

Dothistroma (red-band) needle blight of pines and the dothistromin toxin: a review

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Summary

Dothistroma (red-band) needle blight has been a problem in plantations of exotic pines in the southern hemisphere for many decades. The prevalence of this disease is currently increasing in the northern hemisphere and is now affecting trees in their native ranges. The fungal pathogen *Mycosphaerella pini* with its anamorph *Dothistroma pini*, which is responsible for the disease, produces a toxin, dothistromin, that is closely related to the potent carcinogen, aflatoxin. Understandably this has provoked concern about possible effects on the health of forestry workers. This review gives a broad coverage of literature on both disease and toxin. The fungus has a complicated taxonomy with many synonyms and in most countries only the anamorph is found. It is a necrotrophic pathogen that kills needle tissue and completes its life cycle in the lesion thus formed. Dispersal of the disease is normally by rain splash of conidiospores but there is evidence that long range dispersal has occurred by transport of contaminated plant tissue and by wind/cloud dispersal of spores in air currents. The severity of disease is affected by humidity, temperature and light. There is variation in susceptibility of different *Pinus* species and some achieve increased resistance with age. The current method of control in southern hemisphere plantation forests is through spraying with copper fungicides and, with *P. radiata*, increased disease resistance has been achieved through a breeding programme. The dothistromin toxin is a difuroanthraquinone and is similar in structure to the aflatoxin precursor versicolorin B. Part of a gene cluster encoding dothistromin biosynthetic genes has been cloned and this has confirmed parallels between the dothistromin and aflatoxin biosynthetic pathways. Dothistromin produces damaging oxygen radicals by reductive oxygen activation rather than by photosensitization, but is also thought to exert its toxic effects on specific cellular targets. Studies have shown that dothistromin is a weak mutagen and clastogen and is therefore a potential carcinogen. Although the risks to forest workers are considered very low it is prudent to avoid unnecessary exposure during periods when dothistromin levels are likely to be at their peak.

1 Introduction

Until recently, Dothistroma needle blight was of far more economic concern to foresters in the southern hemisphere than in the north. The disease sped from obscurity to notoriety during the 1960s when commercial pine plantations, predominantly monocultures of susceptible pines planted out of their natural host range, suffered severe economic losses. Plantations in New Zealand, Australia, Chile and Kenya were particularly badly affected (IVORY 1967; GIBSON 1972). In the northern hemisphere a few serious outbreaks of needle blight have been recorded in the USA since the 1960s (COBB and MILLER 1968), and sporadic outbreaks reported in Europe (GIBSON 1974). During the past decade, however, prevalence of the disease has increased in the northern hemisphere and, in a worrying trend, is affecting trees in their native ranges in addition to those planted as exotics. One of the worst affected areas at present is northwest British Columbia, Canada, where a 2002 survey found disease symptoms in 93% of 700 stands of young (<20 years old) lodgepole pines (*Pinus contorta* var. *latifolia*) covering 21 000 ha. Mortality was seen in 6.2% of the

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Table 1. Some new outbreaks/increased incidence of *Dothistroma* needle blight recorded in the northern hemisphere since 1992

Country/ region	<i>Pinus</i> species	Distribution notes	Reference
Poland	<i>P. nigra</i>	First record in Poland	KOWALSKI and JANKOWIAK (1998)
Germany	<i>P. mugo</i>	Outbreak in native ranges at altitudes between 1200 and 1600 m	PEHL and BUTIN (1992), MASCHNING and PEHL (1994)
Portugal	<i>P. pinaster</i> <i>P. pinea</i> <i>P. radiata</i>	First record of teleomorph	FONSECA (1998)
Hungary	<i>P. nigra</i>	Epidemics throughout the country	KOLTAY (2001)
France	<i>P. nigra</i> var. <i>laricio</i>	Considerable foliage damage	LANDMANN (2000)
England	<i>P. nigra</i> var. <i>laricio</i>	Greatly increased incidence	EVANS and WEBBER (2002)
Canada	<i>P. contorta</i>	High incidence and mortality in north-west British Columbia	WESTFALL (2002), WOODS (2003)
Montana, USA	<i>P. flexilis</i> <i>P. albicaulis</i>	First report on limber and whitebark pine in native ranges	TAYLOR and WALLA (1999)
Vermont, USA	<i>P. nigra</i> <i>P. mugo</i> <i>P. ponderosa</i>	First report in New England	PFISTER et al. (2000)

surveyed area (WOODS 2003). The same author reported observations of an additional stand of 55-year-old lodgepole pine with 20% mortality and 60% 'close to death' with <5% of foliage remaining. Since 1992 there have been many other reports of an increased incidence of blight in Europe and the USA: some of these are listed in Table 1. The Forestry Compendium (CABI 2003) lists countries in which *Dothistroma* needle blight has been recorded.

In addition to the increasing prevalence of *Dothistroma* needle blight in the northern hemisphere, another reason for heightened interest in the disease is its associated toxin dothistromin. The similarity of dothistromin to the potent carcinogen aflatoxin has been known since the 1970s but there has been a recent surge of interest in mycotoxins and in the gene clusters that encode them. Toxins are often a key weapon in the armoury of a plant pathogen and once the mode of action is understood, opportunities for disease control may appear. More information may also make it possible to obviate any adverse effects of the toxin on human health.

The first part of this review focuses on taxonomic, pathological and epidemiological aspects of *Dothistroma* needle blight and how it is currently controlled. The second part deals with the dothistromin toxin, starting with an overview of the biochemistry and genetics of toxin biosynthesis and finishing with an assessment of its mode of action and the potential risk to human health. Finally I will speculate on future possibilities for disease control.

2 The pathogen and the disease

2.1 Taxonomy and distribution of the needle blight fungus

The causal agent of *Dothistroma* needle blight is an Ascomycete that, like many fungi, has a tortuous taxonomic history littered with synonyms of both teleomorphic and

anamorphic names (SUTTON 1980; EVANS 1984; ROUX 1984). 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. Both *D. septospora* and *D. pini* are in current use. The teleomorphic form was first described as *Scirrhia pini* Funk and Parker but renamed *Mycosphaerella pini* E. Rostrup apud Monk, although both names are still used. There was an attempt to reclassify the teleomorph into a new genus *Eruptio* on the basis of the diversity in the genus *Mycosphaerella* (BARR 1996), but phylogenetic analysis suggested that *Mycosphaerella* is a more appropriate classification (GOODWIN et al. 2001).

The anamorphic state has been divided into three varieties on the basis of differences in length of conidiospores. THYR and SHAW (1964) distinguished between *D. pini* Hulbary var. *pini* (syn. *D. septospora* var. *septospora*) with conidial lengths of 15.4–28.0 (mean 22.4) μm and *D. pini* Hulbary var. *linearis* (syn. *D. septospora* var. *lineare*) with conidial lengths of 23.0–42.0 (31.9) μm . Another variety *D. pini* Hulbary var. *keniensis* (syn. *D. septospora* var. *keniense*), with intermediate conidial lengths of 13.0–47.5 (28.9) μm , was subsequently proposed (IVORY 1967). EVANS (1984) is of the opinion that *D. pini* is native to the cloud forest regions of Central America, occurring on isolated mountain 'islands' above 1500 m. However, IVORY (1994) maintains that it is also endemic to the Himalayas and suggests that the short spore form *D. pini* var. *pini* (found predominantly in Asia, Australasia and South America) may have originated there whilst the intermediate spored type *D. pini* var. *keniensis* (found predominantly in Africa) may have originated from Central America. The long-spored type *D. pini* var. *linearis* (found in North America and France) may have another origin. However, several leading authorities have questioned whether division into varieties is warranted on the basis of a single characteristic (FUNK and PARKER 1966; GADGIL 1967; SUTTON 1980; EVANS 1984) and classification of isolates at the varietal level has proved difficult (EDWARDS and WALKER 1978; ROUX 1984; BRADSHAW et al. 2000). Furthermore, DNA analysis of a small international collection of isolates did not support distinction between varieties in different geographical locations. Isolates from New Zealand, Europe, South America and parts of North America (Oregon and Canada) shared identical ribosomal spacer (ITS) sequences, whilst other isolates from North America (Minnesota and Nebraska) had a different sequence (BRADSHAW et al. 2000). However, more comprehensive sequence analysis is required with a larger group of isolates, including some from African countries, before firm conclusions can be drawn on the basis of DNA evidence.

The sexual form, *M. pini* (syn. *S. pini*), is less prevalent than the asexual form. Officially the perfect stage of *S. pini* is connected to *D. pini* var. *linearis* and no perfect state has yet been found for *D. pini* var. *pini* or *D. pini* var. *keniensis* (IVORY 1967). There are reports of the teleomorph in Canada (FUNK and PARKER 1966), parts of the USA (COBB and MILLER 1968; PETERSON and HARVEY 1976; PETERSON 1982), Germany (BUTIN and RICHTER 1983), Yugoslavia (KARADZIC 1989), Poland (KOWALSKI and JANKOWIAK 1998) and Portugal (FONSECA 1998). However, there have been no confirmed reports of the teleomorph in countries where *D. pini* has long been a major needle pathogen (Chile, East Africa, Australia, New Zealand) (EVANS 1984; MARKS et al. 1989). The rarity of the sexual state in many parts of the world suggests that the fungus is heterothallic (FUNK 1979), but no investigations of the existence of mating types have been published to date.

2.2 Symptoms and epidemiology of Dothistroma needle blight

The disease is characterized by distinct brick-red bands (1–3 mm wide) around the needles that can appear within weeks of infection and can sometimes still be seen after the needles have died, earning it the alternative name of red-band disease. The red colour is due to production of the mycotoxin, dothistromin, by the fungus (SHAIN and FRANICH 1981).

Black fruiting bodies (stromata) can be seen in the red band in later stages of the disease. 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 (EDWARDS and WALKER 1978; KERSHAW et al. 1988). The disease affects over 60 *Pinus* species in 45 countries (IVORY 1994) but is also reported to affect some *Picea* species (LANG 1987; KARADZIC 1994), as well as *Larix decidua* (BASSETT 1969) and *Pseudotsuga menziesii* (DUBIN and WALPER 1967; KARADZIC 1994). The economic losses as a result of the disease are difficult to measure but early attempts to quantify yield loss are summarized by GIBSON (1972). The most serious impacts are due to retardation of growth due to defoliation of needles, rather than to mortality (which is rare). Wood yield loss is approximately proportional to disease severity, particularly when young photosynthetically active needles (rather than older needles) are affected. An additional study of the effect of the disease on *P. radiata* in New Zealand was made by VAN DER PAS (1981). Disease assessments (percentage of needles showing disease) were made at 3 monthly intervals from age 1–7 years. The mean disease level over that period was compared with the final wood volume. Trees with an average of 20% diseased needles had c. 20% less volume than uninfected trees, whilst those with 40% disease had c. 40% less volume, etc. Severe levels of disease can result in tree death (GIBSON 1974; WOODS 2003).

In efforts to control the disease, scientists have sought to understand the infection process, life cycle and mode of dispersal. Asexual conidiospores from the black fruiting bodies that form in the brick-red bands on a diseased needle are the main source of inoculum. The first conidiospores are generally released in spring from attached needles infected in the previous year. Spore production can continue from infected stands for up to 7 months of the year (KARADZIC 1989) but most infection takes place from late-spring to late-summer (GILMOUR 1981) and is dependent on sufficient temperature and leaf wetness. The spores are passively transported in water droplets on to fresh needles where they germinate and infect current year or 1-year-old needles. There are conflicting reports about whether the direction of germ tube growth is random or targeted towards stomata. But there is a clear tendency for random growth when artificial inoculations are made in the laboratory and in some cases penetration is made directly through the cuticle (GADGIL 1967; PETERSON and WALLA 1978). At the stomatal pore (the usual site of entry) an appressorium-like structure is formed and an infection peg grows into the plant. From this, hyphae branch into intra- and intercellular regions of the mesophyll. Hyphal growth is restricted to 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 defence response. The host cells collapse after 32–114 days (depending on environmental conditions) and needle symptoms and stromata appear (IVORY 1972b; PETERSON 1973). The stromata generally mature and produce conidia the year after infection (PETERSON 1982; BUTIN 1985; KARADZIC 1989), but in some regions of the USA it can take an additional year for the fungus to complete its life cycle (PETERSON 1982; TAYLOR and 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. Conidia are exuded in a mucilaginous mass, are hyaline, generally straight and one to three septate. Further morphological details can be found in EDWARDS and WALKER (1978) and EVANS (1984).

The role of the sexual form in dissemination of the disease is not known. Where present, ascospores are produced in erumpent black stromata, up to 850 μm wide, on necrotic regions of diseased needles. The ascospores are elliptic, hyaline, one septate, generally

8–16 × 3–4 µm and contained as groups of eight in bitunicate asci. Working in Serbia, KARADZIC (1989) proposed they are less important than conidiospores as they are less abundant and produced over a shorter time period (conidia from the beginning of April until the end of October; ascospores from the second half of June until the end of September). Conidiospores and ascospores were sometimes produced simultaneously from the same disease lesion. Other authors suggested that the anamorph is the parasitic stage whilst the teleomorph is a saprophytic stage, with the latter appearing only on older (2- and 3-year old) attached needles in a study of *P. nigra* (BUTIN and RICHTER 1983; BUTIN 1985). Considered alongside the relatively limited global distribution of the sexual stage compared with the anamorphic form, these studies suggest that conidiospores, rather than ascospores, are the inoculum of primary epidemiological importance.

Dothistroma pini has shown a rapid rate of dispersal. Only 7 years after the first discovery of a severe outbreak in Tanzania in 1957, most young *P. radiata* forests in East and Central Africa had been infected (GIBSON 1972). However, the infective conidiospores are only dispersed for short distances (<150 cm) by rain-splash (PETERSON 1973) and spores remaining in stromata on needles lose viability within 4–6 months of the needles falling on the forest floor (GADGIL 1970). In view of this there are two proposed mechanisms to account for the rapid spread of *D. pini* around the world – movement of infected material and cloud/wind spore transport (GIBSON 1974). Probably the most important route, and one that has biosecurity implications, is the transport of infected plant material. There are many reports of outbreaks in nurseries that suggest an origin via contaminated planting material (EVANS and OLEAS 1983; DICK and VANNER 1986; IVORY 1990). The first country in Australasia to find the disease was New Zealand, where it was reported in a North Island plantation in 1964. It seems most likely that infected plant material was responsible as the geographic isolation of the country would seem to preclude infection via wind-blown spores. The discovery that New Zealand isolates, including those obtained both in the 1960s and the 1990s, have very low genetic diversity suggests a single introduction of the pathogen (HIRST et al. 1999). In contrast, the introduction of *D. pini* into south-eastern Australia in the 1970s is thought to have occurred by natural means with conidia being blown across the Tasman Sea in moist airstreams from New Zealand. Strict quarantine regulations in Australia meant that introduction of infected plant material was unlikely (EDWARDS and WALKER 1978; MARKS et al. 1989; MIREKU and SIMPSON 2002).

2.3 Further comments on disease identification

There are several difficulties in identifying *Dothistroma* needle blight. Occasionally the characteristic red-band is not seen (PEHL and BUTIN 1992; IVORY 1994) and infected needles may show various degrees of damage, from clear red-bands around the needle to complete discoloration and death of the needle (EDWARDS and WALKER 1978). Furthermore, the symptoms can vary between host species. Thus, early symptoms on the needles of Austrian pine (*P. nigra*) include deep green bands and yellow/tan spots that later turn brown/red-brown (PETERSON 1982). The similarity of these early symptoms to those of brown spot (*Lecanosticta*) needle blight (see next paragraph) means that more detailed analysis (such as microscopic examination of spores) is required to distinguish between these species on this host. Many other factors can cause needle damage and make diagnosis more difficult. For example, boron and sulphur deficiencies cause needle browning. However, in these cases there is usually uniform damage across entire stands, whole needles or tips of needles, whilst symptoms caused by *D. pini* are more sporadic and may involve only part of a tree or several trees within an otherwise healthy stand. Generally, it is the needle of lower branches that are the first to be infected and the disease gradually moves up the crown, although in some cases the infection starts in inner parts of lower branches, moves up the inner crown then subsequently outwards along the branches (MARKS et al. 1989). The

development of fruiting bodies on the needles is of course a sure way to determine that fungal rather than environmental factors are to blame for the symptoms (EDWARDS and WALKER 1978; HUNT 1995).

Dothistroma pini can be isolated from fruiting bodies and grown in culture where secretion of the red-brown dothistromin toxin provides a useful additional diagnostic aid. However, the fungus is very slow growing (radial growth <1 mm/day at 23°C) and morphological variations are a common occurrence (KARADZIC 1994; HIRST et al. 1999; BRADSHAW et al. 2000). Isolates differ in their ability to sporulate (BRADSHAW et al. 2000) but conidiospores produced in culture are able to germinate and infect plants (GADGIL 1977).

The two fungal diseases most often confused with *Dothistroma* needle blight are brown spot (*Lecanosticta*) needle blight and brown needle disease (*Cercospora* needle blight). Brown spot is caused by *L. acicola* (Thumen) Sydow [teleomorph *S. acicola* (Dearness) Siggers syn. *M. dearnessii* Barr], and occurs in Asia, Europe, Northern and Central America. The pattern of infection is similar and the fruiting bodies similar in shape and size to those of *D. pini*. However, *L. acicola* does not produce dothistromin (hence no red-brown banding of needles), it causes necrosis in spots rather than in bands, and has distinctly greenish-brown rather than hyaline conidiospores. Differences between brown-spot and red-band diseases are summarized (in German) and superbly illustrated by PEHL and WULF (2001). Brown needle disease is caused by *Cercoseptoria pini-densiflorae* (Hori & Nambu) Deighton (syn. *Cercospora pini-densiflorae* Hori & Nambu) (teleomorph *M. gibsonii* Evans) and occurs mainly in Africa and Asia. Infected needles develop light green bands that spread along the needle and turn yellow then grey/brown. Conidiomata are grey to black, with fasciculate conidiophores. Conidia are typically curved rather than straight as in *D. pini* and often have a stiffened or rigid appearance due to melanin granules in the spore walls. Ascstromata are rarely grouped but spread along the needle (EVANS 1984; CROUS et al. 1990). Useful identification keys for *D. pini* are provided by HUNT (1995) and CROUS et al. (1990).

2.4 Factors affecting disease severity

The level of disease is very dependent on environmental conditions, such as humidity and temperature. Spore trap experiments showed that wet weather is required for the release of large numbers of spores (PETERSON 1973). Wet spores will then germinate and penetrate a needle even in dry conditions, but symptom development and the formation of stromata require high humidity (GADGIL 1977). In the northern hemisphere the amount of rainfall in June–September is a good indicator of the severity of disease (PETERSON 1973) and in general a long dry period after infection leads to less severe disease and slower development of stromata than during wet weather (GADGIL 1977), i.e. dry weather can inhibit the progression of infection to disease expression. Temperature is less important than humidity, as infection and development of symptoms can occur between 5 and 26°C, although infection at lower temperature is dependent on an extended period of high humidity (GILMOUR and CROCKETT 1972). In a 3-year controlled field study in New Zealand involving a 2 week exposure of individual seedlings to infection in the field, no disease symptoms developed in trees exposed to infection below 7°C or when the leaf wetness period was <10 h (GILMOUR 1981), although the threshold values for these parameters varied from year to year.

Light intensity has a strong influence on the severity of disease. Although the germination of conidia and early growth is unaffected, the development of symptoms is drastically reduced with a low light intensity (c. 58 W/m²) (GADGIL and HOLDEN 1976). Indeed if foliage is shaded during the period 5–20 days after inoculation then no symptoms are seen (IVORY 1972a), although blight symptoms can develop soon after removal of the

shade treatment. It has been suggested that the reduced disease seen in low light intensities is due to the nature of the host response, rather than that of the fungus or its toxin (GADGIL and HOLDEN 1976). There is no direct evidence to support this suggestion but there is some tentative evidence for the role of photosynthetically active tissue in augmenting the toxicity of dothistromin (see Section 3.2). The effect of light is reminiscent of that seen in *Cercospora* needle blight where the fungal toxin cercosporin is light activated and induces the formation of highly damaging oxygen radicals. However, it appears that dothistromin may act in a different manner to cercosporin (see Section 3.2 on mode of action).

Very little work has been published on the effects of different soil types on susceptibility of pines to *Dothistroma* needle blight. Highest disease levels of *P. radiata* in Australia occurred on poor soils (sulphur-deficient basalt), but there were also influences of other soil and topographic factors (ELDRIDGE et al. 1981). Disease was also more severe in stands in which foliage arginine concentrations were high as a consequence of using high levels of nitrogen fertiliser (400 kg N/ha) (LAMBERT 1986).

Disease susceptibility varies with host species. For example, *P. attenuata*, *P. ponderosa* and *P. radiata* are highly susceptible, although *P. radiata* develops resistance at about 10–15 years of age. Species with very low susceptibility include *P. patula*, *P. taeda* and *P. sylvestris* (GIBSON 1972; KERSHAW et al. 1988). In an attempt to understand how *P. radiata* increases in resistance with age FRANICH et al. (1982) studied the needle monoterpenes that vary in amount and composition between young and mature trees. No simple relationship was found between monoterpene composition and mature tree resistance, although the monoterpenes stimulated spore germination and fungal growth *in vitro*. Subsequently a study was made of resinous material that occludes the stomatal pores of needles on mature trees (FRANICH et al. 1983). As well as forming a physical barrier to penetration of the fungus, oxidized resin acid derivatives found on the needle surface show fungistatic properties *in vitro* and prevent spore germination. However, a similar study comparing surface wax from young needles of different pine species (susceptible *P. nigra* and resistant *P. sylvestris*) concluded that differences in their susceptibility to *D. pini* were not because of differences in the surface wax (WALLA and PETERSON 1976).

2.5 Current methods of control

Dothistroma needle blight in commercial forests of the southern hemisphere is currently controlled by breeding resistant planting stock and by fungicide spraying. Diligent silvicultural practices of thinning and pruning also help considerably in the control of disease by removing infected branches, increasing air circulation and decreasing the inoculum in the forest environment (VAN DER PAS et al. 1984; KERSHAW et al. 1988). Resistant planting stock can be obtained by selecting species with natural resistance such as *P. sylvestris* (GIBSON 1974), or by selecting the most resistant provenances within a species. There is natural variation in resistance within many species including *P. radiata* (WILCOX 1982), *P. ponderosa* (PETERSON 1984) and *P. flexilis* (TAYLOR and SCHWANDT 1998). Field studies with *P. radiata* showed that *Dothistroma* resistance has a moderately high heritability and is a polygenic (quantitative) trait (WILCOX 1982). On the basis of this a breeding programme was established in 1983 to develop a *Dothistroma*-resistant family. This breed was estimated to have 15% less disease, and to cost 56% less in chemical spray costs compared with control trees (CARSON 1989; CARSON et al. 1991; DICK 1989). In New Zealand, breeding carried out by the Radiata Pine Breeding Co-operative is currently focussed on improvement of growth and wood quality traits but *Dothistroma* resistance is one of nine selection traits routinely assessed in their field trials (JAYAWICKRAMA and CARSON 2000). A different approach to increase *Dothistroma* resistance was taken by ADES and SIMPSON (1990), who used established methods for large-scale propagation of rooted cuttings. Based on the knowledge that *P. radiata* trees develop resistance with age they

proposed that rooted cuttings would have increased resistance compared with seedlings, because of their increased maturity. Their hypothesis was supported with a significantly higher mean disease level on 1-year-old seedlings compared with cuttings taken from 4-year-old plants. The authors did not report relative growth characteristics. However, in general cuttings grow more slowly but have straighter stems than trees grown from seedlings.

A range of fungicides, such as Benlate (benomyl), Brestan (fentin acetate + maneb), Daconil 2787 (chlorothalonil) and Dyrene (anilazine) are effective against *D. pini* (GIBSON 1974). But since the 1970s copper oxychloride and cuprous oxide have been the most widely used fungicides on account of their effectiveness in preventing spore germination and relatively low cost (RAY and VANNER 1988). In New Zealand's Radiata pine forests there is a nationally coordinated spray programme and the fungicide is sprayed from fixed wing aircraft fitted with micronair atomizers (KERSHAW et al. 1988). This treatment is usually reserved for stands where at least 50% of the trees have disease symptoms on >25% of the current foliage, which is the point at which the impact of the disease is considered significant in terms of wood loss (KERSHAW et al. 1988). There have been attempts to quantify the benefits of spraying on wood yield. WHYTE (1976) estimated that two sprays per year on severely diseased 5-year-old *P. radiata* would save just 6% of potential yield in the first year of spraying but 29% in the second year. In another trial, the final wood yield of diseased 13-year-old *P. radiata* was estimated to be 30–40 m³/ha more from trees sprayed three times during the rotation than from unsprayed trees (KERSHAW et al. 1988). Despite these figures, some have questioned whether there is a real financial benefit because of the high cost of spraying on a commercial scale (VAN DER PAS et al. 1984). However, spraying is beneficial not only to prevent loss of wood yield but also to minimize potential health hazards posed by dothistromin toxin (ELLIOTT et al. 1989).

Prior to fungicide spraying of commercial forests, surveying for Dothistroma needle blight is carried out on a large-scale. Trained forestry staff initially survey susceptible regions by helicopter. On the basis of premature defoliation and needle browning, the level of stand disease is estimated in 5% increments. Stands rated at >15% are subsequently checked in a ground survey to confirm that symptoms are caused by *D. pini* and to gain a more accurate assessment by estimating the percentage of the crown that is infected in 100–200 individual trees (VAN DER PAS 1981; KERSHAW et al. 1988). New technology being developed in Australia brings the prospect of dramatically improving both the coverage and accuracy of disease detection and monitoring using remote sensing. This exciting development involves detecting alterations in the spectral reflectance characteristics of *P. radiata* when affected by Dothistroma needle blight (STONE et al. 2003).

3 Dothistromin toxin

3.1 The biochemistry and genetics of dothistromin biosynthesis

The possibility of toxin production by *D. pini* was suggested by GADGIL (1967) following his observations of dead mesophyll cells in diseased needles adjacent to regions colonized by hyphae. Dothistromin toxin, distinctively red in colour, was subsequently isolated from lesions of infected pine needles and extracted from a *D. pini* culture grown *in vitro* (BASSETT et al. 1970). Toxicity of thin layer chromatography (TLC)-purified extracts was confirmed using an assay with the unicellular green alga, *Chlorella pyrenoidosa*. The difuroanthraquinone structure of the toxin was determined by mass spectrometry and nuclear magnetic resonance (NMR), and the name dothistromin was proposed. The crystal structure was determined by X-ray diffraction (BEAR et al. 1972) and this confirmed that the anthraquinone and difuran components of the molecule are fused to give a linear

arrangement of five- and six-membered rings. A further study (GALLAGHER and HODGES 1972) described chemical reactions of dothistromin and also showed that 80–90% of the toxin extracted from cultures consists of dothistromin, whilst the remainder is deoxydothistromin. The structural similarity of the difuran moiety to those found in the aflatoxins, sterigmatocystin and versicolorins was also noted. Indeed, the overall structural similarity to versicolorin B is remarkable, although dothistromin is unusual in having 1,4-hydroxylation in the anthraquinone ring system (Table 2). A further examination of *D. pini* cultures (DANKS and HODGES 1974) revealed more anthraquinones, including some with similarity to aflatoxin precursors (averufin, averylthrin and 6-deoxyversicolorin C) as well as some alternative forms of dothistromin (see Table 2). Data from a ¹³C-NMR study of dothistromin provided evidence that the biosynthesis of both anthraquinone and furan moieties is consistent with that seen in aflatoxin biosynthesis (SHAW et al. 1978). Further work on the chemistry of dothistromin has focussed on improving methods to isolate and identify the toxin, the development of a reverse-phase TLC system for improved

Table 2. Dothistromin and related compounds found in species of *Dothistroma* and *Cercospora*

Metabolite identified	Fungal species				Chemical structure
	D	P	A	S	
1a. Dothistromin ¹	✓	✓	✓	✓	
1b. Deoxydothistromin ²	✓				
1c. 4'-Deoxydothistromin			✓		
1d. Bisdeoxydothistromin	✓				
1e. 4',5-Bisdeoxydothistromin			✓		
2a. Bisdeoxydehydrodothistromin	✓				
2b. 3',4'-Dehydro-4'-deoxydothistromin		✓	✓		
3a. Versicolorin B				✓	
3b. 6-Deoxyversicolorin C ³	✓				
4. Versicolorin A	✓				
5a. Averantin			✓		
5b. Averythrin	✓			✓	
6. Averufin	✓	✓	✓	✓	

✓ Metabolite identified as a product of the species indicated.
 D: *D. pini* (GALLAGHER and HODGES 1972; DANKS and HODGES 1974; BRADSHAW et al. 2002).
 P: *C. personata* (GNANAMANICKAM and STOESSL 1986).
 A: *C. arachidicola* (STOESSL 1984; STOESSL and STOTHERS 1985).
 S: *C. smilacis* (ASSANTE et al. 1977).
 Structures of Aflatoxin B₁ and Sterigmatocystin are shown for comparison (STEYN et al. 1980).
¹Five other species of *Cercospora* were also shown to produce dothistromin (ASSANTE et al. 1977).
²May be deoxygenated at opposite side of R₃ ring to that shown (GALLAGHER and HODGES 1972).
³May be the racemate 6-deoxyversicolorin B (DANKS and HODGES 1974).

separation (FRANICH 1981) and a competitive enzyme-linked immunosorbent (ELISA) assay (JONES et al. 1993).

Our understanding of the biosynthesis of dothistromin has been facilitated by its structural similarity to aflatoxins and related compounds. Only a few species of *Aspergillus* are known to produce aflatoxins. However, the highly carcinogenic and toxic nature of this group of compounds, and their widespread occurrence as contaminants in agricultural produce, has fuelled intensive research on their biosynthesis and control (BHATNAGAR et al. 2003; CLEVELAND et al. 2003). Furthermore, the discovery of sterigmatocystin biosynthesis by *A. nidulans* accelerated toxin research because of the relative ease with which genetic studies can be carried out in this model organism. The aflatoxin and sterigmatocystin toxins have a polyketide origin but their biosynthesis involves many additional steps. Because of this complexity genetic studies have been crucial for dissecting the pathways and have complemented biochemical studies to determine how this group of toxins is synthesized. BENNETT (1979) isolated two mutants of *A. parasiticus* that were blocked in aflatoxin biosynthesis and accumulated the brightly coloured intermediate compounds versicolorin or norsolorinic acid. Genetic analysis showed these two mutations to be genetically linked and provided the first hint that aflatoxin genes might be clustered (BRADSHAW et al. 1983). The availability of these mutants paved the way for cloning genes by complementation and accordingly the *ver-1* gene was isolated (CHANG et al. 1992). Because the aflatoxin genes are clustered it was a relatively easy task to identify adjacent genes and currently the *A. parasiticus* aflatoxin gene cluster is thought to contain 24 genes in a 70 kb length of the genome (BHATNAGAR et al. 2003). The sterigmatocystin gene cluster of *A. nidulans* contains a similar number of genes (BROWN et al. 1996). Targeted replacement of toxin genes in these fungi has produced mutants blocked at specific stages in the pathways; many of the stages have been analysed by studying the precursors that accumulate in the mutants and by metabolite feeding studies.

Based on the expectation that dothistromin biosynthesis involves common steps, aflatoxin genes were used as hybridization probes to isolate toxin genes from *D. pini*. One of the genes isolated from *D. pini*, called *dotA*, encodes a putative versicolorin reductase with 80% amino acid identity to the *A. parasiticus ver-1* aflatoxin gene product (BRADSHAW et al. 2002). Using a transformation system developed for *D. pini* (BRADSHAW et al. 1997), a targeted *dotA* gene replacement was made. The *dotA*⁻ *D. pini* mutant produced undetectable levels of dothistromin but accumulated versicolorin A, confirming that *dotA* is a dothistromin pathway gene and supporting the hypothesis that dothistromin shares common biosynthetic intermediates with aflatoxin (BRADSHAW et al. 2002). Eight further dothistromin genes have been identified in *D. pini* so far, including putative polyketide synthase, monooxygenase and toxin transporter genes. Further characterization of the dothistromin gene cluster is expected to yield at least 20 genes, with similarities to most aflatoxin genes except the methyltransferases that are specific to the later stages of aflatoxin biosynthesis. We already have some evidence that the order and orientation of genes is not conserved between these related gene clusters (BRADSHAW et al. 2002). But even between the closely related aflatoxin cluster of *A. parasiticus* and sterigmatocystin cluster of *A. nidulans* there is little conservation in terms of gene arrangement. Unravelling the details of the dothistromin gene cluster will increase our knowledge of how fungal gene clusters evolved and will also provide genetic tools for studying the biosynthesis of dothistromin. Furthermore, as outlined later, dothistromin genes and their specific products may be targets for disease control.

Some fungi of the genus *Cercospora*, including the peanut pathogen *C. arachidicola* also produce dothistromin and related metabolites, including aflatoxin biosynthetic intermediates (Table 2). The presence of these intermediates in other dothistromin-producing fungi, along with the ¹³C-NMR study outlined earlier (SHAW et al. 1978) suggests that the biosynthesis of dothistromin is the same as that of aflatoxin as far as the versicolorins.

Exactly how dothistromin is synthesized will hopefully be elucidated once more dothistromin genes and gene-disrupted mutants are available for analysis. One of many interesting questions is whether versicolorin A, which has an unsaturated bisfuran ring, is a true direct precursor of dothistromin (which has a saturated bisfuran ring), or whether it is a shunt metabolite that accumulated when dothistromin synthesis was blocked in the *D. pini dotA* mutant (for example, by desaturation of versicolorin B). This is of particular interest because it is the unsaturated bisfuran moiety in aflatoxin B₁ that is largely responsible for its extremely potent carcinogenicity when it is activated by microsomal cytochrome P450 to an epoxide form (EATON and GALLAGHER 1994). Indeed unsaturated forms of dothistromin (such as bisdeoxydehydrodothistromin) have been detected as minor metabolites in some dothistromin-producing fungi grown in culture (Table 2) but whether these specific variants are carcinogenic has not been tested.

3.2 The occurrence and mode of action of dothistromin

The amount of dothistromin produced by *D. pini* is highly variable and is affected by many environmental factors. In diseased *P. radiata* needles, an average of about 180 ng of dothistromin can be obtained from a single lesion (DEBNAM et al. 1994). In culture, the toxin is strongly secreted into the growth medium, providing both a useful additional diagnostic aid and a convenient source of toxin for biochemical studies. Toxin production is, however, affected by the composition of the medium, with higher levels being detected in glucose as opposed to peptone media, and with no toxin being produced when ammonium is used as the nitrogen source. This is also the case for sterigmatocystin production in *A. nidulans* (FENG and LEONARD 1998; BRADSHAW et al. 2002). Aerated (shaking) cultures produce more dothistromin than static cultures, and light is not necessary for toxin production. Indeed more dothistromin was detected from *D. pini* isolates grown in the dark compared with those grown in the light (GANLEY 2000), although the fact that dothistromin is degraded in the light (see below) would have influenced levels. Of potentially great significance for forest health is the discovery that isolates from different parts of the world appear to vary greatly in their capacity to produce dothistromin. For example, isolates obtained from high altitude plantations of *P. mugo* in the Bavarian Alps produced between 5- and 500-fold more dothistromin in culture than New Zealand isolates (BRADSHAW et al. 2000). Whether they also differ in dothistromin production in the field is not known. However, it would clearly be wise to exercise caution in transferring isolates between countries and to this end a microsatellite profiling system has been developed that could be used as part of a surveillance system to distinguish between isolates (GANLEY and BRADSHAW 2001).

The role of dothistromin in needle blight is not yet known, although the injection of purified dothistromin into needles of *P. radiata* reproduced the symptoms of disease (SHAIN and FRANICH 1981). Whether dothistromin is required for pathogenicity (essential for disease to occur; SHANER et al. 1992) or virulence (contributes to severity of disease; SHANER et al. 1992) is still uncertain. In a similar study with a different system, 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). However, in the case of the Dutch elm disease toxin cerato-ulmin there was a different outcome. Although cerato-ulmin toxin reproduced disease symptoms on elm seedlings (TAKAI 1974) toxin-deficient mutants of *Ophiostoma ulmi* were still as virulent as the wild type (BOWDEN et al. 1996). Work is in progress to characterize the pathogenicity and virulence of dothistromin-deficient mutants of *D. pini*.

Host defences contribute to the symptoms seen in Dothistroma needle blight. When purified dothistromin is injected into pine needles the red-bands and surrounding

necrotic regions characteristic of the disease are accompanied by a strong host defence response (FRANICH et al. 1986). Benzoic acid, made by the plant, accumulates in high enough concentrations to be inhibitory to *D. pini* and is proposed to be a phytoalexin although it is, at the same time, toxic to plant cells. Purified dothistromin is broken down in the needle tissue to oxalic acid and CO₂, with only 10–20% of the toxin remaining after 24 h. Whether this is plant-induced or photolytic degradation is not known. However, there is evidence that dothistromin is degraded (to unknown products) in the light and in the absence of plant material (HARVEY et al. 1976; BRADSHAW et al. 2000), hence photolytic degradation is certainly possible. As the necrotic lesion continues to expand after most of the dothistromin has been destroyed it was suggested that most of the needle damage is due to the plant's defence response rather than to direct toxicity of the dothistromin (FRANICH et al. 1986). Indeed the amount of benzoic acid produced by the plant was proportional to the length of the necrotic lesion. Further evidence of a strong defence response was seen with increased lignification in cells immediately adjacent to the necrotic regions, a typical plant response to prevent further spread of a fungal pathogen. However, no significant correlation was found between field resistance and length of lesions induced in needles injected with dothistromin (DEBNAM et al. 1994). Moreover, the host is also capable of mounting a defence response to *D. pini* in the absence of dothistromin. *Pinus radiata* cell suspension cultures challenged with a purified cell wall fraction from *D. pini* elicited a rapid (20–40 min) transient oxidative burst, accumulation of phenolics, and induction of phenylpropanoid biosynthetic enzymes (HOTTER 1997). In view of this it seems possible that dothistromin is a virulence factor rather than a pathogenicity factor and that other components of the plant–pathogen interaction are important in the disease process.

In considering the mode of action of dothistromin it is worth digressing to consider the toxin cercosporin with which dothistromin is often compared. Cercosporin is a photosensitizing agent and is one of a group of photoactivated toxins that require light for toxicity to plants. A red-coloured polyketide-based perylenequinone toxin, it is produced by a range of *Cercospora* species and requires light to initiate its production by the fungus as well as its activation (ROLLINS et al. 1993; DAUB and EHRENSHAFT 2000). Cercosporin is converted to an electronically activated (triplet) state by light and then reacts with oxygen molecules to generate reactive oxygen species (ROS) such as singlet oxygen (¹O₂) and superoxide (O₂⁻). The toxicity of cercosporin is predominantly because of singlet oxygen that can react with lipids, proteins and DNA in a very broad range of cell types. In host plants, peroxidation of membrane lipids causes membrane damage, allowing the fungus access to the host cell nutrients. Because of the broad toxicity of the ROS produced, cercosporin is considered a non-host-specific toxin (DAUB and EHRENSHAFT 2000). Species of *Cercospora* that produce cercosporin show a very high level of resistance to the toxin. A variety of mechanisms have been suggested to account for this, including the reversible reduction of cercosporin to a non-phototoxic form in the cell, toxin export by efflux transporters and chemical quenching of singlet oxygen (UPCHURCH et al. 2002).

The toxicity of dothistromin to plant host tissue, and indeed to most other cell types, is dependent on the presence of light, hence there are some clear similarities with cercosporin (SHAIN and FRANICH 1981; STOESSL et al. 1990; DAUB and EHRENSHAFT 2000). However, in the case of dothistromin it is not entirely clear why light is required. In a study of pine needles injected with dothistromin, benzoic acid only accumulated in the presence of light (FRANICH et al. 1986), but at the same time dothistromin was broken down (to CO₂ and oxalic acid) more efficiently in the light (80% breakdown) compared with the dark (5–10% breakdown). Despite the higher levels of toxin remaining in the dark, the needles appeared to be tolerant of the toxin under these conditions. These observations lead to three suggestions to account for the light requirement for dothistromin toxicity: (i) the toxin is

light-activated, (ii) the toxin specifically targets cellular components that function only in the light or (iii) a photolytic breakdown product of the toxin is itself toxic and causes symptoms. The latter possibility seems unlikely as oxalic acid, the predominant breakdown product found in needles, was not directly toxic to plant tissue (FRANICH et al. 1986). There is some information in the literature on the first two possibilities, as discussed below, and a combination of these modes of action seems likely.

Is dothistromin a light-activated toxin, producing ROS in a similar manner to cercosporin? A direct comparison of the oxygen activating abilities of cercosporin and dothistromin was made by YOUNGMAN and ELSTNER (1984). They noted that both toxins cause lipid peroxidation and breakdown of photosynthetic pigments but showed that their mechanisms of oxygen activation are quite different. The predominant feature of cercosporin toxicity is singlet oxygen ($^1\text{O}_2$) that is produced mainly by light-dependent reactions. Dothistromin did not produce $^1\text{O}_2$ and therefore does not show any significant photosensitizing properties. In contrast, the ROS superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) are products of reductive oxygen activation rather than light activation, resulting from reactions between O_2 and an appropriate reductant. Both dothistromin and cercosporin were able to act as reductants: they were reduced in NADPH-dependent reactions and subsequently able to produce O_2^- and H_2O_2 by auto-oxidation. In photosynthetic organisms the light requirement for dothistromin toxicity may be due to reduction of dothistromin by photosynthetic electron transport *in planta* (YOUNGMAN and ELSTNER 1984; HEISER et al. 2002).

An additional possibility for the mode of action of dothistromin is that it specifically targets cellular components that function only in the light. A specific interaction with pine tissue was postulated after it was shown that dothistromin is significantly more toxic to pine embryo tissue than are other minor anthraquinone metabolites isolated from *D. pini* (including bisdeoxydothistromin, nidirufin and dehydrodothistromin) (DEBNAM et al. 1994). The specific toxicity of dothistromin was suggested to lie in the bisfuran moiety and this was confirmed when dothistromin conjugated to mouse albumin (MSA) through the anthraquinone moiety (at the other end of the molecule) was still toxic to pine embryos (JONES et al. 1995). The cellular target(s) of dothistromin are not known, although some progress has been made towards elucidating this. Using MSA-conjugated dothistromin, and a monoclonal antibody to dothistromin as a probe, specific dothistromin-binding sites were identified within small protein-storage vesicles in embryos. Subsequently a 40 kDa peptide was identified in mature embryo extracts that reacted specifically with dothistromin (JONES et al. 1995). The use of anti-idiotypic antibodies that mimic dothistromin also revealed binding to protein-containing vesicles in embryos (JONES et al. 1998). In unpublished work, these authors also found dothistromin-binding sites associated with chloroplasts in mature pine needles (mentioned in JONES et al. 1995).

In summary of its role as a toxin, dothistromin produces ROS by reductive oxygen activation and also appears to have specific targets in some cells. The broad toxicity of dothistromin to many cell types presumably reflects its action as a producer of oxygen radicals. The requirement for light can be explained by reductive activation of dothistromin by photosynthetic electron transport, effects of ROS on photosynthetic pigments or by specific targeting of photosynthetic components. However, none of these account for the light-dependent toxicity to non-photosynthetic organisms (STOESSL et al. 1990). Furthermore, since dothistromin is rapidly degraded in the presence of light the requirement for light in toxicity is far from clear.

In addition to its role as a phytotoxin, dothistromin is weakly mutagenic to a wide range of cell types and has clastogenic (chromosome damaging) properties (see next Section) but its mode of action in these respects is unknown. However, mutagenicity was enhanced by metabolic activation in two studies (MCLARIN and FERGUSON 1985; FERGUSON et al. 1986), suggesting similarities with aflatoxin.

3.3 Is dothistromin a human health risk?

The structural similarity of dothistromin to aflatoxins prompted serious concerns about the health risk associated with *Dothistroma* needle blight, especially for forest workers. Aflatoxin B₁ is the most potent naturally occurring carcinogen known (SQUIRE 1989) and is classified as a group I carcinogen by the International Agency for Research on Cancer. It is also teratogenic (causing deformities in embryos) and toxic. Cancer research with animals showed that a minimum dose of 50 ng aflatoxin B₁/kg body weight/day is necessary to produce tumours, whilst toxicology studies with humans exposed to dietary aflatoxins have suggested the acute lethal dose of aflatoxin B₁ for adults is 10–20 mg (ELLIOTT et al. 1989; BENNETT and KLICH 2003). Aflatoxins are activated to more potent forms by microsomal enzymes. Cytochrome P450 enzymes convert the unsaturated bisfuran ring of aflatoxin B₁ to the highly carcinogenic 8,9-epoxide form that can bind to DNA (EATON and GALLAGHER 1994). Recent work by our group in collaboration with Dr D. Bhatnagar's aflatoxin group (New Orleans SRRC, USDA) has shown no evidence for aflatoxin production by *D. pini* but the acute toxicity and potential genotoxicity of dothistromin remains a concern.

Table 3 summarizes dothistromin toxicology studies that have been carried out with a wide range of organisms and cell types. The reader is also referred to the comprehensive summary of published and unpublished work on this topic by ELLIOTT et al. (1989). Toxicity has been measured by growth inhibition, cell lysis and mitotic index. Dothistromin is toxic to *P. radiata* tissue at low concentrations, but is also highly toxic to a range of bacterial, fungal, plant, animal and human cells (Table 3). In most studies light was required for toxicity, but the brine shrimp *Artemia salina* was susceptible to the effects of dothistromin without light activation (STOESSL et al. 1990). Compared with aflatoxin B₁, dothistromin appeared to have higher toxicity to *Bacillus megaterium*, with concentrations of 5–10 µg/ml aflatoxin B₁ but only 0.5–1.0 µg/ml dothistromin required to completely inhibit growth (HARVEY et al. 1976). However, when a direct comparison of these toxins was made with human lymphocytes using mitotic index as a measure of toxicity, there was little difference between them (FERGUSON et al. 1986), although when aflatoxin was metabolically activated it caused a significant depression of mitotic index (suggesting higher toxicity) compared with that seen with dothistromin.

Does dothistromin cause damage to genetic material and is it a carcinogen? There have been no epidemiological studies reported to date that suggest it is a carcinogen: reviews of cancer incidence in forestry workers showed no evidence to connect dothistromin with lung cancer (ELLIOTT et al. 1989). However, there is evidence that dothistromin is a weak mutagen and clastogen. Dothistromin showed significant mutagenic activity in an Ames assay (FERGUSON 1986; ELLIOTT et al. 1989) and in a screen for specific induced mutations in Chinese hamster fibroblasts (MCLARIN and FERGUSON 1985) but mutagenic activity was weak compared with aflatoxin B₁. In both cases, it appears that metabolic activation enhanced mutagenicity but unfortunately the details of these studies were not published. Clastogenic activity of dothistromin has also been demonstrated with tests for chromosome aberrations and sister-chromatid exchange (SCE) in human cells and with micronucleus assays in rodent cells (MCLARIN and FERGUSON 1985; FERGUSON et al. 1986; ELLIOTT et al. 1989; SKINNIDER et al. 1989). Chromosome aberrations caused by dothistromin were mostly simple gaps and deletions and were not affected by metabolic activation, whilst aflatoxin B₁ (with metabolic activation) induced a higher level of aberrations at equivalent doses, with more complex exchange type aberrations. Similarly the effect of dothistromin as demonstrated by the micronucleus assay with Chinese hamster cells, was weak compared with aflatoxin B₁ and was not dependent on metabolic activation (MCLARIN and FERGUSON 1985). In a study of SCE induced by dothistromin in human cells, SKINNIDER et al. (1989) made the interesting observation that purified lymphocytes show a greatly enhanced sensitivity to dothistromin compared with

Table 3. Toxicology studies with dothistromin

Species/cells tested	Parameter(s) tested	Concentration and effect	Comments	Reference
<i>Chlorella pyrenoidosa</i> <i>Bacillus megaterium</i> Chinese hamster ovary cells	Growth inhibition Growth inhibition Mutagenicity micronucleus assay	1.0 µg/ml (2.7 µM): complete inhibition 0.5 µg/ml: complete inhibition No concentrations given. Mutagenic and clastogenic effects	RNA synthesis inhibited RNA synthesis inhibited Metabolic activation required for mutagenicity	HARVEY et al. (1976) HARVEY et al. (1976) McCLARIN and FERGUSON (1985)
Human lymphocytes	Mitotic index ¹ chromosome aberrations ²	5.0 µg/ml: 50% MI 5.0 µg/ml: 12% aberrations	No effect of metabolic activation	FERGUSON et al. (1986)
Human lymphocytes	Sister-chromatid exchange ³	2.0 µg/ml: SCE frequency = 9 (control SCE = 7)	Higher SCE induction in smokers than non-smokers	SKINNIDER et al. (1989)
Chinese hamster ovary cells	Sister-chromatid exchange	2.0 µg/ml: SCE frequency = 19 (control SCE = 9)		SKINNIDER et al. (1989)
Mouse bone marrow and red blood cells	Micronucleus assay	1 mg/kg injected twice into mouse	Increased micronuclei but not significant	ELLIOTT et al. (1989)
Human red blood cells	Cell lysis	92.5 µg/ml: c. 100% haemolysis in 2 h	Light-dependent and air-dependent	STOESSL et al. (1990)
Beetroot cells	Cell lysis	18.5 µg/ml: c. 100% haemolysis in 24 h	Light-dependent	STOESSL et al. (1990)
Bacteria and fungi (15 spp.)	Growth inhibition	1-30 µg/ml: toxicity to 12 spp.	Most spp. strongly light-dependent	STOESSL et al. (1990)
Fenugreek	Inhibition of root elongation	37 µg/ml: 50-100% inhibition	Light-dependent	STOESSL et al. (1990)
Brine shrimp <i>Pinus radiata</i> embryo tissue	Mortality Inhibition of development	3.7 µg/ml: 100% mortality 0.3 µg/ml: 50% inhibition 1.0 µg/ml: 100% inhibition	Toxicity was not light-dependent Leaf callus growth also inhibited (100%) by 1.0 µg/ml	STOESSL et al. (1990) JONES et al. (1995)

¹Mitotic index (MI), used as a measure of culture toxicity, expressed as percentage of control.

²Chromosome aberrations measured as chromosome gaps, deletions and exchanges.

³Sister-chromatid exchange (SCE) frequency per 25 metaphase spreads.

lymphocytes in whole blood (a threefold increase in SCE with only 1/10 the concentration of dothistromin). This effect was not seen with the mitomycin control and it was therefore postulated that dothistromin may be 'neutralized' by some component in whole blood. There is clearly a lot more to discover about the complexities of how dothistromin might act *in vivo*. The only *in vivo* study on mammals reported in the literature showed an equivalent clastogenic effect of dothistromin and aflatoxin (ELLIOTT et al. 1989) although the increases in clastogenicity seen with these toxins were not significantly greater than a toxin-free control. The authors attributed this lack of statistical significance partly to the genetic diversity of the mice used in the study.

Because dothistromin is highly toxic to a wide range of cell types at low doses it appears that there is a narrow difference between effective doses for mutagenicity/clastogenicity and toxicity, with, in effect, cells being killed before their genetic material is damaged. FERGUSON et al. (1986) wrote that the 'toxicity may be masking any major potential for clastogenicity by dothistromin'. In summary, there is evidence that dothistromin is a weak mutagen and clastogen in mammals, although at higher doses there is more likely to be toxicity to cells rather than an increased risk of genetic damage.

Research from the New Zealand Forest Research Institute (NZFRI) and Department of Health indicated low levels (<7 ng/ml) of dothistromin in air and water from the catchment area of forests with Dothistroma blight (documented by ELLIOTT et al. 1989). Dothistromin levels are higher in wet periods than in dry, and are higher at certain times of the year such as during the summer when the fungus is most active (BRIGGS 1985; FERGUSON 2002). The exposure of forestry workers involved in thinning, low pruning or final clear felling of pine trees with 23–40% mean Dothistroma disease rate was monitored by measuring dothistromin levels in the air, on clothing and on skin (BRIGGS 1985). Although low but quantifiable levels of toxin were detected, there was a wide variation even between workers carrying out the same job on the same day, for example, air filtered from the breathing zone of two workers engaged in pruning contained <0.0009 and 0.1493 ng/ μ l dothistromin. This variation could be accounted for by the collection of a few particles (such as needle fragments) containing relatively high levels of dothistromin, rather than the collection of many particles with low toxin content. Despite the generally accepted low risk to forestry workers nonetheless it is prudent to limit exposure. The New Zealand Dothistroma Control Advisory Committee (ANONYMOUS 1986) advised the following (reproduced with permission from the NZ Farm Forestry Association):

- In dry conditions there is a negligible risk of exposure to dothistromin. In wet stands the recorded incidence of dothistromin was higher but still too low to confirm a real exposure risk to workers.
- Wherever practical, work should be scheduled away from infected stands during wet weather.
- It is important to ensure workers wear protective clothing when working in infected stands in wet weather.
- It is important to correctly implement the spray programme recommended by the Disease Control Advisory Committee (all stands with disease level above 15% and in which workers will be operating over the next 12 months are to be sprayed).

Further advice given by BRIGGS (1985) includes:

- Forestry workers should wear clothing that does not collect dothistromin-containing particles: cotton is preferable to wool.
- Work clothes should be washed frequently and exposed areas of the body such as hands, face and hair should be kept clean to prevent accumulation of dothistromin-containing particles.
- Disposable dust masks should be supplied for use by workers.

However, note that, in New Zealand, after decades of coping with *Dothistroma* needle blight in commercial forests these practices are not adhered to rigorously nowadays as there has been no evidence of detrimental effects on forestry workers.

3.4 Future possibilities for disease control using knowledge of dothistromin

Knowledge of the role played by dothistromin in the development of disease may open up new approaches to disease control. Genetically engineered trees that inactivate, remove or prevent synthesis of the toxin could be developed and four possibilities are outlined here. First, pine trees that express dothistromin-specific antibodies in the needles could prevent normal toxin action. It has already been shown that dothistromin-specific antibodies can protect embryo callus cells from the toxic effects of 20 $\mu\text{g/ml}$ dothistromin (20 times the usual toxic dose) (JONES et al. 1995). Hence, there is some experimental support for this possibility. A second option is to target destruction of the toxin in the plant tissue by enzymatic means. In a study with grapevines, over-expression of an NADPH-dependent aldehyde reductase gene led to enhanced detoxification of the fungal toxin, eutypine (LEGRAND et al. 2003). Further study of dothistromin detoxification mechanisms could lead to candidate genes for this approach. A third area of toxin resistance that has great promise is the deployment of toxin transporter genes. Toxin transporters are found in both fungi and plants and some are related to bacterial multidrug resistance transporters (DEL SORBO et al. 2000; SIMMONS et al. 2003). Toxin transporters are believed to be important in plant-pathogen interactions in two respects: they enable toxins to be secreted by the fungus into the plant tissue and also provide a self-protection mechanism for the fungus against the effects of its own toxin. Because of this latter function they are prime targets for incorporation into host plants. A candidate dothistromin transporter has been found in *D. pini* (BRADSHAW et al. 2002) and relevant studies have already been carried out with other systems. Transfer of both the cercosporin transporter gene to a cercosporin-sensitive fungus, and of the yeast PDR5 transporter gene to tobacco, led to gain of toxin resistance (DEL SORBO et al. 2000; UPCHURCH et al. 2002). A fourth possibility for transgenic disease control would be to specifically target genes involved in dothistromin biosynthesis. For example, we already know that the *dotA* gene product is required for biosynthesis and if its expression were reduced, for example, by RNA interference (COTTRELL and DOERING 2002; OGITA et al. 2003) there could be concomitant loss of toxin production and consequently lower disease.

This speculative discussion is not intended to imply that genetic modification of trees is the best option for disease management. Indeed, in many forests *Dothistroma* infections are currently being kept at a tolerable level with current practices, while in others the replacement of *Dothistroma*-susceptible species with *Dothistroma*-resistant species is a viable option. However, in the long-term, it may well be our detailed knowledge of the molecular biology of the pathogen and its toxin that will provide the basis for sustainable control.

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Résumé

Revue sur la maladie des bandes rouges des pins, causée par Dothistroma, et la toxine dothistromine

La maladie des bandes rouges causée par *Dothistroma* est un problème dans les plantations de pins exotiques de l'hémisphère sud depuis de nombreuses années. La prévalence de cette maladie est en augmentation dans l'hémisphère nord et affecte maintenant les pins dans leurs régions d'origine. Le champignon pathogène *Dothistroma pini*, responsable de la maladie, produit une toxine, la dothistromine, proche de l'aflatoxine qui est un puissant carcinogène. Ceci pose donc la question des effets possibles sur la santé des travailleurs forestiers. Cette revue repose sur une large couverture de la littérature concernant aussi bien la maladie que la toxine. Le champignon a une taxonomie complexe avec de nombreux synonymes, et seul l'anamorphe se rencontre dans de nombreux pays. C'est un champignon nécrotrophe qui tue les tissus de l'aiguille et réalise son cycle biologique dans la lésion ainsi formée. La dissémination de la maladie s'effectue principalement par élaboussures de pluie contenant les conidies mais une dissémination à longue distance a été mise en évidence par transport de matériel contaminé ou par dissémination des spores par le vent ou les nuages dans les courants aériens. La sévérité de la maladie est affectée par l'humidité, la température et la lumière. Il existe des différences de sensibilité entre espèces de *Pinus*, et certaines présentent une résistance accrue avec l'âge. La méthode actuelle de lutte dans les forêts de plantations de l'hémisphère sud consiste à pulvériser des fongicides à base de cuivre ; dans le cas de *Pinus radiata*, une augmentation de la résistance a été obtenue grâce à un programme d'amélioration génétique. La toxine dothistromine est une difuroanthraquinone, similaire en structure à la versicolorine B, précurseur de l'aflatoxine. Une partie d'une batterie de gènes comprenant des gènes de biosynthèse de la dothistromine a été clonée, confirmant les analogies entre les voies de biosynthèse de la dothistromine et de l'aflatoxine. La dothistromine produit des radicaux oxygène nocifs par activation de la réduction de l'oxygène plutôt que par photosensibilisation, mais ses effets toxiques s'exercent aussi probablement sur des sites cellulaires spécifiques. Des études montrent que la dothistromine est un mutagène et clastogène faible, et donc potentiellement carcinogène. Bien que les risques pour les ouvriers forestiers soient considérés comme très faibles, il est prudent d'éviter dans la mesure du possible de s'exposer dans les périodes où les niveaux de dothistromine sont supposés élevés.

Zusammenfassung

Die Dothistroma-Nadelbräune an Kiefern und das Toxin Dothistromin: eine Literaturübersicht

Die *Dothistroma*-Nadelbräune ist in der Südhemisphäre in Plantagen mit exotischen Kiefernarten seit vielen Jahren ein Problem. In der Nordhemisphäre nimmt die Bedeutung dieser Krankheit derzeit zu und sie befällt nun Bäume auch in ihren natürlichen Verbreitungsgebieten. Der Erreger ist der Ascomycet *Mycosphaerella pini* (Anamorphe: *Dothistroma pini*). Der Pilz bildet das Toxin Dothistromin, das eng mit dem hochtoxischen Karzinogen Aflatoxin verwandt ist. Daraus ergab sich die Frage nach möglichen Nebenwirkungen dieser Baumkrankheit auf die Gesundheit von Waldarbeitern. Dieser Review fasst die Information über die Krankheit und das Toxin zusammen. Der Pilz hat eine komplizierte Taxonomie mit vielen Synonymen und in den meisten Ländern wurde nur die Anamorphe nachgewiesen. Er ist ein nekrotrophes Pathogen, das Blattgewebe abtötet, und in den so gebildeten Läsionen seinen Lebenszyklus abschliesst. Der normale Ausbreitungsweg der Krankheit erfolgt über Konidiosporen mit Regentropfen, aber es gibt auch Hinweise auf einen Ferntransport mit infiziertem Pflanzenmaterial und über die Verbreitung von Sporen mit dem Wind bzw. Wolken in Luftströmungen. Die Krankheitsintensität wird durch Luftfeuchte, Temperatur und Licht beeinflusst. Es gibt Unterschiede in der Anfälligkeit zwischen verschiedenen Kiefernarten und manche davon werden mit zunehmendem Alter resistenter. Derzeit werden in Plantagen der südlichen Hemisphäre Kupferfungizide zur Kontrolle dieser Krankheit eingesetzt und für *Pinus radiata* wurde in Züchtungsprogrammen eine erhöhte Resistenz erreicht. Das Toxin Dothistromin ist ein Difuroanthrachinon und ähnelt in seiner Struktur dem Aflatoxin-Präkursor Versicolorin B. Ein Teil des Genclusters, das die Dothistromin-Biosynthese codiert, wurde geklont, und es wurden dabei Parallelen zwischen dem Dothistromin- und dem Aflatoxin-Biosyntheseweg bestätigt. Dothistromin bildet schädliche Sauerstoffradikale (wahrscheinlich eher durch reduktive Sauerstoffaktivierung als durch Photosensibilisierung), es dürfte aber auch auf spezifische Zellkomponenten toxisch wirken. Dothistromin zeigt schwache mutagene und chromosomenschädigende Wirkungen und ist deshalb ein potentielles Karzinogen. Obwohl das Risiko für Waldarbeiter als gering eingeschätzt wird, sollte man in Perioden, in denen der Dothistromingehalt hoch sein dürfte, eine unnötige Exposition vermeiden.

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