

REVIEW ARTICLE

A review of Pinaceae resistance mechanisms against needle and shoot pathogens with a focus on the *Dothistroma*–*Pinus* interaction

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Summary

Dothistroma needle blight (DNB), caused by *Dothistroma septosporum* and *Dothistroma pini*, is a highly damaging disease of pine. DNB was originally considered a problem on exotic *Pinus radiata* plantations in the Southern Hemisphere and on both exotic and native pines in parts of North America in the 1960s. Since the mid-1990s, however, DNB has increased in importance in various parts of the world, including Europe. On susceptible species, DNB causes premature needle drop, a loss of yield and, in some circumstances, mortality. In some areas, DNB is controlled by the application of copper-based fungicides and silvicultural techniques, such as thinning and pruning. In New Zealand, there has also been a long history of selection of more resistant *P. radiata* for use in breeding programmes. A richer understanding of the resistance mechanisms involved in the *Dothistroma*–*Pinus* interaction will play a critical role in helping the development of sustainable integrated DNB management strategies. This review therefore summarizes current knowledge of defence mechanisms involved in the defence of Pinaceae against needle and shoot pathogens and identifies research gaps. Collaborative research efforts from countries directly or indirectly affected by DNB are rapidly generating new knowledge to address these gaps.

1 Introduction

Dothistroma needle blight (DNB; syn. red band needle blight) is an economically important fungal disease that affects more than 80 *Pinus* spp. as well as some other species in the Pinaceae (Watt et al. 2009). Originally considered a problem in the Southern Hemisphere and parts of North America in the middle of the 20th century, DNB is now of increasing importance in various parts of Europe and western Canada (Woods et al. 2005; Brown and Webber 2008). DNB leads to premature needle drop, reductions in growth and yield and, in some cases, tree mortality (Woods et al. 2005).

Dothistroma needle blight is caused by two morphologically indistinguishable species, *Dothistroma septosporum* (Dorog.) M. Morelet and *Dothistroma pini* Hulbary (Barnes et al. 2004). *Dothistroma septosporum* has a worldwide distribution, whereas *D. pini* has, so far, only been found in the USA and areas of Europe, including the Czech Republic, France, Hungary, Russia, Slovenia, Switzerland and Ukraine (Barnes et al. 2008, 2014; Ios et al. 2010; Piškur et al. 2013; Queloz et al. 2014). The native ranges of the pathogens are unknown, although they were initially thought likely to have evolved in either the cloud forests of Central America (Evans 1984) or the Himalayas (Ivory 1994). Gibson (1974) and Evans (1984) both suggested that *Dothistroma* was endemic on pines in parts of Europe and North America. Recent studies based on dendrochronology, herbarium specimens and population genetics have supported this suggestion (Welsh et al. 2009; Dale et al. 2011; Fabre et al. 2012; Barnes et al. 2014). Most work has been done on *D. septosporum* as it is a significant pathogen, especially in the Southern Hemisphere (where *D. pini* has not yet been found). The main exception to this is the major work of Peterson's work, which was undertaken in areas of the USA (mostly Nebraska) where only *D. pini* has been confirmed using molecular methods (Barnes et al. 2004, 2014). This work included investigations into the infection and disease development processes on *Pinus nigra* J.F. Arnold and *Pinus ponderosa* Douglas ex C. Lawson (Peterson and Walla 1978). Research in the western regions of North America, including that of Cobb (Cobb and Libby 1968; Cobb and Miller 1968; Muir and Cobb 2005), Parker (Funk and Parker 1966; Parker and Collis 1966), and Thyr and Shaw (1964), was most likely on *D. septosporum*, as this is the only *Dothistroma* species confirmed using molecular methods in this region.

The life cycle of *Dothistroma* has been well documented (Figs 1–3). With free water available, *Dothistroma* conidia are released from conidiomata and disperse by water splash, throughout the growing season (Gibson et al. 1964; Thyr and Shaw 1964; Peterson 1967, 1973; Gadgil 1977; Rack 1986; Karadžić 1989; Boateng and Lewis 2015). Conidia germinate

within 3 days at an optimum temperature of between 17 and 22°C (Gibson et al. 1964; Gadgil 1967; Ivory 1967) producing numerous germ tubes, which have been observed growing randomly on the needle surface (Gadgil 1967; Ivory 1972; Peterson and Walla 1978; Muir and Cobb 2005) as well as directly towards stomata (Peterson and Walla 1978; Muir and Cobb 2005). Simple germ tubes penetrate needles through stomata and grow in stomatal cavities (Ivory 1972; Muir and Cobb 2005; Kabir et al. 2014). Growth from the epistomatal chamber into the mesophyll sometimes involves an infection peg, and appressoria-like structures have been noted in some cases (Gadgil 1967, 1974; Gadgil and Holden 1976; Peterson and Walla 1978). Gadgil (1967) also observed direct penetration through the cuticle by a mycelial fragment, although direct penetration has not been reported elsewhere. Once the pathogen penetrates the needle, substomatal vesicles may form (Muir and Cobb 2005). Intercellular hyphae grow locally from the site of infection, in mesophyll tissue (Gadgil 1967; Ivory 1972; Peterson and Walla 1978; Kabir et al. 2014), resin canals (Gadgil 1967; Peterson and Walla 1978), endodermal cells (Gadgil 1967; Peterson and Walla 1978) and transfusion and vascular tissues (Peterson and Walla 1978). Gadgil (1967) and Peterson and Walla (1978) also observed intracellular hyphae in the mesophyll and transfusion and vascular tissues. Eventually, erumpent conidiomata will form in lesions, and conidia are released. Gadgil (1977) concluded that, under optimum conditions (20°C day and 12°C night; constant free moisture on needle surface), 19 days is the minimum time required for germination, penetration, hyphal growth in needle tissues and the formation of conidiomata on *Pinus radiata* D. Don needles, although the timing is very variable and the complete life cycle can take up to 12 weeks, even under controlled conditions (Kabir et al. 2014).

Ascospores and ascomata of *D. septosporum*, indicative of the sexual stage, have been observed rarely, while those of *D. pini* have never been reported. Studies of the population genetics of *D. septosporum* suggest, however, that sexual recombination occurs in some areas where both mating types are present (e.g. Dale et al. 2011). Ascospores are released during a shorter period than conidia, from late spring to early summer (Funk and Parker 1966; Karadžić 1989). Although rarely reported, windborne ascospores may be important in establishing pathogen populations in new areas, through long distance dispersal (Dale et al. 2011).

During infection and disease development, *Dothistroma* spp. release the mycotoxin dothistromin (Bassett et al. 1970), which is closely related to the aflatoxin precursor, versicolorin B (Chettri et al. 2013). Dothistromin release leads to disruption of mesophyll tissue in advance of growing *Dothistroma* hyphae (Gadgil 1967; Kabir et al. 2015) and is responsible for the red colour seen on symptomatic needles (Shain and Franich 1981). Dothistromin injection can also generate a hypersensitive-like response in *P. radiata* needles, the plant producing benzoic acid as a phytoalexin and highly lignified lesion-delineating bands (Franich et al. 1986). *Dothistroma septosporum* cell wall elicitors also induce a hypersensitive-like response in suspension-cultured *P. radiata* cells (Hotter 1997). However, dothistromin production is not required for infection of *P. radiata*, as genetically modified *D. septosporum* strains, unable to produce the toxin, are still able to complete their life cycle on this host (Schwelm et al. 2009). Recent research has shown that dothistromin production is a virulence factor that is required for expansion of the necrotic lesions and for normal levels of conidia production on *P. radiata* (Kabir et al. 2015).

Several environmental factors influence *Dothistroma* infection and development, including moisture levels (Gadgil 1974, 1977), temperature (Ivory 1972), light intensity (Gadgil and Holden 1976) and soil fertility (Eldridge et al. 1981). DNB epidemics occur mostly in areas and years with either high levels of summer precipitation or frequent warm rain events. Recent upsurges in disease severity have been linked to climate change induced increases in both summer precipitation and average minimum temperature (Woods et al. 2005; Archibald and Brown 2007; Watt et al. 2009, 2011a; Woods 2011; Welsh et al. 2014).

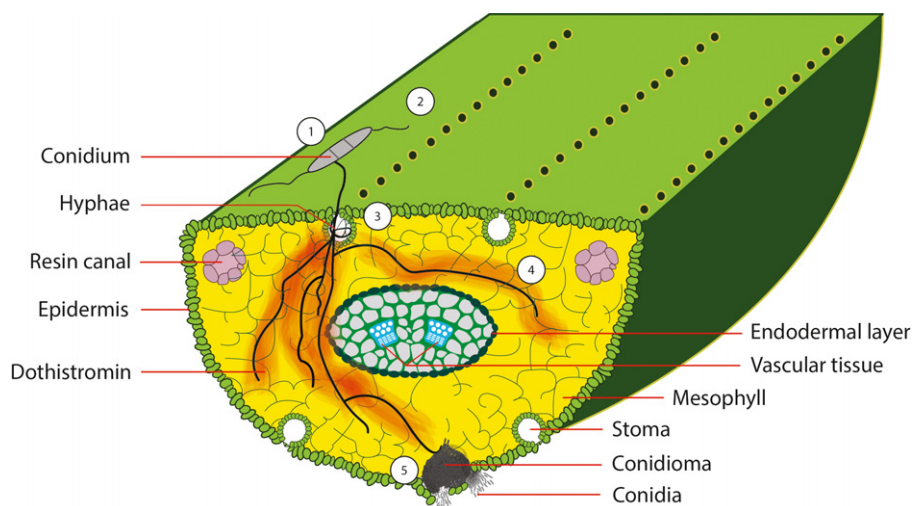


Fig. 1. Infection and disease development of *Dothistroma* on susceptible Pinaceae species. 1. Conidium germinates on the needle surface; 2. hyphae grow across the needle surface and through stomata; 3. hyphae colonize an epistomatal chamber; 4. intercellular hyphae colonize the mesophyll, producing dothistromin in increasing quantities, which kills host tissues ahead of the growing hyphae; 5. conidioma forms and new conidia are produced. Not to scale.

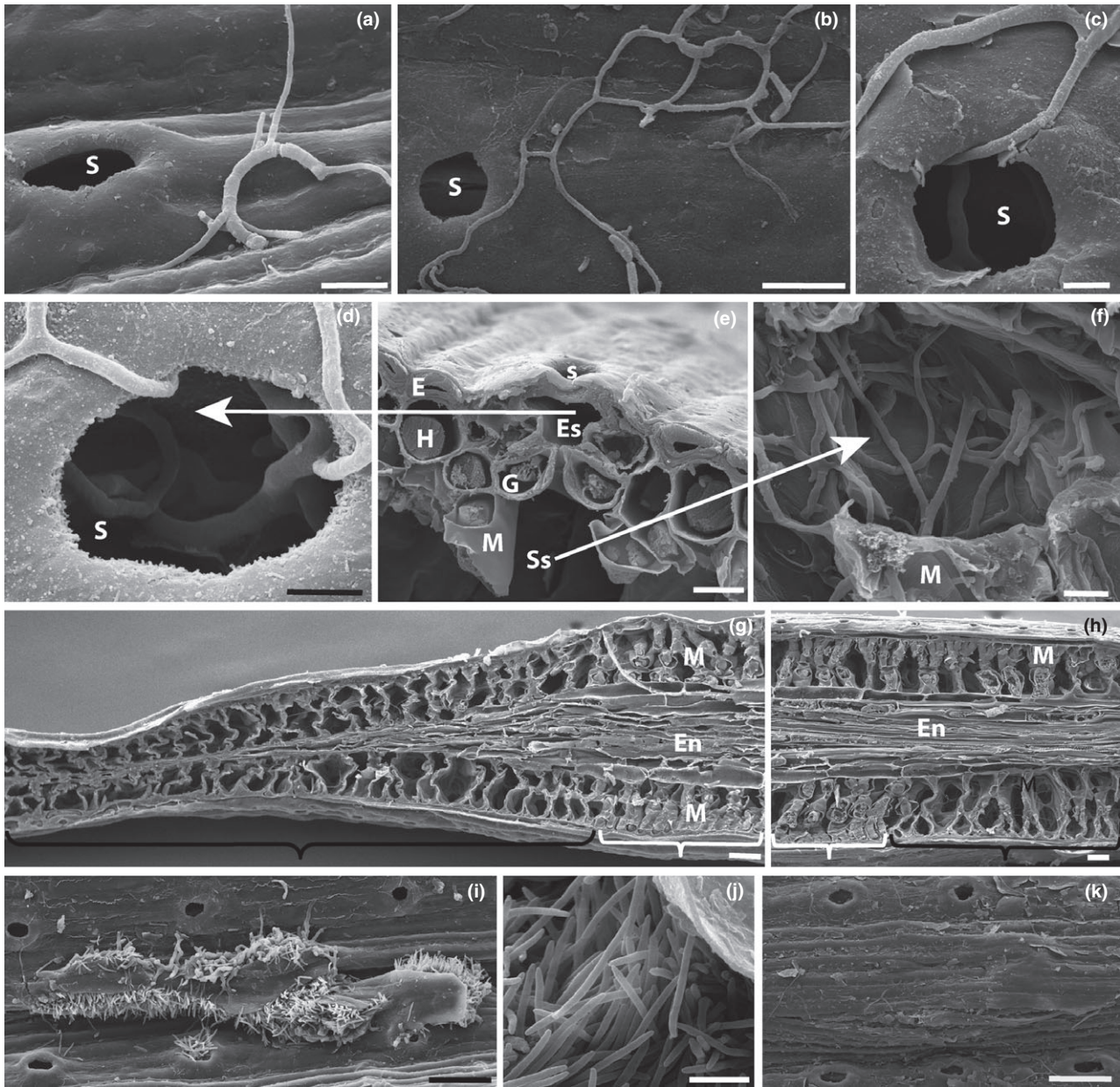


Fig. 2. Scanning electron microscopic (SEM) overview of the *Dothistroma septosporum* life cycle on *Pinus radiata* needles. All SEM pictures show stages of infection of *Pinus radiata* needle with a wild-type (WT) strain of *D. septosporum* (NZE10), except for (h) and (k) which show late stages of infection with a mutant of *D. septosporum* that is unable to produce the virulence factor dothistromin. (a) Germinating conidium with several germ tubes on the needle surface near a stomatal pore (S) at week 1 ai (after inoculation); (b) epiphytic fungal network on pine needle surface at 3 weeks ai. hyphae appeared to grow randomly without targeting towards stomatal pores (S); (c) penetration of a hypha into a stomatal pore (S); (d) fungal hyphae growing inside epistomatal chamber of stoma (S) after penetration from needle surface; (e) partial cross section of an uninfected *P. radiata* needle showing epidermal (E), hypodermal (H), mesophyll (M) and guard (G) cells, epistomatal (Es) and substomatal (Ss) chambers; (f) colonization of the substomatal chamber (Ss) and mesophyll (M); (g) a longitudinal cross section through a needle lesion (black bracket) adjacent to an undamaged green area (white bracket) of needle. The lesion area shows severe damage caused to upper and lower mesophyll (M) and endodermal cells (En) by the presence of the fungus and its toxin dothistromin; (h) A longitudinal cross section through a disease lesion (black bracket) caused by a dothistromin non-producing mutant at the same stage of disease as in (g) shows less extensive tissue death, with some damage to lower mesophyll cells while upper mesophyll (M) and endodermal (En) cells remained intact. The white bracket indicates adjacent undamaged green area of needle; (i) extensive eruption of conidiomata of the WT *D. septosporum* through the needle epidermis; (j) masses of conidia released from WT *D. septosporum* conidioma; (k) in contrast to the WT, the dothistromin non-producing mutant produces weaker eruptions, along with fewer conidiomata and conidia than the WT. Size bars c, d = 5 μm ; a, e, f, j = 10 μm ; b = 20 μm ; g, h, i, k = 50 μm .

The economic impacts of DNB damage have rarely been estimated (Alzamora et al. 2004; Watt et al. 2011b). However, severe losses in wood volume (Whyte 1976; Van der Pas 1981; Woollons and Hayward 1984) and occasional high mortality rates (Parker and Collis 1966; Taylor and Schwandt 1998; Woods et al. 2005; Brown and Clayden 2012) have been

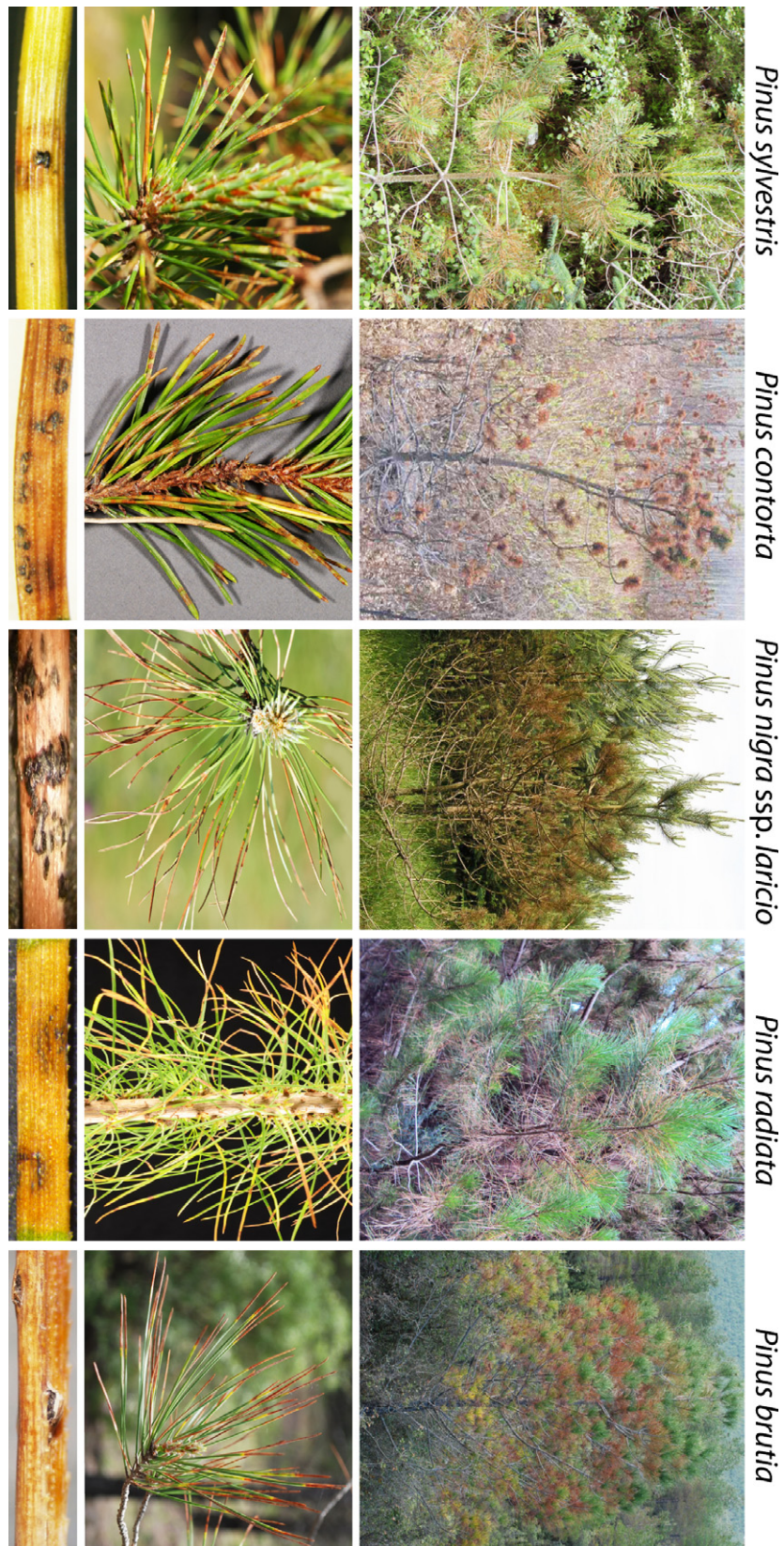


Fig. 3. Typical dothistroma needle blight symptoms on some common host pine species. The top *Pinus contorta* photo was supplied by Alex Woods, all other photos were supplied by the authors.

reported. This, together with the investment in host removal, loss of ecological and sociocultural values, and discouragement of foresters and owners, reinforces the importance of controlling this disease. Several fungicides, including copper-based treatments, have been used to control the disease (Bulman et al. 2013). Nevertheless, as the use of plant protection products is heavily restricted by European Union directives (EU, 2009), other more environmentally friendly methods embodying the principles of integrated pest management are prioritized. The identification of resistance mechanisms against *Dothistroma* will support the identification of less susceptible individuals, families and provenances that can be used for both breeding programmes and the establishment of new plantations.

Only limited amounts of research have been published on resistance mechanisms directly involved in the *Dothistroma*–*Pinus* system, therefore, other similar pathogen–Pinaceae systems are also considered in this review (Table 1). These systems include other foliar pathogens as well as shoot pathogens that infect foliage, such as *Diplodia sapinea* (Desm.) J. Kickx f. (syn. *Diplodia pinea*; Diplodia blight) and *Cronartium ribicola* (J. C. Fisch) [white pine blister rust (WPBR)]. It is important to acknowledge at this point that *Dothistroma* is a hemibiotrophic pathogen (Kabir et al. 2014), whereas the other pathogens considered may be biotrophs or necrotrophs, and as a result, the resistance mechanisms against these pathogens could be quite different. This point is demonstrated by the fact that the hypersensitive response (HR) is an effective resistance mechanism against biotrophic pathogens, but may facilitate development of necrotrophic pathogens (see sections 2.2.1 and 2.2.5). Hemibiotrophic pathogens first establish a biotrophic interaction with a host, before switching to necrotrophy at later disease development stages.

This study reviews the current knowledge of resistance mechanisms that may play a role in the *Dothistroma*–*Pinus* pathogen–host system. In section 2, the mechanisms shown to be involved in the defence of Pinaceae against needle and shoot pathogens are discussed. In section 3, the role of plant associations with mutualist fungi is discussed. In sections 4 and 5, the observed variation in DNB susceptibility is assessed and hypotheses that may explain this variation are introduced. Section 6 covers the progress made in breeding for DNB resistance. In section 7, the implications of the sequencing of both the *D. septosporum* genome as well as that of several host species are considered. Finally, in section 8, some conclusions are drawn and several areas for further research are suggested. The information presented in this review will enable future research needs to be assessed, to fill knowledge gaps and establish a basis for integrated disease management of DNB.

2 Resistance mechanisms involved in the *Dothistroma*–*Pinus* system and other similar pathogen–host interactions

Different defence mechanisms act at various stages within the infection and disease development process. They include both pre- and post-penetration mechanisms and can be categorized as constitutive or induced, chemical or mechanical, systemic or local (Bonello et al. 2001; Bonello and Blodgett 2003; Luchi et al. 2005; Gordon 2006; Eyles et al. 2007; Gould et al. 2008). Defence mechanisms are also categorized based on whether they produce qualitative or quantitative resistance (Heijari et al. 2005; Bonello et al. 2006; Hammerschmidt 2006; Blodgett et al. 2007; Ganley et al. 2008; Krokene et al. 2008; Witzell and Martin 2008; Eyles et al. 2010). It is important to stress that defence and resistance are not one and the same thing; defence mechanisms act against pathogens, but may or may not confer resistance, which is the ultimate outcome of the host–pathogen interaction.

Several different methods have been employed to identify defence mechanisms that act against Pinaceae needle and shoot pests and pathogens. A commonly employed technique is to compare constitutive chemical/morphological traits of species/provenances/individuals that vary in relative susceptibility to a disease (Woo et al. 2001). It is important to recognize, however, that a defence trait is not necessarily linked directly to resistance, even if the two are correlated. Michelozzi et al. (1995), for example, found a link between the relative abundance of β -phellandrene and resistance to fusiform rust (caused by *Cronartium fusiforme* Hedgcock & Hunt ex Cummins) in both *Pinus elliotii* Engelm. and *Pinus taeda* L., but concluded that this terpenoid was not itself directly involved in defence (see section 2.2.3). These resistance ‘markers’ can still be used to screen for resistant individuals. Another similar method is to compare the chemical and morphological response of ‘resistant’ and ‘susceptible’ plants to inoculation with a given pathogen (Jurgens et al. 2003; Luchi et al. 2005; Jacobs et al. 2009). Host tissue cultures have also been used to determine responses to pathogen recognition (Hotter 1997). Finally, *in vitro* experiments have been used to investigate the impact of plant secondary metabolites on pathogen growth and their potential role in plant defence and resistance (Blodgett and Stanosz 1997).

2.1 Prepenetration

Several prepenetration defence mechanisms may play a role in the *Dothistroma*–*Pinus* interaction; these include needle surface topography, the quantity and composition of epi-cuticular and epi-stomatal waxes and other needle surface exudates. Campbell (1972) suggested that older *Pinus nigra* J.F. Arnold ssp. *laricio* (Poir.) Maire needles were more resistant to *Lophodermella sulcigena* (Link) Tubeuf than younger needles because of their thicker cuticle and epidermal cell walls. However, as *Dothistroma* infects needles through stomata it is unlikely that cuticle thickness is an important resistance mechanism in this system. Some morphological differences between the needles of DNB-resistant and DNB-susceptible individuals have been identified, which may be more relevant. Franich et al. (1977), for example, noted that *P. radiata* needle topography became rougher and stomata size decreased with tree age (as does DNB susceptibility, see section 4). Similarly, Woo et al. (2001) observed that *Pinus monticola* Douglas ex D. Don families susceptible to WPBR had wider and larger stomata. Conversely, Peterson and Walla (1978), probably working with *D. pini*, found that the number of stomata did not

Table 1. Defence mechanisms involved in interactions between Pinaceae and needle and shoot pathogens. See text for further details.

Defence mechanism	Pathogen/pest	Disease	Host	References
Defences acting pre penetration				
Needle surface topography	<i>Dothistroma septosporum</i>	<i>Dothistroma</i> needle blight	<i>Pinus radiata</i>	Franich et al. (1977)
Epicuticular wax	<i>Cronartium ribicola</i>	White pine blister rust	<i>P. strobus</i>	Smith et al. (2006a)
	<i>D. septosporum</i>	<i>Dothistroma</i> needle blight	<i>P. radiata</i>	Franich et al. (1983)
Epicuticular resinacids (diterpenes)	<i>D. septosporum</i>	<i>Dothistroma</i> needle blight	<i>P. radiata</i>	Franich et al. (1983)
Epistomatolwax occlusion	<i>C. ribicola</i>	White pine blister rust	<i>P. strobus</i>	Patton (1972), Smith et al. (2006a)
	<i>D. septosporum</i>	<i>Dothistroma</i> needle blight	<i>P. radiata</i>	Franich et al. (1977, 1983)
			<i>P. muricata</i>	Muir and Cobb (2005)
Stomatal morphology	<i>C. ribicola</i>	White pine blister rust	<i>P. strobus</i>	Woo et al. (2001)
Defences acting post-penetration	<i>D. septosporum</i>	<i>Dothistroma</i> needle blight	<i>P. radiata</i>	Franich et al. (1977)
Phenolic compounds	<i>C. ribicola</i>	White pine blister rust	<i>P. strobus</i>	Boyer (1964), Boyer and Isaac (1964), Jurgens et al. (2003), Jacobs et al. (2009)
				Lesney (1989)
	<i>Cronartium fusiforme</i>	Fusiform rust	<i>P. elliotii</i>	Wallis et al. (2008)
	<i>Diplodia sapinea</i>	Diplodia blight of pines	<i>P. nigra</i>	Reglinski et al. (1998)
			<i>P. radiata</i>	Blodgett et al. (2007)
	<i>D. septosporum</i>	<i>Dothistroma</i> needle blight	<i>P. resinosa</i>	Franich et al. (1986), Hotter (1997)
	<i>Elytoderma deformans</i> (Weir) Darker	Elytoderma needle cast	<i>P. radiata</i>	Wallis et al. (2010)
	<i>Lophodermella concolor</i> ;	Lophodermella needle cast	<i>P. contorta</i>	Wallis et al. (2010)
	<i>L. montivaga</i> Petrák		<i>P. contorta</i>	
Resin	<i>Sirococcus conigenus</i>	Sirococcus blight	<i>Picea abies</i>	Bahnweg et al. (2000)
	<i>D. septosporum</i>	<i>Dothistroma</i> needle blight	<i>P. radiata</i> ;	Cobb and Libby (1968)
			<i>P. muricata</i>	
	<i>Lecanosticta acicola</i>	Brown spot needle blight	<i>P. palustris</i>	Verral, 1934 (cited in Cobb and Libby 1968)
Terpenoids	<i>C. fusiforme</i>	Fusiform rust	<i>P. elliotii</i> ; <i>P. taeda</i>	Michelozzi et al. (1995)
	<i>D. sapinea</i>	Diplodia blight of pine	<i>P. nigra</i>	Wallis et al. (2008)
	<i>E. deformans</i>	Elytoderma needle cast	<i>P. resinosa</i>	Blodgett and Stanosz (1997)
	<i>L. concolor</i> ; <i>L. montivaga</i>	Lophodermella needle cast	<i>P. contorta</i>	Wallis et al. (2010)
	<i>C. ribicola</i>	White pine blister rust	<i>P. contorta</i>	Wallis et al. (2010)
Hypersensitive response			<i>P. flexilis</i>	Kinloch and Dupper (2002)
			<i>P. lambertiana</i>	Kinloch et al. (1970), Kinloch and Littlefield (1977)
			<i>P. monticola</i>	Kinloch et al. (1999)
			<i>P. strobiformis</i>	Kinloch and Dupper (2002)
			<i>P. taeda</i>	Wilcox et al. (1996)
			<i>P. taeda</i>	Gray and Amerson (1983)
			<i>P. armandii</i>	Hoff and McDonald (1975)
			<i>P. monticola</i>	Hoff and McDonald (1975)
			<i>P. strobus</i>	Jurgens et al. (2003), Jacobs et al. (2009)
			<i>P. radiata</i>	Hotter (1997)
			<i>P. monticola</i>	Ekramoddoullah et al. (2006), Liu et al. (2013a)
Antimicrobial peptides (AMP)	<i>D. septosporum</i>	<i>Dothistroma</i> needle blight	<i>P. monticola</i>	Liu et al. (2003, 2005, 2010)
	<i>C. ribicola</i>	White pine blister rust	<i>P. strobus</i>	Smith et al. (2006b)
Pathogenesis-related (PR) proteins	<i>C. ribicola</i>	White pine blister rust	<i>P. monticola</i>	McDonald and Hoff (1971), Liu and Ekramoddoullah (2011)
Needle shedding	<i>C. ribicola</i>	White pine blister rust	<i>P. wallichiana</i>	Heimbürger, 1962 (in McDonald and Hoff 1971)
Stationary interface	<i>L. sulcigena</i>	Lophodermella needle cast	<i>P. nigra</i> ssp. <i>laricio</i>	Williamson et al. (1976)
	<i>Plotoderma hedgcockii</i>	Plotoderma needle cast	<i>P. palustris</i>	Jewell (1990)

vary between resistant and susceptible *P. nigra* and *P. ponderosa* needles or between needle bases and tips, despite more symptoms observed at the tips.

Epicuticular waxes and other exudates may also have a role in prepenetration defence against needle and shoot pathogens. Certain epicuticular fatty and resin acids (particularly, 13-hydroxytodocarpic acid) inhibited *in vitro* conidial germination and mycelial growth of *D. septosporum* (Franich et al. 1983). Smith et al. (2006a) identified biochemical differences in the wax composition of *Pinus strobus* L. families resistant and susceptible to WPBR. *In vivo* experiments also showed that *D. septosporum* infection severity was significantly greater on *P. radiata* trees treated to remove needle surface waxes than on control trees (Franich et al. 1983). Similarly, inoculation of resistant *P. strobus* plants, pretreated to remove needle surface waxes, with *C. ribicola* led to the development of the same disease severity as that on susceptible families (Smith et al. 2006a). In contrast, Walla and Peterson (1976) found no relationship between *Pinus sylvestris* L., *P. ponderosa* and *P. nigra* needle surface wax quantities and resistance to either *D. pini* or *D. sapinea*.

It is possible that the results of the two *in vivo* wax removal experiments outlined above may have been due to the removal of epistomatal wax, as the occlusion of needle epistomatal chambers by wax may be a defence mechanism against fungal pathogens (Patton and Johnson 1970). The proportion of stomata occluded with wax is known to vary between species of pine, between provenances and with tree age. Hanover and Reicosky (1971), for example, observed that *P. sylvestris* epistomatal chambers were occluded, whereas those of *P. nigra* were not. Wax occlusion increased with tree age in *P. radiata* and *P. strobus*, as did resistance to DNB and WPBR, respectively (Patton 1972; Franich et al. 1977). Patton (1972) also observed that *P. strobus* secondary needles, which are less susceptible to WPBR than primary needles, had increased numbers of wax-occluded stomata. Muir and Cobb (2005) observed that a more DNB-resistant provenance of *Pinus muricata* D. Don produced more epistomatal wax, whereas a susceptible provenance had no wax-occluded stomata. Likewise, Smith et al. (2006a) observed that a WPBR-resistant *P. strobus* family had a significantly greater proportion of stomata occluded with wax than a WPBR-susceptible family. Woo et al. (2001), however, found no differences in stomata occlusion between WPBR-susceptible and WPBR-resistant *P. monticola* families.

Needle wettability, which is affected by both needle surface morphology and wax quantity and composition, can also impact on host susceptibility. Woo et al. (2001), for example, reported that WPBR-resistant *P. monticola* families had less wettable needles than those of susceptible families. A less wettable surface is more hydrophobic, causing water to run off more rapidly than on a wettable surface. Pathogen spores that disperse within water droplets, such as *Dothistroma* conidia, will, therefore, have less opportunity to adhere to a less wettable needle surface before water droplets run-off. Also, as *D. septosporum* requires foliage to be wet for long periods of time to allow for successful infection and disease development (Gadgil 1974, 1977), it is clear that wettability may be a very important, and, as yet, overlooked factor in the *Dothistroma*–*Pinus* system.

2.2 Post-penetration

Several observations suggest that defence mechanisms against *Dothistroma* infection that act within needles may be more important than those acting on the needle surface. Muir and Cobb (2005), for example, noticed the presence of empty and disintegrating substomatal vesicles in needles of more DNB-resistant *P. muricata* individuals and suggested the importance of post-penetration mechanisms in defence against the pathogen. In the same work, no correlations were found between stomatal penetration by *D. septosporum* and resulting disease severity during artificial inoculation experiments (Muir and Cobb 2005). Several authors have also reported no differences in *Dothistroma* conidia germination, growth and penetration on the needle surfaces of resistant and susceptible individuals (Ivory 1972; Walla and Peterson 1976; Peterson and Walla 1978). Possible post-penetration defence mechanisms include those constitutively present in needles of Pinaceae, such as sequestered metabolites (e.g. phenolic and terpenoid compounds) and physical barriers (e.g. lignified tissues), and those that are induced, such as phytoalexins, antimicrobial peptides (AMPs), PR proteins and the HR. These induced defence mechanisms are triggered by signalling molecules of varied metabolic origin, such as terpenoids, phenolic compounds, alkaloids, certain hormones and pheromones (Zeneli et al. 2006; Gould et al. 2008; Krokene et al. 2008; Symonds and Elgar 2008; Witzell and Martin 2008; Pieterse et al. 2009).

2.2.1 Soluble phenolic compounds

Phenolic compounds have several different roles in defence of plants; many are directly antifungal, while others bind extracellular enzymes produced by pests and pathogens, inactivate fungal toxins and elicitors, and disrupt membranes (Vance et al. 1980; Witzell and Martin 2008). Franich et al. (1986) found that benzoic acid, a simple phenolic compound that is highly fungistatic *in vitro*, accumulated in *P. radiata* needles after dothistromin injection and suggested that it was therefore a phytoalexin. The same authors showed that benzoic acid is also found in natural *D. septosporum* lesions on *P. radiata* needles. Benzoic acid accumulation in the dothistromin-injected needles was moderately correlated with *P. radiata* DNB-field resistance, further suggesting its role as a phytoalexin. Benzoic acid, like other phytoalexins, is also toxic to host tissue and its accumulation may, therefore, be involved in, or be a consequence of, host cell death. Cell death may be involved in a programmed cell death (apoptosis) response as part of a defensive mechanism, or may just be a by-product of the toxicity of benzoic acid. Either way, dead host tissue will provide nutrition for the necrotrophic lifestyle stage of *Dothistroma* spp. (see section 2.2.5).

There is no published work on the possible impact of other soluble phenolic compounds on DNB severity, although work has been reported from other similar systems. Pinosylvin, a constitutive stilbene in pine heartwood, inhibited spore germi-

nation and mycelial growth of both *D. sapinea* and *D. scrobiculata* J. de Wet, B. Slippers & M.J. Wingf., *in vitro* (Blodgett and Stanosz 1997). Further support for the defensive role of soluble phenolics comes from experiments with *P. nigra* where quantitative differences in constitutive phenolic compounds were negatively correlated with susceptibility to *D. sapinea* (Wallis et al. 2008). Moreover, quantities of several constitutive needle phenolic compounds were negatively associated with severity of both Lophodermella and Elytroderma needle cast on *Pinus contorta* Dougl. ex Loud. in the field (Wallis et al. 2010). Wallis et al. (2010), however, found that constitutive flavonoid concentrations were positively associated with susceptibility to Lophodermella needle cast in *P. contorta*, suggesting that these compounds may have other non-defence-related functions.

Plants in the Pinaceae also synthesize and accumulate phenolic compounds in response to pathogen attack, although apart from the role of benzoic acid, discussed above, this process has not been investigated in the *Dothistroma*–*Pinus* interaction. However, the action of phenolic compounds in defence has been studied in other host–pathogen systems. For example, in WPBR-resistant *P. strobus* needles inoculated with *C. ribicola*, phenol-containing vacuoles, that are present constitutively, fragment and release phenolic compounds into the cytoplasm; deposition of phenolic compounds occurs on the host cell wall, leading to encapsulation and death of the fungi (Boyer 1964; Boyer and Isaac 1964; Jurgens et al. 2003; Jacobs et al. 2009). Despite the finding by Wallis et al. (2010) that constitutive flavonoid concentrations were positively associated with *P. contorta* susceptibility to Lophodermella needle cast (noted above), the same authors found that Lophodermella and Elytroderma needle cast field infections induced systemic foliar flavonoid accumulation, potentially indicating a role in defence. Similarly, *Sirococcus conigenus* (DC.) P.F. Cannon & Minter inoculation of *Picea abies* L. and *D. sapinea* inoculation of *P. nigra* both induced accumulation of phenolic compounds in needles (Bahnweg et al. 2000; Wallis et al. 2008). Interestingly, Wallis et al. (2008) also found evidence for systemic induced resistance, as the systemic accumulation of phenolic glycosides and stilbenes in *P. nigra* was negatively correlated with susceptibility to later *D. sapinea* inoculations. Importantly, the same authors found that, together, phenolic glycosides, lignin and stilbenes had a stronger relationship with *P. nigra* susceptibility than any compounds alone. This finding demonstrates that host plants deploy a combination of broad-spectrum defences in response to pathogen attack. As yet, no research has been published on systemic induced resistance in the *Dothistroma*–*Pinus* interaction.

2.2.2 Cell wall-bound phenolic and polyphenolic compounds

In addition to the chemical defences within the needle, physical barriers also have important defensive roles. These include cell wall-bound phenolic and polyphenolic compounds, such as lignin, that can restrict pathogen growth (Vance et al. 1980; Miedes et al. 2014). During *in vitro* experiments, lignin and other cell wall-bound phenolics accumulated in *P. radiata* cell suspension cultures after exposure to *D. septosporum* cell wall elicitors (Hotter 1997). The activity of both phenylalanine ammonia-lyase (PAL) and cinnamyl alcohol dehydrogenase, two enzymes key to the biosynthesis of phenolic compounds, increased dramatically in these cell suspension cultures (Hotter 1997). Hotter (1997) also observed an oxidative burst – the accumulation of hydrogen peroxide (H_2O_2) – which, apart from being implicated in hypersensitive host cell death, mediates cross-linking of phenolic compounds into the host cell wall, increasing resilience against pathogen attack (Levine et al. 1994). *Cronartium fusiforme* mycelial elicitors had a similar effect on *P. elliotii* cell suspensions *in vitro*, leading to elevated lignin-like compounds in the medium and in host cell walls (Lesney 1989). *In planta* studies with purified dothistromin showed that lignin and other cell wall-bound phenolic compounds accumulate in *P. radiata* needles after injection with dothistromin (Franich et al. 1986). Systemic induction of lignin accumulation in needles was also observed in *P. contorta* infected by *Lophodermella* spp. (Wallis et al. 2010) and, along with phenolic glycosides and stilbenes, lignin also accumulated in *P. nigra* after inoculations with *D. sapinea* (Wallis et al. 2008). These increases in lignin content in *D. sapinea* inoculated *P. nigra* were negatively correlated with susceptibility to later *D. sapinea* inoculations (Wallis et al. 2008). Furthermore, salicylic acid application reduced *P. radiata* susceptibility to *D. sapinea* inoculation (Regliński et al. 1998), an effect that was associated with increased PAL activity.

2.2.3 Terpenoid compounds

Terpenoids are the main constituents of plant resins and have long been associated with defence against both pests and pathogens (Sell 2003; Cheng et al. 2007). Production of volatile monoterpenes, such as limonene and the pinenes, is under strong genetic control and was previously used to both characterize the provenances and study the population genetics of different *Dothistroma* hosts, including *P. sylvestris* and *P. contorta* (Forrest 1980a,b; Kinloch et al. 1986). In foliage of the Pinaceae, resin is formed in resin canals that run longitudinally through the needles. The number of resin canals in a needle varies both inter- and intraspecifically (Steven and Carlisle 1959; Cobb and Libby 1968; Wu and Hu 1997). This variation in resin canal number has previously been linked to relative susceptibility of trees to DNB: resin canals were more abundant in needles from less susceptible Guadalupe Island and Cedros Island *P. radiata* populations and *P. muricata* than in needles from the more susceptible Monterey *P. radiata* population (Cobb and Libby 1968). It must be noted, however, that Guadalupe Island and Cedros Island *P. radiata* trees were more susceptible to DNB than the Monterey provenance when planted in New Zealand (Burdon and Bannister 1973). It would be interesting to determine whether the number of resin canals remains stable between sites. *Pinus palustris* Mill. seedlings with greater numbers of resin canals per needle were also less susceptible to *Lecanosticta acicola* (von Thümen) Sydow, the cause of brown spot needle blight, a very similar disease to DNB (Verrall, 1934 cited in Cobb and Libby 1968).

As is the case with certain phenolic compounds, some monoterpenes can inhibit pathogen growth in culture. For example, α -pinene, β -pinene and δ -3-carene inhibited *in vitro* spore germination and mycelial growth of *D. sapinea* and *D. scrobiculata* (Blodgett and Stanosz 1997). Franich et al. (1982), on the other hand, found that, in more realistic *in vitro* experiments with volatile monoterpene mixtures (rather than individual compounds), *D. septosporum* germination was stimulated at most concentrations tested (10–300 ppm), while mycelium growth was only inhibited at higher volatile concentrations (1000 ppm).

Other *in planta* studies suggest that monoterpenes act as defence mechanisms against needle pathogens. Wallis et al. (2010) reported that five *P. contorta* needle monoterpenes were negatively associated with susceptibility to Lophodermella and Elytroderma needle casts. Michelozzi et al. (1995) also reported that high concentrations of β -phellandrene in *P. elliotii* and *P. taeda* were markers for fusiform rust resistance; however, it was suggested that β -phellandrene itself was not toxic to the pathogen, but rather that the gene responsible for this chemotype was likely linked with other resistance genes. Similarly, Aitken (1993) observed a correlation between high β -phellandrene content and resistance in *P. sylvestris* to *Gremmeniella abietina* (Lagerb.) Morelet a shoot pathogen that does not infect through intact needles. Although Franich et al. (1982) found that the proportion of δ -3-carene increased with tree age, volatile monoterpenes were more abundant in foliage from younger *P. radiata* trees than in foliage from more resistant older trees. The same authors also found that the proportion of both β -phellandrene and β -pinene decreased with age. These authors concluded that no simple relationship existed between *P. radiata* age-induced DNB resistance and needle monoterpene abundance.

Needle monoterpene abundance and composition can also respond to pathogen attack and contribute to systemic resistance. Wallis et al. (2008) established that β -pinene accumulation in *P. nigra* after fungal induction was negatively correlated (marginally) with later susceptibility to *D. sapinea*. As with phenolic compounds, the impact of *Dothistroma* infection on the composition and abundance of foliar terpenoids has yet to be investigated.

2.2.4 Pathogenesis-related proteins and antimicrobial peptides

Pathogenesis-related (PR) proteins are either antimicrobial or involved in strengthening of host cell walls. These proteins include hydrolytic enzymes, such as chitinases and β -1,3-glucanases, which attack fungal cell walls (Liu et al. 2005). Host plants also produce peroxidases and laccases that are involved in cross-linking reactions and lignification of the host cell wall (Mayer and Staples 2002). PR proteins have been implicated in the defence reaction against WPBR in both *P. monticola* and *P. strobus*. In *P. monticola* needles, both *C. ribicola* inoculation and needle wounding led to the accumulation of PR10 proteins (PR family 10; Liu et al. 2003). *Cronartium ribicola* inoculation also induced chitinase (PR family 3) and thaumatin-like protein (PR family 5) accumulation in *P. monticola* needles (Liu et al. 2005, 2010). Likewise, in *C. ribicola*-inoculated *P. strobus*, six proteins with homologues with known resistance roles in other plants were upregulated in WPBR-resistant seedlings (Smith et al. 2006b). As of now, no work has been published in this area with *Dothistroma*. For a review of PR proteins in forest tree species, see Veluthakkal and Dasgupta (2010).

Antimicrobial peptides are produced in Pinaceae foliage in response to pathogen attack. AMP genes are upregulated in *P. monticola* needles during the early stage of *C. ribicola* infection (Liu et al. 2013a), and needles from inoculated WPBR-resistant *P. monticola* trees had higher levels of AMPs than those from similarly treated susceptible trees (Ekramoddoullah et al. 2006). AMPs are also induced in roots and shoots of *P. sylvestris* after inoculation with other pathogen species (Adomas and Asiegbu 2006; Adomas et al. 2008). However, there remains a lack of in-depth information on the function and antimicrobial activity of AMPs from *Pinus* spp. (Manners 2009), and no work has been done in this area with *Dothistroma*.

2.2.5 Hypersensitive and hypersensitive-like responses

Pathogen attack can result in the HR, where direct or indirect recognition of a pathogen effector leads to the rapid death of the affected plant cell(s) and containment/inhibition of biotrophic pathogens (Kinloch and Dupper 2002). Delayed HR reactions have been observed in the needles of four WPBR-host pine species, *Pinus lambertiana* Dougl., *Pinus monticola*, *Pinus flexilis* E. James and *Pinus strobiformis* Engelm. in response to inoculations with *C. ribicola* (Kinloch et al. 1970, 1999; Kinloch and Littlefield 1977; Kinloch and Dupper 2002). Mendelian segregation suggests that a single dominant allele conferred resistance in three of these species (*CR1*, *P. lambertiana*; *Cr2*, *P. monticola*; *Cr3*, *P. strobiformis*), and a *Cr4* allele has recently been identified in *P. flexilis* (Schoettle et al. 2014). Host cell death resembling HR has been observed in *P. taeda* embryos in response to *C. fusiforme* infection, the speed of the response correlating with resistance (Gray and Amerson 1983). Furthermore, genomic mapping identified a single dominant allele associated with resistance against *C. fusiforme* in *P. taeda* (*Fr1*, Wilcox et al. 1996). HR-like reactions have also been observed in the needles of WPBR-resistant individuals of *Pinus armandii* Franch and *P. strobus*; rapid host cell death which stops the progression of the pathogen was observed in the immediate area of infection (Hoff and McDonald 1975; Jurgens et al. 2003; Jacobs et al. 2009).

The rapid accumulation of H_2O_2 after infection (mentioned above), as seen in *P. radiata* cell suspension cultures exposed to *D. septosporum* cell wall elicitors (Hotter 1997), is commonly involved in hypersensitive cell death (Levine et al. 1994). To date, however, no HR has been reported in DNB-infected needles, and DNB resistance, in *P. radiata* at least, has been shown to be polygenic (see section 6, below). Recent research revealed that the *D. septosporum* genome contains homologues of *Cladosporium fulvum* Cooke (syn. *Passalora fulva* (Cooke) U. Braun & Crous) effector genes, including *Ecp2* and *Avr4*, encoding effector proteins that are recognized by tomato (*Solanum lycopersicum* L.) resistance proteins, inducing HR in this plant (de Wit et al. 2012). As most plant–fungal pathogen relationships proven to have a gene-for-gene pattern of resistance involve a biotrophic pathogen (de Wit et al. 2009), the presence of effector homologues within the *D. septosporum*

rum genome may seem surprising, as *D. septosporum* is considered to have a hemibiotrophic lifestyle (de Wit et al. 2012; Kabir et al. 2014). Recently, hemibiotrophic pathogens have been found to initially produce effectors to suppress cell death, but these are downregulated at later stages when necrotrophic effectors are induced (Vleeshouwers and Oliver 2014). As necrotrophs are able to exploit oxidative bursts and programmed cell death for nutrition (Vleeshouwers and Oliver 2014), another possible explanation for the presence of effector genes is the hijacking of the plant HR reaction by *Dothistroma* during the necrotrophic stage. These findings demonstrate the importance in distinguishing between necrotrophic, biotrophic and hemibiotrophic pathogens, as pathogen lifestyle has important implications for the effectiveness of different host defence mechanisms.

2.2.6 Stationary interfaces and needle shedding

Other general physical defences may include the production of stationary interfaces, which are areas of cell death and intercellular matrices that physically block the progress of pathogens within needles. These interfaces have been observed in *P. nigra* ssp. *laricio* needles infected with *L. sulcigena* and in *P. palustris* needles infected with *Ploioderma hedgcockii* (Dearn.) Darker (Williamson et al. 1976; Jewell 1990). Williamson et al. (1976) reported that prior to hypertrophy and hyperplasia of cells at the host–parasite interface in *L. sulcigena*-infected *P. nigra* ssp. *laricio*, a stationary interface is formed in response to infection. After the fungus colonizes intercellular areas of mesophyll, endodermis, hypodermis and epidermis, a stationary interface, comprising a fungal-free zone, dead mesophyll cells and an intercellular matrix, is formed. The matrix may provide a physical barrier in needle tissue to prevent the pathogen from advancing into healthy tissue. Jewell (1990) reported similar observations in *P. hedgcockii*-infected *P. palustris* needles; however, no hypertrophy or hyperplasia was observed, nor invasion of the endodermis. In both cases, hyphae grew extensively in diseased tissue, but were not observed in or beyond the matrix into healthy tissue. No similar research on the host morphological response to *Dothistroma* invasion has been published.

An effective plant defence against shoot pathogens is the shedding of infected foliage; the plant sacrifices needles, preventing the pathogen from reaching the branches and stem and potentially girdling and killing them. For example, needle shedding occurs on WPBR-resistant plants of both *P. monticola* and *Pinus wallichiana* A. B. Jacks (Heimbürger, 1962 cited in McDonald and Hoff 1971). Liu and Ekramoddoullah (2011) identified the gene *PmTNL1* as having a possible role in *P. monticola* needle shed. Although a well-known symptom of DNB is premature needle loss, it is unclear whether needle shed has a role in resistance against *Dothistroma*. A similar form of WPBR resistance seen in *P. monticola* is ‘short stem resistance’; *C. ribicola* is able to grow normally throughout the needle until it reaches the short shoot at the needle base where a HR-like response occurs and host cell necrosis results in death of the pathogen (Hoff and McDonald 1971). It is currently unclear what mechanisms are involved in blocking the progression of *Dothistroma* into the stem. Hunt et al. (2011) suggested that *Dothistroma* infection lead to the development of stem lesions on *P. monticola*; however, *Dothistroma* was not isolated from these lesions, and similar symptoms have not been reported by any other authors. Shedding *Dothistroma*-infected needles would also potentially remove a source of inoculum from tree crowns, although conidiomata are usually formed before needles are shed.

2.3 Tolerance

Another type of resistance not yet examined in the *Dothistroma*–pine interaction is tolerance. Tolerance has two definitions. Firstly, tolerance is one of several different types of resistance, where symptom development is inhibited or limited despite pathogen proliferation within the host (Palukaitis 2012). For example, *Rhynchosporium commune* Zaffarano, McDonald and Linde infection without symptom development has been observed recently in barley (*Hordeum vulgare* L.) (Walters et al. 2012). Sometimes, the pathogen might actually be seen as a mutualist or endophyte. For example, some *Verticillium* isolates increase the growth of host individuals despite being pathogens on other host individuals/species (Robb 2007). Similarly, *Cyclaneusma minus* (Butin) DiCosmo, Peredo & Minter, a needle pathogen, is able to infect and act as an endophyte throughout its life cycle on some *P. radiata* genotypes, but acts as a serious pathogen on other genotypes of the same host species (McDougal et al. 2012). Furthermore, latent infection (infection without symptom development) of *P. nigra* and *P. sylvestris* by *D. sapinea* has also been observed (Flowers et al. 2001). Secondly, tolerance may describe a situation where symptoms develop, but yield (i.e. growth or fecundity) is less affected than the average loss for the observed level of disease severity (Walters et al. 2012). Mechanisms include loss of foliage lower in the canopy (which has less effect on plant photosynthetic capacity) and increases in growth or photosynthesis in unaffected parts of the plant. With either definition, a tolerant host will benefit from supporting a pathogen that damages its competitors (if they are susceptible). No specific research has yet been published on the role of tolerance mechanisms on the susceptibility of hosts to DNB, either looking for evidence of *Dothistroma* behaving as an endophyte or variation in the impact of DNB infection/symptom development on yield.

3 The role of endophytic and ectomycorrhizal fungi

Endophytes have co-evolved with their hosts for millions of years, and it is well known that numerous species of needle endophytes are found within the foliage of the Pinaceae (Bernstein and Carroll 1977; Ganley et al. 2004; Sieber 2007). Endophyte species may play a role in host–pathogen interactions and host resistance (Kogel et al. 2006; Ganley et al. 2008; Rodriguez et al. 2009). Endophytes may act in several different ways: through direct antagonism against parasites by com-

petition or parasitism, by production of secondary metabolites that inhibit insects and pathogens, secretion of elicitors that induce the plant to produce inhibitory secondary metabolites, or by stimulating plant growth and vigour (Porras-Alfaro and Bayman 2011). The benefits of endophytes and their potential as tools for biological control are well known (Backman and Sikora 2008). However, other authors cast serious doubt on the mutualistic effect of endophytes (Sieber 2007), particularly in the case of native plants (Faeth 2002), and commercially viable biocontrol products for use in the forest are not yet widely available.

Ganley et al. (2008) found that *P. monticola* seedlings inoculated with certain fungal endophytes prior to inoculation with *C. ribicola* lived longer than endophyte-free seedlings and showed reduction in disease severity. Romeralo et al. (2015) demonstrated that the presence of several endophytes reduced the damage caused by *G. abietina* in *Pinus halepensis* Mill. seedlings. Similar results have been reported for other plant tissues; for example, root inoculation with a non-pathogenic endophytic *Rhizoctonia* sp. lowered *P. sylvestris* susceptibility to later infection by a pathogenic *Rhizoctonia* sp. (Grönberg et al. 2009). Interestingly, Regliński et al. (2012) found that *Trichoderma atroviride* Karsten root endophyte treatments enhanced systemic resistance to *D. sapinea* in *P. radiata*. Benefits of mutualistic associations between ectomycorrhizal fungi and plant roots in reforestation (Kropp and Langlois 1990) and their applications in biocontrol (Duchesne 1994) are well known. In this regard, Garrido et al. (1982) observed that *P. radiata* trees associated with Russulaceae ectomycorrhizae were not attacked by *D. septosporum*, *D. sapinea* or *Armillaria* species. *In vitro* experiments also showed that extracts from these mycorrhizal fungi, or from needles on trees associated with them, produced strong growth inhibition of *Dothistroma* conidia. These findings demonstrate the potential risk of using broad target fungicides to control DNB, as beneficial or mutualistic fungi may also be affected. Experiments in Britain are now underway to assess the impact of fungicides on both needle endophytes and root mycorrhizae (K. Tubby, UK Forestry Commission, personal communication).

In vitro experiments showed that *Trichoderma* isolates have a fungicidal effect on *D. septosporum*, while certain *Bacillus* isolates have a fungistatic effect (McDougal et al. 2011). A recent *in planta* study found that the inoculation of *P. ponderosa* needles with *Penicillium goetzii* J. Rogers, Frisvad, Houbraken & Samson, isolated from *P. ponderosa* root material, before exposure to natural *D. septosporum* infection reduced DNB severity (Ridout and Newcombe 2015). However, the same authors reported that inoculation with four needle endophyte species (*Bionectria ochroleuca* (Schwein.) Schroers & Samuels, *Elytroderma* sp., *Penicillium raistrickii* Smith and *Sydowia polyspora* (Bref. & Tavel) E. Müll) increased DNB severity. Both the interaction between endophytes and *Dothistroma* and the potential use of biological control agents against *Dothistroma* are areas for further research.

4 Observed variation in DNB susceptibility

Variation in susceptibility to DNB infection and disease development has been observed at several different levels. Interspecific variation has been reported for a range of host species (see Table 3 in Watt et al. 2009). Intraspecific variation includes variation between different individuals within a stand, trees of different age classes, and between populations and provenances.

Based on both field observations and experimental trials, several authors have reported interspecific variation in susceptibility to DNB. Published attempts to rank species by relative susceptibility are mostly based on surveys of naturally infected trees in arboreta, field trials and mixed stands (Gibson et al. 1964; Peterson 1967; Cobb and Miller 1968; Muir and Cobb 2005). However, some are based on experimental trials with limited numbers of species and provenances (Gibson et al. 1964; Cobb and Libby 1968; Fraser et al. in press). Of the 89 host species listed by Watt et al. (2009), 26 were classified as slightly susceptible, 22 as moderately susceptible and 16 as highly susceptible. A further 13 pine species have unknown susceptibility, and the remaining 12 pine species were rated differently by different authors.

Between-provenance variation in susceptibility to DNB has been reported for several *Pinus* species, including *Pinus caribaea* Morelet (Ivory 1968), *P. contorta* (Gilmour and Noorderhaven 1969), *P. elliottii* (Gibson 1972), *P. monticola* (Hunt et al. 2011), *P. muricata* (Ades et al. 1992; Muir and Cobb 2005), *P. nigra* (Peterson and Read 1971), *P. ponderosa* (Eldridge et al. 1980; Peterson 1984), *P. radiata* (Cobb and Libby 1968; Burdon and Bannister 1973; Power and Dodd 1984; Ades and Simpson 1991), *P. sylvestris* (Fraser et al. 2014) and *Pinus tecunumanii* Eguluz & J. P. Perry (Rodas et al. 2015). As discussed above, one way of identifying possible resistance mechanisms is to compare the morphology and chemistry of susceptible and resistant provenances. For the examples above, however, no clear evidence for the causal defence mechanisms has yet been found.

Importantly, the relative susceptibility of provenances often varies between sites. Although susceptibility rankings of *P. muricata* provenances were consistent between California (Muir and Cobb 2005) and Australia (Ades et al. 1992), and rankings of *P. radiata* provenances were consistent within the same geographical area, rankings of *P. radiata* were reversed between Oceania (Burdon and Bannister 1973; Ades and Simpson 1991) and California (Cobb and Libby 1968; Power and Dodd 1984). Furthermore, the relative DNB susceptibility of Scottish *P. sylvestris* populations varied between experimental sites within Scotland (Fraser et al. in press). Ideally, reciprocal common garden experiments should be initiated with a variety of species and provenances on a range of moderate-to-high DNB risk sites around the world. It is not currently known whether variation in relative susceptibilities is caused by local adaptation in the host or pathogen, or a combination of the two.

At an early stage, it was recognized that disease incidence and severity on *P. radiata* is related to tree age, that is resistance has an ontogenetic component (Gibson et al. 1964; Gilmour 1967; Ivory 1972). Bassett (1972) observed that, in New Zealand, *P. radiata* became more resistant to DNB after c. 15 years and that there was no need to apply fungicides after this time. Several experiments also showed that rooted *P. radiata* cuttings were less susceptible to DNB than seedlings (e.g.

Burdon and Bannister 1973, 1985; Gadgil and Holden 1976; Power and Dodd 1984; Ades and Simpson 1990). Age-induced (ontogenetic) DNB resistance has also been noted in other pine species. Ivory (1968), for example, reported mature-plant resistance in *P. canariensis* C. Sm. and *P. elliottii*. Ades et al. (1992) also found that, although *P. muricata* trees younger than 4 years old were moderately susceptible to DNB, older trees from three provenances developed a high degree of field resistance. There is also an interaction between age and environment; however, as *P. radiata* trees still showed heavy infection well after 15 years in some areas (Bassett 1972; Ades and Simpson 1991). Other host species, such as *P. nigra* ssp. *laricio* and *P. contorta*, remain susceptible throughout their lives (Woods et al. 2005; Brown and Webber 2008).

There is also an indication that for some species, including *P. nigra* and *P. ponderosa*, older foliage may be more susceptible to DNB than younger foliage (Peterson 1967). However, for other species, including *P. radiata*, there appears to be little relationship between needle age and susceptibility (Gibson et al. 1964).

5 The location of resistant individuals

Several hypotheses have been proposed to predict where hosts showing resistance to DNB should be found. Most of these hypotheses predict that resistant individuals should be found in areas where there has been a long history of high pathogen pressure, which has selected for the expression of resistance mechanisms in the host population. Even for species that are not 'native' hosts of a pathogen species it is reasonable to expect that host populations that have evolved on sites optimal for similar indigenous pathogens will express resistance mechanisms that, being broad-spectrum in nature, may also be effective against other 'alien' pathogens.

One such hypothesis is the 'latitudinal defence hypothesis' (as described in Moles et al. 2011). This hypothesis suggests that expression of host defence mechanisms will be greater at lower latitudes because larger herbivore (and presumably pathogen) populations are supported by higher temperatures and longer growing seasons at these latitudes. There is, however, little empirical evidence for this idea and Moles et al. (2011), who conducted a meta-analysis on the subject, concluded that the literature does not support this hypothesis.

In analogy to the latitudinal defence hypothesis, the 'elevational defence hypothesis' suggests that defence mechanism expression will be greater within host populations from lower elevations because larger pest (and presumably pathogen) populations are supported in these areas. There is some support for this hypothesis within the Pinaceae. For example, crown damage to *P. contorta* caused by *Zeiraphera diniana* Guenée (larch tortrix) was positively associated with provenance elevation (Day et al. 1991). Wu et al. (1996) also found that *P. contorta* provenances from higher disease-risk areas were more resistant to several pests and pathogens (*Cronartium coleosporioides* J.C. Arthur, *Endocronartium harknessii* (J.P. Moore) Y. Hiratsuka, *Lophodermella concolor* (Dearness) Darker and *Synanthedon sequoiae* Edwards), with resistance declining at increasing provenance elevation. In Idaho, USA, susceptibility of *P. contorta* to *L. concolor* also increased with the elevation of both provenance and family (Hoff 1985). Similarly, in Alberta, Canada, Yang et al. (1997) found that high-elevation *P. contorta* families were more susceptible to *E. harknessii*. Interestingly, there was an indication that *P. contorta* provenance susceptibility to DNB in New Zealand may have been related to provenance elevation; some coastal provenances were completely uninfected, whereas one provenance from above 2000 m was almost completely defoliated (Gilmour and Noorderhaven 1969).

Other hypotheses include the hybridization (or introgression) hypothesis. Wu et al. (1996) working in a *P. contorta* provenance trial in Prince George, BC, reported a strong relationship between pest incidence and provenance distance from the western limit of the natural range of *Pinus banksiana* Lamb., with high resistance of provenances in the *P. contorta*-*P. banksiana* hybridization zone. It was hypothesized, therefore, that introgression from *P. banksiana* may have played a significant role in the evolution of resistance in *P. contorta*. Further support for this hypothesis comes from provenance trials on a range of sites (Wu and Ying 1998); *P. contorta* provenances resistant to both *E. harknessii* and *L. concolor* were concentrated in the hybrid zone and adjacent areas and the closer a provenance was to the limit of *P. banksiana* distribution the higher and more stable its resistance to both diseases. Fusiform rust resistance in the western range of *P. taeda* has also been linked to introgression from *Pinus echinata* Mill. (Hare and Switzer 1969). Furthermore, although *Pinus patula* Schiede ex Schltdl. & Cham. is susceptible to *Fusarium circinatum* Nirenberg & O'Donnell, its hybrid with *P. tecunumanii* is resistant (Mitchell et al. 2012). It is clear, however, that hybridization does not always confer resistance, as the *Pinus attenuata* Lemmon X *radiata* hybrid, for example, is highly susceptible to DNB (Cobb and Miller 1968).

There is some support for the hypothesis that geographical isolation of host populations and provenances influences disease susceptibility. In one *P. contorta* seed orchard in interior BC, for example, Wallis et al. (2010) found that isolated provenances were more susceptible to a range of native diseases than those in more continuous stands. This phenomenon is explained by the fact that there would be less inoculum build up in isolated stands and, therefore, less selection for resistance within the populations. The authors noted that, as these isolated stands were surrounded by *P. ponderosa* (highly susceptible to DNB), it was highly unlikely that this would also be the case with DNB susceptibility for the studied populations.

The most important environmental variable known to impact the incidence and severity of DNB (Woods et al. 2005), as well as some other needle diseases (Hoff 1985), is summer precipitation (or warm rain events). Thus, it is hypothesized that areas with a history of high levels of summer precipitation (or frequent warm rain events) will have experienced more needle disease epidemics than areas with lower precipitation and will therefore be more resistant to these pathogens. This trend has been found for other insect pests and diseases, for example in resistance to a number of *P. contorta* insect pests and pathogens, including *E. harknessii* and *L. concolor* (Wu et al. 1996; Yang et al. 1997; Wu and Ying 1998). Furthermore, Wallis et al. (2011) investigated the foliar secondary metabolites of *P. contorta* growing within different biogeoclimatical zones in Central BC and found higher levels in trees growing in rainforest areas than those growing in drier areas. It was

suggested that this trend could be linked to either greater pest and pathogen pressure or longer growth seasons, which allowed the accumulation of greater resources by the trees, in rainforest areas. Ades and Simpson (1991), however, found no relationship between the susceptibility of *P. radiata* provenances to DNB and precipitation in areas of host origin. Similarly, Fraser et al. (2014) found no relationship between the susceptibility of native Scottish *P. sylvestris* populations to DNB and precipitation in their area of origin.

6 Breeding for resistance

Breeding programmes for resistance to DNB and research on the heritability of DNB resistance have focused on *P. radiata*. Early breeding programmes for *P. radiata* DNB resistance were established in East Africa and New Zealand in the 1960s (Ivory and Paterson 1970; Wilcox 1982), and seed of a resulting line with increased DNB resistance has been available for use in New Zealand since the 1980s (Carson 1989). Carson (1989) found that resistance was quantitative (polygenic) and reported a narrow sense heritability mean of 0.24 for DNB severity. Results from Australia also produced similar heritability estimates (Ivković et al. 2010). Ades et al. (1992) reported similar heritability estimates for *P. muricata*. Recently, the *P. radiata* breeding lines were combined to form one New Zealand breeding population where DNB resistance is treated as a non-key trait, selected for in the production population (Kennedy et al. 2014). Because of a strong correlation between DBH (diameter at breast height) and DNB resistance, selection for growth alone increased DNB resistance (Kennedy et al. 2014).

Breeding programmes for disease resistance in *P. radiata* have had a good likelihood of success in New Zealand and Australia because the *D. septosporum* population is clonal, has a small effective population size and shows strong founder effects (Hirst et al. 1999; Groenewald et al. 2007; McDougal et al. 2011; Barnes et al. 2014). The clonal nature of the *D. septosporum* population in New Zealand means that qualitative resistance (if it is found) would be more durable than in more diverse regions or where both mating types are found. The success of breeding for resistance in New Zealand has also been enhanced by the fact that *P. radiata* is usually only susceptible for the first 15 years of growth, and chemical control is employed to reduce the pathogen population size (Bulman et al. 2013). The presence of both *D. septosporum* and *D. pini* mating types in Europe and North America (Groenewald et al. 2007; Barnes et al. 2011, 2014; Dale et al. 2011) will enable the pathogens to evolve more effectively in these areas, complicating efforts to develop resistant trees. Other factors may also hamper breeding efforts in these regions. For example, Brown and Webber (2008) point out that a *P. nigra* ssp. *laricio* breeding programme in the UK will be hindered, as follows: (i) the host species is susceptible to DNB throughout its life; (ii) high inoculum levels build up because of a lack of chemical control; and (iii) sexual recombination occurs within the pathogen population.

As resistance to DNB in *P. radiata* cannot be reliably selected in the field until plants are at least 3 years old (Carson 1989; Ades and Simpson 1991), several authors have looked for more rapidly identifiable markers for resistance. Few, however, have been successful so far. Gibson et al. (1964) could find no morphological features of needles that related to differences in susceptibility. Bergmann et al. (1995) also found no evidence for an association between pine seed embryo traits and DNB resistance. Shain and Franich (1981) found that the introduction of dothistromin into wounds made with a hypodermic needle induced the formation of larger lesions in susceptible *P. radiata* clones than in 'field resistant' clones. However, Franich et al. (1986) reported an opposite effect and therefore concluded that dothistromin-induced lesion length was not a good measure of field resistance in *Pinus radiata*. Devey et al. (2004) were able to identify quantitative trait loci for DNB resistance in *P. radiata* in Australia, which can be used as markers for DNB resistance and should contribute to future resistance breeding.

7 Genome sequencing of *D. septosporum* and host species

Effectors are emerging as important tools for identification of major resistance genes against biotrophic, hemibiotrophic and necrotrophic pathogens in many agricultural and horticultural crops (Vleeshouwers and Oliver 2014). Because of the rapid breakdown in major gene resistance that can occur when pathogens mutate to alter or lose effectors to escape recognition, a current focus is on identification of core effectors that have an essential virulence function for the pathogen and therefore cannot escape recognition without significant cost to the pathogen (Dangl et al. 2013). By stacking R genes that recognize such core effectors, the hope is that resistance will be durable (Dangl et al. 2013), although whether this effect would last over the lifetime of a pine will be a stringent test. The availability of the genome sequence of *D. septosporum* and the discovery of genes encoding putative effectors that elicit an HR in tomato cultivars carrying specific R genes (section 2.2.6; de Wit et al. 2012) leads to the possibility that cognate R genes could be identified in pines by effector screening. So far, more than 170 candidate effector genes encoding short, secreted, cysteine-rich proteins, that is with characteristics typical of apoplastic effectors, have been identified in the *D. septosporum* genome (de Wit et al. 2012). It is also possible that some might be necrotrophic effectors that target susceptibility genes in an inverse gene-for-gene manner. In wheat (*Triticum* L. spp.), susceptibility genes targeted by necrotrophic Tox effectors of the pathogen *Parastagonospora nodorum* (E. Müll.) Hedjar. were bred out of wheat cultivars to provide durable resistance (Vleeshouwers and Oliver 2014).

Thanks to rapid technological advances, large Pinaceae genomes can now be sequenced comparatively quickly and inexpensively, although repetitive sequences, gene duplication and transposable elements mean that their quality, assembly and interpretation can be challenging (Mackay et al. 2012). There are ongoing genome projects for many Pinaceae (Mackay et al. 2012), with genomes released for *Picea glauca* (Moench) Voss (Birol et al. 2013), *Picea abies* (Nystedt et al. 2013) and *Pinus taeda* (Zimin et al. 2014). Genomic resources for other important species such as *Pseudotsuga menziesii* (Mirb.)

Franco, *P. lambertiana*, *Pinus pinaster* Aiton and *P. radiata* are also being developed. By comparing genetic sequences of individuals with a known phenotype, the genetic architecture of these traits may be determined by association mapping. Genetic differences between phenotypes, such as single nucleotide polymorphisms (SNPs), which are putatively associated with the trait, can be validated in a large number of individuals to test for the strength of association. Techniques used to survey the genome and identify potential markers include candidate gene sequencing, whole genome resequencing and transcriptomics (e.g. sequencing expressed genes at different stages of infection). In addition to developing resistance markers, transcriptomics can also be used to follow the hosts' response to infection and attack at the molecular level. For example, Liu et al. (2013b) recorded significant upregulation of *P. monticola* genes involved in the biosynthesis of flavonoid and related compounds in resistant seedlings, compared to susceptible seedlings, when challenged with *C. ribicola*. Despite the wealth of sequence data being generated, however, without high quality phenotypic data collected from trials and natural populations, the genetic variation underlying mechanisms of resistance to pests and pathogens will remain poorly understood (Telford et al. 2014).

8 The future

It is clear from this review that several different defence mechanisms are involved in the defence of Pinaceae foliage against needle and shoot pathogens. It is not yet clear, however, which of these mechanisms are important in the *Dothistroma-Pinus* interaction, and there is huge scope for further research. This work is required to facilitate the identification of more resistant host individuals and provenances that will support the control of these important pathogens. Although research has demonstrated that resistance in this pathogen–host interaction is probably quantitative, the recent finding of putative avirulence effectors in *D. septosporum* will help to determine whether there is also a role for qualitative resistance in this system. Further basic research is needed to investigate the infection and disease development processes of both *Dothistroma* spp., as well as the morphological defence response of different hosts. Very little is also known about the role of phenolic and terpenoid compounds in the defence of pine against *Dothistroma*. For several other systems, the changes induced on infection in the abundance and composition of these compounds have been linked to defence, but the same effect has yet to be shown for *Dothistroma*. Similarly, nothing is known regarding the role of AMPs and PR proteins in the *Dothistroma-Pinus* interaction, and no research has been carried out on the role of signalling molecules, such as salicylic acid or jasmonic acid, in inducing systemic resistance to DNB.

Other areas for further research include the importance of tolerance in this host–pathogen system, the role of other pathogens, endophytes and mycorrhizae in resistance to DNB, the use of biocontrol agents to control the disease, the mechanisms behind observed interactions between relative susceptibility and the environment, the occurrence and importance of systemic induced resistance, the influence of changing climatic scenarios (prior to infection) on the subsequent susceptibility of pine to DNB and maternal environmental effects on progeny resistance. The availability of genomic, transcriptomic, proteomic and metabolomic data for large Pinaceae genomes as well as that of *D. septosporum* will further support this work, as well as the identification of resistance mechanisms critical in the interaction.

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