vbsA* in the synthesis of dothistromin. The expression levels of *dotC* and *tub1* showed no significant correlation with *dotA*, *pksA* or *vbsA*, or with each other, supporting the results reported in Section 3.2.2. The *dotC* expression did not significantly decline after day 3 and appeared to be constitutive in these conditions as seen before.

The production of secondary metabolites by fungi in culture is dependent on the growth media (Buchanan *et al.* 1987; Maggio-Hall *et al.* 2005; Skory *et al.* 1993). To determine whether early production of dothistromin is a media effect, gene expression and toxin biosynthesis were also investigated in PDB medium. PDB is a less rich medium than DB, containing only potato dextrose as nutrients, while DB contains malt and nutrient broth (which consists of meat extract, yeast extract, peptone and NaCl). As seen for the DB media the expression of *dotA*, *pksA* and *vbsA* in PDB occurred at an early stage of growth, but the effect of early gene expression was less pronounced than in DB (Figure 3.7B). However, although the growth was much reduced (Figure 3.6), dothistromin gene expression was at its highest between days 3 and 9 after inoculation (Figure 3.7B). The decline of the gene expression was slower, but levels of dothistromin

synthesis and dothistromin gene expression were in general at a lower level in PDB than DB. Compared to DB the maximum toxin levels (µg dothistromin/mg DW) were approximately 30-fold less in PDB. Furthermore the variation in toxin production between replicates was large and toxin production between days did not differ significantly (Figure 3.6B). The expression of the dothistromin genes was likewise at a lower level in PDB than in DB media. To exclude any run-to-run variation, a direct comparison of *pksA* gene expression using real time RT-PCR was performed for the samples of maximum expression from PDB and DB media. The maximum level of expression in PDB (day 4) was approximately 25 times lower than the maximum expression in DB (day 3) (Figure 3.8).

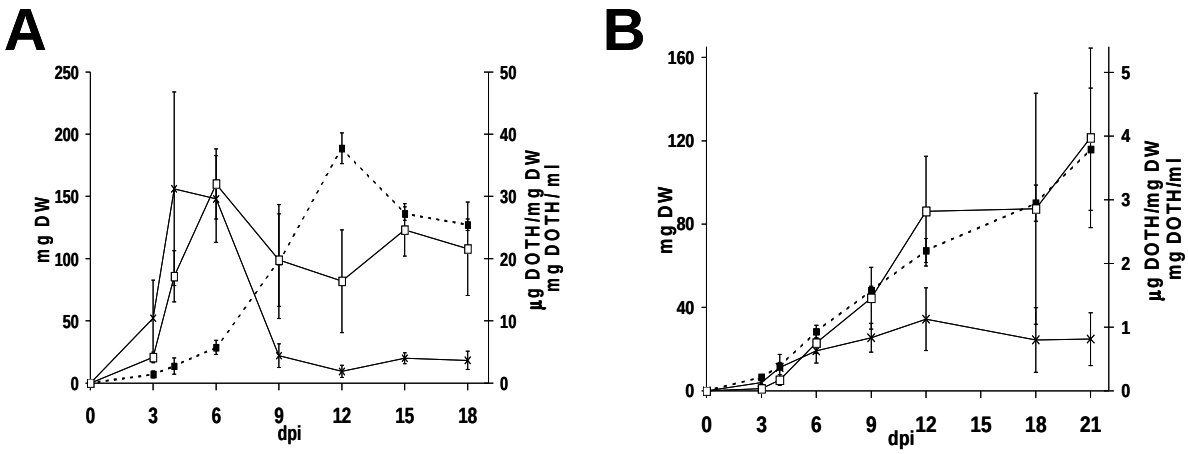


Figure 3.6: Growth and dothistromin production of *D. septosporum* NZE7 in liquid media.
Flasks containing 25 ml of either DB (**A**) or PDB (**B**) medium were inoculated with 10^6 conidia/ml. Growth (-■-) is shown in mg dry weight (mg DW), toxin production (-x-) in µg dothistromin per mg DW (µg DOTH/mg DW) and dothistromin concentration per ml media (□)µg dothistromin per ml media (µg DOTH/ml) for days after inoculation (dpi). The decrease in DW in (**A**) at day 15 and 18 is most likely due to autolysis of the cells or due to the high variability of the growth which might have resulted in an inflated value for the DW at day 12. Values shown are means and standard deviations of three independent samples. Note the different scales in **A** and **B**.

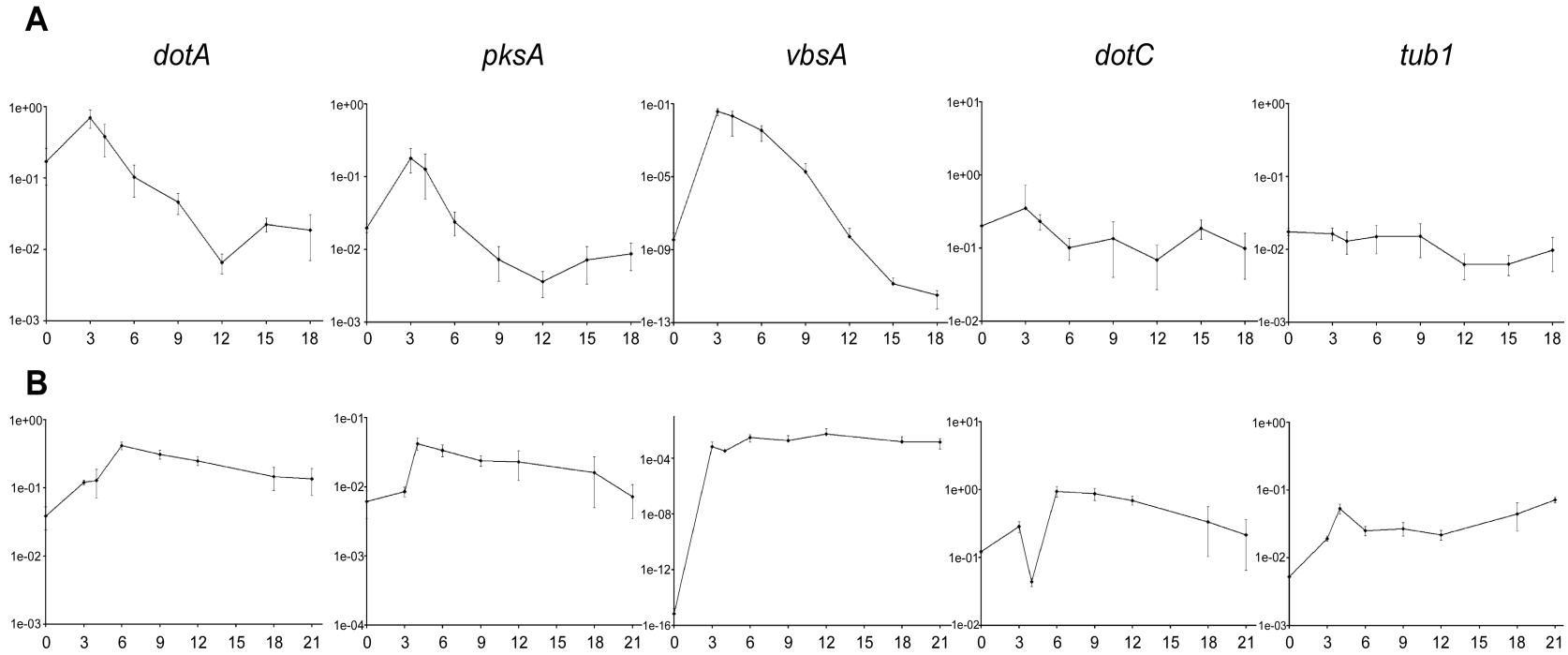


Figure 3.7: Relative gene expression of dothistromin genes over time.

Expression of the dothistromin genes *dotA*, *pksA*, *vbsA* and *dotC* from *D. septosporum* NZE7 in DB (**A**) and PDB (**B**) media of growth experiments shown in Figure 3.6. Shown are days after inoculation (X-axis) and gene expression level relative to ribosomal 18S rDNA (Y-axis). The β-tubulin (*tub1*) was included as a constitutive control. The *dotA*, *pksA* and *vbsA* genes showed high expression at an early growth stage and a similar pattern of expression during culture in DB and PDB. The *dotC* gene appears not to be co-regulated with the other genes. Each data point is the mean and standard deviation of three biological replicates.

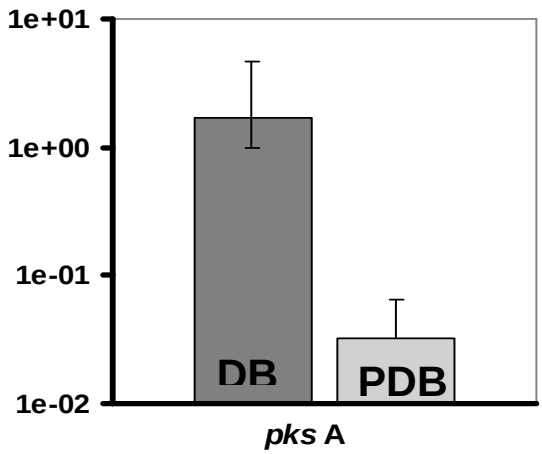


Figure 3.8: Direct comparison of *pksA* gene expression in DB and PDB media.
The maximum gene expression of the *pksA* gene in DB media and PDB media was assessed by real-time RT-PCR. To be able to compare the maximum expression levels directly and exclude variation of different PCR experiments the expression was assessed in the same PCR experiment. The expression in DB (day 3) was approximately 25 times higher than in PDB (day 4). Shown are means ± SD for triplicate biological samples (duplicated PCR).

The synthesis of AF/ST is known to be affected by ambient pH (Keller *et al.* 1997). Furthermore, the needles of the pine hosts of *D. septosporum* are acidic. As the DB media changed from slightly acidic (pH 6) to slightly alkaline (pH 8.3) over the growth period (Figure 3.9 A), the expression of *pksA* and *dotA* was determined in buffered media to test if the dothistromin gene expression is dependent on the ambient pH. In buffered DB media, there was no significant effect of pH on *dotA* and *pksA* expression or on toxin production at 3 days after transfer of mycelium from a seed flask (Figure 3.9 B). Although some variation is seen between the expression at the different pH values ANOVA analyses showed the differences were not significant ($p>0.2$). The pH 8 could not be tested as the media did not stay buffered at this pH.

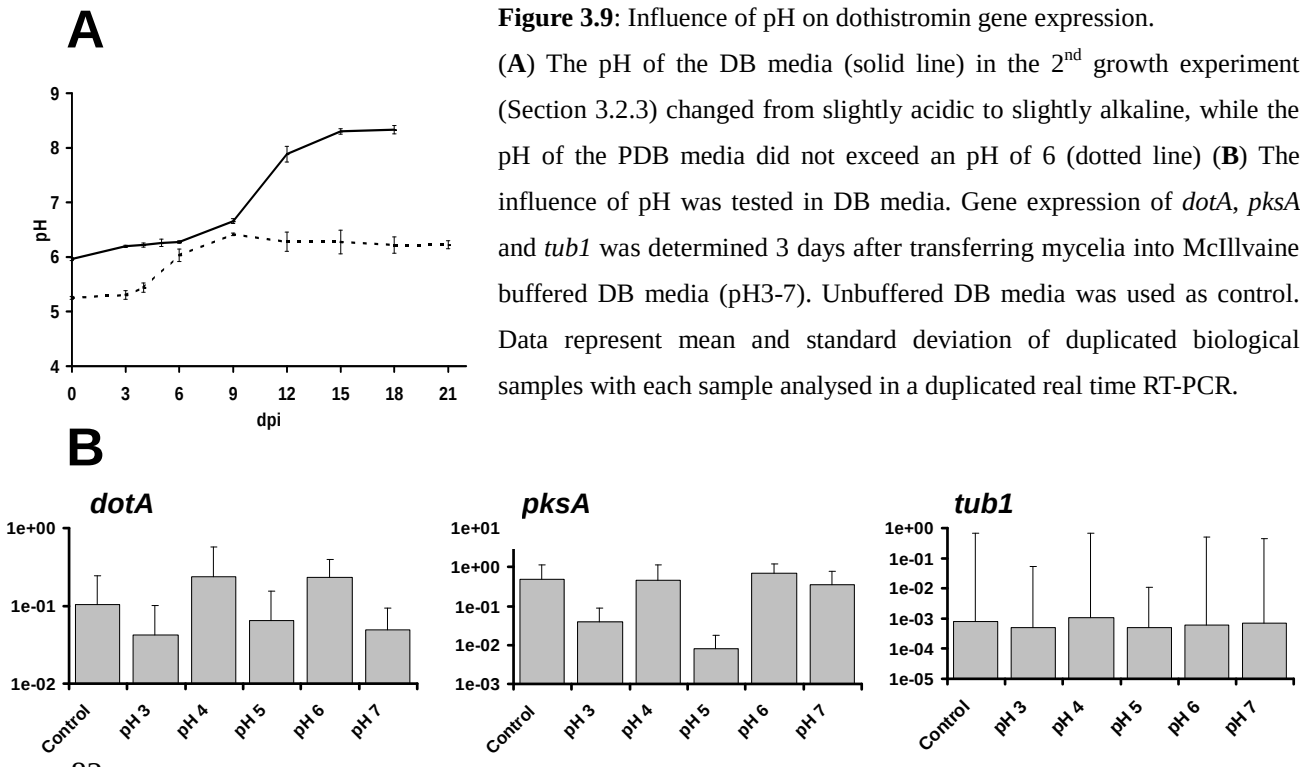


Figure 3.9: Influence of pH on dothistromin gene expression.
(**A**) The pH of the DB media (solid line) in the 2nd growth experiment (Section 3.2.3) changed from slightly acidic to slightly alkaline, while the pH of the PDB media did not exceed an pH of 6 (dotted line) (**B**) The influence of pH was tested in DB media. Gene expression of *dotA*, *pksA* and *tub1* was determined 3 days after transferring mycelia into McIllvaine buffered DB media (pH3-7). Unbuffered DB media was used as control. Data represent mean and standard deviation of duplicated biological samples with each sample analysed in a duplicated real time RT-PCR.

3.3 Discussion

The three mini-clusters containing the eleven identified putative dothistromin genes are located on a 1.3 Mb chromosome (Figure 3.1). As outlined previously, the dothistromin genes are not organised in a continuous cluster (Figure 1.6, Zhang *et al.* 2007), in contrast to the big clusters of AF/ST (Bhatnagar *et al.* 2003; Brown *et al.* 1996).

In the model proposed by Cary and Ehrlich (2006) for the evolution of the AF/ST clusters, the authors speculated that the dothistromin cluster diverged earlier from a common ancestral cluster than the AF/ST clusters. Their proposed basal cluster includes homologs of *pksA* and *hexA* as well as the regulatory genes *aflR* and *aflJ*. Subsequently it is now shown that the *D. septosporum pksA* and *hexA* genes are located in two separate mini-clusters (Zhang et al. 2007) although *aflR* or *aflJ* genes have not been identified. In addition the mini-cluster 3 containing the *pksA* gene also contains the *cypA* gene, whose homolog in the AF cluster is proposed to be recruited at a late stage of the evolution of the AF cluster (Cary & Ehrlich 2006). Therefore the current knowledge about the dothistromin genes does not support the model. However, the relative positions of *pksA* and *hexA* to each other in the *D. septosporum* genome are not known, and the *cypA* gene position could have changed over time. Further it appears to be likely that *D. septosporum* contains an *aflR*-like gene as putative AflR binding sites are present in all three mini-clusters.

It is possible that, in *D. septosporum*, the recruited genes were never formed into a close cluster as in AF/ST. Alternatively, tight clustering may have been an ancestral feature that degenerated over time in *D. septosporum*. However, the location of all so far identified dothistromin genes on a mini-chromosome does suggest that some level of clustering has occurred. Fragmented gene clusters are also known for trichothecene (Kimura *et al.* 2001) and cephalosporin biosynthesis. The cephalosporin genes in *Acremonium chrysogenum* are located on two different chromosomes (Liras & Martin 2006). The linkage of the known dothistromin genes is higher as they are all located on a 1.3 Mb mini-chromosome.

The location of the dothistromin genes on a mini-chromosome is interesting by itself. Mini-chromosomes are often dispensable and contain virulence factors, such as

mini-chromosomes of *Cochliobolus carbonum* and *Nectria haematococca* (reviewed in Covert 1998). *C. carbonum* contains genes necessary for HC-toxin production on either a 2.2 Mb or 3.5 Mb mini-chromosome and loss of the mini-chromosome leads to loss of virulence. *N. haematococca* contains a gene on a 1.6 Mb mini-chromosome necessary for detoxification of the phytoalexin pisatin of garden peas, which has been shown to be a virulence factor. Similarly in *Alternaria alternata* chromosomes of 1.9 Mb or less contain genes necessary for toxin production and pathogenicity (Hatta *et al.* 2002; Johnson *et al.* 2001). It would be interesting to identify if the 1.3 Mb chromosome is always present in *D. septosporum* isolates and if a lack of this would have an influence on virulence or pathogenicity.

Although the dothistromin genes are not as firmly clustered as the AF/ST, a high degree of co-regulation was seen for most of the putative dothistromin genes (Table 3.1). Co-regulation is an important defining feature of clustered secondary metabolite genes and there are many examples of this (Bok *et al.* 2006; Gardiner *et al.* 2004; Gardiner & Howlett 2005). Co-expression of dothistromin genes from all three mini-clusters was observed. This suggests the dothistromin genes are able to function as one unit despite being physically separated. The *pksA*, *moxA* and *cypA* genes of mini-cluster 3 as well as the *dotD* gene from mini-cluster 1 showed the highest co-regulation, with each expression pattern showing significant correlation to the pattern of 6 other putative dothistromin genes. The expression of *dotA* and *avfA* showed high correlation with 5 other dothistromin genes not including *vbsA*. In the experiment described in Section 3.2.2 the expression of *dotA* and *vbsA* was positively correlated but not significant. However, their expression was highly correlated in the subsequent experiment in Section 3.2.3. The statistical analyses in Section 3.2.2 might not be so reliable as each data point was obtained from duplicated PCRs of one pooled cDNA sample, while data in Section 3.2.3 were obtained from 3 independent samples. The high correlations of *vbsA* expression and the other dothistromin genes suggest that the *vbsA* gene is indeed a dothistromin biosynthesis gene. During this study further evidence for an involvement of *vbsA* in dothistromin biosynthesis was obtained by creation of a *vbsA* replacement strain, which was dothistromin deficient (Zhang *et al.* 2007).

The expression patterns of *dotC* and *dotB* appear to be different from that of the other dothistromin genes (Figures 3.2, 3.4, 3.6, Table 3.1). The *dotC* gene encodes a major

facilitator superfamily (MFS) transporter with homology to the *aflT* gene in the AF gene cluster of *A. parasiticus* (Bradshaw *et al.* 2002). The *aflT* gene is also regulated differently to other genes in the AF cluster (Chang *et al.* 2004). The constitutive expression of *dotC* indicates a possible role in secretion of dothistromin. However, *aflT* is not required for AF secretion (Chang *et al.* 2004). Alternatively *dotC* might play a role in self-protection of *D. septosporum* against dothistromin, as seen for sirodesmin in *Leptosphaeria maculans*. The sirodesmin gene cluster in *L. maculans* contains the ABC-transporter gene *sirA* which, although it has been shown not to be required for sirodesmin secretion, is involved in self-protection of the fungi against sirodesmin (Gardiner *et al.* 2005). To clarify the role of *dotC*, a *dotC* knock out mutant has been recently constructed in this lab. Preliminary results suggest that *dotC* is not necessary for dothistromin secretion (Z. Feng, unpublished results).

The predicted *dotB* gene product contains a peroxidase domain and a putative heme-binding site. Although it shows similarity to the product of the ST gene *stcC*, no equivalent is seen in the AF gene cluster (Bradshaw & Zhang 2006b). Furthermore, the role of StcC in *A. nidulans* is unknown as disruption of *stcC* appears to have no effect on production of ST (mentioned in Zhang *et al.* 2005). Therefore *dotB* might not be involved in the biosynthesis of dothistromin although its expression is also highest at an early growth stage (Figure 3.4). To clarify the role of *dotB*, knock out mutants need to be constructed.

The expression of the non-dothistromin genes *DS2* and *DS14* did not correlate with the expression of the dothistromin genes. *DS2* showed high correlation to *tub1* and appears therefore to be constitutively expressed, which would be reasonable for its proposed function as a chitin synthase. Interestingly the expression of the putative amino acid permease gene DS25 appears to be correlated with the expression of the dothistromin genes (with the exception of *avfA*).

The RT-PCR results of the expression of the putative epoxide hydrolase gene, *epoA*, suggest also co-regulation with the other dothistromin genes. As the real time RT-PCR assay for *epoA* was not successful this could not be confirmed. There are no epoxide hydrolases in the AF or ST gene cluster and function of an epoxide hydrolase gene product in the dothistromin synthesis is speculative. The predicted gene product could potentially have either a biosynthetic or a bio-protective role in the fungus. The *D.*

septosporum epoxide hydrolase could detoxify epoxide derivatives of dothistromin that could otherwise damage the fungal cells. Alternatively it may be involved in the last stages of dothistromin biosynthesis (Bradshaw & Zhang 2006b). However, Jin (2005) has shown that *epoA* gene replacement mutants are able to synthesize dothistromin, suggesting EpoA has no biosynthetic role. It is also not known if the *epoA* gene product is functional (Bradshaw & Zhang 2006b; Jin 2005).

The expression of AF and ST genes requires regulatory gene products, AflR and AflJ, encoded by genes within the clusters (Chang 2003; Price *et al*. 2005). So far no homologs of these have been identified in *D. septosporum*, although putative AflR binding sites (5'-TCGN$_5$CGR-3') are found upstream of many of the dothistromin genes, as well as a modified form of the binding site (5'-TCGN$_{11}$CGR-3') (Bradshaw & Zhang 2006b). Interestingly, the *aflT* gene in the AF cluster does not have the 5'-TCGN$_5$CGR-3' AflR binding site in its regulatory region (Cary & Ehrlich 2006) and the *aflT* gene is also differently expressed as the other AF genes (Chang *et al.* 2004). The *aflT* homolog *dotC* in mini-cluster 1 also showed a different expression compared to the other putative dothistromin genes as it was constitutively expressed under the conditions examined. In mini-cluster 1 *dotB* also shows no co-regulation to other dothistromin genes. In contrast the expression of *dotA* and *dotD*, which are divergently transcribed to *dotB* and *dotC* respectively, has a high correlation with the other dothistromin genes. This might be due to the closer location of AflR binding motifs to the coding regions of *dotA* and *dotD* (Figure 3.5). In mini-cluster 3, *avfA* was the only gene with a significant correlation to the expression of *dotB* and no significant correlation to the expression of *DS25*. Interestingly the *avfA* gene does not contain putative AflR binding sites upstream of its ORF, which might account for the slightly different expression compared to the other dothistromin genes. Likewise, the promoter region of the *aflI* homologue of *avfA* in the AF cluster does not contain AflR binding sites (Cary & Ehrlich 2006). The presence of *aflR* binding sites and the different regulation of the dothistromin genes which do not contain the 5'-TCGN$_5$CGR-3' putative *aflR* binding site suggest an *aflR*-like regulation. The involvement of an AflR homologue could explain the co-regulation of the dispersed dothistromin genes in *D. septosporum*. In *A. parasiticus* AflR controls the expression of several genes located outside the AF cluster and each contain AflR binding sites (Price *et al*. 2006). But since *moxA* and *DS25* were co-regulated with other dothistromin genes

despite no putative AflR binding sites, it appears that also other regulator(s) influence the synthesis of dothistromin in *D. septosporum*.

In *A. nidulans* the ST expression is also regulated by LaeA. Regulation by LaeA shows chromosome-location specificity: genes within the ST cluster were regulated but those moved out of the cluster were not. LaeA is a global regulator of secondary metabolite gene expression, and LaeA homologs have been found in other fungal species (Bok *et al.* 2006). An earlier study also showed the expression of the AF gene *aflD* (*nor-1*) to be position dependent (Chiou *et al.* 2002). A position dependent regulation of the dothistromin genes might explain the co-regulation of *DS25* with the dothistromin genes. The ORF of *DS25* is located only 1778 bp away from *moxA* in mini-cluster 3. In contrast *DS2* and *DS14* which are not co-regulated with the dothistromin genes are further away from the dothistromin genes in their clusters. *DS2* is 4653 bp away from the closest co-regulated dothistromin gene *dotA* in mini-cluster 1, while *DS14* is separated by a 5359 bp non-coding region from *vbsA* in mini-cluster 2. Whether a LaeA ortholog exists in *D. septosporum* or if it is involved in regulating expression of the dothistromin genes needs to be clarified.

The production of AF and ST is partly regulated by the pH regulator PacC (Keller *et al.* 1997) but the role of pH regulation in aflatoxin production is not resolved and often contradictory (Calvo *et al.* 2002; Ehrlich *et al.* 2005). The synthesis of pathogenicity factors in *Botrytis cinerea* appears to be influenced by ambient pH (Manteau *et al.* 2003). In the case of dothistromin, as the pH of pine needles is acidic, it was anticipated that pH could be an influencing factor for gene expression and toxin production. In *D. septosporum* putative binding sites for PacC are found upstream of all of the dothistromin genes characterized so far (Bradshaw & Zhang 2006b), although attempts to isolate a *pacC* homologue in *D. septosporum* by degenerate PCR have been unsuccessful (Bradshaw and Barron, unpublished results). In this study no significant effect of the ambient pH between pH 3 and pH 7 on the expression levels of *dotA* and *pksA* was detected (Figure 3.8). This indicates that if the toxin is involved in the disease process its production is most likely not triggered by the acidic pH of the pine needles. However, the gene expression at the different pH levels showed a high variation. Furthermore the results were only obtained from duplicated biological replicates. Therefore it should not be excluded that the ambient pH might influence the synthesis of dothistromin.

In DB media the highest expression of the dothistromin genes coincided with the beginning of exponential growth. The expression declined in mid-exponential phase and this was reflected by the toxin production which was also highest at the onset of exponential growth. Similarly the expression of the genes and production of toxin occurred at an early growth stage in PDB media, although no strong decline in gene expression was observed. AF synthesis in *A. parasiticus* in liquid cultures is dependent on the media source, with a higher level of production in rich media (Skory *et al.* 1993). Approximately six times more dothistromin (mg/ml) is produced in DB media than in PDB (Figure 3.5) and *pksA* expression is about 25 times higher in DB than in PDB (Figure 3.7). However, in both media toxin production and dothistromin gene expression are observed at an early stage of growth (Figure 3.6).

Dothistromin biosynthesis is clearly regulated differently to AF and ST which have maximum levels of production during late exponential/stationary phase (Trail *et al.* 1995). In *A. parasiticus* the transcript of the AF *dotA* homologue *aflM* (*ver-1*) was not detected until near the end of the exponential phase and was still highly expressed during stationary phase in batch fermentation (Skory *et al.* 1993). Likewise the aflatoxin gene *nor-1* in *A. flavus* was induced during the later stage of exponential growth, reaching its maximum at the beginning of the stationary phase when grown on wheat (Mayer *et al.* 2003). In general secondary metabolites like ST and AF accumulate at later stages after primary growth has slowed (Cleveland & Bhatnagar 1990; Payne & Brown 1998; Trail *et al.* 1995). In this study the expression of the dothistromin genes and production of dothistromin were clearly different from this pattern. Considering the similarities between dothistromin and AF/ST in respect of their structures, genes and biosynthetic pathways, the difference in timing of biosynthesis demonstrated here is intriguing.

Secondary metabolism and sporulation are often linked in filamentous fungi, sharing some (but not all) regulatory elements (Calvo *et al.* 2002). Mycotoxin production and sporulation are connected by G-protein signaling pathways (Brodhagen & Keller 2006). In *A. nidulans* ST appears to be important for sporulation, with more conidia accumulating with each progressive conversion in a series of mutants blocked at points along the ST pathway (Wilkinson *et al.* 2004). Decreased sporulation was also seen in one experiment of a *pksA* mutant of *D. septosporum* (Bradshaw *et al.* 2006; Jin 2005), but sporulation rates are very inconsistent in *D. septosporum* cultures. However, AF proteins

including the DotA homologue AflM (Ver-1) and VbsA homologue AflK (VBS) were detected in both vegetative hyphae and conidiospores in *A. parasiticus* (Chiou *et al.* 2004; Lee *et al.* 2004; Lee *et al.* 2002). Furthermore, while sporulation is usually accompanied by AF/ST biosynthesis, the converse is not always true as ST/AF can be produced in growth conditions where sporulation is inhibited (i.e. submerged culture) (Yu & Adams 1998). The high level of dothistromin gene expression at an early stage of growth, however, suggests that the regulation of dothistromin biosynthesis is not directly connected with that of sporulation. It would be interesting to see if a G-protein-mediated signalling pathway regulates dothistromin biosynthesis negatively, as in aflatoxin/sterigmatocystin, or positively, as in penicillin biosynthesis that occurs in *A. nidulans* at a slightly earlier stage of growth (Tag *et al.* 2000).

In summary the results of this chapter showed that the dothistromin genes are not organised in a continuous gene cluster, but they are all located on a mini-chromosome. Co-regulation of the putative dothistromin genes occurs even though the genes are not tighly clustered. An early onset of the dothistromin gene expression was seen and this lead to the question of whether this pattern of expression is relevant to the biological function of dothistromin.

A direct biological function of dothistromin is still unknown and isolates of *D. septosporum* show remarkable variations in levels of toxin production in culture (Bradshaw *et al.* 2000). Whether these differences are relevant for virulence or pathogenicity of *D. septosporum* is not known. It has proven to be particularly difficult to obtain a reliable pathogenicity test in the glasshouse (Barron 2006; Devey *et al.* 2004; West 2004), which is required to test toxin-deficient mutants. However, the unusual expression pattern of dothistromin genes in culture leads to two hypotheses about possible biological functions of dothistromin.

The first hypothesis is that dothistromin has a functional role in an early stage of plant host infection. Pure dothistromin induces disease symptoms (Shain & Franich 1981) and this suggests that early production of toxin on the needle surface could kill the plant tissue or induce a hypersensitive response, therefore facilitating invasion and establishment of the pathogen within the host. A direct effect of a toxin from the pathogen on the host is seen in many pathosystems (Howlett 2006). In other *Mycosphaerella* species necrotic areas were observed in advance of the colonizing hyphae of *M. fijiensis,*

M. eumusae and *M. musicola* in bananas, which led the authors to the conclusion of a diffusible toxin secreted from the fungi (Balint-Kurti *et al.* 2001). Similarly, dothistromin could be secreted from young mycelium, which appears to have a high expression of the toxin genes. Like *D. septosporum*, several plant pathogens in the genus *Cochliobolus* produce toxins at an early growth stage, or even prior to germination in the conidia. For example *C. carbonum* produces and releases HC-toxin as one of the initial morphogenetic events in the infection process and this is thought to aid in the colonization of host tissue (Weiergang *et al.* 1996). Dothistromin could similarly play a role in the early plant-microbe interaction although it differs in being a non-host-specific toxin (Stoessl *et al.* 1990).

A second hypothesis is that the production of dothistromin at an early growth stage could provide a competitive advantage against other microorganisms in the environment. *D. septosporum* itself is very slow-growing and dothistromin is known to be toxic towards a broad range of other organisms (Stoessl *et al.* 1990). Many secondary metabolites have antimicrobial activity (Pelaez 2005), suggesting roles in defence. For *D. septosporum* the early production of dothistromin could, because of its broad toxicity, provide a crucial competitive advantage in the environment. Interestingly the DB medium, which contains yeast extract in the nutrient broth, had much higher gene expression and toxin production than observed for PDB. Similarly in *A. ochraceus*, higher ochratoxin A production was observed in PDB media supplemented with yeast extract than in PDB alone or supplemented with casein (O'Callaghan *et al.* 2006). This might be due to the fungal origin of the yeast extract and therefore a response towards a competing organism instead of being a pure nutritional effect.

Both hypotheses were subsequently tested and preliminary results are shown in the following chapters. The results will shed light on the biological role of dothistromin. The dramatic difference in timing of production of dothistromin compared to AF/ST has implications for our understanding of the evolution of secondary metabolite gene clusters, the biological roles of these metabolites and the fundamental processes involved in regulation of their biosynthesis.

Chapter 4:
Transformation of *D. septosporum* with reporter genes and expression of GFP and DsRed

4.1 Introduction

The unexpected finding of the early expression of the dothistromin genes in culture (Section 3) led to further questions about the role of the toxin in the disease process. Is the early expression also seen in the natural host-fungus interaction? How early are the genes expressed? Reporter gene strains of *D. septosporum* would help to answer these questions. The GFP is particularly useful as a reporter as it does not require substrates or further treatment of the tissue but can instead be visualized in living tissue under UV light (see Section 1.6). The use of GFP as a reporter gene in fungi, under the control of either a constitutive or inducible promoter, has enabled researchers to follow toxin gene expression patterns and development of fungal pathogens on and in their hosts (Balint-Kurti *et al.* 2001; Kaufman *et al.* 2004; Lorang *et al.* 2001; Sexton & Howlett 2001). This chapter describes the generation of GFP reporter transformants of *D. septosporum* for the purpose of investigating the role of the dothistromin toxin.

The optimal choice of which GFP to use as a reporter appears to be dependent on the host (reviewed by Lorang *et al.* 2001, Section 1.6). Two different versions of GFP, the SGFP and EGFP, have been predominantly used as reporters in filamentous fungi. Similar to GFP, a optimised version of the red fluorescent protein DsRed, $DsRed_{express}$, has been successfully expressed in filamentous fungi (Mikkelsen *et al.* 2003). Consequently, the SGFP, EGFP and $DsRed_{express}$ genes were transformed into *D. septosporum* in order to create reporter gene strains.

There were several reasons why *D. septosporum* reporter strains were made. The establishment of a reliable pathogenicity test was impeded by technical difficulties (Barron 2006; West 2004); constitutive GFP expressing *D. septosporum* strains would facilitate observation of the fungal pathogen and optimisation of the pathogenicity test.

Furthermore the infection process would be seen more clearly with GFP-labelled strains, clarifying conflicting reports about the mode of infection of *D. septosporum* (Section 1.1.2, Muir & Cobb 2005).

At the beginning of this study it was thought that the dothistromin toxin is most likely involved in the infection process as a pathogenicity factor (Bradshaw *et al.* 2000). GFP expressing, dothistromin-deficient strains of *D. septosporum* would allow the observation of the infection progress and assist in the identification of dothistromin deficient strains on the host and hence determine if dothistromin is a pathogenicity factor. Therefore GFP genes under control of constitutive promoters were transformed into *D. septosporum* wild-type and dothistromin-deficient strains.

For analysis of dothistromin gene expression, *D. septosporum* was transformed with the *egfp* gene under control of a dothistromin regulated promoter region. A dothistromin regulated GFP transformant strain would allow investigation of toxin gene expression *in planta*, which will be particularly useful for investigating the role of the toxin in the disease process. At the begin of the study only the *dotA* gene had been proven to be involved in dothistromin biosynthesis by gene replacement (Bradshaw *et al.* 2002). Therefore a GFP reporter vector was constructed which contained a *gfp* gene fused to the regulatory region of the *dotA* gene (P*dotA*).

GFP fusion proteins for evaluating gene expression and protein localization have been successfully used in other filamentous fungi to localize proteins of interest (i.e. Akao *et al.* 2006; Chung *et al.* 2002; Fernández-Ábalos *et al.* 1998). The intracellular localization of proteins involved in the biosynthesis of dothistromin may reveal new clues about the function of dothistromin and auto-protection of the fungi against the toxin. For this study a vector was created which contained the P*dotA* region and a *dotA*::*egfp* fusion gene with the EGFP gene translationally fused to the C-terminus of the *dotA* gene. The DotA-EGFP fusion protein should allow the intracellular localization of the native DotA protein. This vector was also transformed into the *dotA* replacement strain FJT2 in order to complement the $dotA^-$ phenotype.

4.2 Construction of reporter gene vectors

Constitutive GFP expressing *D. septosporum* strains were created by transformation with the available plasmids pPN82 (Tanaka *et al.* 2006) and pCT74 (Lorang *et al.* 2001). The plasmid pPN82 contains the *egfp* gene under control of the constitutive promoter P*gpd*A of *A. nidulans* and plasmid pCT74 contains the *sgfp* gene under control of the P*toxA* gene promoter from *Pyrenophora tritici-repentis.* In addition a constitutive DsRed reporter vector (pR237), derived from Pgpd-DsRed express (Mikkelsen *et al.* 2003) with the DsRed gene under control of P*gpdA,* was constructed by introducing a hygromycin selection marker into the reporter vector.

Regulated GFP vectors in this study included a *dotA* regulated *egfp* vector (pR242) derived from pPN82. This vector was constructed to observe the *dotA* expression *in vivo*. Another vector, pR261, was made containing the EGFP fused to the C-terminal end of the DotA protein with its expression under the control of the *dotA* promoter region (P*dotA*). This was constructed to identify the possible localization of the native DotA protein.

4.2.1 Construction of the DsRed vector pR237

The *hph* gene was cloned into the existing DsRed vector pPgpd-DsRed (Mikkelsen *et al.* 2003) as a selection marker for fungal transformation. The DsRed vector pR237 (7.8 kb) was constructed by ligation of a 1.4 kb *Hin*dIII fragment of pPN82 containing the *hph* gene into the single *Hin*dIII restriction site of plasmid pPgpd-DsRed (Figure 4.1). Plasmid pPgpd-DsRed (6.4 kb, Appendix A3) contains the $DsRed_{express}$ gene, a variant of DsRed from *Discosoma* sp. placed under the control of the constitutive *A. nidulans* glyceraldehyde 3-phosphate promoter (P*gpd*A) and the *A. nidulans trp*C transcriptional terminator (T*trp*C), which has been successfully expressed in filamentous fungi (Mikkelsen *et al.* 2003).

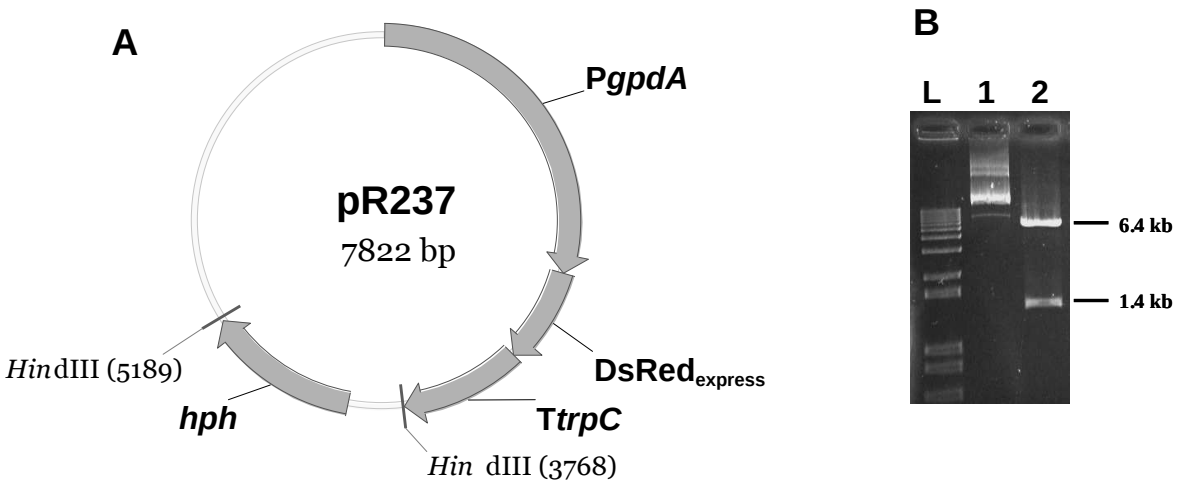


Figure 4.1: Outline of plasmid pR237.

Plasmid pR237 (**A**) was obtained by cloning the 1.4 kb *hph Hin*dIII fragment of pPN82 (Figure 4.2) into pPgpd-DsRed (Mikkelsen *et al.* 2003). (**B**) *Hin*dIII digestion of the newly created plasmid pR237 confirmed the insertion. The original 6.4kb pPgpd-DsRed and the 1.4kb *Hin*dIII insert were recovered. Lane 1 shows the undigested plasmid pR237 and Lane 2 shows pR237 after *Hin*dIII digestion. Lane L shows the 1kb$^+$ ladder.

4.2.2 Construction of the *dotA* regulated GFP reporter vector pR242

The constitutive promoter region of an existing *egfp* vector was replaced with the *dotA* gene regulatory region P*dotA*.

The *dotA* regulatory region P*dotA* was amplified by PCR from gDNA using primers pdotA-rev and pdotA-fw (Table 2.2). The pdotA-rev primer contained a mismatch relative to the genomic sequence to introduce a downstream *Rca*I restriction site encompassing the ATG start site. The pdotA-fw primer contained mismatches relative to the genomic sequence to remove an *Rca*I restriction site of the original sequence to facilitate the subsequent ligations. The P*dotA* PCR product was cloned into pGEMTeasy (Promega) to generate pR241 (Figure 4.2) and sequenced as described in Section 2.7. Compared to the original published sequence the product was missing a G at position 4729 in three independent PCRs, suggesting an error in the original sequence. The *dotA* regulated *egfp* plasmid pR242 (6.6 kb, Figure 4.2) was prepared by replacing the 2.3-kb *Eco*RI/*Nco*I P*gpdA* fragment of pPN82 with a 971 bp *Eco*RI/*Rca*I fragment of

plasmid pR241. The *Eco*RI/*Rca*I fragment includes the 958 bp *dotA* regulatory region P*dotA* (nucleotides 4479-5436 of the previously published sequence AF448056 (Bradshaw et al. 2002)).

Box 4.1: Sequence alteration through PCR amplification for construction of pR242

Original sequence of P*dotA*

5437→ **←4479**

gctgagcgacgaagattcatgataga …….. acaccacctcatctcccata*atg*tccgt

*Rca*I

Sequence after amplification with pdotA-rev and pdotA-fw

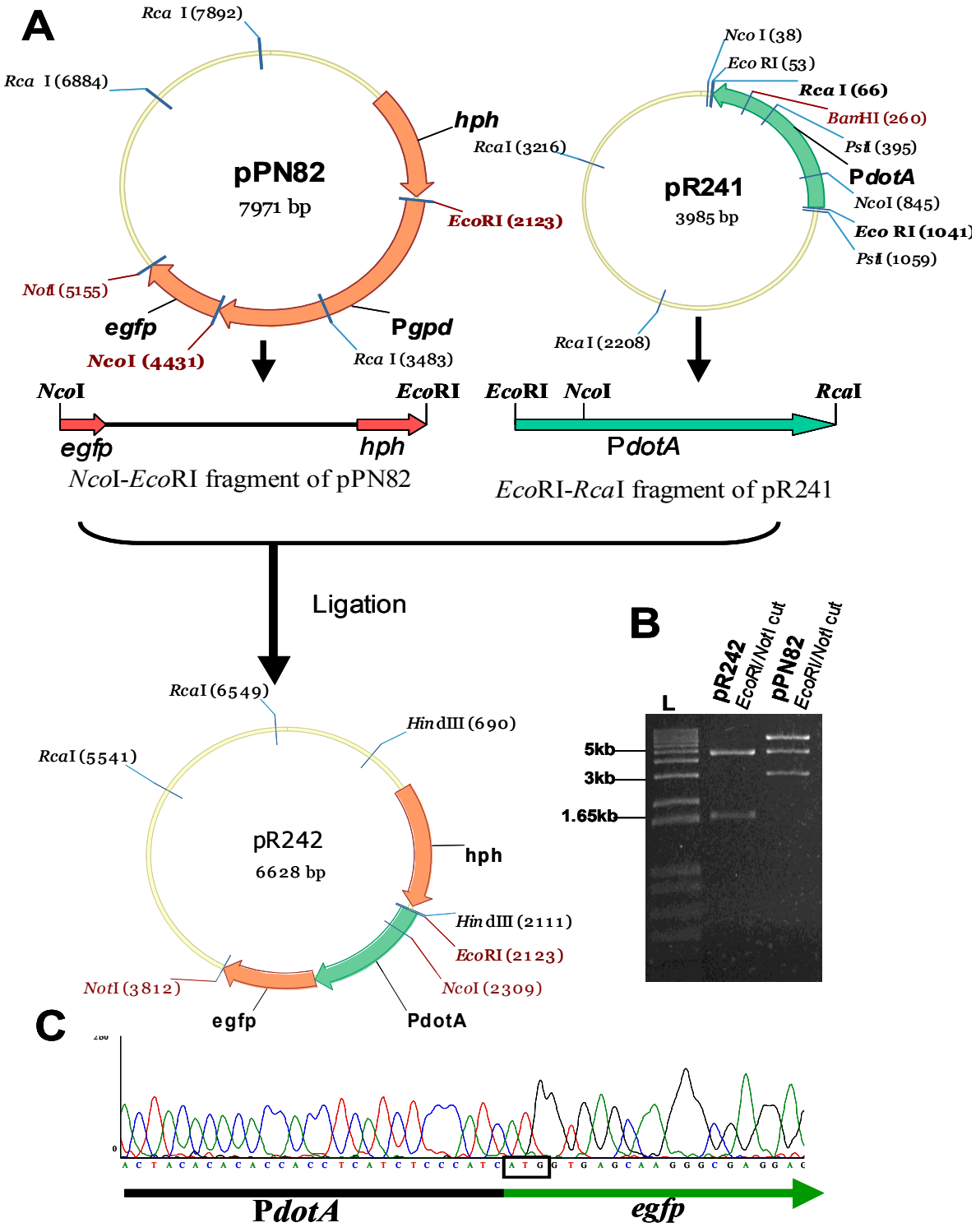
gctgagcgacgaagattc**tt**a**g**aga ……..... acaccacctcatctccat**c***atg***a**ccgt

*Rca*I

Changes to the original sequence due to mismatch primers are bold. The *Rca*I restriction sites are underlined. The translation start for the *dotA* in the original sequence and *egfp* in the altered sequence are in italics. Shown is the coding strand. Numbers are equivalent to the nucleotide numbers of the original published sequence AF448056 (Bradshaw *et al.* 2002).

Figure 4.2: Construction of P*dotA*::*egfp* reporter vector pR242.

(**A**) For construction of pR242 the *Eco*RI/*Nco*I fragment of pPN82 (Tanaka *et al.* 2006) containing the P*gpdA* promoter region was cut out and replaced by a *Eco*RI/*Rca*I fragment of pR241, which contained the *dotA* regulatory region P*dotA* amplified from genomic DNA. The *Rca*I and *Nco*I restriction sites are compatible, but ligation results in removal of the recognition sequences for either of them. (**B**) An *Eco*RI/*Not*I digest of pR242 resulting in the expected fragments of 4.9kb and 1.68kb. *Eco*RI/*Not*I digest of pPN82 is shown as comparison resulting in the expected fragments of 4.9kb and 3kb. Digestion of pPN82 was not complete resulting in a third band of 7.9kb that represents the linearised vector. Lane L shows the 1kb$^+$ ladder. (**C**) The sequence chromatogram of vector pR242. Shown is the fusion site of the P*dotA* region and the *egfp* gene of pPN82. The box marks the ATG start codon of the *egfp*.


A
RcaI (7892)
RcaI (6884)
pPN82
7971 bp
hph
RcaI (3216)
EcoRI (2123)
NotI (5155)
egfp
NcoI (4431)
Pgpd
RcaI (3483)
NcoI
EcoRI
egfp
hph
NcoI-EcoRI fragment of pPN82
NcoI (38)
EcoRI (53)
RcaI (66)
BamHI (260)
PsiI (395)
pR241
3985 bp
PdotA
NcoI (845)
EcoRI (1041)
PsiI (1059)
RcaI (2208)
EcoRI
NcoI
RcaI
PdotA
EcoRI-RcaI fragment of pR241
Ligation
RcaI (6549)
HindIII (690)
RcaI (5541)
pR242
6628 bp
hph
HindIII (2111)
EcoRI (2123)
NotI (3812)
NcoI (2309)
egfp
PdotA
B
L
pR242 EcoRI/NotI cut
pPN82 EcoRI/NotI cut
5kb
3kb
1.65kb
C
PdotA
egfp

4.2.3 Construction of the fusion protein vector pR261

A vector containing the *egfp* gene fused to the complete genomic *dotA* gene under the control of the *dotA* promoter region was also created. The *egfp* gene was amplified from vector pPN81 (Tanaka, A.; Appendix A3) which is identical to pPN82 (Figure 4.2) but without the 2.3 kb P*gpdA* promoter region. The translation of the P*dotA*::*dotA*::*egfp* gene provides a DotA protein with EGFP joined at the C-terminus. This fusion protein was constructed to gain information about the intracellular location of the native DotA protein. The P*dotA* promoter was used in order to use the fusion protein strains also as regulated reporter gene strain.

The *PdotA::dotA::egfp* gene was created in a three step PCR as outlined in Figure 4.3. In PCR1, the P*dotA*::*dotA* region was amplified from gDNA using primers PdotA-fusion (P1) and egfp-dotA-connect (P2). This PCR amplified an 1130 bp region of P*dotA* and the ORF of the *dotA* gene, resulting in a 2061 bp PCR product. The primers dotA-egfp-connect (P3) and TrpCfusion (P4) were used to amplify the *egfp* from pPN81 in PCR2, resulting in a 736 bp product. P2 and P3 are exactly complementary and each contain a 18 bp region of both the *egfp* and *dotA* gene, resulting in overlapping ends of the PCR1 and PCR2 products, but with the stop codon of the *dotA* gene replaced by the start codon of the *egfp*. PCR products were gel-purified (Section 2.3.3). In PCR3 aliquots of PCR1 and PCR2 products were used as template with primer P1 and P4, resulting in the 2797 bp P*dotA::dotA::egfp* product. The *PdotA::dotA::egfp* product was purified, A-tailed and ligated into pGEMTeasy to form vector pR260.

The vector pR260 was sequenced as outlined in Figure 4.3.C. There were no errors in the sequence compared to either the *egfp* gene or the P*dotA::dotA* region, apart from missing a G at position 4729 of the original published P*dotA* sequence (AF448056 (Bradshaw et al. 2002)) as seen previously in the construction of pR242 (Section 4.2.2). The 1.4kb *Hind*III fragment of pPN82 containing the *hph* gene was ligated into the single *Hin*dIII restriction site of pR260 as a selection marker. This resulted in plasmid pR261, which can be used for the transformation of the wild type in the future. The ligation of the *hph* fragment from pPN82 reduces the P*dotA* region from 1130 bp to 1079 bp.

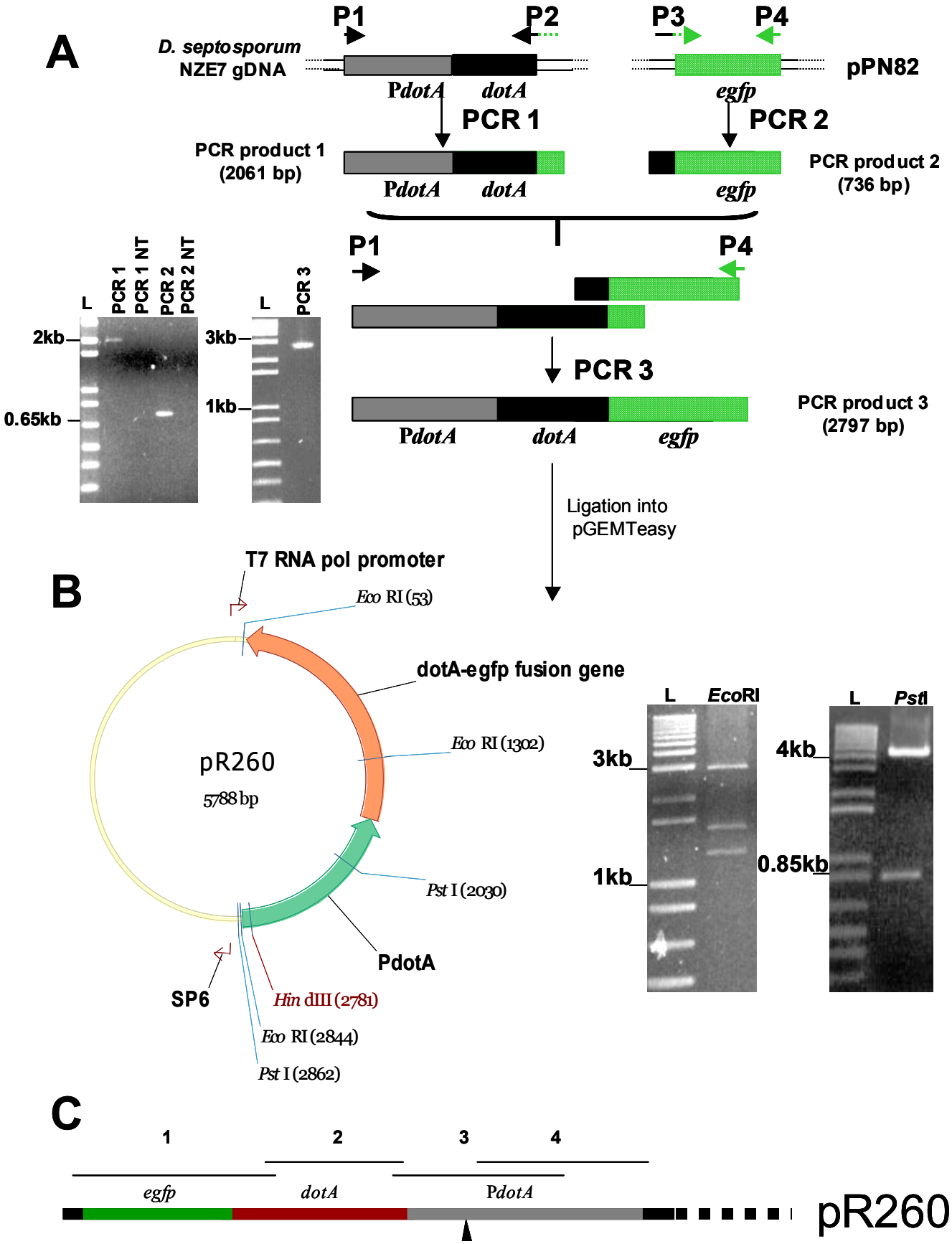

A
P1
P2
P3
P4
D. septosporum
NZE7 gDNA
PdotA
dotA
egfp
pPN82
PCR 1
PCR 2
PCR product 1
(2061 bp)
PCR product 2
(736 bp)
L
PCR 1
PCR 1 NT
PCR 2
PCR 2 NT
2kb
0.65kb
L
PCR 3
3kb
1kb
PCR 3
PCR product 3
(2797 bp)
Ligation into
pGEMTeasy
B
T7 RNA pol promoter
Eco RI (53)
dotA-egfp fusion gene
pR260
5788 bp
Eco RI (1302)
Pst I (2030)
PdotA
SP6
Hin dIII (2781)
Eco RI (2844)
Pst I (2862)
L
EcoRI
3kb
1kb
L
PstI
4kb
0.85kb
C
1
2
3
4
egfp
dotA
PdotA
pR260

Figure 4.3: Construction and analysis of vector pR260.

(**A**) The P*dotA*::*dotA*::*egfp* gene was constructed in a three step PCR as described in the text. The PCR products of PCRs 1-3 are shown separated on a 1% TBE agarose gel on the left. L shows the 1 kb+ ladder, NT labelled lanes show non-template controls. (**B**) The constructed P*dotA*::*dotA*::*egfp* gene was cloned into pGEMTeasy to obtain plasmid pR260. Integration was tested with an *Eco*RI and *Pst*I digest of pR260 resulting in the expected bands. (**C**) The P*dotA*::*dotA*::*egfp* insert of pR260 was checked by overlapping sequencing using primers T7 (1), RTCexon3 (2), JdrKC1 (3) and SP6 (4). Shown is a diagram of the linearised plasmid pR260 starting on the left with nucleotide 1 of the annotation of the circular vector shown in (B). An arrowhead indicates the position of the missing G in comparison to the original published sequence.

4.3 Transformation of *D. septosporum*

To obtain GFP expressing *D. septosporum* strains a protoplast based transformation was performed (Section 2.14). Vectors pPN82 (Figure 4.2) and pCT74 (Appendix A3) were transformed into *D. septosporum* NZE7 to obtain constitutive GFP expressing reporter strains.

D. septosporum NZE7 was also successfully transformed with plasmid pR242, containing the *egfp* gene under control of the *dotA* regulatory region P*dotA*. Two P*dotA* regulated GFP expressing transformants were obtained, FJT24 and FJT26

Dothistromin deficient GFP expressing strains were obtained by co-transformation. The dothistromin deficient mutants FJT2 (*dotA*$^-$) (Bradshaw *et al.* 2002; Seconi 2001) and FJT3 (*pksA*$^-$) (Bradshaw *et al.* 2006; Jin 2005) already contain a hygromycin resistance marker as these strains were constructed by gene replacement with the *hph* gene. Therefore plasmid pBC-phleo (Silar 1995), containing a phleomycin resistant marker, was co-transformed with pPN82 to introduce the *egfp* gene into FJT3 and FJT2. Co-transformation lead to the GFP expressing *dot*A$^-$ strain FJT29 and the GFP expressing *pks*A$^-$ strain FJT30.

FJT2 was also successfully co-transformed with pBC-phleo and the plasmid pR261 containing the *dotA::egfp* fusion gene under control of the P*dotA* regulatory region. The strains FJT31 and FJT32 showed successful expression of the *dotA::egfp* fusion gene. It was hoped to complement the dothistromin deficiency due to the replacement of the *dotA* gene in FJT2.

Although all transformants described here were obtained by PEG protoplast transformation, transformation of *D. septosporum* was not successful in every assay. The transformation efficiency ranged from 0-80 transformants per μg transformed DNA. Two rounds of single spore isolation were performed as described in Section 2.14.3 to purify successful transformants.

4.4 Characterization of *D. septosporum* reporter gene transformants

All transformants were tested for integration of the reporter gene into the genome by Southern hybridisation. Growth rates, sporulation and germination rates of representative transformants were tested to see if there was an effect on the general fitness of the GFP strains compared to the wild type. An overview of the GFP reporter strains obtained in this study is shown in Table 4.2.

4.4.1 Southern analysis of GFP transformants

Southern hybridisations (Section 2.11) were carried out to test the integration of the transformed *sgfp*, *egfp*, P*dotA*::*egfp* and *dotA*::*egfp* genes transformed into *D. septosporum* NZE7.

Preliminary Southern analyses that were performed to check the integration of the *gfp* genes into the genome are shown in Appendix A8. FJT20 was the only successful pCT74 (P*toxA*::*sgfp*) transformant and contained a single copy of plasmid pCT74 (Appendix A8).

Successful *D. septosporum* pPN82 transformants all contained more than one copy of pPN82 (Appendix A8). For further experiments the pPN82 transformants FJT21 and FJT22 were chosen. FJT21 was chosen because of its very bright fluorescence, which is most likely due to the high copy number of the plasmid inserted. FJT22 was chosen as it only has 2 copies of the plasmid inserted but showed reasonable fluorescence. Both

strains showed pigment production on media plates indicating dothistromin production. Strains FJT34-FJT37 were also successful EGFP-expressing transformants containing pPN82, but not used in further experiments. Stocks of all strains were made.

FJT29 was the only successful pPN82 transformant derived from the *dotA* replacement strain FJT2 (Bradshaw *et al.* 2002). It also had several copies of the *egfp* inserted into the genome and showed bright fluorescence. Out of eight successful transformants of FJT2 with pR260, containing the P*dotA*::*dotA*::*egfp* fusion gene, FJT31 and FJT32 were chosen as they showed the darkest pigmentation on DM plates and were therefore thought to be possible *dotA*$^{-}$ complementation strains.

Out of eight pPN82 transformants of the *pksA* replacement strain FJT3 (Bradshaw *et al.* 2006), FJT30 was chosen for further use as it had the brightest fluorescence and would therefore be most easily detected in pathogenicity trials.

Southern hybridisations for the regulated P*dotA*::*egfp* strains FJT24 and FJT26 are shown in Figure 4.4 B. The genomic DNA of NZE7 and the transformants were digested with *Bam*HI and *Pst*I, which have only one restriction site in the pR242 vector (Figure 4.4 A) and should result in one hybridisation band per integration. The Southern blots were hybridised with two probes: a part of the *egfp* gene and the P*dotA* promoter region (Figure 4.4 A). The *egfp* probe was not expected to show a hybridisation band with the NZE7 wild type DNA (Figure 4.4 B). Both the *Bam*HI and *Pst*I digested gDNA of FJT24 and FJT26 showed one hybridisation band for FJT24 and two bands for FJT26. The P*dotA* probe was seen to hybridise to a native 3.8 kb *EcoR*I fragment in NZE7 as expected. The pR242 transformants FJT24 and FJT26 showed additional bands confirming the integration of 1 and 2 copies of the P*dotA*::*egfp* gene into the gDNA respectively (Figure 4.4 C).

Southern analyses using the *egfp* for the constitutive GFP transformants used in further experiments are shown in Figure 4.4 D. An *egfp* hybridisation band was expected in the GFP transformants but, as seen, not for the *dotA* mutant FJT2 and the *pksA* mutant FJT3. All strains transformed with the constitutive *egfp* vector pPN82 (FJT21, FJT22, FJT29, and FJT30) contained several copies of the vector.

Figure 4.4: Southern analyses of the subsequent used GFP reporter strains.

(**A**) Shown is a linear outline of the *egfp* vectors used for transformation and the positions of the DIG labelled *egfp* and P*dotA* probes used for hybridisation. The letters E, B and P indicates the *Eco*RI, *Bam*HI *and Pst*I restriction sides respectively. DNA size ladders are assigned with L (1 kb^+ ladder) and EH (*Eco*RI/*Hin*dIII digested λ–DNA).

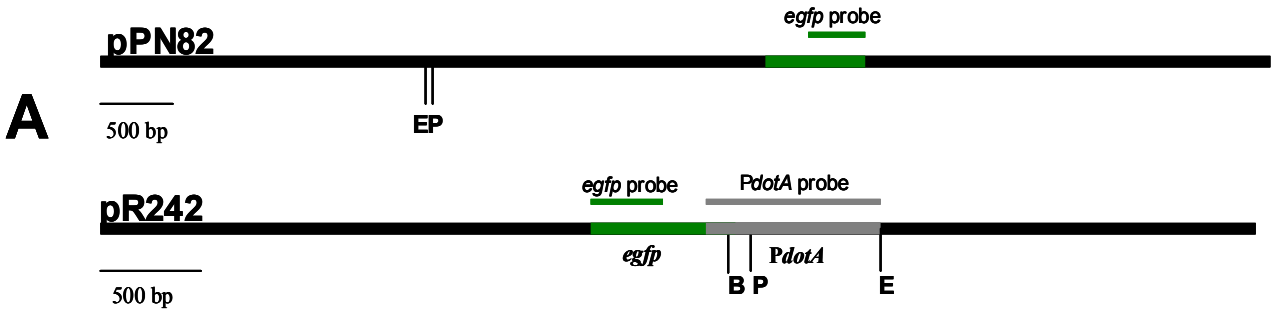
(**B-D**) Shown are separations of the digested gDNAs on a 0.8 % TBE-agarose gel and hybridisation bands of the P*dotA* probe and *egfp* probe of the corresponding Southern blots. The gel pictures (left) are grouped with their corresponding Southern analyses (right). Blots were hybridised with DIG-dUTP labelled PCR products derived from pPN82 (*egfp* probe) and pR242 (P*dotA* probe). Ladders are indicated by L (1 kb^+ Ladder) and EH (*Eco*RI/*Hin*dIII digested λ–DNA)

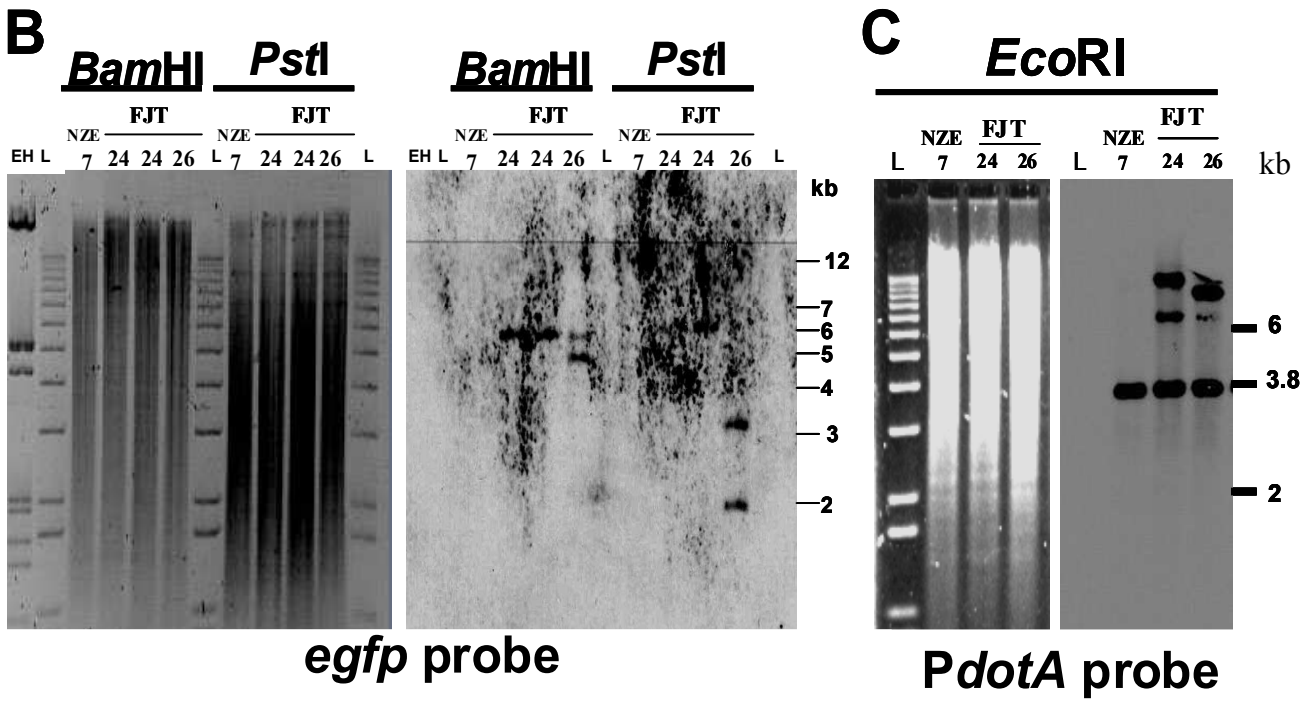
(**B**) Genomic DNA of *D. septosporum* wild type NZE7 and the pR242 transformants FJT24 and FJT26 were digested with *Bam*HI and *Pst*I and hybridised with the *egfp* probe. One hybridisation band was seen for FJT24 in both digestions, while FJT26 DNA showed two hybridisation bands. As expected the NZE7 wild type had no hybridisation band with the *egfp* probe.

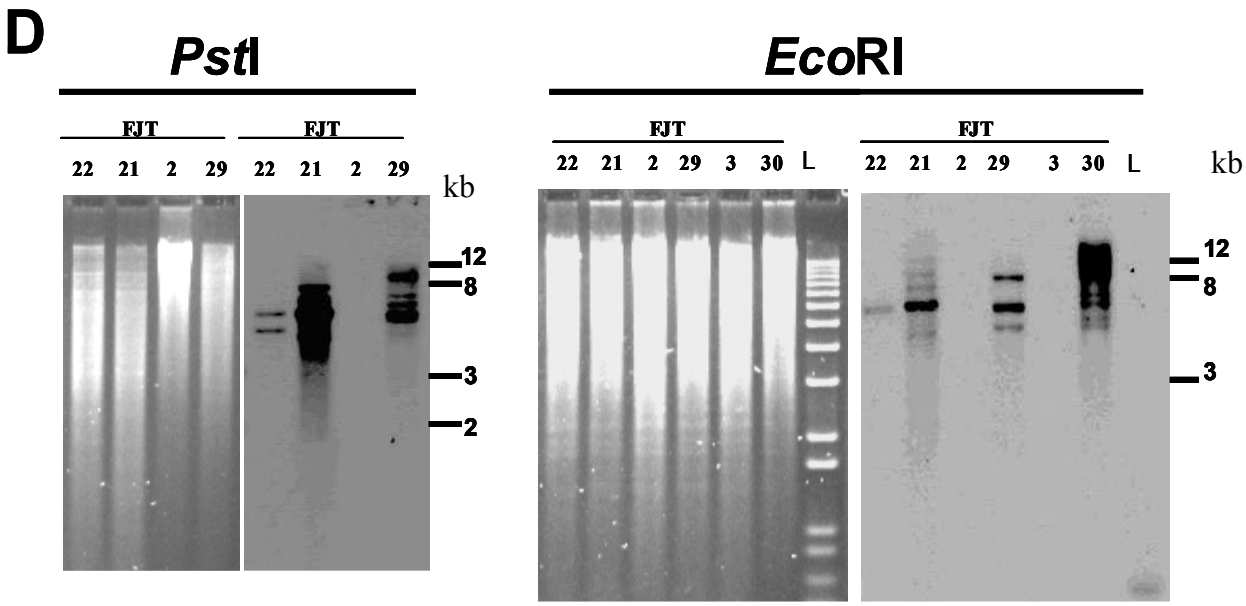
(**C**) Hybridisation bands with the P*dotA* probe of the *EcoR*I digested DNA is shown on the right panel. The wild type DNA shows the expected 3.8 kb hybridisation fragment. FJT24 has one additional band, while FJT26 has two additional bands (where one is only partly seen due to an air bubble between Southern gel and membrane). Both FJT24 and FJT26 show also a weaker band at approximately 6.5 kb which could represent the pR242 vector (6.6 kb) or might be due to a tandem integration of the vector. However, the *egfp* probed Southern blots did not show a band at the putative vector size. Therefore it is assumed that the vector is completely integrated into the genome of FJT24 and FJT26.

(**D**) Southern analyses of the constitutive GFP strains.

Genomic DNA of FJT22 (pPN82) and FJT21 (pPN82); FJT2 (*dotA*$^-$) and its derived transformant FJT29 (pPN82) were digested with *Pst*I and hybridised with the *egfp* probe (left panel). The *egfp* probe hybridised with two fragments of FJT22, while it hybridised with several fragments of FJT21 and FJT29. As expected FJT2 DNA did not show any hybridisation with the *egfp* probe. The left panel shows *Eco*RI digested DNA of FJT22, FJT21, FJT2 and FJT29 as well as FJT3 (*pksA*$^-$) and its derived transformant FJT30 (pPN82). *Eco*RI hybridisation bands with the *egfp* probe are shown on the right side. FJT30 showed several hybridisation bands with the *egfp* probe, as seen for FJT21 and FJT29. As expected the FJT2 and FJT3 did not show hybridisation bands with *egfp*.


A
pPN82
egfp probe
500 bp
EP
pR242
egfp probe
PdotA probe
egfp
PdotA
500 bp
B P
E


B
BamHI
PstI
BamHI
PstI
NZE
FJT
EH L 7 24 24 26 L 7 24 24 26 L
EH L 7 24 24 26 L 7 24 24 26 L
kb
12
7
6
5
4
3
2
egfp probe
C
EcoRI
L NZE 7 FJT 24 26 L NZE 7 FJT 24 26
kb
6
3.8
2
PdotA probe


D
PstI
FJT
22 21 2 29 22 21 2 29
kb
12
8
3
2
EcoRI
FJT
22 21 2 29 3 30 L
22 21 2 29 3 30 L
kb
12
8
3

4.4.2 Growth, sporulation and germination of GFP strains

To assess if the introduction of the GFP reporter genes into *D. septosporum* reduces the fitness of the transformants the growth rates on DM and PDA media were determined. The growth was determined in mm increase of colony diameter per day for the growth periods 3-25 d and 25-47 d. Colony growths of *D. septosporum* transformants were compared to that of the wild type and statistical analyses (ANOVA) were carried out using Excel (Microsoft Office, 2003). Significant ANOVA data were further analysed with Tukeys HSD using an online statistics calculator (http://graphpad.com/quickcalcs/posttest1.cfm) to determine pairwise significant differences. On DM media the transformants FJT 20, 34, 35 and 37 had significantly higher growth than the wild type NZE7 between day 3 and 25 (n=5, P<0.05) (Table 4.1). But none of the transformants seemed to be inhibited in growth by the transformation with the *gfp* vectors (Table 4.1).

Table 4.1: Radial growth of wild type and transformants

Strain	PDA (mm/day)		DM (mm/day)	
	3-25 d	25-46 d	3-25 d	25-46 d
constitutive GFP strains				
NZE 7	0.50 ± 0.02	0.08 ± 0.03	0.50 ± 0.20 ^{A} 0.80 ± 0.03 ^{B}	0.23 ± 0.04^{A} 0.20 ± 0.06 ^{B}
FJT 20 (P*toxA*::*sgfp*)	0.48 ± 0.04	0.09 ± 0.04	*0.70 ± 0.08 ^{A}	0.26 ± 0.18 ^{A}
FJT 21 (P*gpdA*::e*gfp*)	0.54 ± 0.01	0.09 ± 0.04	0.81 ± 0.02 ^{B}	0.30 ± 0.03 ^{B}
FJT 22 (P*gpdA*::e*gfp*)	0.49 ± 0.04	0.12 ± 0.04	0.71 ± 0.03 ^{B}	0.26 ± 0.06 ^{B}
FJT 34 (P*gpdA*::e*gfp*)	0.46 ± 0.02	0.04 ± 0.03	*0.82 ± 0.05 ^{A}	0.25 ± 0.09^{A}
FJT 35 (P*gpdA*::e*gfp*)	0.50 ± 0.08	0.08 ± 0.02	*0.80 ± 0.04 ^{A}	0.15 ± 0.06^{A}
FJT 36 (P*gpdA*::e*gfp*)	0.49 ± 0.04	0.09 ± 0.02	0.83 ± 0.04 ^{B}	0.19 ± 0.08 ^{B}
FJT 37 (P*gpdA*::e*gfp*)	0.50 ± 0.07	0.08 ± 0.02	*0.73 ± 0.02 ^{A}	0.26 ± 0.05 ^{A}
regulated GFP strains				
NZE 7	0.48 ± 0.04	0.19 ± 0.05	0.70 ± 0.03	0.31 ± 0.02
FJT 24 (P*dotA::egfp*)	0.53 ± 0.02	0.13 ± 0.02	0.65 ± 0.04	0.28 ± 0.04
FJT 26 (P*dotA::egfp*)	0.48 ± 0.08	0.24 ± 0.05	0.70 ± 0.10	0.26 ± 0.08

Values given are means ± SD (n=5)
^{A,B} different DM media batch
* statistically significant difference to NZE7 (n=5; P<0.05) on the same media batch

The main growth period of the fungi is between days 3-25, while between days 25-46 only marginal growth is seen. In some cases, as seen for NZE7 the growth of *D. septosporum* can be very variable, showing differences both between media batches and within the same media batch (see NZE7 on DM). This is a frequent phenomen observed in this lab. Therefore the growth rates can only provide an indication of the general fitness of the transformants. However, on both PDA and DM media plates the GFP transformants did not appear to be impaired in growth relative to the wild type.

The germination rate was also similar for the transformants FJT21, FJT22, FJT24, FJT26 and the wild type. This was seen before for FJT21 and FJT22 (Barron 2006) with over 70% spores germinated after 72h on water-agar cellophane plates. Further, Barron (2006) showed that spores of the *D. septosporum* transformants FJT21 and FJT22 germinate on the *P. radiata* needle surface. In this study the germination of FJT24 and FJT26 spores was assessed with spores of NZE7, FJT21 and FJT22 as controls. In this trial the germination rate for all strains was much lower with only 15-25% germinating after 48h. After 120h 38% of FJT24 and FJT26 were germinated and 33% of FJT22, but only 15 % of FJT21 and 24% of the wild type NZE7. In a third trial over 72% of FJT24, FJT21 and NZE7 spores were germinated after 48h, while FJT22 had 65% spores germinated. The high variation between different germination trials precluded further analysis of the results, but germination of the regulated GFP strains was in general not lower than for the wild type. Therefore it was suggested that the germination of the reporter strains is not inhibited by the transformation.

Attempts were made to test if the transformation of the reporter genes into *D. septosporum* impairs the sporulation. However, as seen in Table 4.2 the differences in sporulation for NZE7 or FJT35 in independent experiments were enormous. In view of this variability the sporulation rate was not determined for other transformants as it appeared to be an unreliable indicator. Furthermore it has been noticed in this lab that the number of spores obtained from DSM sporulation plates was very inconsistent between replicates and between trials. The level of inconsistency in sporulation was similar for transformants and wild type, so the effect of GFP insertion on sporulation was considered negligible.

Table 4.2: Summary of characterization of *D. septosporum* transformants and strains.

D. septosporum strain/transformant	Transformed with (Reference)	Characteristics	Copy No. of reporter gene	GFP expression	Sporulation rate[1] (spores/mm^2) n=5
NZE 7	-	**wild type, DOTH+**	-	-	**1.51 ± 1.02** [A] **0.24 ± 0.42** [B]
FJT2 (Seconi 2001)	pR208 (Bradshaw *et al.* 2002)	*dotA* replacement strain; DOTH-	-	-	nd
FJT3 (P6) (Bradshaw *et al.* 2006)	pR226 (Bradshaw et al. 2006)	*pksA* replacement strain; DOTH-	-	-	nd
FJT20	pCT74 (Lorang et al. 2001))	derived from NZE 7 DOTH+ P*toxA*::*sgfp*	1	constitutive GFP +	0.55 ± 0.34
FJT21	**pPN82 (Tanaka et al. 2006)**	**derived from NZE 7 DOTH+ P*gpdA*::*egfp***	**3-4**	**constitutive GFP + + +**	**2.95 ± 1.4**
FJT22	**pPN82 (Tanaka et al. 2006)**	**derived from NZE 7 DOTH+ P*gpdA*::*egfp***	**2**	**constitutive GFP + +**	**0.66 ± 0.47**
FJT34	pPN82 (Tanaka et al. 2006)	derived from NZE 7 DOTH+ P*gpdA*::*egfp*			1.27 ± 0.65
FJT35	pPN82 (Tanaka et al. 2006)	derived from NZE 7 DOTH+ P*gpdA*::*egfp*			1.5 ± 0.75 [A] 0.89 ± 0.33 [B]
FJT36	pPN82 (Tanaka et al. 2006)	derived from NZE 7 DOTH+ P*gpdA*::*egfp*			0.63 ± 0.42
FJT37	pPN82 (Tanaka et al. 2006)	derived from NZE 7 DOTH+ P*gpdA*::*egfp*			1.89 ± 0.81
FJT24	**pR242 (Section 4.2.2)**	**derived from NZE 7 DOTH+ P*dotA*::*egfp***	**1**	**regulated GFP + +**	**nd**
FJT26	**pR242 (Section 4.2.2)**	**derived from NZE 7 DOTH+ P*dotA*::*egfp***	**2**	**regulated GFP + +**	**nd**
FJT27	pR237 (Section 4.2.1)	derived from NZE 7 DOTH+ P*gpdA*::*dsred*	nd	constitutive DsRed	nd
FJT29	**pPN82 (Tanaka et al. 2006) pBC-Phleo (Silar 1995)**	**derived from FJT2 *dotA* replacement strain; DOTH- P*gpdA*::*egfp***	**3**	**constitutive GFP + +**	**nd**
FJT30	**pPN82 (Tanaka *et al.* 2006) pBC-Phleo (Silar 1995)**	**derived from FJT3 *pksA* replacement strain; DOTH- P*gpdA*::*egfp***	**4**	**constitutive GFP + + +**	**nd**
FJT31	**pR260 (Section 4.2.3) pBC-Phleo (Silar 1995)**	**derived from FJT2 *dotA* replacement strain; DOTH- P*gpdA*::*dotA*::*egfp***	**nd**	**regulated DotA-GFP fusion + +**	**nd**
FJT32	**pR260 (Section 4.2.3) pBC-Phleo (Silar 1995)**	**derived from FJT2 *dotA* replacement strain; DOTH- P*gpdA*::*dotA*::*egfp***	**nd**	**regulated DotA-GFP fusion + +**	**nd**

Strains further used in this study are indicated in **bold**.
[1]Determined as described in Chapter 2.15.3
+ - +++ gives an estimation about fluorescence intensity
[A, B] indicate repetitive trials
nd = non determined

4.5 Reporter gene expression

The GFP and DsRed reporter genes are successfully expressed in *D. septosporum*. GFP expression was observed in all transformants containing pCT74 (P*tox*A::*sgfp*), pPN82 (P*gpdA*::*egfp*), pR242 (P*dotA*::*egfp*) and pR261 (P*dotA*::*dotA*::*egfp*). Fluorescence of the constitutive GFP and DsRed reporter gene transformants was clearly distinguishable from the auto-fluorescence of the wild type NZE7 (Figure 4.5). Constitutive reporter gene expression was seen on over whole colonies, for both GFP and DsRed transformants (Figure 4.6).

4.5.1 Constitutive GFP reporter strains

At a whole colony level, transformants with EGFP driven by the constitutive promoter P*gpdA*, EGFP in the wildtype-derived FJT21 and FJT22 strains and the dothistromin-deficient transformants FJT29 (*dotA*$^-$) and FJT30 (*pksA*$^-$) showed strong GFP expression evenly distributed over the whole colony (Figure 4.6). The transformant strain FJT20 with the SGFP driven by the constitutive promoter P*toxA,* showed also GFP expression over the whole colony, although GFP fluorescence appeared to be less (Figure 4.6). The expression of the GFP in the constitutive P*gpdA*::*egfp* strains was seen in all hyphae and in spores as shown in Figure 4.7.

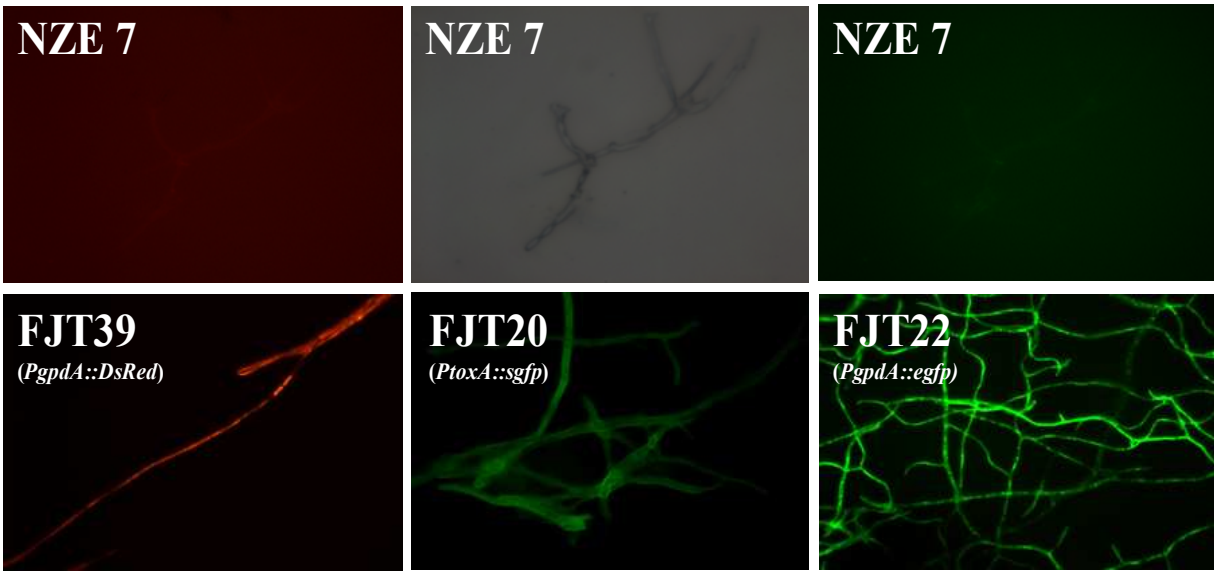


Figure 4.5: Fluorescence of *D. septosporum* wild type and reporter gene strains.
Shown is fluorescence of wild type NZE7 and reporter gene strains FJT39, FJT20 and FJT22. Exposure time for the NZE7 fluorescence was 5 times longer for the DsRed exposure and 9 times longer in case of the GFP exposure.

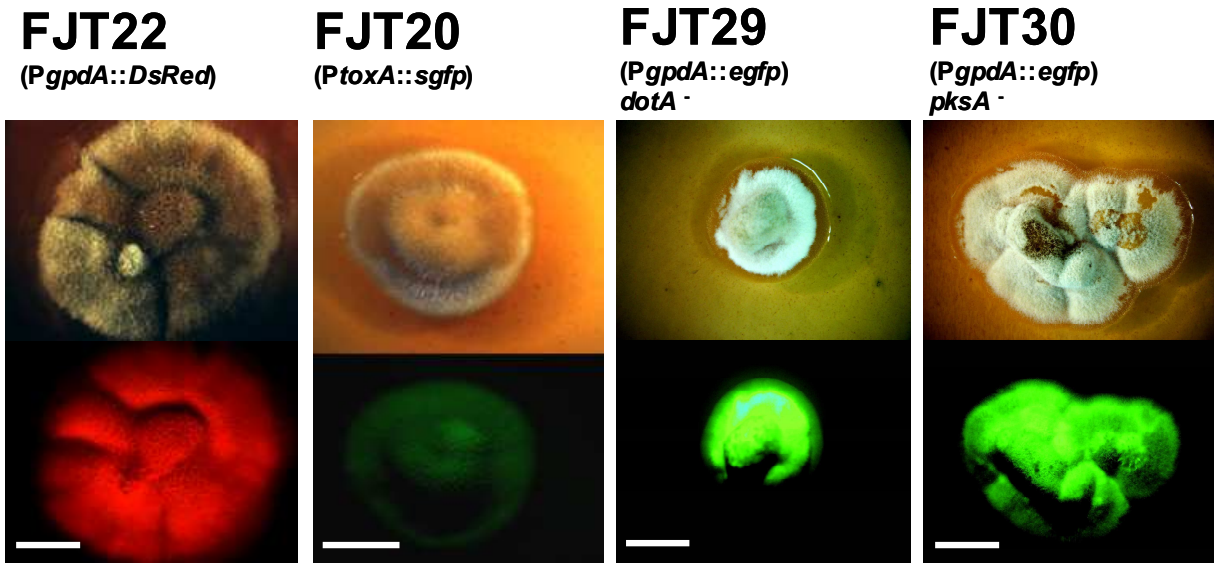


Figure 4.6: Expression of DsRed and SGFP in *D. septosporum* wild type and EGFP in dothistromin deficient mutants. Colonies are shown in visible light (upper part) and UV light for GFP expression (lower part). Bars indicate 5 mm.

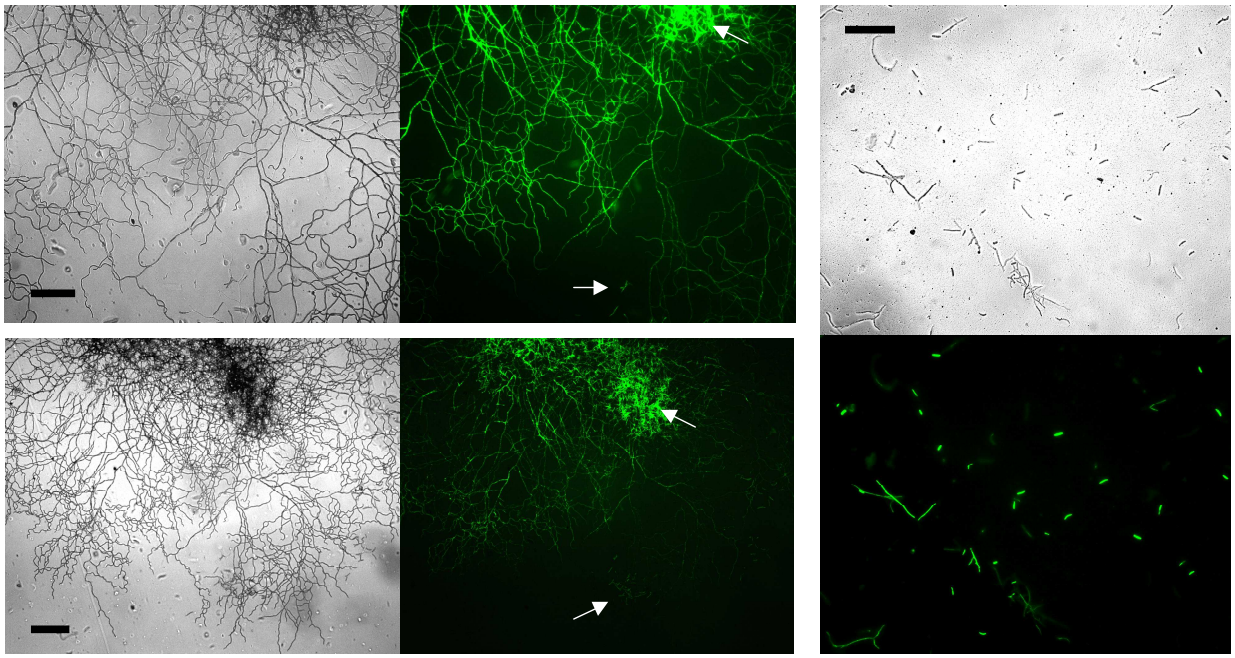


Figure 4.7: GFP expression of the constitutive (P*gpdA*) EGFP expressing strain FJT22.
GFP expression is seen in all mycelia and spores. Regions of spores accumulations are indicated with arrows. Mycelium is shown in visible light and UV light for GFP expression. Size bars in the visible light pictures indicate 20 µm.

4.5.2 Expression of P*dotA* regulated EGFP

The expression of the P*dotA* regulated EGFP expressing transformants was compared to the expression of the constitutive EGFP transformants. On a colony level the constitutive GFP expressing control strain FJT22 showed GFP fluorescence over the whole colony (Figure 4.8). In contrast the strongest fluorescence of EGFP was detected at the outer margin of colonies in P*dotA::egfp* transformants FJT24 and FJT26 (Figure 4.8). The centre of colonies grown on either DM media or PDA media was usually free of GFP expression or showed only a marginal level of expression. The expression of EGFP was in general very even around the edge of the colonies although some sectoring was observed where no EGFP expression seemed to occur (Figure 4.8). Those sectors usually also showed a different morphology and secreted less pigment into the media.

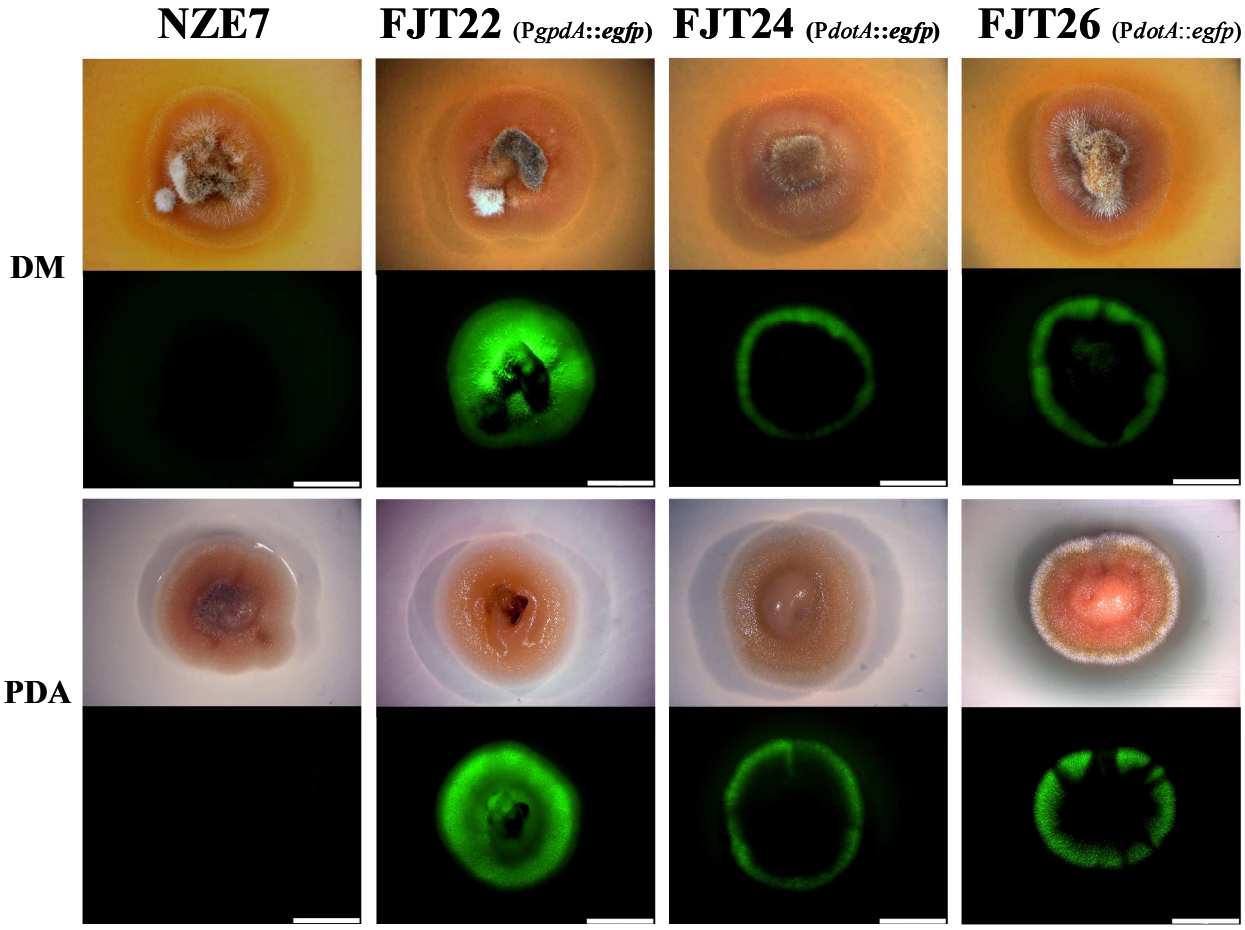


Figure 4.8:GFP expression of *D. septosporum* constitutive and P*dotA* regulated GFP transformants.
12 day old colonies of *D. septosporum* wild type isolate NZE7, constitutive GFP expressing transformant FJT22 (P*gpdA*::*egfp*) and *dotA* regulated GFP expressing transformants FJT24 and FJT26 (P*dotA*::*egfp*) on DM and PDA. Colonies are shown in visible light (upper part) and UV light for GFP expression (lower part). Bars indicate 5 mm. The sectoring seen in the regulated GFP strains FJT24 and FJT26 was also observed in wild type strains and is discussed in Appendix A7.

The GFP expression on the margin of the colonies is consistent with the early onset of expression of the dothistromin genes observed in Chapter 3. Based on the early expression of the dothistromin genes a higher GFP fluorescence at the margin of colonies (Figure 4.8) could be expected, as the growing and therefore younger mycelia are at the colony margin.

In an experiment in which FJT24 was used to inoculate liquid DB cultures, out reaching hyphae of growing mycelium showed higher GFP fluorescence (Figure 4.9). Although not all outreaching hyphae showed expression of GFP, the expression pattern was similar to that observed in the colonies on plates.

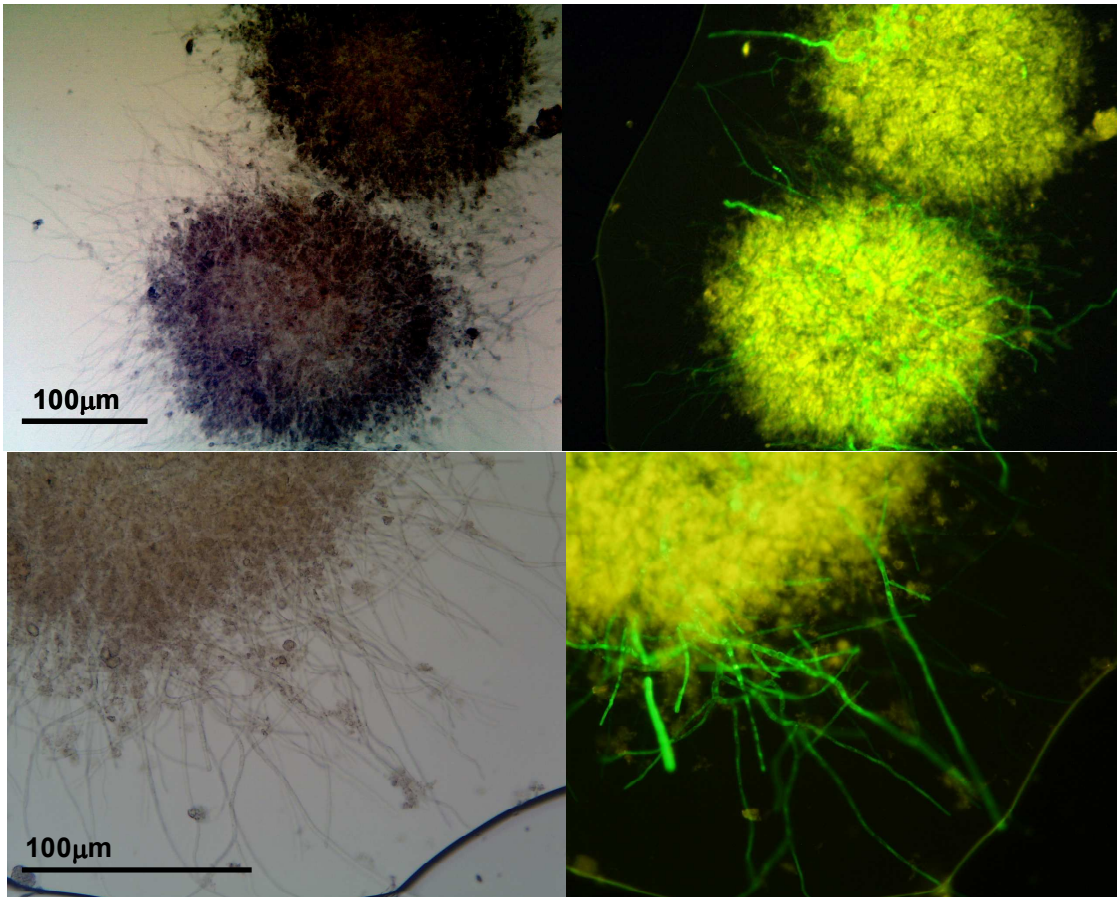
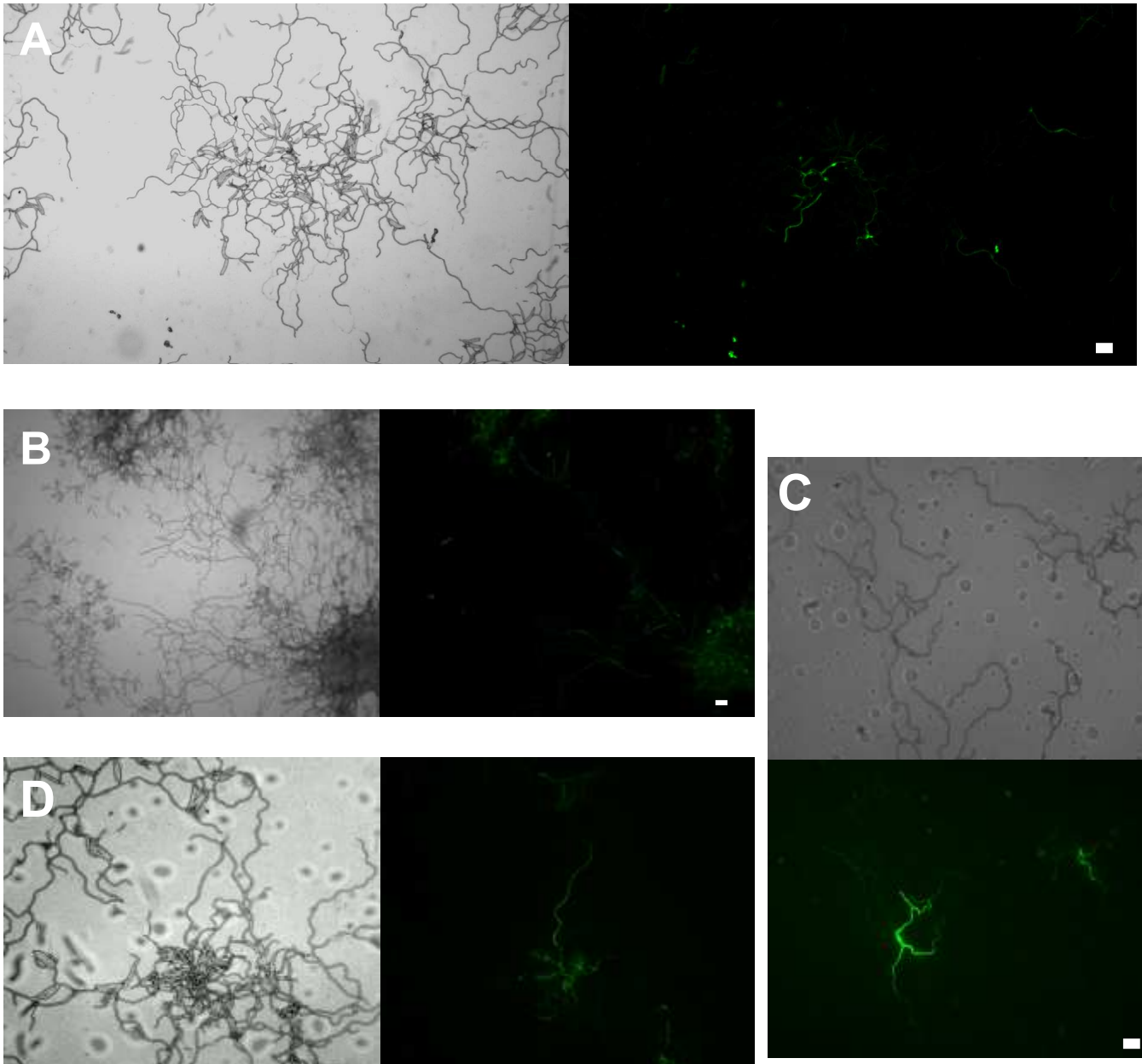


Figure 4.9: GFP expression of *D. septosporum* FJT24 in liquid DB culture.
Shown is mycelium harvested from liquid culture 6 dpi in visible light (left) and UV light for GFP detection (right). Highest GFP expression of the P*dotA*::*egfp* strain FJT24 is detected at outreaching hyphae of mycelium, which formed "balls" due to the shaking. Note that not every outreaching hypha shows GFP fluorescence.

More detailed observation of GFP expression in single hyphae of FJT24 and FJT26 gave somewhat unexpected results when mycelium was grown on cellophane water agar plates (Figure 4.10). On the basis of the results of Chapter 3 and the GFP expression pattern of the FJT24 and FJT26 colonies (Figure 4.8) it was expected that a higher fluorescence might occur on outreaching hyphae of growing colonies. However the GFP expression appeared to be mosaic-like. In contrast to the ubiquitous EGFP expression in spores and mycelium of the constitutive EGFP expressing strains (Figure 4.7) the P*dotA*-regulated strains showed high expression of EGFP in only a few spores and parts of mycelium. However, whenever EGFP expression was clearly seen it appeared to be restricted to germinating spores or young mycelium. Older mycelium was usually low in expression of GFP. Even in younger tissue hyphae often showed a pattern of GFP expression where sections of high fluorescence were interspersed with regions of non-fluorescence (Figure 4.10 F).

Figure 4.10: GFP expression in hyphae and spores of FJT24 and FJT26.
Pictures show mycelium in visible light (left) and GFP fluorescence (right). Scale bars indicate 10 µm.
(**A-F**)The expression of the GFP is (in contrast to the constitutive GFP expressing strains (Figure 4.7)) only detectable in some parts of *D. septosporum* mycelium. A clear pattern of the GFP expression was not detected. Instead GFP expression is seen in spores (**E,F**), germtubes (**E**) and conidiophores (**D**), but not every spore, germtube or conidiophore shows GFP expression. (**F**) Hyphae also show GFP fluorescence with interrupting parts showing no GFP expression.


A
B
C
D
E
F

The early expression of the toxin genes (Chapter 3) also suggested that that the dothistromin genes are not regulated as for AF and ST in *Aspergillus* where AF/ST biosynthesis usually coincides with sporulation. Therefore it might have been expected that the regulated P*dotA*::*egfp D. septosporum* strains show no GFP fluorescence in conidiophores or conidiogenous cells. But when examining conidiophores from FJT24 and FJT26, high GFP fluorescence was seen in some while others did not show fluorescence (Figure 4.11). GFP fluorescence was mainly detected in the cytoplasma with vacuoles appear to be free of fluorescence (Figure 4.11B).

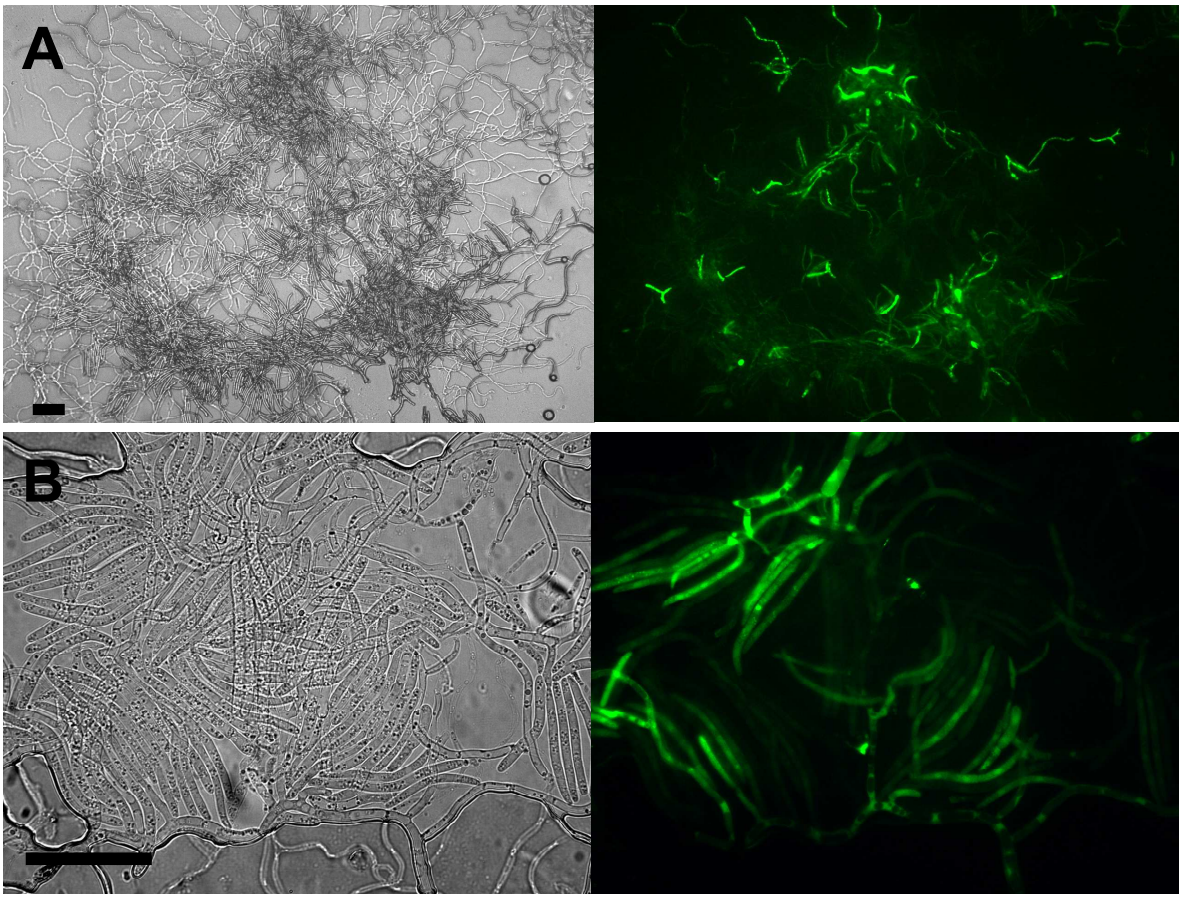


Figure 4.11: GFP expression in conidiophores, condinogenous cells and spores of FJT24.
(**A**) Germinating spores of the regulated P*dotA*::*egfp* strains appear often to have high fluorescence, although this is not seen for every germinating spore. Older mycelium is free from, or low in, GFP fluorescence. (**B**) Conidiophores and spores show a high degree of variation of fluorescence, some showed high GFP fluorescence while other showed none. The EGFP appears to be in the cytoplasm and is absent from vacuoles. Bars indicate 10 μm.

The work just described showed that although expression of the EGFP in the P*dotA* regulated reporter strains gave a clear defined pattern with glowing edges of colonies on plates (Figure 4.8), the expression of EGFP appeared to be variable in individual hyphae and cells (Figure 4.9, 4.10 and 4.11). Therefore the expression of the P*dotA* regulated *egfp* gene and the *dotA* gene in the transformant strains FJT24 and FJT26 was determined to confirm that the expression of the EGFP is indeed correlated with expression of the native *dotA* gene. A one-step RT-PCR (Section 2.12.5) was performed using RNA obtained from FJT24 and FJT26 in liquid culture (Section 2.2.2.2). Figure 4.12 shows co-expression patterns of the P*dotA* regulated GFP and the native *dotA* gene for both strains in liquid culture, confirming a similar pattern of regulation. Furthermore the expression of both the *dotA* and the *egfp* gene was consistent with the expression observed in the wild type (Chapter 3) with maximum expression detected at day 3-4 and declining thereafter.

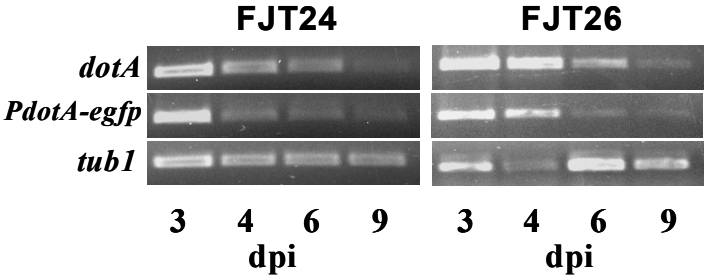


Figure 4.12: Co-regulation of *egfp* and native *dotA* expression in reporter gene transformants FJT24 and FJT26.
GFP transformants FJT24 and FJT26, contain the *egfp* gene under regulation of the *dotA* regulatory region P*dotA*. Shown are RT-PCR products from a equivalent of 100 ng total RNA 3, 4, 6 and 9 days after inoculation (dpi) on a 1 % TBE agarose gel for the native *dotA*, *egfp* and β-tubulin gene (*tub*1*)* genes

4.5.3 DotA-EGFP fusion transformants

The transformants FJT31 and FJT32, derived from the *dotA*⁻ strain FJT2, contain the DotA-EGFP fusion protein. The pattern of GFP fluorescence at colony level was similar to the pattern observed for FJT24 and FJT26, in that the main fluorescence was detected at the colony margin (Figure 4.13). However, more fluorescence was detected in the colony centre of FJT31 and FJT32 in comparison to FJT24 and FJT26 (Figure 4.8).

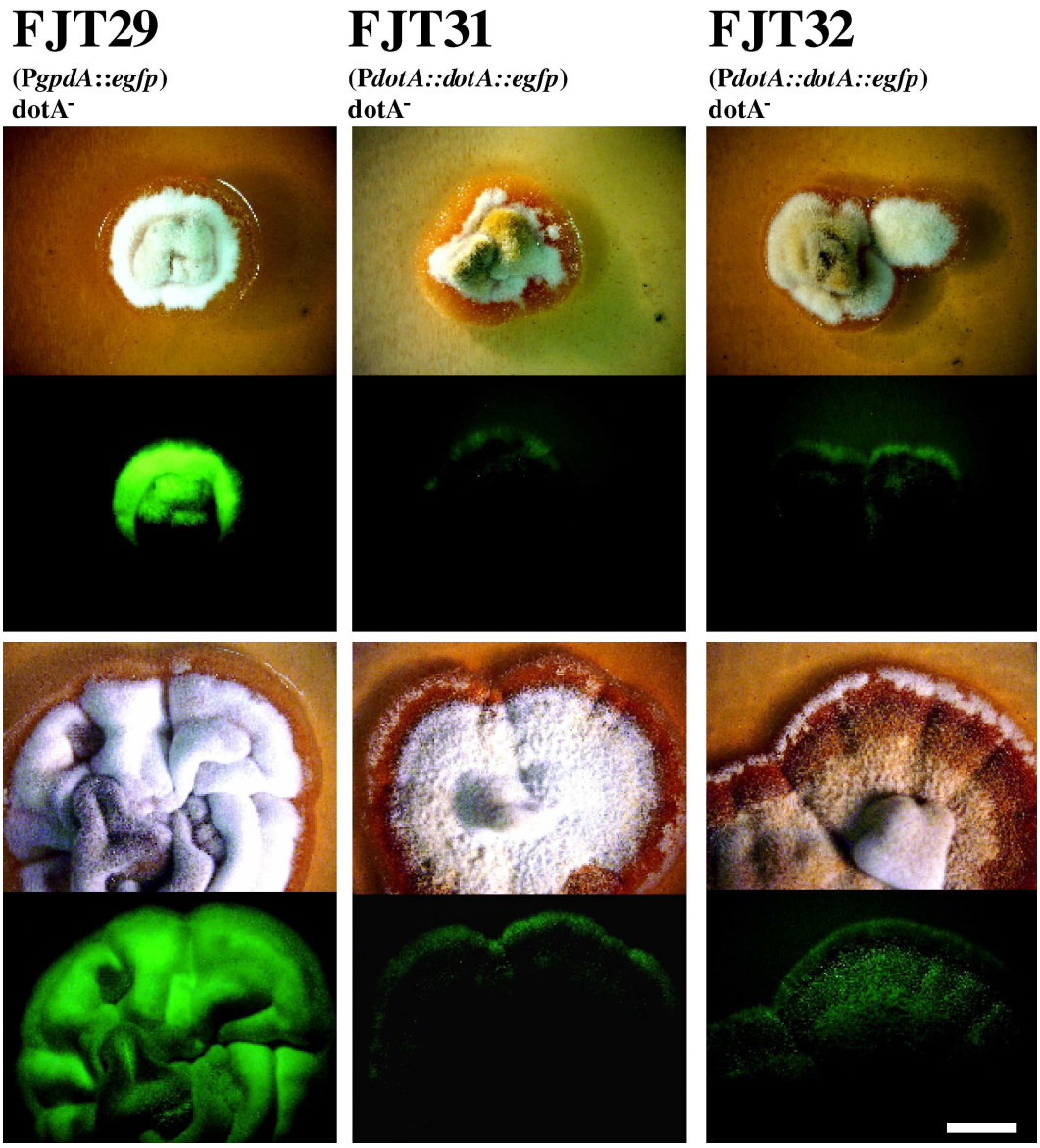


Figure 4.13: GFP expression of transformants of the *dotA* mutant FJT2.

Shown is the GFP fluorescence of the constitutive EGFP *dotA* mutant FJT29 and of FJT31 and FJT32, which were transformed with the pR262 containing the P*dotA*::*dotA*::*egfp* gene. Colonies were grown on DM media plates for 12 (upper part) and 32 days (lower part). Bars indicate 2mm.

When hyphae were examined in more detail the GFP expression of the P*dotA* regulated DotA-EGFP fusion strains was different from that seen in FJT24 and FJT26. GFP fluorescence appeared to be more uniform in the fusion strains, although the intensity varied (shown in the example of FJT32 in Figure 4.14). GFP fluorescence was also detected in hyphae closer to the colony centre in contrast to what was seen in FJT24 and FJT26. Furthermore the GFP signals in hyphae seemed to be different than in FJT24 and FJT26, where GFP fluorescence was seen in the cytoplasm and absent in the vacuole like structures. In FJT31 and FJT32 GFP fluorescence is seen in a more defined pattern, which suggested a different localisation of the DotA-EGFP in comparison to the EGFP.

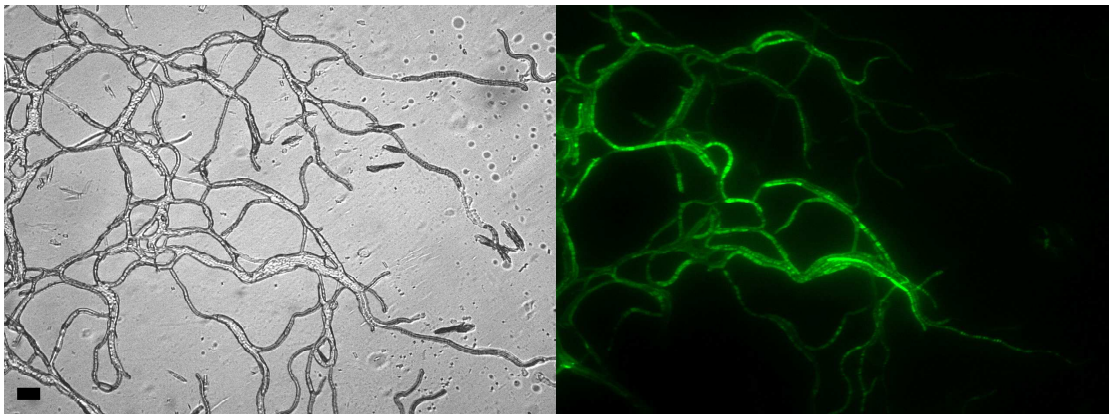


Figure 4.14: GFP expression of the DotA-EGFP fusion strain FJT32 in single hyphae.
The left side shows mycelium in visible light and the right side GFP expression under UV light. Bar indicates 10 μm.

It was further tested if the transformation of the P*dotA*::*dotA*::*egfp* gene did restore the ability of the dotA$^-$ mutant strain FJT2 to produce dothistromin. ELISA analyses showed no detectable dothistromin in media where FJT31 and FJT32 had been grown. This result was supported by TLC analyses of FJT31 and FJT32 extract which lacked the band related to dothistromin. (Figure 4.15). Therefore it is concluded that the transformation did not complement the dothistromin deficiency of the original FJT2 strain.

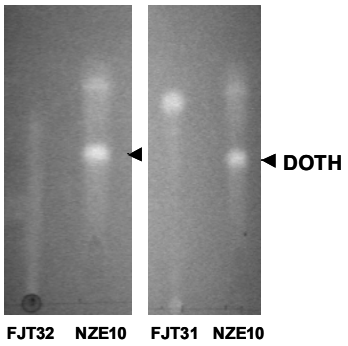


Figure 4.15: TLC plates of *D. septosporum* wild type NZE10 and GFP fusion strains. Shown is the separation of chloroform extract of FJT31 and FJT32 in comparison to NZE10 wild type extracts. The dothistromin (DOTH) band (indicated with an arrowhead) is missing in the FJT32 and FJT31 lanes.

4.5.4 Intracellular location of the GFP reporter proteins

GFP-fusion proteins can be used to determine the localisation of the protein in cells. The EGFP in both constitutive and P*dotA* regulated *D. septosporum* transformants was mainly located in the cytoplasm and did not seem to be incorporated into vacuoles. In contrast the DotA-EGFP fusion protein appeared to be differently located in the hyphae. Patches of GFP fluorescence were observed in distinct intracellular structures. In Figure 4.16 vesicles are seen that harbour the GFP fluorescence, while the cytoplasm appears to be devoid of GFP. An endocytosis-dependent vacuole staining of the FJT22 strain (P*gpdA*::*egfp*), FJT31 (P*dotA*::*dotA*::*egfp*) and FJT32 (P*dotA*::*dotA*::*egfp*) strains was performed. The FM4-64 is a red fluorescent stain which is taken in by endocytosis and accumulates in the vacuoles. The vacuole staining confirmed the absence of the EGFP from vacuoles in FJT22, supporting the suggestion that it is located in the cytoplasm. The intracellular localisation of the DotA-EGFP appears to be more organised. Fluorescence of the DotA-EGFP in the FJT31 and FJT32 strains showed a higher pattern match with the fluorescence of the stain. However, patches of GFP fluorescence were observed in structures which were not stained by FM4-64 (Figure 4.16). This suggested that the DotA-EGFP protein might be located in organelles but not necessarily vacuoles. But those results need to be confirmed and further investigation about the kind of organelles need to be done.

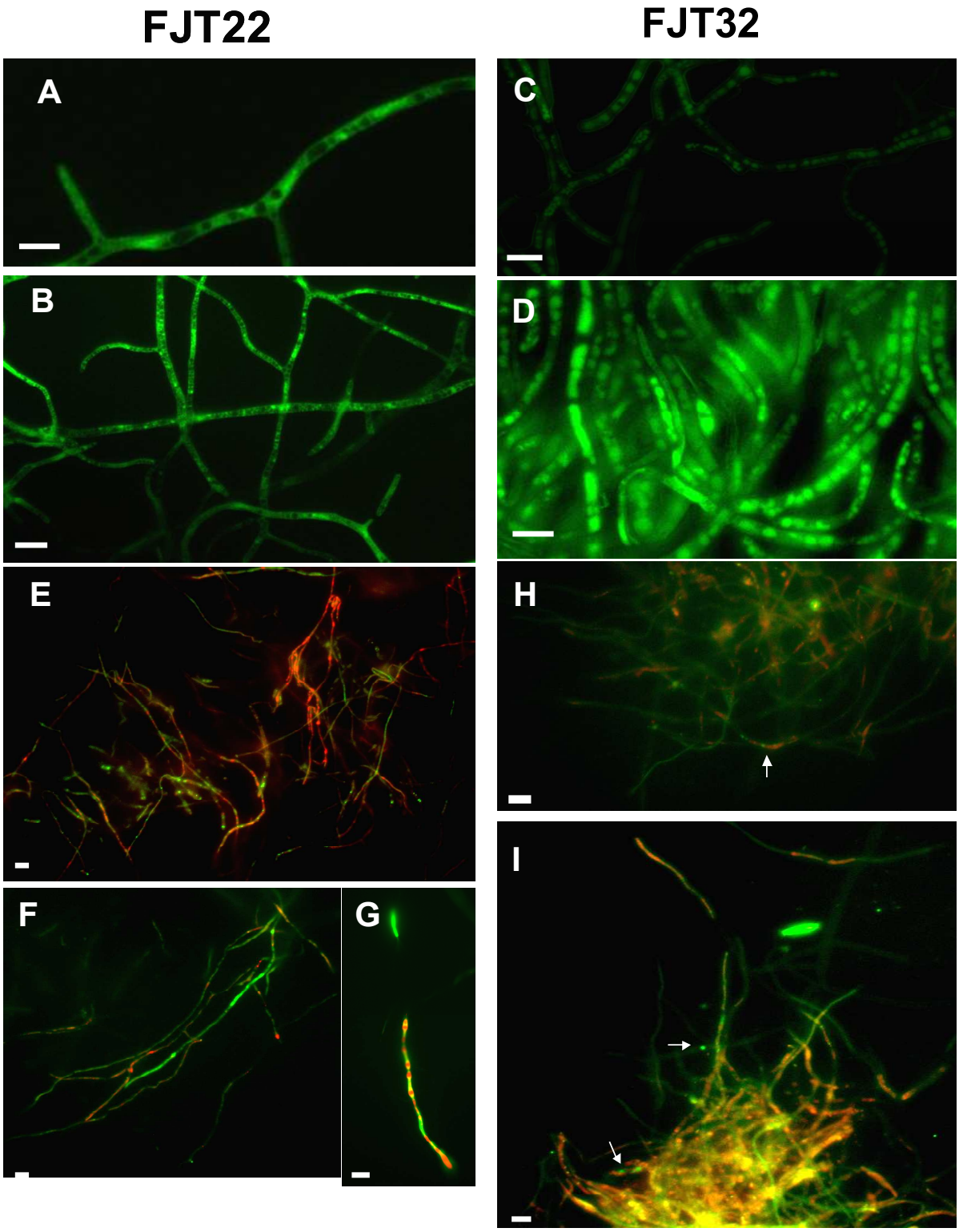


Figure 4. 16: Intracellular localization of the EGFP and DotA-EGFP fusion protein.
(**A,B**) The EGFP fluorescence of FJT22 is located in the cytoplasm. Vesicle-like structures appear to be free from GFP expression. (**C,D**) The fluorescence of the DotA-EGFP fusion protein in FJT32 is clearly seen in patches and vesicle like structures and is different from FJT22. Some vesicles free of fluorescence are seen. (**E,F,G**) The red fluorescence of the vacuole stain does not overlap with the green fluorescence of the EGFP in FJT22. (**H,I,**) The fluorescence of the vacuole stain does partly overlap with the fluorescence of the DotA-EGFP fusion protein. However, EGFP expression is also seen in non-stained mycelium and structures (arrows) in FJT32.

A preliminary analysis to define the putative organelles to which the DotA protein might be targeted was performed using the online pTARGET program (http://bioinformatics.albany.edu/~ptarget/, Guda and Subramaniam 2005), a computational method to predict the subcellular localization of proteins from animals, fungi and metazoans. Predictions are carried out based on the occurrence of patterns of protein functional domains and amino acid compositional differences in proteins from different subcellular locations. This method is able to predict proteins targeted to nine distinct subcellular locations, including cytoplasm, endoplasmic reticulum, extracellular/secreted, Golgi, lysosomes, mitochondria, nucleus, peroxisomes and plasma membrane. Predictions are based on Pfam database version 19.0 (Guda and Subramaniam 2005).

The intracellular localisations for the predicted protein sequences of the genes in the dothistromin mini-clusters were predicted using pTARGET. The native DotA protein was predicted to be a peroxisomal protein with 100 % confidence. Similarly the DotD and PksA proteins are predicted to be localized in the peroxisomes, although with lower confidence. All other putative dothistromin proteins are predicted to be localized in other cell organelles, except for HexA for which only a partial sequence is known (Table 4.3). The predictions are speculative and no putative peroxisomal targeting signals PTS1 and PTS2 were identified in the predicted amino acid sequence of DotA using SignalP 3.0 analyses (Bendtsen *et al.* 2004).

Table 4.3: Prediction of dothistromin biosynthesis protein localizations.

Gene	Putative protein function	Predicted protein localization	Confidence for prediction %
dotA	**ketoreductase**	Peroxisomes	**100**
dotB	**oxidase**	ER	**81.4**
dotC	**toxin pump**	Plasma membrane	**100**
dotD	**thioesterase**	Peroxisomes	**87.6**
hexA[1]	**fatty acid synthase**	Cytoplasm	**93.9**
vbsA	**versicolorin B synthase**	Mitochondria	**93.9**
pksA	**polyketide synthase**	Peroxisomes	**93.9**
cypA	**averufin monooxygenase**	ER	**87.6**
avfA	**oxidase**	Lysosomes	**87.6**
epoA	**epoxide hydrolase**	Golgi apparatus	**62.6**
moxA	**hydroxyversicolorone monooxygenase**	Lysosomes	**87.6**
DS25	amino acid permease	Plasma membrane	75.1
DS31 (StcT)	translation elongation factor gluthathione S-transferase	Peroxisomes	75.1

The pTARGET online program (Guda and Subramaniam 2005) was used to predict the localization of the dothistromin biosynthesis gene products (bold). DS25 was included because of its similar gene expression pattern to the dothistromin genes (Chapter 3). DotC and DS31 were included because of their similarities to other AF/ST cluster genes. ER = endoplasmic reticulum.

[1] only partial sequence was available

4.5.5 GFP expression of FJT24 *in planta*

Spores of the P*dotA*-regulated GFP transformant FJT24 were used to inoculate *P. radiata* seedlings to assess GFP expression on needles. The inoculum was checked for GFP expression on the needles for a time period of 2 weeks. In this experiment slight GFP expression was observed on the needle. The GFP expression was not very strong and most spores did not germinate. (Figure 4.17). However, the expression of the P*dotA* regulated GFP on needles does suggest early expression of the dothistromin genes on needles.

The experiment was conducted over 14 days. The pine seedlings died after 14 days but no Dothistroma needle blight symptoms were seen, suggesting that the death was not caused by the inoculation with FJT24. A limited time frame for this study and lack of suitable pine seedlings at the time required did not allow the experiment to be repeated.

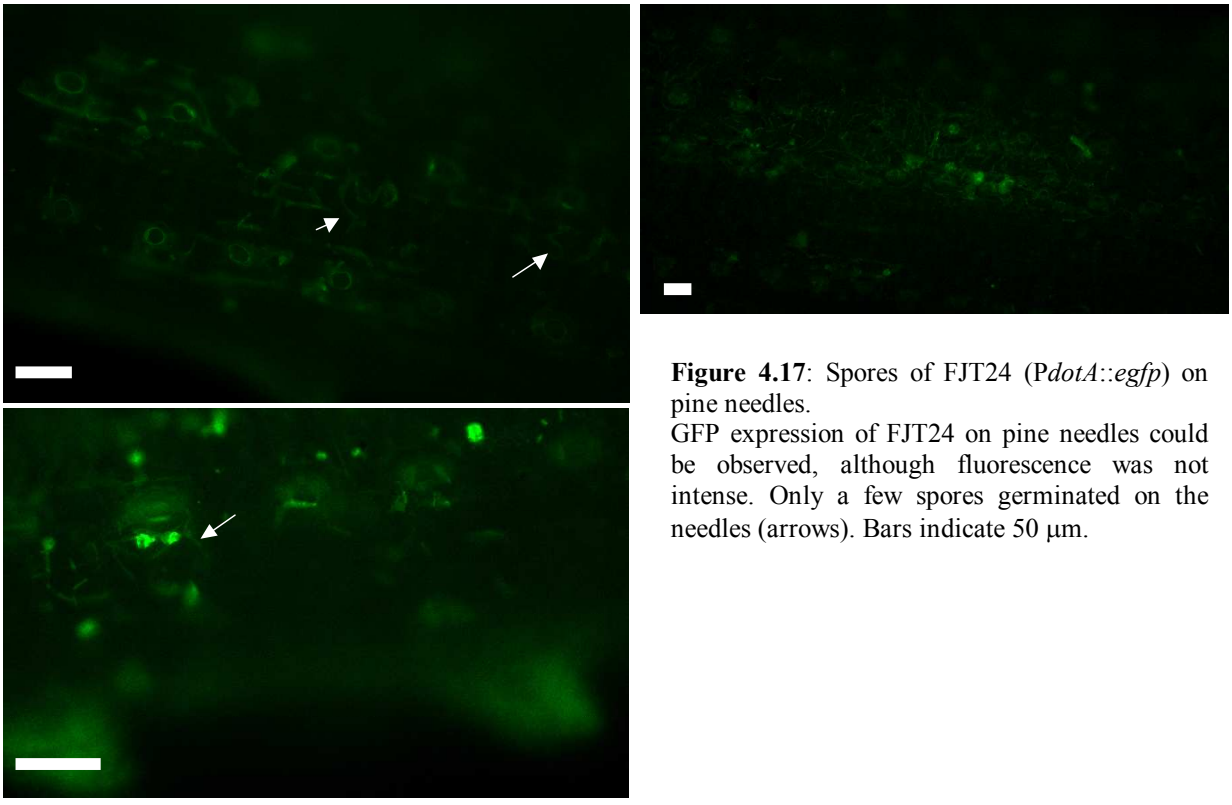


Figure 4.17: Spores of FJT24 (P*dotA*::*egfp*) on pine needles.
GFP expression of FJT24 on pine needles could be observed, although fluorescence was not intense. Only a few spores germinated on the needles (arrows). Bars indicate 50 µm.

4.6 Discussion

D. septosporum expresses EGFP, SGFP and $DSRed_{express}$ which makes them useful tools as reporters in this organism. The transformants with the mammalian codon optimised *egfp* under control of the *A. nidulans* promoter P*gpdA* showed brighter fluorescence than to the single transformant obtained with pCT74, which contained the plant codon optimised *sgfp* under the control of the P*toxA* promoter. In some fungi the SGFP does not appear to be successfully expressed (Pöggeler *et al.* 2003). In this study it was not determined if the lower fluorescence was due to the SGFP itself, to the P*toxA* promoter or to the single gene introduction in the only one transformant obtained. For the aim of obtaining GFP reporter strains, the transformants expressing the EGFP were satisfactory.

A parallel study (Barron 2006) highlighted the use of these constitutive GFP reporter strains in the *D. septosporum*/ pine system as it revealed important information about the disease process and the role of dothistromin as a pathogenicity factor. The constitutive GFP reporter strain FJT21 strain facilitated the observation of the early infection process. Germ tubes of *D. septosporum* enter the pine needle through stomatal openings (Figure 4.18), but no directed growth towards stomata was observed (Barron 2006) as suggested by Muir and Cobb (2005).

Further the constitutive GFP expressing, dothistromin deficient strain FJT30 (derived from the *pksA* mutant FJT3) and FJT29 (derived from the *dotA* mutant FJT2) were successfully used to infect *P. radiata* seedlings (Figure 4.18, Barron unpublished). GFP fluorescence was detected in fruiting bodies in newly formed lesions (Figure 4.18), suggesting that the infection was caused by FJT30 and not by strains which might have been on the host prior to inoculation with FJT30. Thus, the construction of FJT29 and FJT30 led to the important conclusion that dothistromin is not a pathogenicity factor in the disease.

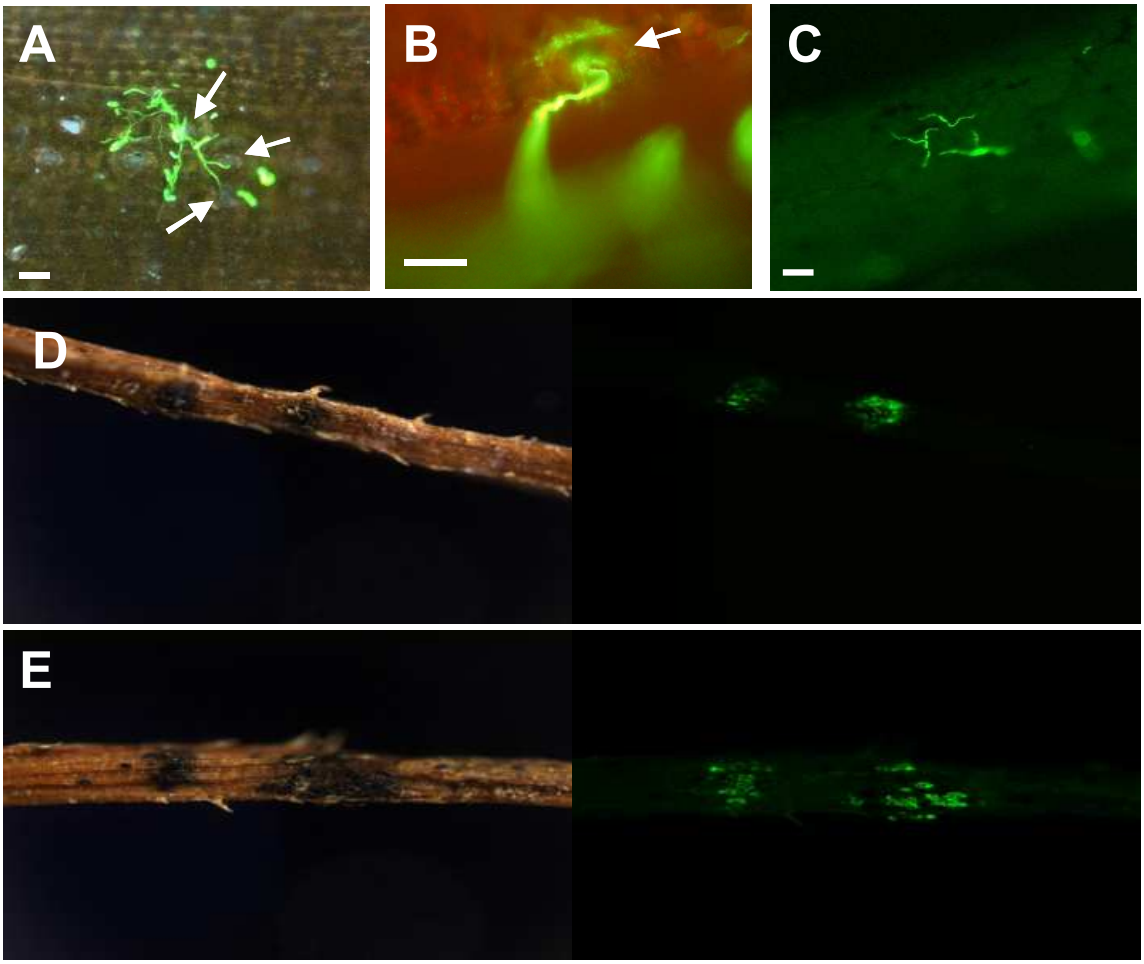


Figure 4.18: Use of GFP transformants, developed in this study, in pathogenicity trials.
(**A, B**) FJT21 (wild type; *PgpdA::egfp*) facilitates the observation of the infection process on needles. Penetration of hyphae through stomata are marked by arrows. (**C**) Dothistromin deficient strain FJT30 (*pksA*-; P*gpdA*::*egfp*) was also easily observed on needles. Bars in **A,B** and **C** indicate 10 µm. (**D**) Pine needles infected with FJT30 showed disease symptoms. GFP fluorescence of the fruiting bodies identified that the FJT30 caused the disease symptoms and identity of FJT30 in the lesion was subsequently confirmed by PCR. Results shown here are from experiments conducted by Naydene Barron (2006, unpublished).

The successful expression of the *egfp* gene in *D. septosporum* allowed the construction of the P*dotA* regulated *egfp* reporter strains FJT24 and FJT26. In those strains the GFP was expressed and the gene expression patterns of the native *dotA* and the *pdotA::egfp* appeared to be co-regulated (Figure 4.12). The expression pattern for both *dotA* and *egfp* is also consistent with the dothistromin gene expression observed for the wild type strains in culture (Chapter 3, Figure 4.12). Therefore the expression of the EGFP in those strains appears to be a reliable reporter for the expression of the *dot*A gene in FJT24 and FJT26.

The unusual early expression of dothistromin genes (Chapter 3) was also seen in FJT24 and FJT26 colonies, which showed highest GFP fluorescence in younger mycelium. While constitutive GFP expressing strain FJT22 showed ubiquitous GFP fluorescence over the whole colony, *dotA*-regulated strains FJT24 and FJT26 showed GFP expression predominantly at the growing margin of the colony (Figure 4.8). This is consistent with elevated P*dotA*-driven gene expression in young hyphae and the early onset of *dotA* expression seen in liquid culture (Chapter 3). The same pattern of GFP expression has been observed on both PDA and DM media, suggesting that this is not a media effect, although the expression in DB and PDB media was different in liquid cultures (Section 3.2.3). The detection of GFP in spores and germinating spores of FJT24 and FJT26 (Figures 4.10, 4.11) confirms the early onset of dothistromin gene expression observed in liquid culture as reported in Chapter 3, where dothistromin gene expression was also detected in the spore inocula.

The high level of dothistromin gene expression at an early stage of growth (Chapter 3) also suggests that the regulation of dothistromin biosynthesis is not directly connected with that of sporulation which usually occurs at a late stage of growth. High expression of EGFP in FJT24 and FJT26 was seen in some but not all conidiophores (Figure 4.11). This indicates that some dothistromin synthesis might occur parallel to the sporulation but is not necessarily co-regulated. Interestingly the expression of secondary metabolite genes at an early development stage (depending on the environmental conditions) has been observed in other species and does not appear to be unique in *D. septosporum*. The *pksP* gene, encoding a polyketide synthase is involved in both pigment biosynthesis and virulence of *A. fumigatus*. A GFP-PksP fusion protein was only found in phialides and conidia *in vitro* indicating a developmentally controlled expression of the gene. But *pksP-egfp* expression was also detected in hyphae of germinating conidia isolated from the lungs of immunocompromised mice (Langfelder *et al.* 2001).

The high GFP expression at the margin of the colony supports the hypothesis that dothistromin is involved in the early plant pathogen interaction, where a toxin is secreted in advance of the growing mycelia (Gadgil & Holden 1976, Section 1.2.2). But as the

infection with the dothistromin deficient strains FJT29 and FJT30 showed, the toxin does not appear to be necessary for pathogenicity (Barron 2006). However, GFP expression was also detected in spores of FJT24 on pine needles (Figure 4.17), suggesting the genes are expressed early *in planta*, even though this experiment was not repeated and GFP fluorescence was weak. As the expression of dothistromin genes was also detected in the spore inocula in Chapter 3, the different levels of GFP fluorescence in the conidiospores might represent different metabolic states, with some spores about to germinate while others are not.

In single hyphae GFP expression in FJT24 and FJT26 was detected in outreaching hyphae in DB cultures but not seen in colony centres (Figure 4.9). However, when grown on cellophane plates of the GFP fluorescence in the *dotA*-regulated GFP transformants FJT24 and FJT26 was very inconsistent (Figure 4.10), in contrast to the constitutive GFP expressing strains FJT21 and FJT22 (Figure 4.7). While high GFP expression was often seen in germinating spores or small mycelium fragments, other hyphae of similar size or germination status did not show fluorescence. As seen in Figure 4.10F strong fluorescent parts were dispersed between non-fluorescent parts even in single hyphae. This suggests that the loss of GFP fluorescence is not due to a loss of the P*dotA*::*egfp* gene. Further, insertion of the *PdotA::egfp* gene into the FJT24 and FJT26 genome was shown by Southern analyses (Figure 4.4) and two rounds of single spore isolation purified the transformants. This makes it unlikely that the observed mosaic-like GFP expression in FJT24 and FJT26 is caused by the loss of the P*dotA*::*egfp* gene. A similar non-uniform pattern of distribution for AF proteins has been seen in areas of colonies in *A. parasiticus* that are presumably the same age and in a similar environment (Lee *et al.* 2004). The detection of AF proteins by immunoelectron microscopy showed intensely labelled, weakly labelled, and unlabelled cells adjacent to each other in the same thin layer section. This suggests that the extent and timing of aflatoxin synthesis also varies from cell to cell. Likewise a similar observation was made for other genes in *Aspergillus niger*. Using GFP reporter genes, it was shown that "exploration hyphae" differentiate, with some strongly expressing the glucoamylase gene *glaA*, while others hardly express it at all (Vinck *et al.* 2005). Those findings indicate that a fungal mycelium is highly

differentiated, even though hyphae in the exploration zone are exposed to the same nutritional conditions (Vinck *et al.* 2005).

The GFP expression pattern of the P*dotA*::*dotA*::*egfp* transformants FJT31 and FJT32 showed high GFP fluorescence at the margin of colonies as well as in spores and hyphae (Figures 4.13, 4.14). In contrast to FJT24 and FJT26 the GFP was detected more evenly over the colony as well as in older hyphae. This suggests that the protein might be stable and active after translation. This would be a possible explanation for the delayed maximum of toxin production in comparison to the maximum of expression of dothistromin genes seen in Section 3.

Transformants expressing the DotA-EGFP fusion protein also revealed some clues on the intracellular localization of dothistromin biosynthesis. While the EGFP in the *D. septosporum* transformants appears to be located in the cytosol, the DotA-EGFP fusion protein appeared to be in certain cellular compartments in the fusion strains FJT31 and FJT32. The dappled nature of the DotA-EGFP signal suggests that this fusion protein is localized within cytoplasmic vesicles. While vacuole staining showed some overlapping of GFP signals and vacuolar stain, GFP signals were also observed in unstained vesicles. However, those experiments need to be repeated. On a side note it should be said that the FM4-64 stain used in this study is an endocytosis dependent stain. This is interesting as endocytosis by hyphae of filamentous fungi is still in debate (Fuchs & Steinberg 2005; Read & Kalkman 2003). However, the uptake of FM4-64 and incorporation into vacuoles (Figure 4.16) suggest that endocytosis occurs in *D. septosporum*.

Observations of the location of the DotA-EGFP fusion protein has been made near the completion of this study and need to be further investigated. There are several things to be considered. Firstly, the fusion protein is not proven to be functional. Different folding might cause non-functionality and therefore the observed localisation of the protein may be an artefact. Further, the fusion protein has been transformed into the *dotA* replacement strain FJT2 and not into the wild type, and the DotA-EGFP fusion protein did not complement the *dotA*$^{-}$ strain FJT2. Since morphological changes have been correlated with the production of secondary metabolites the lack of dothistromin, or metabolites derived from it, might cause alterations in FJT2 compared to the wild type.

Nevertheless, it would be very interesting to see if the synthesis of dothistromin indeed occurs in certain cell organelles. This would imply a regulated mechanism for dothistromin secretion. The identification of such a mechanism might result in new targets for the disease control, if spatial organisation of dothistromin biosynthesis is correlated to auto-protection of the fungi against dothistromin.

The DotA-EGFP fusion protein will facilitate the identification of the organelles of dothistromin sythesis. Although dothistromin antibodies are available (Jones 1993), immunoelectron microscopy to detect the intracellular accumulation of dothistromin is difficult due to technical limitations in the preparation of samples (Jones, personal communication). In contrast, the determination of the intracellular localisation of the DotA-EGFP fusion protein should be more accessible using confocal microscopy. This should allow the identification of organelles required for dothistromin biosynthesis in future work.

The localization of the DotA protein in organelles is supported by the pTARGET analysis, which predicted DotA localization in peroxisomes. Peroxisomes might represent the organelles in which GFP expression is detected in absence of the vacuole stain. Interestingly most of the dothistromin gene products identified so far are predicted to be in cell organelles (Table 4.3). It can be speculated that dothistromin biosynthesis is highly spatially organized and this might play a role in auto-protection. In *Cercospora nicotianae*, it was shown that the PDX1 protein is localized in cellular vesicles of unknown function. PDX1 is thought to have a role in auto-protection against cercosporin, as it is required for growth in the presence of cercosporin and other photosensitizers, and the localization of PDX1 might be important for this (Chung *et al.* 2002). Because of dothistromin's toxic properties it might be favourable for *D. septosporum* if its synthesis is localized in certain cell organelles to keep the intracellular concentration of toxin low. Lee et al. (2002) observed a similar phenotype for the AF protein OmtA in *A. parasiticus*. OmtA converts ST into o-methylsterigmatocystin and is a protein of the late biosynthesis steps of AF. Cells labeled with OmtA polyclonal antibodies showed patches of fluorescence within fungal cells, suggesting that OmtA is confined to subcellular compartments. In a further study (Lee *et al.* 2004) the authors determined the localization of OmtA mainly to basal vacuole organelles, while the protein AflM (Ver-1) and AflD

(Nor-1), which are involved in the earlier steps of aflatoxin synthesis are mainly cytoplasmic. Interestingly the AflM protein is the homologue of the DotA protein. But the DotA protein is involved in the late state of dothistromin biosynthesis (Bradshaw *et al.* 2002) after the dothistromin and AF pathway are proposed to diverge (Henry & Townsend 2005, Figure 1.7).

The localization of proteins involved in fungal secondary metabolism in organelles has also been observed for penicillin synthesis in *Penicillium chrysogenum* (Müller *et al.* 1991) and localization of prehelminthorospol in organelles was observed in *Bipolaris sorokina* (Akesson *et al.* 1996). The intracellular organization might therefore in general play an important role in the synthesis of secondary metabolites.

The intracellular localisation of the DotA-EGFP protein might also explain the differences of GFP fluorescence between the P*dotA*::*egfp* reporter strains FJT24 and FJT26 and the fluorescence of the P*dotA*::*dotA*::*egfp* transformants FJT30 and FJT31. Vinck *et al*. (2005) reported that differences in fluorescence using cytoplasmic GFP reporter proteins, and GFP fused to the native protein of interest, were due to cytoplasmic streaming of the GFP in the former case. Similarly the distribution of the cytoplasmic EGFP in FJT24 and FJT26 and the intracellular localization of DotA-EGFP in FJT31 and FJT31 might be responsible for the differences in GFP fluorescence.

In consideration of the similarities of the AF/ST and dothistromin pathways it is very interesting that the dothistromin proteins DotA, DotD, PksA are predicted to be located in peroxisomes. Peroxisomal β–oxidation appears to have an important role for the synthesis of AF/ST, depending on the kind of fatty acids available, and the AF/ST precursor norsolorinic acid was located in peroxisomes (Maggio-Hall *et al.* 2005). Polyunsaturated fatty acids can be converted to oxylipins. Oxylipins appear to interfere in the perception, metabolism and signalling between host and microbe of each other and are necessary for secondary metabolite production and seed colonisation by *Aspergillus* species (Tsitsigiannis & Keller 2006; Tsitsigiannis & Keller 2007). If it can be confirmed that the synthesis of dothistromin is located in peroxisomes and therefore connected with the fatty acid metabolism it is feasible that oxylipins play a role as signals or external regulatory molecules, which influence the production of dothistromin, as seen for the AF/ST synthesis in *Aspergillus*. Further experimental work about this aspect needs to be

done. The identification of possible signalling molecules for dothistromin production will improve our understanding of the putative roles of dothistromin.

In summary, the construction of *D. septosporum* GFP reporter strains led to the following results:

- *D. septosporum* invades *P. radiata* needles through stomata. The toxin is not necessary for pathogenicity, although the toxin genes appear to be expressed *in planta*.
- The *dotA*-regulated GFP reporter strains confirmed that the dothistromin genes are highly expressed at an early stage of growth, as seen previously in submerged culture (Section 3). The expression is different in each cell, as also seen for other genes in *Aspergillus* (Lee *et al.* 2004; Vinck *et al.* 2005) although the overall expression of the dothistromin genes appears to decrease with age (Section 3, Figure 4.7).
- The DotA-EGFP protein appears to be at least occasionally located in organelles and the same is predicted for other dothistromin genes. This suggests that the synthesis of dothistromin is spatially organised, as seen for other mycotoxins (Akesson *et al.* 1996; Lee *et al.* 2004; Müller *et al.* 1991).

The early toxin production was supported by the *D. septosporum* GFP reporter strains, but it was shown that dothistromin is not a pathogenicity factor. Therefore no support was seen for the first hypothesis, implying a role for dothistromin in the early infection process, outlined at the end of Chapter 3.

The second hypothesis, implying a role of dothistromin in competition, is tested in the next chapter.

Chapter 5:
A role for dothistromin in competition?

5.1 Introduction

Dothistromin has previously been assumed to be a pathogenicity factor as dothistromin can be isolated from the red bands in infected needles (Bassett *et al.* 1970) and the injection of purified dothistromin reproduced disease symptoms (Shain & Franich 1981). However, the high variation in dothistromin production levels by different isolates of *D. septosporum* and *D. pini* (Bradshaw *et al.* 2000) questions the role of dothistromin as a pathogenicity factor. Furthermore, red bands are not always seen in *Dothistroma*-infected needles and dothistromin content of lesions is not correlated to the length of lesion (Section 1.2). The infection of *P. radiata* seedlings with the dothistromin deficient *D. septosporum* GFP mutant FJT30 (Chapter 4) suggests that dothistromin is not a pathogenicity factor. The question remains: why is *D. septosporum* producing the toxin *in planta*, if it is not necessary for the pathogenicity? This chapter studies the hypothesis that dothistromin inhibits the growth of other fungi which may compete with *D. septosporum* for the host.

Fungal secondary metabolites often have antifungal, antibiotic, insecticidal or anti-mammalian properties (Pelaez 2005; Seo & Yu 2005). The broad toxicity of dothistromin (Section 1.2) and the unusual early growth stage expression of the dothistromin genes (Chapters 3 + 4, Schwelm *et al.* 2007) led to the hypothesis that dothistromin might play a role in competition with other microorganisms. Because *D. septosporum* is a slow growing fungus, the early production of a toxin which inhibits the growth of other microorganisms in the same environment, would be beneficial for the fungus. It would allow *D. septosporum* to compete with other fungi in colonizing the needle tissue. Similarly, it is proposed that *Streptomyces* spp. evolved to secrete the toxins FK506, ascomycin and rapamycin to inhibit the growth of competing yeast and fungi (Arndt *et al.* 1999; Challis & Hopwood 2003).

The interaction of competing organisms is a complex issue and the unreliability of pathogenicity tests for *D. septosporum* did not allow for testing the role of dothistromin in competition on needles. Instead, dothistromin-producing strains and dothistromin-deficient strain FJT3 of *D. septosporum* were challenged with competitor fungi in plate tests to determine whether there is an advantage of toxin production. The competitor fungi were selected on the basis of their frequent occurrence in pine forests (*Lophodermium conigenum, L. pinastri, Cyclaneusma minus, Strasseria geniculata*), as a producer of sterigmatocystin (ST), a toxin similar to dothistromin (*A. nidulans*), because they are aggressive plant pathogens in their own right (*Eutypa lata, Fusarium graminearum, Glomerella cingulata, Alternaria alternata*) or because they are biocontrol agents with proven antifungal qualities (*Trichoderma harzianum, T. viride*). Competition test were conducted on PDA plates as all competitors used in the experiment were known to grow on PDA media. Furthermore, expression of the dothistromin genes was more or less constitutive in PDB media (Section 3) so induction of the GFP-expression in the reporter strains would be easier to detect.

Since the expression of the dothistromin genes was much higher in DB media, which contained yeast extract in the nutrient broth, than in PDB (Section 3) it was speculated that expression of the genes might have been induced by the fungal components in the media. In a study on maize it was shown that co-inoculation of *Aspergillus* spp and *Fusarium* spp. can enhance the production of fumonisin by *Fusarium*, which is thought to be a defence response (Marin *et al.* 1998). In *A. flavus* the microorganisms *Bacillus amylloliquefaciens* and *Hyphopichia burtonii* can increase the synthesis of AF (Cuero *et al.* 1987). Further serendipitous observations described in the results section of this chapter also suggested a possible induction effect. Therefore, it was investigated if competing fungi induce dothistromin biosynthesis. GFP fluorescence in plate cultures of challenging fungi and *D. septosporum* FJT24, containing the P*dotA*::*egfp* gene, were used for a visual estimation of enhanced dothistromin gene expression as a response to competitors. Liquid cultures of wild-type *D. septosporum* challenged with a yeast “elicitor” broth or broth in which competitor species had previously been grown were also used to determine if there is an effect on dothistromin production.

5.2 Results

5.2.1 Plate competition with *D. septosporum* wild type and dothistromin deficient mutant

The effect of dothistromin production on the growth of competitive fungi was tested in culture as described in Section 2.15.

In an initial trial (Trial 1) the growth rates for *L. conigenum, L. pinastri, S. geniculata*, *A. nidulans*, *E. lata* and *G. cingulata* were assessed on plates containing dothistromin-producing (dot^+) *D. septosporum* strains. These growth rates were compared to those on plates containing the dothistromin-deficient (dot^-) strain FJT3. The differences between growth of each competitor towards and away from *D. septosporum* were used to estimate the inhibition of growth caused by *D. septosporum* (as described in Section 2.15). Table 5.1 summarizes the result of this competition trial. In each case the growth inhibition of the competing fungus was higher on plates with dothistromin producing fungi than on plates with the FJT3 dot^- strain. The FJT3 dot^- strain was quickly overgrown by each competitor during the time period investigated, whilst the dothistromin producing strain was not overgrown until a later stage if at all.

The effect of inhibition is highly dependent on the competitor species. For example, *L. pinastri* and *L. conigenum* are highly affected by the *D. septosporum* wild type, with over 70% inhibition of growth by the wild type 15 and 11 days after inoculation respectively. At the same time the FJT3 (dot^-) was overgrown by the *Lophodermium* spp.. In contrast the growth of *S. geniculata* was only mildly affected by the presence of the *D. septosporum* (dot^+) in comparison to the FJT3 (dot^-) strain; although the growth of *S. geniculata* was initially slowed down by the presence of the *D. septosporum* dot^+ wild type it started to overgrow the *D. septosporum* dot^+ colony after 4 days.

Figure 5.1 shows some typical results obtained in this competition trial. At the time the pictures were taken the competitors had not made contact with the wild type

strain NZE7 whereas the competitor had overgrown the dothistromin deficient strain FJT3 in each case.

Table 5.1: Growth inhibition of competitor species by *D. septosporum* (Trial1).

Competitor Species	dpi of competitor	% inhibition of competitor by wildtype (dot^+) *D. septosporum*[1] (mean ± SD; n ≥3)	% inhibition of competitor by FJT3 (dot^-) *D. septosporum*[1] (mean ± SD, n=2)
Aspergillus nidulans	6 11 15	42.6 ± 7.1 58.7 ± 4.9 43.9 ± 34.1 (partly overgrown[2])	20.4 ± 12.8 overgrown [2] overgrown[2]
Eutypa lata	3 11 15	No inhibition[3] 67.2 ± 7.2 23.7 ± 41.1 (mostly overgrown)	overgrown overgrown overgrown
Glomerella cingulata	3 4 6	27.8 ± 26.5 40.8 ± 17.2 30.0 ± 33.1 (mostly overgrown)	0.6 ± 3.5 overgrown overgrown
Lophodermium pinastri	6 11 15	53.6 ± 6.6 66.7 ± 1.7 74.0 ± 5.8	15.6 ± 13.5 overgrown overgrown
Lophodermium conigenum	4 6 11	37.1 ± 10.5 54.4 ± 7.1 73.1 ± 4.2	No inhibition overgrown overgrown
Strasseria geniculata	3 4	18.2 ± 10.9 overgrown	overgrown overgrown
Cyclaneusma minus	4 6 15	41.4 ± 28.3 42.6 ± 18.9 46.7 ± 34.1	16.9 ± 8.6 overgrown overgrown

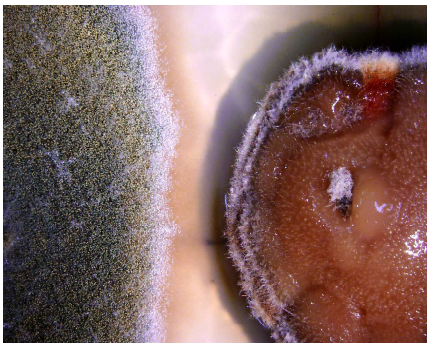
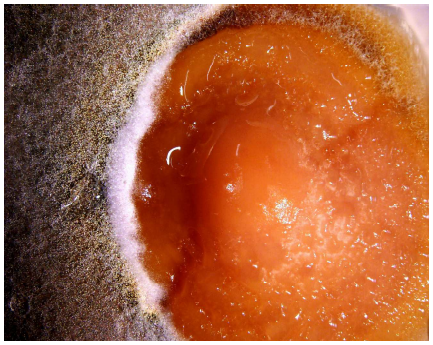
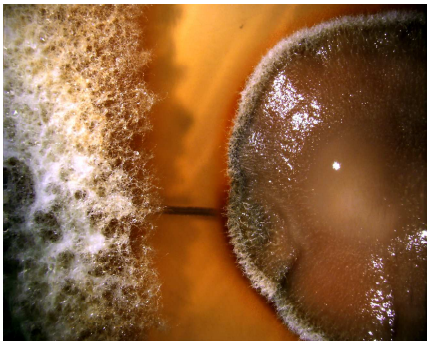
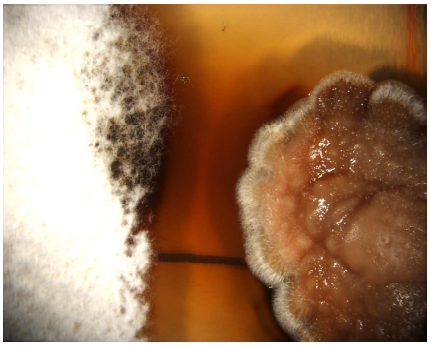
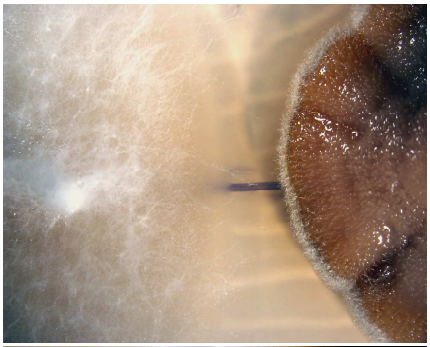
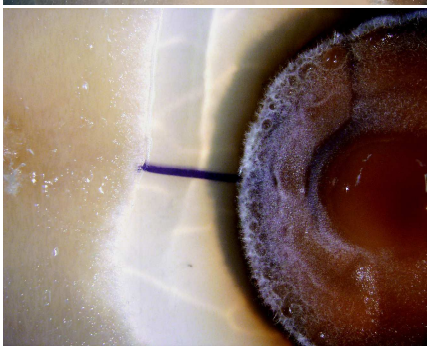
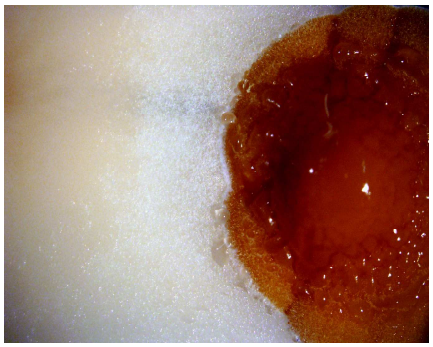
Shown is the inhibition of competitor growth by *D. septosporum* dothistromin producing wild type and FJT3. Shown are the values for at least two time points which vary between competitors due to their different growth rates. In general the last time point for each competitor was the last measurement before all wild type colonies made contact with the competitor.

[1] % inhibition of radial growth calculated as I = (G-C)/C x 100 % as described in Section 2.15

[2] Overgrown indicates that *D. septosporum* colony made contact with the competitor, was overgrown or passed by the competitor

[3]No inhibition means the growth towards *D. septosporum* was identical to, or higher than the growth away from *D. septosporum*.

Figure 5.1: Plate competition assay with *D. septosporum* wild type and dothistromin deficient strain FJT3. Colonies of NZE7 and FJT3 were grown on PDA plates for 15 days and challenged with different competitors which were inoculated 1.5 cm away from the colony margin of *D. septosporum* on the same plate. The competitors are seen on the left side of the pictures. Corresponding pictures of NZE7 wild type and FJT3 were taken at the same time point for each competitor, when the NZE7 wild type colonies and the competitors' colonies had not made contact. In contrast, the *D. septosporum* FJT3 colonies were at least partly overgrown in each case. Pigmentation in the PDA media plates (most obvious for the competition plates with *L. conigenum* and *C. minus*) is seen for all NZE7 plates suggesting secretion of dothistromin.

Competitor	NZE7	FJT3
A. nidulans		
L. conigenum		
C. minus		
E. lata		
L. pinastri		

To validate the inhibition of competitor growth by *D. septosporum* wild type strains a second trial (Trial 2) was carried out using competitiors *L. conigenum, C. minus, S. geniculata, A. nidulans, E. lata, F. graminearum, G. cingulata* and the biocontrol fungi *T. harzianum* and *T. viride*. The experiment was repeated with at least 5 replicates for each *D. septosporum*/competitor combination. In addition the overall growth diameters were determined. This work was carried out with the assistance of a summer student Justine Baker.

In Trial 2 (Table 5.2), a significant difference between inhibition of competitors by dothistromin-producing and non-producing *D. septosporum* was recorded for only two species: *A. nidulans* (6 dpi) and *C. minus* (8 dpi) (Table 5.2). However, for all competitor species the mean percentage of inhibition was higher in competition with the dothistromin producing strains than with the dot$^-$ strain FJT3. The lack of significance might be due to the high standard deviations obtained in this experiment. On the last day of measurements, no growth inhibition was detected for *T. harzianum, T. viride* or *S. geniculata*, while growth inhibition ranged between 40 – 50% for *A. nidulans, C. minus, E. lata, F. graminareum* and *L. conigenum* in the presence of the *D. septosporum* dot$^+$ wild type. In contrast, none of the competitors' growth was inhibited by the dothistromin-deficient strain FJT3, which had been grown over (or past) by the competitors. T-tests could not be performed as numerical data were only collected when there was a gap between the *D. septosporum* strains and competitors.

The overall growth rate was also measured by taking a diameter perpendicular to the competition axis. The overall growth rate was lower on plates with the *D. septosporum* (dot$^+$) wild type in comparison to the plates with FJT3 (dot$^-$) or competitor alone, except for the case of *T. viride, A. nidulans* and *S. geniculata* (Table 5.3).

Representative photographs of the plate competition assay (Trial 2) are shown in Figure 5.2. The inhibition of the growth can be seen by the gap between the colonies of *D. septosporum* NZE7 and *A. nidulans, C. minus, E. lata, F. graminareum* or *L. conigenum*. In contrast the colonies of *T. harzianum, T. viride* and *S. geniculata* started to overgrow the *D. septosporum* colonies (Figure 5.2 A). It was also noted that, in most cases, the pigmentation of the PDA media appeared in most cases higher on the side between NZE7 (dot$^+$) and competitor than on the opposite side of NZE7. The FJT3

(dot$^-$) strain was overgrown or passed by all competitors investigated (Figure 5.2 B). The photographs also show the overall growth of the competitors on plates without *D. septosporum*, to illustrate the inhibited overall growth by *D. septosporum* wild type (dot$^+$) (Figure 5.2 C).

Table 5.2: Inhibition of competitor species by *D. septosporum* (Trial 2).

Competitor Species	dpi of competitor[1]	% inhibition of competitor by wildtype (dot$^+$) *D. septosporum*[2] (mean ± SD)	% inhibition of competitor by FJT3 (dot$^-$) *D. septosporum* (mean ± SD)	Two-tailed P value[3]
Aspergillus nidulans	6 8	**31.5 ± 9.5** 40.8 ± 6.8	**14.5 ± 10.1** overgrown [4]	0.01
Eutypa lata	3 6	16.5 ± 20.9 45.0 ± 4.3	5.8 ± 9.9 overgrown	0.28
Fusarium graminearum	2 3	29.3 ± 11.9 44.2 ± 16.9	10.3 ± 20.8 overgrown	0.08
Cyclaneusma minus	8 15	**20.2 ± 9.7** 50.6 ± 4.6	**5.5 ± 9.8** overgrown	0.03
Lophodermium conigenum	3 6	19.1 ± 14.9 47.7 ± 4.4	14.4 ± 10.8 overgrown	0.55
Strasseria geniculata	2 3	21.1 ± 19.1 overgrown	19.8 ± 19.1 overgrown	0.91
Trichoderma harzianum	2 3	18.4 ± 13.0 overgrown	12.5 ± 10.5 overgrown	0.40
Trichoderma viride	2 3	11.6 ± 6.5 overgrown	6.1 ± 8.4 overgrown	0.24

Shown is the inhibition of competitor growth by *D. septosporum* (dot$^+$) wild type and FJT3 (dot$^-$).

[1] dpi= day after inoculation; The second time-point for each competitor represents the last day on which measurements were taken

[2] % inhibition of radial growth calculated as I = (G-C)/C x 100 % as described in Section 2.15

[3] T-test values were calculated using the unpaired T-test calculator on www.graphpad.com/quickcalcs, testing the null hypothesis of no difference in inhibition between competitors challenged with dot$^+$ and dot$^-$ *D. septosporum*

T-test values were not calculated when *D. septosporum* was overgrown by the competitor, as no dependable numerical data were available.

Inhibition values in bold are significantly different between the *pksA* mutant FJT3 and corresponding wild-type (P value < 0.05)

[4] Overgrown indicates that *D. septosporum* colony made contact with the competitor, was overgrown or passed by the competitor

Table 5.3: Final diameters of competitor colonies.

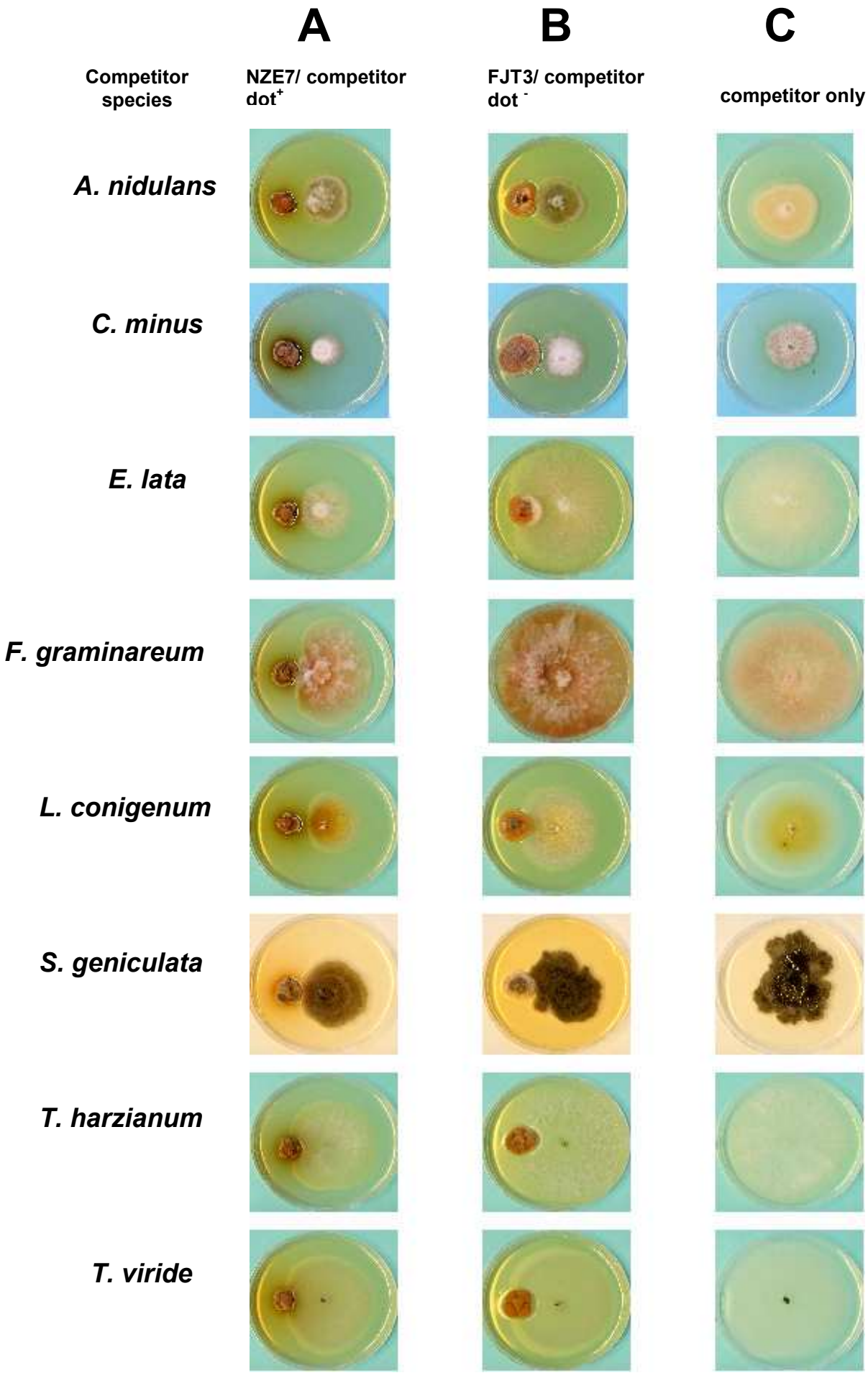
Competitor Species	dpi of competitor[1]	Final diameter of competitor (mm) with wildtype (dot$^+$) *D. septosporum* (mean ± SD)	Final diameter of competitor (mm) with FJT3 (dot$^-$) *D. septosporum* (mean ± SD)	Two-tailed P value[3]
A. nidulans	8	30.1 ± 4.9	34.8 ± 1.8	0.07
E. lata	6	**37.8 ± 14.1**	**83.3 ± 3.5**	**< 0.01**
F. graminearum	3	**52.4 ± 12.1**	**85.0[2] ± 0**	**< 0.01**
C. minus	15	**20.8 ± 3.4**	**28 ± 6.5**	**0.04**
L. conigenum	6	**41.3 ± 2.5**	**50.7 ± 2.6**	**< 0.01**
S. geniculata	3	48.7 ± 5.3	54.6 ± 18.6	0.47
T. harzianum	2	**65.5 ± 6.0**	**85.0[2] ± 0**	**< 0.01**
T. viride	2	62.8 ± 5.6	63.3 ± 1.2	0.81

Shown are the final diameters of competitor colonies on plates with *D. septosporum* (dot$^+$) wild type and FJT3 (dot$^-$).
[1] dpi= day after inoculation; Final diameters were measured on the last day of measurements
[2] A diameter of 85mm was given when the fungi had grown over the entire plate
[3] For each competitor species, T-test values were calculated to compare differences between colony diameters on plates with *D. septosporum* (dot$^+$) wild type and FJT3 (dot$^-$), using an unpaired T-test (www.graphpad.com/quickcalcs). Values in bold indicate a significant difference (P value < 0.05).

An inhibition of the growth of *D. septosporum* itself by the competitors was not seen. However, an inhibition of *D. septosporum* by the competitors cannot be ruled out as the growth of *D. septosporum* is very slow and therefore inhibition effects might not have been evident during the time scale of this experiment.

Figure 5.2: Comparison of competitor growth challenged with *D. septosporum* (dot$^+$) wild type and FJT3 (dot$^-$) and alone.

Shown are representative results of Trial 2 (shown in Table 5.2 and Table 5.3). Inhibition of the competitor growth by *D. septosporum* (dot$^+$) wild type is higher than inhibition by FJT3 (dot$^-$) in all cases (compare columns A + B), even though the inhibition was not always significant (Table 5.2). The overall growth of the competitors is also inhibited by the presence of *D. septosporum* (dot$^+$) wild type (compare columns A + C), while the overall growth is usually less inhibited by the presence of FJT3 (dot$^-$) on the same plate (compare columns B + C). Corresponding photos (A/B/C) were taken at the same time point for each competitor. Photos were taken by Justine Baker.


A
B
C
Competitor species
NZE7/ competitor dot+
FJT3/ competitor dot -
competitor only
A. nidulans
C. minus
E. lata
F. graminareum
L. conigenum
S. geniculata
T. harzianum
T. viride

5.2.2 Plate assay of toxin induction with reporter strain FJT24

The GFP fluorescence of the P*dotA* regulated reporter strain FJT24 was used in a plate competition assay to estimate if competing fungi induce the production of dothistromin. Preliminary trials were also made to assess if the production of dothistromin by *D. septosporum* could be elicited by exposure to media in which competitors had previously been grown. For those results see Appendix A6.

During the study of the FJT24 strain a serendipitous observation was made that suggested that dothistromin biosynthesis might be triggered by the presence of other microorganisms. A contaminating fungus appeared to enhance the expression of the P*dotA* regulated GFP and toxin accumulation was also seen in some other contaminated plates (Figure 5.3).

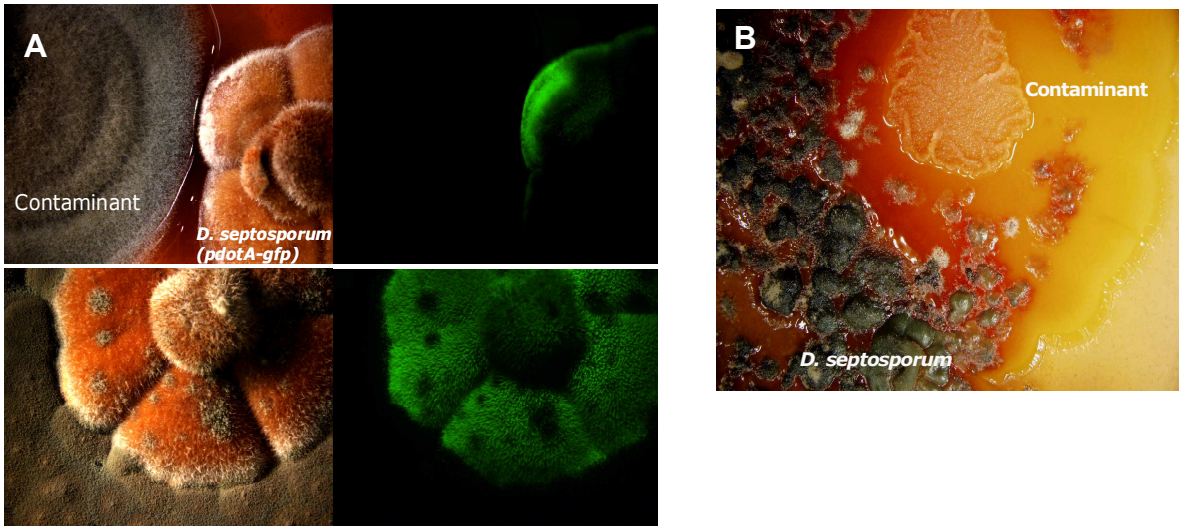


Figure 5.3: Toxin gene expression induced by contaminants?
(A) The P*dotA* regulated GFP strain FJT24 showed higher GFP expression at the contact zone with a contaminant (upper part). The GFP expression was seen over the entire FJT24 colony after the contaminant colony grew over the whole plate (lower part) (B) High secretion of toxin around towards a contaminant on a *D. septosporum* sporulation plate. This observation led to the hypothesis that other competitors might induce toxin production in *D. septosporum*.

The induction of dothistromin gene expression was further investigated in a plate competition assay using competitors and the P*dotA*::*egfp* reporter strain FJT24. GFP fluorescence was observed as an indication of gene expression.

In an initial trial the GFP fluorescence of the FJT24 strain appeared to be higher on the side towards some competitors, indicating a possible induction of the *dot* gene production by the presence of the competitor (Figure 5.4 and 5.5). Furthermore, the secretion of toxin often appeared to be directed towards a competitor. Also the margin of colonies sometimes appeared to accumulate red crystals and had a more intense red coloration on the colony side towards the competitors, suggesting a dothistromin accumulation (Figures 5.4 - 5.7). Initial trials suggested increased fluorescence on the *D. septosporum* FJT24 colonies towards *E. lata*, *G. cingulata* (Figure 5.4), *F. graminearum*, *A. alternata* (Figure 5.5, 5.6) and *C. minus* (Figure 5.7). As observed before for the wild type (Section 5.2.1), the presence of the dot$^+$ *D. septosporum* strain FJT24 inhibited the growth of the competitive species used in this trial. An example is shown in the competition with *C. minus* and FJT24 in Figure 5.7. More detailed observations are noted in the figure legends for figures 5.4-5.7.

In some cases red crystalline structures were observed in areas with increased GFP expression and pigmentation (Figure 5.8). Crystals were seen in both media and mycelium. Interestingly the crystals did not accumulate in all hyphae, and in some hyphae red crystals appeared in a similar punctuated pattern as observed for the GFP fluorescence of the DotA-GFP fusion protein (Section 4.5.4; compare Figure 4.16), suggesting that those crystalline structures represent dothistromin and are located punctuated similarly as seen for the vesicle like structures containing the DotA-GFP protein.

However, the colonies of *D. septosporum* FJT24 frequently showed sectoring on plates. This limits the interpretation of the results as different sectors showed different morphologies and GFP expression, independent of whether sectors were directed towards or away from the competitor colonies, or even without competitors on the plates (see also Appendix A7).

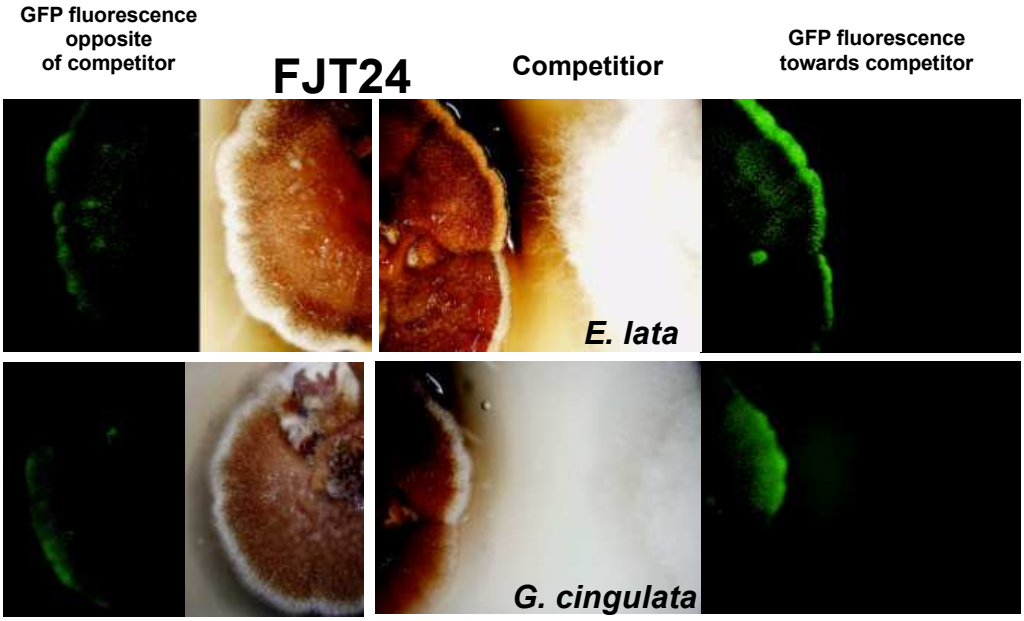


Figure 5.4: Possible induction of dothistromin by competitive fungi 1.
Shown are *D. septosporum* FJT24 (P*dotA*::*egfp*) reporter strains colonies in competition with *E. lata* and *G. cingulata*. In both cases a higher pigmentation and GFP expression is seen on the side of the FJT24 colony closest to the competitor. The white light pictures (centre) show the FJT24 colony side closest and furthest from the competitor. Pictures paired in a row are obtained from the same colony. UV pictures show the GFP fluorescence of the colony margins taken with identical settings.

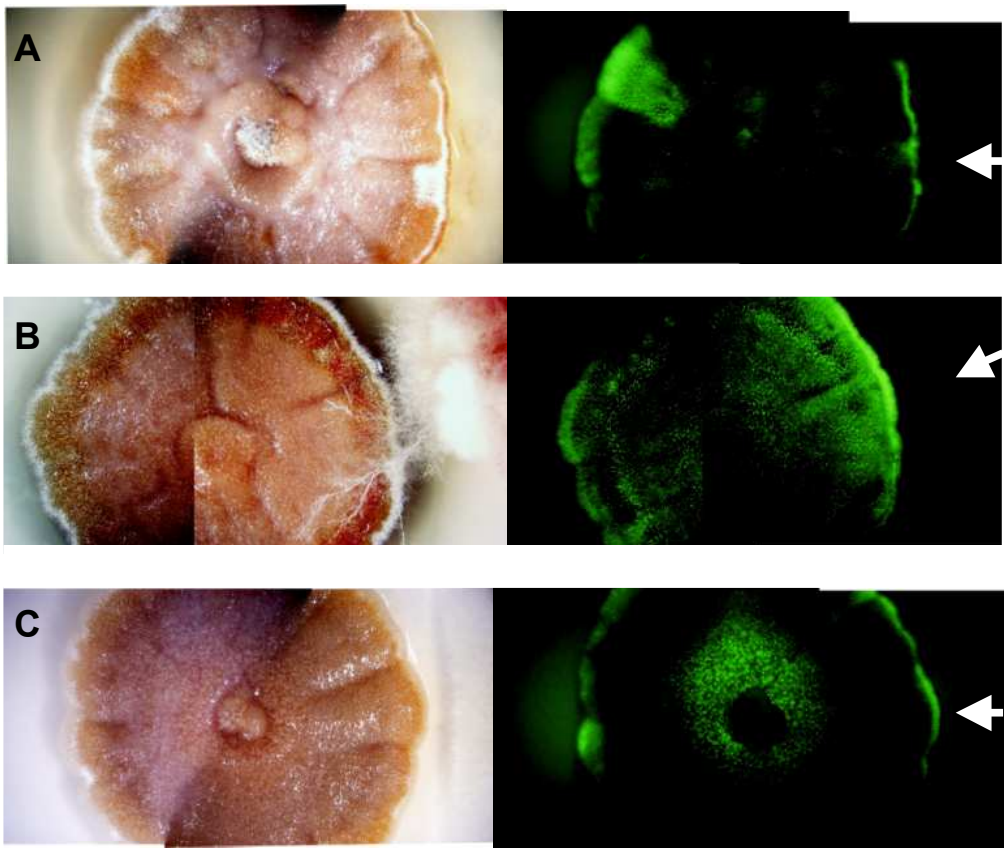


Figure 5.5: Possible induction of dothistromin by competitive fungi 2.
Shown are *D. septosporum* FJT24 (P*dotA*::*egfp*) reporter strain colonies in competition with (**A**) *E. lata* (**B**) *F. graminearum* and (**C**) *A. alternata*. The competitors were located on the right side as indicated with arrows in the GFP fluorescence pictures. The GFP expression appears to be more intense (**A**,**C**) or increased (**B**) on the side closest to the competitor colonies. Pictures in white light are shown in the left column with the according GFP expression pictures in the right column.

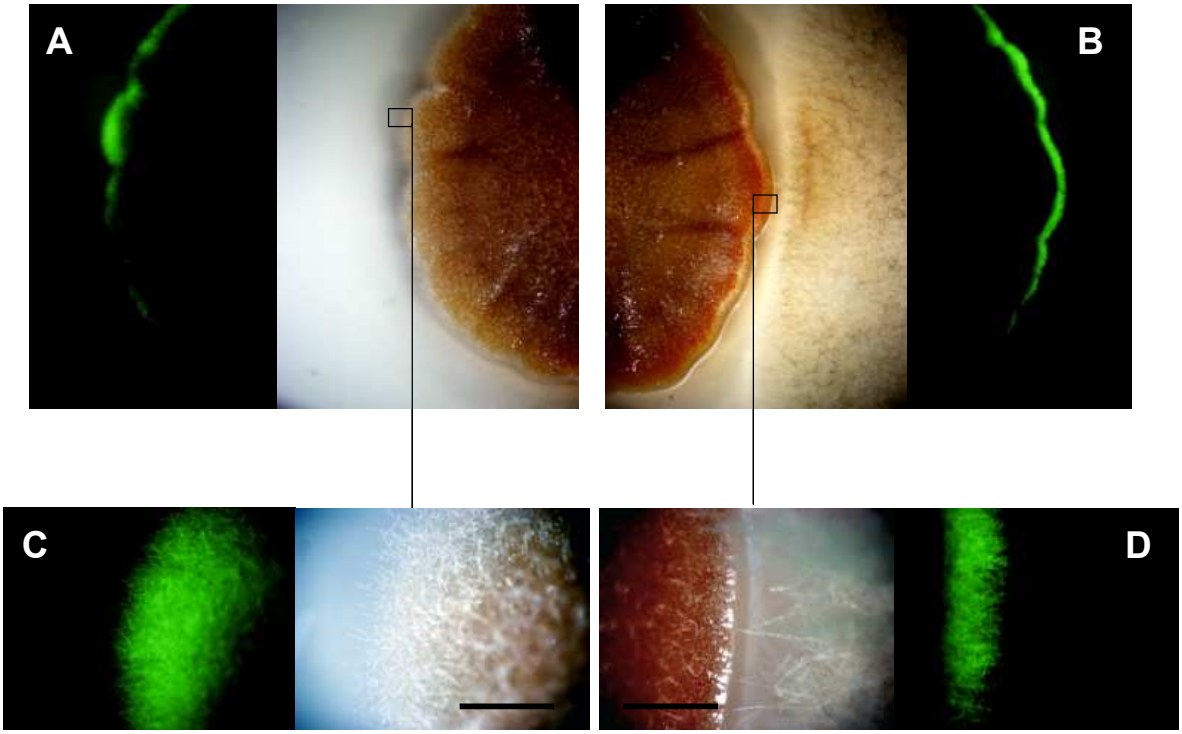
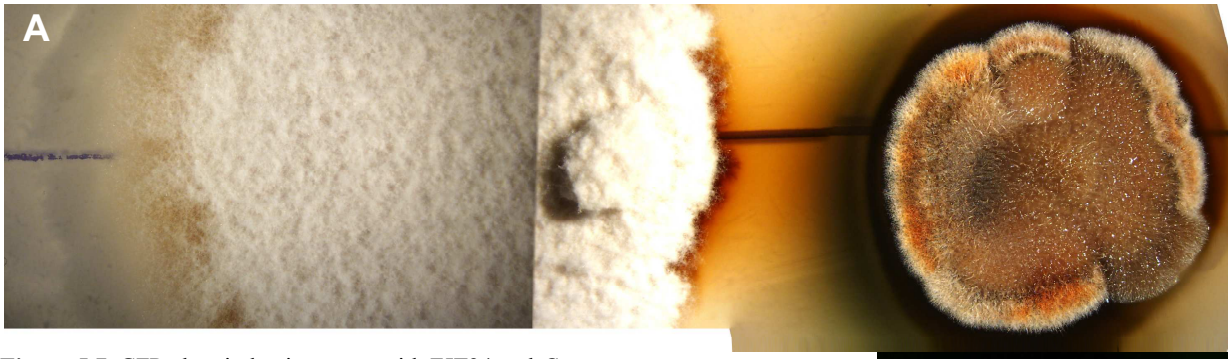


Figure 5.6: GFP plate induction assay with *D. septosporum* FJT24 and *A. alternata*.
(**A**) GFP fluorescence and colony margin of FJT24 furthest from the *A. alternata* colony. (**B**) Colony of FJT24 and GFP fluorescence at the contact zone with the *A. alternata* colony. (**A, B**) The *D. septosporum* colony appears to secrete more toxin on the side closest to the *A. alternata* colony (B) than on the side furthest away. (A). A red pigmentation is also seen in the edge of the colony itself at the side towards *A. alternata* (B). The GFP expression appears also to be more intense at the colony edge of FJT24 towards *A. alternata* (B) than at the margin opposite the colony. (**C**) Close-up of the colony margin framed in (A). (**D**) Close-up of the FJT24/*A. alternata* contact zone framed in (B). (**C, D**) Higher magnifications of the colony margins reveal that the more intense GFP fluorescence observed in (B) compared to (A) might be caused by a more dense mycelium of the FJT24 colony towards *A. alternata*. However a more intense red pigmentation of the mycelium in (D) compared to (C) suggests a higher dothistromin production in the contact zone with *A. alternata*. Bars in C and D indicate 1mm.



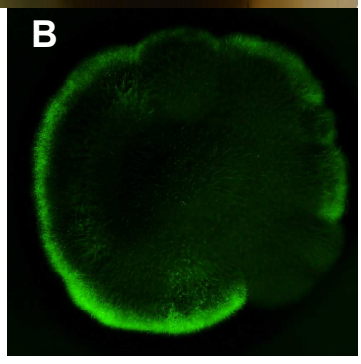


Figure 5.7: GFP plate induction assay with FJT24 and *C. minus*.
(**A**) Shown is the inhibition effect of the dot$^+$ strain FJT24 on *C. minus*. The *C. minus* colony grew away from the FJT24 colony. It appears that more red toxin is produced towards the side of *C. minus*. Pigment accumulates at the margin of the *C. minus* colony and is likely to cause the growth inhibition of *C. minus*. The morphology of the *D. septosporum* colony also suggests a higher production of toxin towards *C. minus* as red crystals accumulate at the side towards *C. minus*. However some sectoring of the FJT24 colony is seen. It is therefore not possible to determine if the different pigment accumulation is caused by the sectoring or by the presence of competitors.
(**B**) The GFP fluorescence pattern also suggests an induction of GFP expression by *C. minus*. GFP fluorescence is higher towards the side of *C. minus*, but the expression pattern might be caused by the different metabolic states of the sectors of FJT24.

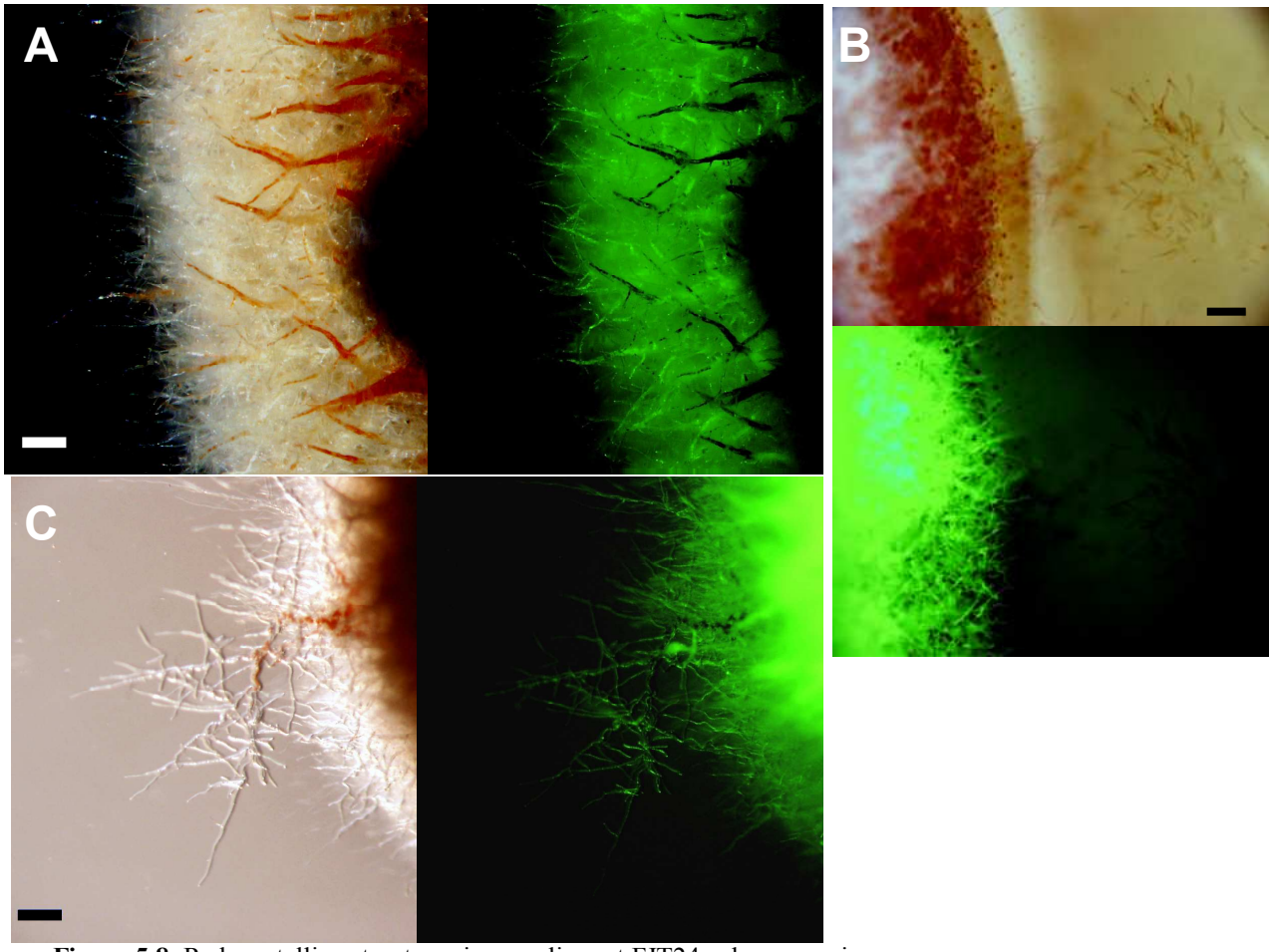


Figure 5.8: Red crystalline structures in mycelium at FJT24 colony margin.

(**A**) Crystalline structures observed in colony margin mycelium of FJT24 on a competition plate closest to *L. conigenum*. The visible light picture (left) shows the accumulation of a red pigment in some hyphae. The GFP fluorescence of the according mycelium (right) is high suggesting dothistromin production in those cells and the red crystals to be dothistromin. Further the crystals are mostly located in a punctuated pattern the hyphae and appear to quench UV radiation. Bar indicates 200µm. (**B**) FJT24 in competition with *A. alternata*. The red crystals appear to be secreted into the media and appear to be taken up by *A. alternata*. Again a punctuated localization of the crystals, high GFP fluorescence and UV absorbance is seen. Bar indicates 250 µm. (**C**) Outgrowing mycelia on colony edge of FJT24 closest to the *L. conigenum* competitor. Most hyphae show GFP expression indicating the synthesis of dothistromin in those hyphae. However the hyphae appear to be metabolically different as red crystals have been accumulated in some but not all hyphae. Bar indicates 100µm.

Although some increased GFP fluorescence of FJT24 towards some competitors was seen, an artefact was noticed due to the uneven distribution of the UV light over the specimen in the fluorescence microscope used. The GFP fluorescence depended on the orientation of the colony toward the light source as demonstrated in Figure 5.9.

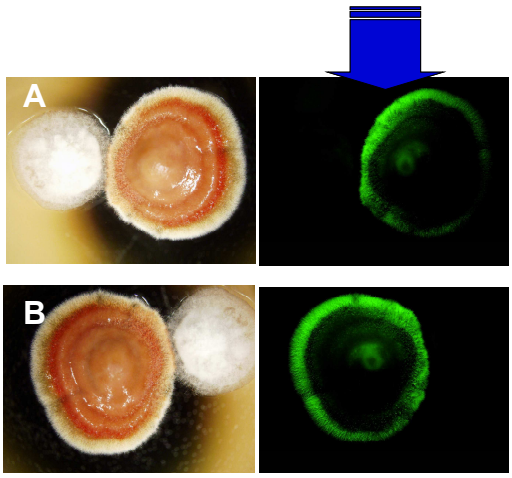


Figure 5.9: Influence of the orientation of the specimen on the GFP fluorescence.
GFP fluorescence detected on colonies depended on the orientation of the colonies towards the light source (blue arrow). (**A**) The GFP fluorescence appears to show a induction by the competitor colony. (**B**) The same specimen as in (**A**) but 180° rotated shows a evenly fluorescence around the *D. septosporum* colony.

To limit the influence of this artefact the experiment was repeated with smaller colonies of FJT24 which were located in the centre of the UV exposure region. Increased GFP expression of FJT24 towards the competitor was detected for some plates but that was not always seen for all replicates (Figures 5.10-5.12). The biggest limitation in this experiment was the morphological instability of *D. septosporum*. Sectoring was frequently observed, and sectors of the colonies showed a very different level of GFP expression. Therefore it was not possible to define if the level of GFP fluorescence was due to induction or repression by the competitor or due to the sectoring of *D. septosporum*. Furthermore the red crystalline pigment showed some quenching (Figure 5.8) so that GFP fluorescence would not be detectable if mycelium parts are covered by it. Replicates are shown for all competitors studied so variability can be clearly seen. More detailed observations are noted in the figure legends of Figures 5.10-5.12.

No strong evidence of consistent induction of GFP expression was seen when FJT24 competed with the species used in this trial under the conditions investigated. The observations made with the GFP regulated *dotA* reporter strain did not support the hypothesis that *dotA* expression and dothistromin production are induced by competitor fungi.

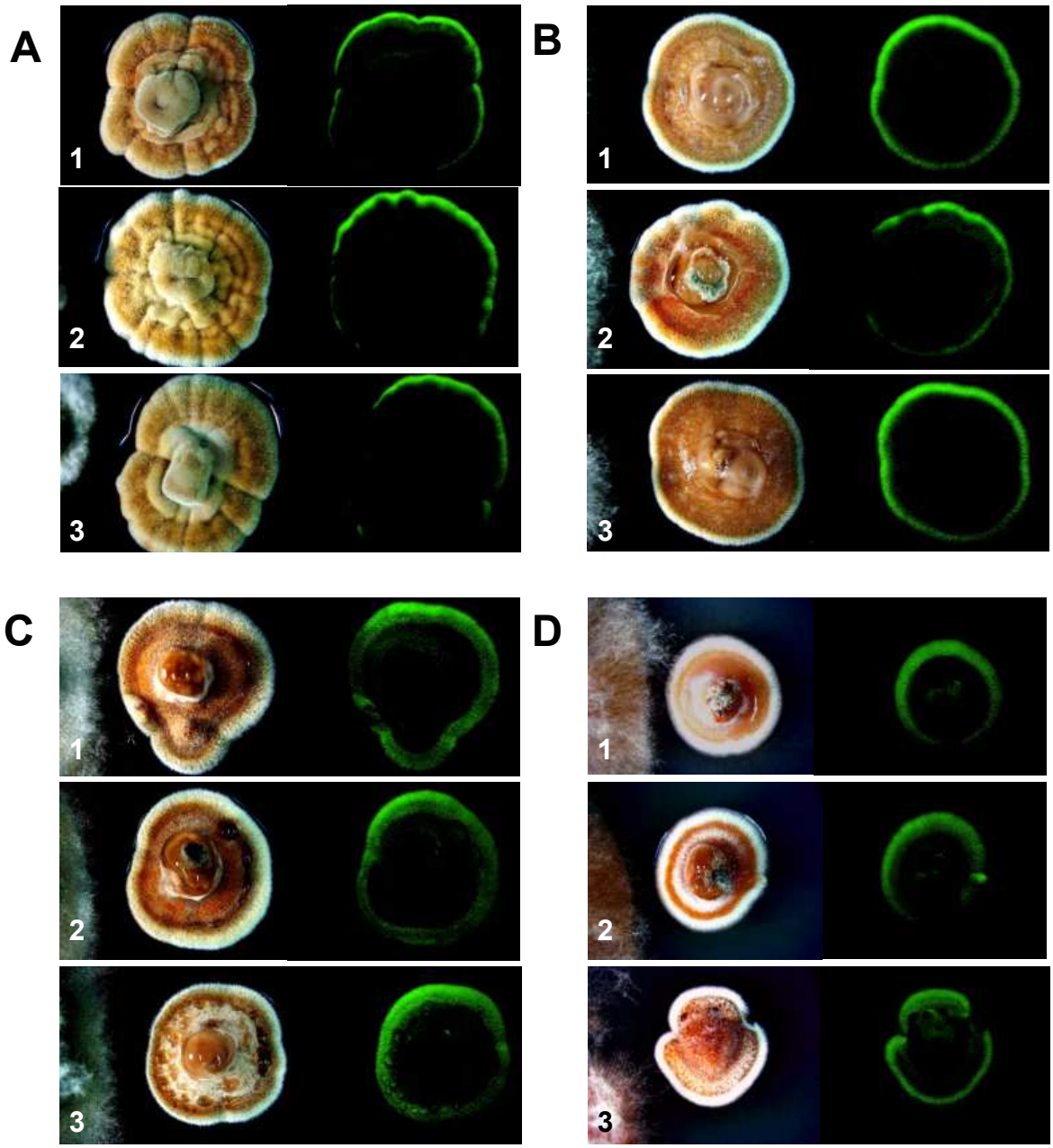

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Figure 5.10 (previous page): Plate test for induction of dothistromin gene expression by competitors. GFP expression of FJT24 (PdotA::egfp) was monitored to assess if dothistromin gene expression is induced by competitors. Competitors shown here are *C. minus* (**A**), *E. lata* (**B**), *A. nidulans* (**C**), *F. graminearum* (**D**), and *L. conigenum* (**E**). Shown are triplicate plates (1-3) for each competitor with the competitor located just off to the left side in each picture. Adjoined pairs are visible light (left) and UV light (right). (**A**) FJT24 in competition with *C. minus* showed no enhanced GFP expression at the side closest *C. minus*. The expression towards the *C. minus* colony even appeared lower in all three replicates. (**B**) FJT24 in competition with *E. lata*. Replicates 1 and 3 showed putative higher expression of GFP closest to *E. lata*. The GFP fluorescence of replicate 2 is lower on the side towards *E. lata*, but instead has red crystals accumulated in the mycelium. As the red crystals absorb UV light in the GFP channel, the GFP fluorescence might not be detectable in this area. (**C**) FJT24 in competition with *A. nidulans* only showed in replicate 3 a putative induction of GFP. GFP fluorescence in replicate 1 was lower at the side closest to *A. nidulans*, but this area had also a higher accumulation of the red crystals. (**D**) In competition with *F. graminearum* the FJT24 replicates 1 and 2 showed higher GFP fluorescence closer to the *F. graminearum* colony. (**E**) Competition of FJT24 with *L. conigenum*. Shown are three replicates (1-3) at 4 days (upper pictures) and 6 days (lower picture). Replicates 2 and 3 show putative enhanced GFP fluorescence towards the *L. conigenum* colony. However, some sectoring is seen with different GFP expression. Replicate 1 did not show enhanced GFP expression.

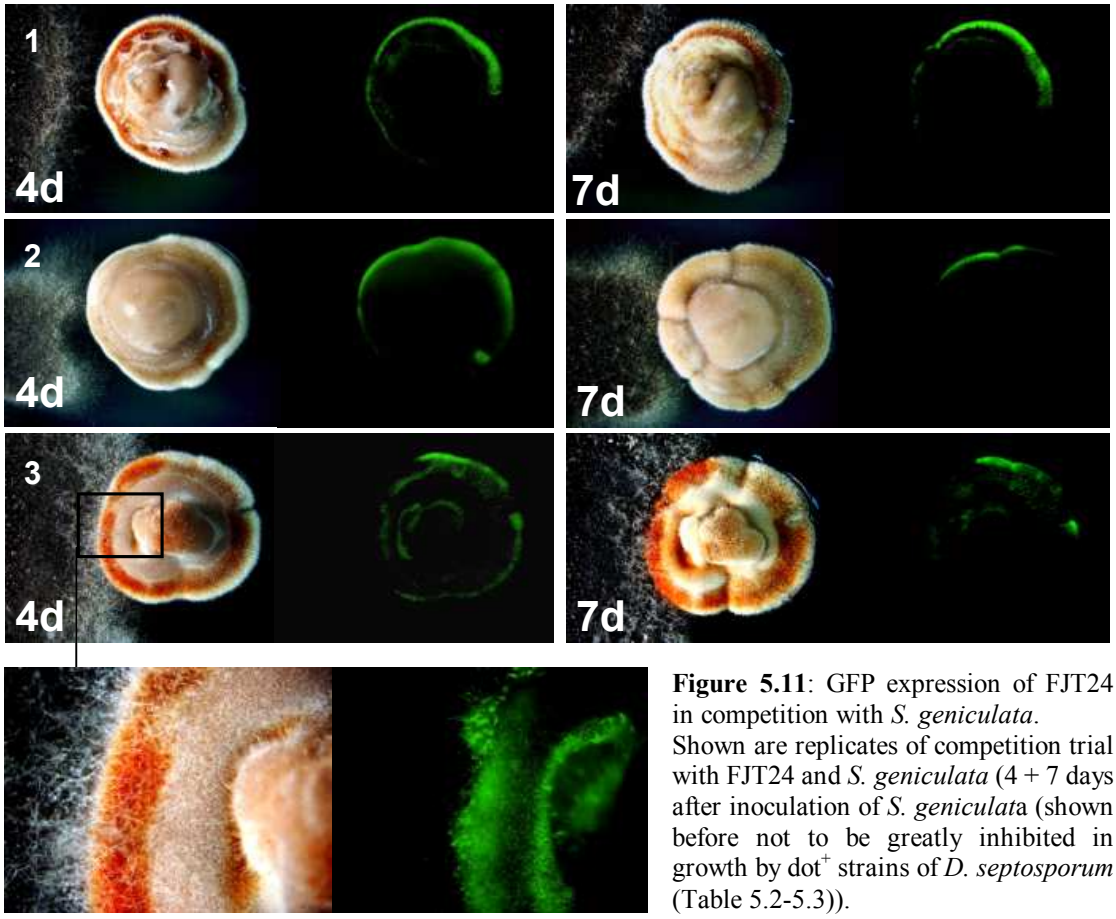


Figure 5.11: GFP expression of FJT24 in competition with *S. geniculata*. Shown are replicates of competition trial with FJT24 and *S. geniculata* (4 + 7 days after inoculation of *S. geniculat*a (shown before not to be greatly inhibited in growth by dot$^+$ strains of *D. septosporum* (Table 5.2-5.3)).

Replicates 1 and 3 show accumulation of pigments towards the *S. geniculata* colony but no obvious enhanced GFP fluorescence. In replicate 1 this might be an effect of the sectoring of FJT24. The close-up of replicate 3 shows that areas of FJT24 overgrown by *S. geniculata* lose GFP fluorescence, which might be caused through the accumulation of the red pigment or through death of the *D. septosporum*. Replicate 2 showed a different morphology, no enhanced GFP fluorescence or accumulation of pigment toward the *S. geniculata* colony.

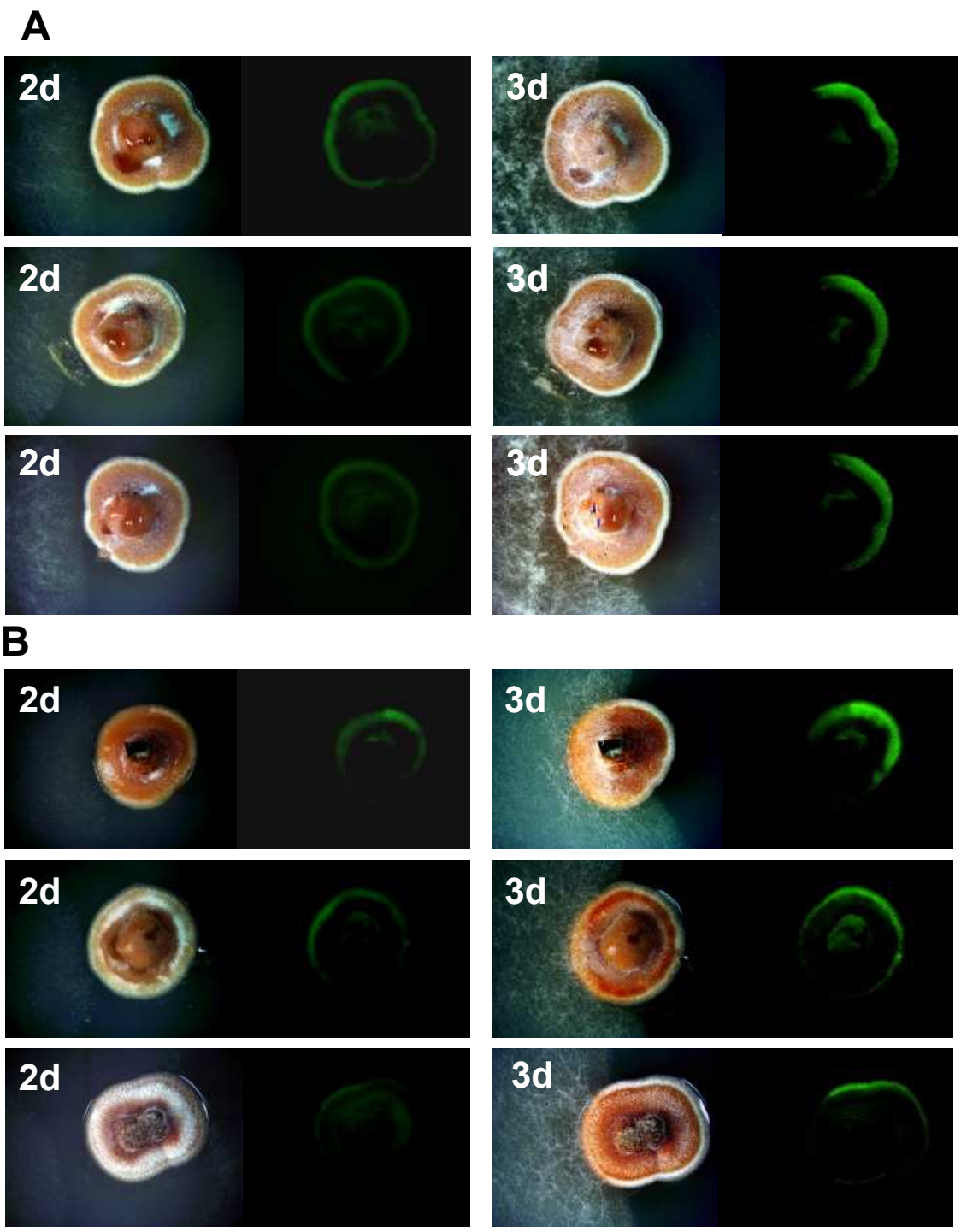


Figure 5.12: Plate competition assay with FJT24 and *Trichoderma* biocontrol species.
Trichoderma spp. were shown not to be inhibited in growth by *D. septosporum* dot^{+} strains (Table 5.2). The pictures show the competition of (**A**) *T. harzianum* and (**B**) *T. viride* with FJT24 2 and 3 days after inoculation with *Trichoderma*. (**A**) The FJT24 colonies show even GFP fluorescence around the margins of the colonies 2d after inoculation suggesting no induction of dothistromin synthesis by the presence of *T. harzianum*. After 3d *T. harzianum* grew over the FJT24 colonies without appearing to be inhibited. Areas of the FJT24 colonies grown over by *T. harzianum* lost the GFP fluorescence at the colony margin while not overgrown areas still showed GFP expression. This suggests that *T. harzianum* kills the *D. septosporum* mycelium when it overgrows FJT24. (**B**) Colonies of FJT24 also showed fluorescence at the colony margin 2d after inoculation with *T. viride*. No strong evidence for an induction of GFP is seen. However the FJT24 colonies showed an accumulation of red pigment in the mycelium after 3d of competition with *T. viride*, suggesting a putative increase of synthesis or secretion of dothistromin. At 3d the *T. viride* overgrew the FJT24 colonies and the overgrown mycelia showed a weaker GFP expression, suggesting that *T. viride* also kills the *D. septosporum* colonies but less effectively than seen for *T. harzianum*.

5.3 Discussion

The results obtained in this study suggest that the synthesis of dothistromin does have an advantage for *D. septosporum*, when challenged with other fungi. However, the results do not imply that dothistromin production is increased in a defence-like response when challenged with other fungi or elicitors of fungal origin in those preliminary experiments.

In the plate competition assay, the growth of all competitor species tested in this study was more inhibited in competition with the dothistromin producing strains than with the dot$^-$ mutant FJT3 (Tables 5.1-5.3, Figures 5.1 and 5.2). The FJT3 mutant did not significantly differ in radial growth compared to the wild type (Jin 2005), suggesting that the intake of nutrients from the media by FJT3 and wild type strains was comparable. Therefore the inhibition of the growth of the competitors was most likely an effect of dothistromin or dothistromin intermediates secreted by the wild type and not of a lower availability of nutrients.

The plate inhibition assay has several limitations. The different fungal species have different growth rates on the PDA media used in this study. As long as dothistromin is not degraded, slow growing species are more likely to be exposed to a higher concentration of dothistromin as it accumulates in time. The results might be different on other media with optimal growth conditions for the competitor species. However, growth conditions in nature are usually sub-optimal and *D. septosporum* grows sub-optimally on PDA media (Ganley 2001). Another limitation of this assay is that the inhibition of growth of the competitors caused by the *D. septosporum* dot$^+$ has only been investigated in one environmental condition. Studies in other systems showed that the outcome of competition between different fungal species is highly dependent on environmental factors (Marin *et al.* 1998a; Marin *et al.* 1998b; Ramakrishna *et al.* 1996a; Ramakrishna *et al.* 1996c; Velluti *et al.* 2000). Therefore the inhibitions observed in this study might not occur under different conditions, and investigations of the interaction *in planta* might lead to different results. Furthermore, in this study the effects of the *D. septosporum* wild type (dot$^+$) were compared to those of the FJT3 *pksA* (dot$^-$) mutant. Although FJT3 is dothistromin deficient the replacement of the *pksA* gene also resulted in a loss of all intermediates in the dothistromin biosynthetic pathway. While it has been shown that the

pksA gene is involved in the biosynthesis of dothistromin (Bradshaw *et al.* 2006; Jin 2005), it cannot be excluded that other biosynthetic pathways are influenced by the disruption (Firn & Jones 1999) of the *pksA* gene in FJT3. Therefore the inhibition of growth could be caused by other metabolites than dothistromin. Indeed a preliminary test showed also growth inhibition of the competitors caused by the presence of the *dotA* disruption mutant FJT2 (J. Baker, unpublished results). However, although FJT2 is dothistromin deficient, it synthesizes and secrets versicolorin A (Bradshaw *et al.* 2002), which also has toxic properties (Mori *et al.* 1985). To confirm that the inhibition of the competitors are indeed caused by dothistromin, pure dothistromin should be tested for its ability to inhibit the competitor growth.

Trichoderma species *T. harzianum* and *T. viride*, but also the forest species *S. geniculata*, appeared to be resistant to dothistromin. The preliminary results using strains of *D. septosporum* marked with fluorescent reporter genes further suggest that these resistant competitor species began to inhibit or kill *D. septosporum* once they made contact, as suggested by the loss of GFP fluorescence at the margin of overgrown FJT24 mycelium (Figure 5.11, 5. 12). *S. geniculata* is a forest pathogen, which is able to infect several pine species (Vujanovic *et al.* 1997) and therefore might share the same ecological niche as *D. septosporum* and *D. pini*. Therefore the limited effect of dothistromin production is interesting and might be caused by an co-evolutionary adaption towards dothistromin of *S. geniculata*. However, the knowledge and research on *S. geniculata* is very limited. From a biocontrol point of view the fast overgrowth of *D. septosporum* by *T. viride* and *T. harzanium* is very interesting. *Trichoderma* spp, which are mycoparasites and plant symbionts (Harman et al. 2004), are frequently used as biocontrol agents of fungal diseases (Benitez *et al.* 2004; Harman 2000) and might be suitable for control of Dothistroma needle blight. However, the results were obtained in an artificial environment and the interaction *in planta* was not assessed. Since it was shown that dothistromin is not a pathogenicity factor (Section 4.5), it is not a suitable direct target for disease control. But if biocontrol agents are considered as an option to control *D. septosporum* outbreaks in future, those agents should be screened for resistance against dothistromin.

Although initial observations suggested that the production of dothistromin and dothistromin gene expression might be induced by the presence of competing fungi, this could not be concluded from the results of this study. On the plate induction assay using the P*dotA*::*egfp* reporter strain FJT24, some colonies showed possible induction of GFP expression through the presence of competitors, suggesting a induced induction of dothistromin genes. However several limitations in this assay did hinder to conclude an induction. Firstly, a putative induction was not seen for all replicates in the competition trials. Secondly, the production of the red pigment absorbs the UV light in the GFP channel, so that the GFP fluorescence would not be detected in mycelium where the pigment is accumulated. The red crystalline pigment is most likely dothistromin as the red colour suggests. Dothistromin is also the main metabolite produced by *D. septosporum* in culture and needles (Bassett *et al.* 1970). Frequently the pigmentation of FJT24 colonies was directed towards the competitor colony. This would support the secretion or synthesis of dothistromin as a response to the presence of the competitors. However, it was not tested that the red pigment is indeed dothistromin. The third main difficulty was the frequent sectoring seen in *D. septosporum* cultures. The FJT24 sectors differ in pigment production, morphology and level of GFP fluorescence. Therefore it is difficult to exclude that an increased expression of GFP is not due to the sectoring of the FJT24. A fourth limitation is that the induction the GFP fluorescence was only observed at certain time points. Therefore a short-term induction might have been missed. Short-term induction could be detected in a long-term movie of the GFP fluorescence. Finally, an induction of dothistromin gene expression was only tested on one media. The competitor species might produce different metabolites on different media or *in planta*, including possible elicitor(s) of dothistromin biosynthesis. As seen in other studies *in vitro* studies on solidified media of competing fungi are not necessarily able to predict the outcome of competition *in planta* (Simpson *et al.* 2004). Therefore field/ *in planta* studies are compulsory to make solid conclusions about a role of dothistromin in competition.

To avoid some of the limitations of the plate induction assay the dothistromin production and *dot* genes expression was determined in submerged culture before and after adding “elicitor” broths. Those preliminary results did not support an induction of dothistromin synthesis (Appendix A6).

The results of this chapter suggest that even though the toxin production is not triggered in an elicitor kind of response, dothistromin producing strains have an advantage in competition with other fungi. This is not an unexpected outcome as dothistromin was previously shown to have a broad toxicity (Stoessl *et al.* 1990), but in view of the early growth-stage synthesis of dothistromin and dispensability for pathogenicity, these results support a biological role for dothistromin in competition.

It was previously shown that different isolates of a worldwide collection of *D. septosporum* and *D. pini* have high variations in the amount of dothistromin they produce (Bradshaw *et al.* 2000). *Dothistroma* strains which were isolated from the wild and kept in culture for some time do lose the ability to produce dothistromin. This is a frequent phenomenon for fungal producers of secondary metabolites and might be related to aging (Kale *et al.* 1994; Wang *et al.* 2005).

In nature the production of dothistromin might give *D. septosporum* an advantage against certain competitive fungi as it inhibits or slows down the growth of competitors. The GFP expression of the FJT24 reporter strain does suggest the production of dothistromin occurs mainly at the colony margin, protecting the colony centre by a ring of toxin producing cells. The production of dothistromin might have also a protective role for *D. septosporum* in and on the needle. Therefore strains which produce more dothistromin are more likely to complete their lifecycle and to reproduce. However, since dothistromin is not effective against all competitors, it is proposed that, in certain environments (with no competitors, as in culture, or with competitors which are not affected by dothistromin) the production of dothistromin would not be advantageous for *D. septosporum*, so no selection for dothistromin occurs. Therefore the ability to produce dothistromin could be lost without consequences for the strain. This might explain the high variation of dothistromin production in different *Dothistroma* isolates (Bradshaw *et al.* 2000). However, whether the need to produce dothistromin in nature is actually caused by competing organisms, or other environmental circumstances, is not known and needs to be tested *in planta* and field trials.

Pine needles contain a variety of different endophytes and many of them are unknown (Ganley *et al.* 2004; Ganley & Newcombe 2006). *C. minus* and *L. pinastri* can occur as endophytes in symptomless needles on pines (Sieber *et al.* 1999). While *L.*

pinastri appears to be non-pathogenic, *C. minus* can also cause needle cast (Bulman & Gadgil 2001). Field studies showed that 35-40% of needles are colonized by endophytes, including *C. minus* and *L. conigenum*, in the European Alps (Sieber *et al.* 1999). Gourbiere *et al*. (2001) showed that the colonisation of *P. sylvestris* by *C. minus* and *L. pinastri* increased with altitude. Interestingly very high levels of dothistromin production have been seen for the *D. septosporum* high altitude isolates of the German Alps (Bradshaw *et al.* 2000). In the current study both *C. minus* and *L. pinastri* were inhibited by dothistromin-producing strains but not by the dothistromin deficient strain (Table 5.1-5.3). *Lophodermium* spp. and *C. minus* are pine endophytes and therefore have a different ecological niche than *D. septosporum*, but interaction of those species in the needle is likely once *D. septosporum* has invaded the host. Both *C. minus* and *Lophodermiun* spp. were shown to be able to overgrow and compete with dot$^-$ *D. septosporum* but not with dot$^+$ *D. septosporum* (Section 5.2.1). Therefore, if *D. septosporum*, *C. minus* and *L. pinastri* compete for the same host at high altitudes it could be speculated that it would be beneficial for *D. septosporum* to produce dothistromin to compete with fungi like *L. pinastri* or *C. minus*.

Field studies which correlate the occurrence of red bands (or amount of dothistromin produced) in infected needles with the number or kind of other fungi would provide more information about a role of dothistromin in competition. The availability of dothistromin producing and dothistromin deficient strains should also allow this hypothesis to be tested in the laboratory, with co-inoculation trials including competitors.

There are several examples of the effects of competitor species on toxic metabolite production in fungi. Under certain conditions increased fumonisin production by *Fusarium* spp. is seen when co-inoculated with different species as *Aspergillus* or *Penicillium* (Marin *et al.* 1998b). Similarly AF production by *A. flavus*, and toxin production by *Penicillium* spp. are each affected (increased or decreased) by the presence of other fungi (Cuero *et al.* 1988; Ramakrishna *et al.* 1996a; Ramakrishna *et al.* 1996b; Ramakrishna *et al.* 1996c; Wicklow *et al.* 1980). The toxin production and outcome of competition in all these studies was very dependent on environmental factors (temperature, incubation period, water accessibility). Therefore dothistromin production

in *D. septosporum* might indeed be induced by the presence of competitors, but the conditions of the competition studies in this work did not allow detection of an effect.

There are several other reasons why it would be beneficial for *D. septosporum* to produce dothistromin in nature. Although dothistromin appears not to be a pathogenicity factor in the disease it might be a virulence factor. This appears likely as the dothistromin toxin is clearly able to interact with, and cause damage in, pine tissue (Jones *et al.* 1995; Shain & Franich 1981). But forests that have a high incidence of disease often show mature stromata on diseased needles without red-bands (A. Woods, M. Dick, personal communication), which may suggest that dothistromin is not a virulence factor either.

Dothistromin fluorescences under UV light and therefore it might be simply a UV protectant for the fungi (see also Figure 5.8). In *A. nidulans* the production of ST is connected with light through the regulator protein VeA (Calvo *et al.* 2004; Stinnett *et al.* 2007). Therefore the levels of UV radiation might have an influence on dothistromin production. This could also explain the much higher levels of dothistromin produced by isolates obtained from the German Alps in comparison with isolates from lower altitude as determined by Bradshaw *et al*. (2000).

As pointed out by Vining (1990), the ultimate functions of secondary metabolites appear to benefit the producer, but secondary metabolites appear not to have a single universal function. As stated by Challis and Hopwood (2003), when "assuming a selective advantaged for secondary metabolite production, such an advantage for many of the compounds is likely to be conditional, or sporadic". This probably applies to the production of dothistromin by *D. septosporum*. This study showed that in certain situations (plate competition) it is beneficial for the fungi to produce dothistromin as it reduces competitor growth. Other research showed toxic properties of dothistromin towards a broader range of organisms, including bacteria (Harvey *et al.* 1976; Stoessl *et al*. 1990), which might compete with *D. septosporum* in nature. Shain and Franich (1981) showed that dothistromin is able to kill pine needle tissue and therefore could facilitate the establishment of the fungus in the necrotic tissue. While the extent of these effects of dothistromin in nature are not known, the combination of these effects might be so beneficial that evolutionary pressure forces *D. septosporum* to keep and express the dothistromin genes in some environments, while in other environments the investment of

energy to produce dothistromin is not beneficial and *D. septosporum* which produce less dothistromin outgrow high toxin producers.

But as discussed above to certify a competitive advantage of dothistromin producing strains, or to determine any induction of toxin production in the natural environment, it is compulsory to test for both *in planta*.

Chapter 6:
Summary and future directions

The mycotoxin dothistromin produced by *Dothistroma* spp. is structurally similar to the AF/ST precursor versicolorin B. Genes involved in the biosynthesis of dothistromin have been identified and show high similarities to genes involved in the synthesis of AF/ST in *Aspergillus* spp. (Bradshaw *et al.* 2002; Bradshaw *et al.* 2006; Zhang *et al.* 2007). While the genes involved in AF/ST biosynthesis are organized in big continuous gene clusters (McDonald *et al.* 2005; Yu *et al.* 2004; Zhang *et al.* 2005), the genes involved in the biosynthesis of dothistromin appear to be dispersed and are localized in three mini-clusters interspersed with genes not thought to be involved in dothistromin biosynthesis (Zhang *et al.* 2007). Although the relative positions of those mini-clusters to each other is not known, this study showed that the genes are localized on a 1.3 Mb mini-chromosome (Section 3.2). The finding that the dothistromin genes are located on a mini-chromosome is interesting. Mini-chromosomes in filamentous fungi are often conditionally dispensable, but contain many functional genes (Covert 1998) and the loss of those mini-chromosomes in plant pathogenic fungi often results in non-pathogenic strains (Hatta *et al.* 2002; Johnson *et al.* 2001). Therefore it would be interesting to determine if this mini-chromosome is always present in *Dothistroma* spp. isolates and if loss of the mini-chromosome results in non-pathogenic isolates. While we could show that dothistromin is not a pathogenicity factor (this study, Barron 2006), other genes on the mini-chromosome might be involved in pathogenicity and the dothistromin production in infected pine needles might be coincidental because of the location of the dothistromin genes.

The similarities of dothistromin genes to AF/ST genes of *Aspergillu*s spp. imply a common evolutionary history. Therefore the identification of further genes involved in the biosynthesis of dothistromin would be interesting in respect of the evolution of the AF/ST gene cluster. The relative positions of the three so far identified mini-clusters of dothistromin genes (Zhang *et al.* 2007) should be determined. The detection of further dothistromin genes might reveal important information to determine if the dothistromin gene cluster is ancestral, as proposed by Cary & Ehrlich (2006) or if, instead, the

dothistromin genes might be originated from the ST/AF cluster and developed from there in a different direction. In each case this would help to resolve the evolutionary history of the ST/AF cluster.

The gene expression study revealed that although the dothistromin genes are not organized in a tight cluster they are co-regulated in culture (Section 3.3). The surprising outcome of these studies was the early toxin production and dothistromin gene expression (Section 3.3), which was seen in both submerged cultures and in P*dotA* regulated EGFP reporter strains, which showed highest expression at the outer margin of colonies on plate cultures (Chapter 4). Secondary metabolites are usually produced in the late exponential growth phase and this is certainly the case for AF in *Aspergillus* (Trail *et al.* 1995). In contrast the dothistromin genes are highly expressed during early exponential growth which is also the main toxin production period, although expression differs depending on the growth media (Section 3.3). Analyses of the intergenic regions of the dothistromin genes suggest the involvement of an AflR like regulatory protein, as seen for AF/ST biosynthesis. However the absence of putative binding sites for AflR in the upstream region of *moxA* which is also co-regulated with the other dothistromin genes, suggests that other regulatory proteins may be involved in co-regulation. In *D. septosporum* candidate dothistromin regulatory genes have not been identified yet. The identification of regulatory genes for the biosynthesis of dothistromin should be a key area for future research. The early onset of gene expression and dothistromin production implies a different regulation of the biosynthesis compared to the very similar AF/ST genes. Therefore it would be very interesting to define if the same regulatory elements known for the AF/ST biosynthesis, including *aflR* and *laeA*, regulate the synthesis of dothistromin in *D. septosporum*. The identification of the regulatory mechanism in *D. septosporum* might therefore give new information about the control of AF/ST synthesis and contribute to a better control of AF food contamination by *Aspergillus* spp.. The identification of regulatory factors for the biosynthesis of dothistromin might also give new clues about the biological function of dothistromin (and AF/ST).

The early expression of the dothistromin genes was speculated to be related to a biological function of dothistromin. It was thought that the early production of dothistromin could facilitate the infection of the host by *D. septosporum* or that the early

production of dothistromin might give *D. septosporum* a competitive advantage against other microorganisms in the same environment. Biological roles of secondary metabolites are often unknown and discussed controversially (Challis & Hopwood 2003; Firn & Jones 2000; Vining 1990). Dothistromin has been previously suspected to be a pathogenicity factor in pine needle blight, but technical difficulties did not previously allow this hypothesis to be tested in pathogenicity trials (West 2004). To overcome those difficulties GFP reporter strains were constructed in this study. This study is the first report of reporter genes used in *D. septosporum*, with the SGFP and EGFP variation of the GFP and the DsRed reporter successful expressed in *D. septosporum*. The constitutive GFP expressing strains facilitated the study of the infection process *in planta* carried out by a Masterate student in a parallel study and helped to overcome the difficulties in the pathogenicity trials (Barron 2006).

In this study the dothistromin-deficient GFP strain FJT30, derived from the *pksA* mutant FJT3, which does not produce any known dothistromin intermediates (Jin 2005), and the GFP reporter strain FJT29, derived from the versicolorin A accumulating *dotA* mutant FJT2 (Bradshaw *et al.* 2002), were constructed. The construction of FJT30 allowed the conclusion that dothistromin is not a pathogenicity factor (Chapter 4). Barron (2006) showed that dothistromin is not required for infection and subsequent colonisation of *P. radiata*, as disease incidence of FJT30 was comparative to that of the wild type. Further research is required to show if dothistromin is a virulence factor, as there may be some role of dothistromin in the infection process. The availability of the *D. septosporum* reporter gene strains will be helpful in defining a role of dothistromin in virulence. Further, a DsRed wild type dothistromin producing strain is now available. The co-inoculation of the dothistromin producing DsRed strain with one of the dothistromin deficient GFP strains *in planta* might help to determine a role of dothistromin as a virulence factor. It should be easy to differentiate between infections caused by dothistromin-deficient GFP strains and dothistromin producing DsRed strains.

As it was shown that dothistromin is not a pathogenicity factor it was speculated that the production of dothistromin might play a role in competition with other micro-organisms. This appeared feasible as dothistromin was shown in earlier studies to be toxic against a broad range of organisms (Harvey *et al.* 1976; Skinnider *et al.* 1989;

Stoessl *et al.* 1990). Plate competition assays showed an advantage of the dothistromin producing strains NZE7 and NZE10 when compared to the dothistromin deficient strain FJT3. Inhibition of growth was seen towards a range of other fungal species including other inhabitants of pine trees. Therefore the production of dothistromin might indeed be advantageous for *D. septosporum*. However, the results were only obtained in culture, where growth conditions do not represent the situation in nature. Under different conditions the growth rates and metabolic profile of all species are likely to be different. Therefore it is advised to investigate the competition of *D. septosporum* and different species *in planta.* The detection and identification of *D. septosporum* in those experiments should be facilitated by the GFP reporter strains obtained in this study.

It was further investigated if the production of dothistromin and dothistromin gene expression is induced by the presence of competitors, but no clear support was obtained for an induction effect by the competitors used in this study. There were limitations in this study partly due to the morphological instability, including sectoring, of *D. septosporum* as discussed in Chapter 5. Induction assays in submerged culture might overcome some of the technical limitations. Initial trials using submerged cultures to check for induction of dothistromin production were performed (Appendix A6) but the preliminary data provide no clear evidence of induction.

The sectoring of the *D. septosporum* colonies (see also Appendix A7) is interesting as morphologically distinct sectors usually differed in pigmentation and dothistromin gene (P*dotA*::*egfp*) expression. It would be interesting to determine if those differences are caused by a loss of dothistromin biosynthetic genes or maybe regulatory elements. Secondary metabolite gene clusters are often located close to the telomere ends of chromosomes, which are rapidly evolving DNA regions (Zhang *et al.* 2005). If the dothistromin genes are close to the telomeric ends of the 1.3 Mb mini-chromosome this could explain the frequent sectoring of the colonies observed in culture.

Reporter strains, including those with a dothistromin gene regulated GFP reporter (P*dotA*::*egfp*) and containing a DotA-EGFP fusion protein were constructed. The P*dotA*::*egfp* reporter strains FJT24 and FJT26 confirmed the early dothistromin gene expression with colonies showing highest GFP fluorescence at the colony margin. The expression pattern in single hyphae was somewhat inconclusive in that only a few

showed expression of GFP and also sometimes only in hyphal sectors. This suggests that the expression differs in individual hyphae even in a constant environment, as observed for AF genes and other genes in *Aspergillus* spp. (Lee *et al.* 2004; Vinck *et al.* 2005). The P*dotA*-regulated GFP appears to be expressed the same as the native *dotA* gene and the expression of GFP was shown in germinating spores, germtubes and vegetative hyphae. While these observations are interesting it should kept in mind that the results in this thesis are mainly obtained in culture. Therefore it is not known if the same expression would be seen in natural conditions. Although preliminary results showed that the P*dotA*-*egfp* is early expressed *in planta* (Section 4.5.3), confirmation of this is a priority for future work.

The DotA-EGFP fusion protein indicated a spatial localisation of the DotA protein and therefore of the synthesis of dothistromin in vesicles. There is also some evidence for intracellular spatial organisation of the AF synthesis of *Aspergillus* spp.. Confocal microscopy should be performed to investigate the intracellular localisation of the DotA-EGFP protein in more detail and further experiments should be made to identify the organelles in which the DotA-EGFP is located. This might identify a secretion system for dothistromin in *D. septosporum*. This could also have implications for the control of AF contamination, as the candidate transporter gene *aflT* for AF secretion was shown not to play a major role as had previously been thought (Chang *et al.* 2004).

In summary this study revealed that dothistromin production and expression of dothistromin genes occur unusually early in culture. This is fundamentally different from AF and most other secondary metabolites, and might have implication for current thinking about secondary metabolites.

One possible explanation for an early onset of dothistromin biosynthesis is required for an early stage of needle penetration or colonisation. However, using GFP strains it was shown that dothistromin is not a pathogenicity factor in Dothistroma needle blight.

The early onset of dothistromin biosynthesis also led to the suggestion that dothistromin may have a role in competition with other fungi, and this hypothesis was

supported by studies in culture. This might have a possible practical outcome as, in contrast to most of the competitor fungi tested, biocontrol species such as *Trichoderma* showed resistance against dothistromin producing strains. Therefore further studies might lead to a new way of disease control, which might be of special interest given the recent epidemics in the Northern hemisphere and the current reliance on chemical sprays for control in the Southern hemisphere.

Appendices

Appendix 1: Media

All media were prepared with Milli-Q water and sterilized by autoclaving at 121°C for 15 minutes. Media were cooled to approximately 50°C before addition of antibiotics.

A1.1 *E. coli* media

Luria Broth (LB) (g/L): Tryptone (Becton, Dickison and company), 10; NaCl, 5; Yeast extract (Becton, Dickison and company), 5

LB solid media (g/L): Tryptone, 10; NaCl, 5; Yeast extract, 5; Agar, 15.

Selective LB media

Supplements were added at the following final concentrations.

(µg/mL): Ampicillin, 100; isopropylthio-β-D-galactoside (IPTG), 30;

5-bromo 4-chloro 3-indolyl-β-D-galactoside (X-gal), 60

A1.2 Fungal growth media

***D. septosporum* media** (DM)

(g/L): Malt extract (Oxoid), 50.0; Nutrient agar (Oxoid), 28.0.

***D. septosporum* Broth** (DB)

(g/L) Nutrient broth (Oxoid), 23; Malt extract (Oxoid), 23.

***D. septosporum* Sporulation Media** (DSM)

(g/L) Malt extract (Oxoid), 15; Yeast extract (BD), 5; Agar, 20.

Potato dextrose broth (PDB)

Yeast extract for elicitor trial:

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

McIlvaine Buffer for pH trial

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

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

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

A1.3 *D. septosporum* transformation media

***D. septosporum* Top media** (DM Top)
(g/L): Malt extract, 50.0; Nutrient Agar, 11.2: Sucrose, 273.9 (0.8 M).
Osmotically Stabilised DM (DM Suc)
(g/l): Malt extract, 50.0; Nutrient Agar, 28.0: Sucrose, 273.9 (0.8 M).
Selective DSM and DM
Media used to select for hygromycin resistant *D. septosporum* transformants contained 70 μg/ml. Hygromycin B (Sigma),
Media used to select for phleomycin resistant *D. septosporum* transformants contained 7 μg/ml Phleomycin (Sigma).

Appendix 2: Buffers and solutions

All solutions were prepared with Milli-Q water and sterilized by autoclaving at 121^{o}C for 15 minutes, unless otherwise stated.

A2.1 Common buffers and solutions

TE Buffer
10 mM Tris-HCl and 1mM Na_2EDTA (TE 10:1) prepared from 1 M Tris-HCl (pH 8.0) and 250 mM Na_2EDTA (pH 8.0) stock solutions

1 x TBE Buffer
89 mM Tris-HCl, 2.5 mM Na_2EDTA and 89 mM Boric acid (pH 8.3).

Ethidium Bromide
Agarose gels were stained in ethidium bromide prepared as follows: 1 μl of 10 mg/ml stock per 10 ml of Milli-Q water to give a final concentration of 1 μg/ml.

RNAseA (DNAse free)
10 mg/mL RNAse was dissolved in 0.01M Sodium acetate (pH 5.2) and placed in a boiling water bath for 15 minutes. This was cooled slowly to room temperature and 0.1 volumes of Tris-HCl (pH 7.4) added and stored at -20^{o}C.

Gel Loading Dye

2 M Urea, 50% (v/v) glycerol, 50 mM Tris acetate, 0.4% (w/v) bromophenol blue and 0.4% (w/v) xylene cyanol.

1×TNE buffer

10 mM Tris-HCl, 1 mM Na_2EDTA and 100 mM NaCl, pH 7.4

Fluorometer DNA standard

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

Hoechst dye stock solution

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

Fluorometer working solution

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

Acid and Base

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

A2.2 Genomic DNA isolation buffers

Lysis buffer

40 mM Tris-Acetate pH 7.8; 20 mM Sodium Acetate; 1 mM EDTA; 1% SDS

CTAB buffer

2% CTAB (w/v), 1% PVP40 (w/v), 5 M NaCl, 0.5 M EDTA, 1 M Tris-HCl

A2.3 Reagents for *D. septosporum* transformation

OM buffer: 1.4 M $MgSO_4 \cdot 7H_20$, with 10 mM Na_2HPO_4/100 mM NaH_2PO_4 buffer, pH 5.8

ST buffer: 1.0 M Sorbitol, 100 mM Tris-HCl (pH 8.0).

STC Buffer: 1.0 M Sorbitol, 50 mM Tris-HCl (pH 8.0), 50 mM $CaCl_2$.

40% PEG: 40 g PEG 6000 in 100 ml STC buffer

Glucanex: 10 mg/ml in OM buffer

A2.4 Reagents for Southern blotting and hybridisation

Denaturing Solution: 500 mM NaOH, 500 mM NaCl

Neutralising Solution: 500 mM Tris (pH 7.4), 2 M NaCl

20 × SSC: 3 M NaCl, 0.2 M tri-sodium citrate, pH 7.0

Radioactive labelling:

100× Denhardts buffer: 1% Ficoll, 1% BSA, 1% PVP40

Prehybridisation buffer (made fresh)

6 × SSC, 2 × Denhardts buffer, 0.1% SDS, 0.1% pre-boiled salmon sperm DNA (Sigma).

Wash Solution I: 2 x SSC, 0.1% SDS

Wash Solution II: 0.5 × SSC, 0.1% SDS

DIG-labelling:

Buffer I: 100 mM Tris, 150 mM NaCl, pH 7.5

Buffer II : 1% blocking reagent (Roche Applied Science), in Buffer I

Antibody solution: Anti-Digoxigenin AP diluted 1:10000 in Buffer II

Buffer III: 100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5

Hybridisation buffer: 5 x SSC, 50% formamide (Sigma), 0.02 % SDS, 2% blocking reagent, 0.1%sodium lauroylsarcosine

Stripping buffer: 0.2 M NaOH, 0.1% (v/v) SDS

A2.5 Reagents for Northern blotting and hybridisation

DEPC-H_2O: 0.01% DEPC in MilliQ H_2O, sterilise twice.

10 ×MOPS buffer: 200 mM Na-MOPS, 50mM NaOAc, 10 mM EDTA

BPB Loading dye: 0.2% bromophenol blue dissolved in 1 × MOPS buffer.

20 × SSC: M NaCl, 0.2 M tri-sodium citrate in 0.01% DEPC-treated H_2O, pH 7.0.

100× Denhardts buffer: 1% Ficoll, 1% BSA, 1% PVP40

Prehybridisation buffer (made fresh)

6 × SSC, 2 × Denhardts buffer, 0.1% SDS, 0.1% pre-boiled salmon sperm DNA (Sigma).

A2.6 Reagents for ELISA

Phosphate-buffered saline (PBS 10X):

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

Phosphate-buffered saline plus 0.1% Tween 20 (PBST):

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

Skim milk powder:

1% skim milk powder (Pams), 1% PBS and 1% thiomersal (BDH)

Dilution buffer (per ELISA plate):

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

Dilution buffer and broth (per ELISA plate):

Dilution buffer/growth media 1:1

Working buffer (per ELISA plate):

Dilution buffer 10 ml, DMSO 15 µl.

Working buffer and broth (per ELISA plate):

Dilution buffer 20 ml, growth media 20 ml, DMSO 40 µl.

labeled peroxidase (per ELISA plate):

Dilution buffer 198 µl, 10C12 (dothistromin antibody) 2 µl makes 1/100 dilution. Add 40 µl of 1/100 labeled peroxidase to dilution buffer 12 ml (makes 1/300 dilution).

Appendix 3: Plasmid maps

Maps of plasmids used in this thesis that were not constructed or shown before in this study.

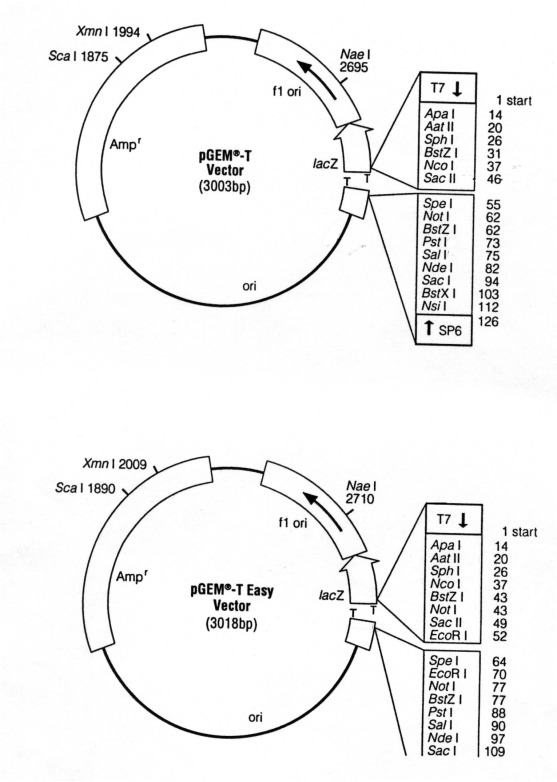


Figure A3.1: Cloning vectors pGEM-T and pGEM-T Easy (Promega)

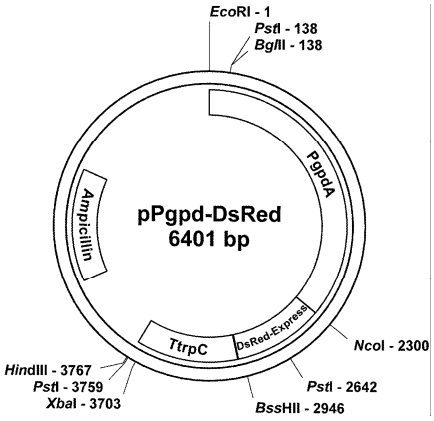


Figure A3.2: DsRed vector pPgpd-DsRed. (Mikkelsen et al., 2003)

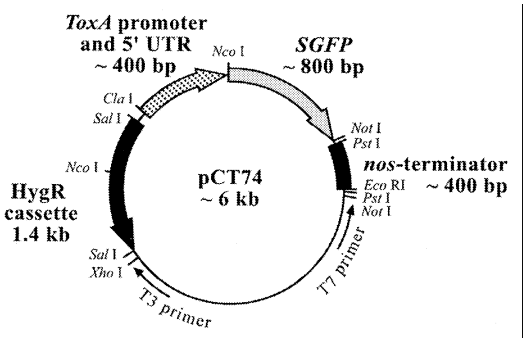


Figure A3.3: SGFP vector pCT74 (Lorang et al., 2001)

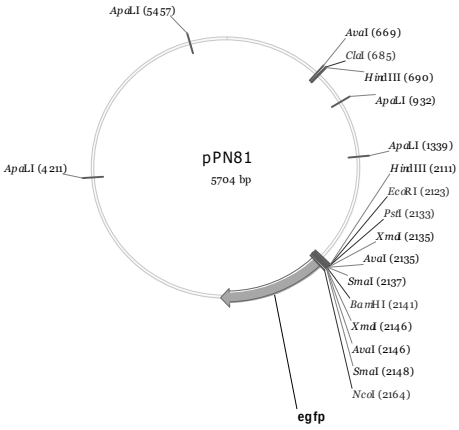


Figure A3.4: EGFP vector pPN81 (A. Tanaka, Centre of Functional Genomics, Massey University)

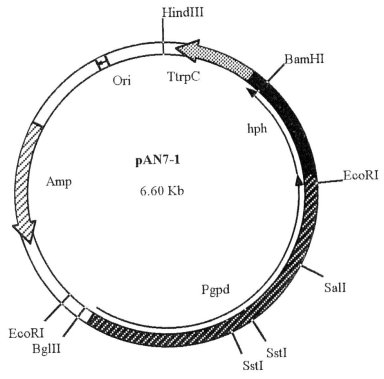


Figure A3.5: Transformation control vector pAN7-1 (Punt and van Hondel, 1992)

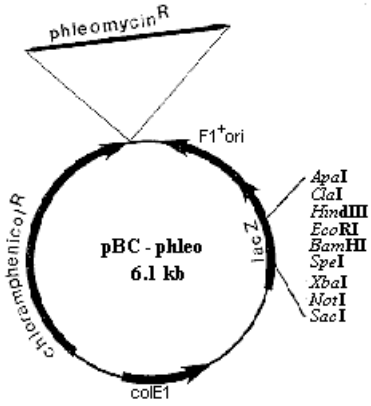


Figure A3.6: Transformation vector pBC-Phleo (Silar, 1995)

Appendix 4:
High Pressure Liquid Chromatography (HPLC) assay

As mentioned in Section 2.13, initial trials were performed to establish an HPLC assay as an alternative to the quantification of dothistromin by ELISA. The assay was based on previous work done by P. Debnam (Forest Research, Rotorua, NZ).

Dothistromin was extracted from growth media with ethyl acetate including 1% formic acid. One part media was mixed with the same volume of ethyl acetate + 1% formic acid in a microcentrifuge tube. Samples were vortexed and mixed for additional 10 min on a benchtop shaker. For phase separation the mix was centrifuged for 5 min at maximum speed in a microcentrifuge and the ethyl acetate phase was transferred into a new centrifugation tube. This was repeated 2 more times and the ethyl acetate phases were combined. The ethyl acetate was evaporated in a speedvac. Samples were resolved in an appropriate volume of methanol prior to HPLC.

A reverse phase HPLC assay was performed using a Dionex Summit (Dionex Corporation, CA, USA) HPLC system equipped with a Luna (Phenomenex, CA, USA) C18 column (5 µm particle size, 4.6 × 250 mm). Peak detection was performed using a photo diode array detector reading absorbance at 483 nm with a reference beam set at 550 nm. Running solvent used was acetonitrile (ACN) in ddH_2O, with 5 min 15% ACN, continuously increasing to 75% ACN for 30 min, hold at 75% ACN for 5 min and continuous decreasing down to 5% ACN with a flow rate of 1 ml/min.

Sample pictures of the peak analyses for the standard and media samples are shown in Figure A4.1. The putative dothistromin peak has a broad shoulder which complicated the analyses of low concentrations (= small main peak). Further the dothistromin peak varied in its retention time (by ± 0.2 min), which made a clear identification difficult if the dothistromin peak was low and “surrounded” by peaks resulted from other substances. This, together with the long retention time of the dothistromin peak, made it impractical to use the HPLC method for the experiments in this study. Furthermore, results obtained did not justify the amount of chemical waste (ACN) accumulated by the analysis of each sample. However, it would be recommended to develop an HPLC assay with a shorter retention time and better resolution which would facilitate the quantification of dothistromin. HPLC analyses should be more accurate than values determined by the ELISA.

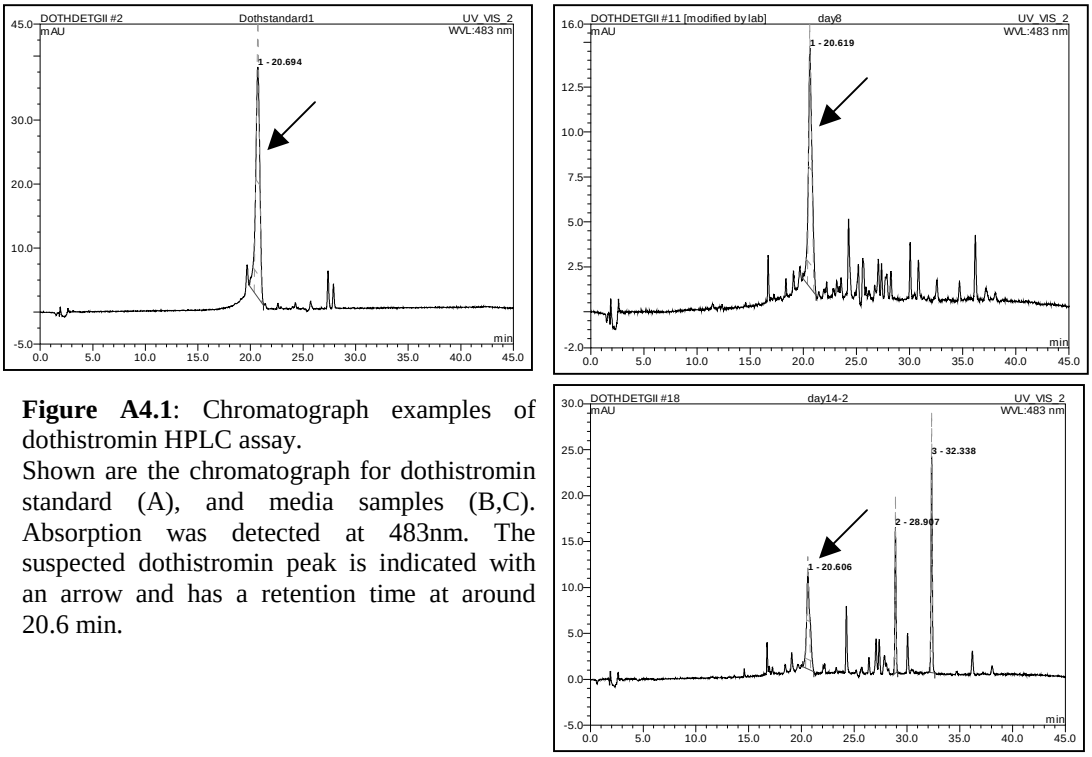


Figure A4.1: Chromatograph examples of dothistromin HPLC assay.
Shown are the chromatograph for dothistromin standard (A), and media samples (B,C). Absorption was detected at 483nm. The suspected dothistromin peak is indicated with an arrow and has a retention time at around 20.6 min.

However quantitative analyses of the dothistromin production using the peak area of the putative dothistromin peaks, showed the same trend of early toxin production and similar values (Figure A4.2) as observed in the experiments, where dothistromin was quantified using ELISA (Figure 3.6). The variability of the values obtained by HPLC appears to be less than for ELISA (lower standard deviations at low concentration (day3)).

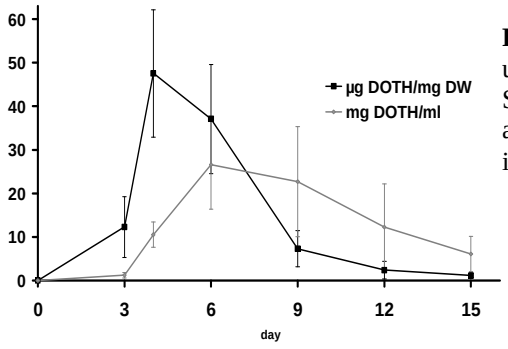


Figure A4.2: Dothistromin quantification using HPLC.
Shown are the values obtained by HPLC analyses for the growth experiment shown in Figure 3.2 (Section 3.1).

Appendix 5: Real-time PCR data example

Typical data for real time PCR and workflow analyses are shown using the example of the *pksA* gene.
The *pksA* cDNA was created in a RT-PCR (Section 2.15) using the same primer pair as used in subsequent *pksA* real-time RT-PCR. A tenfold dilution series was made and amplified in a real-time PCR. Using the Roche Light Cycler 3.0 software, melting curves and melting peaks (Figure A5.1) were analysed. Crossing points were determined using the fit points method (Figure A5.2) and standard curves were created. The same procedure was performed for the 18s DNA reference gene. The data obtained from the standard curve runs were then imported to the Roche RelQuant 1.1.1 software to create a coefficient file, which compares the standard curves of target gene (*pksA*) and reference gene (18s DNA). This coefficient file was saved and used in the analyses of the sample runs.

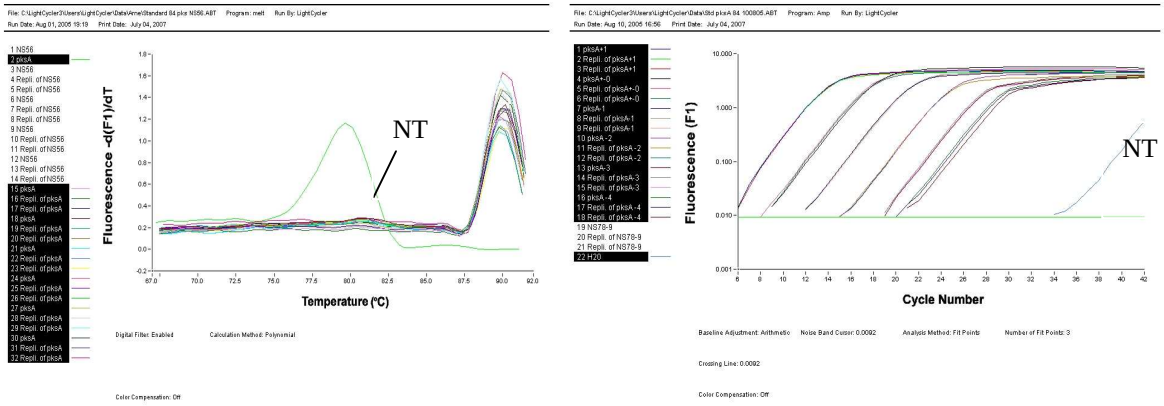


Figure A5.1: Standard curve for *pksA* cDNA.
Left panel shows the melting peaks of the PCR products. The NT labelled lines resulted from the non template rune included and fluorescence peak is most likely due to primer dimmers. The right panel shows the distribution of the *pksA* cDNA dilution series which were used to create standard curve. each sample is done in triplicate.

For each sample to be tested, amplification of the reference gene (18S rDNA) was carried out simultaneously with amplification of the target gene. In addition each experimental run contained calibrator for the target gene (*pksA*) gene and reference gene (18S rDNA) of known concentrations. Melting curve analyses were performed to assure the absence of unwanted products. Data of each run were then imported into the RelQuant 1.1.1 software to analyse the relative amount of *pksA* cDNA to the amount of 18s rDNA in each sample. The coefficient file created out of the standard curves was used to correct for run to run differences, as the software calculated those out of the data obtained from the calibrators included in each run.

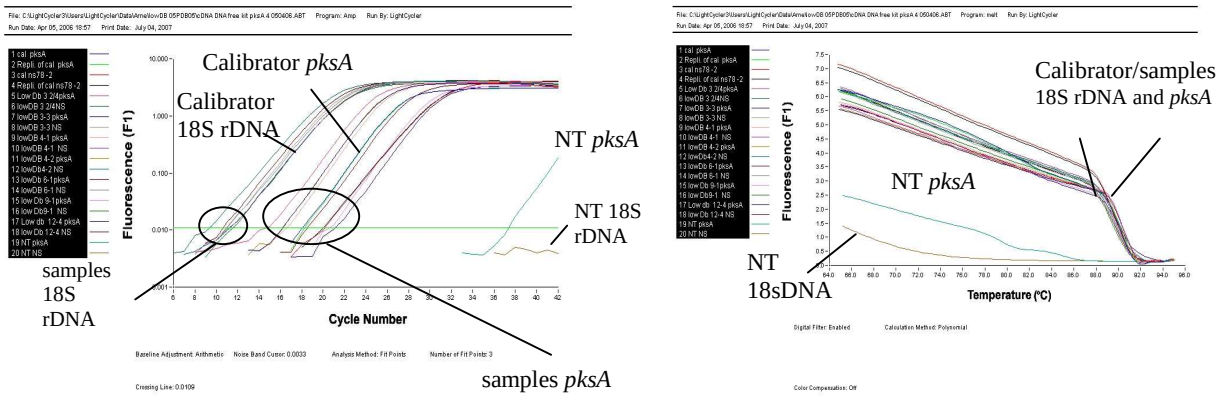


Figure A5.2: Sample run for quantitative gene expression analyses for the *pksA* gene.
The left panel shows the amplification analyses. The 18s DNA samples are more concentrated and amplify earlier than *pksA*. The equivalent *pksA* sample cDNA is amplified at a higher cycle number. Calibrator of both the 18S rDNA and *pksA* cDNA were amplified in the same run. Data obtained were then analysed with the RelQuant 1.1.1 software. The right panel shows the melting curve analysis of the light cycler run. Lines labelled with NT show the non-template controls for each the *pksA* and 18s DNA reactions.

Appendix 6: Dothistromin induction by elicitor broth in culture (preliminary results)

Parallel to the GFP plate induction assays it was attempted to induce dothistromin production of *D. septosporum* using NZE7 and NZE10 wild type strains with extracts from competitors' growth media. As discussed in Chapter 3, it was speculated that the higher expression of the *dot* genes and the higher dothistromin production in the DB media compared to PDB was caused by the presence of the components of fungal origin in DB. Therefore it was tested if adding an elicitor made from yeast could induce dothistromin production and dothistromin gene expression in PDB media. PDB media was chosen as the dothistromin genes are expressed at a relatively low and constant level (Section 3.2.2) and therefore an induction should be detectable.

"Elicitor" broths were created using similar methods as described by Kim *et al*. (2001). The elicitor broth created from yeast was obtained by dissolving 10 g yeast extract (BD) in 80 ml H_2O, 320 ml of 95% ethanol were subsequently added and the solution was left overnight at 4ºC. The liquid was poured off and the precipitate that formed was allowed to air dry and dissolved in a minimal amount of distilled water. The mixture was lyophilized and re-dissolved in 50 ml distilled water. For each sample 5 ml of the solution was filter sterilized before adding to the growth media.

Flasks containing 20 ml PDB media were inoculated with 10^6 wild-type *D. septosporum* spores/ml. After 6 days growth with shaking (180 rpm) at 22°C a 5 ml volume of "yeast elicitor" broth was added to each flask. As controls 5 ml sterile water and 5 ml PDB were added to three replicated flasks. Mycelium was harvested after 48 h and growth, dothistromin production (Section 2.2.2.2) and dothistromin gene expression (Section 2.13) were determined. The results were compared to control samples taken at day 6 and shown in Figure A.6.1 and A6.2. The other used "elicitor" broths consisted of autoclaved and filtered PDB media in which fungal competitor species had been inoculated and grown for 5 days with shaking (180 rpm) at 22°C. Three replicate *D. septosporum* flasks were harvested for each type of "elicitor" broth 3 h after addition and a further three flasks harvested 24 hours after addition of the "elicitor" broth. Liquid broth from each *D. septosporum* flask was assayed for dothistromin concentration using an ELISA assay as described previously (Section 2.13). Mycelium was harvested and divided to calculate the dry weight and to extract RNA as previously described in Section 2.2.2.2. Gene expression of *dotA*, *dotC*, *pksA* and *vbsA* was assessed by real time RT-PCR as described in Section 2.10.4.

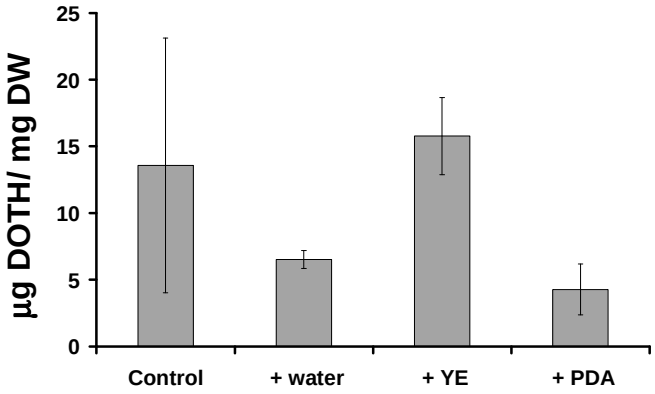
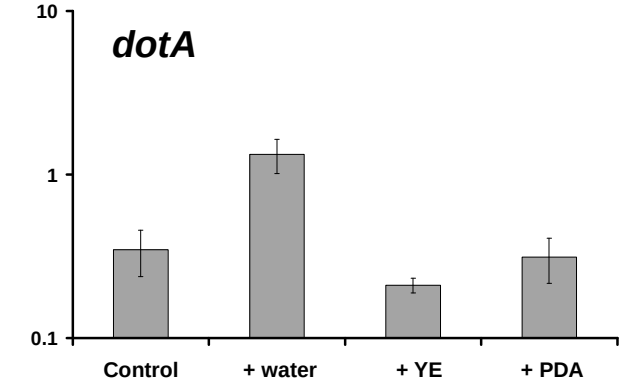
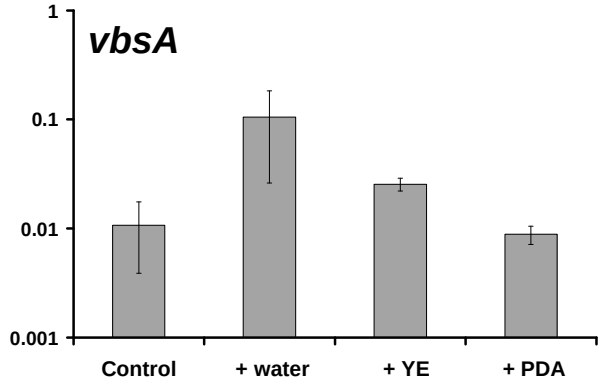
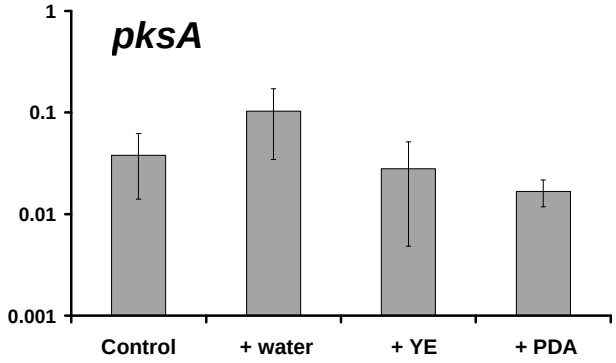


Figure A6.1: Dothistromin synthesis in yeast elicitor trial.

Shown is the dothistromin synthesis in µg dothistromin per mg DW for the control (before adding media) and 48h after adding water, yeast elicitor (YE), and potato dextrose media (PDA). The toxin concentration was highest in the YE media and control flasks, although an increase between the YE and control sample was not seen. The addition of water and PDA to the samples resulted in lower level of dothistromin production than with YE. Each data point is the mean ± standard deviation of three replicate samples.







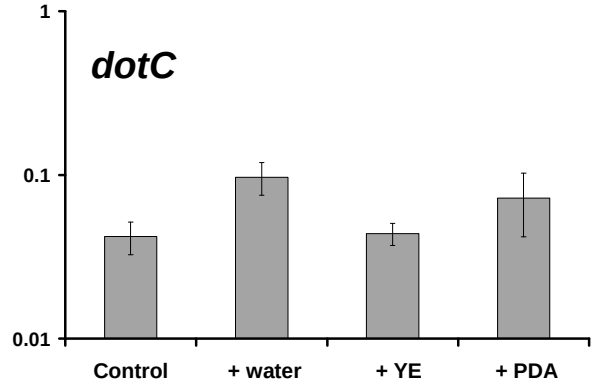
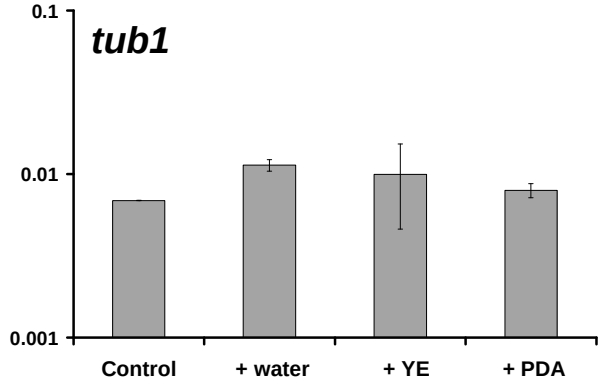


Figure A6.2: Relative gene expression of dothistromin genes in yeast elicitor trial.

Shown are gene expression levels relative to ribosomal 18S rDNA (Y-axis) for the control (before adding media) and 48h after adding water, yeast elicitor (YE), and potato dextrose media (PDA). Although the toxin concentration was highest in the YE media (Figure A6.1) no induction by YE of gene expression for *dotA*, *pksA*, *vbsA* or *dotC* was detected. The β-tubulin gene expression (*tub1*) was included as a constitutive control. Each data point is the mean ± standard deviation of three replicate samples.



An additional experiment was performed, this time using “yeast elicitor” and “ elicitor broth” consisting out of autoclaved PDB media in which *A. alternata* and *Phoma glomerata* had been grown for 6 days. *P. glomerata* was a contaminating fungus which frequently grew on needles of seedlings used in the pathogenicity trials (Barron 2006). In this trial only the toxin production was determined. Samples were taken 3 h and 24 h after adding the elicitor broth, as the high toxin production in the “yeast elicitor” media in the previous experiment (Figure A6.1) and the relatively low gene expression in those samples (Figure A6.2) suggested that an induction might have occurred at an earlier time. Results are shown and discussed in Figure A6.3.

These preliminary results did not allow detection of induction of dothistromin synthesis or gene expression by the yeast extract used by Kim *et al*. (2001) to induce secondary metabolites in ginseng cell cultures. This suggests that the higher dothistromin production in DB media (Chapter 3) is not due to the presence of material of fungal origin. Further no significant increase of dothistromin production was seen using an elicitor broth generated from *P. glomerata*. However, the *A. alternata* broth might have induced the production of dothistromin (Figure A6.3)

The liquid culture assay also had several limitations. As for the plate induction assay discussed in Chapter 5, a temporary increase of gene expression could have been missed as only a few time points were tested. However, an increased dothistromin concentration would reveal an earlier induction of dothistromin gene expression or secretion of the toxin. Further as discussed in Section 5.3, the PDB media might restrict the synthesis of some metabolites. Therefore the elicitor broths might not contain the substances which induce toxin production in *D. septosporum*. Furthermore the elicitor media were autoclaved and certain substances might have been destroyed; volatile compounds, which have been shown to play a major role in interactions of micro-organisms and their environment (Wheatley 2002), would have been lost in the process.

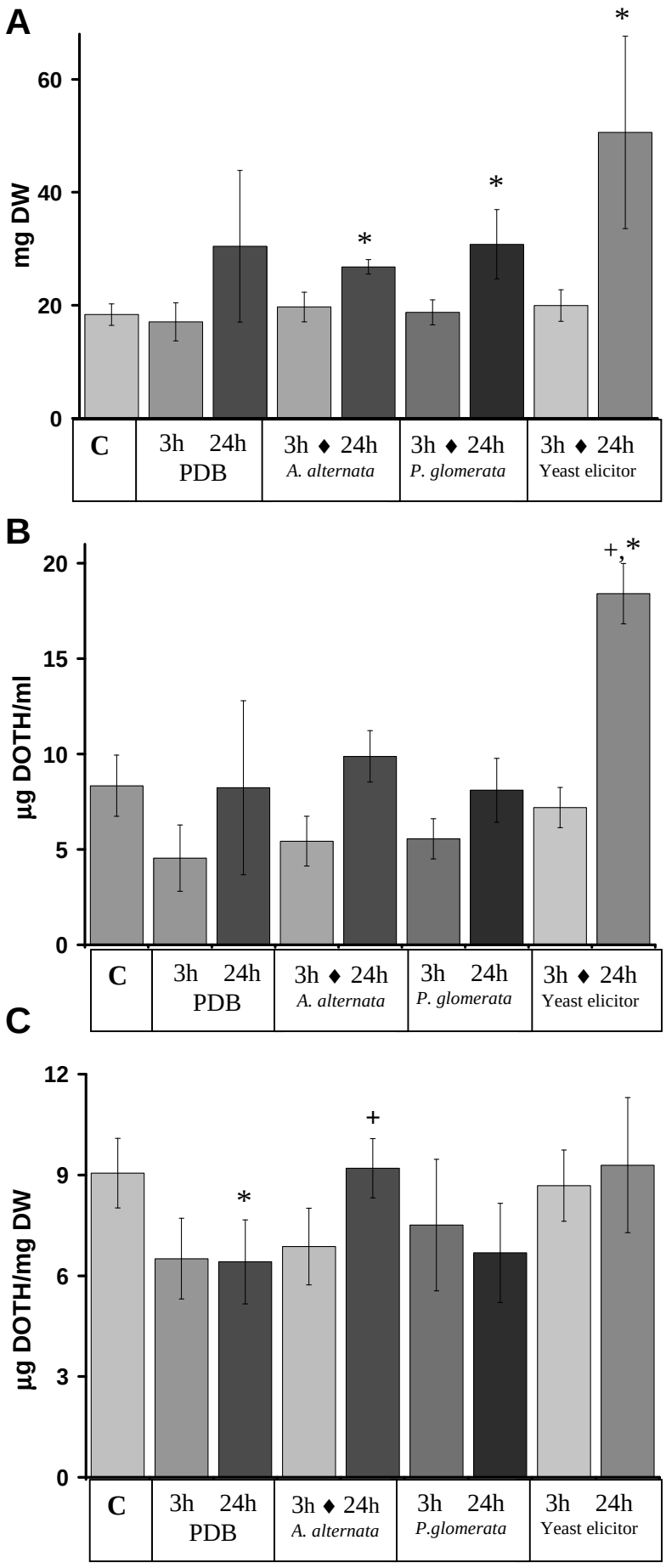


Figure A.6.3: Growth and dothistromin production of *D. septosporum* in elicitor media.
Shown are the values obtained from the control flasks (before adding elicitor broth or additional PDB media) **C**, and after addition of PDB, elicitor broth of *A. alternata*, elicitor broth of *P. glomerata* and yeast elicitor.
The first column of each pair represents values obtained 3h and the second column values obtained 24h after addition of media.
Significant different values from the control flask **C** are indicated with an * (n=3, $p<0.05$). Significant differences between each pair are indicated with an ♦ in the legend (n=3, $p<0.05$). and a significant difference between the elicitor sample and the PDB control is indicated with an + (n=3, $p<0.05$).

(A) Biomass per flask in mg DW. A significant increase in growth compared to the control **C** and between the 3h and 24h time point is seen for all three tested elicitor media.

(B) The concentration of dothistromin in the media is shown in µg DOTH/ml. Only the 24h yeast elicitor sample showed significant difference between the control **C** and its equivalent 24h PDB control. A significant increase of dothistromin concentration is seen between the 3h and 24h samples of both the yeast elicitor and *A. alternata* elicitor media.

(C) The dothistromin synthesis is shown in µg DOTH/mg DW. A significant increase for each pair is only seen for the 3h and 24h sample of the *A. alternata* elicitor. The 24h *A. alternata* elicitor sample showed also significant difference between the equivalent 24h PDB control. Further, those data suggest the significant increase of dothistromin concentration of the 24h yeast elicitor seen in (B) is solely due to the increase of biomass (compare (A)) as no significant difference is seen compared to the control **C** and to the 3h yeast elicitor sample

Appendix 7: Sectoring and morphological instability of *D. septosporum* in culture

During this study the morphological instability of *D. septosporum* complicated the investigations. *D. septosporum* frequently changed its morphology as well as its sporulation rate. Strains used in this study demonstrated variable morphologies also on the same media plates, different relative growth rates in liquid and plate cultures and sectoring of colony morphologies on plates. Likewise attenuation of dothistromin production has been noticed during serial cultivation (Shaw 1975) and Bradshaw et al. (2000) showed inconsistent levels of dothistromin biosynthesis of the New Zealand *D. septosporum* NZE3 isolate. Morphological variability was also noted by Barnes *et al*. (2004). Colony sectoring, morphological instability and chromosome rearrangements are all common phenomena in fungi, particularly asexual plant pathogens. Moreover, taking a plant pathogen out of its natural environment and into axenic culture is expected to lead to instability and morphological differences. These phenomena may be due in part to the presence of mycoviruses and/or transposable elements in the genome or ageing (Daboussi 1997; Wang et al. 2005). On one occasion where the sporulation rate of the isolate NZE5 was very low 5 different morphologies were isolated and grown up from a single sporulation plate. Those 5 isolates showed distinctively different colony morphologies on different and the same media plates (Figure A7.1). The pigment production in the media was also very different.

In addition to the morphological variability of whole colonies *D. septosporum* showed frequent sectoring in both wild type and transformants. In the P*dotA*::*egfp* transformants the different sectors were very distinguishable by both pigmentation of the colony and GFP expression. This supports the suggestion that the pigment is dothistromin and that GFP expression is co-regulated with the production of dothistromin. Further it suggests that the sectoring in the GFP expression pattern was not due to gene loss. Further some non-pigmented/GFP-fluorescence sectors appeared to regain GFP expression. It is proposed that the differences are due to epigenetic changes, such as alterations in gene regulation. Preliminary PCR (Figure A7.3) tests also suggest that the sectoring is not due to a loss of the dothistromin genes (Figure A7.4).

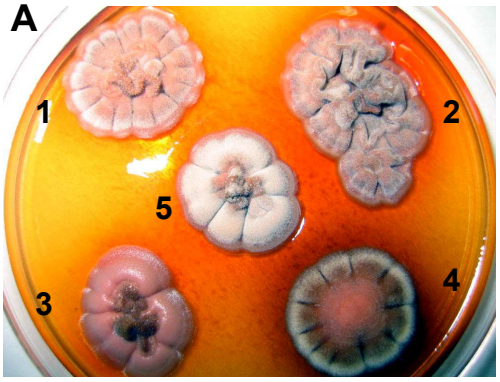
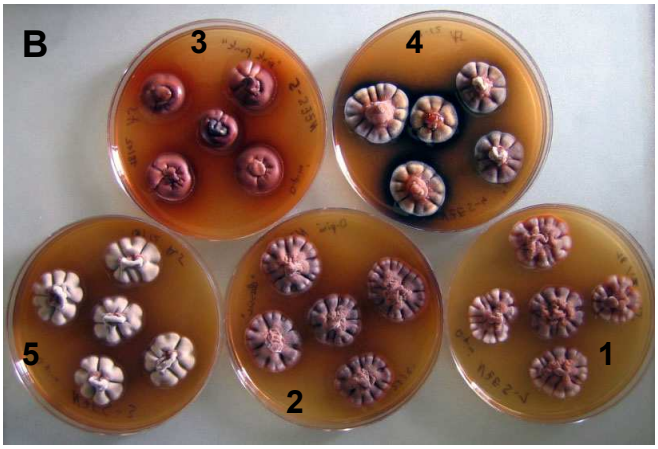


Figure A7.1: Morphological variations of *D. septosporum*.
(**A, B**) Shown are 5 distinctive morphologies (1-5) isolated from one media plate. Morphology does not appear to depend on the environmental conditions as differences are seen when grown together on one plate (**A**) or in different plates (**B**). Each morphology type was confirmed to be *D. septosporum* by its ITS sequence.
(**C**) Differences of pigment production on DM plates. Each column represents one subcultured isolate with plates in each row having the identical age (1st subculturing at the top and 5th at the bottom). Missing plates were contaminated by other microorganisms at the time the picture was taken.



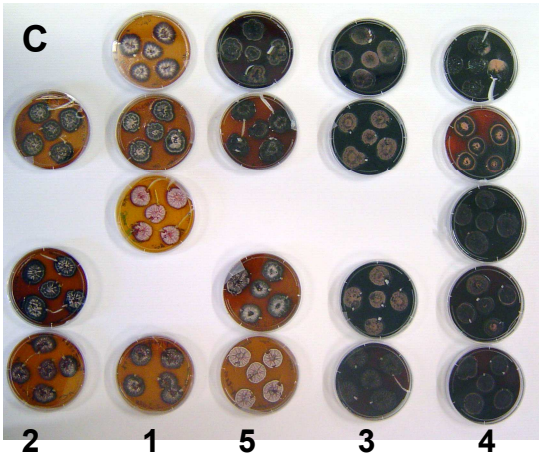
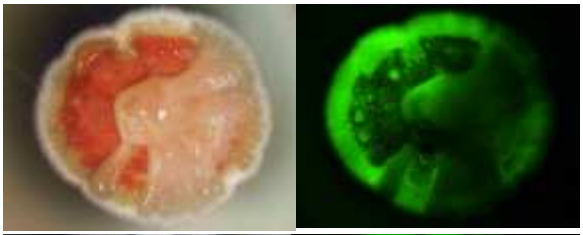
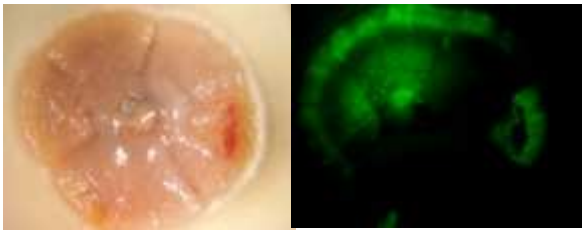


Figure A7.2: Sectoring in the P*dotA::egfp* strain FJT26.
Sectoring was frequently observed and was most obvious in some colonies of FJT26, which had intense pigmentation in some but none in other sectors. The levels of GFP fluorescence mirrored the differences in pigmentation.





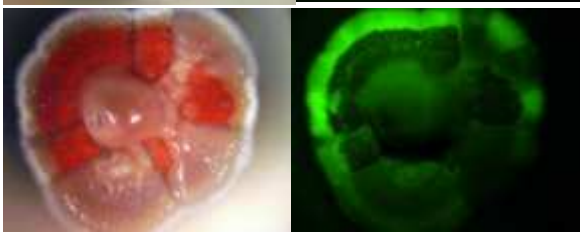
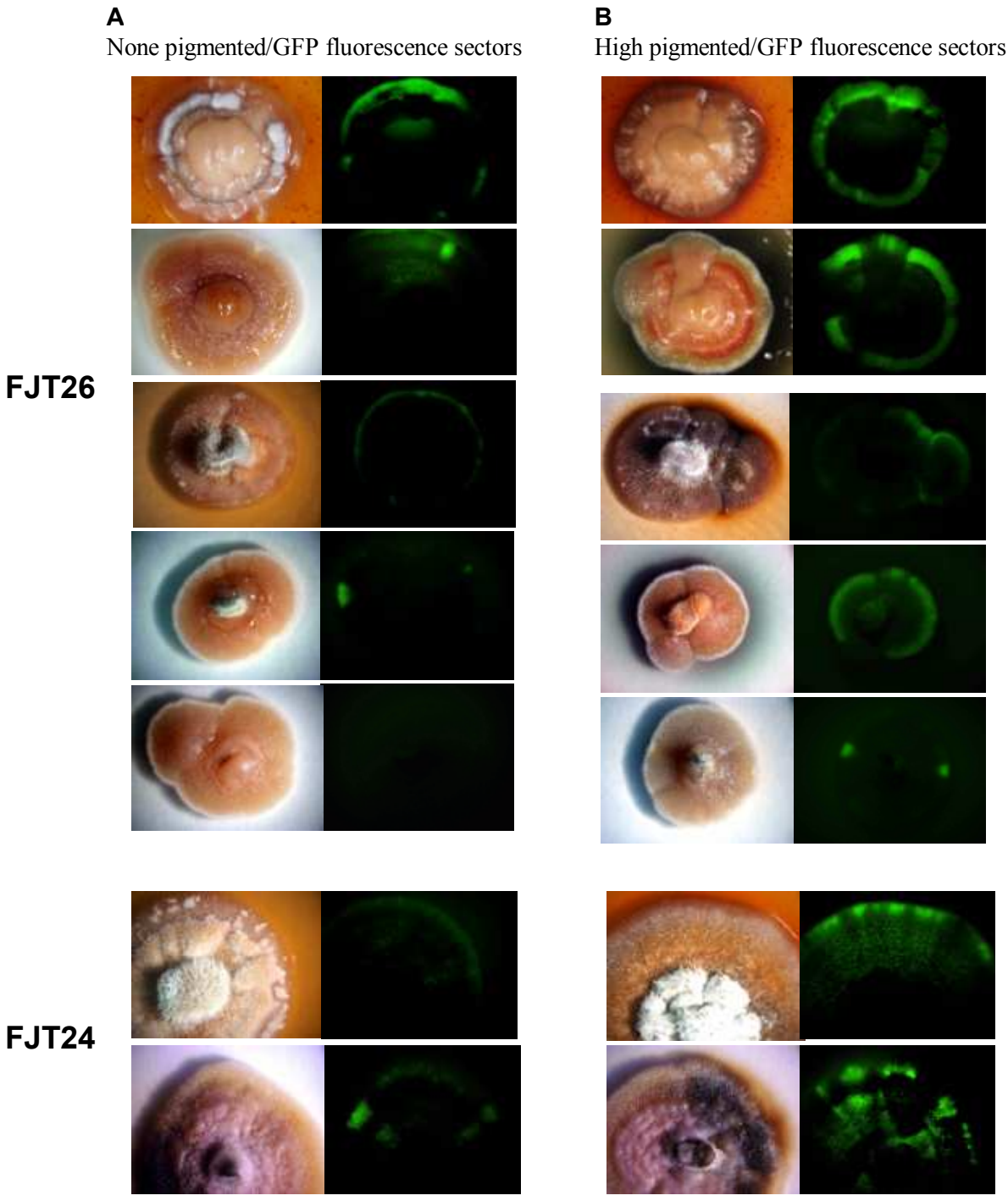


Figure A7.3: Sub-culturing of sectors with lack of and high pigmentation/GFP fluorescence.
Plates were inoculated with FJT24 and FJT36 with mycelium taken from highly pigmented and non-pigmented sectors of colonies similar to those shown in A7.2 inoculated on different plates. (A) The non-pigment/-GFP fluorescence sectors showed less GFP expression and pigmentation which also appeared to occur in sections. (B) The highly pigmented sectors showed a high variety in extent of pigmentation and GFP fluorescence.



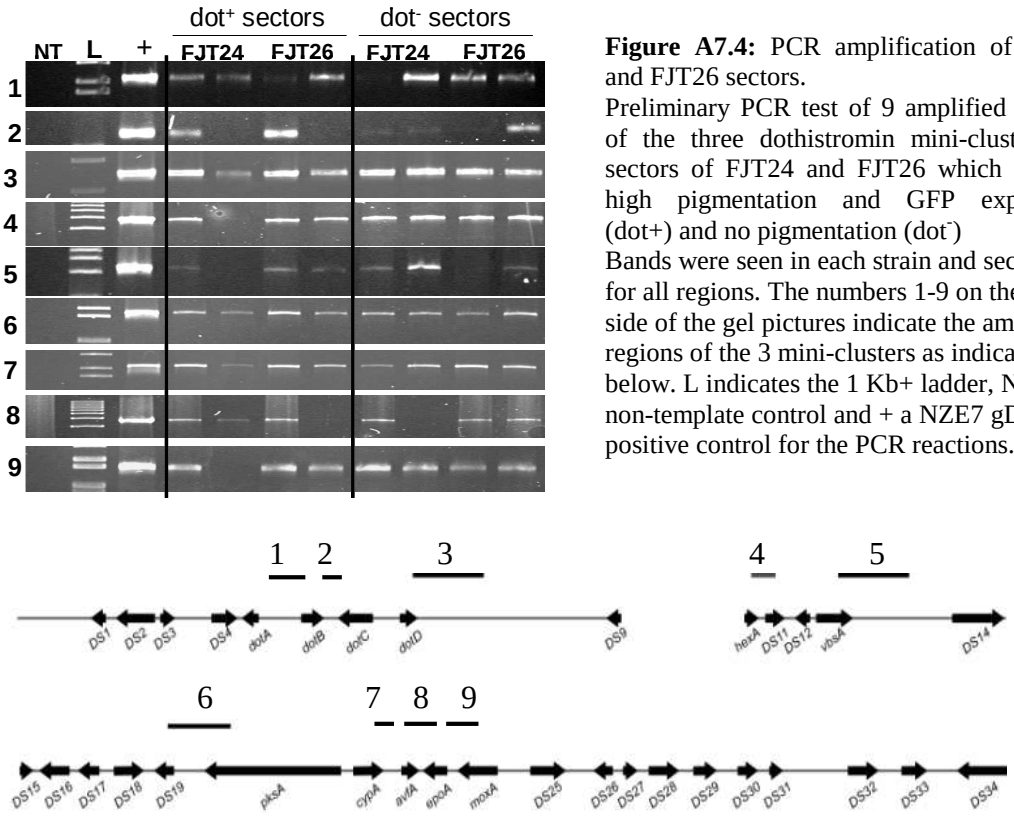


Figure A7.4: PCR amplification of FJT24 and FJT26 sectors.
Preliminary PCR test of 9 amplified regions of the three dothistromin mini-clusters for sectors of FJT24 and FJT26 which showed high pigmentation and GFP expression (dot+) and no pigmentation (dot$^-$)
Bands were seen in each strain and sectors for all regions. The numbers 1-9 on the left side of the gel pictures indicate the amplified regions of the 3 mini-clusters as indicated below. L indicates the 1 Kb+ ladder, NT is a non-template control and + a NZE7 gDNA positive control for the PCR reactions.

Preliminary PCR tests of 9 amplified regions of the 3 dothistromin mini-clusters for sectors of FJT24 and FJT26 which showed high pigmentation and GFP fluorescence expression, or no pigmentation were performed. DNA was extracted directly from plate cultures as described in Section 2.3.1.1. PCR products were seen in each strain in both high and non pigmented/GFP fluorescent sectors. However some samples did not produce a PCR product which is most likely due to the low DNA of some of the samples and not caused by the absence of the gene in the sector. Once high quality DNA from the different sectors is obtained, further investigations should be made to define differences in the dothistromin gene clusters between the sectors which might be responsible for the distinctive phenotypes of the sectors.

Appendix 8: Additional Southern of GFP transfomants

Figure A8.1: Additional GFP Southerns.

Shown are additional Southern analyses for the constitutive *egfp* strains (Table 4.1) obtained in this study. Genomic DNA was digested with HindIII (H) and EcoRI (E). Shown are separations of the digested gDNAs on a 0.8 % TBE-agarose gel (top) and hybridisation bands of the *egfp* probe of the corresponding Southern blots (bottom). Blots were hybridised with labelled PCR products derived from pPN82 (*egfp* probe). Ladders are indicated by L (1 kb^+ Ladder) and λ (*Eco*RI/*Hin*dIII digested λ–DNA). Except for FJT20 all transformants show more than one insertion of the GFP reporter gene. Wild type strains NZE does not show a GFP hybridisation band as expected.

Appendix 9: Publications and conference presentations

Main parts of this study have been published in the following peer reviewed publication:

Schwelm A, Barron NJ, Zhang S and Bradshaw RE, 2007.
Early expression of aflatoxin-like dothistromin genes in the forest pathogen *Dothistroma septosporum.* *Mycological Research.* **in press**

Results of obtained in this study contributed to the following peer reviewed publications:

Bradshaw RE, Jin H, Morgan B, Schwelm A, Teddy O, Young C and Zhang S, 2006.
A polyketide synthase gene required for biosynthesis of the aflatoxin-like toxin, dothistromin. *Mycopathologia.* **161:** 283-294.

Zhang S, Schwelm A, Jin H, Collins LJ and Bradshaw RE, 2007.
A fragmented aflatoxin-like gene cluster in the forest pathogen *Dothistroma septosporum.* *Fungal Genetics and Biology.* **accepted manuscript**.

Further work was published in the IMC8 Conference Proceedings as:

Schwelm A and Bradshaw RE, 2006. Early expression of aflatoxin-like genes.
In *8th International Mycological Congress* (Eds, Meyer W and Pearce C)
Medimond S.r.l., Cairns, Australia 20-25 August 2006, pp. 265-268

Oral presentation of this work have been made at the following conferences/meetings

8th International Mycological Congress 2006
A. Schwelm and R.E. Bradshaw
Early Toxin Biosynthesis in a Forest Pathogen
(August 20-25, 2006, Cairns, Australia)

This work was represented as poster presentation at the following conferences/meetings:

7th European Conference on Fungal Genetics 2003
17-20th April, Copenhagen, Denmark

New Zealand Microbiology Society conference 2004
17th to 19th November 2004, Palmerston North, New Zealand

Queenstown Molecular Biology Meeting 2005
Queenstown, New Zealand

References:

Akao T, Yamaguchi M, Yahara A, Yoshiuchi K, Hiroya Fujita, Yamada O, Akita O, Ohmachi T, Asada Y, Yoshida T, 2006. Cloning and expression of 1,2-α-mannosidase gene (*fman*IB) from filamentous fungus *Aspergillus oryzae*: *in vivo* visualization of the FmanIBp-GFP fusion protein. *Bioscience, Biotechnology, and Biochemistry* 70, 471-479.

Akesson H, Carlemalm E, Everitt E, Gunnarsson T, Odham G, Jansson H-B, 1996. Immunocytochemical localization of phytotoxins in *Bipolaris sorokiniana*. *Fungal Genetics and Biology* 20, 205-216.

Al-Samarrai TH, Schmid J, 2000. A simple method for extraction of fungal genomic DNA. *Letters in Applied Microbiology* 30, 53-56.

Arndt C, Cruz MC, Cardenas ME, Heitman J, 1999. Secretion of FK506/FK520 and rapamycin by *Streptomyces* inhibits the growth of competing *Saccharomyces cerevisiae* and *Cryptococcus neoformans*. *Microbiology-Sgm* 145, 1989-2000.

Assante G, Camarda L, Merlini L, Nasini G, 1977. Dothistromin and 2-epidothistromin from *Cercospora smilacis*. *Phytochemistry.* 16, 125-126.

Balint-Kurti PJ, May GD, Churchill ACL, 2001. Development of a transformation system for *Mycosphaerella* pathogens of banana: a tool for the study of host/pathogen interactions. *FEMS Microbiology Letters* 195, 9-15.

Barnes I, Crous PW, Wingfield BD, Wingfield MJ, 2004. Multigene phylogenies reveal that red band needle blight of *Pinus* is caused by two distinct species of *Dothistroma*, *D. septosporum* and *D. pini*. *Studies in Mycology* 50, 551-565.

Barnes SE, Dola TP, Bennett JW, Bhatnagar D, 1994. Synthesis of sterigmatocystin on a chemically defined medium by species of *Aspergillus* and *Chaetomium*. *Mycopathologia* 125, 173-178.

Barron N, 2006. Optimizing *Dothistroma septosporum* infection of *Pinus radiata* and the development of red-band disease, *IMBS*. Massey University, Palmerston North, New Zealand.

Basset C, 1969. *Larix decidua* a new host for *Dothistroma pini*. *Plant Disease Reporter* 53, 706.

Bassett C, 1972. The Dothistroma situation, 1972. *Farm Forestry* 14, 47-52.

Bassett C, Buchanan M, Gallagher RT, Hodges RL, 1970. A toxic difuroanthraquinone from *Dothistroma pini*. *Chemistry and Industry.* 26 December 1970, 1659-1660.

Beck V, 2003. Advances in life sciences and bioterrorism. *EMBO reports* 4 (Supp1), S53-S56.

Bendtsen JD, Nielsen H, Heijne Gv, Brunak. S, 2004. Improved prediction of signal peptides: SignalP 3.0. *Journal of Molecular Biology* 340, 783-795.

Benitez T, Rincon AM, Limon MC, Codon AC, 2004. Biocontrol mechanisms of *Trichoderma* strains. *International Microbiology* 7, 249-260.

Bennett JW, Klich MA, 2003. Mycotoxins. *Clinical Microbiology Reviews* 16, 497-516.

Bhatnagar D, Ehrlich KC, Cleveland TE, 2003. Molecular genetic analysis and regulation of aflatoxin biosynthesis. *Applied Microbiology and Biotechnology* 61, 83-93.

Bidlake AT, 1996. Development of an efficient transformation system for *Dothistroma pini*, *IMBS*. Massey University, New Zealand, Palmerston North.

Bok JW, Balajee SA, Marr KA, Andes D, Nielsen KF, Frisvad JC, Keller NP, 2005. LaeA, a regulator of morphogenetic fungal virulence factors. *Eukaryotic Cell* 4, 1574-1582.

Bok JW, Keller NP, 2004. LaeA, a regulator of secondary metabolism in *Aspergillus* spp. *Eukaryotic Cell* 3, 527-535.

Bok JW, Noordermeer D, Kale SP, Keller NP, 2006. Secondary metabolic gene cluster silencing in *Aspergillus nidulans*. *Molecular Microbiology* 61, 1636-1645.

Bowden CG, Smalley E, Guries RP, Hubbes M, Temple B, Horgen PA, 1996. Lack of association between cerato-ulmin production and virulence in *Ophiostoma novo-ulmi*. *Molecular Plant-Microbe Interactions* 9, 556-564.

Bradshaw RE, 2004. Dothistroma (red-band) needle blight of pines and the dothistromin toxin: a review. *Forest Pathology* 34, 163-185.

Bradshaw RE, Bhatnagar D, Ganley RJ, Gillman CJ, Monahan BJ, Seconi JM, 2002. *Dothistroma pini*, a forest pathogen, contains homologs of aflatoxin biosynthetic pathway genes. *Applied and Environmental Microbiology* 68, 2885-2892.

Bradshaw RE, Bidlake A, Forester N, Scott DB, 1997. Transformation of the fungal forest pathogen *Dothistroma pini* to hygromycin resistance. *Mycological Research* 101, 1247-1250.

Bradshaw RE, Ganley RJ, Jones WT, Dyer PS, 2000. High levels of dothistromin toxin produced by the forest pathogen *Dothistroma pini*. *Mycological Research* 104, 325-332.

Bradshaw RE, Jin H, Morgan B, Schwelm A, Teddy O, Young C, Zhang S, 2006. A polyketide synthase gene required for biosynthesis of the aflatoxin-like toxin, dothistromin. *Mycopathologia* 161, 283-294.

Bradshaw RE, Zhang S, 2006a. An aflatoxin-like gene cluster in pieces?, in: Meyer W, Pearce C (Eds), *8th International Mycological Congress*. Medimond S.r.l., Cairns, Australia, pp. 139-142.

Bradshaw RE, Zhang S, 2006b. Biosynthesis of dothistromin. *Mycopathologia* 162, 201-213.

Brodhagen M, Keller NP, 2006. Signalling pathways connecting mycotoxin production and sporulation. *Molecular Plant Pathology* 7, 285-301.

Brown A, 2005. Seeing Red! *Forestry and British Timber* July 2005, 16-18.

Brown DW, Yu J-H, Kelkar HS, Fernandes M, Nesbitt TC, Keller NP, Adams TH, Leonard TJ, 1996. Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*. *Proceedings of the National Academy of Sciences of the United States of America* 93, 1418-1422.

Bu'lock J, 1965. *The biosynthesis of natural products*. McGraw-Hill, New York.

Buchanan RL, Jones SB, Gerasimowicz WV, Zaika LL, Stahl HG, Ocker LA, 1987. Regulation of aflatoxin biosynthesis: assessment of the role of cellular energy status as a regulator of the induction of aflatoxin production. *Applied and Environmental Microbiology* 53, 1224-1231.

Bullock WO, Fernandez JM, Short JM, 1987. XL1-Blue - A high-efficiency plasmid transforming *rec*A *Escherichia coli* strain with beta-galactosidase selection. *Biotechniques* 5, 376-378.

Bulman L, Gadgil P (Eds), 2001. *Cyclaneusma needle cast in New Zealand*. New Zealand Forest Research Institute Lmtd.

Burns C, Gregory KE, Kirby M, Cheung MK, Riquelme M, Elliott TJ, Challen MP, Bailey A, Foster GD, 2005. Efficient GFP expression in the mushrooms *Agaricus bisporus* and *Coprinus cinereus* requires introns. *Fungal Genetics and Biology* 42, 191-199.

Butin H, Kowalski T, 1989. Schüttepilze der Kiefer. *Merkblätter der forstlichen Versuchs- und Forschungsanstalt Baden-Würtenberg* 39.

Calvo AM, Bok J, Brooks W, Keller NP, 2004. *veA* is required for toxin and sclerotial production in *Aspergillus parasiticus*. *Applied and Environmental Microbiology* 70, 4733-4739.

Calvo AM, Wilson RA, Bok JW, Keller NP, 2002. Relationship between secondary metabolism and fungal development. *Microbiology and Molecular Biology Reviews* 66, 447-459.

Cary JW, Ehrlich KC, 2006. Aflatoxigenicity in *Aspergillus*: molecular genetics, phylogenetic relationships and evolutionary implications. *Mycopathologia* 162, 167-177.

Challis GL, Hopwood DA, 2003. Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* species. *Proceedings of the National Academy of Sciences of the United States of America* 100, 14555-14561.

Chang P-K, Yu J, Yu J-H, 2004. *aflT*, a MFS transporter-encoding gene located in the aflatoxin gene cluster, does not have a significant role in aflatoxin secretion. *Fungal Genetics and Biology* 41, 911-920.

Chang PK, 2003. The *Aspergillus parasiticus* protein AFLJ interacts with the aflatoxin pathway-specific regulator AFLR. *Molecular Genetics and Genomics* 268, 711-719.

Chen HQ, Lee MH, Daub ME, Chung KR, 2007. Molecular analysis of the cercosporin biosynthetic gene cluster in *Cercospora nicotianae*. *Molecular Microbiology* 64, 755-770.

Chiou C-H, Lee L-W, Owens SA, Whallon JH, Klomparens KL, Townsend CA, Linz JE, 2004. Distribution and sub-cellular localization of the aflatoxin enzyme versicolorin B synthase in time-fractionated colonies of *Aspergillus parasiticus*. *Archives of Microbiology* 182, 67-79.

Chiou C-H, Miller M, Wilson DL, Trail F, Linz JE, 2002. Chromosomal location plays a role in regulation of aflatoxin gene expression in *Aspergillus parasiticus*. *Applied and Environmental Microbiology* 68, 306-315.

Chung K-R, Ehrenshaft M, Daub ME, 2002. Functional expression and cellular localization of cercosporin-resistance proteins fused with the GFP in *Cercospora nicotianae*. *Current Genetics* 41, 159-167.

Cleveland TE, Bhatnagar D, 1990. Evidence for *de novo* synthesis of an aflatoxin pathway methyltransferase near the cessation of active growth and the onset of aflatoxin biosynthesis in *Aspergillus parasiticus* mycelia. *Canadian Journal of Microbiology* 36, 1-5.

Cobb FW, Uhrenholst B, Krohn RF, 1969. Epidemiology of *Dothistroma pini* needle blight on *Pinus radiata*. *Phytopathology* 59, 1021-1022.

Cole RJ, Cox RH, 1981. *Handbook of Fungal Metabolites*. Academic Press, New York.

Cotty PJ, 1989. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. *Phytopathology* 79, 808-814.

Covert SF, 1998. Supernumerary chromosomes in filamentous fungi. *Current Genetics* 33, 311-319.

Cuero R, Smith JE, Lacey J, 1988. Mycotoxin formation by *Aspergillus flavus* and *Fusarium graminearum* in irradiated maize grains in the presence of other fungi. *Journal of Food Protection* 51, 452-456.

Daboussi MJ, 1997. Fungal transposable elements and genome evolution. *Genetica* 100, 253-260.

Denison SH, 2000. pH regulation of gene expression in fungi. *Fungal Genetics and Biology* 29, 61-71.

Devey ME, Groom KA, Nolan MF, Bell JC, Dudzinski MJ, Old KM, Matheson AC, Moran GF, 2004. Detection and verification of quantitative trait loci for resistance to Dothistroma needle blight in *Pinus radiata*. *Theoretical and Applied Genetics* 108, 1056-1063.

Dick AMP, 1989. Control of *Dothistroma* needle blight in the *Pinus radiata* stands of Kinleith forest. *New Zealand journal of forestry science* 19, 171-179.

Doyle J, Doyle J, 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19, 11-15.

Edwards DW, Walker J, 1976. *Dothistroma septospora* on *Pinus* in Australia. *APPS Newsletter* 5, 25.

Ehrlich KC, Cotty PJ, 2002. Variability in nitrogen regulation of aflatoxin production by *Aspergillus flavus* strains. *Applied Microbiology & Biotechnology* 60, 174-178.

Ehrlich KC, Montalbano B, Boue SM, Bhatnagar D, 2005a. An aflatoxin biosynthesis cluster gene encodes a novel oxidase required for conversion of versicolorin A to sterigmatocystin. *Applied and Environmental Microbiology* 71, 8963-8965.

Ehrlich KC, Montalbano BG, Cotty PJ, 2003. Sequence comparison of *aflR* from different Aspergillus species provides evidence for variability in regulation of aflatoxin production. *Fungal Genetics and Biology* 38, 63-74.

Ehrlich KC, Montalbano BG, Cotty PJ, 2005b. Divergent regulation of aflatoxin production at acidic pH by two *Aspergillus* strains. *Mycopathologia* 159, 579-581.

Elliott GS, Mason RW, Ferry DG, Edwards IR, 1989. Dothistromin risk assessment for forestry workers. *New Zealand Journal of Forestry Science.* 19, 163-170.

Engelbrecht C, 2006. Dothistroma needle blight of pine, *Horticulture and Home Pest News*, *http://www.ipm.iastate.edu/ipm/hortnews/2006/2-8/dothistroma.html*. Iowa State University.

Evans H, Webber J, 2003. Pest and diseases, *Forest Research Annual Reports and Accounts 2002-2003*. The Stationary Office, Edinburgh, pp. 30-39.

Evans HC, 1984. *The genus Mycosphaerella and its anamorphs Cercoseptoria,Dothistroma and Lecanosticta on pines*, 1st ed. Commonwealth Agricultural Bureaux., Surrey.

Feng Z, 2007. Further studies of dothistromin toxin genes in the fungal forest pathogen *Dothistroma septosporum, IMBS*. Massey University, Palmerston North, New Zealand.

Ferguson LR, Parslow MI, McLarin JA, 1986. Chromosome damage by dothistromin in human peripheral blood lymphocyte cultures: a comparison with aflatoxin B1. *Mutation Research* 170, 47-53.

Fernández-Ábalos J, Fox H, Pitt C, Wells B, Doonan JH, 1998. Plant-adapted green fluorescent protein is a versatile vital reporter for gene expression, protein localization and mitosis in the filamentous fungus, *Aspergillus nidulans*. *Molecular Microbiology* 27, 121-130.

Firn RD, Jones CG, 1999. Secondary metabolism and the risks of GMOs. *Nature Correspondence* 400, 13-14.

Firn RD, Jones CG, 2000. The evolution of secondary metabolism - a unifying model. *Molecular Microbiology* 37, 989-994.

Firn RD, Jones CG, 2003. Natural products - a simple model to explain chemical diversity. *Natural Product Reports* 20, 382-391.

Fleetwood DJ, Scott B, Lane GA, Tanaka A, Johnson RD, 2007. A complex ergovaline gene cluster in *Epichloe* endophytes of grasses. *Applied and Environmental Microbiology* 73, 2571-2579.

Fonseca N, 1998. *Mycosphaerella pini* (= *Scirrhia pini*), the perfect state of *Dothistroma septospora*: first observation in Portugal, in: Laflamme G, Berube JA, Hamelin RC (Eds), *Foliage, Shoot and Stem Diseases of Trees*. Canadian Forest Service, Quebec, pp. 25-31.

Franich RA, 1988. Chemistry of weathering and solubilisation of copper fungicide and the effect of copper on germination, growth, metabolism, and reproduction of *Dothistroma pini*. *New Zealand Journal of Forestry* 18, 318-328.

Franich RA, Carson MJ, Carson SD, 1986. Synthesis and accumulation of benzoic acid in *Pinus radiata* needles in response to tissue injury by dothistromin, and correlation with resistance of *P. radiata* families to *Dothistroma pini*. *Physiological and Molecular Plant Pathology* 28, 267-286.

Franich RA, Gadgil PD, Shain L, 1983. Fungistatic effects of *Pinus radiata* needle epicuticular fatty and resin acids on *Dothistroma pini*. *Physiological Plant Pathology* 23, 183-195.

Fuchs U, Steinberg G, 2005. Endocytosis in the plant-pathogenic fungus *Ustilago maydis*. *Protoplasma* 226, 75-80.

Gadgil PD, 1967. Infection of *Pinus radiata* needles by *Dothistroma pini*. *New Zealand Journal of Botany* 5, 498-503.

Gadgil PD, 1968. Artificial inoculation of Douglas fir with *Dothistroma pini*. *New Zealand Journal of Forestry* 13, 123-124.

Gadgil PD, 1974. Effect of temperature and leaf wetness period on infection of *Pinus radiata* by *Dothistroma pini*. *New Zealand journal of forestry science* 4, 495-501.

Gadgil PD, 1977. Duration of leaf wetness periods and infection of *Pinus radiata* by *Dothistroma pini*. *New Zealand journal of forestry science* 7, 83-90.

Gadgil PD, Holden G, 1976. Effect of light intensity on infection of *Pinus radiata* by *Dothistroma pini*. *New Zealand journal of forestry science* 6, 67-71.

Gallagher RT, Hodges R, 1972. The chemistry of dothistromin, a difuroanthraquinone from *Dothistroma pini*. *Australian Journal of Chemistry.* 25, 2399-2407.

Ganley RJ, 2001. Characterisation of a global collection of *Dothistroma pini* isolates., *IMBS*. Massey University, Palmerston North, New Zealand.

Ganley RJ, Brunsfeld SJ, Newcombe G, 2004. A community of unknown, endophytic fungi in in western white pine. *Proceedings of the National Academy of Sciences of the United States of America* 101, 10107-10112.

Ganley RJ, Newcombe G, 2006. Fungal endophytes in seeds and needles of *Pinus monticola*. *Mycological Research* 110, 318-327.

Gardiner DM, Cozijnsen AJ, Wilson LM, Pedras MSC, Howlett BJ, 2004. The sirodesmin biosynthetic gene cluster of the plant pathogenic fungus *Leptosphaeria maculans*. *Molecular Microbiology* 53, 1307-1318.

Gardiner DM, Howlett BJ, 2005. Bioinformatic and expression analysis of the putative gliotoxin biosynthetic gene cluster of *Aspergillus fumigatus*. *FEMS Microbiology Letters* 248, 241-248.

Gardiner DM, Jarvis RS, Howlett BJ, 2005. The ABC transporter gene in the sirodesmin biosynthetic gene cluster of *Leptosphaeria maculans* is not essential for sirodesmin production but facilitates self-protection. *Fungal Genetics and Biology* 42, 257-263.

Gibson IAS, 1972. Dothistroma blight of *Pinus radiata*. *Annual Review of Phytopathology* 10, 51-72.

Gibson IAS, 1974. Impact and control of *Dothistroma* blight of pines. *European Journal of Forest Pathology* 4, 89-100.

Gilmour JW, 1981. The effect of season on infection of *Pinus radiata* by *Dothistroma pini*. *European Journal of Forest Pathology* 11, 265-269.

Gourbiere F, van Maanen A, Debouzie D, 2001. Associations between three fungi on pine needles and their variation along a climatic gradient. *Mycological Research* 105, 1101-1109.

Groenewald M, Barnes I, Bradshaw RE, Brown A, Dale A, Groenewald JZ, Lewis KJ, Wingfield BD, Wingfield MJ, Crous PW, 2007. Characterization and worldwide distribution of the mating type genes in the Dothistroma needle blight pathogens. *Phytopathology* 97, 825-834.

Guda C, Subramaniam S, 2005. pTARGET: a new method for predicting protein subcellular localization in eukaryotes. *Bioinformatics* 21, 3963-3969.

Haarmann T, Machado C, Lubbe Y, Correia T, Schardl CL, Panaccione DG, Tudzynski P, 2005. The ergot alkaloid gene cluster in *Claviceps purpurea*: Extension of the cluster sequence and intra species evolution. *Phytochemistry* 66, 1312-1320.

Harman GE, Howell CR, Viterbo A, Chet I, Lorito M, 2004. *Trichoderma* species - Opportunistic, avirulent plant symbionts. *Nature Reviews Microbiology* 2, 43-56.

Harvey AM, Batt RD, Pritchard GG, 1976. Inhibition of RNA synthesis in *Chlorella pyrenoidosa* and *Bacillus megaterium* by the pine-blight toxin, dothistromin. *Journal of General Microbiology* 96, 268-276.

Hatta R, Ito K, Hosaki Y, Tanaka T, Tanaka A, Yamamoto M, Akimitsu K, Tsuge T, 2002. A conditionally dispensable chromosome controls host-specific pathogenicity in the fungal plant pathogen *Alternaria alternata*. *Genetics* 161, 59-70.

Heiser I, Koehl J, Elstner EF, 2002. Oxygen activation by fungal and bacterial toxins, in: Upadhyay RK (Ed), *Advances in microbial toxin research and its biotechnological exploitation*. Kluwer Academic/Plenum Publishers, New York, pp. 63-79.

Henry KM, Townsend CA, 2005a. Ordering the reductive and cytochrome P450 oxidative steps in demethylsterigmatocystin formation yields general insights into the biosynthesis of aflatoxin and related fungal metabolites. *Journal of the American Chemical Society* 127, 3724-3733.

Henry KM, Townsend CA, 2005b. Synthesis and fate of *o*-carboxybenzophenones in the biosynthesis of aflatoxin. *Journal of American Chemical Society* 127, 3300-3309.

Hirst P, Richardson TE, Carson SD, Bradshaw R, 1999. *Dothistroma pini* genetic diversity is low in New Zealand. *New Zealand journal of forestry science* 29, 459-472.

Hoffmeister D, Keller NP, 2007. Natural products of filamentous fungi: enzymes, genes, and their regulation. *Natural Product Reports* 24, 393-416.

Hohn T, McCormick S, Desjardins A, 1993. Evidence for a gene cluster involving trichothecene-pathway biosynthetic genes in *Fusarium sporotrichioides*. *Current Genetics* 24, 291-295.

Horowitz S, Freeman S, Sharon A, 2002. Use of green fluorescent protein-transgenic strains to study pathogenic and nonpathogenic lifestyles in *Colletotrichum acutatum*. *Ecology and Population Biology* 92, 743-749.

Hotter GS, 1997. Elicitor-induced oxidative burst and phenylpropanoid metabolism in *Pinus radiata* cell suspension cultures. *Australian Journal of Plant Physiology* 24, 797-804.

Howlett BJ, 2006. Secondary metabolite toxins and nutrition of plant pathogenic fungi. *Current Opinion in Plant Biology* 9, 371-375.

Hulbary RL, 1941. A needle blight of Austrian pines. *Natural History Survey Bulletin* 21, 231-236.

Isshiki A, Ohtani K, Kyo M, Yamamoto H, Akimitsu K, 2003. Green fluorescent detection of fungal colonization and endopolygalacturonase gene expression in the interaction of *Alternaria citri* with citrus. *Phytopathology* 93.

Ivory MH, 1972. Infection of *Pinus radiata* foliage by *Scirrhia pini*. *Transactions of the British Mycological Society* 59, 365-375.

Jayawickrama KJS, Carson MJ, 2000. A breeding strategy for the New Zealand radiata pine breeding cooperative. *Silvae Genetica* 49, 82-90.

Jin HP, 2005. Further characterization of dothistromin genes in the fungal forest pathogen *Dothistroma septosporum*, *IMBS*. Massey University, Palmerston North, New Zealand.

Johnson LJ, Johnson RD, Akamatsu H, Salamiah A, Otani H, Kohmoto K, Kodama M, 2001. Spontaneous loss of a conditionally dispensable chromosome from the *Alternia alternata* apple pathotype leads to loss of toxin production and pathogenicity. *Current Genetics* 40, 65-72.

Jones WT, Harvey D, Jones SD, Sutherland PW, Nicol MJ, Sergejew N, Debnam PM, Cranshaw N, Reynolds PHS, 1995. Interaction between the phytotoxin dothistromin and *Pinus radiata* embryos. *Phytopathology* 85, 1099-1104.

Jones WT, Harvey D., Jones S.D., Fielder, S., Debnam, P., and Reynolds, P.H.S., 1993. Competitive ELISA employing monoclonal antibodies specific for dothistromin. *Food & Agricultural Immunology* 5, 187-197.

Kale SP, Bhatnagar D, Bennett JW, 1994. Isolation and characterization of morphological variants of *Aspergillus parasiticus* deficient in secondary metabolite production. *Mycological Research* 98, 645-652.

Kaufman G, Horwitz BA, Hadar R, Ullmann Y, Berdicevsky I, 2004. Green fluorescent protein (GFP) as a vital marker for pathogenic development of the dermatophyte *Trichophyton mentagrophytes*. *Microbiology* 150, 2785-2790.

Keller NP, Hohn TM, 1997. Metabolic pathway gene clusters in filamentous fungi. *Fungal Genetics and Biology* 21, 17-29.

Keller NP, Nesbitt C, Sarr B, Phillips TD, Burow GB, 1997. pH regulation of sterigmatocystin and aflatoxin biosynthesis in *Aspergillus* spp. *Phytopathology* 87, 643-648.

Keller NP, Turner G, Bennett JW, 2005. Fungal secondary metabolism - from biochemistry to genomics. *Nature Reviews Microbiology* 3, 937-947.

Kim CY, Im HW, Kim HK, Huh H, 2001. Accumulation of 2,5-dimethoxy-1,4-benzoquinone in suspension cultures of *Panax ginseng* by a fungal elicitor preparation and a yeast elicitor preparation. *Applied Microbiology and Biotechnology* 56, 239-242.

Kimura M, Anzai H, Yamaguchi I, 2001. Microbial toxins in plant-pathogen interactions: biosynthesis, resistance mechanisms, and significance. *Journal of General and Applied Microbiology* 47, 149-160.

Kirsitis T, Cech TL, 2006. Entwickelt sich die Dothistroma-Nadelbräune zu einem Forstschutzproblem in Östereich? *Forstschutz Aktuell* 36, 20-27.

Koltay A, 2001. Incidence of *Dothistroma septospora* (Dorog.) Morlet in the Austrian pine (*Pinus nigra* Arn.) stands in Hungary and results of chemical control trials. *Novenyvedelem* 37, 231-235.

Kowalski T, Jankowiak R, 1998. First record of *Dothistroma septospora* (Dorog.) Morelet in Poland: a contribution to the symptomology and epidemiology. *Phytopathologia Polonica* 16, 15-29.

Landmann G, 2000. Forest health in France: assessment for 1998 and new facts. *Revue Forestière Francaise* 52, 9-22.

Lang KJ, 1987. *Dothistroma pini* an jungen Fichten (*Picea abies*). *European Journal of Forest Pathology* 17, 316-317.

Langfelder K, Phillipe B, Jahn B, Latge J-P, Brakhage AA, 2001. Differential expression of the *Aspergillus fumigatus pksP* gene detected *in vitro* and *in vivo* with green fluorescent protein. *Infection and Immunity* 69, 6411-6418.

Lee L-W, Chiou C-H, Klomparens K, Cary J, Linz J, 2004. Subcellular localization of aflatoxin biosynthetic enzymes Nor-1, Ver-1, and OmtA in time-dependent fractionated colonies of *Aspergillus parasiticus*. *Archives of Microbiology* 181, 204-214.

Lee L-W, Chiou C-H, Linz JE, 2002. Function of native OmtA *in vivo* and expression and distribution of this protein in colonies of *Aspergillus parasiticus*. *Applied and Environmental Microbiology* 68, 5718-5727.

Liras P, Martin JF, 2006. Gene clusters for beta-lactam antibiotics and control of their expression: why have clusters evolved, and from where did they originate? *International Microbiology* 9, 9-19.

Liu BH, and Chu, F.S., 1998. Regulation of *aflR* and its product, AflR, associated with aflatoxin biosythesis. *Applied and Environmental Microbiology* 64, 3718-3723.

Lorang JM, Tuori RP, Martinez JP, Sawyer TL, Redman RS, Rollins JA, Wolpert TJ, Johnson KB, Rodriguez RJ, Dickman MB, Ciuffetti LM, 2001. Green fluorescent protein is lighting up fungal biology. *Applied and Environmental Microbiology* 67, 1987-1994.

Lu Z, Tombolini R, Woo S, Zeilinger S, Lorito M, Jansson JK, 2004. *In vivo* study of *Trichoderma*-pathogen-plant interactions, using constitutive and inducible green fluorescent protein reporter systems. *Applied and Environmental Microbiology* 70, 3073-3081.

Lubeck M, Knudsen IMB, Jensen B, Thrane U, Janvier C, Jensen DF, 2002. GUS and GFP transformation of the biocontrol strain *Clonostachys rosea* IK726 and the use of these marker genes in ecological studies. *Mycological Research* 106, 815-826.

MacLaren J, 1993. Radiata pine growers' manual. *FRI Bulletin* 184.

MAF, 2003. *New Zealand's contribution to Montreal process overview report*. Ministry of Agriculture and Forestry New Zealand.

MAF, 2006. Situation and outlook for New Zealand agriculture and forestry. *Ministry of Agriculture and Forestry, New Zealand* July 2006.

MAF, 2007. *http://www.maf.govt.nz/statistics/primaryindustries/forestry/trade/june-export-highlights/june-2005-exports/export-of-forestry-products-june-2005.htm*. . Ministry of Agriculture and Forestry, New Zealand.

Maggio-Hall LA, Wilson RA, Keller NP, 2005. Fundamental contribution of beta-oxidation to polyketide mycotoxin production *in plant*a. *Molecular Plant-Microbe Interactions* 18, 783-793.

Manteau S, Abouna S, Lambert B, Legendre L, 2003. Differential regulation by ambient pH of putative virulence factor secretion by the phytopathogenic fungus *Botrytis cinerea*. *FEMS Microbiology Ecology* 43, 359-366.

Maor R, Puyesky M, Horwitz BA, Sharon A, 1998. Use of green fluorescent protein (GFP) for studying development and fungal-plant interaction in *Cochliobolus heterostrophus*. *Mycological Research* 102, 491-496.

Marin S, Sanchis V, Ramos AJ, Vinas I, Magan N, 1998a. Environmental factors, in vitro interactions, and niche overlap between *Fusarium moniliforme*, *F. proliferatum*, and *F. graminearum*, *Aspergillus* and *Penicillium* species from maize grain. *Mycological Research* 102, 831-837.

Marin S, Sanchis V, Rull F, Ramos AJ, Magan N, 1998b. Colonization of maize grain by *Fusarium moniliforme* and *Fusarium proliferatum* in the presence of competing fungi and their impact on fumonisin production. *Journal of Food Protection* 61, 1489-1496.

Markham JE, Hille J, 2001. Host-selective toxins as agents of cell death in plant-fungus interactions. *Molecular Plant Pathology* 2, 229-239.

Maschning E, Pehl L, 1994. Threat to native *Pinus mugo* by *Dothistroma*. *AFZ, Allgemeine Forst Zeitschrift* 49, 249-252.

Mayer Z, Farber P, Geisen R, 2003. Monitoring the production of aflatoxin B1 in wheat by measuring the concentration of *nor-1* mRNA. *Applied and Environmental Microbiology* 69, 1154-1158.

McDonald T, Noordermeer D, Zhang Y-Q, Hammond TM, Keller NP, 2005. The ST cluster revisited: Lessons from a genetic model, in: Abbas HK (Ed), *Aflatoxin and Food Safety*. Taylor & Francis Group, LLC, Boca Raton, FL, pp. 117-136.

Mikkelsen L, Roulund N, Lubeck M, Jensen DF, 2001. The perennial ryegrass endophyte *Neotyphodium lolii* genetically transformed with the green fluorescent protein gene (*gfp*) and visualization in the host plant. *Mycological Research* 105, 644-650.

Mikkelsen L, Sarrocco S, Lubeck M, Jensen DF, 2003. Expression of the red fluorescent protein DsRed-Express in filamentous ascomycete fungi. *FEMS Microbiology Letters* 223, 135-139.

Miller MJ, Linz JE, 2006. Genetic mechanisms involved in regulation of mycotoxin biosynthesis, in: al.] KSe (Ed), *Food biotechnology*. CRC Press/Taylor & Francis, Boca Raton, FL.

Monahan BJ, 1998. Identification of putative dothistromin biosynthetic genes, *IMBS*. Massey University, Palmerston North, New Zealand.

Morelet M, 1968. De Aliquibus in Mycologia Novitatibus (3 note). *Bulletin de la Société des Sciences Naturelles et d'Archéologique de Toulon et du Var* 177, 9.

Mori H, Kitamura J, Sugie S, Kawai K, Hamaski T, 1985. Genotoxicity of fungal metabolites related to aflatoxin B1 biosynthesis. *Mutation Research* 143, 121-125.

Müller W, van der Krift T, Krouwer A, Wösten H, van der Voort L, Smaal E, Verkleij A, 1991. Localization of the pathway of the penicillin biosynthesis in *Penicillium chrysogenum*. *EMBO Journal* 10, 489-495.

Muir JA, Cobb FW, 2005. Infection of radiata and bishop pine by *Mycosphaerella pini* in California. *Canadian Journal of Forest Research* 35, 2529-2538.

Narasaiah KV, Sashidhar RB, Subramanyam C, 2006. Biochemical analysis of oxidative stress in the production of aflatoxin and its precursor intermediates. *Mycopathologia* 162, 179-189.

Neveu B, Labbe C, Belanger RR, 2007. GFP technology for the study of biocontrol agents in tritrophic interactions: A case study with *Pseudozyma flocculosa*. *Journal of Microbiological Methods* 68, 275-281.

O'Callaghan J, Stapleton PC, Dobson ADW, 2006. Ochratoxin A biosynthetic genes in *Aspergillus ochraceus* are differentially regulated by pH and nutritional stimuli. *Fungal Genetics and Biology* 43, 213-221.

Oren L, Ezrati S, Cohen D, Sharon A, 2003. Early interactions in the *Fusarium verticillioides*-maize interaction characterized by using a green fluorescent protein-expressing transgenic isolate. *Applied and Environmental Microbiology* 69, 1695-1701.

Parker AK, 1972. Artificial inoculation of *Pinus radiata* with *Scirrhia* (*Dothistroma*) *pini*: effect of relative humidity and temperature on incubation. *Phytopathology* 62, 1160-1164.

Payne GA, Brown MP, 1998. Genetics and physiology of aflatoxin biosynthesis. *Annual Review of Phytopathology* 36, 329-362.

Pehl L, Butin H, 1992. *Dothistroma septospora*- Ein neuer Schadpilz an der Bergkiefer *Dothistroma septospora*, a new fungus pest on *Pinus mugo*. *Allgemeine Forst Zeitschrift* 47, 758-760.

Pelaez F, 2005. Biological activities of fungal metabolites, in: An Z (Ed), *Handbook of Industruial Mycology*. Marcel Dekker, New York, pp. 49-92.

Penalva MA, Arst HN, Jr., 2002. Regulation of gene expression by ambient pH in filamentous fungi and yeasts. *Microbiology and Molecular Biology Reviews* 66, 426-446.

Penalva MA, Arst J, Herbert N., 2004. Recent advances in the characterization of ambient pH regulation of gene expression in filamentous fungi and yeast. *Annual Review of Microbiology* 58, 425-451.

Peterson GW, 1973. Infection of Austrian and Ponderosa pines by *Dothistroma pini* in Eastern Nebraska. *Phytopathology* 63, 1060-1063.

Peterson GW, 1982. Dothistroma needle blight of pines. Forest Insect and Disease Leaflet 143. *[Broomall, PA:] U.S. Dept. of Agriculture, Forest Service, Northern Area State and Private Forestry.*

Peterson GW, Walla JA, 1978. Development of *Dothistroma pini* upon and within needles of Austrian and Ponderosa pines in eastern Nebraska. *Phytopathology* 68, 1422-1430.

Pitt JI, Hocking AD, 2006. Mycotoxins in Australia: biocontrol of aflatoxin in peanuts. *Mycopathologia* 162, 233-243.

Pöggeler S, Masloff S, Hoff B, Mayrhofer S, Kück U, 2003. Versatile EGFP reporter plasmids for cellular localization of recombinant gene products in filamentous fungi. *Current Genetics* 43, 54-61.

Price MS, Conners SB, Tachdjian S, Kelly RM, Payne GA, 2005. Aflatoxin conducive and non-conducive growth conditions reveal new gene associations with aflatoxin production. *Fungal Genetics and Biology* 42, 506-518.

Price MS, Yu J, Nierman WC, Kim HS, Pritchard B, Jacobus CA, Bhatnagar D, Cleveland TE, Payne GA, 2006. The aflatoxin pathway regulator AflR induces gene transcription inside and outside of the aflatoxin biosynthetic cluster. *FEMS Microbiology Letters* 255, 275-279.

Proctor RH, Brown DW, Plattner RD, Desjardins AE, 2003. Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. *Fungal Genetics and Biology* 38, 237-249.

Punt PJ, van den Hondel CAMJJ, 1992. Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers. *Methods in Enzymology* 216, 447-457.

Ramakrishna N, Lacey J, Smith JE, 1996a. *Aspergillus flavus* colonization and aflatoxin B1 formation in barley grain during interaction with other fungi. *Mycopathologia* 136, 53-63.

Ramakrishna N, Lacey J, Smith JE, 1996b. Colonization of barley grain by *Penicillium verrucosum* and ochratoxin A formation in the presence of competing fungi. *Journal of Food Protection* 59, 1311-1317.

Ramakrishna N, Lacey J, Smith JE, 1996c. The effects of fungal competition on colonization of barley grain by *Fusarium sporotrichioides* on T-2 toxin formation. *Food Additives and Contaminants* 13, 939-948.

Read N, Kalkman E, 2003. Does endocytosis occur in fungal hyphae? *Fungal Genetics and Biology* 39, 199-203.

Rohel EA, Payne AC, Fraaije BA, Hollomon DW, 2001. Exploring infection of wheat and carbohydrate metabolism in *Mycosphaerella graminicola* transformants with differentially regulated green fluorescent protein expression. *Molecular Plant Microbe Interactions* 14, 156-163.

Rosewich UL, Kistler HC, 2000. Role of horizontal gene transfer in the evolution of fungi. *Annual Review of Phytopathology* 38, 325-363.

Roux C, 1984. The morphology of *Dothistroma septospora* on *Pinus canariensis* from South Africa. *South African Journal of Botany* 3, 397-401.

Sagner G, Goldstein C, 2001. Principles, workflows and advantages of the new LightCycler relative Quantification Software. *ROCHE MOLECULAR BIOCHEMICALS Biochemica* 3, 15-17.

Sakuno E, Wen Y, Hatabayashi H, Arai H, Aoki C, Yabe K, Nakajima H, 2005. *Aspergillus parasiticus* cyclase catalyzes two dehydration steps in aflatoxin biosynthesis. *Applied and Environmental Microbiology* 71, 2999-3006.

Sambrook J, Fritsch EF, Maniatis T, 1989. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schiestl F, Steinebrunner F, Schulz C, von ReuÃŸ S, Francke W, Weymuth C, Leuchtmann A, 2006. Evolution of ˜pollinator"- attracting signals in fungi. *Biology Letters* 2, 401-404.

Schwelm A, Barron NJ, Zhang S, Bradshaw RE, 2007. Early expression of aflatoxin-like dothistromin genes in the forest pathogen *Dothistroma septosporum*. *Mycological Research* in press; doi:10.1016/j.mycres.2007.03.018.

Seconi JM, 2001. Confirmation of the presence of a dothistromin biosynthesis gene cluster in the fungal forest pathogen *Dothistroma pini*, *IMBS*. Massey University, Palmerston North, New Zealand, p. 143.

Seo J-A, Yu J-H, 2005. Toxigenic fungi and mycotoxins, in: An Z (Ed), *Handbook of industrial mycology*. Marcel Dekker, New York, pp. 689-721.

Sexton AC, Howlett BJ, 2001. Green fluorescent protein as a reporter in the *Brassica-Leptosphaeria maculans* interaction. *Physiological and Molecular Plant Pathology* 58, 13-21.

Shain L, Franich RA, 1981. Induction of *Dothistroma* blight symptoms with dothistromin. *Physiological Plant Pathology* 19, 49-55.

Shaw GJ, 1975. The biosynthesis of dothistromin. Massey University, Palmerston North, New Zealand.

Shaw GJ, Chick M, Hodges R, 1978. A ^{13}C NMR study of the biosynthesis of the anthraquinone dothistromin by *Dothistroma pini*. *Phytochemistry*. 17, 1743-1745.

Sieber TN, Rys J, Holdenrieder O, 1999. Mycobiota in symptomless needles of *P. mugo* ssp. *uncinata*. *Mycological Research* 103, 306-310.

Silar P, 1995. Two new easy to use vectors for transformations. *Fungal Genetics Newsletter* 42.

Simpson DR, Thomsett MA, Nicholson P, 2004. Competitive interactions between *Microdochium nivale* var. *majus*, *M. nivale* var. *nivale* and *Fusarium culmorum in planta* and *in vitro*. *Environmental Microbiology* 6, 79-87.

Skadsen RW, Hohn TA, 2004. Use of *Fusarium graminearum* transformed with *gfp* to follow infection patterns in barley and Arabidopsis. *Physiological and Molecular Plant Pathology* 64, 45-53.

Skinnider L, Stoessl A, Wang J, 1989. Increased frequency of sister-chromatid exchange induced by dothistromin in CHO cells and human lymphocytes. *Mutation Research* 222, 167-170.

Skory CD, Chang PK, Linz JE, 1993. Regulated expression of the *nor-1* and *ver-1* genes associated with aflatoxin biosynthesis. *Applied and Environmental Microbiology* 59, 1642-1646.

Southern EM, 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* 98, 503-517.

Stinnett SM, Espeso EA, Cobeno L, Araujo-Bazan L, Calvo AM, 2007. *Aspergillus nidulans* VeA subcellular localization is dependent on the importin alpha carrier and on light. *Molecular Microbiology* 63, 242-255.

Stoessl A, Abramowski Z, Lester HH, Rock GL, Towers GHN, 1990. Further toxic properties of the fungal metabolite dothistromin. *Mycopathologia* 112, 179-186.

Stoessl A, Stothers JB, 1985. Minor anthraquinoid metabolites of *Cercospora arachidicola*. *Canadian Journal of Chemistry* 63.

Sutton BC, 1980. The Coelomycetes: fungi imperfecti with pycnidia, acervuli and stromata. *Kew,Surrey, UK: Commonwealth Mycological Instititute*.

Takai S, 1974. Pathogenicity and cerato-ulmin production in *Ceratocystis ulmi. Nature.* 252, 124-126.

Tanaka A, Christensen MJ, Takemoto D, Park P, Scott B, 2006. Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic interaction. *Plant Cell* 18, 1052-1066.

Tanaka A, Shiotani H, Yamamoto M, Tsuge T, 1999. Insertional mutagenesis and cloning of the genes required for biosynthesis of the host-specific AK-toxin in the Japanese pear pathotype of *Alternaria alternata*. *Molecular Plant-Microbe Interactions* 12, 691-702.

Taylor J, Walla J, 1999. First report of *Dothistroma septospora* on native limber and whitebark pine in Montana. *Plant Diseases* 83, 590.

Taylor JE, Schwandt JW, 1998. Dothistroma needle blight of limber pine in Montana. *United States Department of Agriculture, Forest Service, Northern Region* 1998 (4).

Teddy OR, 2004. Further characterisation of the dothistromin gene cluster of *Dothistroma pini*, *IMBS*. Massey University, Palmerston North, New Zealand, p. 180.

Thines E, Aguirre J, Foster AJ, Deising HB, 2006. Genetics of phytopathology: Secondary metabolites as virulence determinants of fungal plant pathogens, *Progress in Botany*. Springer-Verlag, Berlin Heidelberg, pp. 134-161.

Tillett D, Neilan B, 1999. n-butanol purification of dye terminator sequencing reactions. *Biotechniques* 26, 606.

Trail F, Mahanti N, Linz J, 1995. Molecular biology of aflatoxin biosynthesis. *Microbiology-Uk* 141, 755-765.

Tsien RY, 1998. The green fluorescent protein. *Annual Review of Biochemistry* 67, 509-544.

Tsitsigiannis DI, Keller NP, 2006. Oxylipins act as determinants of natural product biosynthesis and seed colonization in *Aspergillus nidulans*. *Molecular Microbiology* 59, 882-892.

Tsitsigiannis DI, Keller NP, 2007. Oxylipins as developmental and host-fungal communication signals. *Trends in Microbiology* 15, 109-118.

Tudzynski B, Hölter K, 1998. Gibberellin biosynthetic pathway in *Gibberella fujikuroi*: evidence for a gene cluster. *Fungal Genetics and Biology* 25, 157-170.

Upchurch RG, Walker DC, Rollins JA, Ehrenshaft M, Daub ME, 1991. Mutants of *Cercospora kikuchii* altered in cercosporin synthesis and pathogenicity. *Applied and Environmental Microbiology.* 57, 2940-2945.

Valdez-Taubas J, Harispe L, Scazzocchio C, Gorfinkiel L, Rosa AL, 2004. Ammonium-induced internalisation of UapC, the general purine permease from *Aspergillus nidulans*. *Fungal Genetics and Biology* 41, 42-51.

Velluti A, Marin S, Bettucci L, Ramos AJ, Sanchis V, 2000. The effect of fungal competition on colonization of maize grain by *Fusarium moniliforme*, *F. proliferatum* and *F. graminearum* and on fumonisin B1 and zearalenone formation. *International Journal of Food Microbiology* 59, 59-66.

Vinck A, Terlou M, Pestman WR, Martens EP, Ram AF, van den Hondel CAMJJ, Wösten HAB, 2005. Hyphal differentiation in the exploring mycelium of *Aspergillus niger*. *Molecular Microbiology* 58, 693-699.

Vining LC, 1990. Functions of secondary metabolites. *Annual Review of Microbiology* 44, 395-427.

Vujanovic V, St-Arnaud M, Neumann P, 1997. Seven new *Pinus* hosts for *Strasseria geniculata* (Berk. & Br.) Höhn, in: Laflamme G, Berube JA, Hamelin RC (Eds), *Proceedings of the IUFRO WP*. Laurentian Forest Centre, Quebec, Canada, pp. 48-53.

Walton JD, 2000. Horizontal gene transfer and the evolution of gene clusters in fungi: an hypothesis. *Fungal Genetics and Biology* 30, 167-171.

Wang CS, Butt TM, St Leger RJ, 2005. Colony sectorization of *Metarhizium anisopliae* is a sign of ageing. *Microbiology-Sgm* 151, 3223-3236.

Wardle DA, Parkinson D, 1992. The influence of the herbicide glyphosate on interspecific interactions between four soil fungal species. *Mycological Research* 96, 180-186.

Weiergang I, Dunkle LD, Wood KV, Nicholson RL, 1996. Morphogenic regulation of pathotoxin synthesis in *Cochliobolus carbonum*. *Fungal Genetics and Biology* 20, 74-78.

Weiergang I, Wood KV, Dunkle LD, Nicholson RL, 2004. In vivo growth and pathotoxin production by the maize pathogen *Cochliobolus carbonum*. *Physiological and Molecular Plant Pathology* 64, 273-279.

Wen Y, Hatabayashi H, Arai H, Kitamoto HK, Yabe K, 2005. Function of the *cypX* and *moxY* genes in aflatoxin biosynthesis in *Aspergillus parasiticus*. *Applied and Environmental Microbiology* 71, 3192-3198.

West PJ, 2004. Development of a pathogenicity testing system for the pine pathogen *Dothistroma pini*, *IMBS*. Massey University, Palmerston North, New Zealand.

Wheatley RE, 2002. The consequences of volatile organic compound mediated bacterial and fungal interactions. *Antonie van Leeuwenhoek* 81, 357-364.

White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in: Innis MA, Gelfand DH, Sninsky JJ, White TJ (Eds), *PCR protocols*. Academic Press, San Diego, pp. 315-322.

Wicklow DT, Hesseltine CW, Shotwell OL, Adams GL, 1980. Interference competition and aflatoxin levels in corn. *Phytopathology* 70, 761-764.

Wilkinson HH, Ramaswamy A, Sim SC, Keller NP, 2004. Increased conidiation associated with progression along the sterigmatocystin biosynthetic pathway. *Mycologia* 96, 1190-1198.

Wingfield MJ, Slippers B, Roux J, Wingfield BD, 2001. Worldwide movement of exotic forest fungi, especially in the tropics and the southern hemisphere. *BioScience* 51, 134-140.

Woods AJ, Coates DK, Hamann A, 2005. Is an unprecedented *Dothistroma* needle blight epidemic related to climate change? *BioScience* 55, 761-769.

Yabe K, Nakajima H, 2004. Enzyme reactions and genes in aflatoxin biosynthesis. *Applied Microbiology & Biotechnology* 64, 745-755.

Yang TT, Cheng L, Kain SR, 1996. Optimized codon usage and chromophore mutations provide enhanced sensitivity with the green fluorescence protein. *Nucleic Acids Research* 24, 4592-4593.

Young CA, Bryant MK, Christensen MJ, Tapper BA, Bryan GT, Scott B, 2005. Molecular cloning and genetic analysis of a symbiosis-expressed gene cluster for lolitrem biosynthesis from a mutualistic endophyte of perennial ryegrass. *Molecular Genetics and Genomics* 274, 13-29.

Young CA, Felitti S, Shields K, Spangenberg G, Johnson RD, Bryan GT, Saikia S, Scott B, 2006. A complex gene cluster for indole-diterpene biosynthesis in the grass endophyte *Neotyphodium lolii*. *Fungal Genetics and Biology* 43, 679-693.

Youngman RJ, Elstner EF, 1984. Photodynamic and reductive mechanism of oxygen activation by the fungal phytotoxins, cercosporin and dothistromin. Walter de Gruyter & Co., Berlin New York, pp. 501-508.

Yu J-H, Adams TH, 1998. Coordinate control of secondary metabolite production and asexual sporulation in *Aspergillus nidulans*. *Current Opinion in Microbiology* 1.

Yu J-H, Keller N, 2005. Regulation of secondary metabolism in filamentous fungi. *Annual Review of Phytopathology* 43, 437-458.

Yu J, Bhatnagar D, Cleveland TE, 2004. Completed sequence of aflatoxin pathway gene cluster in *Aspergillus parasiticus*. *FEBS Letters* 564, 126-130.

Yu J, Chang PK, Cary JW, Wright M, Bhatnagar D, Cleveland TE, Payne GA, Linz JE, 1995. Comparative mapping of aflatoxin pathway gene clusters in *Aspergillus parasiticus* and *Aspergillus flavus*. *Applied and Environmental Microbiology* 61, 2365-2371.

Zhang S, Monahan BJ, Tkacz JS, Scott B, 2004. Indol-diterpene gene cluster from *Aspergillus flavus*. *Applied and Environmental Microbiology* 70, 6875-6883.

Zhang S, Schwelm A, Jin H, Collins LJ, Bradshaw RE, 2007. A fragmented aflatoxin-like gene cluster in the forest pathogen *Dothistroma septosporum*. *Fungal Genetics and Biology* 44, 1342-1354.

Zhang Y, Keller N, Tsitsigiannis D, Wilkinson HH, 2005. Secondary metabolite gene clusters, in: An Z (Ed), *Handbook of Industrial Mycology*. Marcel Dekker, New York, pp. 355-385.

Zubrik M, Kunca A, Turcani M, Vakula J, Leontovyc R, 2006. Invasive and quarantine pests in forests in Slovakia 1. *EPPO Bulletin* 36, 402-408.