VARIATION IN SECONDARY METABOLITE SYNTHESIS IN LODGEPOLE PINE (*PINUS CONTORTA*) AS A DEFENCE AGAINST *DOTHISTROMA SEPTOSPORUM*

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ABSTRACT

Dothistroma septosporum Dorog. Morelet is an emergent fungal pathogen of pine responsible for increasingly severe and widespread damage in British Columbia. Native lodgepole pine (*Pinus contorta* var. *latifolia*) is considered highly susceptible, but has been extensively planted due to its commercial value. Current management options involve either avoidance or species diversification. Quantitative resistance to *Dothistroma* has been observed in several pine species, and further identification of resistant lodgepole pine varieties is important for future management of the disease. My objectives were to determine the historical climatic influences on constitutive foliar terpene levels, identify foliar secondary metabolites associated with resistance and historical disease pressure, and to ascertain the *in vitro* effects of these compounds. I quantified foliar secondary metabolites from both seed orchard and resistance trial lodgepole pines, many of which were identified as terpenes. I quantified the *Dothistroma* outbreak history of lodgepole pine provenances using dendrochronological methods, and the climate history of these provenances using an interpolated historical climate model. I found that constitutive foliar levels of specific terpenes significantly correlated ($P<0.05$) with climate at provenance origin, *Dothistroma* resistance, and provenance outbreak history. I assessed the *in vitro* effects of the sesquiterpene alcohol (E,E)-farnesol on various *D. septosporum* isolates. Temperature normals at provenance origin were significantly negatively correlated with foliar terpene levels in orchard trees. Family crown retention variables were significantly positively correlated with levels of a number of sesquiterpenes and sesquiterpene alcohols. Provenance *Dothistroma* outbreak history was significantly positively correlated with levels of a wide range of monoterpenes, two sesquiterpenes, and

two sesquiterpene alcohols. *In vitro* effects of the sesquiterpene alcohol (E,E)-farnesol were complex, with the compound conferring growth inhibition at high concentrations and stimulation at low concentrations. My results indicate that foliar terpenes play an important role in quantitative resistance of lodgepole pine to *Dothistroma septosporum*, and this work provides a basis for future investigation into the defensive role of these compounds. A more comprehensive knowledge of the *Dothistroma*-*Pinus* relationship is required for future identification of resistant lodgepole pine varieties, and to further our understanding of the forces driving emergence of this damaging fungal pathogen in British Columbia.

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0. Introduction

0.0 Abstract

Dothistroma septosporum (Dorog.) Morelet is a fungal pathogen with a worldwide distribution, and the causal agent of the defoliating pine disease red band needle blight. The severity of disease outbreaks has been increasing in lodgepole pine (*Pinus contorta* var *latifolia*) of northwestern British Columbia, largely due to changes in climate which favour development and spread of the pathogen (Woods et al. 2005; Welsh et al. 2014). While outbreak severity can be reduced by species diversification in replanted stands, development and implementation of resistant lodgepole pine varieties will play an important role in forest management going forward (McCulloch and Woods, 2009; Bulman et al. 2016). Resistant varieties have been identified, but the basis of this observed resistance is not yet understood in lodgepole pine, and poorly understood in other host species. Resistance to *D. septosporum* is likely to be quantitative and multifactorial. A number of potential resistance mechanisms have been identified, but few have been investigated in the context of the *Dothistroma-Pinus* pathosystem. Foliar terpenes have been associated with resistance of lodgepole pine to other fungal defoliators (Wallis et al. 2010), but the contribution of terpenes to *Dothistroma* resistance has been identified as a potential knowledge gap. Investigation into the basis of *Dothistroma* resistance is hindered by difficulties in both field assessment of resistance and the hugely complex nature of quantitative resistance systems in pines. In this chapter I provide a context for the research presented in this thesis, and outline my objectives for investigation into the role of terpenes in *Dothistroma-Pinus* defence interactions.

0.1 Context of forestry in British Columbia

For the duration of human history, forest ecosystems have played a major role in many aspects of society. Forests directly provide food, fuel, and building materials, in addition to a wide range of indirect benefits such as biodiversity, climate regulation, and water catchment (Shvidenko et al, 2005). In northern British Columbia forestry is a major contributor to the local economy, accounting for over a quarter of total income in the northern interior (Horne, 2009). Much of the region is highly vulnerable to forest sector downturns (B.C. Ministry of Forests, Mines and Lands 2010). As British Columbia's abiotic and biotic environment is altered by changes in use and climate, increasing stress is placed on forest ecosystems (Lewis and Lindgren, 2000; Haughian et al, 2012). Altered temperature and precipitation regimes directly impact the forest tree species which support the region's economy (Hamann and Wang, 2006), but also affect disturbance agents that can reduce the ability of forests to provide services (Lewis and Lindgren 2000; Bentz et al. 2010).

Climate has been a constant factor in the evolutionary history of the organisms which make up the world's forests, but the pace of recent changes makes it difficult for relatively long lived tree species to adapt (Hamann and Wang, 2006). One early example of the direct impacts of a changing climate on forest trees is thought to be yellow cedar decline, in which reduced snowpack is hypothesized to expose shallow roots of coastal yellow cedar (*Chamaecyparis nootkatensis* [D. Don] Spach) to freezing damage, leading to tree mortality. Widespread mortality of yellow cedar in affected areas was first observed in 1909, with initial deaths thought to occur subsequent to the Little Ice Age (Hennon et al. 2008). However, the effects of changing abiotic conditions are not always so obvious. Both

increased summer temperature and decreased precipitation can cause decreased basal area in lodgepole pine (Chhin et al, 2008), a change that may not be visually evident to the casual observer.

More recent and noticeable changes in British Columbia‘s forests are associated with biotic disturbance agents. Not only are climate-stressed trees often more vunerable to attack (Mattson and Haack, 1987; Waring and Pitman, 1985; Wargo 1996), but insects and pathogens are themselves affected by climate, which can influence both their incidence and their severity (Woods et al. 2005; Welsh et al. 2009; Welsh et al. 2014). Bark beetles such as the mountain pine beetle (*Dendroctonus ponderosae* Hopkins) and spruce beetle (*Dendroctonus rufipennis* [Kirby]) experience lower winter mortality and generation time under warmer temperature regimes (Bentz et al. 2010). A major factor contributing to the extent of the recent mountain pine beetle outbreak in British Columbia was low incidence of the extended cold periods required for insect winter mortality (Stahl et al. 2006). Where these temperature and precipitation changes favor disease development, native pathogens that were previously considered benign can emerge as seriously damaging (Welsh et al. 2009). Fungi are responsible for almost a third of these emergent plant diseases (Anderson 2004). *Dothistroma septosporum* (Dorog.) Morelet is a fungal disease agent of growing importance in British Columbia is, and is responsible for the disease red band needle blight (Bradshaw 2004). As a pathogen native to this province that cooevolved with its pine host, *D. septosporum* was historically only a management concern in exotic pine plantations of the southern hemisphere (Gibson 1972). Though the fungus is considered one of the most destructive pathogens of pines in the world (Bradshaw 2004), it was only with recent changes

in climate that its damage potential was realized in this province (Woods et al. 2003; Woods et al, 2005; Welsh et al. 2014).

As British Columbia's climate changes, the economic impact of forest pathogens is likely to increase. While forest trees such as lodgepole pine can adapt in range or genetics in response to changing conditions, there are inherent limitations to the speed and efficiency of these processes (Bunnell and Kremsater, 2012). Short life cycles give fungi such as *D. septosporum* an adaptive advantage over their longer-lived hosts in rapidly changing climates (Watt et al, 2009; Sturrock et al, 2011). The effects of climate change on forests are both numerous and interacting (Williamson et al. 2009), and there are few models developed specifically for the prediction of climatic effects on pathogens (Sturrock 2011). Without accompanying changes in management, the stress these interacting factors place on our forests will compromise their ability to provide the economic and social services that northern British Columbia depends on (Lewis and Lindgren, 2000). Coping with these changes requires a more comprehensive understanding of the forces driving disturbances in forest ecosystems. In the case of pathogens, this understanding is predicated on detailed knowledge of the complex interactions between tree hosts and their attackers, knowledge that is often lacking in native pathogens that were previously considered benign (Welsh et al. 2009).

0.2 The fungal pathogen *Dothistroma septosporum*

Native pathogens often go unnoticed due to their usually low impact on coevolved host species (Parker and Gilbert 2004; Loo 2009; Jousimo et al. 2014), typically receiving more initial study outside of their native range (Desprez-Loustau et al. 2016). This is the case for *Dothistroma septosporum*, which was initially identified in Europe in 1911, but only recognized as a serious disease of pines in 1957, when plantations in the region now known as Tanzania (then Tanganyika) were affected. The disease was not initially attributed to *Dothistroma*, and only after later and more serious incidents was *D. septosporum* identified as the cause (Gibson 1972). In 1964 *Dothistroma* needle blight symptoms were identified in plants located in Chile and New Zealand, both countries having a sizable forestry industry. It is thought that the disease had most likely been occurring at lower intensity for several years prior (Gibson 1972). The first report of *Dothistroma* in British Columbia was in 1963 (Parker and Collis, 1966), although dendrochronological reconstruction of outbreaks indicate the pathogen has been present in the province as early as 1831 (Welsh et al. 2009), and genetic studies indicate an even earlier presence (Dale et al. 2011). *Dothistroma* blight is caused by two genetically-distinguishable species, *D. septosporum* and *Dothistroma pini* Hulbary, though the latter is restricted in range and has seen little study (Barnes et al. 2004). In many cases identification of the pathogen may have been delayed by isolation difficulties related to its slow growth relative to saprophytic fungi, and by the long latent periods which can occur in its lifecycle (Gibson 1972; Drenkhan et al. 2013).

The *Dothistroma* lifecycle begins with germination of an ascospore or conidiospore on the host needle surface. Growth of emerging hyphae can be either random (Gadgil 1967; Kabir et

al. 2014) or directed towards stomata (in *D pini* - Peterson 1969; and Peterson and Walla, 1978), depending on host species and environment (Gibson 1972; Muir and Cobb, 2005). Once a stomatal pore is penetrated by growing hyphae the fungus continues to grow as an epiphyte within the epistomatal chamber. In *D. septosporum* this asymptomatic epiphytic period can last as long as eight weeks before the mesophyll is penetrated and the pathogen begins necrotrophic growth (Kabir et al. 2014). The necrotrophic phase corresponds with formation of a necrotic lesion 1-3 mm in length, often accompanied by a distinctive red coloration indicating the presence of the phytotoxic secondary metabolite dothistromin (Gadgil 1967; Kabir et al. 2014). While production of this compound is not required for infection (Schwelm et al. 2009), it is toxic to the host and is thought to act as a virulence factor (Shain and Franich, 1981; Kabir et al. 2015). Six to twelve weeks after initial infection fruiting bodies (stromata) emerge through the needle cuticle, facilitating spore release (Kabir et al. 2014). Both production of stromata and spore dispersal are highly dependent on moisture (in *D. pini* - Peterson 1973; Gadgil 1977), as the conidia are spread through the canopy by rain splash (Boateng and Lewis 2015). Progressive defoliation occurs as the disease spreads through the crown, resulting in growth reduction due to loss of photosynthetic potential (Gibson 1974). Severe defoliation can result in death, and while this has historically been confined to young trees and exotic plantations (Gibson 1972), more recently mortality has been observed in mature trees in north western British Columbia (Woods et al. 2003).

This change in disease severity within the pathogen's native range is largely driven by changes in precipitation and temperature, a consequence of the pathogen's rain splash spore

dispersal mechanism. More numerous warm rain events in northwestern British Columbia since the 1970s have been associated with greater severity and extent of *Dothistroma* outbreaks (Woods et al. 2005; Welsh et al. 2014). Conidia spread is highly correlated with these precipitation events (Boateng and Lewis, 2015), and spore germination rate increases with temperature (Gadgil 1974). Consequently, disease severity increases with increasing humidity and length of rainfall events at favourable temperatures (Gadgil 1974; Gadgil 1977), leading to greater damage to susceptible species under a warmer and wetter climate (Woods et al. 2005). The effect of these changes in British Columbia's climate has been compounded by a forest management preference for commercially valuable lodgepole pine (Woods et al. 2003), a species that is considered highly susceptible to the disease (Watt et al. 2009). As spore load decreases exponentially with distance (Boateng and Lewis, 2015), host density increases are likely to be directly associated with disease severity, and may lead to outbreaks in areas where resistance development has been suppressed (Ennos 2015).

Current management strategies for controlling *Dothistroma* in British Columbia are largely limited to managing species composition (McCulloch and Woods, 2009), but further identification of resistant lodgepole pine varieties may expand available options (Ukrainetz et al. 2013). A diverse range of control strategies are used for management of *D. septosporum* around the world (Bulman et al. 2016). These are based on altering one of the three factors required for disease occurrence: virulent pathogen, susceptible host, and a conducive environment (Agrios 2005). Strategies targeting the pathogen through exclusion or eradication are often impossible due to its worldwide distribution and the ease with which it can spread (Bulman et al. 2016). Longer range dispersal of the pathogen can occur through

airborne dispersal of ascospores (Dale et al. 2011), and movement of infected stock, which has spread the pathogen to every continent but Antarctica (Gibson 1974; Watt et al. 2009). At this point occurrence of the disease is thought to be limited more by the range of susceptible host species (Watt et al. 2009). Chemical control strategies are widely used in exotic pine plantations, typified by the spraying of *Pinus radiata* D. Don in New Zealand with copper based fungicides (Gibson 1974; Bulman et al. 2016). High cost aerial spraying in these areas is justified by the financial impact of the disease (Watt et al. 2011), the concentration of commercial forests in areas separate from native forests, and the mature tree resistance of *P. radiata* that limits treatment requirements to early in the rotation period (Gibson 1972). While the abiotic environment is largely beyond the control of forest managers, silvicultural practices such as thinning can be used in managed plantations to increase the distance between adjacent host trees and promote airflow (reducing the humidity which would otherwise contribute to disease development - Bulman et al. 2016). Chemical and silvicultural control strategies have been successfully implemented in intensively managed plantations, but these methods are not possible in native forests, for both environmental and financial reasons. Chemical treatments have the potential to impact non-target organisms when indiscriminately applied (Giller et al. 1998; Zwieten et al. 2004), and the lack of mature tree resistance in lodgepole pine makes aerial spraying of this species relatively costly (McCulloch and Woods, 2009). Control measures in British Columbia involve either promotion of species diversity or replacement with non-host species where disease development is likely. Current guidelines recommend that forest managers limit pine establishment to less than 20% of total stand density where risk of *Dothistroma* outbreak is high, with western hemlock [*Tsuga heterphylla* (Raf.) Sarg.], western redcedar (*Thuja plicata*

Donn), and Douglas-fir [*Pseudotsuga menziesii (*Mirb.) Franco] suggested as a replacement species where conditions are suitable (McCulloch and Woods, 2009). Identification of resistant varieties is important for host-focused control, and has shown promise in several pine species (Carson 1989; Ukrainetz et al. 2013), but little is currently known about the basis of *Dothistroma* resistance. Identification and characterization of the defence mechanisms involved in the *Dothistroma-Pinus* interaction is important for continued development of resistance (Fraser et al. 2015), and consequently for future forest management in this province.

0.3 Plant defence in the *Dothistroma-Pinus* interaction

Pines, and plants in general, deploy a sophisticated array of defences against their numerous attackers (Franceschi et al. 2005; Huber and Bohlmann, 2005). In the case of pathogens, chemical and physical plant defence mechanisms can confer quantitative resistance by hindering host penetration or inhibiting growth (Fraser et al. 2015), and qualitative resistance through recognition of pathogen elicitors (Rivas and Thomas, 2005). Defensive compounds are maintained at a constitutive level in the absence of threats, but can be upregulated when a threat is detected (Johnson et al. 1989; Miller et al. 2005; Wallis et al. 2008), often conferring long lasting defence through systemic acquired resistance (Bonello et al. 2001; Bonello et al. 2006). This diversity of defence mechanisms is a response to the equally diverse threats posed by pathogens and insect herbivores, the attacks of which are often infrequent and irregular (Welsh et al. 2009; Hrinkevich and Lewis, 2011).

The specific defence mechanisms involved in observed resistance of pine species to *D. septosporum* are poorly understood (Fraser et al. 2015), which is typical of the complex quantitative resistance systems seen in plants (Neale and Kremer, 2011). Data from *P. radiata* breeding trials indicates that *Dothistroma* resistance is likely to be quantitative and multifactorial, involving the contribution of multiple defence mechanisms (Carson 1989). Early work on identifying these mechanisms focused on the mature tree resistance of this species (Gibson 1972), which was shown to depend on occlusion of stomata by cuticular wax (Franich and Gadgil, 1983). This occlusion increases with tree age, impeding penetration by fungal hyphae in older trees (Franich et al. 1977). Cuticular waxes were also found to inhibit growth of the fungus *in vitro*, suggesting that these compounds are active in constitutive

chemical defence (Franich and Gadgil 1983).

Identification of other *Dothistroma* resistance mechanisms has been more elusive, due in part to the difficulties involved in assessing resistance in the field (Franich et al. 1986; Carson 1989) but also the fact that a reliable pathogenicity assay was developed only recently (Kabir et al. 2013). Both benzoic acid and lignin have been suggested as potential contributors to resistance based on *in vitro* experiments. Artificially induced lesions produced by injection of needle tissue with the *Dothistroma* mycotoxin dothistromin have been shown to contain high concentrations of benzoic acid, produced by host cells surrounding the lesion (Franich et al. 1986). *In vitro* tests on *D. septosporum* showed that this compound was highly fungistatic. Areas surrounding these lesions were found to be highly lignified, leading to the hypothesis that lignin is produced as an induced physical defence to restrict lesion extension (Franich et al. 1986). Defensive lignin production was further investigated by *in vitro* tests on *P. radiata* cell culture. Addition of *D. septosporum* cell wall elicitors led to increased activity of lignin biosynthesis pathway enzymes, and elevation of lignin levels over a 96-hour period (Hotter 1997).

The diversity observed in other host-pathogen interactions suggests that these mechanisms do not represent the complete spectrum of defences contributing to *Dothistroma* resistance. In their review of defence mechanisms relating to the *Dothistroma-Pinus* interaction, Fraser et al. (2015) identify a number of potential avenues of future research, including the contributions of avirulence factors recently discovered in the *D. septosporum* genome (de Wit et al. 2012) and foliar terpenes that have been associated with resistance to other foliar pathogens (Wallis et al. 2010). The qualitative resistance conferred by host recognition of

pathogen produced avirulence factors is a common goal in the breeding and modification of intensively managed crops (Hulbert et al. 2001). This class of resistance is typified by the interactions of the dothideomycete pathogen *Cladosporum fulvum* and its tomato (*Lycopersicon esculentum* Mill.) host (de Wit and Joosten 1999; Rivas and Thomas 2005). While a number of *C. fulvum* effector gene homologs are present and expressed in *D. septosporum* (de Wit et al. 2012; Bradshaw et al. 2016), no qualitative resistance has been observed in any host species. Fraser et al. (2015) suggest that the pathogen may instead hijack host hypersensitive responses to enable its necrotrophic phase. If qualitative resistance to *Dothistroma* is achievable it is likely to be less durable than quantitative resistance due to the specific nature of the gene-for-gene interactions involved (Fraser et al. 2015; Bulman et al. 2016). This limits applicability in British Columbia, where *D. septosporum* is highly genetically diverse (Dale et al. 2011), but qualitative resistance may be useful in areas where pathogen diversity is low, such as New Zealand (Hirst et al. 1999).

While highly specific gene-for-gene resistance systems have been well characterized (Neale and Kremer, 2011), assessment of the defence contribution of terpenes is complicated by their diversity, both in chemistry and function (Franich et al. 1982). Plants produce a huge variety of these compounds in their bark and foliage, but secondary metabolites were historically considered waste products of primary metabolism and saw little investigation into their biological roles (Hartmann 1996; Hartmann 2007). It was not until a landmark publication by Swain (1977) that their role in defence was recognized, and subsequent research has shown that these compounds are in fact the product of highly regulated biosynthetic pathways formed through positive selection of their function (Hartmann 1996;

Qi et al. 2004; Benderoth et al. 2006).

The defensive functions of these secondary metabolites in plants can be highly diverse. Emission of volatile compounds from foliage can protect plant species from heat stress (Sakasi et al. 2007), and terpenes in particular are involved in defence against a wide range of organisms. Terpene levels have been shown to affect selection of ponderosa pine (*Pinus ponderosa* Douglas ex C. Lawson) bark by Alberts squirrel (*Sciurus aberti* Woodhouse - Snyder 1992), and in insects terpenes can have inhibitory, repellant, or antifeedant properties (Isman 2000). These properties may be related to their acetylcholinesterase inhibitor activity (Lopez and Pascual-Villalobos 2010), but in many cases terpene resin production and release acts to physically repel or entrap attacking insects (Francheschi et al. 2005). Terpenes have been associated with fungal disease resistance in a variety of conifer species. β-phellandrene levels have been linked to conifer resistance against *Cronartium fusiforme* Hedgcock & Hunt ex Cummins (in slash pine [*Pinus elliottii* Englm. var. *elliottii*] - Michelozzi et al. 1990; in loblolly pine [*Pinus taeda* L.] - Michelozzi et al. 1995) and *Gremmeniella abietina* (T. Lagerb.) M. Morelet (in Scots pine [*Pinus sylvestris* L.] - Aitken 1993). In lodgepole pine constitutive monoterpene levels have been correlated with resistance to foliar fungal pathogens *Lophodermella concolor* (Dearn.) Darker, *Lophodermella montivaga* Petre., and *Elytroderma deformans* (Wier) Darker (Wallis et al. 2010).

While terpenes have not been linked to resistance against *Dothistroma* in previously published literature, the action of these compounds *in vitro* makes such a defence contribution plausible. Monoterpenes have been shown to inhibit germination of *D. septosporum* spores *in vitro,* and steam distilled extracts from *P. radiata* containing

predominantly terpenes inhibited fungal growth in broth culture (Franich et al. 1982). This is consistent with the broadly inhibitory *in vitro* effects of terpenes on other pathogenic microorganisms (Andrews et al. 1980; Taniguchi et al. 1988; Wedge et al. 2000; Cakir et al. 2005; Derengowski et al. 2009; Portillo et al. 2005; Jabra-Rizk et al. 2006; Bakkali et al. 2008; Gazim et al. 2008; Combrink et al. 2011). The broad antimicrobial effects of terpene compounds are most likely due to the damage they cause to cellular membranes. Permeability of these membranes is increased in exposed bacteria (Carson et al. 2002; Jabra-Rizk et al. 2006), and exposure of fungi to sesquiterpene alcohols can cause upregulation of DNA repair genes, potentially indicating internal membrane damage (Bakkali et al. 2005). Specific compounds can also induce apoptosis in some species (*Aspergillus nidulas* - Semighini et al. 2006; *Fusarium graminearum* - Semighini et al. 2008), Alternatively, terpenes may sensitize microorganisms to other defence chemicals, as observed with the sesquiterpene aldehyde polygodial (Kubo and Taniguchi, 1988).

The complement of terpene compounds found in pine foliage varies greatly, both between and within pine species. While all terpenes are made up of isoprene subunits, the precursors and pathways responsible for this biosynthesis depend on their class. Monoterpenes are synthesized from dimethylallyl pyrophosphate precursors produced by the melvonate pathway; sesquiterpenes from the methyl-erithritol 4-phosphate pathway (Zulak and Bohlmann 2010; Vranova et al. 2013). Production of these compounds is relatively costly to the plant (Gershenzon 1994), and their emission can represent a sizable percentage of total fixed carbon (Monson and Fall 1989; Llusia and Penuelas 2000). It is little surprise then that the secondary metabolite profile of conifer species is highly plastic (Huber et al. 2004).

Terpene levels are upregulated above constitutive levels when needed; in some cases induction of a defence response results in a changes in production and emission of more than an order of magnitude (Martin et al. 2003). However, the hemibiotrophic lifecycle of *Dothistroma* indicates that it initially avoids detection by its host (Fraser et al. 2015), implying that constitutive defence may be more effective. Constitutive levels of defensive secondary metabolites are largely genetically determined (Hanover 1966; Hamilton et al. 2001), but are influenced by a wide range of biotic and abiotic factors (Ramakrishna and Ravishankar, 2011). Environmental conditions such as light level, temperature, and precipitation contribute to give a 'constitutive plus' level of foliar secondary metabolites (Constable et al. 1999; Sallas et al. 2003; Stamp 2003; Mayrhofer et al. 2005; Lavoir et al. 2009; Holopainen et al. 2013). In pines these constitutive terpenes levels have been shown to be highly variable between species and provenance, even in a consistent environment (Pureswaran et al. 2004; Wallis et al. 2010; Wallis et al. 2011; Ioannou et al. 2014). This implies that there are variable defence contributions from terpenes in different pine provenances, indicating that these provenances may have variable levels of resistance to foliar fungi such as *Dothistroma*.

0.4 Objectives and structure

While there has been considerable study of *D. septosporum* virulence factors (Gallagher and Hodges 1972; Shain and Franich 1981; Bradshaw et al. 2000; Bradshaw et al. 2002; Schwelm et al. 2009; Chettri et al. 2012; de Wit et al. 2012; Kabir et al. 2015), there is little published work on how host secondary metabolites affect the pathogen, or on resistance to *Dothistroma* in general (Fraser et al. 2015). The pine hosts of the disease produce a wide variety of defence-related secondary metabolites in their foliage, at great cost to the plant. The ability to produce of these defensive compounds can be rapidly lost from populations where they are no longer required (Gershenzon 1994; Tian et al. 2003), implying that their continued production provides some benefit. Previous study has associated variation in foliar terpene levels of lodgepole pine with varying resistance to fungal defoliators (Wallis et al. 2010). While tolerance of fungi to terpenes varies by species (Wedge et al. 2000; Bakkali et al. 2008), terpenes have been shown to inhibit growth and germination of *D. septosporum in vitro* and may also play a role in resistance to this pathogen (Franich et al. 1982). Both continued development of resistance and prediction of future outbreaks is dependent on our understanding of pathogen-host interactions (Welsh et al. 2009), and this understanding is lacking for *Dothistroma*. In this study I aim to fill established knowledge gaps relating to the resistance contribution of foliar terpenes in the *Dothistroma-Pinus* interaction (Fraser et al. 2015). My investigation of terpene defenses in this context will be based on the following broad chapter objectives:

Chapter 1 – Determine the influence of historical climate at provenance origin on the constitutive foliar secondary metabolite levels of lodgepole pine. Emergence of *D.*

septosporum in British Columbia is closely linked to climate. Understanding the relationship between constitutive foliar terpene levels and the historical climate of host provenances may give insight into drivers of variation between these provenances. In this chapter I quantified and correlated foliar secondary metabolite level data from seed orchard trees with 1961-1990 interpolated climate model data from ClimateWNA (Wang et al. 2016). A number of broad and significant correlations were found between constitutive secondary metabolite levels and historical climate variables, with temperature showing a strong influence.

Chapter 2 – Investigate the relationship between individual foliar terpene levels and observed *D. septosporum* resistance in lodgepole pine. *Dothistroma* resistant lodgepole pine varieties have been identified (Ukrainetz et al. 2013), but the mechanistic basis of this resistance is unknown. Understanding the basis of resistance in this species may aid in future development of resistant varieties. In this chapter I quantified foliar secondary metabolite levels from lodgepole pine families that exhibited varying levels of crown retention in a *Dothistroma* resistance trial. I correlated levels of these secondary metabolites with crown retention measures to identify compounds associated with *Dothistroma* resistance. A number of putatively identified sesquiterpenes and sesquiterpene alcohols were found to significantly correlate with family crown retention, indicating that these compounds may contribute to resistance.

Ch 3 – Identify effects of resistance-related foliar secondary metabolites on the growth of *D. septosporum in vitro*. While sesquiterpene alcohols have been shown to inhibit growth of pathogenic fungi *in vitro*, no inhibition testing with this class of compounds has been performed on *D. septosporum*. Determining the effect of these compounds *in vitro* may shed

light on their resistance contribution. In this chapter I assayed the *in vitro* growth effects of a farnesol isomer potentially linked to resistance in Chapter 2. While inhibition was seen in some isolates at high farnesol concentrations, more striking was the significant growth promotion seen at lower concentrations.

Ch 4 – Investigate the influence of *Dothistroma* disease history on constitutive secondary metabolite levels of lodgepole pine. Lodgepole pine provenances which have coevolved with *D. septosporum* have potentially higher resistance (Burdon et al. 2013), and if foliar terpenes play a role in resistance then their levels may also be higher in these provenances. In this chapter I used dendrochronological methods to quantify the outbreak history of the lodgepole pine provenances assessed in Chapter 1, which I compared to secondary metabolite levels quantified in that chapter. Monoterpene levels were broadly positively correlated with *Dothistroma* outbreak history, as were levels of several sequiterpenes and sesquiterpene alcohols.

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1. Specific foliar secondary metabolite levels of plantation lodgepole pine are strongly influenced by provenance origin climate variables

1.0 Abstract

There has been a great deal of research into the defense chemistry of lodgepole pine (*Pinus contorta* var *latifolia* Engelmann) and conifers in general, but how climate has influenced the genetics of secondary metabolism in this species is still unclear. In this study I explored relationships between historical climate at provenance origin and observed variation in genetically determined constitutive levels of volatile foliar secondary metabolites. I collected visually uninfected foliage from plantation lodgepole pine and quantified volatile components by GC-FID. I found that the secondary metabolite profiles of sampled trees were consistent with previously published studies, and that levels of most quantified compounds significantly correlated ($P<0.05$) with a wide range of climate variables using nonparametric methods. Plantation foliage correlations with temperature variables were found to be the opposite of those previously seen in naturally established lodgepole pine stands, indicating that historical pest prevalence plays a lesser role than growing season in genetic variation of constitutive secondary metabolite levels. This has important implications for future investigation into the involvement of secondary metabolism in disease resistance, as previous work on lodgepole pine has often focused on constitutive rather than induced defense.

1.1 Introduction

Pines, and conifers in general, produce a diverse array of volatile secondary metabolites with a wide variety of functions. Lodgepole pine (*Pinus contorta* var. *latifolia* Engelmann*)* is no exception, and previous studies have shown a broad range of compounds are present in its bark and foliage (Wallis et al. 2010; Wallis et al. 2011; Ioannou et al. 2014). This great chemical variety and the complex nature of plant-environment interactions makes conclusively identifying functional roles for secondary metabolites difficult (Seybold et al. 2006). As a result, some compounds are know to play a role in defense, but the biological function of many others remains unknown.

Research into secondary metabolism began in earnest with the advent of radiochemical labelling experiments in the early 1950's; however, there was relatively little early investigation into the function of secondary metabolites (Hartmann 2007). These compounds were historically viewed as waste products (Hartmann 1996), and it was not until much later that their role in defense was widely accepted (Swain 1977). We now know that the huge diversity seen in plant secondary metabolites is not random, but rather the result of a complex and highly regulated set of biochemical pathways developed through evolutionary processes (Hartmann 1996). These pathways form by positive selection of genes responsible for biosynthesis of compounds with important ecophysiological roles, such as those involved in plant defense (Qi et al. 2004; Benderoth et al. 2006).

Investigation into the effects of plant secondary metabolites on insect pests and pathogens has driven much of the work on now well-characterized classes of secondary metabolites.

Plants maintain a baseline level of defensive compounds, such as terpenes and alkaloids, that act as constitutive chemical defenses, but their levels can be increased when attacked (Johnson et al. 1989). Induction of chemical defenses by disease or wound signaling pathways will result in upregulation of biosynthesis and heightened levels of target metabolites in the short term, allowing the tree to defend against immediate threats. Systemic induced resistance is a longer-term response, where herbivory or infection induces a defense response throughout the tree that can confer resistance to future attack (Bonello et al. 2006). Both constitutive and induced defenses in plants are hugely diverse (Franceschi et al. 2005; Fraser et al. 2015). This diversity is a response to the diversity of organisms attacking them - plant defenses must act against numerous pathogenic microorganisms, insect pests, and vertebrate herbivores. These threats may be concurrent or consecutive in both time and space, which adds to the overall complexity of the response.

Plants utilize both the chemical and physical properties of secondary metabolites to defend themselves against this plethora of attackers. Toxic secondary metabolites can inhibit the growth of pathogenic microorganisms (Bakkali et al. 2008), but physical inhibition of infection and growth also plays a role in defense (Fraser et al. 2015). For example, foliar terpenoids have been shown to inhibit growth of the fungal foliar pathogen *Dothistroma septosporum* (Dorog.) Morelet. *in vitro* (Franich et al. 1982), indicating direct toxicity, whereas cuticular wax and lignin form physical barriers to infection and growth (Franich and Gadgil 1983; Franich et al. 1986). Terpenes in particular are known to play a role in the lodgepole pine - mountain pine beetle (*Dendroctonus ponderosae* Hopkins) interaction, with terpene oleoresin flow acting as both a physical and chemical defense (Franceschi et al.

2005). These isoprene-based secondary metabolites have shown inhibitory, repellant, or antifeedant properties against a number of insect species (Isman 2000), possibly due to their inhibitory activity against acetylcholinesterase enzymes involved in neurotransmission (Lopez and Pascual-Villalobos 2010). For vertebrate herbivores, mechanisms such as postingestive feedback and social learning allow vertebrates to identify plants containing toxic compounds and reduce consumption (Bryant et al. 1991). For example, Abert's squirrel shows a preference for ponderosa pine bark based on the type and concentration of terpenes in xylem oleoresin, with trees producing lower levels of β-pinene and β-phellandrene being preferentially targeted (Snyder 1992). Similarly, the snowshoe hare selectively consumes mature Alaska paper birch internodes over juvenile internodes based on the latter's high papyriferic acid content (Reichardt et al. 1984). The emission of volatiles can act as an indirect defense by attracting predators, though the same compounds can also act as olfactory attractants for insect herbivores (Brilli et al. 2009) and the resulting complex interaction between numerous species makes predator attractant effects difficult to study (Dicke and Baldwin 2010). Volatile secondary metabolites can also protect the plant against abiotic influences: Sakasi et al. (2007) found that overexpression of *Populus alba* isoprene synthase in genetically modified *Arabadopsis* conferred elevated thermotolerance, with high levels of isoprene emission resulting in lower leaf temperature under heat stress relative to wild type plants.

In the absence of pathogens and other pests, constitutive levels of defense-related secondary metabolites are largely determined by genetics (Hanover 1966; Hamilton et al. 2001). However, no plant is free from all external influence, and the incompletely developed and

fragmented nature of plant defense theory makes it difficult to predict the response of a plant to what can be a complex and varying environment (Loehle 1987; Stamp 2003). A number of plant defense hypotheses attempt to explain the relationship between environmental variables and secondary metabolite levels, but no comprehensive theory of plant defense exists. Consequently, it is difficult to provide a physiological basis for the observed impact of variable environmental conditions on secondary metabolite levels. Terpene levels in the foliage and boles of pines have been shown to vary both between species (Ioannou et al. 2014) and provenance (Pureswaran et al. 2004), and previous studies have linked this variation to climate and disease history (Wallis et al. 2010; Wallis et al. 2011).

The wide variety of endogenous and exogenous effects influencing the terpene profile of conifers make it challenging to study the individual factors affecting constitutive secondary metabolite production. Conifers have a high level of phenotypic plasticity - a defense response can involve both upregulation of secondary metabolite biosynthesis and differentiation of specific defense structures such as traumatic resin ducts (Huber et al. 2004). This response can profoundly change defense-related compound levels in the affected tree. Tests by Martin et al. (2002) showed that artificial induction of a defense response in Norway spruce (*Picea abies* L. Karst) with methyl jasmonate – a physiological analog of jasmonic acid, which is a phytohormone - can lead to as much as a 12-fold increase in wood monoterpene levels and a 38-fold increase in wood diterpene levels. Levels of biosynthetic enyzmes and their products also vary based on environmental factors such as light level and air temperature (Mayrhofer et al. 2005). Elevated temperature has been shown to significantly increase needle monoterpene concentration in Scots pine seedlings (Sallas et al.

2003) and increase monoterpene emission in ponderosa pine (Constable et al. 1999). Foliar monoterpene levels were found to increase in drought stressed Norway spruce (Holopainen et al. 2013), though emissions from holm oak under drought conditions were reduced (Lavoir et al. 2009). How levels of these compounds vary in the plant is a function of both biosynthesis rates and compound-specific chemistry. Higher volatility compounds, such as monoterpenes, will have higher emission rates and faster turnover than those of lower volatility, such as sesquiterpenes (Holopainen et al. 2013).

While some of these compounds have well defined physiological roles, many have no defined basis for their production beyond that of their overall chemical group. However, the effectiveness of plant defense is more often determined by the levels of specific compounds than the sum of chemical classes (Adler et al. 1995; Moore et al. 2014). The screening hypothesis defined by Moore et al. (2014) suggests that plants produce such a large variety of secondary metabolites to hedge their bets against the unpredictable array of pests and pathogens they may encounter over sometimes long lifetimes with no opportunity for spatial escape. Thus some of the wide array of secondary metabolites found in their foliage have specific roles in defense that have simply not yet been identified.

In this chapter, I investigated the effect of historic climate variation on levels of specific foliar secondary metabolites in plantation offspring from different lodgepole pine provenances. This study expands on previous work by Wallis et al. (2010), which established a connection between levels of specific monoterpenes in plantation lodgepole pine foliage and resistance to foliar pathogens, and Wallis et al. (2011), which linked broader foliar secondary metabolite group levels of naturally occurring stands to seasonal and yearly

climate variables. The aim of my study was to identify potential physiological bases for observed variation in lodgepole pine foliar monoterpenes and sesquiterpenes, based on their relationship with historical climate. The objectives of my study were to: 1) quantify levels of volatile secondary metabolites in foliage samples from uninfected orchard lodgepole pine; 2) correlate specific secondary metabolite levels with historical monthly climate variables of the provenance origin locations; and 3) discuss the potential physiological basis for these correlations, and implications for plant defense. The identification of strong correlations between historical climate at provenance origin and secondary metabolite levels of plantation offspring indicates that constitutive secondary metabolism is under strong genetic control in this species, and that these compounds have important physiological roles.

1.2 Methods

I followed the methods of Wallis et al. (2010) for foliage sampling and analysis. I collected foliage samples from randomly ordered lodgepole pine trees in the Bulkley No. 228 seed orchard at the Prince George Tree Improvement Station (PGTIS) during late August and early September 2011. Provenance origins of sampled trees and the location of the PGTIS site are given in Figure 1.1 and Table 1.1. Where possible I collected from a minimum of three clones from each of the 13 provenances, with three trees sampled per clone. Trees with obvious disease symptoms were excluded from sampling. I collected foliage samples at breast height from north, west, and south facing aspects of each tree. Only visibly disease-free green needles from the previous year's growth (2010) were taken. Collected needles were snap frozen in liquid nitrogen immediately after sampling, transported on dry ice, and stored at -80°C until processed.

Needle samples were ground to a fine powder under liquid nitrogen using a mortar and pestle. Approximately 100 mg of ground needle material from each sample was transferred into pre-weighed 1.7 mL microcentrifuge tubes, and reweighed to give fresh weight values. 500 µL of HPLC-grade dichloromethane (Fisher Scientific, Waltham, MA, USA) containing 0.1% analytical grade n-pentadecane (Sigma-Aldrich, St. Louis, MO, USA) was transferred into each vial, and incubated at 4°C for 24 hours. Samples were then centrifuged at 16,100 RCF for 15 minutes, and the supernatant transferred to fresh microcentrifuge tubes. The centrifugation was repeated, and the supernatant transferred to glass autosampler vials. All extracts were stored at -20°C prior to analysis.

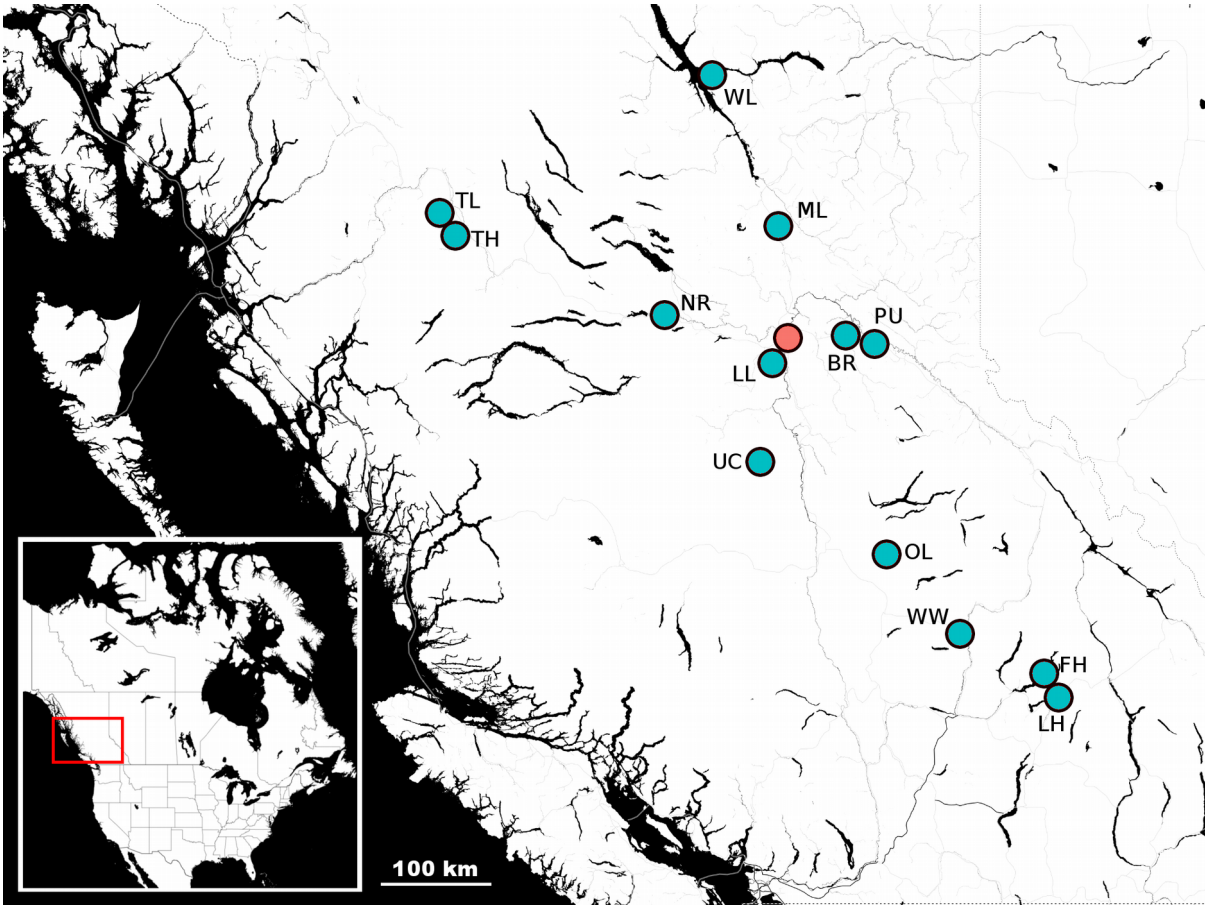

Figure 1.1. Location of provenance origin sites (blue) and PGTIS site (pink) within British Columbia. Map tiles by Stamen Design, under CC BY 3.0. Data by OpenStreetMap, under OdbL.

Table 1.1. GPS coordinates of provenance origin locations and PGTIS site, from Wallis et al. (2010).

ID	Site	UTM coordinates
PU	Purden	10 U, 583292 m E, 5969431 m N
BR	Bowron River	10 U, 565705 m E, 5972859 m N
TH	Telkwa High	9 U, 601125 m E, 6055118 m N
WL	Williston Lake	10 U, 450042 m E, 6200804 m N
ML	McLeod Lake	10 U, 509639 m E, 6074401 m N
TL	Telkwa Low	9 U, 625814 m E, 6057591 m N
LL	Lynx Lake	10 U, 502203 m E, 5944582 m N
NR	Nechako River	10 U, 400626 m E, 5986440 m N
OL	Oie Lake	10 U, 623523 m E, 5762726 m N
WW	Wentworth	10 U, 687239 m E, 5649504 m N
LH	Larch Hill	11 U, 345818 m E, 5618738 m N
FH	Fly Hills	11 U, 327316 m E, 5621872 m N
UC	Udy Creek	10 U, 485465 m E, 5874147 m N
PGTIS	Prince George Tree Improvement Station	10 U, 518367 m E, 5958279 m N

Identification of extracted secondary metabolites was carried out by running a representative sample on a Shimadzu QP2010S GC-MS (Shimadzu, Kyoto, Japan) with a 5% phenylmethylsiloxane column. Compounds were identified by automated fragmentation pattern library lookup, comparison of relative retention time with previous studies, and comparison of retention time with analytical standards where available. Identified compounds were quantified by FID using a Shimadzu GC2010 gas chromatograph with a 5% phenylmethylsiloxane column. Quantified amounts were normalized to the n-pentadecane internal standard and expressed as n-pentadecane equivalents.

Averaged tree peak area of identified compounds was compared with monthly climate variables from ClimateWNA (http://climatewna.com/climatewna_map/; Wang et al. 2012). Coordinates and elevations of origin sites were taken from Wallis et al (2010), and monthly climate variables calculated based on 1961-1990 normals. Monthly normal values for variables mean maximum temperature (T_{max}), mean minimum temperature (T_{min}), mean temperature (T_{av}), precipitation (Prec), degree-days below 0°C (DD<0), degree days above 5°C (DD>5), degree days below 18°C (DD<18), degree days above 18°C (DD>18), number of frost free days (NFFD), precipitation as snow (PAS), reference atmospheric evaporative demand (Eref), and climatic moisture deficit (CMD) were compared with mean tree levels of each quantified compound using R 3.3.3 (R Core Team 2017). Variables were checked for normality using Shapiro-Wilk and Lilliefors test (Venables and Ripley 2002; Gross and Ligges 2015; Meyer et al. 2017). Pearson's correlation coefficients, Spearman's ρ, and Kendall's τ were calculated for each combination of monthly climate variable and peak area. The intercorrelation of temperature averages and precipitation was also tested.

1.3 Results

From the GC analysis, 24 peaks were quantified across 249 samples. Of these high level peaks, 14 were putatively identified based on MS fragmentation pattern library matches and elution order, four were tentatively identified but did not give strong library matches, and six remained unidentified. A further five peaks were included in the analysis based on correlations with resistance established in chapter 2; these are listed as Unknowns 2.2-2.6 in Table 1.2.

Levels of individual compounds often varied greatly between provenance, clone, tree, and branch. The standard deviations of differences between sample peak area totals and mean totals at tree, provenance, and overall levels were 1.09E6, 1.69E6, and 2.04E6, respectively. Despite this variability, a number of significant correlations with climate variables were established. Figures 1.2 through 1.4 give P-values and measures of association for these variables. All quantified peaks showed at least some significant correlation ($P<0.05$) with monthly climate variables. Results were generally consistent between parametric and nonparametric methods, though the relative strength of the correlations varied. The majority of the compounds showed strong negative correlations with temperature variables representing higher temperature, and strong positive correlations with those representing lower temperature: areas of most peaks were negatively correlated with T_{max}, T_{av}, DD>5, DD>18, and Eref year round. Correlations with NFFD were mostly negative and significant ($P<0.05$) in the first half of the year only, whereas significant negative CMD correlations were mostly seen in the second half of the year.

Table 1.2. High level volatile compounds extracted from orchard lodgepole pine foliage and quantified using GC-FID, listed by elution order.

	Compound	RT[a]	ID[b]	Peak area[c]	Shapiro[d]	Lilliefor[e]
1	α-pinene	9.923	95	74,938	8.52E-13	1.18E-16
2	Unknown 1.1	11.639		59,139	0.000404	0.0448
3	β-pinene	11.639	97	238,720	5.50E-13	4.50E-17
4	Unknown 1.2	12.255		37,965	5.35E-08	2.26E-07
5	Δ-3-carene	12.672	90	10,460	4.09E-14	1.80E-20
6	Δ-limonene	13.264	96	34,475	3.15E-08	6.33E-08
7	β-phellandrene	13.603	95	880,792	1.15E-08	2.49E-08
8	Para-cymene	14.018		3,935	8.14E-14	3.42E-28
9	β-linalool	17.889	96	13,538	5.37E-12	5.75E-14
10	Anisole	20.422	97	12,184	1.72E-11	7.97E-16
11	Unknown 1.3	22.464		65,918	1.12E-19	3.63E-55
12	2,4-dimethylbenzaldehyde	22.534	97	674,268	2.39E-07	2.72E-05
13	Unknown 1.4	22.957		1,986	1.63E-18	4.77E-40
14	α-copaene	23.024	88	8,188	5.78E-10	3.50E-08
15	Unknown 1.5	23.120		3,818	3.57E-15	2.49E-24
16	Unknown 1.6	23.203		5,733	4.09E-10	7.90E-13
17	Germacrene D	23.949	90	2,975	3.30E-10	1.29E-19
18	β-elemene	24.195	95	2,687	2.67E-14	3.59E-25
19	Unknown 2.2*				8.68E-11	5.03E-11
20	α-muurolene	26.639		29,621	1.17E-10	1.43E-10
21	Unknown 2.3*				1.88E-09	2.15E-08
22	Unknown 2.4*				3.54E-12	1.58E-13
23	γ-muurolene	27.276		96,954	3.80E-17	1.62E-28
24	Δ-cadinene	27.330	95	82,389	7.13E-09	4.90E-05
25	Unknown 2.5*				4.62E-19	4.16E-52
26	Δ-cadinol	28.890		20,625	3.53E-10	1.04E-10
27	Unknown 2.6*				1.37E-11	3.93E-14
28	Cubenol	30.355	85	22,040	3.48E-10	4.30E-07
29	Germacrene D-4-ol	30.593	90	585,097	2.16E-10	1.44E-08

[a] Retention time (minutes).
[b] MS library lookup match score.
[c] Average peak area across all samples.
[d] Shapiro-Wilk test for normality, P-value.
[e] Lilliefors test for normality, P-value.
* Unknown compound identified in Ch. 2.

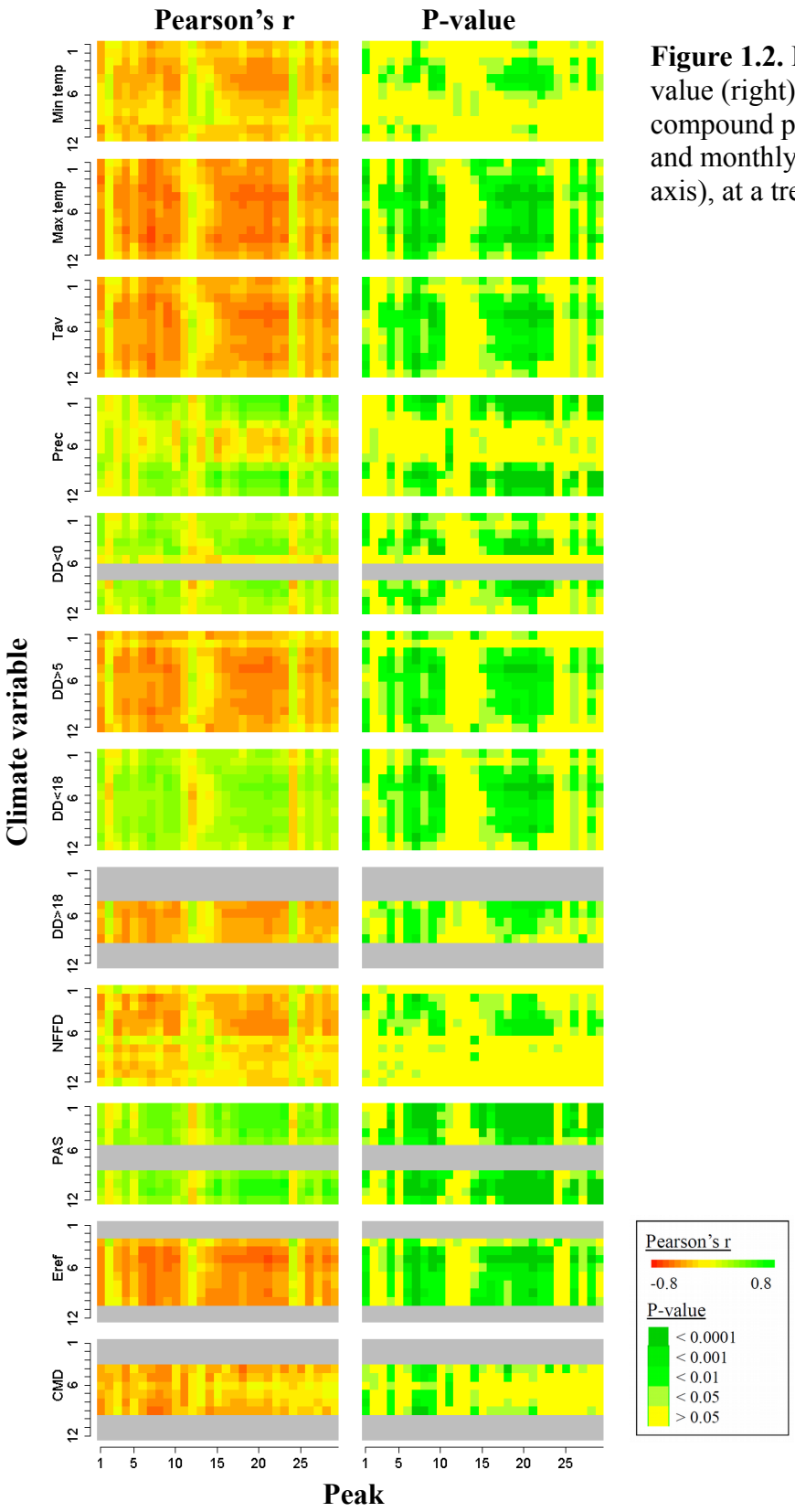

Figure 1.2. Pearson's r (left) and P-value (right) for correlations between compound peak area (1-29; x-axis) and monthly climate variables (y-axis), at a tree level.

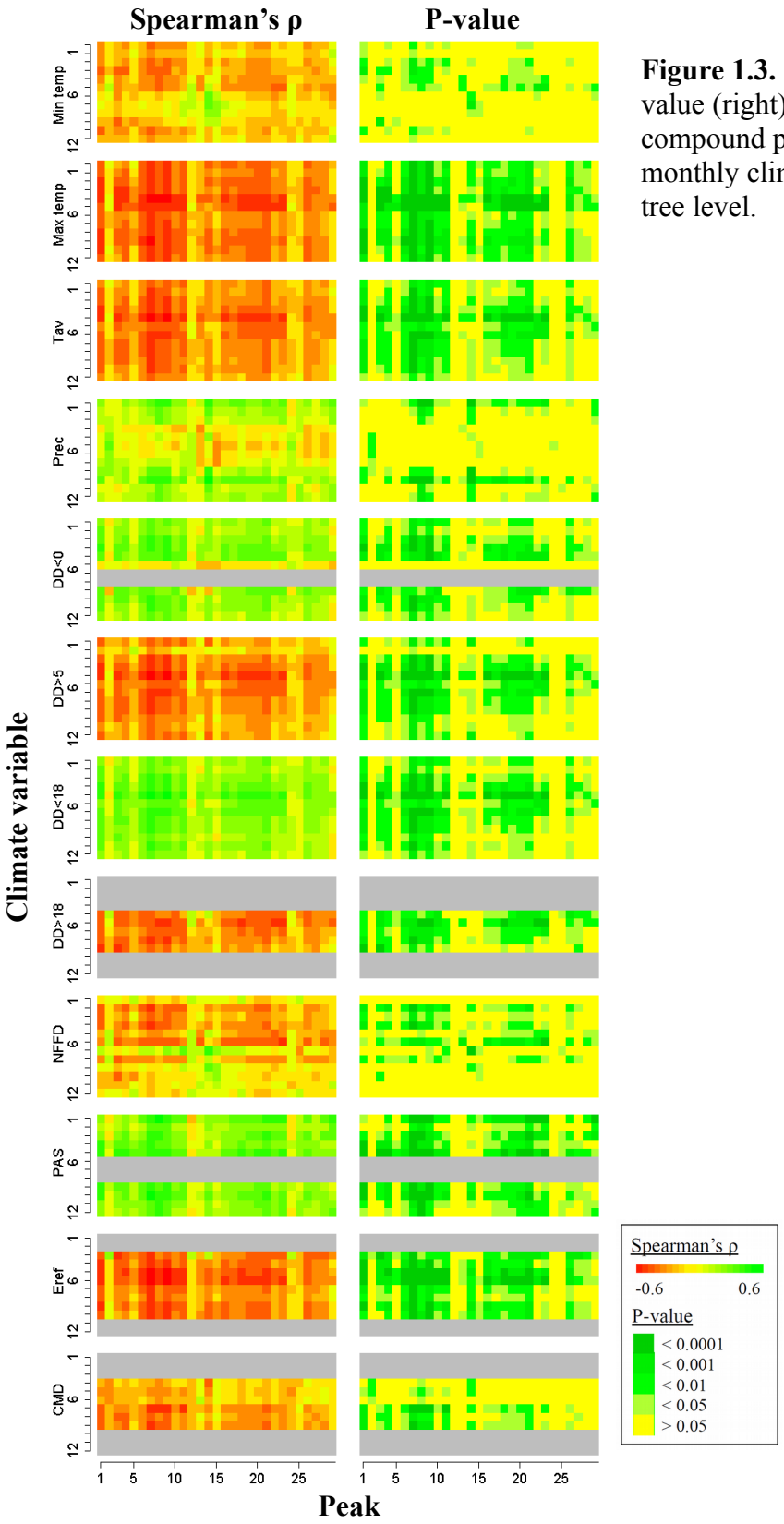

Figure 1.3. Spearman's ρ (left) and P-value (right) for correlations between compound peak area (1-29; x-axis) and monthly climate variables (y-axis), at a tree level.

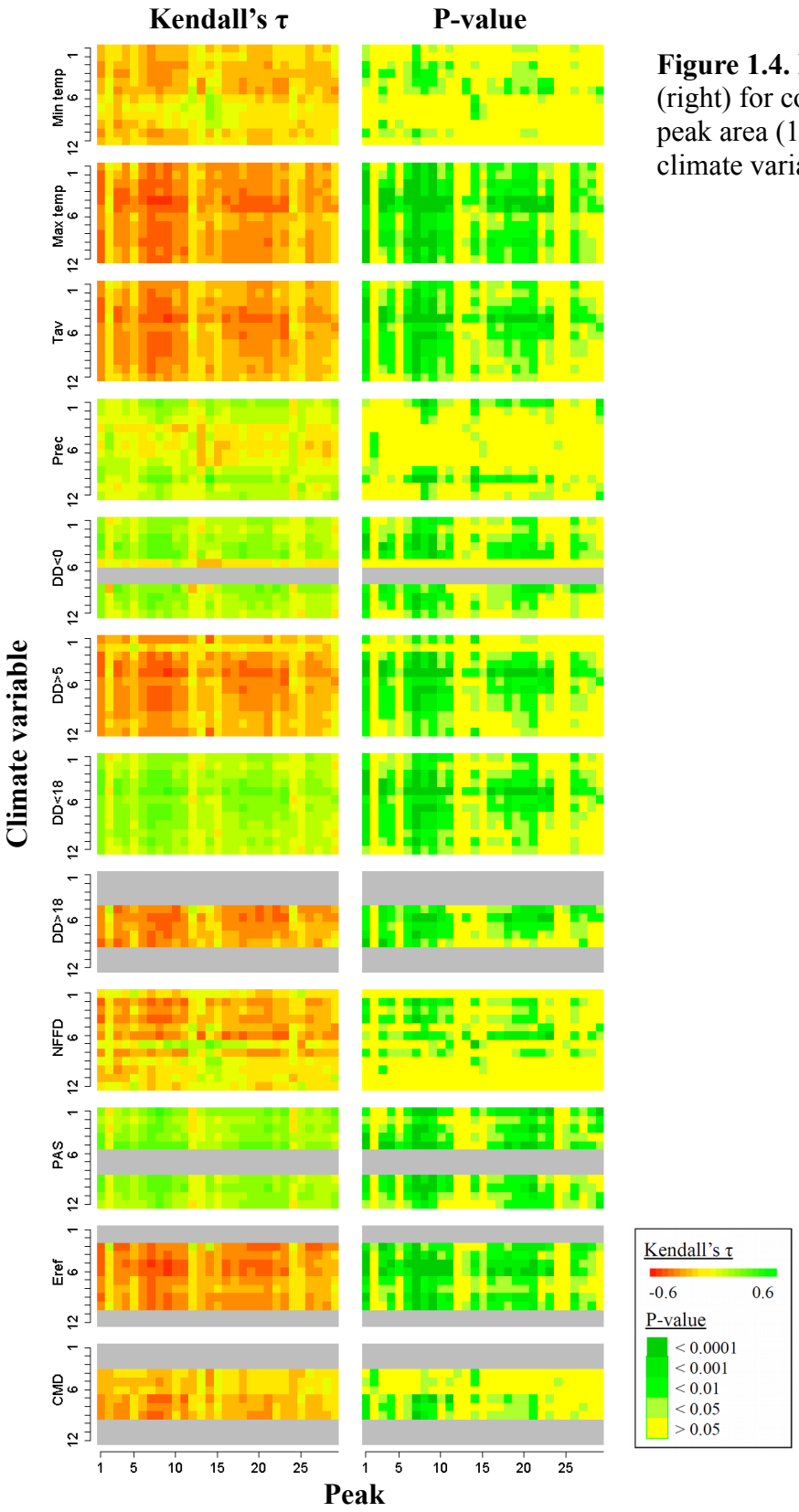

Figure 1.4. Kendall's τ (left) and P-value (right) for correlations between compound peak area (1-29; x-axis) and monthly climate variables (y-axis), at a tree level.

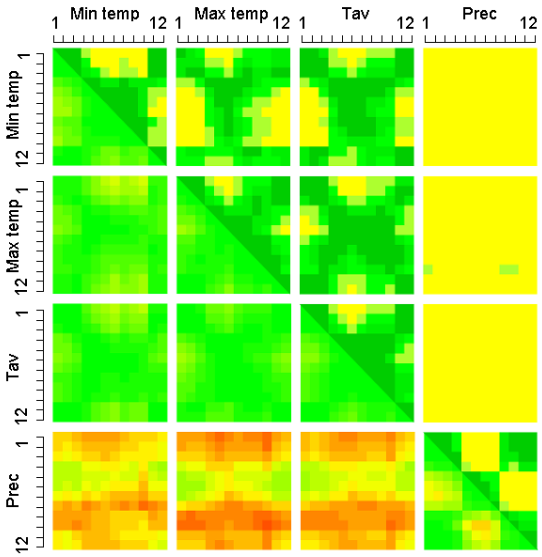

Figure 1.5. Pearson's r (bottom left) and P-value (top right) for inter-correlations between monthly climate variables, at a provenance level.

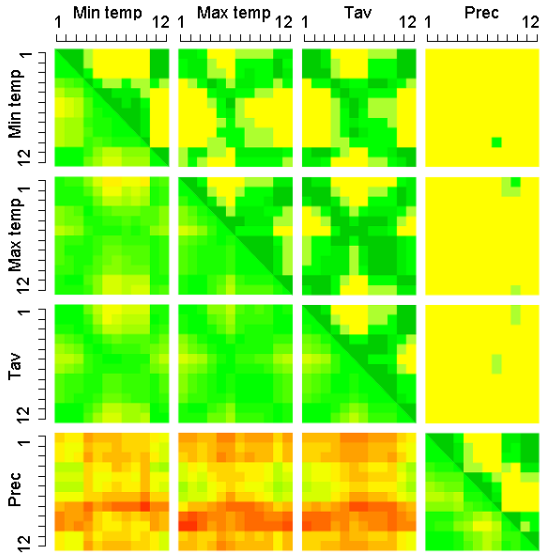

Figure 1.6. Spearman's ρ (bottom left) and P-value (top right) for inter-correlations between monthly climate variables, at a provenance level.

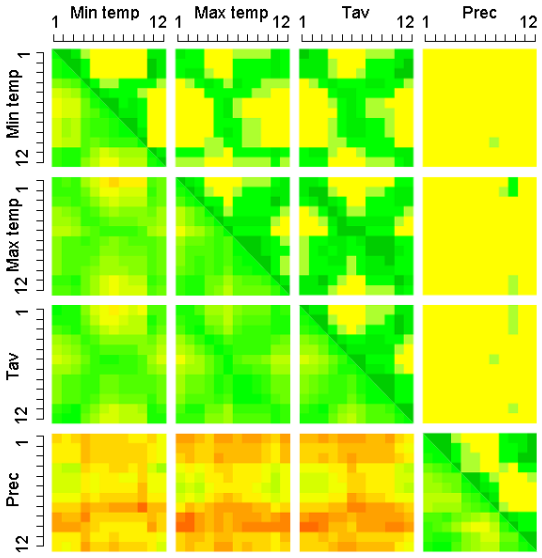

Figure 1.7. Kendall's τ (bottom left) and P-value (top right) for inter-correlations between monthly climate variables, at a provenance level.

Correlations with precipitation and PAS were strongly positive in most cases. Positive correlations with January and October precipitation were especially strong.

A number of compounds showed different patterns of correlation. Three peaks (2 – unknown 1.1; 12 – 2,4-dimethylbenzaldehyde; 24 – Δ-cadinene) showed little response to the majority of the climate variables tested, though peak 2 showed a significant positive correlation with summer precipitation. A further three peaks (11 – unknown 1.3; 14 – α-copaene; 15 – unknown 1.5) showed strong positive responses to minimum mean temperatures of June to September.

Climate variables showed strong patterns of intercorrelation (Figures 1.5-1.7). T_{av} and T_{max} were significantly correlated almost year-round, whereas T_{min} was only loosely correlated with these variables (and itself) outside of the same season. August to October precipitation showed some significant correlation with temperature variables, and weak (non-significant) correlations were seen between summer T_{av}/T_{max} and January precipitation.

1.4 Discussion

Overall, foliar levels of identified compounds were consistent with previously published work, with β-pinene and β-phellandrene being the two of the largest components of most samples (Pauly and von Rudloff 1971; Wallis et al. 2011; Ioannou et al. 2014). While levels of most compounds were within the expected range, several were quite different to that seen in previous publications on lodgepole pine. On average, germacrene D-4-ol (peak 29) made up a much higher proportion of total quantified metabolites than in previous studies, and the compound 2,4-dimethylbenzaldehyde (peak 12) has not been identified in conifers in any prior literature. The latter compound has been detected in the extracts of other plants (*Camillia sinensis* L. - Mick and Schreier 1984; *Capsicuum annuum* L. - Guadayol et al. 1997). The high average peak areas seen for germacrene D-4-ol and 2,4-dimethylbenzaldehyde are partly due to their presence above the threshold of quantification in all samples. This suggests that these compounds could be environmental or extraction contaminants, but I identified no plausible contamination source for these chemicals based on a search of published literature. 2,4-dimethylbenzaldehyde shows little significant correlation with any of the monthly climate variables tested, but germacrene D-4-ol shows a similar response to other terpenes: This indicates that 2,4-dimethylbenzaldehyde could be a contaminant, but germacrene D-4-ol is unlikely to respond in this way by chance. Only trace levels of these compounds were present in extraction blanks, consistent with residual sample carryover from the GC column. Baking out between samples would likely reduce carryover in future analyses, but was not feasible in this study due to the large number of samples tested in the given timeframe. Since analytical standards of these compounds were not available,

their identity cannot be determined with absolute certainty by this study. More in depth identification work carried out in Chapter 2 indicates that germacrene D-4-ol may be misidentified by library-based identification due to the highly similar fragmentation patterns of related terpenes, but the peak is otherwise likely to be a sesquiterpene alcohol. In this study I identified compound peaks primarily by automated matching of MS fragmentation patterns to mass spectral libraries and relative retention times, as per previously established methods (Wallis et al. 2008; Wallis et al. 2010; Wallis et al. 2011). While this approach is likely to be accurate for commonly quantified compounds with readily available standards, for higher retention time compounds with very similar fragmentation patterns, such as sesquiterpene alcohols, identities are only tentative. I attempted identification of a number of these compounds in Chapter 2, but without authentic standards conclusive identification may require a combination of data from multiple analytical techniques as well as via synthetic chemistry (Lee et al. 2007; Svatos and Attygalle 1997).

While the methods of this study assume that I collected completely disease free lodgepole pine foliage, it is possible that latent infections, or infections by endophytes, were present. Pathogen symptoms are not always visible or obvious, especially in early infection, as the lifecycle of plant pathogens can include an asymptomatic (latent) phase. In the fungal pine pathogen *Dothistroma septosporum* this phase can be as long as 8 weeks (Kabir et al. 2014), but the presence of fungal elicitors could still lead to rapid local upregulation of secondary metabolite levels before disease symptoms are visible (Hotter et al. 2007). Conversely, secondary metabolite levels could remain elevated long after infection or symptoms have disappeared: Systemic defense responses in conifers are long lasting (Bonello et al. 2001)

and include increased levels of monoterpenes and sesquiterpenes (Miller et al. 2005; Wallis et al. 2008). Differences in attack history could explain some of the variability in compound peak area between clonal trees, where different levels of induced or systemic defense responses result in variable secondary metabolite production above constitutive levels. While it may be possible to measure and normalize defense responses (e.g. by measuring levels of signaling molecules such as salicylic acid and methyl jasmonate: Wilbert et al. 1998; Engelberth et al. 2006; Phillips et al.2006), this was beyond the scope of this study. The high measures of association seen in figures 1.3 and 1.4 indicates that the potential lack of statistical power commonly associated with nonparametric tests was not a problem in this study, likely due to the large sample size (Bishara and Hittner 2012).

The high correlation coefficients I observed with historic climate conditions at provenance origin indicate that constitutive levels of the foliar secondary metabolites quantified are under strong genetic control. This is consistent with previous literature on the heritability of secondary metabolite levels in a variety of plant species (Hanover 1966; Hamilton et al. 2001). A number of theories of plant defense attempt to explain the relationship between environmental conditions and defensive compound abundance; however, the complex nature of these systems and lack of a unified theory of plant defense makes them difficult to apply to my results (Stamp 2003). For the purposes of this study the various hypotheses of plant defense [Optimal Defense (OD); Carbon:Nutrient Balance (CNB); Growth Rate (GR); and Growth:Differentiation Balance (GDB)] make limited predictions on the relationship between levels of defensive compounds and a diverse and difficult to quantify range of factors, such as maximal growth rate, herbivory, and light levels. For example, the

Carbon:Nutrient Balance (CNB) hypothesis predicts that for trees with a plastic defense response a change in relative carbon:nitrogen availability can result in a change in levels of defensive compounds. Those adapted to shaded and low-nutrient sites will have a slower growth rate and less plastic phenotype, and will show less response to C:N ratio changes. This change in defense applies to total rather than individual defensive compound levels, and is relative to the baseline genetic defense of the plant at maximal growth rate and under ideal conditions (Stamp 2003). The CNB hypothesis does not make predictions on the levels of individual metabolites, and as neither optimal growth rate, total defense, nor baseline genetic defense are easily quantifiable its predictive value is limited in this type of study. Furthermore, CNB is thought not to apply to terpenes (Gershenzon 1994), and there is debate about its usefulness considering its narrow applicability (Hamilton et al. 2001). The OD, GR, and GDB hypotheses are all similarly constrained with respect to this work; while it is possible to use these hypotheses to make broad justifications for the observed results, there is no way to directly test their predictions from the data collected.

While heat stress and drought are known to affect production and emission of volatile secondary metabolites (Monson and Fall 1989; Llusia and Penuelas 1998; Grote et al. 2009; Lavoir et al. 2009; Loreto and Schnitzler 2010), their effects are not obvious in my results. Plants emit secondary metabolites both deliberately to protect against extreme temperatures, and as a consequence of their volatility (Singsaas et al. 1997; Sallas et al. 2003; Sakasi et al. 2007). Provenances that historically experienced high temperature stress could be expected to produce greater levels of these secondary metabolites, but no significant positive correlations were observed with climate variables relating to high temperature. This could indicate that

heat stress does not have a strong selective influence on the provenances tested, or that variation in production of the quantified compounds as a response to temperature is largely plastic rather than genetically fixed. That foliar terpene production is dynamically regulated as a response to temperature is consistent with published literature on plant volatile production and emission. The vapor pressure of terpenes increases rapidly with increasing temperature (Pickett and Peterson, 1929), with both foliar levels and emissions of terpenes peaking during summer months (Yatagai et al. 1995). The emission of these volatiles represents a sizeable investment of fixed carbon (Monson and Fall 1989; Llusia and Penuelas 2000); considering the high level of control conifers display over terpene production (Huber et al. 2004) they are unlikely to allocate these resources wastefully. Similarly, drought or water stress seems unlikely to have strong effects on constitutive secondary metabolite levels. There is no substantial physiological basis for historical drought increasing constitutive foliar secondary metabolite levels, and while significant correlations were seen with Eref and CMD, measures of association were lower than that for temperature. As both are derived variables (Eref is calculated from temperature, and CMD is the total monthly difference between Eref and Prec; Wang et al. 2012) temperature is more likely to be the driving factor in these correlations.

Based on their preliminary identification, many of the quantified compounds are highly chemically similar. Compounds of the same chemical class are generally derived from the same biosynthetic pathway, and could respond similarly to changes in levels of a precursor. The monoterpenes and sesquiterpenes quantified in this study are synthesized from dimethylallyl diphosphate produced through the melvonate and methyl-erithritol 4-phosphate

pathways, respectively (Zulak and Bohlmann 2010; Vranova et al. 2013). That their levels correlate so similarly across many different climate variables could indicate that variability in their levels stems from a pooled precursor, or that the same factors shape regulatory or genetic variability of their late stage biosyntheses. Variation in their expression may be a factor of vigor, with plants under more suitable conditions having greater excess photosynthetic capacity to allocate to production of secondary metabolites. This is the explanation favored by Wallis et al. (2011), who quantified lodgepole pine secondary metabolite levels from naturally established lodgepole pine stands and compared them with seasonal climate variables at the same location. The authors found statistically significant correlations that are largely the opposite of those seen in this study, despite studying lodgepole pine provenances with a similar range. Monoterpenes and sesquiterpenes were found to positively correlate with summer, winter, and annual temperature, and monoterpenes showed negative correlation with summer precipitation. Wallis et al. (2011) hypothesized that the longer growing season associated with increased temperature allows increased photosynthetic capacity, but also favors the growth of pests, necessitating greater levels of defensive compounds. That I found trees from colder climates produce greater levels of monoterpenes and sesquiterpenes when environmental conditions are equalized favors the growing season length hypothesis over the influence of pests. Trees with shorter growing seasons would be expected to produce secondary metabolites faster in an attempt to maintain adequate levels, whereas trees conditioned to longer growing seasons could easily attain greater levels with slower production. An increase in constitutive secondary metabolite levels driven by historically greater exposure to insects and disease due to more conducive temperature would be expected to give the opposite result to that shown in this study. While

it is likely that temperature influence on insect and disease abundance has driven some level of constitutive secondary metabolite level variation in lodgepole pine, any detectable genetic effect on constitutive levels appears to be overshadowed by the influence of climate variables. Conifers can respond rapidly to attack by plant pests (Miller et al. 2005), and induced or systemic responses to these threats may be more efficient than maintaining high constitutive levels of volatile compounds. Pathogen and insect attacks on stands can be infrequent and irregular (Welsh et al. 2009; Hrinkevich and Lewis 2011), and there are metabolic costs associated with the storage and maintenance of high levels of volatile terpenes (Gershenzon 1994). To separate the role of disease and insect pest incidence from that of climate variables requires that the former be directly quantified, rather than derived from the latter. This can be done through dendrochronological methods for a number of tree pests and pathogens, including western spruce budworm (*Choristonerua occidentalis* Freeman; Swetnam and Lynch 1989) and *D. septosporum* (Welsh et al. 2009). Using these techniques, a more in depth investigation on the relationship between disease history and constitutive secondary metabolite levels was carried out in chapter 4.

The strong positive correlations with snowfall and precipitation are likely the result of some combination of climate inter-correlation, growing season length, and plant pest influence. Precipitation and snowfall have a complicated relationship with plant growth and secondary metabolite production; precipitation is a limiting factor in the growth of some northern conifer species (Griesbauer and Green 2010), but pathogens can also respond positively to increased precipitation (Gadgil 1974; Woods et al. 2005). Provenances that are growth-limited by precipitation have the capacity to produce greater constitutive levels of defensive

compounds (if precipitation limits growth more than differentiation; Herms and Mattson, 1992), but the opposite effect is seen in my study. Alternatively, precipitation as snow may affect metabolite levels by varying the length of the growing season. Increased snowpack can insulate forest soil from frost at warmer temperatures (Campbell et al. 2011), but also prolong thawing where temperatures are low enough for soil to freeze (Hardy et al. 2001); the presence of this soil frost in spring can postpone growth (Fahey and Lang 1975). Conversely, January snowfall can improve tree growth, with higher snowpack generating increased spring snowmelt (Wang et al. 2006). Snowfall has also been shown to affect the carbon:nitrogen ratio in some species of plants (Welker et al. 2005) due to changes in soil microbial activity and nitrogen leaching (Brooks and Williams 1999; Schimel et al. 2004). Despite being non-nitrogenous, terpenoids can respond positively to increases in nitrogen levels (Bjorkman et al. 1991), possibly because differentiation of resin producing structures is nitrogen-limited. Despite these potential physiological influences, some inter-correlation between August-October precipitation and temperature variables was seen, meaning that much of the observed correlation with precipitation could be derivative of temperature correlations. Weak inter-correlation with January precipitation was also observed. Further research will be required to quantify the effects of these various factors in isolation.

While these climate correlations were seen with the majority of quantified compounds, a number showed notably different responses. Some showed little to no correlation with any climate variables tested (e.g. peaks 2, 12, and 24), whereas others had a different pattern of correlation (e.g. peaks 11, 14, and 15). As previously discussed, contaminant peaks would be expected to have no correlation with environmental variables other than that caused by Type I

error, but non-correlating peaks cannot be classified as contaminants on this basis alone. It is possible that these peaks respond strongly to some untested variable which overrides any response to the variables tested. Plant secondary metabolites respond to a wide variety of biotic and abiotic factors (Ramakrishna and Ravishankar 2011), many of which were not tested in this study. Alternatively, these peaks may be induced by some local factor or elicitor, with elevated induced levels eliminating any observable variation in constitutive levels.

Peaks 11, 14, and 15 showed strong positive responses to summer minimum temperature and NFFD relative to their responses to other climate variables. The measures of association for peak 14 were particularly high in the hottest months of the year (July, August, and September). While summer minimum temperature has an important relationship with surface humidity (Kimball et al. 1996), correlation coefficients with precipitation variables in these months were low. Summer minimum temperature is also important for recruitment near the tree line (Wang et al. 2006), but what negative effect this compound would have on low temperature resistance (or positive effect at higher minimum temperatures) is not known. That these compounds both respond differently than the majority of other compounds in this study, despite putative chemical similarity, and are maintained at such high levels suggests that their variation is controlled by factors not tested in this study, and that they may have a specific function. Terpenoids are metabolically expensive to produce, and their levels are reduced when they are no longer required (Gershenzon 1994). Any further investigation into these compounds should start with their conclusive identification by comparison of GC-MS fragmentation pattern and retention time to analytical standards. Identification of these compounds may shed light on their potential function.

This work forms the basis for several other potential avenues of investigation. This study found temperature to be a strong determinant of constitutive foliar secondary metabolite levels, and indicates that this is due to variable growing season length. However, growing season length is often only weakly correlated with seasonal temperature variables (Linderholm 2006). Growing season length in these provenances could be determined directly using sapflow measurements (Barnard et al. 2017) and correlated with both plantation metabolite level results from this study and natural stand data from previously published studies (Wallis et al. 2011). Quantifying the disease history of the provenances studied using dendrochronological methods (Swetnam and Lynch 1989; Welsh et al. 2009) would enable determination of the relative influence of growing season length and disease history on constitutive secondary metabolite levels.

The effect of constitutive vs induced secondary metabolite levels on disease is of great importance to current work on plant resistance. In the case of economically important pine pathogens such as *D. septosporum*, little is known about the relative importance of these methods of defense (Fraser et al. 2015). Current work on the volatile defensive compounds of lodgepole pine has focused on levels in the absence of visible disease (Wallis et al. 2010; Wallis et al. 2011); if resistance has a greater dependence on induced defence than constitutive defense then the implications for disease susceptibility will be very different. In chapter 2 I correlate crown retention values for resistance trial lodgepole pine with variable resistance to *D. septosporum* with secondary metabolite levels in the foliage of uninfected replicates. Comparison of foliar secondary metabolite levels between genetically identical infected and uninfected trees could give some indication of their relative ability to respond to

pathogens with induced secondary metabolites. Resistance to this pathogen is likely to be multifactorial (Carson, 1989), with both constitutive and induced levels of secondary metabolites playing a role in defense.

The high correlation coefficients observed between foliar volatile secondary metabolite levels of plantation lodgepole pine and the climate of its provenance origin indicate that production of these compounds is under strong genetic control, and invites further investigation into multi-generational factors influencing their constitutive and induced production in different environments. The complex interaction between the different environmental variables tested makes it difficult to form concrete conclusions on the physiological basis of the observed correlations; however this work indicates that temperature is the primary factor influencing genetic variation in constitutive foliar secondary metabolite production for these lodgepole pine provenances. That this response is the opposite of that seen in natural lodgepole pine stands suggests that this response is mainly due to the influence of temperature on growing season length rather than pest prevalence. While the majority of quantified compounds responded similarly to climate variables, the fact that some showed entirely different patterns of correlation despite putative chemical similarity indicates that levels of these compounds may respond to some yet undetermined environmental factor or factors. Future work should focus on firmly establishing the effect of growing season length on constitutive defense levels, identifying that relative effect of constitutive and induced levels of defensive compounds on pathogen resistance, and identifying unknown compounds with potentially important physiological roles.

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2. Constitutive foliar secondary metabolites levels of lodgepole pine and correlation with crown retention in a *Dothistroma* resistance trial

2.0 Abstract

Foliar secondary metabolites are broadly associated with plant defense, and are involved in resistance to a wide range of herbivores and plant pathogens. However, little study has been done on the specific interactions between *Dothistroma septosporum* (Dorog.) Morelet. and the foliar secondary metabolites of its pine hosts. In this study I aimed to identify volatile foliar secondary metabolites from lodgepole pine (*Pinus contorta* var. *latifolia* Dougl. ex Loud.) which contribute to *Dothistroma* resistance. I quantified 61 foliar volatiles from the foliage of apparently uninfected lodgepole pine using GC-FID, and correlated levels of these metabolites with family crown retention variables from a *Dothistroma* resistance trial. Levels of foliar volatiles were highly variable between samples, though intra-tree variation was lower than intra-family and inter-family variation. Relative levels of identified secondary metabolites were consistent with previous work and values found in literature. Levels of seven quantified compounds significantly correlated with crown retention variables using Kendall's τ or Spearman's ρ at an alpha level of 0.05, with a further fifteen significantly correlated with $\alpha = 0.10$. Three correlated compounds were putatively identified as α-cadinene [(1S,4aR,8aR)-4,7-dimethyl-1-propan-2-yl-1,2,4a,5,6,8a-hexahydronaphthalene], germacrene D-4-ol [(2Z,7Z)-1,7-dimethyl-4-propan-2-ylcyclodeca-2,7-dien-1-ol], and an isomer of farnesol [3,7,11-trimethyldodeca-2,6,10-trien-1-ol] based on their linear retention indices and GC-MS fragmentation patterns. There is a strong physiological basis for sesquiterpene alcohols acting as a chemical defense against fungal pathogens, and this work

forms a foundation for further investigation into these compounds as contributors to *Dothistroma* resistance.

2.1 Introduction

Chemical defense mechanisms play an important role in resistance of plant hosts to fungal pathogens (Fraser et al. 2015). The effectiveness of these defenses varies with the pathogen, and knowing which compounds are involved is an important step in understanding their contribution to host resistance. In the case of lodgepole pine (*Pinus contorta* var. *latifolia* Dougl. ex Loud.) and *Dothistroma septosporum* (Dorog.) Morelet, there has been considerable study of pathogen-produced virulence factors (Gallagher and Hodges 1972; Shain and Franich 1981; Bradshaw et al. 2000; Bradshaw et al. 2002; Schwelm et al. 2009; Chettri et al. 2012; de Wit et al. 2012; Kabir et al. 2015), but little published work on how host secondary metabolites affect the pathogen, or on resistance to *Dothistroma* in general. In a recent comprehensive review of the literature on *Dothistroma* the authors cited only five publications on Pinacea defense mechanisms specific to that pathogen (Fraser et al. 2015). To fill these knowledge gaps, further research is required on the defense interactions between the host and the pathogen.

Conifers produce myriad chemical and physical defenses against the numerous pathogens and herbivores that beset them (Franceshci et al. 2005; Huber and Bohlmann, 2005). How these defenses act on *Dothistroma* spp. specifically is largely unknown. However, defenses have been studied in related host-pathogen systems, and may act similarly on *Dothistroma*. Levels of defenses and defense-related compounds have been found to vary greatly between different species and provenances (and to a lesser extent stands and trees) based on their abiotic and biotic environment (Wallis et al. 2011; Ioannou et al. 2014). Consequently, trees differ in their ability to defend against specific pathogens and herbivores, with species and

provenances showing varying degrees of resistance or susceptibility (Watt et al. 2009; Wallis et al. 2010). Study of resistant individuals or populations is a common approach to understanding plant defense, and much of the early study on *Dothistroma* resistance focused on resistant age classes of *Pinus radiata* D. Don.

It was suggested by Franich et al. (1977) that resistance observed in these individuals was a result of increasing occlusion of stomatal pores by epicuticular wax. This would hypothetically inhibit germ tube penetration of the needle surface, and this hypothesis was supported by similar work on white pine blister rust resistance in *Pinus monticola* Dougl. (Woo et al. 2001), which showed a correlation between stomatal pore size and resistance (though wax occlusion did not vary significantly in that species). Franich et al. (1977) found that the proportion of occluded stomata increases with tree age, suggesting that this physical defense could be responsible for observed resistance in mature *P. radiata* (Gibson 1972). Further research showed that components of epicuticular and stomatal pore wax could act as constitutive chemical defenses (Franich and Gadgil 1983). While previous study (Walla et al. 1976) found crude pine needle waxes had no effect on *D. septosporum* cultures, selected resin and fatty acid components were shown to be highly fungistatic *in vitro.* Removal of needle surface waxes with acetone significantly increased infection rate in an *in vivo* infection assay, strongly indicating that the chemical or physical properties of needle surface wax contributed to mature tree resistance in *P. radiata.*

It has been hypothesized that mature tree resistance is influenced by foliar terpenes, as levels of these compounds vary with tree age. However, in a study by Franich et al. (1982) there was no correlation between tree age and *in vitro* inhibitory activity of crude foliar volatile

extracts containing predominantly α-pinene (2,6,6-trimethylbicyclo[3.1.1]hept-2-ene), β-pinene (6,6-dimethyl-2-methylene-bicyclo[3.1.1]heptane), 3-carene (3,7,7-trimethylbicyclo[4.1.0]hept-3-ene), limonene [1-methyl-4-(1-methylethenyl)-cyclohexene], and β-phellandrene (3-methylidene-6-propan-2-ylcyclohexene). Tests showed a complex relationship between mycelial growth and volatile extract concentration, with lower levels of crude extract promoting mycelial growth (10 – 300 ppm) and inhibition only occurring at levels approximating those found in needles (1000 ppm or 0.1%). Spore germination assays showed increased germination in the presence of crude extract, but most of the purified monoterpenes tested were found to significantly inhibit germination. This, along with more recent work on other fungal foliar pathogens (Wallis et al. 2010), indicates that while foliar terpenes could contribute to resistance, the relationship is complex, and likely to be dependent on individual chemical components. This complexity, the logistical challenges involved in assessing *Dothistroma* resistance in the field (Franich et al. 1986; Carson 1989), and the difficulty associated with developing a reliable *Dothistroma* pathogenicity assay (Kabir et al. 2013) have likely contributed to the relative paucity of research in this area.

The inherent limitations of working with long-lived, forest tree species *in vivo* have led to attempts at representative *in vitro* assays. Franich et al (1986) investigated artificial lesion induction using the *Dothistroma* mycotoxin dothistromin, and hypothesized that lesion length could be used as an indicator of resistance. While lesion length was not a reliable marker, induced lesions were found to contain high levels of benzoic acid. Benzoic acid was consequently shown to be produced by cells surrounding the lesion, and was demonstrated to be both fungistatic to *D. septosporum in vivo* and toxic to *P. radiata* needle tissue. Areas

surrounding both artificially-induced and infection-induced lesions were also highly lignified, suggesting that lignin production is an induced, physical, defense in response to *Dothistroma* infection (Franich et al. 1986). Induction of lignin production *in vitro* was further investigated by Hotter (1997), who showed that the presence of cell-wall elicitors from *D. septosporum* can induce a variety of defense responses in host cells. Addition of crude cell wall extracts to *P. radiata* cell cultures resulted in a short-term oxidative burst response, and increased activity of lignin biosynthesis pathway enzymes phenylalanine ammonia lyase and cinnamyl alcohol dehydrogenase. Lignin levels were elevated over the 96-hour period tested.

While these studies illustrate that *P. radiata* deploys a number of different defense mechanisms against *D. septosporum,* these are unlikely to represent the entire complement of defenses contributing to observed resistance. Breeding studies performed on *P. radiata* suggest that resistance in that species is multifactorial - a combination of small improvements from a variety of defense mechanisms and the interactions between them (Carson 1989). While study of resistance in lodgepole pine has been relatively lacking (in part due to the recently emergent nature of the disease in British Columbia; Woods et al. 2005), the similarities in both foliar chemistry (Ioannou et al. 2014) and resistance (Watt et al. 2009) between the two species make it likely that similar defense mechanisms are responsible for observed *Dothistroma* resistance. While some of these have been identified and described above, there remain a large number of known plant defense mechanisms that have not yet been investigated in the context of the *Pinus-Dothistroma* pathosystem. Fraser et al. (2015) identified numerous gaps in current knowledge, including needle wettability, the role of

recently discovered *D. septosporum* avirulence factors (de Wit et al. 2012), and the chemical defense contribution by terpenes - the latter of which is the subject of this study.

In this chapter I describe how I investigated the chemical basis for *Dothistroma* resistance in *P. contorta*, focusing specifically on terpenes. Investigations of physiological or chemical differences between resistant and susceptible varieties or age classes have previously identified potential resistance mechanisms to *D. septosporum* (Franich et al. 1977; Franich and Gadgil 1983) and other fungal foliar pathogens (Lawrence et al. 2000; Woo et al. 2001; Tivoli et al. 2006). Recent work on *P. contorta* resistance trials by the British Columbia Ministry of Forests, Lands, Natural Resource Operations & Rural Development (MOFLNRORD, hereafter referred to as MOF) allowed me to directly study resistant varieties. The aim of my study was to identify volatile foliar secondary metabolites with a potential contribution to *D. septosporum* resistance.

The objectives of the work described in this chapter were to: 1) quantify a wide range of secondary metabolites from foliage of susceptible and resistant lodgepole pine; 2) identify foliar secondary metabolites that correlate with crown retention; and 3) propose physiological explanations for identified metabolites that are related to crown retention.

2.2 Methods

The B.C. MOF *Dothistroma* resistance trial (Figure 2.1), established near Hazelton B.C. in the Interior Cedar/Hemlock biogeoclimatic zone (Meidinger and Polar, 1991), featured two replicate sites: the high-infection 1200A site (55.508164°N, -127.960066°W), which was assessed for crown retention and growth by the MOF, and the 1200C site (55.504443°N, -127.975914°W), which showed negligible *D. septosporum* symptoms by visual assessment (Ukrainetz et al. 2013). I used two crown retention variables in my study, crown retention percentage (CRN12: percentage of live crown relative to total tree height at age 12) and live crown (LIVCRN: CRN12 multiplied by the percentage of healthy internodes in the crown at age 12, expressed as CRN12*[NOD12/TNOD12]). The methods used for CRN12 and LIVCRN assessment are defined by the B.C. MOF (2005), which uses CRN12 as a measure of *Dothistroma* resistance in lodgepole pine families and LIVCRN for assessment of *Dothistroma* damage to regenerating stands.

I collected needles from a total of 19 families at the 1200C site (to target constitutive defence compounds), taking three needle samples from each of three half sib (sibling) trees per family for a total of 171 collected samples. I selected the 19 families of interest out of 111 total families to include high crown retention families (Figure 2.2: 3043, 3055, 3061, 3096, 3089, 3107, 3113, 3127, 3140, and 3143), low crown retention families (Figure 2.2: 3078, 3079, 3108, 3110, and 3145), high growth/high crown retention families (Figure 2.2: 3043, 3052, 3061, 3127, and 3143), and families under study by the MOF (Figure 2.2: 3034, 3043, 3050, 3055, 3061, 3078, 3108, 3110, 3113, and 3142).

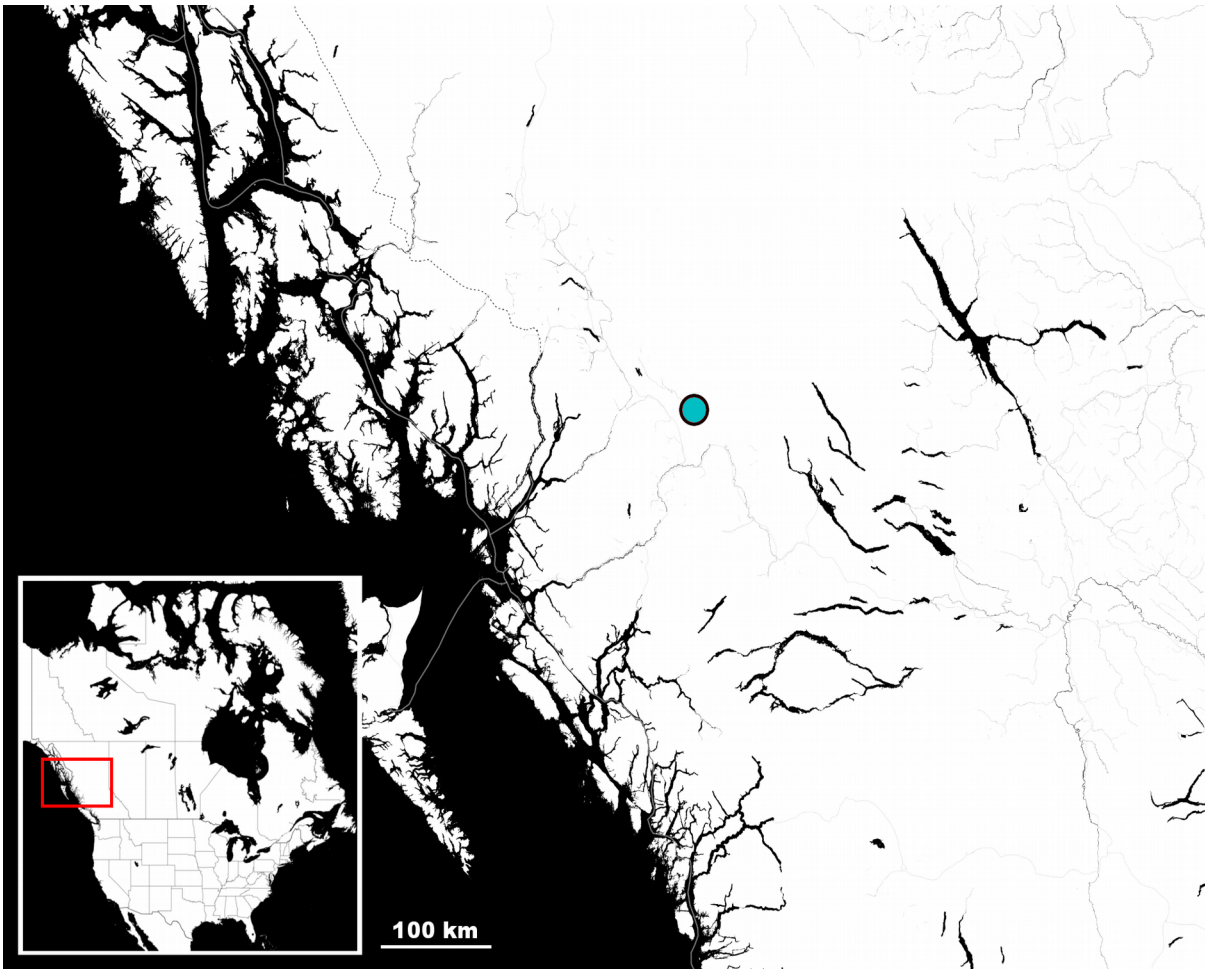

Figure 2.1. Location of MOF *Dothistroma* resistance trial north of Hazelton, B.C. (blue). Map tiles by Stamen Design, under CC BY 3.0. Data by OpenStreetMap, under OdbL.

I carried out foliage sampling over the period of one day in November 2014. I collected visibly disease-free needle samples at breast height from north, west, and south facing aspects of each tree (to control for directional effects), snap froze them in liquid nitrogen, and stored them on dry ice for transport. I subsequently stored all samples at –80°C until they were processed. I excluded samples that were damaged or compromised in transport, leaving 157 samples for secondary metabolite quantification, representing 56 trees.

I extracted volatile foliar secondary metabolites as per the methods of Wallis et al. (2008). I ground samples under liquid nitrogen using a mortar and pestle, and added approximately 100 mg of ground, frozen tissue to a preweighed microcentrifuge tube. I then reweighed the tube and added 1000 µL of HPLC-grade dichloromethane (99.97%; EMD Millipore, Billerica, MA, USA) containing 0.1% analytical grade n-pentadecane (99.9%; Supelco Analytical, Bellefonte, PA, USA). The samples were then incubated at 4°C for 48 hours. Following extraction, I removed 200 µL of supernatant and transferred it to a gas chromatography (GC) vial containing a 200 µL insert. Samples were quantified on a Varian CP-3800 gas chromatograph (Palo Alto, CA, USA) equipped with a Varian CP-8400 autosampler or Varian CP-8410 autoinjector, Varian 1177 injector, Phenomenex VF-5ms column (Torrance, CA, USA), and flame ionization detector (FID). Inline mass spectroscopy was carried out using a separate, sequential injection into a Varian 1079 injector, Phenomenex ZB-5ms column, and Varian Saturn 2200 MS. N-pentadecane (99.9%; Supelco Analytical), n-heptadecane (99%; Alfa Aesar, Ward Hill, MA, USA), and n-octadecane (99%; Alfa Aesar) were used as retention index standards for my preliminary linear retention index calculations. I used the GC temperature gradient program of Wallis et al. (2008) without

modification. This program starts with an oven temperature hold of 40°C for 2 minutes, ramping to 240°C at 10°C per minute, then ramping at 50°C min^{-1} to 300°C, and ending with a 5-minute temperature hold. Injector and detector temperatures were set at 220°C, and a split injection mode was used with a split ratio of 12:1. Flow rate of the hydrogen carrier gas was set to a constant 2.1 ml min^{-1}. Total run time was 28.2 minutes.

I quantified FID peaks above the baseline area of 500 units, and each peak area was normalized to both the fresh weight of the sample and the mean peak area of the n-pentadecane internal standard. Analysis of both sample peak areas (n=157) and tree mean peak areas (n=56) was carried out using R 3.3.3 (R Core Team 2017) with modules nortest (Gross and Ligges, 2015), e1071 (Meyer et al. 2017), MASS (Venables and Ripley, 2002), and ggplot2 (Wickham 2009). I checked tree mean peaks areas for normality using Shapiro-Wilk and Lilliefors tests (Gross and Ligges, 2015), and attempted normal transformation of this data with square root, square root of square root, logarithmic, Box-Cox (Box and Cox, 1964), inverse hyperbolic sine (Burbidge et al. 1988), and mirroring transforms. Pearson product-moment correlation coefficient, Kendall's τ, and Spearman's ρ were calculated between peak areas (sample and tree mean) of all quantified compounds at the 1200C site and the family mean of crown retention variables (CRN12 and LIVCRN) provided by the MOF for trees at the 1200A site. While most peak areas were not normally distributed, Pearson correlation coefficients were included in the analysis to allow comparison with previous work (Wallis et al. 2010). I selected six peaks showing significant ($P<0.05$) correlation to crown retention variables in initial tests for identification and further investigation (U1-6). I attempted identification of these peaks (U1-6) using a combination of

relative retention time, MS fragmentation pattern analysis (automated and manual), linear retention index, and comparison with authentic analytical standards (where available). I examined the MS spectra of peaks U1-6 for overlap using AMDIS (Automated Mass-spectra Deconvolution and Identification System; http://www.amdis.net/).

2.3 Results

Lodgepole pine families assessed by the MOFLNRORD were consistent in their relative mean crown retention percentage (CRN12) and live crown (LIVCRN; e.g. families with high CRN12 also showed high LIVCRN - Figure 2.2). Low standard errors for family CRN12 and LIVCRN indicate that within-family variability was low (Figure 2.2).

A total of 61 FID peaks were quantified across 157 samples. Mean tree-level peak areas and standard errors for quantified compounds are shown in Table 2.1. The peak tentatively identified as β-phellandrene (based on retention time and library match) showed the greatest mean peak area, though variation between samples was high (as seen in Chapter 1 of this thesis). Many low-level compounds were below the limit of quantification in a large number of samples. Peak area histograms and normality tests showed that distribution of peak areas was non-normal in most cases, and was largely resistant to transformation. This was often due to a large number of lower-than-threshold samples skewing the distribution of peak areas low or a small number of high peak area samples skewing the distribution high. Measures of normality for tree-level means of quantified compounds are given in Table 2.1.

Peak areas of quantified compounds were highly variable between samples (see Table 2.1), though the standard error (SE) of differences between sum peak area of individual samples and group means (Sum_{sample}-Sum_{group}; n=157) indicated that intra-tree (SE 21540) and intra-family variability (SE 30983) were lower than inter-family variability (SE 33886). Variability of individual peaks among samples was largely independent of variation in total peak area.

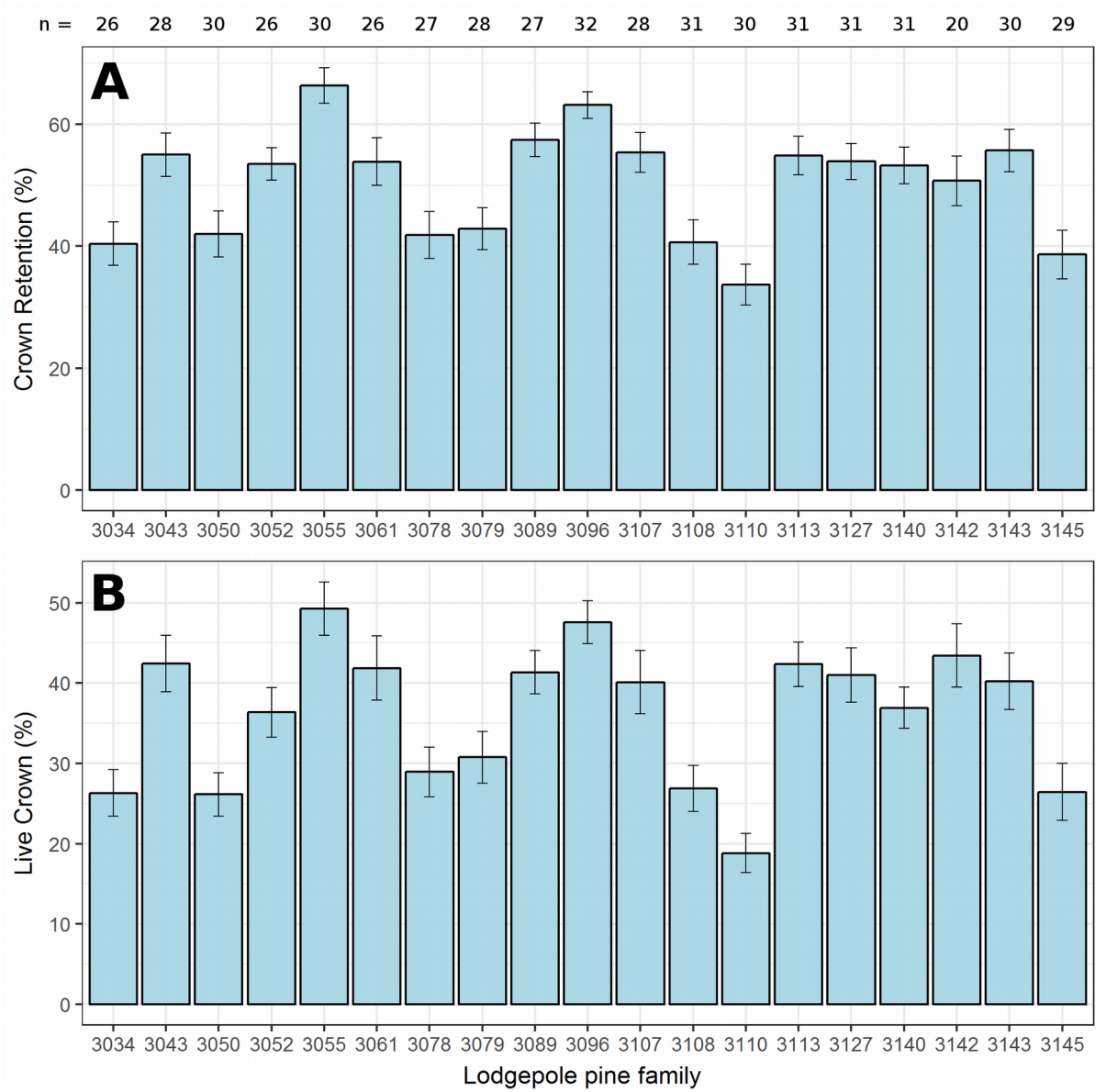

Figure 2.2. Mean **A)** crown retention (CRN12) and **B)** live crown (LIVCRN) percentages for lodgepole pine families assessed at age 12 in the Kispiox 1200A *Dothistroma* resistance trial conducted by the Ministry of Forests, Lands and Natural Resource Operations (Ukrainetz et al. 2013). Error bars show standard error.

Table 2.1. Mean peak area, variance, and normality of GC-FID quantified compounds from the foliage of Kispiox 1200C resistance trial trees, listed by retention time (n=56).

	ID[a]	RT[b]	Peak Area[c]	SE[d]	Shapiro[e]	Lilliefor[f]
1		2.486	5900	2794	0.0000	0.0000
2	Unknown 1	2.507	5331	2344	0.0000	0.0000
3		3.490	2962	205	0.0328	0.1124
4		4.050	6009	561	0.0009	0.0008
5		4.078	1609	108	0.1072	0.1926
6		4.338	632	95	0.0000	0.0000
7		4.679	9752	728	0.0000	0.0597
8		4.981	1759	131	0.4420	0.6837
9	α-pinene	6.322	13356	1535	0.0004	0.0356
10		6.622	1436	166	0.0002	0.0275
11		6.917	510	59	0.0005	0.0010
12		7.006	1911	220	0.0007	0.0145
13	β-pinene	7.102	38358	9417	0.0000	0.0000
14		7.258	8282	883	0.0003	0.1297
15		7.498	2238	118	0.1266	0.1745
16		7.576	4968	308	0.0349	0.1379
17		7.716	587	63	0.0035	0.0270
18	β-Phellandrene	7.981	226624	23944	0.0018	0.1390
19		8.020	3840	797	0.0000	0.0000
20		8.640	3490	155	0.3953	0.1548
21		8.706	3015	148	0.1990	0.1792
22		10.623	3332	713	0.0000	0.0000
23		10.951	331	129	0.0000	0.0000
24		11.075	919	60	0.1937	0.0455
25		11.172	1050	361	0.0000	0.0000
26		11.321	15458	14942	0.0000	0.0000
27		11.554	1470	1008	0.0000	0.0000
28		11.832	1825	284	0.0000	0.0000
29		11.937	6574	490	0.0000	0.0000
30		12.055	5281	180	0.5822	0.3547
31		12.169	4130	153	0.5506	0.4022
32		13.243	1563	166	0.0002	0.0009
33		13.671	234	102	0.0000	0.0000
34	Unknown 2	13.939	1933	106	0.2683	0.2399
35		14.051	4239	168	0.4244	0.3060
36		14.167	3722	222	0.0000	0.0000
37		14.268	18791	1436	0.1000	0.4649
38		14.646	5238	549	0.0115	0.1650
39		14.711	14638	752	0.6425	0.5617
40		14.849	13054	1370	0.0000	0.0000
41		14.888	23859	2336	0.0002	0.0013
42	Unknown 3	15.035	5442	303	0.0011	0.3424
43	Unknown 4	15.152	4096	167	0.0374	0.5494
44		15.307	1464	99	0.0183	0.1160
45	Unknown 5	15.624	15944	1877	0.0002	0.0023
46		16.536	7977	794	0.0000	0.0047
47		16.792	48802	3306	0.1136	0.1073
48		16.855	5900	569	0.0000	0.0010
49	Unknown 6	17.121	11553	696	0.0510	0.7565

50	17.221	5728	355	0.6325	0.2343
51	17.315	6149	616	0.0000	0.0000
52	17.433	15041	811	0.7787	0.9377
53	17.554	28671	1930	0.0000	0.0330
54	17.657	8171	733	0.0000	0.0000
55	17.774	7898	435	0.7231	0.8181
56	18.288	11692	1139	0.0000	0.0000
57	18.710	6221	858	0.0000	0.0000
58	19.290	10778	898	0.0000	0.0635
59	19.556	9723	904	0.0035	0.0206
60	19.588	14182	2128	0.0000	0.0000
61	20.131	17513	1301	0.0408	0.7632

[a] Putative identity or assigned name.
[b] Retention time (minutes).
[c] Mean peak area.
[d] Standard error.
[e] Shapiro-Wilk test for normality, P-value.
[f] Lilliefors test for normality, P-value.

Table 2.2.1. Sample level correlations between crown retention (CRN12) of 1200A families and peak areas of GC-FID quantified compounds from lodgepole pine at the 1200C resistance trial (n = 157).

	ID[a]	RT[b]	Kendall's τ[c]		Pearson's r[d]		Spearman's ρ[e]	
			p-value	τ	p-value	r	p-value	ρ
2	Unknown 1	**2.507**	0.060	-0.111	**0.048**	-0.158	0.062	-0.150
19		**8.020**	0.162	0.084	**0.035**	0.168	0.143	0.117
26		11.321	0.096	0.105	0.292	-0.085	0.097	0.133
27		11.554	0.071	0.109	0.314	-0.081	0.072	0.144
34	Unknown 2	**13.939**	**0.024**	0.126	0.078	0.141	**0.024**	0.180
42	Unknown 3	**15.035**	0.052	0.107	**0.021**	0.184	0.054	0.154
43	Unknown 4	**15.152**	**0.049**	0.109	0.085	0.138	0.050	0.156
45	Unknown 5	**15.624**	**0.048**	-0.109	**0.005**	-0.223	**0.048**	-0.158
48		**16.855**	0.122	0.087	**0.017**	0.189	0.140	0.118
49	Unknown 6	**17.121**	**0.036**	0.115	**0.017**	0.190	**0.045**	0.160
50		17.221	0.138	0.083	0.064	0.148	0.139	0.119
51		**17.315**	0.143	0.082	**0.019**	0.187	0.149	0.116
52		17.433	0.125	0.084	0.059	0.151	0.136	0.119
53		17.554	0.071	0.099	0.153	0.115	0.066	0.147
54		**17.657**	0.082	0.096	**0.020**	0.185	0.089	0.136
55		17.774	0.071	0.100	0.067	0.147	0.077	0.141
56		**18.288**	0.053	0.106	**0.006**	0.216	0.071	0.144
58		**19.290**	0.081	0.097	**0.016**	0.192	0.088	0.136

Table 2.2.2. Sample level correlations between live crown (LIVCRN) of 1200A families and peak areas of GC-FID quantified compounds from lodgepole pine at the 1200C resistance trial (n = 157).

	ID[a]	RT[b]	Kendall's τ		Pearson's r		Spearman's ρ	
			p-value	τ	p-value	r	p-value	ρ
2	Unknown 1	2.507	0.082	-0.103	0.082	-0.140	0.072	-0.144
4		4.050	0.098	-0.092	0.453	-0.060	0.112	-0.127
13	β-pinene	7.102	0.923	-0.005	0.092	-0.135	0.889	-0.011
26		11.321	0.079	0.111	0.284	-0.086	0.086	0.138
33		13.671	0.072	0.119	0.226	0.097	0.071	0.145
41		**14.888**	0.098	-0.091	**0.022**	-0.183	0.115	-0.126
42	Unknown 3	15.035	0.084	0.095	0.059	0.151	0.101	0.131
43	Unknown 4	15.152	0.084	0.095	0.125	0.123	0.091	0.135
45	Unknown 5	**15.624**	**0.035**	-0.116	**0.003**	-0.238	**0.037**	-0.167
48		16.855	0.350	0.052	0.055	0.153	0.366	0.073
49	Unknown 6	17.121	0.228	0.067	0.071	0.144	0.231	0.096
51		17.315	0.329	0.054	0.053	0.155	0.348	0.075
53		17.554	0.076	0.098	0.142	0.118	0.094	0.134
54		17.657	0.239	0.065	0.059	0.151	0.260	0.090
56		**18.288**	0.199	0.071	**0.015**	0.193	0.202	0.102
58		19.290	0.320	0.055	0.056	0.153	0.328	0.079

[a] *Putative identity or assigned name.* [b] *Retention time (minutes).*

Bold *indicates P<0.05 in one or more statistical tests, all others P<0.10.*

Table 2.3.1. Tree level correlations between crown retention (CRN12) of 1200A families and peak areas of GC-FID quantified compounds from lodgepole pine at the 1200C resistance trial (n = 56).

ID[a]		RT[b]	Kendall's τ[c]		Pearson's r[d]		Spearman's ρ[e]	
			p-value	τ	p-value	r	p-value	ρ
19		8.020	0.078	0.172	0.085	0.232	0.076	0.239
34	Unknown 2	**13.939**	0.052	0.181	0.059	0.254	**0.040**	0.275
42	Unknown 3	**15.035**	0.084	0.161	**0.029**	0.293	0.082	0.234
43	Unknown 4	**15.152**	**0.049**	0.184	0.079	0.236	**0.045**	0.269
45	Unknown 5	15.624	0.240	-0.110	0.062	-0.251	0.249	-0.157
48		**16.855**	0.130	0.142	**0.023**	0.304	0.135	0.202
49	Unknown 6	**17.121**	**0.044**	0.188	**0.020**	0.310	**0.050**	0.263
50		17.221	0.170	0.128	0.083	0.234	0.133	0.203
51		**17.315**	0.113	0.148	**0.019**	0.314	0.091	0.228
52		17.433	0.170	0.128	0.074	0.241	0.169	0.186
53		**17.554**	**0.041**	0.191	0.114	0.214	**0.050**	0.263
54		**17.657**	0.092	0.157	**0.017**	0.317	0.078	0.238
55		17.774	0.116	0.147	0.094	0.226	0.099	0.223
56		**18.288**	**0.011**	0.238	**0.001**	0.426	**0.010**	0.341
57		**18.710**	**0.049**	0.184	0.158	0.191	0.052	0.261
58		**19.290**	0.100	0.153	**0.020**	0.311	0.099	0.223

Table 2.3.2. Tree level correlations between live crown (LIVCRN) of 1200A families and peak areas of GC-FID quantified compounds from lodgepole pine at the 1200C resistance trial (n = 56).

ID[a]		RT[b]	Kendall's τ[c]		Pearson's r[d]		Spearman's ρ[e]	
			p-value	τ	p-value	r	p-value	ρ
1		2.486	0.106	-0.158	0.175	-0.184	0.080	-0.236
2	Unknown 1	2.507	0.237	-0.112	0.083	-0.234	0.209	-0.170
29		11.937	0.087	0.160	0.720	-0.049	0.115	0.213
33		13.671	0.051	0.214	0.365	0.123	0.050	0.263
42	Unknown 3	15.035	0.276	0.102	0.071	0.243	0.293	0.143
43	Unknown 4	15.152	0.095	0.156	0.107	0.218	0.114	0.214
44		15.307	0.089	0.159	0.057	0.256	0.103	0.220
45	Unknown 5	**15.624**	0.246	-0.108	**0.040**	-0.275	0.233	-0.162
48		16.855	0.223	0.114	0.063	0.250	0.263	0.152
49	Unknown 6	17.121	0.251	0.107	0.073	0.242	0.262	0.153
53		17.554	0.087	0.160	0.103	0.220	0.118	0.211
54		17.657	0.257	0.106	0.050	0.263	0.258	0.154
56		**18.288**	**0.030**	0.202	**0.003**	0.387	**0.034**	0.284
58		19.290	0.263	0.105	0.060	0.252	0.295	0.143

[a] *Putative identity or assigned name.* [b] *Retention time (minutes).*

Bold *indicates P<0.05 in one or more statistical tests, all others P<0.10.*

Areas of seven peaks were significantly correlated ($P<0.05$) with CRN12 or LIVCRN variables in nonparametric tests at a sample level (Tables 2.2.1 and 2.2.2) or tree level (Tables 2.3.1 and 2.3.2), with the majority of correlations being positive. Relative retention times indicated that none of the peaks significantly correlated at this alpha level (0.05) in nonparametric tests were monoterpenes. Twenty-two peaks were significantly correlated with CRN12 or LIVCRN at an alpha level of 0.10.

Six peaks were chosen for further analysis, with preference placed on the lower retention time peaks whose correlations were first identified. These were labeled unknown 1 through to unknown 6 (U1-6). For positively correlated unknown compounds (U2-4 and U6) a linear trend showed that a 0.8 to 2% increase in sample peak area was associated with a 1% increase in CRN12. Negatively correlated unknown compounds (U1 and U5) showed a 3-8% decrease in sample peak area for each 1% increase in CRN12.

The low retention time of unknown 1 (2.507 min) indicated that this peak did not represent a terpene, and its elution close to the solvent peak meant that a modified method would be required to identify it with GC-MS. Therefore, analysis of this compound was discontinued.

Unknowns 2 through 6 were tentatively identified as either sesquiterpenes or sesquiterpene alcohols by automated MS fragmentation pattern library matches and manual fragmentation pattern analysis (Figures 2.3.1-5), but more specific identification was not possible based on MS data alone due to the highly similar fragmentation patterns of related terpene isomers. Comparison of retention time to that of n-alkane standards allowed tentative calculation of linear retention index values for these compounds, but published retention index figures for

sesquiterpenes and sesquiterpene alcohols were highly variable between different literature sources, making conclusive identification difficult from retention index alone (Adams, 2000; Kant et al. 2004; Babushok et al. 2011; Ioannou et al. 2014).

Analytical standard compounds ordered based on preliminary linear retention index identification allowed elimination of some candidate compounds, but no conclusive identification was possible due to the lack of commercially available standards. Standards were not readily available for α-cadinene, germacrene D-4-ol, and three of the four farnesol isomers. An analytical standard of (E,E)-farnesol was compared to unknown 6 in retention index and MS spectra, and was found to represent a minor shoulder peak visible in a small number of samples. MS spectra of U2-6 are given in Fig 2.2.1-5. Putative identifications based on retention index and MS fragmentation pattern are summarized in Table 2.4.

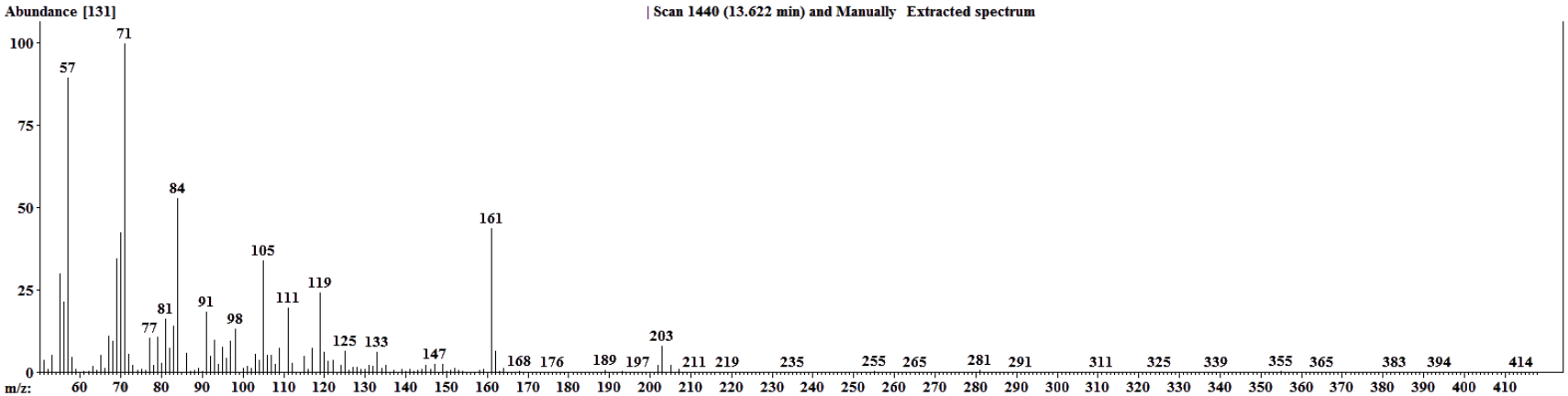

Figure 2.3.1. The GC-MS fragmentation pattern of unknown compound 2 (GC-FID RT: 13.939, GC-MS RT 13.622) shows a potential molecular ion peak at m/z 203 and a strong peak at m/z 161, the latter of which is seen in many sesquiterpenes and sesquiterpene alcohols. Analysis of individual ion peaks with AMDIS shows that this peak potentially co-elutes with a non-terpene compound, though only one peak was visible in FID chromatograms.

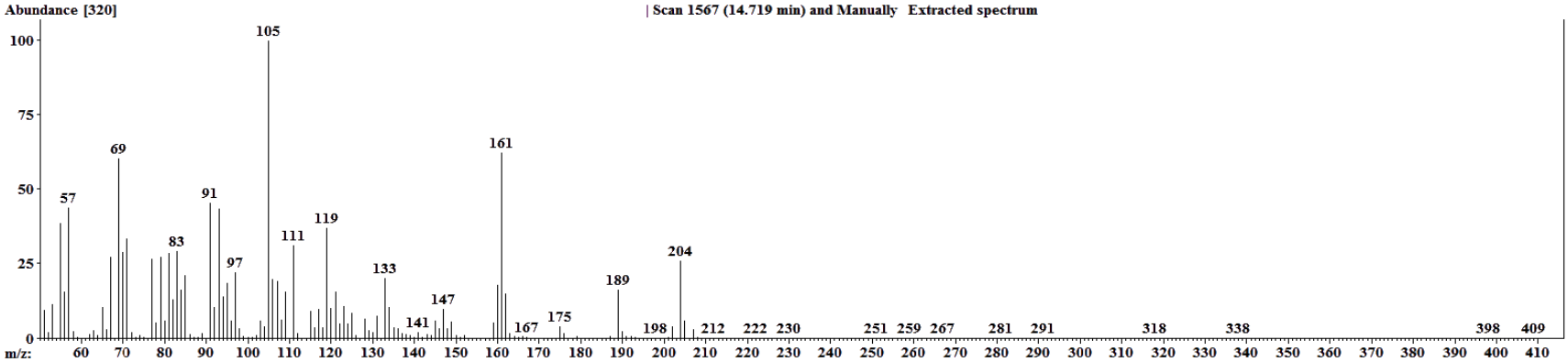

Figure 2.3.2. GC-MS fragmentation pattern of unknown compound 3 (GC-FID RT: 15.035, GC-MS RT: 14.719). Unknown 3 has a molecular ion peak at m/z 204, and also shows a strong m/z 161 peak. The MS spectra of this peak is very similar to published spectra for α-cadinene ((1S,4aR,8aR)-4,7-dimethyl-1-propan-2-yl-1,2,4a,5,6,8a-hexahydronaphthalene – Adams 2007), and the calculated linear retention index falls within the range of published values for this compound (Table 2.2; Babushok et al. 2011). Ion peak analysis using AMDIS indicates that this compound eluted separately from nearby peaks.

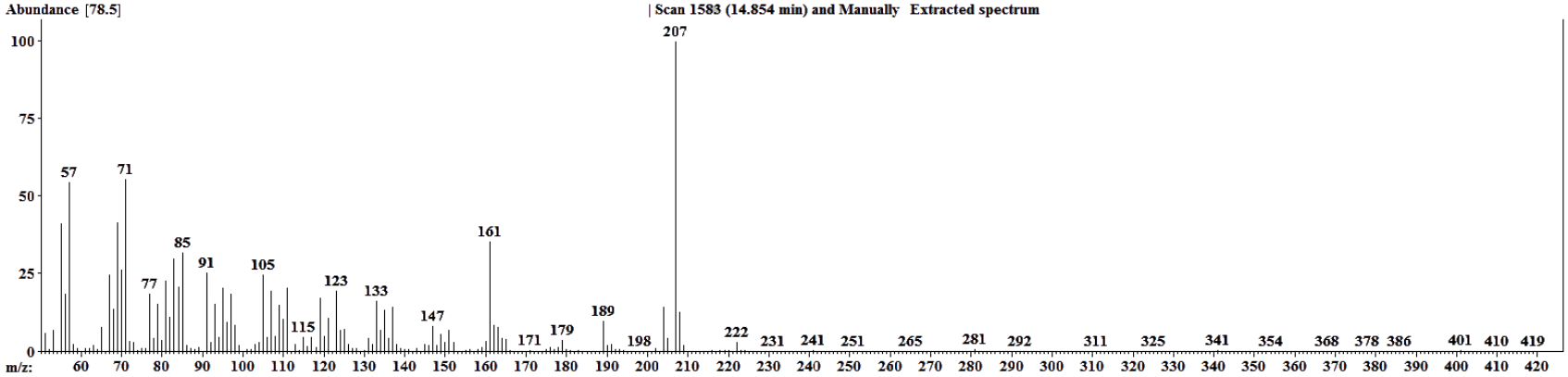

Figure 2.3.3. The GC-MS fragmentation pattern of unknown 4 (GC-FID RT: 15.152, GC-MS RT: 14.854) shows a weak molecular ion peak at m/z 222 and a stronger m/z 204 peak in its GC-MS fragmentation pattern, suggesting that this compound is a sesquiterpene alcohol. A strong peak at m/z 207 may be indicative of column bleed (Ahn et al. 2007), in which case the low abundance of the overall GC-MS peak may make this individual ion peak appear relatively high. Some peak overlap was seen with a lower RT non-terpene peak. This overlap was visible in FID chromatograms and overlapping peaks were manually split in affected samples prior to quantification.

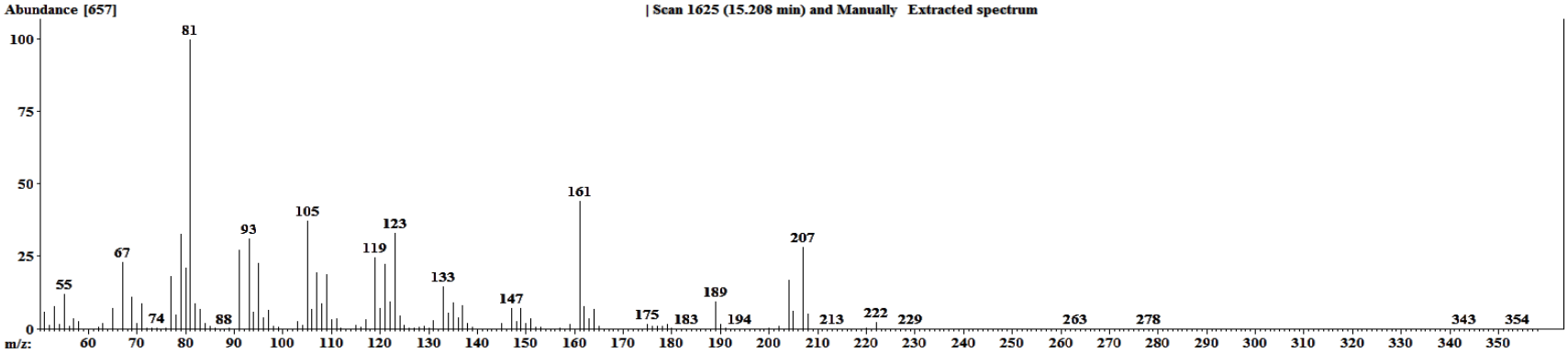

Figure 2.3.4. GC-MS fragmentation pattern of unknown compound 5 (GC-FID RT: 15.624, GC-MS RT: 15.208). As with Unknown 4, Unknown 5 shows a weak molecular ion peak at m/z 222 and a stronger m/z 204 peak, indicating that this is also a sesquiterpene alcohol. The MS spectrum of this compound is very similar to published spectra for germacrene D-4-ol ((2Z,7Z)-1,7-dimethyl-4-propan-2-ylcyclodeca-2,7-dien-1-ol) from Adams (2007), and the NIST library. Potential column bleed was seen at m/z 207, though this ion is also seen in published spectra. The library reverse match factor that I obtained (881) is high enough to suggest that this identification is correct on this basis alone (Wallis et al. 2008), however the calculated linear retention index of this peak (1592) falls outside the range of values commonly seen in literature (90% CI 1560-1582; Babushok et al. 2011).

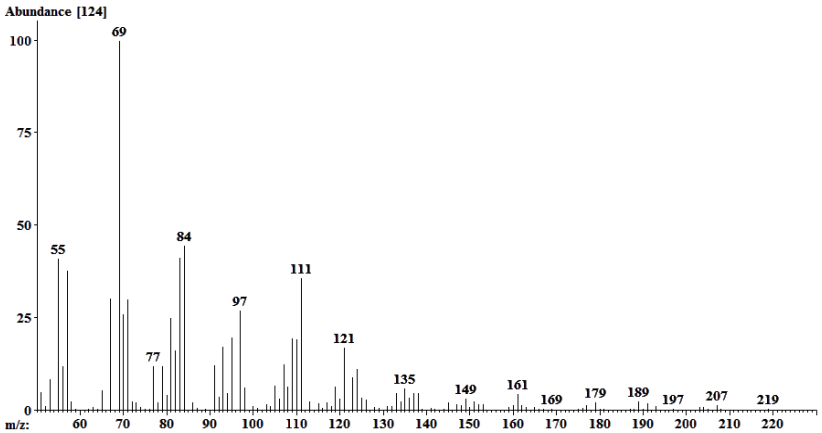

Figure 2.3.5. GC-MS fragmentation pattern of unknown compound 6 (GC-FID RT: 17.121, GC-MS RT: 16.828). While the spectra is dominated by low m/z ions, it strongly resembles that of the four farnesol (3,7,11-trimethyldodeca-2,6,10-trien-1-ol) isomers; (2E,6E)-farnesol, (2E,6Z)-farnesol, (2Z,6E)-farnesol, and (2Z,6Z)-farnesol (Adams 2007; Lee et al. 2007). The m/z 69 peak may represent isoprene (Hornby et al. 2001).

Table 2.4. Putative identities of unknown compounds 2-6 based on linear retention index and GC-MS fragmentation pattern.

Compound	RT[a]	RI[b]	Putative ID	RI 90% CI[c]
Unknown 2	13.939	1446	Unidentified sesquiterpene	
Unknown 3	15.035	1541	α-cadinene	1503–1541
Unknown 4	15.152	1551	Unidentified sesquiterpene alcohol	
Unknown 5	15.624	1592	Germacrene D-4-ol	1560–1582
Unknown 6	17.121	1723	Unidentified farnesol isomer	1678–1700 (2Z,6Z); 1702–1722 (2E,6Z); 1715–1732 (2Z, 6E)

[a] *Retention Time.*

[b] *Linear Retention Index.*

[c] *Linear Retention Index 90% Confidence Interval of putatively identified compounds in literature (from Babushok et al. 2011).*

2.4 Discussion

Overall, the relative levels of volatile secondary metabolites quantified were consistent with that of previous work (Chapter 1 of this thesis) and published literature for lodgepole pine (Pauly and von Rudloff, 1971; Ioannou et al. 2014). While peak areas were highly variable, the relative consistency and repeatability of GC-FID quantification methods indicates that the quantification step was probably not a major source of variation (Zika et al. 1994; Smith et al. 2015). Quantifying variation introduced during extraction is more difficult, as the extraction process is destructive, meaning that repeats cannot be carried out on the exact same tissue. However, variation in peak areas due to varying extraction efficiency would be expected to be consistent across all compound peaks, and while variation in total peak areas showed some correlation with individual peak areas, it does not explain the majority of individual peak area variation. Intra-tree variation was lower than intra-family and inter-family variation, as seen in Chapter 1 of this thesis. This is likely a result of the strong genetic control over secondary metabolite levels seen in pines and other plants (Hanover 1966; Hamilton et al, 2001). While it is plausible that levels of foliar secondary metabolites could vary within a tree based on environmental differences due to aspect (e.g. light levels - Mayrhofer et al. 2005), these tissues should be genetically indistinguishable and have the same genetic contribution to constitutive secondary metabolite levels. By comparison, trees from the half-sib families tested (which are naturally pollinated, meaning only one parent tree is known) are likely related by only a single parent tree, and trees of families originating from geographically distinct sites are likely to be even more genetically distant. Consequently, these more genetically distinct trees are likely to also have more distinct constitutive

secondary metabolite levels.

The nonparametric statistical methods (Kendall's τ and Spearman's ρ) I used to determine correlations with crown retention variables involve a rank-based correlation analysis that is less sensitive to outliers or skewed data, but is typically thought to give lower statistical power. However, for highly kurotic data, such as peak areas of low level compounds, rank based methods such as Spearman's ρ can give both lower type I error rate and higher power than Pearson's r when the sample size is sufficiently large (Bishara and Hittner, 2012). Despite the large sample size in this study, high variability in peak area between samples limited statistical power, leading to potential type II errors. While the use of a standard 0.05 alpha level with non-parametric tests gave only a small number of significantly correlated peaks (seven), using $\alpha = 0.10$ greatly increased this number (to twenty-two). Further investigation into the identity of these compounds is required to determine whether their potential effects on crown retention variables are biologically significant. The relatively high retention times of these additional peaks indicates that they may be sesquiterpenes or sesquiterpene alcohols, but further investigation of their identities was outside the scope of this study.

While identified compounds were all either sesquiterpenes and sesquiterpene alcohols, monoterpenes were the original focus of this study due to reports of antifungal effects in previous publications (Franich et al. 1982; Wallis et al. 2010). Monoterpenes are a widely studied class of isoprenoid-based compounds, with a range of published research on their biosynthesis and function, in both conifers and other organisms. There is a comparative paucity of research on sesquiterpene alcohols, which complicates both their identification and

further investigation into their biosynthesis. While three of the six unknown compounds selected for further investigation were putatively identified based on MS fragmentation pattern and linear retention index (Table 2.4), for the other unknown compounds no specific identification was possible. Identification of these compounds would likely require a further purification step (such as two-dimensional GC) or possibly modifications to the GC method (such as a slower temperature ramp) that would increase separation of the target peak from neighboring peaks, followed by expert examination of the MS fragmentation pattern and/or synthesis of authentic standards for retention time matching. Nuclear Magnetic Resonance (NMR) could also be used for identification, either on a collected fraction (Pretorius et al 2003), or using an inline apparatus (Lacey et al. 2001).

Complete separation of the peak of interest would be required to ensure that it consists of only a single compound. Coelution or overlap of peaks is almost unavoidable when analyzing such a wide range of chemicals. While GC-based methods are developed to give adequate separation of the compounds studied, methods can only rarely if ever be optimized to fully prevent overlap in all peaks in complex mixtures. Any visible peak doublets or shoulders were split when peak areas were integrated, but coelution with compounds around or under the limit of quantification is difficult to detect by visual examination of the FID chromatogram. MS chromatograms were analyzed in AMDIS to identify peak overlap in U2-6 by visualizing individual MS ion peaks, which allows closely eluting peaks with different component ions in their MS spectra to be resolved. However, GC-MS is not a common quantification method for diverse mixtures of compounds where standards are not available, as fragment abundance is highly dependent on structure (and consequently unique to each

compound analyzed), and production of ions does not correspond linearly to compound abundance. This results in highly variable MS response between even closely related compounds, making relative quantification difficult (Bicchi et al. 2008). While peak overlap or co-elution can be difficult to identify and correct in complex FID chromatograms, this quantification method has the advantage that detector response is largely proportional to chain length, though some variation based on structure can occur. The peak areas shown in this study are expressed as n-pentadecane response equivalents, as they were normalized to the 0.1% n-pentadecane internal standard incorporated into the extraction solvent, in addition to sample weight. Because this quantification is only relative to the internal standard, and not based on direct comparison to analytical standards for each compound, it is not an absolute quantification (IOFI Working Group on Methods of Analysis, 2011).

Preliminary identification of U2-6 was not possible from retention time or pattern alone, so alkane standards were used to calculate a linear retention index. This method involved the standardization of retention times for a particular type of column and method, allowing accurate comparison to published retention indices. However, published linear retention indices for the relevant compounds were found to be highly variable (Table 2.4). This was partly due to minor variations in GC conditions, but errors in identification and measurement also played a role (Babushok et al. 2011). This variation was well illustrated by the four farnesol isomers, which Babushok et al. (2011) found varied in both retention time and elution order in their meta-analysis of GC linear retention index publications. Reliable identification of the individual farnesol isomers is thought to require both MS and FTIR (Fourier Transform Infrared Spectroscopy) data (Lee et al. 2007; Svatos and Attygalle, 1997).

Based on the data I collected, unknown 1 is unlikely to be a terpene, as it elutes well before any other identified terpenes. Prior literature indicates that the monoterpenes α-thujene and α-pinene should be the first lodgepole pine terpenes to elute under this GC separation method (Babushok et al. 2011; Ioannou et al. 2014). This is consistent with my observations in this study (see Table 2.1) and previous work (Chapter 1 of this thesis). The putative α-pinene peak identified in this study has a much higher retention time than U1. The early retention time of U1 also means that it elutes close to the solvent peak, and therefore before the MS filament is turned on in this GC-MS method. Development of a modified GC-MS method would be required to adequately separate this specific peak from the solvent front. While this compound is one of few to show a negative correlation with CRN12 or LIVCRN (Tables 2.2.1 and 2.2.2), the absence of a significant non-parametric correlation at a tree level (Tables 2.3.1 and 2.3.2) may indicate that its correlation at a sample level is the result of a Type I error, and as it remains unidentified the physiological basis of any potential correlation cannot be evaluated.

Like unknown 1, unknown 5 was negatively correlated with crown retention variables (CRN12 and LIVCRN) at a sample level ($P<0.05$ in nonparametric tests; Tables 2.2.1 and 2.2.2), but was not significantly correlated in non-parametric tests at a tree level (Tables 2.3.1 and 2.3.2). However, I was able to putatively identify this compound as germacrene D-4-ol based on its high MS library reverse match factor with a reference spectrum in the NIST database. The weak molecular ion peak (m/z 222) and stronger M-18 peak seen in Figure 2.3.4 is characteristic of alcohols, which easily lose the -OH group to form M-18 fragments (Silverstein et al. 1980). A negative correlation with crown retention means that U5 would be

unlikely to have an inhibitory effect on *Dothistroma* growth *in vivo,* but its presence in foliar extracts could be partly responsible for observed stimulation of mycelial growth *in vitro* (Franich et al. 1982). Alternatively, U5 may be a sesquiterpene alcohol that does not have any direct effect on *Dothistroma*, but competes for precursors with a sesquiterpene or sesquiterpene alcohol that does. While not all of the specific enzymes responsible for sesquiterpene alcohol biosynthesis in conifers are known or characterized, these enzymes can compete for the same pool of precursor molecules, as seen in other species (Zulak and Bohlmann, 2010), and are often produced by analogous biosynthetic enzymes (Martin et al. 2004; Keeling and Bohlmann, 2006). Increased efficacy of a certain enzyme, or a change in product profile, could increase production of one terpene at the expense of another. Further work will be required to determine whether correlations of crown retention variables with putatively identified germacrene D-4-ol are the result of a genuine host-pathogen interaction or simply a Type I error.

Unknowns 2, 3 (putative α-cadinene), 4, and 6 (putative farnesol isomer) were positively correlated with crown retention variables (Tables 2.2.1-2.3.2), indicating a potential link to *Dothistroma* resistance. The 1200A site was heavily infected by *D. septosporum* at the time of crown retention assessment; needle retention and health in trees at this site is likely to be directly associated with *Dothistroma* resistance. That I found some peak areas significantly correlate (U2, U4, and U6 $P<0.05$; U3 $P<0.10$) with crown retention variables in non-parametric tests at both sample and tree levels indicates that these compounds may either contribute directly to resistance, or act as markers for resistance. There is a well-defined physiological rationale for sesquiterpene alcohols acting as chemical defenses against foliar

fungal pathogens such as *D. septosporum*, as they are widely reported to inhibit fungal growth (Kusumoto and Shibutani 2015), or have other specific effects on fungi. Farnesol isomers in particular have been widely studied in systems as diverse as bacteria (Jabra-Rizk et al. 2006), and mice (Crick et al. 1995). (E,E)-farnesol produced by *Candida albicans* has been found to act as a quorum sensing molecule, mediating its morphology in culture (Hornby et al. 2001). In other fungal species farnesol has a more direct negative effect. Extracellular (*E,E*)-farnesol was found to induce apoptosis in *Aspergillus nidulans* (Semighini et al. 2006) and *Fusarium graminearum* (Semighini et al. 2008). Inhibition of growth was observed in these species and in *Paracoccidioides basiliensis* (Derengowski et al. 2009) at farnesol concentrations as low as 10-25 μM. The mechanism by which farnesol acts to inhibit fungal growth is not well understood. Farnesol has also been hypothesized to directly disrupt cell membranes (Jabra-Rizk et al. 2006), and scanning electron microscopy analysis of farnesol-treated *P. basiliensis* cells by Derengowski et al. (2009) showed disruption of internal cellular structures. However, a G-protein SfaD knockout strain of *A. nidulans* was found to have complete resistance to farnesol, indicating that G-protein signaling is necessary for farnesol-induced apoptosis (Semighini et al. 2006). Assays described in Chapter 3 of this thesis indicate that (E,E)-farnesol can either inhibit or promote growth of *D. septosporum* depending on concentration, but farnesol isomers can have diverse effects (Shchepin et al. 2003). Further investigation will require synthesis of authentic farnesol isomer standards both for conclusive identification and *in vitro* inhibition testing.

Discussion of the potential physiological basis for resistance conferred by the sesquiterpenes U2 and U3 (putative α-cadinene) is complicated by the lack of a positive identification and

the paucity of published literature for these compounds, respectively. Whether U2 and U3 have antifungal effects on *Dothistroma* or merely show a significant correlation to crown retention as precursors to inhibitory compounds cannot be determined solely from the data collected in this study. However, the relative abundance of literature on the antifungal effects of sesquiterpene derivatives over that on unmodified sesquiterpenes favors the latter hypothesis. While plant volatile extracts containing predominantly sesquiterpenes have been found to inhibit fungal growth (Cakir et al. 2005; Gazim et al. 2008), I found no reports of unmodified sesquiterpenes ($C_{15}H_{24}$) showing antifungal effects. In contrast, cadinene derivatives have been reported to show antifungal effects (Kundu et al. 2013), as have sesquiterpene lactones (Luo et al. 2005; Wedge et al. 2000; Erasto et al. 2006), sesquiterpene aldehydes (Kubo and Taniguchi, 1988; Taniguchi et al. 1988), and the aforementioned sesquiterpene alcohols.

Previous work (unpublished results) showed inhibition of *Dothistroma* growth in broth media by individual monoterpenes, though no identified compounds of this class showed significant correlation with crown retention (α=0.05) in this study. This may indicate that the seven compounds correlated at this alpha level have a specific, more potent, antifungal effect against *Dothistroma*, but this cannot be determined without further *in vitro* inhibition testing. It is also possible that some or all of the compounds correlated with crown retention instead act as markers for resistance, rather than having a direct effect on chemical defense. Michelozzi et al. suggest that a correlation between β-phellandrene and fusiform rust resistance in slash (1990) and loblolly (1995) pine is due to a β-phellandrene levels being associated with an as of yet unidentified inhibitory factor. While β-phellandrene was shown

to be have antifungal effects in media, they were not significantly different from the antifungal effects of monoterpenes that did not correlate with resistance. To perform a similar study on the compounds of interest in this study would require conclusive identification of correlated compounds, then chemical synthesis or purification of quantities sufficient for inhibition assays to be conducted. Any study of this type makes the assumptions that the *in vitro* assay is representative of conditions *in planta*, and that the defense relationship is a simple one, involving a single compound, rather than sensitization (as observed with the sesquiterpene aldehyde polygodial; Kubo and Taniguchi 1988). Conclusively establishing a connection between correlated compounds and *Dothistroma* resistance in a more representative *in vivo* test would be challenging, and would likely involve resistance assays on greenhouse raised lodgepole pine clones modified for increased or decreased production of these compounds with otherwise identical foliar secondary metabolite profiles. This would first require complete identification and characterization of the (likely numerous) biosynthetic enzymes involved in production of compounds of interest in lodgepole pine, and is not likely to be feasible in the near future.

This work suggests that at least some of the putatively identified compounds are involved in resistance of lodgepole pine to *Dothistroma septosporum*, but further work is required to assess the potential effects of currently unidentified compounds. Levels of seven foliar secondary metabolites were found to significantly correlate with family crown retention variables CRN12 or LIVCRN in a *D. septosporum* resistance trial at an alpha level of 0.05, with twenty-two correlated at a 0.10 level. Of these correlated compounds, a number were identified as sesquiterpenes and sesquiterpene alcohols, suggesting a potential contribution

from this class of compounds to *Dothistroma* resistance. While unmodified sesquiterpenes have not been shown to have any effect on fungi, there is a strong physiological basis for sesquiterpene alcohols influencing resistance to fungal pathogens. Future work should seek to both establish the identity of currently unidentified compounds correlated with resistance, and determine the effects of putatively identified sesquiterpene and sesquiterpene alcohol compounds on the growth of *Dothistroma in vitro*.

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3. *In vitro* effects of (E,E)-farnesol on growth of the fungal pathogen *Dothistroma septosporum*

3.0 Abstract

Lodgepole pines produce a wide array of secondary metabolites in their foliage, in part to protect against damage by herbivores and pathogens. Of the fungal foliar pathogens that affect pine *Dothistroma septosporum* (Dorog.) Morelet is considered the most damaging, both in British Columbia and worldwide. Previous work has linked *Dothistroma* resistance seen in some lodgepole pine (*Pinus contorta* var *latifolia* Engelmann) provenances to production of foliar sesquiterpenes alcohols. Levels of an unidentified isomer of farnesol (unknown 2.6) were found to correlate with both crown retention in a *D. septosporum* resistance trial of lodgepole pine (Chapter 2 of this thesis), and *Dothistroma* outbreak history of lodgepole pine provenances determined by dendrochronology (Chapter 4 of this thesis). On that basis, I tested the effect of varying (E,E)-farnesol concentrations on the growth of geographically distinct *D. septosporum* isolates *in vitro*. (E,E)-farnesol was found to significantly stimulate *Dothistroma* growth *in vitro* at concentrations similar to those found in foliage, with inhibition only observed at higher levels. This may indicate that farnesol isomers play a previously unknown role in the *Dothistroma*-*Pinus* interaction, but also points to a complex relationship between terpene levels and disease resistance.

3.1 Introduction

Fungal pathogens of trees have adapted the ability to avoid, overcome, or tolerate host defense mechanisms that include both physical and chemical defenses (Franich et al. 1977; Franich et al. 1982; Franich and Gadgil, 1983; Fraser et al. 2015). Pathogens that attack conifer foliage have to penetrate a protective cuticle and deal with potentially toxic compounds that may exist in the needles prior to attack, or be induced as a result of attack by pathogens (Wallis et al. 2008). *Dothistroma septosporum* (Dorog.) Morelet is a pathogen of many species of pine worldwide (Watt et al. 2009), and its success as a pathogen is due in part to its ability to overcome the various levels of constitutive and induced defenses produced by the host.

Chemical defenses in conifer foliage include volatile secondary metabolites commonly produced and stored in dedicated resin ducts (Wu and Hu, 1997; Zulak and Bohlmann, 2010). While these stored compounds play a diversity of roles over a tree's long lifespan (Constable et al. 1999; Holopainen et al. 2013), their primary function is considered to be constitutive defense. The broad range of foliar secondary metabolites produced by hosts of *Dothistroma* show inhibitory effects on a variety of fungal species *in vitro* (Andrews et al. 1980; Franich et al. 1982; Jabra-Rizk et al. 2006; Semighini et al. 2006; Bakkali et al. 2008; Semighini et al. 2008; Derengowski et al. 2009), but to this date no foliar terpenes of pine have been shown to contribute to *Dothistroma* resistance. However, the lifecycle of the fungus puts it in close proximity to foliar resin canals associated with terpene production and storage (Gadgil 1967; Peterson and Walla, 1978; White and Nilsson, 1984; Kabir et al. 2014), and it is likely to be directly exposed to foliar resins when these canals are penetrated or disintegrated during

lesion formation. While *Dothistroma* spp. are likely to have developed tolerance to many of these chemical defenses (Franich et al. 1982), there is a wide range in the identity and levels of compounds produced that depend on many factors for any host species, including host provenance. Compounds that show inhibitory effects *in vitro* may contribute to the quantitative resistance observed in some pine species and provenances (Carson 1989; Ukrainetz et al. 2013).

The lifecycle of the fungus starts with germination of an ascospore or conidiospore on a host needle, followed by hyphal growth on the needle surface until a stomatal pore is penetrated (Kabir et al. 2014). Epiphytic growth continues within the epistomatal chamber before mesophyll penetration and growth. The pathogen then undergoes a necrotrophic phase corresponding with lesion formation and extension. Finally, fruiting bodies erupt through the needle surface allowing spore release (Gadgil 1967). Infected needles are dropped prematurely by the tree, resulting in growth losses and, in extreme cases, mortality (Woods et al. 2003). In British Columbia, *D. septosporum* outbreaks are thought to commonly start from ascospores arriving at the site via long range airborne dispersal (Dale et al. 2011). After the initial introduction the fungus reproduces asexually, spreading short distances within the tree and site by rainsplash dispersal of conidia. While the range of conidia dispersal by rainsplash is limited (Boateng and Lewis 2015) the fungus is still able to establish new infection sites in areas when the population is clonal (Hirst et al. 1999). Longer range spread of the disease may occur through cloud/wind dispersal or movement of infected stock (Gibson 1974).

Because of the ease with which it is distributed, *Dothistroma* is found on every continent but Antarctica (Watt et al. 2009), and the diversity of the methods used to control it correspond to

the diversity of the forest environments in which it lives (Bulman et al. 2016). Bulman et al. (2013) discuss six general strategies for management of *Dothistroma* needle blight: avoidance (avoiding planting susceptible species in locations with conditions suitable for disease development), exclusion (preventing introduction of the disease to an area), eradication (removal of the disease from an area), protection (chemical or biological control), resistance (breeding for resistance or planting of resistant species), and integrated disease management (the use of silviculture practices to reduce the impact of the disease on forest productivity). Exclusion and eradication based strategies are area-specific, as they are only applicable to highly isolated locations where the disease is either not present or only present in early stages. Strategies involving protection by application of control agents or silviculture are less practical outside of managed plantations. Copper-based fungicides are commonly used to control the disease in New Zealand pine plantations (Bulman et al. 2016), but their application in British Columbia would be costly and environmentally unacceptable. Lodgepole pine does not show the mature tree resistance to *Dothistroma* seen in *Pinus radiata* D. Don (Gibson 1972), so controls would have to be applied for the entire rotation period, and the broad application of fungicides to forests would negatively affect non-target fungal species in the area (McCulloch and Woods 2009). Because of these limitations, avoidance and resistance are the preferred strategies in British Columbia. Forest managers are advised to avoid planting pine species in high risk areas (McCulloch and Woods 2009) and resistance breeding programs for lodgepole pine have been successful in identifying resistant families (Ukrainetz et al. 2013).

The resistance observed in *Dothistroma* specific pine breeding programs is quantitative, and

likely to be multifactorial (Carson 1989). While related dothideomycetes have qualitative gene-for-gene resistance mechanisms in their pathogen-plant interaction (typified by the *Cladosporum fulvum – tomato* [*Lycopersicon esculentum* Mill.] model pathosystem; de Wit and Joosten 1999; Rivas and Thomas 2005), no qualitative resistance to *Dothistroma* species has been observed. *D. septosporum* contains a number of homologs of *C. fulvum* effector genes (de Wit et al. 2012), and analysis of their expression dynamics by high-throughput sequencing shows several are up-regulated during mesophyll colonization and early lesion formation (Bradshaw et al. 2016). Others either show negligible expression, or are pseudogenized (nonfunctional); a possible indication of host adaptation by the fungus (de Wit et al. 2012). Complete resistance to *D. septosporum* is likely to be unattainable through breeding (Bulman et al. 2016), and research has therefore focused on quantitative resistance.

In conifers, quantitative resistance to pathogenic fungi generally involves chemical or physical mechanisms which either inhibit or physically prevent host penetration and growth of the pathogen (Fraser et al. 2015). While there has been some study of the defense mechanisms involved in the *Dothistroma*-*Pinus* interaction, there has been little conclusive identification of the specific mechanisms involved in observed *Dothistroma* resistance. Early research on *Pinus radiata* D. Don strongly linked the mature tree resistance seen in that species to occlusion of stomatal pores by epicuticular wax (Franich et al. 1977; Franich and Gadgil 1983), but resistance seen in other species and varieties has not been linked to a specific defense mechanism or mechanisms. My recent work on resistant varieties of lodgepole pine has connected constitutive foliar levels of putatively identified sesquiterpenes alcohols with both crown retention and *Dothistroma* disease history at a provenance level

(Chapters 2 and 4 of this thesis), but these compounds have not yet been conclusively linked to resistance. Once potential contributors to chemical defense have been identified, a common next step is to determine their effects on fungal growth *in vitro*. Previous studies have used growth assays in media to determine the inhibitory potential of foliar terpenes on *D. septosporum*, but work has focused on overall terpene levels using steam-distilled foliar extracts (Franich et al. 1982). In this chapter I investigated the effects of the sesquiterpene alcohol (E,E)-farnesol (trans, trans-3,7,11-Trimethyl-2,6,10-dodecatrien-1-ol) on *D. septosporum in vitro*, with the aim of determining the potential for farnesol isomers to contribute to observed resistance in lodgepole pine.

The objectives of the work described in this chapter were to: 1) Quantify the effect of (E,E)-farnesol on geographically diverse *D. septosporum* isolates *in vitro*; and 2) Contrast the effect of (E,E)-farnesol with that of a steam-distilled foliar extract.

3.2 Methods

Lodgepole pine needles with *D. septosporum* lesions and mature fruiting bodies were collected from three geographically distinct locations in British Columbia (Figure 3.1). *D. septosporum* was isolated by spore extraction from excised fruiting bodies, and streak plating on *Dothistroma m*edia (2.5% malt extract [BD Bacto; Becton, Dickenson and Company, Sparks MD, USA]/2.3% nutrient agar [BD Difco; Becton, Dickenson and Company]) containing 100 µg/mL kanamycin (Mullet and Barnes 2012). Three single colonies from each site were transferred to 50 mL flasks containing 30 mL of *Dothistroma* Broth (DB; 2.5% malt extract [BD Bacto; Becton, Dickenson and Company, Sparks MD, USA]/1.3% nutrient broth [Oxoid CM0001; Oxoid Ltd, Basingstoke, Hampshire, England]; Schwelm et al. 2008) liquid media and incubated on a Lab-Line orbit shaker (Lab-Line Instruments Inc., Melrose Park, IL, USA) at 22°C for 14 days to increase culture biomass prior to inoculation of inhibition test broths.

Inhibition testing was carried out in triplicate, with three replicates of each of the three isolates from the three test sites, for a total of 27 tests at each of the six farnesol concentrations treatments as well as for the positive and negative controls. Where growth of a fungal isolate was visually inadequate in pre-test flask broth cultures, the highest growth isolate from that site was tested twice, with a minimum of two separate isolates tested per site. Between (E,E)-farnesol and steam distilled foliar extract tests a total of 240 culture growth tubes were assayed, including positive and negative controls.

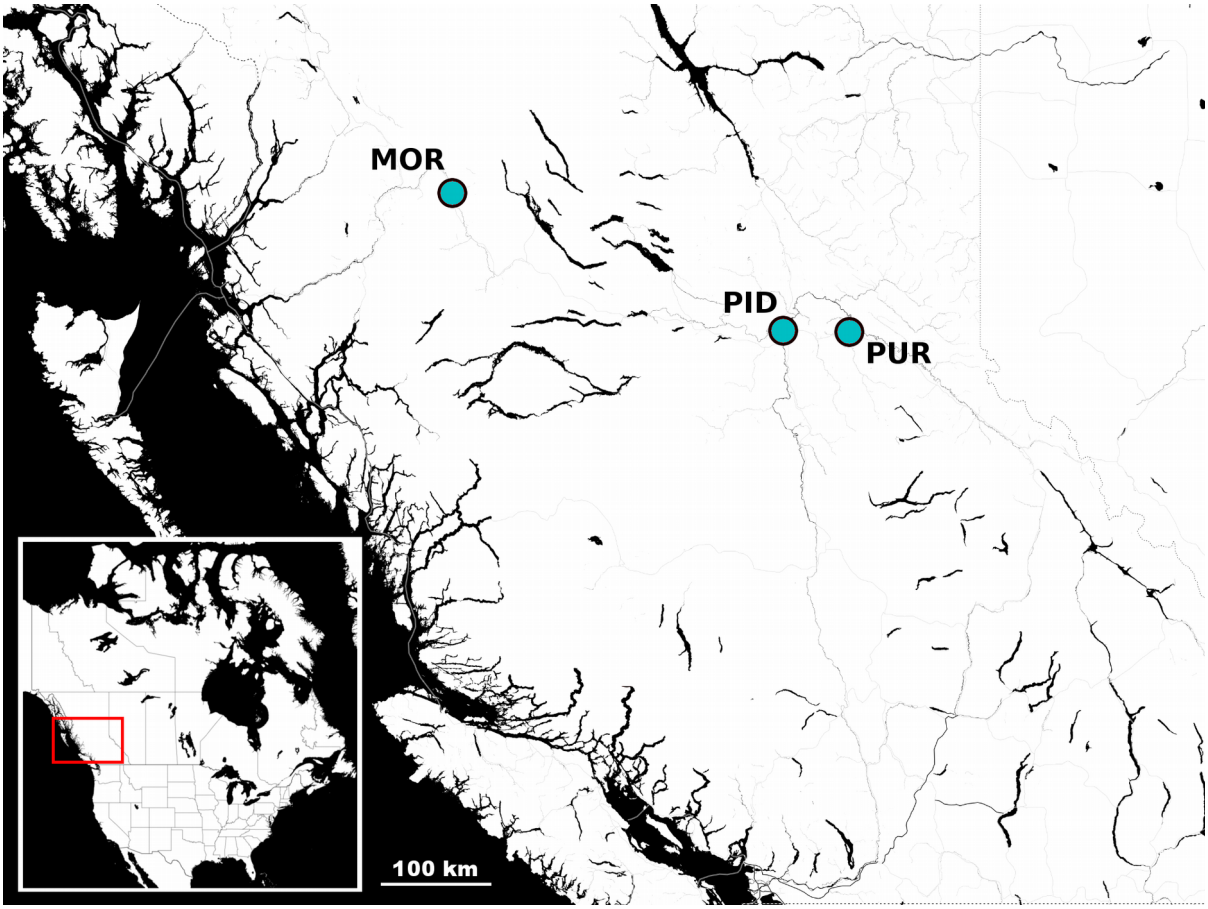

Figure 3.1. Map of British Columbia, Canada, showing sampling sites where *D. septosporum* isolates were collected for use in fungal inhibition testing. MOR: Moricetown (UTM 9N 606470.1E/6103557.2N). PID: Pidherny, Prince George (UTM 10 N 509153.6E/5982045.4N). PUR: Purden Lake (UTM 10 N 568391.2E/5973023.5N).

For each set of inhibition tests on a specific isolate, 5 mL of the 30 mL flask culture was transferred to a sterile 15 mL centrifuge tube and homogenized using a Vortex Genie 2 vortex mixer (Scientific Industries Inc., Bohemia, New York, United States). 100 µL aliquots of this homogenized broth culture were used to inoculate each inhibition test broth culture tube. Inhibition testing was performed in 16x125 mm culture tubes containing a total volume of 5 mL Dothistroma broth, amended with 0.5% Triton X-100 (Sigma-Aldritch, St. Louis, MO, USA) to increase volatile compound retention. Use of smaller 5 mL cultures for inhibition tests allowed a larger number of replicates to be performed in a given timeframe, relative to broth sizes used in previously published tests (100 mL; Franich et al. 1982).

Inhibition test cultures had 96% (E,E)-farnesol (Sigma-Aldritch) or steam distilled foliar extract added as a dilution series to an equal final culture volume of 5 mL. The final concentrations of the dilution series (5E-1%, 5E-2%, 5E-3%, 5E-4%, 5E-5%, and 5E-6% v/v) were based on a range around the foliar concentration of the unknown farnesol isomer quantified in Chapter 2 of this thesis (Unknown 2.6; 0-0.039%, mean 0.0065%). Steam distilled foliar extracts were obtained from roughly chopped needles of even aged lodgepole pine trees as per the methods of Clevenger (1928), but low yield in steam distillations limited replication to one isolate (as seen in Franich et al. [1982]). Negative controls were made with 20% v/v 95% ethanol in media; positive controls had no (E,E)-farnesol or steam distilled extract added.

Test broths were incubated for 15 days on a Lab-Line orbit shaker (Lab Line Instruments Inc.), then homogenized using a vortex mixer. One mL of each broth was removed and added to a microcentrifuge tube, which was centrifuged at 16,100 g for 10 minutes on an Eppendorf

5415 D centrifuge (Eppendorf, Hamburg, Germany). The supernatant was removed and 1 mL of sterile deionized water was added. This centrifugation and wash step was repeated a further two times, resulting in a cell suspension in 1 mL sterile deionized water. The resuspended pellets were transferred to a 96 well bead beater plate with one stainless steel bead per well, then agitated at 350 cycles per minute for 5 minutes on a SPEX Certiprep Geno/Grinder 2000 (SPEX Certiprep, Metuchen, NJ, USA). The homogenized mixture in each well was sequentially transferred to a cuvette for 595 nm absorbance measurement on a Bio-Rad Smartspec Plus spectrophotometer (Bio-Rad Laboratories, Hercules, California, USA). Sample absorbance was normalized to a deionized water absorbance control, and mixed by pipetting prior to absorbance measurement.

To control for variation in growth rate between isolates in farnesol inhibition tests, absorbance values were normalized to the growth of the positive control before final analysis (Figure 3.3 and Table 3.1; Figure 3.2 shows data before normalization). The mean absorbance of the positive control across the three replicates used for each isolate test set (Pos_{test}; n=3) was divided by the mean positive control absorbance of all positive control replicates ($Pos_{overall}$; n=27). This gave a ratio indicating the growth rate of that isolate in a specific test relative to the mean growth rate of all isolates in all tests. To normalize test results, individual absorbance values from inhibition tests (Abs_{raw}) were divided by this ratio, then the mean negative control absorbance for that test (Neg_{test}; n=3) was subtracted. This normalization process can be represented by the formula $Abs_{norm} = Abs_{raw}/[Pos_{test}/Pos_{overall}]-Neg_{test}$. Statistical analyses were carried out on these individual normalized values (Abs_{norm}) using R 3.4.3 (R Core Team 2017), with the package ggplot2 used to create Figures 3.2-3 (Wickham 2009).

3.3 Results

After centrifugation a distinct, transparent, layer was often visible above the cell pellet in cultures containing 0.05% (E,E)-farnesol. A smaller volume was occasionally observed in 0.5% (E,E)-farnesol tests. The colour of this layer varied from clear to bright orange, with approximate volume of up to 50 μL.

Absorbance results were highly variable, even between identical replicates (Fig. 3.2), though negative controls showed much lower variability (overall Abs. SD 0.055 vs 0.195-0.472). While the majority of isolates showed strong growth in 30 mL growth flask cultures, growth was often weaker in 5 mL inhibition test cultures. This indicates that use of larger volume test cultures in future inhibition tests may give less variable results, but but would result in fewer replicates in the same timeframe. Inhibition of growth was only seen at (E,E)-farnesol concentrations of 5E-2% and higher, but many low growth cultures showed no discernible response. Low concentrations of (E,E)-farnesol (5E-3% to 5E-6%) often showed greater absorbance than that of the control, indicating that low levels of the compound promoted growth of some *Dothistroma* isolates (Fig. 3.2 A-C).

Addition of steam distilled foliar volatiles to broth media did not appear to inhibit growth at any of the concentrations tested, with higher concentrations appearing to promote growth (Fig. 3.2 D).

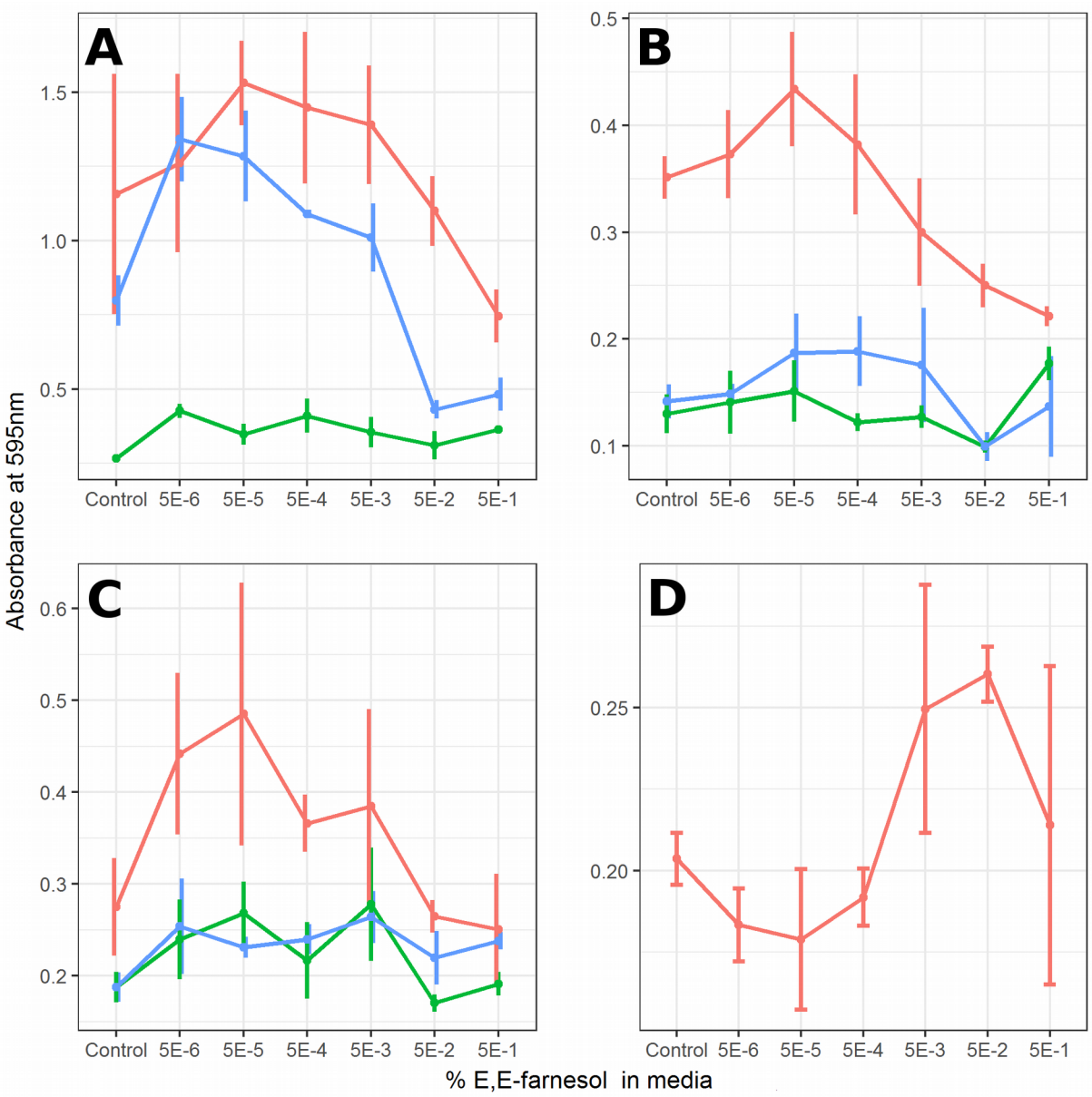

Figure 3.2. Absorbance at 595 nm of washed, homogenized, *D. septosporum* broth cultures with varying media concentrations of **A-C)** E,E-farnesol and **D)** steam distilled foliar volatiles from lodgepole pine. Culture isolates used were as follows: **A)** red – PID A, green – PID B, and blue – PID C. **B)** red – PUR B, green – PUR C, and blue – PUR B. **C)** red – MOR B, green – MOR C, and blue – MOR C. **D)** red – MOR C. Samples were grown for 14 days, and n = 3 for all data points.

A summary of normalized absorbance for all sites and isolates at each tested farnesol concentration is shown in Figure 3.3 (n=27). Analysis of variance of normalized data showed a significant difference in absorbance between tested (E,E)-farnesol levels, across all sites and isolates (n=27; Table 3.1 A). Pairwise t-tests indicated that high levels (5E-1%-5E-2%) of (E,E)-farnesol did not show significant inhibition, but low levels (5E-3%-5E-6%) showed significant promotion of growth relative to the positive control (Table 3.1 B).

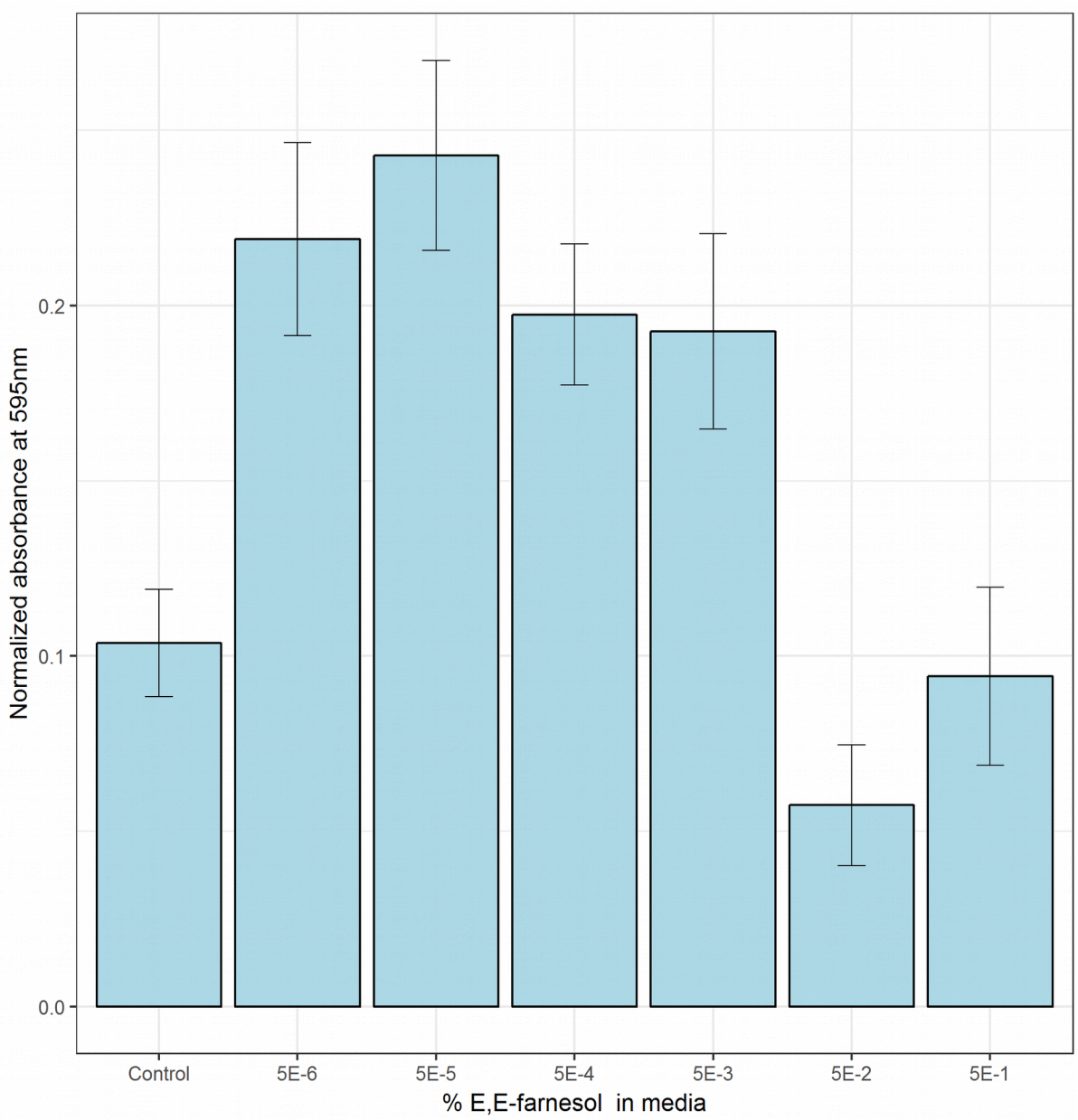

Figure 3.3. Mean, normalized, absorbance at 595 nm of washed, homogenized, *D. septosporum* broths containing varying concentrations of E,E-farnesol, at 14 days. Isolates were collected from three geographically distinct sites in northern British Columbia (n = 27).

Table 3.1. A) Analysis of variance of normalized culture absorbance at varying E,E-farnesol levels, indicating a significant effect of E,E-farnesol concentration on *D. septosporum* growth in broth media. **B)** P-values for pairwise t-tests between normalized culture absorbance values at varying E,E-farnesol levels. Bold P-values indicate significant associations ($P<0.05$). No multiple testing correction was performed (n = 27).

A	**Df**	**Sum Sq**	**Mean Sq**	**F-val**	**P**
Fac	6	0.8305	0.13842	9.332	**6.37E-9**
Res	182	2.6995	0.01483		

B	**0**	**5e-6**	**5e-5**	**5e-4**	**5e-3**	**5e-2**
5e-6	**0.00064**	-	-	-	-	-
5e-5	**4.3E-5**	0.47252	-	-	-	-
5e-4	**0.00524**	0.51717	0.17273	-	-	-
5e-3	**0.00804**	0.42741	0.13143	0.88373	-	-
5e-2	0.16466	**2.4E-6**	**8.2e-8**	**3.8E-5**	**6.9E-5**	-
5e-1	0.77504	**0.00023**	**1.3e-5**	**0.00216**	**0.00342**	0.26890

3.4 Discussion

The results of this work are not conclusive with regard to the contribution of farnesol isomers to *Dothistroma* resistance. Growth inhibition was observed, but only in some isolates, and only at concentrations greater than those of the resistance-linked farnesol isomer identified in lodgepole pine foliage in Chapter 2 of this thesis (Unknown 2.6; 0-0.039%, mean 0.0065%). Concentrations of (E,E)-farnesol approximating levels of the unknown farnesol isomer found in lodgepole pine foliage resulted in stimulation of growth relative to controls. This pattern of growth inhibition at lower concentrations and stimulation at higher concentrations has been termed hormesis (Cutler 2013). The concentrations of (E,E)-farnesol at which inhibitory effects were observed were broadly similar to those seen in tests of other plant volatiles on related fungal species (reviewed in Bakkali et al. 2008), and previous tests on *D. septosporum* (Franich et al 1982). While these compounds are not structurally identical, comparison of the growth promoting effect of (E,E)-farnesol with that of steam distilled foliar volatiles indicates that the former is effective at much lower concentrations. More study of the effect of terpenes on *Dothistroma* growth is required to understand both the physiological basis of these effects and their implications for the host-pathogen relationship.

The observed variability in absorbance between replicates is likely due to a combination of differences in isolates and growth rate, rather than variability in the assay itself. While otherwise identical replicates often gave highly variable absorbances, in most assays negative controls were extremely consistent. This indicates that fungal inoculation methods and measurement of absorbance were not major contributors to the observed variability. That different isolates from the same site showed such differences in growth is not surprising

given the genetic diversity of the pathogen in B.C. and the demonstrated variability of culture morphology between isolates (Dale et al. 2011; Mullet and Barnes, 2012). However, the use of nominally clonal single colony *D. septosporum* isolates should eliminate the contribution of genetic variability and that growth rate differences were also observed between clonal subcultures indicates that the cause is not entirely genetic. Non-genetic variability in growth rate has been observed in other microorganisms (Caten and Jinks, 1968), and quantified in other fungal systems (Inoue et al. 2001). While colony morphology of *Dothistroma* spp. can vary considerably between subcultures of the same isolate (Barnes et al. 2004), no formal reports of growth rate differences have been made. Use of larger broths (i.e. 100 mL; Franich et al. 1982) for future inhibition testing work may reduce variation between replicates, but will be time-consuming for larger numbers of replicates or treatments.

Franich et al. (1982) performed a similar series of tests on a clonal isolate of *D. septosporum* from New Zealand (Hirst et al. 1999), using steam distilled foliar extracts from *Pinus radiata* D. Don. The results show a similar pattern of *D. septosporum* growth inhibition and promotion at high and low concentrations. The steam-distilled extracts were found to only inhibit growth in broth media at the highest concentration tested (0.1% v/v), and growth was stimulated at lower concentrations, peaking at 0.01% v/v. While Franich et al. did not test the effects of individual terpenes on growth, monoterpenes were found to inhibit spore germination whereas steam-distilled extracts promoted spore germination. The differences in assay conditions, tree species, and isolates make it difficult to directly compare my results with that of Franich et al. (1982), but the differences in growth response I observed between (E,E)-farnesol and steam-distilled extract tests support the authors findings, in that the

component compounds of foliar resins can have diverse effects on the fungus.

While toxicity of terpenes is often demonstrated *in vitro*, these effects are rarely linked to resistance due to the difficulty involved in demonstrating a genuine contribution to defense (Gershenzon and Dudareva, 2007). While I observed inhibitory effects on some isolates at high concentrations of (E,E)-farnesol, on average the effect was non-significant at concentrations normally found in homogenized foliage. The mean concentration of the farnesol isomer identified as potentially contributing to quantitative resistance in Chapter 2 was 0.0065% v/w (fresh weight), and did not reach potentially inhibitory levels in any of the tested samples. While this indicates that the inhibitory effects of farnesol isomers are unlikely to have defensive activity at this level, the level of foliar terpenes experienced by *Dothistroma in vivo* may be higher than the mean level quantified from homogenized needle tissue. Pines store pure foliar resins in dedicated structures, potentially leading to highly variable concentrations of component compounds between different needle tissues (White and Nilsson, 1984; Wu and Hu, 1997). This may result in exposure of the fungus to locally elevated levels of these secondary metabolites during lesion formation (Gadgil 1967; Peterson and Walla, 1978; Kabir et al. 2014). While this work does not establish a clear contribution to defense by farnesol isomers based on *in vitro* toxicity, it also cannot be ruled out without knowing the exact exposure of *Dothistroma* during infection and lesion formation. This may prove difficult to quantify based on the scales involved.

This ambiguity in the effects of farnesol isomers is largely due to the many differences between the *in vitro* assay performed and the pathogens environment *in vivo*. *D. septosporum* undergoes a multi-phasic cycle of growth within the complex environment of the pine host

(Kabir et al. 2014), which is poorly emulated by broth media. The fungus has been shown to undergo distinct stages of growth *in vivo* accompanied by genome wide changes in gene expression (Bradshaw et al. 2016), but little is known about this aspect of *D. septosporum in vitro*. The effects of plant secondary metabolites on fungi can vary based on growth phase. For instance *Candida albicans* in the exponential growth phase is highly sensitive to the inhibitory effects of volatile plant secondary metabolites, whereas it is more tolerant in the stationary phase (Bakkali et al. 2005). The authors speculate that this effect may be mediated by the thinner cell walls of budding yeast cells in the exponential phase, which allow cellular penetration by inhibitory compounds. How this would affect filamentous fungi like *D. septosporum* is unknown.

Triton X-100 was added to broth media as a surfactant, and this may have affected the inhibitory properties of farnesol, either positively or negatively. My preliminary tests showed that some terpenes were rapidly lost from broth media over the test period, even if sealed with parafilm as per the methods of Franich et al. (1982). I found that addition of 0.5% Triton X-100 adequately reduced evaporative losses of terpenes from the medium, but had a negative effect on fungal growth, requiring a doubling of growth time relative to previous study (Schwelm et al 2008). Triton X-100 at similar concentrations has been used for this purpose in previous work on fungi (Combrinck et al. 2011), nematodes (Park et al. 2007), and insects (Koschier et al. 2003), but how it interacts with farnesol and other terpenes is unknown.

Because of limitations on the commercial availability of specific sesquiterpenes and sesquiterpene alcohols, (E,E)-farnesol was used for inhibition testing instead of the specific

sesquiterpene, sesquiterpene alcohols, and farnesol stereoisomer identified as being correlated with inhibition in Chapter 2 and provenance disease history in Chapter 4. While chemically similar, different farnesol stereoisomers may have variable effects on the fungus. While Hornby et al. (2001) initially found (E,E)-farnesol to have an equivalent effect on *Candida albicans* germ tube formation to that of a mix of farnesol isomers, further testing showed that (E,E)-farnesol alone was responsible for quorum sensing effects (Shchepin et al. 2003). Other isomers were found to potentially have the opposite effect, and chemical derivatives of (E,E)-farnesol all showed much lower activity if any was observed at all. This is consistent with the stereoisomer specificity exhibited by cellular processes involving farnesol (Bates et al. 1963), and indicates that if farnesol has an effect on *Dothistroma* other than that of general quantitative inhibition it may be stereoisomer specific.

While terpenes and other foliar volatile components have been shown to exhibit broadly inhibitory effects on fungi and other microorganisms, the mechanisms underlying these effects are varied and not fully understood (Bakkali et al. 2008). Because of the diversity of their components, foliar resins are unlikely to have only one mechanism of antifungal action (Carson et al. 2002). Farnesol isomers specifically have been shown to inhibit growth of a variety of fungal species (Semighini et al. 2006; Semighini et al. 2008; Derengowski et al. 2009), potentially due to disruption of internal cellular structures (Jabra-Rizk et al. 2006). Plant extracts predominantly made up of terpenes have been shown to induce expression of genes involved in DNA repair in *Saccharomyces cerevisiae*, though extracts were not found to cause nuclear damage directly (Bakkali et al. 2005). The authors hypothesized that damage was caused by reactive oxygen species formed or released as a result of extract exposure.

Similarly, Carson et al. (2002) found that terpene components of tea tree (*Melaleuca alternifolia*) oil reduced salt tolerance and 260 nm absorbance of *Staphylococcus aureus* at sub-bactericidal levels, indicating damage to cellular membranes.

That (E,E)-farnesol can either inhibit or promote growth of *D. septosporum* in media depending on concentration may indicate that this compound has multiple mechanisms of action in the pathogen. While inhibition could be explained by quantitative inhibitory effects detailed above, one potentially explanation for growth promotion is that the compound has a function in plant-pathogen signalling. Secondary metabolites such as flavenoids, lactones, and phenolics are known to play a signalling role in plant interactions with pathogens (Steinkellner et al. 2012) and epiphytes (Hashidoko 2005). Some of these compounds have been shown to stimulate hyphal growth *in vitro* (Chalbot et al. 1992). The signalling role (E,E)-farnesol plays in quorum sensing of *Candida albicans* has been well studied since its discovery (Hornby et al. 2001;), and is specific to this isomer (Shchepin et al. 2003). While farnesol inhibits hyphal growth in *C. albicans,* functioning as a host-produced autoregulator (Sato et al. 2004), quorum sensing molecules are thought to control fungal dimorphism in a number of species (Nickerson et al. 2006). Quorum sensing effects have been observed in the fungal pathogen *Ceratocystis ulmi* (causal agent of Dutch elm disease), though the effect was not mediated by farnesol, and the authors suggest that the effect is common in dimorphic fungi (Hornby et al. 2004). These morphological changes are often important for fungal pathogenicity (Gow et al. 2002). While *D. septosporum* is not dimorphic (having no yeast form), little is known about what induces observed changes in the pathogen's growth state (Kabir et al. 2014; Bradshaw et al. 2016). That farnesol also acts as a signalling molecule in

diverse mammalian (Forman et al. 1995; Joo et al. 2010) and insect (Ronderos et al. 2014) systems makes it plausible that it could play a similar role in control of the lifecycle of the *D. septosporum* within its host. The effect of (E,E)-farnesol on the lifecycle of *D. septosporum* could potentially be assessed through transcriptomic studies. Genome-wide expression in farnesol-exposed cultures could be broadly compared to that of established infection stages to determine if farnesol isomers induce a response similar to that *in vivo* (Bradshaw et al. 2016).

Alternatively, farnesol isomers may be involved in hyphal chemotaxis. Hyphal extension in fungal pathogens in often highly directed, both pre- and post-penetration (Brand and Gow, 2009). Pre-penetration growth of *Dothistroma* on the needle surface is directional on some host species (*Pinus ponderosa* Laws. and *Pinus nigra* Arnold – Peterson 1969; Peterson and Walla, 1978) and random on others (*Pinus radiata* D. Don - Gadgil 1967; Kabir et al.2014, *Pinus muricata* D. Don – Muir and Cobb, 2005). These differences are thought to be based on host species and infection conditions (Gibson 1972), with seedlings in high humidity infection chambers often having random growth (Muir and Cobb, 2005). If hyphal directionality is based on chemotaxis then differences between hosts could be explained by their varying foliar secondary metabolite product profiles (Ioannou et al. 2014). *P. radiata* is unlikely to be a natural host of *D. septosporum* due to its range (Central California), and consequently the pathogen may not be adapted to the foliar chemistry of this pine species, especially given the clonal nature of the New Zealand *D. septosporum* population. Directional hyphal growth has been seen in *Dothistroma pini* Hulbary on *P. radiata* (Muir and Cobb, 2005); potentially this species of *Dothistroma* is adapted to hosts within its native range. Less directional hyphal growth in New Zealand exotic *P. radiata* could explain mature

tree resistance observed in that species, which has been associated with occlusion of stomata by cuticular wax (Franich et al. 1977; Franich and Gadil, 1983). Reduction of stomatal pore size may provide more of a defense in species where hyphal growth is random.

Microscopic examination of *Dothistroma* hyphae *in vitro* shows a characteristic zig-zag pattern during initial growth (Mullet and Barnes, 2012). This pattern of growth is similar to that of *teaA* and *teaR* deletion mutants of the related fungal pathogen *Aspergillus nidulans*, which lack genes involved in maintaining hyphal polarity (Takeshita et al. 2008). This zig-zagging pattern could results from the lack of a chemotactic factor in the media to orient hyphal growth. However, chemotropism in plant pathogens is relatively rarely observed (Brand and Gow, 2009), and directional growth towards stoma is not seen in the related pathogen *Cladosporium fulvum* (de Wit 1977). To determine whether volatile compounds from lodgepole pine foliage have any chemotactic effect on *D. septosporum* hyphal growth will require further study on their effect *in vitro*. Chemotactic growth could be assessed using a chemotaxis chamber assay at a variety of (E,E)-farnesol concentrations (Morris et al. 1998).

The consistent presence of a denser than water liquid layer in 0.05% farnesol exposed *Dothistroma* cultures may indicate production of an unidentified compound by the fungus, but at this point its origin is unknown. Fungi produce a variety of volatile organic compounds with diverse functions (Morath et al. 2012); pathogenicity of the saprophyte *Botrytis cinerea* is mediated by fungally produced sesquiterpenes (Collado et al. 2007). The relative density of the unknown compound indicates that it is unlikely to be E,E-farneosl, and its volume makes it unlikely that it is precipitated Triton X-100. Future study will involve collecting a larger sample of this liquid to determine whether it is a genuine product of *D. septosporum* or an

artifact of the experimental design.

This work has illuminated previously unknown chemical interactions between *D. septosporum* and its pine host. Significant growth promotion was observed at (E,E)-farnesol levels as low as 0.000005%. The strength of this effect at such a low level indicates that this chemical could play an important role *in vivo,* potentially as a signaling molecule or chemotactic factor. Determining the basis of this effect will require further study. Conversely, while this study has shown potential inhibitory effects of (E,E)-farnesol on some *D. septosporum* isolates, the broadly significant growth promotion seen at lower concentrations makes it difficult to draw any straightforward conclusions about the contribution of farnesol to *Dothistroma* resistance. (E,E)-farnesol was shown to inhibit *in vitro* growth of some *Dothistroma* isolates at media concentrations of 0.5 and 0.05% v/v, but these levels are higher than those seen *in vivo*, indicating that this inhibitory effect may not be physiologically relevant. The contribution of these disparate effects to the *Dothistroma*-*Pinus* interaction may be reconciled by further work on foliar terpenoids.

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4. The influence of *Dothistroma* outbreak history on constitutive foliar secondary metabolite production in lodgepole pine provenances

4.0 Abstract

The changing climate in British Columbia has brought with it a new threat of emergent forest disease. While the fungal pathogen *Dothistroma septosporum* Dorog. Morelet has been historically benign within the province, current climate conditions and forest management practices have led to conditions allowing increased severity of outbreaks, to the point of mature tree mortality in host lodgepole pine (*Pinus contorta* var *latifolia* Engelmann). Previous work has linked foliar disease resistance in lodgepole pine to constitutive foliar terpene levels (Wallis et al. 2010; Chapter 2 of this thesis). To determine whether *D. septosporum* outbreak history has influenced constitutive terpene production by lodgepole pine in British Columbia, I used dendrochronological methods to predict past *Dothistroma* outbreaks at provenance origin sites. I then generated a quantitative measure of outbreak history for each site, and compared this with previously quantified foliar metabolite levels. I found that outbreak history significantly positively correlated ($P<0.05$) with levels of a wide range of monoterpenes. Several sesquiterpenes and a sesquiterpene alcohol also showed significant positive correlation, including two previously identified in work on *Dothistroma* resistance (Chapter 2 of this thesis). The broad, significant, and positive correlation of constitutive foliar terpene levels with *D. septosporum* outbreak history may indicate an evolved response to disease pressure, and that these compounds potentially confer quantitative resistance to the disease.

4.1 Introduction

While historical climate data has established that the abiotic and biotic environment of northern British Columbia is changing, corresponding records on forest disease prevalence are less detailed (Woods et al. 2017). Consequently, little is known about how disease pressure has shaped defence adaptation of forest tree species at a provenance level. One disease of particular concern in British Columbia is red-band needle blight, caused by the defoliating fungal pine pathogen *Dothistroma septosporum* (Dorog.) Morelet. With its extensive distribution (Watt et al. 2009) and direct impact on growth rate (Watt et al. 2011), *D. septosporum* is considered to be the most damaging foliar disease of pine worldwide (Bradshaw 2004). While outbreaks of the fungus have not historically resulted in major forest productivity losses in this province, it has been a serious problem in exotic pine plantations in the southern hemisphere, where chemical control is now widely used to control the disease (Gibson 1974; Bulman et al. 2016). *Dothistroma* outbreaks have been reported in lodgepole pine stands in British Columbia dating back to 1963 (Parker and Collis, 1966), but the pathogen was not considered a major concern until recent outbreaks in northwestern B.C. resulted in widespread tree mortality (Woods 2003). Subsequent examination of the dendrochronological record showed that *Dothistroma* outbreaks have increased in both extent and severity since the 1940's (Welsh et al. 2009). Investigation into the factors driving emergence of the disease has revealed a link to precipitation and temperature, suggesting that damage caused by *D. septosporum* in British Columbia will increase as the climate becomes warmer and wetter (Woods et al. 2005; Welsh et al. 2014).

In general, the occurrence of forest disease is dependent on three factors; availability of a

susceptible host, presence of the pathogen, and a conducive environment (Agrios 2005). Of these three elements, the emergent nature of *D. septosporum* in northwestern British Columbia is the result of both an increasingly conducive abiotic environment and greater host abundance (Woods et al. 2005). Correlation between precipitation and outbreaks of the disease is directly due to the biology of the pathogen, rather than the effect of the environment on the host. Early work on *D. septosporum* in *Pinus radiata* D. Don showed that spore germination increases linearly with temperature, and that infection severity was strongly associated with increased humidity (Gadgil 1974). Production of stromata is dependent on moisture (Gadgil 1977), as is subsequent dispersal of conidiospores within the canopy (Peterson 1973). Conidia dispersal occurs by rainsplash, and is strongly associated with precipitation (Boateng and Lewis 2015). The length of these rainfall events is correlated with outbreak severity (Gadgil 1977). The spread of the disease through the tree results in progressive defoliation, reducing diameter growth through loss of photosynthetic potential. In *P. radiata* defoliation must exceed 25% before diameter growth rate is appreciably changed (Gibson 1974), with tree mortality only occurring when defoliation is especially severe.

While increased precipitation and temperature has contributed to *Dothistroma* outbreaks (Woods et al. 2005; Welsh et al. 2009; Welsh et al. 2014), the current outbreak is the cumulative result of both changes in climate and a forest management preference for lodgepole pine (Woods et al. 2003). Resistance to *Dothistroma* varies between pine species, but lodgepole pine is considered highly susceptible (Watt et al. 2009) and has been planted extensively throughout northern British Columbia (Woods et al. 2003). In species that have shown resistance to the fungus the effect is quantitative and multifactorial (Carson 1989),

potentially involving a range of chemical and physical defences (Fraser et al. 2015). Of these defensive compounds, terpenes are the most numerous in conifers (Bohlmann and Croteau, 1999), but their foliar composition varies widely between species (Ioannou et al. 2014) and provenance (Wallis et al. 2010; Wallis et al. 2011; Chapters 1 & 2). Variability in terpene levels has been associated with varying resistance to fungal foliar pathogens (Wallis et al. 2010; Chapter 2), implying that some of the variation seen in foliar secondary metabolite composition may be an adaptation to disease pressure. While conifer species are long lived, they are potentially more genetically adaptable than their lifespan would imply (Buschiazzo et al. 2012).

Forest trees are often highly adapted to local climate (Sakai 1983; Holliday et al. 2008) and disease pressures (Sork et al. 1993). Comprehensive historical records are available for quantifying provenance climate history, but linking observed disease resistance to historical disease pressure is generally complicated by a lack of complete and quantitative disease histories. However, dendrochronological methods have been successfully used to quantitatively assess forest disease history at a stand level both for *D. septosporum* (Welsh et al. 2009; Welsh et al. 2014) and insect defoliators (Swetnam and Lynch 1989; Swetnam et al. 1995; Boulanger and Arseneault 2004). Ring width series collected from forest trees represent the cumulative effect of a variety of factors influencing tree growth, including both climate and disturbances (Cook 1985). Biotic disturbance agents include defoliating plant pathogens such as *D. septosporum*, which influence growth as a result of reduced photosynthetic potential, changing ring width over a period of years. In the case of *Dothistroma,* this unique pattern of ring width changes can be detected using statistical

methods, allowing species specific defoliation events associated with outbreaks to be identified long after the outbreak has passed (Welsh et al. 2009).

As a fungal pathogen native to British Columbia, *Dothistroma* was previously considered benign relative to more damaging forest pests and pathogens. We therefore lack the detailed knowledge of the *Dothistroma-Pinus* interaction that is required to fully understand the drivers behind this emergent disease (Welsh et al. 2009). Associating foliar secondary metabolite levels with historical disease pressure may prove valuable in identifying host defence compounds that are important in this interaction. In this chapter I compared the *D. septosporum* outbreak history of lodgepole pine provenances to their constitutive foliar secondary metabolite levels quantified in Chapter 1. Host secondary metabolites that are significantly elevated in disease-exposed provenances may play a role in defence.

The objectives of the work described in this chapter were to: 1) Obtain a quantitative measure of *Dothistroma septosporum* outbreak history at previously defined provenance origin sites using dendrochronological methods; 2) Identify previously quantified compounds that correlate with disease history; and 3) Discuss the potential physiological basis for activity of correlated compounds in defence.

4.2 Methods

Tree cores were collected from host (lodgepole pine) and non-host (spruce; white spruce [*Picea glauca* {Moench} Voss], Engelmann spruce [*Picea engelmannii* Parry ex Engelm.], or hybrid spruce) at provenance origin sites for tree families studied in Chapter 1 (Figure 4.1). Samples were collected as close to established GPS coordinates as possible (Table 4.1), up to a maximum distance of 5 km horizontally and 200 m difference in elevation. Up to twenty standing trees of each species at each provenance origin site were cored at 1.3 m height from two sides to avoid decay at the base of the tree. I selected mature trees based on their proximity to the site centre coordinates, with larger diameter (and presumably older) trees preferentially selected to give the greatest chronology depth. I avoided sampling adjacent trees where practical to broaden the sampling area and ensure that selection was representative of overall site conditions. Dead-standing lodgepole pine with evidence of mountain pine beetle attack made up the vast majority of lodgepole pine trees present at many sampling sites. At sites where live mature pine trees were not present three younger live trees were sampled to allow crossdating to the sampling year. Cross-sectional discs were taken where dead-fallen trees or stumps were available.

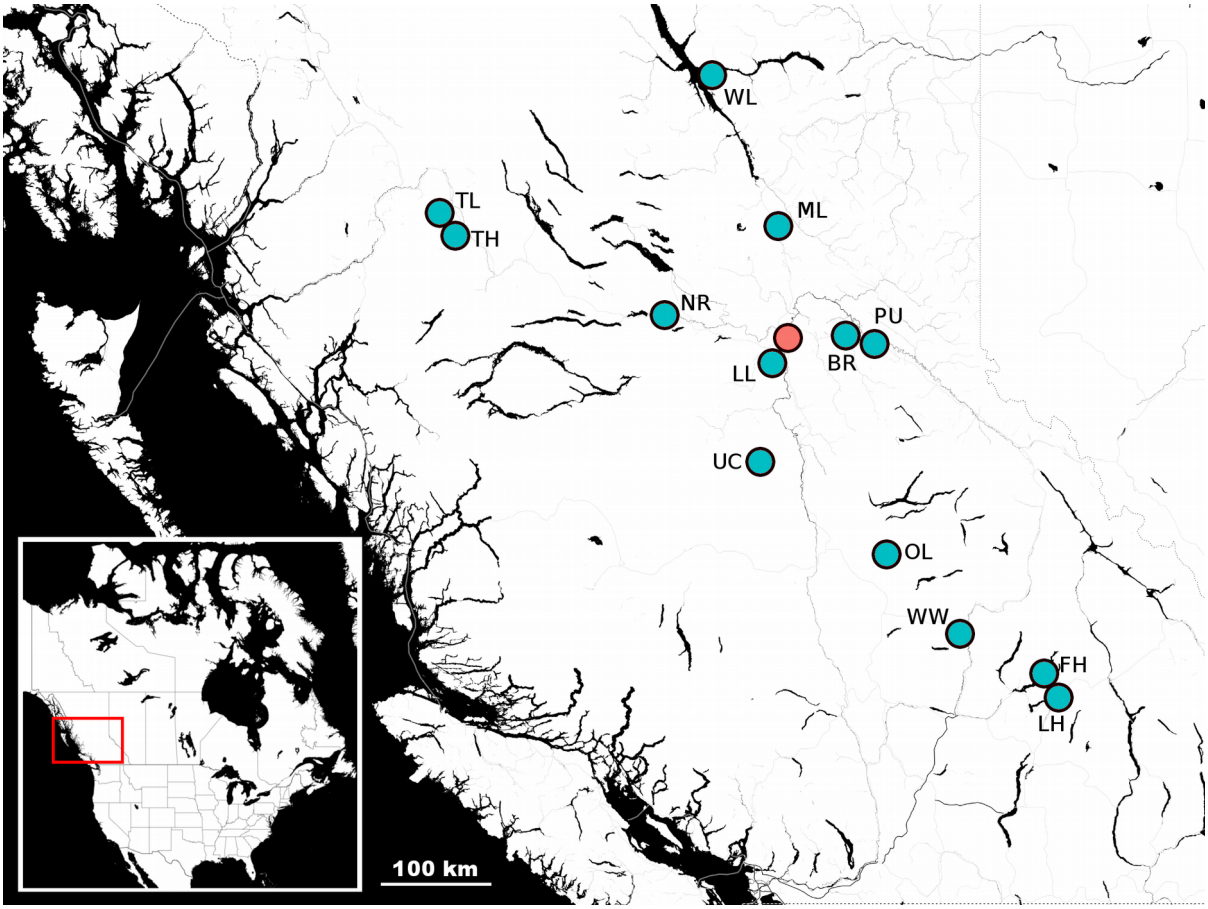

Figure 4.1. Location of tree core sampling sites at provenance origin (blue) and PGTIS site (pink) within British Columbia. Map tiles by Stamen Design, under CC BY 3.0. Data by OpenStreetMap, under OdbL.

Table 4.1. GPS coordinates of tree core sampling sites at provenance origin and PGTIS site, from (Wallis et al. 2010).

ID	Site	UTM coordinates
PU	Purden	10 U, 583292 m E, 5969431 m N
BR	Bowron River	10 U, 565705 m E, 5972859 m N
TH	Telkwa High	9 U, 601125 m E, 6055118 m N
WL	Williston Lake	10 U, 450042 m E, 6200804 m N
ML	McLeod Lake	10 U, 509639 m E, 6074401 m N
TL	Telkwa Low	9 U, 625814 m E, 6057591 m N
LL	Lynx Lake	10 U, 502203 m E, 5944582 m N
NR	Nechako River	10 U, 400626 m E, 5986440 m N
OL	Oie Lake	10 U, 623523 m E, 5762726 m N
WW	Wentworth	10 U, 687239 m E, 5649504 m N
LH	Larch Hill	11 U, 345818 m E, 5618738 m N
FH	Fly Hills	11 U, 327316 m E, 5621872 m N
UC	Udy Creek	10 U, 485465 m E, 5874147 m N
PGTIS	Prince George Tree Improvement Station	10 U, 518367 m E, 5958279 m N

Cores were mounted, and all samples were progressively sanded to 600 grit as per Stokes and Smiley (1968). All cores were visually crossdated using the list method (Yamaguchi 1991), followed by memorization of marker years. Cores and discs were scanned using an Epson Expression 1640 XL scanner (Seiko Epson Corporation, Suwa, Nagano Prefecture, Japan), and ring widths were digitally measured using WinDendro 2012b (Regent Instruments, Ville de Quebec, QC, Canada). Crossdating was statistically verified using COFECHA 6.06P (Holmes 1983). Dated ring width series were averaged and standardized in ARSTAN 6.05P (Cook and Peters, 1981; Cook 1985; Cook and Holmes, 1999) using a cubic smoothing spline with a 50% frequency response cutoff of 40 years, as per Welsh et al. (2009).

Statistical analysis was carried out in R 3.4.3 (R Core Team 2017) with packages dplR (Bunn 2008; Bunn 2010; Bunn et al. 2017), ggplot2 (Wickham 2009), and reshape2 (Wickham 2007). Corresponding host/non-host chronologies that were insufficiently correlated by Pearson's r-value and visual examination were removed from analysis. Published non-host chronologies from the International Tree Ring Data Bank (ITRDB) were used where available, as they provided deeper chronologies than those from collected spruce cores, which were often limited in length by heartwood decay. A climate corrected index was created from host series and non-host chronologies using the software program OUTBREAK 6.00P (Holmes and Swetnam, 1996). *D. Septosporum* outbreaks were inferred using OUTBREAK 6.00P with the parameters established by Welsh et al. (2009); an index threshold of -1.2 SD, duration of 5-10 years, and a 70% minimum reduction for the initiating year. As per Welsh et al. (2009) inferred outbreaks that affected less than 40% of the sample host trees were discounted to eliminate minor outbreaks and alternate causes of defoliation.

Inferred outbreaks at Lynx Lake, and Udy Creek sites centred on 1998 were confirmed against concurrent known outbreaks in Cinema and Cottonwood (Welsh et al. 2009).

A quantitative measure of disease history was generated for each site using the sum of yearly affected tree percentages within outbreaks that reach the 40% threshold, divided by the length of the chronology in years (mS/Y5). A minimum chronology depth of 5 trees was used for the analysis. mS/Y5 values were correlated with compound peak areas from Chapter 1 in R 3.4.3 (R Core Team 2017) using Spearman's r, Kendall's tau, and Spearman's rho. While some compound peak areas showed a non-normal distribution that was resistant to normal transformation, necessitating a nonparametric analysis (Chapter 1 of this thesis), parametric results are included for comparison to previously published work (Wallis et al. 2010; Wallis et al. 2011).

4.3 Results

All sites had extensive evidence of both mountain pine beetle attacks and industrial forestry activity, making sampling of large, live trees within defined sampling areas difficult at some sites. Sufficient material for construction of site chronologies was obtained at twelve of thirteen sampling sites, with Wentworth (WW) being excluded from further analysis. While most sites showed strong correlation between host and non-host chronologies, the Telkwa High (TH) site was excluded from analysis because of its lack of correlation with the non-host spruce chronology. Some sites showed non-significant r-values (TL and UC; Table 4.2), but visual examination indicated that differences in response between the two chronologies were likely due to growth releases from forestry activity. This difference was only possible at sites where host and non-host sampling was spatially separated due to the absence of mixed species stands at the site centre. While a sufficiently widespread growth release in the non-host could potentially be misidentified as an outbreak, I did not observe this in any of my non-host chronologies, meaning that growth releases are unlikely to have influenced outbreak prediction.

Table 4.2. Pearson's r and P-value for correlation between host and non-host chronologies of each provenance.

Host ID	Non Host	r	P
PU	BRS	0.522	**0.00216**
BR	BRS	0.494	**8.46×10^{-11}**
TH	cana091 + SMS*	0.012	0.949
WL	WLS	0.346	**0.00360**
ML	MLS	0.234	**0.00658**
TL	cana091 + SMS*	0.197	0.113
LL	LLS	0.294	**0.00249**
NR	NRS	0.453	**0.00330**
OL	OLS	0.425	**1.42×10^{-5}**
LH	cana161**	0.395	**4.78×10^{-5}**
FH	cana161**	0.521	**4.51×10^{-7}**
UC	LLS	0.134	0.176

Bolded P values indicate are statistically significant at $P<0.05$.

* cana091: ITRDB, Smithers Ski area, white spruce, Schweingruber, F. H., supplemented with additional spruce cores (SMS).

** cana161: ITRDB, Adams Lake, Engelmann spruce, Parish R.

OUTBREAK output showed numerous defoliation events in trees at all sites, although the 40% threshold was not exceeded at all of the sites (Figure 4.2). Synchronicity was seen between outbreaks at a number of sites. Five sites reported outbreaks exceeding the 40% threshold in the 1941-1950 period, out of a total of eight sites with adequately deep chronologies. The most widespread inferred outbreak was seen in seven of eleven reporting sites during the 1974-1983 period. The limited length of most site chronologies made it difficult to conclusively determine whether outbreak synchronicity within the study area was increasing, as seen by Welsh et al. (2009), though the limited extent of the predicted outbreak centred on 2000 indicates that this is not the case.

Numerous significant positive correlations were found between mS/Y5 values and the foliar compound peak areas quantified and putatively identified in Chapter 1 of this thesis (Table 4.3). Levels of all but one of the putatively identified foliar monoterpenes quantified were significantly positively correlated with mS/Y5 by the nonparametric statistical tested performed (Table 4.3; compounds 1, 3, 5-7). Most compounds with higher retention times showed no correlation, however sesquiterpenes tentatively identified as α-muurolene and α-copaene in Chapter 1 showed signficant correlation, as did compounds putatively identified as α-cadinene (unknown 2.3) and farnesol (unknown 2.6) in Chapter 2. All significant correlations were positive; no significant negative correlations were observed with any of the quantified compounds.

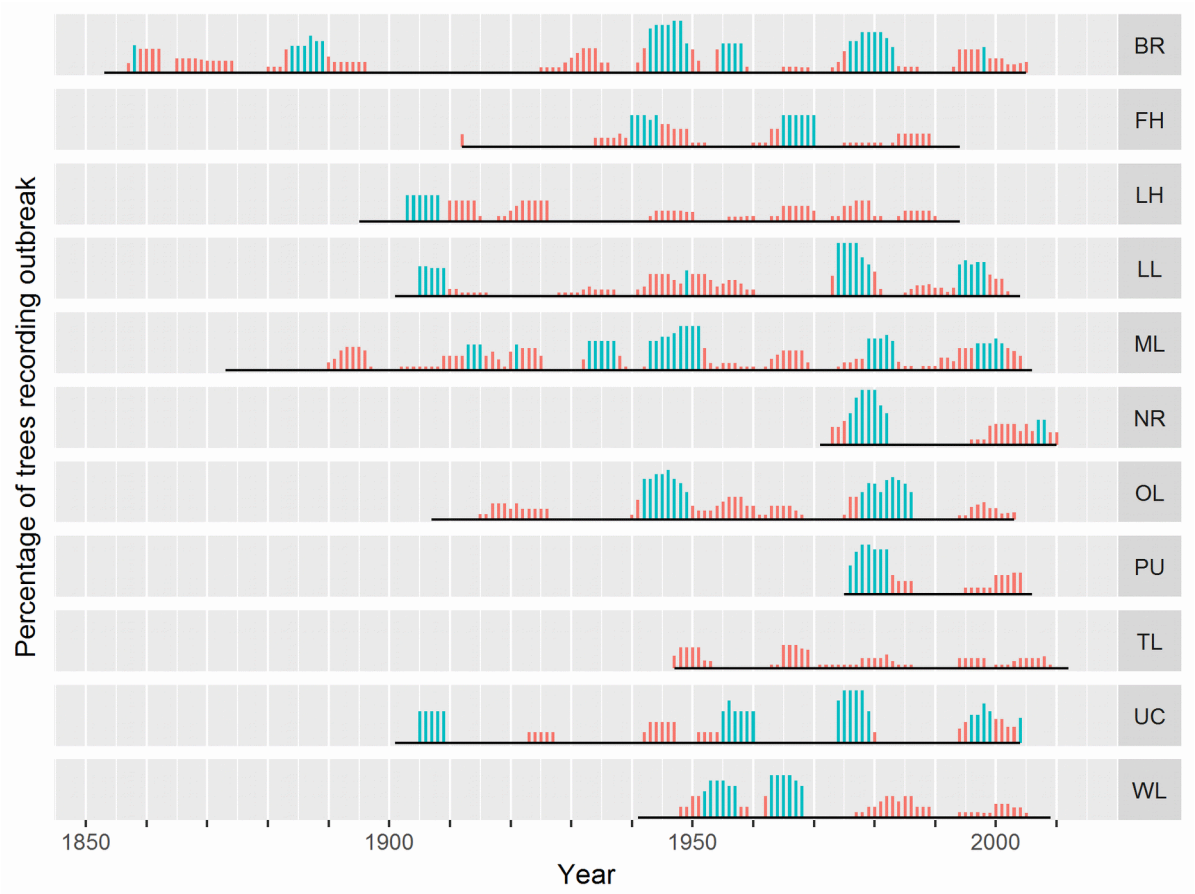

Figure 4.2. Percentage of provenance trees reporting outbreaks at each chronology year. Blue bars indicate outbreak years over the 40% threshold, the black baseline represents the chronology period which meets the minimum depth of 5 trees.

Table 4.3. Correlation between foliar secondary metabolite levels quantified and putatively identified in Chapter 1 and provenance outbreak history, expressed as sum tree percentages affected over outbreak periods, per year of chronology at a minimum depth of 5 trees.

	Compound	Pearson's r		Spearman's ρ		Kendall's τ	
		p-value	r	p-value	ρ	p-value	τ
1	α-pinene	**0.007**	0.305	**0.002**	0.361	**0.003**	0.257
2	Unknown 1.1	0.415	0.078	0.349	0.082	0.337	0.060
3	β-pinene	0.102	0.200	**0.008**	0.307	**0.013**	0.207
4	Unknown 1.2	**0.001**	0.367	**0.001**	0.370	**0.001**	0.282
5	Δ-3-carene	0.126	0.169	0.059	0.222	0.053	0.176
6	Δ-limonene	0.058	0.227	**0.027**	0.266	**0.025**	0.196
7	β-phellandrene	**0.013**	0.285	**0.001**	0.365	**0.001**	0.269
8	Para-cymene	**0.025**	0.256	0.132	0.196	0.133	0.155
9	β-linalool	0.282	0.141	**0.006**	0.317	**0.005**	0.242
10	Anisole	0.860	0.048	**0.008**	0.311	**0.007**	0.239
11	Unknown 1.3	**0.028**	0.241	**0.001**	0.339	**0.000**	0.288
12	2,4-dimethylbenzaldehyde	0.906	-0.022	0.341	0.091	0.274	0.074
13	Unknown 1.4	0.933	-0.013	0.107	-0.180	0.112	-0.146
14	α-copaene	0.054	0.221	**0.013**	0.300	**0.014**	0.218
15	Unknown 1.5	0.582	0.103	0.772	0.063	0.823	0.044
16	Unknown 1.6	0.906	0.012	0.292	0.139	0.245	0.114
17	Germacrene D	0.658	0.081	0.340	0.133	0.300	0.107
18	β-elemene	0.401	0.127	0.378	0.127	0.345	0.102
19	Unknown 2.2*	0.592	0.102	0.126	0.193	0.088	0.153
20	α-muurolene	0.708	0.088	0.065	0.226	**0.045**	0.174
21	Unknown 2.3*	0.103	0.196	**0.011**	0.299	**0.007**	0.226
22	Unknown 2.4*	0.335	-0.013	0.656	0.079	0.653	0.054
23	γ-muurolene	0.925	0.074	0.398	0.123	0.351	0.096
24	Δ-cadinene	0.710	-0.054	0.276	0.100	0.247	0.075
25	Unknown 2.5*	0.637	0.074	0.529	0.106	0.530	0.087
26	Δ-cadinol	0.993	0.039	0.132	0.191	0.094	0.150
27	Unknown 2.6*	0.120	0.190	**0.042**	0.239	**0.036**	0.179
28	Cubenol	0.752	0.031	0.912	0.038	0.938	0.024
29	Germacrene D-4-ol	0.974	0.053	0.747	-0.006	0.695	-0.012

* Resistance-correlated compounds identified in Chapter 2 of this thesis. Bold P values are statistically significant at $P<0.05$.

4.4 Discussion

This work shows broadly significant positive correlations between the constitutive foliar terpene levels of the lodgepole pine provenances tested and their inferred *D. septosporum* outbreak history. This may indicate that these compounds are involved in resistance to this pathogen. Host populations are often highly adapted to local disease pressures (Burdon and Jarosz 1991; Sork et al. 1993; Lively and Jokela 1996), and terpenes are active in disease resistance in other host-pathogen systems (Aitken 1993; Michelozzi et al. 1995; Wallis et al. 2010). Conversely, provenances from locations where *Dothistroma* is not historically prevalent are likely to be more susceptible to the disease, due to the costs associated with maintaining resistance (Gershenzon 1994; Tian et al. 2003). While monoterpenes in general showed significant positive correlations with disease history, only four of the quantified sesquiterpenes and sesquiterpene alcohols significantly correlated. Interestingly, this included unknowns 2.3 (putatively identified as α-cadinene [{1S,4aR,8aR}-4,7-dimethyl-1-propan-2-yl-1,2,4a,5,6,8a-hexahydronaphthalene]) and 2.6 (putatively identified as an isomer of farnesol [3,7,11-trimethyldodeca-2,6,10-trien-1-ol]), levels of which were found to significantly correlate with lodgepole pine crown retention in Chapter 2 of this thesis. That foliar terpene levels also positively correlate with inferred disease history supports the hypothesis that these compounds play a role in resistance to this fungal pathogen, but comparison of this data to the results of previous studies suggests that the relationship is complex. Monoterpene levels were not significantly correlated with *Dothistroma* resistance in Chapter 2 of this thesis, and work on lodgepole pine by Wallis et al. (2010) did not identify sesquiterpenes as significant contributors to foliar pathogen resistance.

Despite this apparent conflict, terpenes have been broadly associated with defence against pathogens in previous research. Monoterpenes, sesquiterpenes, and foliar volatile mixtures have all been shown to affect *Dothistroma in vitro* (Franich et al. 1982; Chapter 3 of this thesis), and foliar chemistry plays an important role in the *Dothistroma* resistance of related pine species (Franich and Gadgil 1983). While terpenes have not been extensively studied in the context of the *Dothistroma-Pinus* pathosystem (Fraser et al. 2015), they are important in many other plant defence interactions (Michelozzi et al. 1995; Franceschi et al. 2005; Wallis et al. 2008). Terpenes are broadly inhibitory to pathogenic fungi *in vitro* (Andrews et al. 1980; Cakir et al. 2005; Combrinck et al. 2011), likely due to nonspecific disruption of internal cellular structures (Bakkali et al. 2005; Jabra-Rizk et al. 2006; Derengowski et al. 2009).

Activity of terpenes in plant defence is well documented in published literature, which supports the results of this chapter, but it seems that there is no simple relationship between terpene levels and resistance (Franich et al. 1982). There are notable differences between results in previous work by Wallis et al. (2010), Chapter 2 of this thesis, and this study. Wallis et al. (2010) assessed damaged caused by *Lophodermella concolor* (Dearn.) Darker, *Lophodermella montivaga* Petre., and *Elytroderma deformans* (Wier) Darker in the PGTIS Bulkey No. 228 orchard, and found that the severity of these foliar diseases was significantly negatively correlated with the foliar concentrations of specific monoterpenes. No correlation was found with overall levels of quantified sesquiterpenes. Conversely, chapter 2 of this thesis showed correlation of specific sesquiterpene and sesquiterpene alcohol levels with crown retention at a *Dothistroma* resistance trial in Kispiox, but no correlations were seen

with monoterpene levels.

These inconsistencies are likely due to differences in the pathogens and hosts tested, and could possibly be resolved by a study with increased statistical power. While the pathogen species involved are all ascomycete defoliators of lodgepole pine, *Dothistroma* is genetically distant from the more closely related *Lophodermella* and *Elytroderma* species (Tehler et al. 2003). Tolerance of fungi to terpenes is highly variable between species (Wedge et al. 2000; Bakkali et al. 2008), so it is reasonable that these pathogens would have distinct tolerances or susceptibilities to terpenoid host defences. As for differences in the host, the Kispiox resistance trial families tested in Chapter 2 are from north western British Columbia, representing a smaller geographical range than that sampled in this study and one that has experienced high levels of *Dothistroma* activity (Woods et al. 2003). Provenances which have coevolved with the disease would likely have a higher average level of resistance and lower variability in resistance than provenances at the PGTIS (Burdon et al. 2013), which represent a far greater geographical range, often with little reported history of the disease, and greater variation in climate (Wallis et al. 2010; Wallis et al. 2011). It is therefore possible that Kispiox resistance trial families would simply have higher levels of some defensive compounds and lower level variability, giving them consistently higher quantitative resistance (relative to PGTIS provenances) but lowering crown retention correlation indices for these compounds. Monoterpenes are only one class in a wide array of chemical defences produced by lodgepole pine and the contribution of other defence mechanisms (including that of sesquiterpenes) could mask the effect of monoterpene variation on crown retention in Chapter 2 families. Resistance to *Dothistroma* is thought to be multifactorial (Carson 1989),

with a number of foliar compounds thought to contribute to *Dothistroma* resistance. This includes foliar phenolics (Wallis et al. 2010), lignin, and benzoic acid (Franich et al. 1986). If observed variation in resistance was substantially due to other factors the contribution of monoterpenes to crown retention may not have been detectable in Chapter 2 without increased statistical power, or a site representing a greater range of resistance phenotypes.

The latter limitation is largely unavoidable when it is necessary to sample from mature trees while limiting environmental variability. The selection of sampling sites for this study was constrained by the availability of provenances represented at the PGTIS Bulkley No. 228 seed orchard (Wallis et al. 2010). To ensure that dendrochronological data was representative of the historical disease pressure experienced by these provenances it was necessary to sample as close to the provenance origin locations as possible. This spatial sampling limitation represents an unfortunate but necessary conflict with the priorities of tree ring sampling. Ideally, trees would be sampled from an undisturbed sites that provide the oldest live trees possible (to extend the chronology as far back as possible; Speer 2010), and sampling sites with previously documented outbreaks would be selected (to allow confirmation of predicted outbreaks; Welsh et al. 2009). However, the requirement to sample at the exact provenance origin sites meant that not only was disease history largely unavailable, but it was also difficult to avoid stands affected by disturbances. Provenance testing has historically been carried out to optimize wood production (Aitken et al. 2008), and it is likely that the origin sites were originally chosen for provenance trial sampling because they were conveniently accessible from forest service roads. The majority of stands sampled were affected in some way by industrial forestry; mostly subsequent to 2000 based

on tree death dates. This activity largely removed older standing lodgepole pine from sampling sites, though in many cases discs could be recovered from slash or stumps. In areas where forestry activity was less recent, samples from these slash or stumps were often not available because of decay, and chronology depth was limited at these sites as a result. The effects of mountain pine beetle (*Dendroctonus ponderosae* Hopkins) were also evident at all sampling sites, with both beetle galleries and blue staining visible in many samples. This is not unexpected based on the extent of the mountain pine beetle outbreak relative to the study area (Pederson 2003), but the decay seen in standing trees killed by MPB can limit the recovery of viable ring data (Lewis et al. 2006) and was likely a factor in the increased forestry activity observed. It may be possible to select sites based on chronology availability, but to limit costs and project length secondary metabolite quantification would have to be performed on either uneven aged trees at climatically variable sampling sites, or on immature greenhouse seedlings representing said sites. Sampling done at geographically variable sites would represent a 'constitutive plus' level of foliar secondary metabolites (Stamp 2003), where the contribution of abiotic and biotic conditions is uncontrolled. Sampling of immature seedlings may not be representative of terpene levels in mature trees (Franich et al. 1982). Neither scenario is ideal, so it is likely that future studies of this type will be similarly limited to established provenance trials.

Despite these sampling limitations, sufficient samples for chronology construction were collected at all but one site (Wentworth), and a number of predicted outbreaks were confirmed against known outbreaks from Welsh et al. (2009), indicating that these predictions were accurate. That other outbreaks predicted by this work were not reported in

forest disease surveys is more likely due to the difficulty involved in complete and accurate surveying than inaccuracy of the OUTBREAK predictions. While *D. septosporum* was first noted in British Columbia in 1963 (Parker and Collis, 1966), both dendrochronological records and population genetics studies indicate that the disease has a long history in the province (Welsh et al. 2009; Dale et al. 2011). Initial identification of the disease may have been delayed by both the long latent period of the pathogen during unfavourable weather conditions and the difficulty of isolating such a slow growing pathogen as an endophyte (Drenkhan et al. 2013). The paucity of subsequent reports on *Dothistroma* outbreaks in provincial disease surveys could be because outbreaks were not detected, or because the disease was not considered sufficiently damaging to report. In the native range of pathogens, where they have coevolved with their host, damage is often minor enough that the disease goes unnoticed (Parker and Gilbert 2004; Loo 2009; Jousimo et al. 2014; Desprez-Loustau et al. 2016). Even in severe outbreaks, the bottom up defoliation that is characteristic of *Dothistroma* may make it difficult to detect by aerial overview.

The use of a non-host species as a baseline allows host-specific growth factors to be separated from the influence of climate (Trotter et al. 2002), but requires the climate response of both species to be similar. Previous work has shown this to be true for the pine/spruce comparison used (Welsh et al. 2009), and this was confirmed in this work by visual and statistical analysis (Table 4.2). While some amount of noise is always present in the corrected index for each tree due to minor response or environment variations (Swetnam and Lynch 1989), the effect of noise from individual trees on outbreak prediction is largely eliminated by the 40% affected threshold (Welsh et al. 2009). The criteria developed by Welsh et al.

(2009) are also demonstrably effective in removing inferred outbreaks caused by other defoliators or host-nonhost response differences. The authors found that the pattern of growth reduction caused by *Dothistroma* is distinct from that of previously studied insect defoliators (Welsh et al. 2009), which normally affect only latewood production in the first year of growth loss (Harper 1913; Jardon et al. 1994; Krause and Morin 1995; Muzika and Liebhold 1999), and is unique to fungal defoliators. While *D. septosporum* is one of a number of fungal pathogens responsible for defoliation of lodgepole pine in British Columbia, other common pine defoliators do not show such a severe pattern of defoliation. In mature trees affected by *Dothistroma*, the first detectable reduction in annual increment corresponds to the spread of the disease from older to younger (and more photosynthetically active) needles (Gibson 1972). This causes an abrupt reduction in width of the first affected annual growth ring, and is the basis of the 70% threshold criteria of the OUTBREAK method used (Welsh et al. 2009). Other fungal defoliators commonly reported within the study area (*Lophodermella concolor, Lophodermella montivaga,* and *Elytroderma deformans*) infect only current year needles, and have a substantially longer lifecycle than *D. septosporum* (Laurent 1962; Childs 1971; Minter 1993; Worrall et al. 2012). These defoliators cause a gradual decrease in ring width as defoliation increases over successive years of infection, meaning that initial growth loss in outbreaks of these pathogens is unlikely to meet the OUTBREAK prediction criteria. This is illustrated by a severe outbreak of *Lophodermella* reported at Fly Hills in 1985, with over 85% of new lodgepole pine needles infected within a 50 ha outbreak area (Erickson and Ferris 1986). While some trees at the Fly Hills sampling site report an outbreak over this time period (Figure 4.2), the number does not exceed the 40% threshold. While the exact extent of the *Lophodermella* outbreak was not reported, this does suggest that the 40% threshold is

successful at eliminating weaker alternative defoliation sources.

Inferred outbreaks were often highly synchronous between sites, as reported by Welsh et al. (2009). This has been attributed to synchronicity of exogenous forces between sites, namely climate, through the Moran effect (Moran 1953; Myers 1998; Welsh et al. 2009). The outbreak centred on 1980 shows the greatest site synchronicity in our data and affected the most trees (Figure 4.2). More recent outbreaks (centred on 2000) show a similar extent, but lesser severity. This is contrary to results by Welsh et al. (2009), who show the latter outbreak having a greater extent and link increasing outbreak severity to an increasingly conducive climate. It is possible that severity of recent inferred outbreaks may be underreported, as only surviving trees are represented in the dendrochronological record (Swetnam 1989). However, as mature tree mortality from *Dothistroma* is thought to be historically rare in British Columbia, the effect on disease history measurements is likely to be negligible (Woods et al. 2003). Instead, this difference is more likely to be explained by the difference in study area. Welsh et al. (2009) predominantly sampled in northwestern British Columbia, where summer precipitation has increased, positively affecting outbreak synchronicity (Woods et al. 2005; Welsh et al. 2014). By contrast, this study includes areas in the interior of B.C. where precipitation may have decreased over the study period (Hamann and Wang 2006), likely decreasing synchronicity.

While significant correlation with defence associated variables (crown retention and disease history) in two separate studies strongly suggests that foliar terpenes play an important role in the *Dothistroma-Pinus* interaction, a great deal more work is needed before we can fully understand the nature of this relationship and its place in quantitative resistance. While some

single gene resistance systems have been well characterized, in most cases resistance is complex, quantitative, and poorly understood (Neale and Kremer 2011). Field based studies on naturally variable provenance genotypes cannot easily eliminate confounding variables. To do this would require resistance trials of deletion mutants for specific genotypes (Schwelm et al. 2009), or study of the effects of specific compounds *in vitro* (Franich and Gadgil 1983). However, these avenues of investigation will require in-depth knowledge of the genetics underlying biosynthesis of these compounds in lodgepole pine and authentic analytical standards. The latter are largely unavailable for sesquiterpene alcohols, and while knowledge of conifer secondary metabolite synthesis has been greatly expanded by work on insect herbivores of conifers (Huber and Bohlmann, 2005; Zulak and Bohlmann, 2010) and the sequencing of conifer genomes (Zimin et al. 2014; Stevens et al. 2016), the likely numerous enzymes responsible for sesquiterpene alcohol biosynthesis are still largely unidentified and uncharacterized. While the genetics underlying monoterpene biosynthesis are better understood (Keeling and Bohlmann, 2006; Vranova et al. 2013), and analytical standards of these compounds are readily available, the link between monoterpenes and *Dothistroma* resistance is more tenuous than that for sesquiterpenes and sesquiterpene alcohols. While this work showed broadly significant positive correlations between monoterpene levels and disease history at provenance origin, Chapter 2 showed no significant correlation of levels of any monoterpenes with crown retention.

In spite of these challenges, furthering knowledge of lodgepole pine resistance to *Dothistroma* is necessary based on its practical importance to forestry. Approximately 30% of emergent plant diseases are caused by fungi (Anderson 2004). Where these pathogens are native

they often go unnoticed prior to their emergence, with initial knowledge often coming from outside of their natural range (Desprez-Loustau et al. 2016). This is clearly the case for *Dothistroma septosporum* in British Columbia, where knowledge of the host-pathogen relationship in lodgepole pine is lacking due to the historically benign effects from the disease (Welsh et al. 2009). While diseases can persist at a moderate level for long periods of time in the absence of exogenous disturbances (May and Anderson, 1983), many areas of B.C. are rapidly moving to temperature and precipitation regimes that are conducive to disease development (Woods et al. 2005; Hamann and Wang, 2006). This change is mirrored by the changes that have occurred in forest species composition, brought about by industrial forestry practices which commonly favour more commercially valuable tree species such as lodgepole pine. This preference necessarily decreases species diversity, while increasing density of host trees above historical levels (Ennos 2015). Increased density directly affects disease severity based on spore dispersal mechanics (Boateng and Lewis 2015), but also leads to outbreaks in hosts where low density has previously suppressed resistance development (Ennos 2015). As an emergent disease, *Dothistroma* has a high potential for damage (McCulloch and Woods, 2009), both physically and economically (Watt et al. 2011). Northwestern B.C. has been seen the most widespread reporting of *Dothistroma* emergence, but global ecoclimatic suitability modelling also identifies regions in eastern and southwestern B.C. as optimal for disease occurrence (Watt et al. 2009). While future work could be done to identify areas at risk of disease emergence using interpolated predictive climate models (Wang et al. 2016), a better understanding of the basis of quantitative *Dothistroma* resistance is required for ongoing management and control of the pathogen.

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5. Synthesis

The overall goal of my research was to investigate the contribution of foliar secondary metabolites to resistance of lodgepole pine (*Pinus contorta* var. *latifolia*) against *Dothistroma septosporum* (Dorog.) Morelet, with a focus on terpenes. The emergent fungal pathogen *D. septosporum* has a widespread distribution (Watt et al. 2009), and a high potential for damage in British Columbia (McCulloch and Woods 2009). The predominant host of the disease in this province is lodgepole pine, a species that has been widely planted due to its wide ecological amplitude and commercial value in British Columbia and other jurisdictions (Lotan And Critchfield 1990). It is considered highly susceptible to the *D. septosporum* (Watt et al. 2009). Host density has contributed to the increased severity and extent of recent outbreaks in northwestern B.C., but the predominant driver has been an increasingly conducive climate for disease development (Woods et al. 2005; Welsh et al. 2014). Current options for disease management in the province are limited to avoidance and species diversification, but resistant lodgepole pine varieties will play an important role in management of the disease going forward (McCulloch and Woods 2009; Bulman et al. 2016). While *Dothistroma* resistance has been identified in pine species such as *Pinus radiata* D. Don (Carson 1989) and lodgepole pine (Ukrainetz et al. 2013), the mechanistic basis of this resistance is not well understood. Resistance is thought to be both quantitative and multifactorial (Carson 1989), the cumulative result of contributions from a number of plant defence mechanisms (Fraser et al. 2015). However, conclusively demonstrating that these mechanisms contribute to resistance is challenging (Gershenzon and Dudareva 2007), and consequently the physiological basis of observed *Dothistroma* resistance is relatively

unknown. A comprehensive knowledge of the interactions between *D. septosporum* and its pine host is required to fully understand the forces driving emergence of this damaging foliar disease (Welsh et al. 2009). My work shows that a number of terpene compounds are active in *Dothistroma* resistance, and provides a basis for further study into terpenes as constitutive chemical defences against *D. septosporum*.

Previous studies have implicated terpenes in the resistance of pines to foliar fungal pathogens (Franich et al. 1982; Wallis et al. 2010; Wallis et al. 2011), and my research strongly suggests that these compounds also play a role in resistance to *D. septosporum*. Wallis et al. (2011) found that in naturally occurring lodgepole pine stands, foliar constitutive levels of monoterpenes and sesquiterpenes were positively correlated with 1961-1990 temperature normals. The authors suggested that this may be due to either the longer growing season at warmer sites allowing trees to accumulate greater levels of secondary metabolites or warmer sites being more conducive to pests and pathogens that the tree must defend against. By contrast, I identified strong negative correlations between levels of a wide range of secondary metabolites in the foliage of plantation lodgepole pine (including monoterpenes and sesquiterpenes) and 1961-1990 temperature normals at provenance origin (Chapter 1 of this thesis). As constitutive terpene levels are under strong genetic control (Hanover 1966; Hamilton et al. 2001), this suggests that the genetics of lodgepole pines from colder provenances may be optimized for faster production of terpenes relative to trees from warmer provenances with consequently longer growing seasons. This could pose a problem for assisted migration of the species, as the results of my other thesis chapters and previous work suggests that constitutive secondary metabolite levels are important for resistance to foliar

fungal diseases such as *D. septosporum* (Wallis et al. 2010; Chapters 2 and 4 of this thesis). If lodgepole pine species from warmer climates are migrated to sites with climates that are currently colder and have growing seasons that are shorter, they may be unable to produce adequate levels of defensive foliar secondary metabolites, leaving them susceptible to attack by these pathogens. The potential for damage in this scenario is highly dependent on the relative contribution of these defence mechanisms to quantitative resistance in migrated trees, and consequently a more comprehensive knowledge of the interaction between host and pathogen is required to make any damage predictions. Future work could involve direct quantification of growing season length at provenance origin sites to conclusively determine the effect of this variable on constitutive secondary metabolism, but further and more general investigation into the contribution of pine defences to *Dothistroma* resistance is needed to predict the future impact of the disease in British Columbia.

My quantitative analysis of foliar secondary metabolites in resistance trial lodgepole pine suggests that sesquiterpenes and sesquiterpene alcohols play a role in observed resistance to *D. septosporum*, but little correlation was seen with monoterpene levels (Chapter 2 of this thesis). I found that a wide range of volatile foliar secondary metabolites correlated with crown retention variables, indicating possible involvement in resistance, but the relative retention times of these compounds suggested that few were monoterpenes. Compounds selected for further analysis were tentatively identified as either sesquiterpenes or sesquiterpene alcohols. There is a strong physiological basis for sesquiterpene alcohols contributing to fungal pathogen resistance, as these compounds are often inhibitory *in vitro* (Bakkali et al. 2005; Semighini et al. 2006; Semighini et al. 2008), but there is little

published literature on fungal growth inhibition by sesquiterpenes. This could indicate that sesquiterpenes linked to resistance are precursors to defensively active sesquiterpene alcohols, but investigation of this relationship will require development of a more detailed knowledge of the biosynthesis of a variety of sesquiterpene alcohols in conifers.

Two resistance correlated compounds - α-cadinene (a sesquiterpene), and an isomer of farnesol (a sesquiterpene alcohol) - were also positively correlated with dendrochronologically determined *Dothistroma* outbreak history (Chapter 4 of this thesis). Lodgepole pine provenances coevolved with *D. septosporum* would be expected to produce defensive compounds active against the pathogen. These constitutive secondary metabolite defences are metabolically expensive to produce and maintain (Gershenzon 1994), and it is unlikely that trees would persist in producing high levels of these compounds without selective pressure to do so. While monoterpene levels were not associated with defence in Chapter 2 of this thesis, they were broadly positively correlated with outbreak history (Chapter 4 of this thesis). This is consistent with previous work by Wallis et al. (2010), which linked monoterpene levels to resistance against the foliar fungi *Lophodermella concolor* (Dearn.) Darker, *Lophodermella montivaga* Petre., and *Elytroderma deformans* (Wier) Darker. Correlation with disease history in Chapter 4 of this thesis suggests that these compounds also act as a defence against *D. septosporum*, which is supported by the *in vitro* inhibitory effects of monoterpenes on a wide range of fungi (Bakkali et al. 2008). That monoterpenes had little association with crown retention in resistance trials (Chapter 2 of this thesis) could simply indicate that monoterpene defences only play a small role the multifactorial resistance of the families tested. Further investigation of the role of these

compounds in the host-pathogen interactions could involve *in vitro* testing their effects on the growth of *Dothistroma*. Monoterpene standards have been shown to inhibit germination of *Dothistroma* spores, and steam distilled extracts of *P. radiata* containing monoterpenes can inhibit *Dothistroma* growth at high concentrations in media (Franich et al. 1982). I found that monoterpenes can inhibit growth of *Dothistroma* at high concentrations *in vitro* (unpublished results), but testing with a wider range of compounds and concentrations may shed light on their defensive contribution.

I carried out *in vitro* growth assays on *D. septosporum* in Chapter 3 of this thesis using (E,E)-farnesol, and found that this compound was either inhibitory or stimulatory to fungal growth depending on concentration (high and low, respectively). This result is similar to that obtained by Franich et al. (1982), who also observed growth stimulation with assays using low concentrations of terpene containing *P. radiata* extracts. The physiological basis of this stimulatory effect on growth of *D. septosporum* is uncertain, but could be related to transition of the fungus between biotrophic and necrotrophic growth stages or chemotaxis towards stomata during infection. Testing the former hypotheses could involve assessing the transcriptome of the fungus when exposed to (E,E)-farnesol, as changes in the lifecycle of stage of *D. septosporum in planta* has been associated with its expression profile (Bradshaw et al. 2016), and chemotactic effects of the compound could be tested using a chemotaxis chamber assay (Morris et al. 1998).

While the isomer of farnesol putatively identified in Chapter 2 (Unknown 2.6) was shown to not be (E,E)-farnesol, the chemical similarity of these compounds makes it possible that they have similar effects. Alternatively, interactions may be isomer-specific, as seen with quorum

sensing effects of *Candida albicans* (Shchepin et al. 2003). This uncertainty can only be resolved by testing authentic standards of the four farnesol isomers, but these are currently unavailable in sufficient quantities for inhibition testing, meaning further work on this compound is likely to involve synthetic chemistry. This is also true for identification and analysis of other currently unidentified compounds associated with *Dothistroma* resistance in lodgepole pine (Chapter 2 of this thesis) or provenance outbreak history (Chapter 4 of this thesis).

My work has demonstrated a significant link between constitutive levels of foliar terpenes and resistance of lodgepole pine to *Dothistroma septosporum*. Foliar terpenes have been positively correlated with crown retention variables in a *Dothistroma* resistance trial and dendrochronologically determined *Dothistroma* outbreak history at a provenance level, strongly suggesting that these compounds play a defensive role in the host-pathogen interaction. While *in vitro* testing suggests that the relationship between terpene levels and resistance is complex, this work provides a basis for further *in vitro* investigation of mechanisms underlying terpene defences. A number of significant knowledge gaps remain in this relationship (Fraser et al. 2015). As yet unidentified foliar compounds have been correlated with resistance and disease history; future work should involve identification of these chemicals and assessment of their effects on *Dothistroma in vitro*. Genetically determined constitutive terpene levels have also been linked to historical climate at provenance origin, which has implications for assisted migration strategies in a changing climate, but a more complete knowledge of the host-pathogen relationship is needed before useful predictions can be made. While this knowledge is still lacking, my work in this thesis

represents a useful step forward in our understanding of the *Dothistroma-Pinus* pathosystem.

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