Production of defensive metabolites by *Pinus patula* X *Pinus tecunumanii* hybrids in response to *Fusarium circinatum* infection

by

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Submitted in partial fulfilment of the requirements for the degree of

Magister Scientiae

Microbiology

in the

Faculty of Natural & Agricultural Sciences

Department of Biochemistry, Genetics, and Microbiology

UNIVERSITY OF PRETORIA

27 May 2022

SUMMARY

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The fungal pathogen *Fusarium circinatum,* causal agent of pitch canker disease, is currently one of the biggest threats to pine health worldwide. Symptomatic infection is associated with high mortality rates and reduced growth volume, resulting in significant annual losses for the forestry industry. Pines respond to insect damage and fungal infection by forming traumatic resin ducts, as well as significantly upregulating the production of defence compounds. These phytochemicals include terpenes, the main chemical constituents of pine resin, and phenolics, produced in specialized cells of the secondary phloem. Many of the compounds belonging to these two phytochemical groups are known to have inhibitory or lethal effects on pine pests and pathogens.

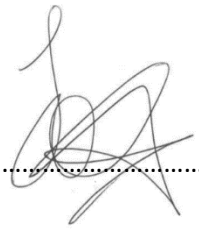
Although most *Pinus* species are susceptible to *F. circinatum* infection, there is significant variation in susceptibility to this pathogen among the different species and their interspecific hybrids. Resistance of a species to the pitch canker fungus is a major determining factor in its value to the pine industry, however, the underlying mechanisms of this phenomenon are poorly understood. Research in the role of phytochemicals in pine defence against infections by *F. circinatum* could aid in breeding resistant trees for commercial exploitation.

Therefore, the aim of this MSc study was to explore chemical defence response of susceptible and resistant *Pinus* hybrid crosses after inoculation with *F. circinatum*. Gas- and liquid-chromatography coupled to mass-spectrometry were used to characterize the phytochemical changes in young *P. patula* X *P. tecunumanii* hybrid clones in response to *F. circinatum* infection. A significant increase was observed in terpene and phenolic production in infected saplings between five and 14 days post-inoculation, compared to mechanically wounded plants. However, more resistant hybrid plants with less severe disease symptoms produced significantly lower concentrations of defensive phytochemicals, both in response to wounding and to *F. circinatum* infection. These findings suggest that increased concentrations of terpenoid oleoresin and phenolics are not part of the defence strategy of pine against infection by *F. circinatum.*

Keywords: defence, phytochemical, terpene, phenolic, pine tree.

DECLARATION

I, Lenteli van Zyl, declare that the dissertation/thesis, which I hereby submit for the degree Master of Science in Microbiology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature: 

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following individuals, who have been instrumental in the completion and success of this dissertation. Thank you all kindly for your support, assistance, and guidance:

My supervisor, Dr Almuth Hammerbacher, for your tireless effort, mentorship, and understanding. Through your guidance, I have already learned and grown far more than I could have imagined. Thank you for believing in me, and for supporting and leading me through this difficult life path.

My supervisors, Dr Emma Steenkamp and Dr Felix Fru, for lending your time, expertise, and support towards the completion of my dissertation. I appreciate your efforts sincerely.

My parents, Francois van Zyl and Lucille van Zyl, for your continued emotional and financial support. Thank you for your love, and for your unwavering faith in my abilities. Thank you for giving me the future I had dreamed of for myself, even throughout the hardest times. To David Joubert, for your emotional and financial support, and for your help and kindness during difficult times.

My friend and roommate, Mandy Messal, for your advice, motivation, understanding. Thank you for being there for me in uncertain times, and for talking me through my doubts. Your mentorship and presence in my life has been invaluable.

Johannes Christoff Joubert, for your instrumental assistance in the lab and with data analysis. Without your knowledge, patience, and generosity, my dissertation would not exist.

My fellow FABIans and lab mates, especially Dineo Mailula, Ongeziwe Mbhele, Tanay Bose, and Jan Nagel, who have assisted kindly and generously with my inoculation trials, helped me find my feet around the lab, and provided me with emotional support.

The van Coller family, for all your unconditional love, admiration, and for truly believing in me and my abilities. Without your support (be it financial, emotional, or just teaching me how to change a tire) I would have been truly lost. Thank you for being my safety and my home-away-from-home when I needed it the most.

My friends: To Lara Prinsloo for your love and support, and for helping me turn my scribbled drawings into beautiful and professional looking images. Your artistic talent and generosity has helped me immensely! To Coléne Storm, for being my inspiration and teaching me to be calm, for lending me perspective, and for knowing my worth.

Research funds were provided by the TPCP (Tree Protection Cooperative Programme) and SIF (Sector-specific Innovation Fund) from the Department of Trade, Industry and Competition. Thank you to FABI (Forestry and Agricultural Biotechnology Institute) and the University of Pretoria for the continued support.

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CHAPTER 1:

Conifer defences against pathogens with specific reference to interactions between pines and the fungal pathogen, *Fusarium circinatum*

The *Pinus* genus has endured, evolved, and flourished in forest ecosystems since its first appearance in the Mesozoic era *ca.* 100 – 200 million years ago (Millar, 1993, Keeley, 2012). Pine wood is coveted globally for its affordability and broad range of uses, and commercial pine plantations are now established on almost every continent (Sabie Forestry, 2006, Norfleet Quality, 2021). In South Africa alone, it is estimated that *ca.* 1 million hectares of land is used for pine silviculture (Sabie Forestry, 2006). As an unfortunate result of the success of this resource, the worldwide distribution of pine seeds and planting stock, timber products, and soil often goes unregulated. Trade of contaminated products leads to the simultaneous distribution and introduction of pine pests and diseases into environments outside of their native ranges (Dick, 1998, Wingfield *et al.*, 2008). One such disease that has been introduced into South Africa and subsequently became a major threat to the local pine industry is the fungal pathogen *Fusarium circinatum* (Wingfield *et al.*, 2008, Mitchell *et al.*, 2011). As the causal agent of pitch canker disease, it affects the growth and wood quality of established plantation trees, causes high post-planting mortality in young plants used for plantation establishment, and severely impacts seedling emergence and root development in young nursery stock (Nirenberg & O'Donnell, 1998, Storer *et al.*, 1998, Wingfield *et al.*, 2008, Mitchell *et al.*, 2011). The pitch canker fungus has been reported from almost all pine nurseries in South Africa (Wingfield *et al.*, 2008).

Pine have evolved sophisticated mechanisms to defend themselves against organisms that infest and infect them (Franceschi *et al.*, 2005). These defence mechanisms can be broadly categorized as constitutive and induced defences, of which both can be further divided into physical and chemical defences (Franceschi *et al.*, 2005, Bonello *et al.*, 2006). Constitutive physical and chemical defences are pre-emptive – they are present in the tree throughout its normal growth and development, and serve as a first line of defence upon the initiation of infection or insect attack (Franceschi *et al.*, 2005, Bonello *et al.*, 2006). Such defences include the rigid, highly-lignified outer bark, and constitutive levels of phytochemicals in the resin and in specialized cells (Blanchette & Biggs, 1992, Phillips & Croteau, 1999, Franceschi *et al.*, 2005). Induced physical and chemical defences are activated upon the detection of damage such as insect feeding or fungal hyphal penetration (Karban & Baldwin, 1997, Franceschi *et al.*, 2005). This damage is communicated to the tree via signalling hormones, which allows reactions such as the formation of wound periderms around penetration sites and an upregulation in the production of phytochemicals to take place (Christiansen *et al.*, 1999, Krekling *et al.*, 2000, Halitschke & Baldwin, 2004, Howe, 2004, Franceschi *et al.*, 2005).

The two most important groups of secondary compounds thought to be involved in pine defence are terpenes and phenolics. Terpenes are a large group of compounds, which includes the monoterpenes, diterpenes, and sesquiterpenes. These terpenes are found in pine resin – a viscous substance produced throughout the tree to flush out and kill invading organisms (Michelozzi, 1999, Phillips & Croteau, 1999, Nagy *et al.*, 2000, Franceschi *et al.*, 2005, Krokene, 2015). Phenolics are primarily produced in specialized cells referred to as polyphenolic parenchyma cells, which make up a large proportion of the secondary phloem near the outer layers of the tree (Franceschi *et al.*, 2000,

Franceschi *et al.*, 2005). Both terpenes and phenolics are upregulated in response to damage to the tree, and several compounds belonging to these groups have notable antifungal and insecticidal properties (Krekling *et al.*, 2000, Krauze-Baranowska *et al.*, 2002, Franceschi *et al.*, 2005, Sherwood & Bonello, 2013, Keefover-Ring *et al.*, 2016).

Throughout the long history of pines on earth, environmental pressures have allowed members of this genus to speciate and expand across continents to occupy many different niches (The Plant List, 2013, GBIF Secretariat, 2021). As a result, *Pinus* species evolved to have varying levels of resistance to certain pests or pathogens such as *F. circinatum* (Viljoen *et al.*, 1995, Blakeslee & Rockwood, 1999, Hodge & Dvorak, 2007). Genetic differences are thus likely to be responsible for the relative susceptibility of species such as *Pinus patula, P. sylvestris,* and *P. radiata* to *F. circinatum* infection (Drenkhan *et al.*, 2020). Research on the defence responses in pines against *F. circinatum* infection specifically, and by extension the chemical defence response against this pathogen, is limited. For example, it is known that certain genes involved in the regulation of primary and secondary metabolism, such as those encoding enzymes in the phenylpropanoid pathway, are upregulated in response to *F. circinatum* infection (Carrasco *et al.*, 2017). However, other aspects of the defence reaction against this pathogen such as the roles of specific secondary metabolites are unknown. Through research conducted on pines and members of other conifer genera closely related to *Pinus,* it is clear that certain secondary metabolites play a significant role in defence against numerous types of fungal pathogens (Wallis *et al.*, 2008, Sherwood & Bonello, 2013, Kusumoto *et al.*, 2014). Whether defensive secondary metabolites are significantly involved in the relative resistance of certain *Pinus* species to *F. circinatum,* remains to be elucidated.

Pine-based forestry in South Africa (and many other countries globally) is increasingly threatened by the presence of *F. circinatum* in plantations and nurseries (Wingfield *et al.*, 2008). With the effects of this devastating pathogen exacerbated by the transport of contaminated goods and plant matter and the worsening impact of climate change, solutions to improve pine defence are becoming more and more essential (Anderson *et al.*, 2004, Wingfield *et al.*, 2008, Fisher *et al.*, 2012, Stenlid & Oliva, 2016). Research on natural anti-fungal defences will thus enable the improvement of tree breeding strategies and the selection of more robust and disease-resistant trees for cultivation and plantation establishment.

1.1 The genus *Pinus*

The genus arose in the Mesozoic era ca. 100 – 200 million years ago (Millar, 1993, Keeley, 2012). It belongs to the subfamily Pinoideae within the Pinaceae family, and *Pinus* has since diversified into 126 distinct species, of which 35 are still unresolved (The Plant List, 2013). Members of this genus are native to the northern hemisphere, where most species are still found today (Price *et al.*, 1998). A few species such as *P. tecunumanii* and *P. patula* have also evolved to grow in tropical regions of the southern hemisphere (Jackson *et al.*, 2022). Pine grows in a diverse range of habitats – from rainforests to semi-arid deserts and at elevations up to 5 200 meters above sea level (GBIF Secretariat, 2021).

Several species are valued commercially for their timber and wood pulp, and are grown extensively in plantations outside of their native ranges (Sabie Forestry, 2006). Some pines are also cultivated as ornamental plants or for products that can be derived from their bark and needles, as well as for the nutritional value of their seeds or 'pine nuts' (Norfleet Quality, 2021, WebMD, 2021). Apart from their economic benefits, pine forests and plantations serve as efficient carbon sequesters, release oxygen into the atmosphere, play an important role in the water cycle, and are the natural habitats for numerous species of forest-dwelling organisms (DeLucia *et al.*, 1999, Furtado, 2016, Nix, 2017)

Pine trees have relatively long lifespans compared to other plants, ranging from 20 years to more than 4,800 years (Loehle, 1988, Rocky Mountain Tree-Ring Research, 2021). Pines therefore face many adverse conditions during their lives, such as damage by insects and microbial pathogens, as well as prolonged drought stress, poor soil quality, and natural phenomena such as storms and earthquakes (Turtola *et al.*, 2003, Franceschi *et al.*, 2005, Ormeño *et al.*, 2008). However, this resilient genus still dominates some forest landscapes today, due in part to their highly effective defence strategies against biotic and abiotic stresses.

1.1.2 Pests and pathogens of pine trees

There are several biotic challenges in the form of pests and pathogens that threaten pine health and the global pine industry. Some *Pinus* species are more susceptible to certain pests and pathogens, while others are more resistant (Rockwood *et al.*, 1988, Viljoen *et al.*, 1995, Hodge & Dvorak, 2007, Wingfield *et al.*, 2008, Drenkhan *et al.*, 2020). Consequently, the prevalence of pine pests and pathogens in an area depends on the species of pine, the endemism of a pest or pathogen, and the prevailing environmental conditions (Anderson *et al.*, 2004, Wingfield *et al.*, 2008, Stenlid & Oliva, 2016). Native pine forests and non-native pine plantations alike are susceptible to damage by these pests and pathogens.

Some of the most devastating threats to global pine health are fungal pathogens. For example, infection by *F. circinatum*, the causal agent of pitch canker disease, can result in seedling losses in nurseries, newly established pine plantations and adult trees (Wingfield *et al.*, 2008, Mitchell *et al.*, 2011, Gordon *et al.*, 2015, Fru *et al.*, 2019). This pathogen is believed to have originated in lower North America, likely Mexico, and has subsequently spread via international trade of seed and wood products to numerous other countries across the globe (O'Donnell *et al.*, 1998, Wikler & Gordon, 2000, Wingfield *et al.*, 2008, Kvas *et al.*, 2009, Drenkhan *et al.*, 2020). Today, *F. circinatum* can be found in South Africa, as well as in several countries in Europe, the Americas, and Asia (Supplemental Figure 1) (Wingfield *et al.*, 2008, Drenkhan *et al.*, 2020).

Another canker-causing fungal pathogen, *Cronartium ribicola*, causes white pine blister rust in *Pinus strobus* and other five-needle pines (Centre for Agriculture and Bioscience International, 2019). Native to central- and eastern Eurasia, this pathogen was initially introduced to North America in the early 20th century. Now, *C. ribicola* causes major damage in most regions where white pines are grown commercially (Supplemental Figure 1) (Kinloch Jr, 2003, Centre for Agriculture and Bioscience International, 2019). White pine blister rust also occurs in Europe, although some

European and Asian white pines are more resistant to the pathogen (Bingham, 1972, Heimburger, 1972, Centre for Agriculture and Bioscience International, 2019).

Fusiform rust, caused by the fungal pathogen *Cronartium quercuum* f. sp. *fusiforme,* has also become a problem in the southern United States since the mid-20th century and is now one of the most economically important pine diseases in this region (United States Department of Agriculture, 2014, Enebak, 2019). Fusiform rust severely affects *P. elliottii* and *P. taeda*, although all southern pines are susceptible to infection by this pathogen (Supplemental Figure 1) (United States Department of Agriculture, 2014, Enebak, 2019).

Fungal pathogens that cause needle- and tip blight also pose serious threats to pine tree health in several parts of the world. For example, *Pinus nigra* and *P. ponderosa* are severely affected by Dothistroma needle blight, caused by the fungal pathogens *Dothistroma pini* and *D. septosporum* (Barnes *et al.*, 2004, Research, 2021). Also known as red band needle blight, this disease is one of the most important foliar diseases of pine trees in the world (Barnes *et al.*, 2004, Watt *et al.*, 2009). Although originating in the high-altitude rain forests of South America, high levels of infection now occur in various areas of the northern hemisphere in Asia and Europe. This disease has been shown to occur on 95 different *Pinus* species or subspecies, spanning across 76 countries and a wide range of climatic conditions and geographic locations (Supplemental Figure 1) (Barnes *et al.*, 2004, Drenkhan *et al.*, 2016, Woods *et al.*, 2016). Due to the particular susceptibility of *P. nigra*, these trees are no longer planted commercially in the United Kingdom (Research, 2021). For similar reasons, *P. radiata* plantations are no longer established in East African countries (Gibson, 1974, Watt *et al.*, 2009).

Non-fungal biotic threats to pine health are also a serious concern globally. One example is the causal agent of pine wilt disease. Although native to North America, this disease was first observed in the early 20th century in Japan (Mamiya, 1988, Proença *et al.*, 2017). More than 60 years later, in 1971, the pinewood nematode (*Bursaphelenchus xylophilus*) was confirmed to be the causal agent of this devastating disease (Yano, 1913, Kiyohara & Tokushige, 1971, Mamiya, 1988). Since its discovery, *B. xylophilus* has spread to pine plantations in China, Korea, and Taiwan in the 1980s, and thereafter to several parts of Europe, and has more recently been reported in Mexico and Nigeria (Supplemental Figure 1) (Mamiya, 1988, Khan & Gbadegesin, 1991, Dwinell, 1993, Mota *et al.*, 1999, Proença *et al.*, 2017). Pinewood nematodes are transmitted between pine trees through insect vectors, such as pine sawyer beetles from the genus *Monochamus* (Mamiya & Enda, 1972, Linit, 1988, Evans *et al.*, 1996, Naves *et al.*, 2001, Proença *et al.*, 2017). The pinewood nematode most commonly infects *P. sylvestris*, *P. mugo*, *P. banksiana*, *P. resinosa* and *P. nigra* (Donald *et al.*, 2003).

In North America, mountain pine beetles (*Dendroctonus ponderosae*) and southern pine beetles (*D. frontalis*) are major pests and have destroyed millions of hectares of *P. contorta* and *P. ponderosa* forests over the last few decades (DiGuistini *et al.*, 2011, Erbilgin *et al.*, 2014). Among the mycobiota associated with mountain pine beetles, the pathogenic fungus *Grosmannia clavigera* is a critical component contributing to the destructiveness of these forest pests (Lee *et al.*, 2006, DiGuistini *et al.*, 2011). These beetles also attack *Pinus contorta*, *P. banksiana*, and numerous other pine species and their hybrids (Supplemental Figure 1) (Erbilgin *et al.*, 2014).

This introduction of harmful forest pests and pathogens into new regions represents one of the greatest concerns for pine-based forestry worldwide (Stenlid & Oliva, 2016). In South Africa, introduced pests such as the Deodar weevil (*Pissodes nemorensis*) and the Sirex woodwasp (*Sirex noctilio*) and introduced pathogens such as *Diplodia pinea, F. circinatum,* and *Dothistroma* sp. cause widespread tree mortality (Gebeyehu & Wingfield, 2003, Wingfield *et al.*, 2008, Watt *et al.*, 2009, Bihon *et al.*, 2011, Hurley *et al.*, 2012). The amount of damage to plantations caused specifically by such invasive tree pathogens has increased dramatically over the past century, mostly due to globalization, the trade of goods across geographical borders, and climate change (Fisher *et al.*, 2012, Santini *et al.*, 2013, Trumbore *et al.*, 2015, Stenlid & Oliva, 2016). Contaminated plant material, soil, and lumber products are introduced to new countries mainly due to unregulated trade (Dick, 1998, Wingfield *et al.*, 2008) and a changing climate enables pests and pathogens to migrate into new areas in which they were previously unable to survive due to inhospitable climatic conditions (Agrios, 1988, Anderson *et al.*, 2004). Climate change in the geographical ranges where pines are native can also weaken tree defences against native pests and diseases (Anderson *et al.*, 2004).

The influx of harmful pests and pathogens is of great concern to commercial growers – especially those in the southern hemisphere, where all pine plantations are non-native and are therefore at risk of suffering greatly from introduced pests and pathogens (Anderson *et al.*, 2004, Stenlid & Oliva, 2016). This is particularly concerning, as most of the varieties and species grown in these regions have been selected for their fast growth and timber quality, rather than for their resistance to pests and diseases (Liebhold *et al.*, 2017). Pine plantations also typically consist of only one species of pine, and these large monocultures suffer an increased risk of infestation and infection from native pests and pathogens present in nearby natural forests (Branco *et al.*, 2015, Wingfield *et al.*, 2015). Therefore, research on pine resistance to introduced and native pests and pathogens alike is important in order to preserve these species for use in future plantation forestry.

1.2 Anatomical features of pines – important defences against insects and pathogens

To protect the nutrient-rich tissues of their needles, branches and stems against pest and pathogen attack, pines have evolved sophisticated anatomical defence mechanisms. Certain anatomical structures and cells in different tissues of the tree are responsible for the production of defensive antimicrobial and insecticidal chemicals. The defence strategies used by pine trees therefore rely largely on the physical durability of these anatomical structures (Figure 1).

The outer layer of the pine stem, or the tree bark, consists of layers of protective periderm and secondary phloem (Franceschi *et al.*, 2000)(Figure 1). The periderm, forming the first line of defence of the stem against biotic and abiotic threats, consists of a bifacial meristem called cork cambium (or phellogen) (Ragni & Greb, Campilho *et al.*, 2020). The cork cambium gives rise to cork (phellem) on the outer side of the tree, and cork parenchyma (phelloderm) towards the inside. Polymeric macromolecules such as suberin accumulate in the layers of phellem, forming a hydrophobic barrier against attacking organisms (Campilho *et al.*, 2020).

A second layer of bifacial meristematic tissue, the vascular cambium, lies underneath the cork cambium. The vascular cambium is responsible for the growth of the secondary phloem outwards, and secondary xylem inwards (Li, 2011)(Figure 1). The secondary phloem tissue, thus located directly between the vascular cambium and cork parenchyma, contains phloem parenchyma cells involved the synthesis of defence chemicals (Cheniclet *et al.*, 1988, Franceschi *et al.*, 1998). This tissue also contains sieve cells responsible for the transport of photosynthates throughout the tree (van Bel *et al.*, 2002, Franceschi *et al.*, 2005).

The secondary xylem makes up the woody tissue of the tree (Li, 2011). The innermost wood is referred to as heartwood, with the lighter, newer sapwood forming the region closest to the vascular cambium. While xylem cells initially remain alive after forming, they eventually die and lose their cellular contents to become highly lignified transporter cells, responsible for moving water and nutrients throughout the tree (Franceschi *et al.*, 2005, Li, 2011).

Resin-producing and storing structures, known as resin ducts, are crucial components of pine anatomy. They are found in the needles, branches, xylem, and secondary phloem of the stem (Christiansen *et al.*, 1987, Franceschi *et al.*, 2005). These resin ducts transport phytochemicals through the tissues of the tree, thus enabling one of the most crucial components of pine defence against attacking pests and pathogens (Franceschi *et al.*, 2005).

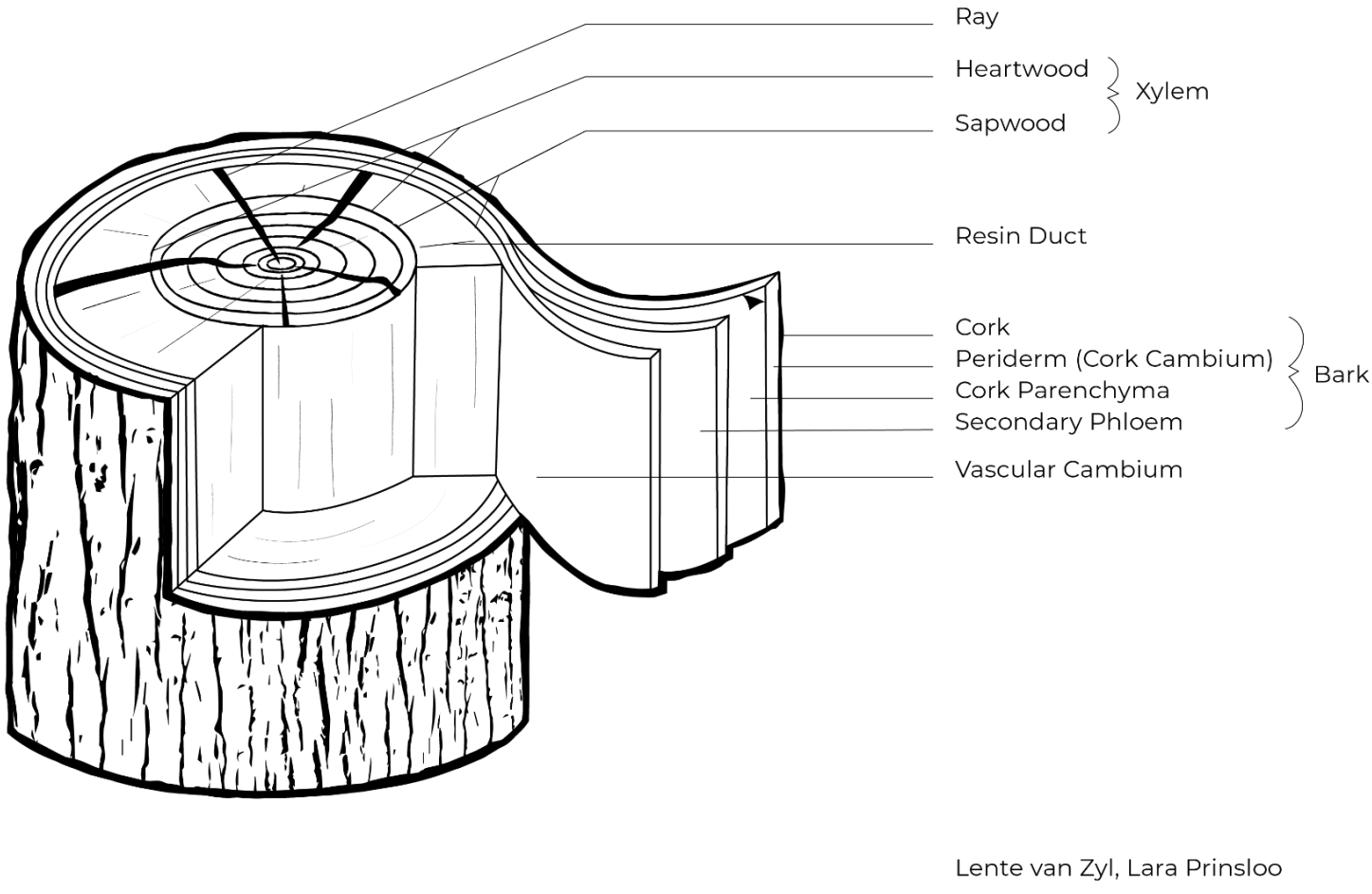


Figure 1. Anatomical structures of the pine stem and woody tissues

1.3 General defences of pines in response to biotic attack

Pine trees employ a multifaceted defence strategy against attack by pests and fungal pathogens, using constitutive and inducible defences. These strategies allow conifers, including pines, to physically and chemically deter, kill, inhibit, or exclude invading pests and pathogens (Franceschi *et al.*, 2005, Schmidt *et al.*, 2005, Wallis *et al.*, 2008).

1.3.1 The roles of constitutive defence, induced defence, resistance, and hormone signalling in conifers

Pines use constitutive defence strategies, which are present in its tissue before any pest or pathogen attempts infection or infestation, and act to repel or inhibit attackers during the early phases of attack (Franceschi *et al.*, 2005, Bonello *et al.*, 2006, Keeling & Bohlmann, 2006, Wallis *et al.*, 2008). The constitutive defence system is made up of pre-existing physical and chemical barriers, which typically defend the tree against a wide range of potential pest or pathogen invaders (Table 1).

Failure of the tree's constitutive defence to ward off pest and pathogen attack can cause the activation of a second set of defence mechanisms (Bonello *et al.*, 2006). These defence mechanisms are induced upon detection of a new threat, allowing the tree to react directly to the pathogen or pest's attack (Hammerschmidt & Nicholson, 1999, Bonello *et al.*, 2006). There are two main classifications of inducible plant defences: local induced resistance (LIR) and systemic induced resistance (SIR). LIR occurs in the tissues around the site of the initial infection or infestation, and allows the tree to limit the damage or proliferation of the pest or pathogen to a small area surrounding the site of attack. SIR is a type of increased resistance that the tree develops in response to an infection or infestation, and is somewhat analogous to the immune system of animals. SIR becomes systemic throughout the tree, allowing it to respond more readily and effectively against future attacks by the same pest or pathogen (Bonello *et al.*, 2006, Wallis *et al.*, 2008). SIR enables an increase in the production of phytochemicals such as terpenes and phenolics. It is also linked to the formation of traumatic resin ducts and an upregulation in resin flow, increased lignin deposition, and the accumulation of defensive phenolic compounds (Bonello & Blodgett, 2003, Blodgett *et al.*, 2007).

In conifers, the formation of physical defences and the biosynthesis of phytochemicals such as phenolics and terpenes occurs in response to information relayed by the signalling molecules that are produced when the tree suffers damage (Halitschke & Baldwin, 2004, Howe, 2004). These signalling molecules are primarily plant hormones such as jasmonates, salicylic acid, abscisic acid, and ethylene. Methyl jasmonate (MeJA), jasmonic acid (JA), and its isoleucine conjugate (JA-Ile) activate plant defence mechanisms in response to pathogen attack, insect-wounding, and environmental stressors and also regulate a diverse array of other plant growth processes (Cheong & Choi, 2003). Studies conducted on the effects of MeJA showed that application of this hormone to the stems of Norway spruce (conifers closely related to members of the *Pinus* genus) results in the formation of traumatic resin ducts, activation of the epithelial cells lining pre-formed resin ducts, increased activity of phenolic-producing cells, and the induction of transcription of genes involved in the biosynthesis of chemical defences and many other defence-induced physiological changes (Martin *et al.*, 2002). Similar studies on *F. circinatum*-infected *P. patula* seedlings showed that the application of MeJA and other SIR activators resulted in a reduction in disease symptom severity (Fitza *et al.*, 2011). It has also

been shown that ethylene is partially responsible for mediating JA-dependent signal transduction (Hudgins & Franceschi, 2004, Keeling & Bohlmann, 2006). Jasmonates and ethylene thus play a crucial role in driving the LIR and SIR responses in conifers, by relaying short- and long-range signals within a tree after an attack has occurred and eliciting appropriate defence mechanisms. The roles of other defence-related plant hormones, such as salicylic acid and abscisic acid, have not been extensively studied in conifers. However, salicylic acid likely assists in triggering the pine immune response, while abscisic acid could play a role in stress tolerance under water deficiency (Pashkovskiy *et al.*, 2019, Ding & Ding, 2020).

1.3.2 Constitutive and induced physical defences

1.3.2.1 Constitutive physical defences

Constitutive physical defences protect the tree by preventing pests and pathogens from penetrating its nutrient-rich tissues (Franceschi *et al.*, 2005). The cortex, which is produced during primary development of the stem, is an important physical barrier in the early stages of tree development (Table 1; Figure 1). The defensive function of the cortex is replaced by that of the secondary phloem as phloem layers are laid down through the years, and the cortex is eventually crushed or shed as development progresses (Franceschi *et al.*, 2005).

In mature trees, the outermost layer of the periderm serves as the first physical barrier against pests and pathogens (Table 1; Figure 1) (Franceschi *et al.*, 2005). The phellem of the periderm is made up of mostly dead cells with lignified or suberized walls (Franceschi *et al.*, 2005). Lignin, a rigid organic polymer, lends thickness and toughness to these cells, allowing them to serve as a solid, impenetrable barrier (Blanchette & Biggs, 1992, Bonello *et al.*, 2006).

The secondary phloem is a key site in pines for physical defence mechanisms (Table 1; Figure 1). Polyphenolic parenchyma cells (or 'PP cells') are the most abundant living component of the secondary phloem of pines, and are primarily involved in chemical defence due to their synthesis and accumulation of phenolics (Franceschi *et al.*, 2005). PP cells also have thickened cell walls and occur in multiple compact layers in the secondary phloem, and therefore provide a thick physical barrier to prevent penetration of the bark (Franceschi *et al.*, 2000, Franceschi *et al.*, 2005). Additionally, in members of the Pinaceae, calcium oxalate crystals occur as intracellular deposits within the phenolic bodies of specialized PP cells (Hudgins *et al.*, 2003, Franceschi *et al.*, 2005). Once mature, the cell walls of these specialized PP cells contain a suberin layer and are dead, unlike typical PP cells (Franceschi *et al.*, 2005). Calcium oxalate crystals might also be found next to the vacuoles of regular, unspecialized parenchyma cells (Franceschi *et al.*, 1998, Krokene, 2015). The physical toughness of calcium oxalate crystals points to their role in the deterrence of bark-boring insects and chewing herbivores and are thought unlikely to play a role in anti-fungal defence due to their chemical inertness (Hudgins *et al.*, 2003, Franceschi *et al.*, 2005).

The secondary phloem is also partially composed of sclerenchyma tissue, which is composed of cells with lignified secondary wall thickenings. These lignified sclerenchyma cells can also exist as large, irregularly-shaped sclereids or 'stone cells' (Franceschi *et al.*, 2005). Sclereids can occur scattered throughout the secondary phloem as

individual cells, or in large cell-clusters, and are important in protecting against bark-boring organisms through their physical hardness (Wainhouse *et al.*, 1998, Franceschi *et al.*, 2005).

Also contributing to the impenetrable barrier of the secondary phloem are layers of dead sieve cells. These dead cells are no longer capable of assimilate translocation throughout the secondary phloem (Franceschi *et al.*, 2000, Franceschi *et al.*, 2005). They collapse progressively over time under the pressure of newly developing cells, and provide good physical resistance against fungal hyphal penetration and chewing insects (Franceschi *et al.*, 1998, Franceschi *et al.*, 2000, Franceschi *et al.*, 2005).

Resin producing and storing structures are important defence structures not only in pines, but in conifers in general. These structures include resin ducts in the bark and woody tissue of the genera *Larix, Picea* and *Pinus,* and less-complex resin blisters in *Abies, Cedrus,* and *Tsuga* (Franceschi *et al.*, 2005, Bonello *et al.*, 2006). Resin, or 'oleoresin', is a viscous substance that serves important functions in both physical and chemical defence (Phillips & Croteau, 1999). In pines, resin ducts are primarily differentiated based on their orientation in the plant tissue: axial ducts, commonly found in the sapwood xylem; and radial ducts, occurring in the secondary phloem (Table 1; Figure 1) (Krokene, 2015). In young pines, cortical resin ducts in the outermost layer of bark are important in constitutive defence against invading insects (Franceschi *et al.*, 2005, Krokene, 2015). Mature pines also have cortical resin ducts, which can be found in the inner, central, and outer parts of the cortex (Wu & Hu, 1997, Lieutier *et al.*, 1999, Krokene & Nagy, 2012). Resin ducts are lined with plastid-rich epithelial cells, responsible for the synthesis and subsequent secretion of resin into the extracellular lumen where it accumulates under pressure (Charon *et al.*, 1987, Gershenzon & Croteau, 1990, Nagy *et al.*, 2000, Franceschi *et al.*, 2005). Upon damage to the outer layers of the stem, the pressurized resin flows from the axial resin ducts towards the radial ducts and is released to repel, flush out, or kill invading insects or pathogens (Nagy *et al.*, 2000, Franceschi *et al.*, 2005, Krokene, 2015). Non-volatile components in the resin polymerize and harden to seal and sterilise the site of penetration, preventing further access by any invading organisms (Franceschi *et al.*, 2005, Krokene, 2015).

1.3.2.2 Induced physical defences

Upon detection of an attacking organism, pines respond physically through the formation of callus tissue. This tissue can become imbued with phenolic compounds, or hardens through lignification and suberization (Table 2) (Franceschi *et al.*, 2005). The callus tissue acts as a barrier against attacking organisms and prevents fungal proliferation into other tissues of the tree (Franceschi *et al.*, 2005). Wound periderms serve a similar function as callus tissue (Table 2). Produced by PP cells in the secondary phloem or by callus tissue in the case of severe mechanical wounds, wound periderms are formed at the boundaries of lesions produced by fungal and insect attack (Christiansen & Kucera, 1999, Franceschi *et al.*, 2005). The damaged or infected tissue is then isolated from water and nutrient supplies, and eventually dies if not already killed by the invading pathogen. A loss of nutrients at the infection site then prevents fungal pathogens from further proliferation (Franceschi *et al.*, 2005).

In response to mechanical damage or fungal infection, PP cells also accumulate phenolic compounds a few days after attack or infection and, as a result, can swell up to four times their size (Table 2) (Franceschi *et al.*, 1998, Franceschi *et al.*, 2000, Krokene *et al.*, 2003, Franceschi *et al.*, 2005). As the PP cells swell, surrounding dead sieve cells become crushed and form a compact layer of cells. This wall of collapsed cells further prevents the growth of invading fungal pathogens, as hyphal growth can no longer occur through the empty sieve cell lumen (Franceschi *et al.*, 2000, Krokene, 2015). New PP cells develop from undifferentiated parenchyma cells in the secondary phloem roughly 15 weeks after attack or infection, most likely as a form of SIR due to the slow differentiation process of these cells (Krekling *et al.*, 2000, Krokene *et al.*, 2003, Krekling *et al.*, 2004, Krokene, 2015).

Traumatic resin ducts typically appear in the secondary phloem and xylem after 2 to 4 weeks, in the area surrounding the mechanical damage or infection point (Table 2) (Alfaro, 1995, Alfaro *et al.*, 1996, Tomlin *et al.*, 1998, McKay *et al.*, 2003, Franceschi *et al.*, 2005, López-Villamor *et al.*, 2021). In Norway spruce, traumatic resin ducts can contain up to four times as much resin as a preformed duct of similar length, and neighbouring ducts often merge to form much wider ducts (Hudgins *et al.*, 2003, Krokene *et al.*, 2008, Krokene, 2015). An increase in resin production and accumulation due to traumatic resin duct formation results in an increase in resin flow, which can flush out or kill the attacking organisms more readily (Martin *et al.*, 2002, Franceschi *et al.*, 2005, Miller *et al.*, 2005). In addition to newly formed traumatic ducts, the epithelial cells lining constitutive resin ducts can also be induced to increase their resin production in response to infection and insect attack (Krokene, 2015). In some *Pinus* species, axial resin ducts are refilled to twice their original capacity in the days following wounding of the outer tree tissues (Ruel *et al.*, 1998, Lombardero *et al.*, 2000, Krokene, 2015). Resin-soaked or hardened wounds on a tree also serve as physical barriers against new microbial or insect invaders (Franceschi *et al.*, 2005).

1.3.3 Constitutive and induced chemical defences

1.3.3.1 Constitutive chemical defences

Constitutive chemical defences are preformed chemicals that allow the tree to kill invading organisms or prevent the proliferation of an attacking fungal pathogen. The most abundant preformed chemical defences are the terpenes and phenolics.

Radial and axial resin ducts are important sites of chemical defence in mature pines (Table 1). Resin is primarily composed of compounds known as terpenes, which are important in pine defence as they are toxic or inhibitory to numerous species of pine-dwelling insects and pathogens, and occur in large quantities despite their high metabolic cost to the tree (Gershenzon, 1994, Keeling & Bohlmann, 2006, Krokene, 2015). The chemical composition of resin depends on intrinsic factors such as tree species and age, or extrinsic stressors such as drought, air pollution, nutrient availability, and fungal infection (Kainulainen *et al.*, 1993, Raffa & Smalley, 1995, Turtola *et al.*, 2002, Turtola *et al.*, 2003, Keeling & Bohlmann, 2006). Radial and axial resin ducts connect through networks of plasmodesmata, allowing for the transport of terpene precursors and other secondary metabolites through the tree, from the bark to the sapwood (Benayoun & Fahn, 1979, Fahn, 1979, Krokene, 2015).

In addition to their other properties, PP cells also contain enlarged vacuoles in which phenolic inclusion bodies are found (Table 1) (Franceschi *et al.*, 1998, Beckman, 2000, Krekling *et al.*, 2000, Franceschi *et al.*, 2005). These highly abundant cells are also responsible for the storage of starches and/or lipids, and are therefore a target for invading fungi and insects (Krekling *et al.*, 2000, Franceschi *et al.*, 2005). Defence phenolics produced within these cells are therefore likely able to both protect the individual storage cell as well as prevent fungal penetration of the surrounding tissues (Franceschi *et al.*, 2005). The cell walls of PP cells contain large amounts of plasmodesmata, which are thought to aid in the exchange of information for defence signalling (Krekling *et al.*, 2000, Franceschi *et al.*, 2005).

Conifers also produce a wide variety of different phenolics in unspecialized parenchyma cells of the roots, bark, stem, and needles (Table 1) (Franceschi *et al.*, 2005). For example, the phellem of the periderm contains stored phenolic compounds (Franceschi *et al.*, 2005). The cells making up the phelloderm of the periderm have also been found to contain phenolics such as flavan-3-ols, in some non-coniferous tree species (Feucht & Treutter, 1990). Radial rays in the xylem are responsible for the transport of phenolic compounds and their precursors to allow for the development of phenol-impregnated heartwood (Shain, 1967, Franceschi *et al.*, 2005). Finally, in young, developing trees, the cortex aids in chemical defence due to the high concentrations of phenolic compounds in the vacuoles of cortical parenchyma cells (Franceschi *et al.*, 2005).

1.3.3.2 Induced chemical defences

Inducible chemical defences are more complex than constitutive chemical defences, as the latter can only be non-selective whilst the former allows the tree to employ both specific and broad-spectrum defensive measures (Karban & Baldwin, 1997, Franceschi *et al.*, 2005). The hypersensitive response is a form of inducible chemical defence (Table 2). Upon detection of an invading pathogen, cells around the infection site will begin to produce reactive oxygen species, leading to rapid localized cell and tissue death as a means to kill and detain the invading pathogen (Bleiker & Uzunovic, 2004).

Phenolic compounds within the vacuoles of PP cells are not static throughout the cell's lifetime – cell contents have been found to change in response to induced defence signals, as well as on a seasonal basis (Krekling *et al.*, 2000). PP cells are also induced to produce larger quantities of phenolic compounds in response to defence signals after an attack (Table 2). These induced phenolics can be more toxic or more specific to the invading organism than constitutive phenolics (Brignolas *et al.*, 1995, Bonello & Blodgett, 2003, Franceschi *et al.*, 2005).

Traumatic resin ducts also produce resin with a different terpene composition compared to that of constitutively formed resin (Table 2) (Nault & Alfaro, 2001, Martin *et al.*, 2002, Miller *et al.*, 2005). The resin produced in traumatic resin ducts may be more toxic due to the changes in terpene composition. Traumatic resin ducts could be involved in SIR, allowing the tree to respond more readily to future attack by the same organism (Christiansen *et al.*, 1999, Nagy *et al.*, 2000, Nault & Alfaro, 2001, Martin *et al.*, 2002, Krokene *et al.*, 2003, Franceschi *et al.*, 2005, Miller *et al.*, 2005). Traumatic resin ducts may also increase the toxicity of the newly produced resin through the addition of phenolic compounds (Nagy *et al.*, 2000).

While non-protein chemicals such as phenolics and terpenes are some of the main inducible phytochemicals in pines, protein-based defences also play an important role in pine defence (Franceschi *et al.*, 2005, Keeling & Bohlmann, 2006). Enzymes such as chitinases can degrade fungal cell walls, and enzyme inhibitors such as proteinase inhibitors interfere with the attacking organism's ability to utilize resources obtained from the tree tissue (Franceschi *et al.*, 2005, Schaller, 2008, Eyles *et al.*, 2010). The induced production of enzymes such as laccases and peroxidases can also occur – these enzymes toughen cell walls through the promotion of lignification and the catalysis of crosslinking reactions, allowing the tree to physically inhibit the proliferation of the invading pathogen (Davis *et al.*, 2002, Franceschi *et al.*, 2005). Many inducible protein-based defences are broad-spectrum, allowing the tree to attack all manner of invading organisms, while other inducible proteins target organisms more specifically (Karban & Baldwin, 1997, Franceschi *et al.*, 2005). Research on Norway spruce trees showed that although these trees are capable of producing numerous types of chitinases, only a few selected chitinases were transcribed upon attack by a particular fungal pathogen (Hietala *et al.*, 2004, Nagy *et al.*, 2004). This indicated that these trees selectively induce their production of defensive enzymes to maximise their efficiency.

Table 1. Constitutive defences in pines and other conifers

Anatomical structure	Physical and chemical defence strategies	In pine trees	In other conifers
Cortex	Physical barrier in early stages of development. Contains resin ducts. Produced when tree is young but can remain functional for years. Is eventually crushed or shed as tree development progresses. Antifungal phenolic compounds present in vacuoles of cortical parenchyma cells and resin ducts with protective oleoresin contents.	The cortex in young pines contains axial resin ducts, serving as the primary source of resin in the stems. Mature pines also have cortical resin ducts, occurring in the inner, central, and outer parts of the cortex (Wu & Hu, 1997, Lieutier *et al.*, 1999, Krokene & Nagy, 2012). Also contains parenchyma cells filled with protective phenolics (Franceschi *et al.*, 2005).	In young conifers such as *Abies*, the cortex can have axial resin ducts (primary source of resin in young conifers) as well as parenchyma cells filled with phenolics (Franceschi *et al.*, 2005, Krokene & Nagy, 2012).
Periderm	Phellem of the periderm consists of dead cells with rigid, lignified, and suberized walls. Phellem also accumulates polymeric macromolecules such as suberin. Phenolic compounds are present within these dead cells.	The periderm of pines are characterized by layers of dead, flattened suberized cells, as well as layers of cells containing phenolic bodies (Hudgins *et al.*, 2005). Rigidity is lent to the periderm through suberin and lignin (Franceschi *et al.*, 2005, Campilho *et al.*, 2020).	The periderm of conifers such as *Picea* spp. also contains cells that are lignified, suberized, or filled with protective phenolic compounds (Franceschi *et al.*, 2005).
Secondary phloem	Polyphenolic parenchyma (PP) cells: multiple layers of PP cells in secondary phloem provide physical barriers to prevent penetration of bark. Phenolic bodies found within PP cells. Sclerenchyma tissue: composed of cells with lignified secondary wall thickenings, which serve as mechanical defence. Lignified sclerenchyma cells can exist as 'stone cells', protecting bark through their physical hardness.	PP cells have been observed in some pine species, but not studied extensively (Murmanis & Evert, 1967, Hudgins *et al.*, 2003, Hudgins *et al.*, 2005, Nagy *et al.*, 2006). Stone cells have not been studied extensively in pines but appear to be found in the outermost bark in the mature stems of at least some pine species (Sacher, 1954,	PP cells have been identified and studied extensively in conifers such as *Picea* (Franceschi *et al.*, 1998, Krekling *et al.*, 2000, Hudgins *et al.*, 2003). They have also been observed in *Larix* and *Pseudotsuga* species (Hudgins *et al.*, 2003). Sclerenchyma tissue is common in all conifers, and appear as stone cells in some genera (*Picea, Tsuga*) or rows of

	Calcium oxalate crystals: intracellular deposits within PP bodies; physical toughness deters insect and herbivore feeding. Contains radial resin ducts, and resin blisters and cells filled with protective oleoresin. Resin ducts are lined with plastid-rich epithelial cells, which synthesize and secrete terpenoid resins into an extracellular lumen. Resin in ducts and in blisters is stored under pressure and released upon damage to the tree to flush out the invading insect or pathogen. Volatile components in the resin evaporate upon exposure to the outside of the tree; non-volatile components harden to seal and sterilize the penetration site. The largest portion of resin is composed of terpenes – compounds with known antifungal properties.	Patel, 1975, Lev-Yadun & Sederoff, 2000). In pines, calcium oxalate crystals are compartmentalised as intercellular deposits within the vacuole of some phloem parenchyma cells (Hudgins *et al.*, 2003, Franceschi *et al.*, 2005). Pines have complex preformed resin ducts (Bannan, 1936). The radial resin ducts in the secondary phloem synthesize and accumulate protective resin (Franceschi *et al.*, 2005).	fibres in others (*Larix, Taxodium*) (Franceschi *et al.*, 2005, Li *et al.*, 2007). Calcium oxalate crystals are found in many different conifer families, and occur in all members of the Pinaceae. In this family, the individual crystals in cells of the phloem parenchyma are the largest of any conifer families thus far examined (Hudgins *et al.*, 2003). Non-Pinaceae conifers have little resin-based stem defences, and therefore rely more heavily on constitutive bark defences such as PP cells, sclerenchymatic fibre cells, and extracellular calcium oxalate crystals. In Pinaceae such as *Picea, Larix,* and *Pseudotsuga*, complex pre-formed radial resin ducts are present in the secondary phloem (Bannan, 1936, Franceschi *et al.*, 2005). In genera such as *Abies, Cedrus,* and *Tsuga*, preformed resin ducts do not exist in the woody tissue. These genera have multicellular resin blisters which accumulate resin under pressure and burst upon damage or wounding by an invading organism (Phillips & Croteau, 1999, Franceschi *et al.*, 2005).

Secondary xylem	Secondary xylem parenchyma is involved in the storage (and synthesis) of terpenoid resin, phenolic compounds, and other secondary compounds. Xylem radial rays responsible for transport of phenolic compounds and their precursors for the development of phenol-impregnated heartwood. Resin ducts in xylem contribute to resin flow in tree.	Many pine species have axial resin ducts in the xylem, to allow for transport of defensive resin (Hudgins *et al.*, 2005). Pines also have radial resin ducts, oriented within the radial rays of the secondary xylem (Krokene & Nagy, 2012). Connections between the radial and axial - resin ducts within the xylem create interconnected reservoirs for the pine resin stores (Bannan, 1936, Krokene & Nagy, 2012).	Other conifer species also possess axial resin ducts in their xylem. In some species such as *Picea abies*, developing xylem lacks constitutive axial resin ducts (Martin *et al.*, 2002).

Table 2. Inducible defences in pines and other conifers

Anatomical structure	Physical and chemical defence strategies	In pine trees	In other conifers
Periderm	Wound periderms develop at the borders of lesions created by insect and fungal attack. They can prevent the further spread of disease. Hypersensitive response is activated upon the detection of invading pests and pathogens; cells around infection site begin to produce reactive oxygen species, leading to rapid localized cell death. Can kill or detain the invading organism.	Wound periderms form in response to attack, at the margin between necrotic and living tissue in pines such as *P. monticola* (Struckmeyer, 1951, Hudgins *et al.*, 2003, Hudgins *et al.*, 2005). Pines such as *P. taeda* have been found to respond to wounding and fungal inoculation with hypersensitive reactions (Paine, 1984, Cook & Hain, 1985).	Wounding on the bark of conifers including those in the genera *Larix, Picea, Pseudotsuga,* and *Taxus* results in the development of wound periderm around the wound site (Hudgins *et al.*, 2003). The hypersensitive response occurs in many plant families, including those in the genus *Abies* (Bleiker & Uzunovic, 2004, Franceschi *et al.*, 2005).
Secondary phloem	Wound periderms, forming upon activation of PP cells in the secondary phloem, develop at the borders of lesions formed by fungal attack. They prevent the further spread of disease. New PP cells typically develop in the secondary phloem 15 weeks after induction. PP cells in this tissue produce defensive phenolic compounds in response to insect and pathogen attack. Hypersensitive response also activated here. Traumatic resin ducts form in response to attack from invading fungi and insects. Traumatic resin ducts can produce resin with different terpenoid composition than that of constitutive resin.	Pines can be induced by mechanical wounding, insect damage, fungal inoculation and MeJA application to produce larger quantities, and different concentrations, of phenolic compounds than can be found in the tree constitutively (Klepzig *et al.*, 1995, Lieutier *et al.*, 1996, Bonello & Blodgett, 2003, Franceschi *et al.*, 2005). Tissue damage by biotic or abiotic factors induces the formation of secondary resin in pines, most of which is produced by newly formed traumatic resin ducts (Bannan, 1936, Hudgins *et al.*, 2005, Krokene & Nagy, 2012). Epithelial cells lining ducts that typically produce preformed resin can be induced to produce more resin in response to insect or fungal wounding (Ruel *et al.*, 1998, Lombardero *et al.*, 2000,	Qualitative differences in phenolic compounds have been found in many members of the *Pinaceae* following harm such as wounding and fungal inoculations (Kusumoto & Suzuki, 2003, Nagy *et al.*, 2004, Franceschi *et al.*, 2005). Terpenoid accumulation and the induction of prenyltransferases and terpene synthases have been observed in conifers such as *P. abies* (Martin *et al.*, 2002). In conifers such as *Abies spp.,* which do not produce pre-formed resin ducts, the formation of traumatic resin cavities or blisters have been noted upon damage by insect attack (Berryman, 1969).

		Krokene & Nagy, 2012).	
Vascular cambium	Early lignification of fibres. Formation of traumatic resin ducts also occur here.	Early lignification of fibres might be present in pines but have not been studied conclusively thus far (Hudgins *et al.*, 2003).	Conifers such as *Taxus brevifolia* experience early lignification of fibre layer in the cambium upon harm (Hudgins *et al.*, 2003). Fibres are more common in non-Pinaceae conifers, and typically become fully lignified at c.a. 3 years of age. Induction by MeJA and wounding can lead to early lignification (Hudgins *et al.*, 2003, Hudgins *et al.*, 2004, Franceschi *et al.*, 2005).
Secondary xylem	Formation of traumatic resin ducts. Phenolic compounds and terpenes as well as other phytochemicals are formed in response to insect or pathogen attack.	Tissue damage by biotic or abiotic factors induces the formation of secondary resin in pines, most of which is produced by newly formed traumatic resin ducts (Bannan, 1936, Hudgins *et al.*, 2005, Krokene & Nagy, 2012).	In conifers such as *Picea spp.*, de-novo formation of traumatic resin ducts is observed in the secondary xylem upon mechanical wounding, insect attack, and fungal inoculation (Alfaro, 1995, Martin *et al.*, 2002, Franceschi *et al.*, 2005).

1.3.4 Chemical defence compounds – terpenes and phenolics

1.3.4.1 Terpenes

Resin is mainly composed of terpenes – a large family of approximately 30 000 known compounds with considerable structural and functional diversity. Among these, only a small subset can be found in pine resin (Michelozzi, 1999, Phillips & Croteau, 1999). Most of the resin produced by conifers is composed of roughly equal parts of monoterpenes and diterpenes, with sesquiterpenes making up the smallest portion (Keeling & Bohlmann, 2006). Constitutively produced terpenes, present in primary resin, exist in a tree before an attack occurs as the first line of defence against pests and pathogens (Bonello *et al.*, 2006). Secondary resin is synthesized by the tree in response to an attack, with its composition differing from that of preformed resin – especially in the composition of monoterpenes (Cates, 1996, Michelozzi, 1999). When resin flows from a damaged area on the tree and comes into contact with air, volatile monoterpenes and sesquiterpenes evaporate, leaving behind non-volatile diterpene resin acids to seal and sterilize the wound (Langenheim, 2003, Keeling & Bohlmann, 2006).

The biosynthesis of mono-, sesqui-, and diterpenes begins with the formation of one of three prenyl diphosphate precursors – geranyl diphosphate (GDP), farnesyl diphosphate (FDP), and geranylgeranyl diphosphate (GGDP) respectively (Wise, 1998, Cane, 1999, Davis & Croteau, 2000, Martin *et al.*, 2004, Keeling & Bohlmann, 2006). Each of these three precursors are derived from the condensation of dimethylallyl diphosphate (DMADP) with one, two, or three isopentyl diphosphate (IDP) units in a head-to-tail fashion, with specific prenyltransferases catalysing each reaction (Koyama, 1999, Keeling & Bohlmann, 2006). In pines, IDP and DMADP are formed by one of two biosynthetic pathways – the methylerythritol phosphate pathway in the plastid, or the mevalonate pathway in the cytosol (Chappell, 1995, Bochar *et al.*, 1999, Lichtenthaler, 1999).

The prenyl diphosphate precursors are transformed by specific terpene synthase enzymes to form a diversity of linear and cyclic hydrocarbon compounds. These enzymes, although all having high sequence similarity in the active site and substrate binding domain, differ in the products that they produce. Some synthases produce multiple products, while others produce only one major compound (Keeling & Bohlmann, 2006). It is thought that the great diversity of terpenes could be due to mutations close to the active site or at the substrate binding sites (Bohlmann *et al.*, 1999, Dudareva *et al.*, 2003, Keeling *et al.*, 2008, Zulak & Bohlmann, 2010). Conifer mono- and sesquiterpenes are not modified after being produced by terpene synthases, but diterpenes can be further modified through oxidation by cytochrome P450 enzymes to form diterpene resin acids (Keeling & Bohlmann, 2006, Zulak & Bohlmann, 2010).

A: Monoterpenes

The most abundant monoterpenes produced by pines are α-pinene, camphene, β-pinene, Δ3-carene, and limonene (Table 3) (Radwan *et al.*, 1982, Lewinsohn *et al.*, 1993, Klepzig *et al.*, 1995, Tognetti *et al.*, 1997, Manninen *et al.*, 2002). Constitutive and induced levels of these compounds differ in composition in different pine species. In addition, there are a number of minor terpenes only found in specific species or in a narrow range of taxonomic groups. Induced monoterpene profiles can differ quantitatively and qualitatively when challenged by different biotic and

abiotic stressors. For example, monoterpene accumulation induced upon inoculation of mycelium of a pathogenic fungus on wounded stems lead to much more extensive and long-lasting terpene accumulation compared to mechanical wounding alone (Cheniclet, 1987, Raffa & Smalley, 1995, Michelozzi, 1999).

Studies on the antifungal properties of the monoterpenes in pine resin have indicated that resin with higher concentrations of certain monoterpenes possess higher antifungal activity (Krauze-Baranowska *et al.*, 2002). In one such study, the constitutive resin of *P. ponderosa* had the strongest ability to inhibit the growth of *Fusarium* spp. among three *Pinus* species tested. *Pinus ponderosa* resin had the highest concentration of β-pinene, and was the only *Pinus* species among the three tested with resin containing the monoterpene Δ3-carene (Krauze-Baranowska *et al.*, 2002). High levels of β-pinene have been associated with antifungal activity, and Δ3-carene has both antifungal and antibacterial properties. The resin of *P. strobus,* which contained the lowest amounts of monoterpene hydrocarbons but contained α-pinene as the dominant monoterpene, showed the lowest antifungal activity. The resin of *P. resinosa,* with moderate antifungal activity, was rich in myrcene (Krauze-Baranowska *et al.*, 2002). In similar studies, it has been found that oxygenated monoterpenes contribute more to the antifungal activity of the resin than monoterpene hydrocarbons (Zhang *et al.*, 2016). For example, oxygenated monoterpenes showed far stronger inhibitory effects against the mycelial growth of the pathogen, *Heterobasidion parviporum,* than monoterpene hydrocarbons (Kusumoto *et al.*, 2014, Zhang *et al.*, 2016). The study showed that oxygenated monoterpenes bornyl acetate and α-terpineol had stronger inhibitory effects compared to other monoterpenes (Kusumoto *et al.*, 2014). In contrast, (+)-α-pinene had modest antifungal activity, but it still showed a statistically higher inhibition of mycelial growth when compared to its enantiomer, (-)-α-pinene (Kusumoto *et al.*, 2014). The higher relative toxicity of oxygenated monoterpenes could be due to the higher evaporation rate of monoterpene hydrocarbons (Zhang *et al.*, 2016).

Table 3. Studies investigating monoterpenes in pine stems

Species of pine tested	Most common monoterpenes in stem*	Details of study	Reference
Constitutive resin			
Pinus ponderosa (Ponderosa pine)	1. Δ3-Carene + myrcene (38.25 %) 2. α-Pinene (37.01 %) 3. Fenchyl alcohol (9.46 %) 4. Limonene (3.07 %) 5. β-Pinene + sabinene (3.03 %) 6. Δ-Terpinene (2.4 %) Note: These compounds were detected in *P. ponderosa* from Coconino national forest, central Arizona	Seedlings were selected from natural stands in nine North American national forests, covering most of the range for this species in the western United States. A total of 24 monoterpenes were present in identifiable amounts. The most abundant monoterpenes were monoterpene hydrocarbons, and composition of the resin differed markedly by source, with those in the same geographical range showing the greatest similarity in monoterpene composition. For example, *P. ponderosa* seedlings from northern sources had higher concentrations of Δ3-carene + myrcene and Δ-terpinene, while seedlings from southern sources were higher in α-pinene and fenchyl alcohol.	(Radwan *et al.*, 1982)
Pinus contorta (Lodgepole pine)	1. β-Pinene (44.7 %) 2. β-phellandrene (33.6 %) 3. Δ3-Carene (10.5 %) 4. α-Pinene (8.2 %) 5. Myrcene (1.2 %) 6. Limonene (1.0 %)	One-year old *P. contorta* and two-year old *Abies grandis* (grand fir) seedlings were analysed for the mono- and diterpenes in their constitutive oleoresin. Grand fir seedlings were found to produce considerably less constitutive oleoresin than lodgepole pine seedlings, and was less complex in the types of monoterpenes they produced.	(Lewinsohn *et al.*, 1993)
Pinus sylvestris (Scots pine)	1. α-Pinene (50.5 %) 2. Δ3-Carene (19.4 %) 3. Limonene (15.6 %) 4. β-Pinene (4.8 %) 5. Myrcene (3.5 %) 6. Δ-Terpinene (3.1 %) 7. Sabinene (1.5 %)	Seedlings from natural stands in seven geographical locations in Finland, and two geographical locations in Estonia, were tested for the terpene production in their needles and stems. The most northern Muonio provenance and most southern Saaremaa provenance had the highest total monoterpene concentrations in the needles and wood, respectively, with the total monoterpene concentration being overall higher in the needles than in the wood.	(Manninen *et al.*, 2002)

	Note: There compounds were detected in *P. sylvestris* from Muonio, Finland	The amount of Δ3-carene was higher in the southern provenances, and α-pinene concentration increased in the northern provenances.	
Pinus halepensis (Aleppo pine)	1. α-Pinene (50.67 %) 2. Δ3-Carene (24.74 %) 3. β-Myrcene (15.07 %) 4. β-Pinene (4.12 %) 5. Limonene (1.3 %) 6. Camphene (0.71 %) Note: These compounds were detected in *P. halepensis* from Vico del Gargano, Italy	Two year old seedlings in greenhouses from six origins representing this species' natural geographic range in Italy were tested for their hydraulic architecture and terpene composition. Terpene composition was related to the different geographical distribution of the Aleppo pine provenances. In all provenances tested, α-pinene (average percentage composition among six provenances of 40.70%), myrcene (31.04%), and Δ3-carene (19.70%) were the major constituents. Minor constituents were β-pinene (4.10%), and an unknown compound (1.92%). None of the other monoterpenes detected represented more than 1% of the total terpene fraction of the oleoresin.	(Tognetti *et al.*, 1997)
Induced resin			
Pinus resinosa (Red pine)	1. α-Pinene (72.6 %) 2. β-Pinene (22.8 %) 3. Δ3-Carene (2.4 %) 4. Limonene (0.9 %) 5. Camphene (0.7 %) 6. Myrcene (0.5 %) 7. Note: These compounds were measured one week after mechanically wounding the seedling stems	Seedlings were tested for their monoterpene production, specifically by testing the seedlings' response to aseptic mechanical wounding, compared to wound inoculation with the fungal pathogen *Leptographium terebrantis.* The concentrations of total monoterpenes, including α-pinene, camphene, β-pinene, Δ3-carene, myrcene, and limonene were higher in stem tissue inoculated with the fungus than in mechanically wounded or constitutive stem tissue. Mechanically wounded and non-wounded tissue did not differ in total monoterpene concentrations, although α-pinene, camphene, limonene, and myrcene concentrations were higher in wounded tissue than in non-wounded tissue.	(Klepzig *et al.*, 1995)

*Percentage of total monoterpenes shown in brackets

B: Diterpenes

The most abundant diterpenes found in pines are the tricyclic diterpene resin acids such as abietic-, neoabietic- and dehydroabietic acid, as well as pimaric-, levopimaric-, and isopimaric acid (Table 4) (Keeling & Bohlmann, 2006). The composition and concentration of constitutive and induced diterpenes differ, and the amounts and types of diterpenes produced also differ from species to species (Table 4) (Anderson *et al.*, 1969, Lewinsohn *et al.*, 1993, Lange *et al.*, 1994, Manninen *et al.*, 2002).

Diterpenes play an important role in the induced chemical defence system of conifers, in part due to their antifungal properties (Kusumoto *et al.*, 2014, Mason *et al.*, 2015, Keefover-Ring *et al.*, 2016). Some studies have found that diterpene resin acid production is higher in response to fungal attack compared to mono- and sesquiterpene production (Keefover-Ring *et al.*, 2016). Additionally, a study on the properties of pine terpenes against the fungal conifer pathogen *Heterobasidion parviporum* concluded that the inhibition of this fungus by two diterpene resin acids, abietic- and dehydroabietic acid, was more than twofold higher when compared to inhibition by certain monoterpenes (Kusumoto *et al.*, 2014). In agreement with previous studies, the authors observed that the inhibition rates of these diterpene resin acids increased with concentration but plateaued at higher concentrations (Kusumoto *et al.*, 2014). Other diterpene resin acids found in pines such as levopimaric and palustric acid, are also known to inhibit the mycelial growth of fungi (Henriks *et al.*, 1979, Kusumoto *et al.*, 2014). These resin acids are, however, unstable, and therefore readily oxidise into abietic acid. Abietic acid will then largely oxidize into dehydroabietic acid in the presence of air (Enoki, 1976, Pastorova *et al.*, 1997, Kusumoto *et al.*, 2014).

In a study on the production of defensive terpenes in *P. ponderosa*, the trees' chemical response to inoculation with the bark beetle-associated fungus, *G. clavigera,* was investigated (Keefover-Ring *et al.*, 2016). The study found that diterpene resin acids were the most abundant class of terpenes produced constitutively in the phloem, and that concentrations of diterpenes increased 35-fold in response to wounding and inoculation (Keefover-Ring *et al.*, 2016). The induced diterpene production in *P. ponderosa* was consistently much stronger in response to a combination of mechanical wounding and fungal inoculation than to mechanical wounding alone, which indicates that conifers show stronger defence responses to biotic elicitation compared to physical elicitation (Franceschi *et al.*, 2005, Raffa *et al.*, 2005, Keefover-Ring *et al.*, 2016). In similar studies, the absolute amount of diterpenes were higher in *C. polonica*-resistant Norway spruce trees compared to susceptible trees, providing further support for the importance of diterpenes in the chemical defence of conifers (Zhao *et al.*, 2010, Kusumoto *et al.*, 2014).

Table 4. Studies investigating diterpenes in pine stems

Species of pine tested	Most common diterpenes in stem*	Details of study	Reference
Constitutive resin			
Pinus contorta (Lodgepole pine)	1. Levopimarate (36.8 %) 2. Palustrate (16.5 %) 3. Isopimarate (14.1 %) 4. Neoabietate (12.4 %) 5. Abietate (11.7 %) 6. Dehydroabietate (5.8 %)	One-year old *P. contorta* and two-year old *Abies grandis* (grand fir) seedlings were analysed for the mono- and diterpenes in their constitutive oleoresin. Grand fir seedlings were found to produce considerably less constitutive oleoresin than lodgepole pine seedlings, and the diterpene fraction of grand fir seedlings were simple in composition compared to lodgepole pine seedlings: in some of the seedlings tested, abietic and dehydroabietic acid comprised more than 97% of the diterpene fraction in the induced oleoresin.	(Lewinsohn *et al.*, 1993)
Pinus jeffreyi (Jeffrey pine)	1. Abietic acid (31 %) 2. Levopimaric/palustric (18 %) 3. Dehydroabietic (13 %) 4. Neoabietic (10 %) 5. Pimaric (9 %) 6. Isopimaric (9 %) Note: These diterpenes were detected in the heartwood of *P. jeffreyi* trees	The sapwood and heartwood of *P. jeffreyi* trees was investigated for their resin acid content. In this study, the resin composition of closely-related *P. jeffreyi* and *P. ponderosa* (both belonging to the subgenus Diploxylon) was compared. While the resin acid composition was comparable between the two species, certain hydrocarbons such as *n*-heptane were only present in *P. jeffreyi.*	(Anderson *et al.*, 1969)
Pinus ponderosa (Ponderosa pine)	1. Abietic acid (29 %) 2. Levopimaric/palustric (25 %) 3. Neoabietic (18 %) 4. Isopimaric (10 %) 5. Pimaric (8 %) 6. Dehydroabietic (5 %)	The sapwood and heartwood of *P. ponderosa* trees was investigated for their resin acid content. In this study, the resin composition of *P. jeffreyi* and *P. ponderosa* was compared. Certain hydrocarbons such as *n*-heptane were absent in *P. ponderosa.*	(Anderson *et al.*, 1969)

Pinus sylvestris (Scots pine)	1. Abietic (44.0 %) 2. Levopimaric/palustric (35.9 %) 3. Neoabietic (11.9 %) 4. Pimaric (3.4 %) 5. Dehydroabietic (2.6 %) 6. Sandaracopimaric (1.3 %) Note: These compounds were detected in *P. sylvestris* from Kinnula, Finland	Seedlings from natural stands in seven geographical locations in Finland, and two geographical locations in Estonia, were tested for the terpene production in their needles and stems. It was found that the needles contained both tricyclic and labdane-type resin acids. The woody tissue contained only tricyclic resin acids. However, the tricyclic resin acid content in the wood was four times higher than in the needles. The wood in the most northern Muonio provenance had the lowest total resin acid concentration, with provenances towards the south having the highest total resin acid concentration.	(Manninen *et al.*, 2002)
Pinus heldreichii (White bark pine)	1. Levopimaric/palustric (48.9 %) 2. Abietic acid (18.2 %) 3. Neoabietic (9.8 %) 4. Isopimaric (7.1 %) 5. Dehydroabietic (7.0 %) 6. Sandaracopimaric (0.6 %)	The oleoresin of 50-year old trees from the mountains of Prokletije, Serbia, was tapped and tested to determine their diterpene content. Abietic-type resin acids were found to be the main components of the diterpene resin acids.	(Lange *et al.*, 1994)

*Percentage of total diterpenes shown in brackets

C: Sesquiterpenes

Although research on the composition and concentration of sesquiterpenes produced by pines is not extensive, a study done on *P. sylvestris* found that the most abundantly produced sesquiterpenes are germacrene D and B, β-caryophyllene, α-muurolene, longifolene and β-elemene (Table 5) (Manninen *et al.*, 2002).

A few studies have been conducted on the production of sesquiterpenes in pines when infected by fungi. For example, although the constitutive phloem tissue of *P. ponderosa* contained lower concentrations of sesquiterpenes than the other terpene classes, their concentrations showed the greatest increase upon mechanical wounding and fungal inoculation (Keefover-Ring *et al.*, 2016). The sesquiterpene longifolene accounted for more than 50% of the sesquiterpene component in the constitutive samples, and longifolene levels in the wounded phloem represented more than 70% of total sesquiterpenes. The overall sesquiterpene diversity was the lowest of all three terpene classes, and their diversity decreased even further in wounded and inoculated tissue (Keefover-Ring *et al.*, 2016). In a similar study, terpene production in mature Norway spruce trees upon inoculation with the blue-stain fungus, *E. polonica*, was investigated (Viiri *et al.*, 2001). The authors concluded that the concentrations of germacrene, bicyclogermacrene, α-muurolene, γ-muurolene, β-caryophyllene, and δ-cadinene, with germacrene and δ-cadinene making up the largest fraction, were significantly higher in the phloem of trees inoculated with this fungus compared to unwounded and mechanically wounded trees (Viiri *et al.*, 2001).

Table 5. Studies investigating sesquiterpenes in pine stems

Species of pine tested☼	**Most common <u>sesquiterpenes</u> in stem***	**Details of study**	**Reference**
Constitutive resin			
Pinus sylvestris (Scots pine)	1. Germacrene D (2.0 %) 2. α-Muurolene (1.2 %) 3. β-Caryophyllene (0.8 %) 4. Germacrene B (0.7 %) 5. β-Elemene (0.4 %) 6. δ-Cadenine (0.4 %) Note: These compounds were detected in *P. sylvestris* from Korpilahti, Finland	*P. sylvestris* seedlings from natural stands in seven geographical locations in Finland, and two geographical locations in Estonia, were tested for the terpene production in their needles and stems. The number of sesquiterpenes in the needles were found to be overall higher than in the wood, with the dominating sesquiterpenes in the wood being germacrene D, β-caryophyllene, and α-muurolene.	(Manninen *et al.*, 2002)

*Percentage of the total TERPENES identified shown in brackets

☼Note: not much research is available on the sesquiterpene production and composition in pines

1.3.4.2 Phenolics

Phenolics are a large group of structurally diverse organic compounds that have one or more aromatic ring in their structure. These compounds play important roles in the interactions of plants with their environment, and facilitate numerous physiological processes (Aron & Kennedy, 2008, Hammerbacher *et al.*, 2020). Phenolics are also known to play important roles in pine defence against fungal attack (Wallis *et al.*, 2008, Sherwood & Bonello, 2013). Conifers produce a wide array of different phenolics in parenchyma cells in their roots, bark, stem, and needles, as well as in specialised PP (polyphenolic parenchyma) cells (Hammerbacher *et al.*, 2020). Upon wounding and pathogen attack, the phenolic compounds within PP cells accumulate, helping the tree to respond to an attack more successfully (Franceschi *et al.*, 2000, Hudgins *et al.*, 2004, Li *et al.*, 2012).

A: Lignans and lignin polymers

Lignans are a group of monolignol C_6-C_3 dimers, some of which have been shown to play a role in antifungal defence in conifers (Céspedes *et al.*, 2006, Conde *et al.*, 2013). For example, pines produce fungistatic (inhibiting the growth of fungi) or fungicidal (destroying or killing fungi) lignans such as hydroxymatairesinol and matairesinol, which inhibit fungal pathogens such as *Heterobasidion annosum* (Shain, 1971, MacRae & Towers, 1984, Gabaston *et al.*, 2020). The compound nortrachelogenin has been shown to exert antifungal activity through the disruption of fungal membranes (Lee *et al.*, 2016, Gabaston *et al.*, 2020). Some of the fungistatic activity of lignans might be attributable to their ability to inhibit extracellular fungal enzymes such as laccases, polygalacturonases, and cellulases (Johansson *et al.*, 1976).

Lignin, a cross-linked phenolic polymer, impregnates the spaces in the plant's cell wall between the cellulose, hemicellulose, and pectin components. This abundant biopolymer provides rigidity, and enables upright growth and the transport of water throughout the plant (Boerjan *et al.*, 2003, Martone *et al.*, 2009, Weng & Chapple, 2010). Lignin plays an important role in conifer defence against insect herbivory, as it increases the physical toughness and indigestibility of the plant tissue. Lignin also increases conifer resistance to fungal pathogens in several ways (Lattanzio *et al.*, 2006). Lignified cell walls have improved resistance against mechanical pressure exerted by the hyphae and infection structures of fungal pathogens. Lignin also shields the cell walls from toxins and enzymes produced by fungal pathogens, and inhibits fungal growth and enzymatic processes by limiting the availability of water and nutrients in the tissue (Millett *et al.*, 1976, Vance *et al.*, 1980, Lattanzio *et al.*, 2006, Sherwood & Bonello, 2013). A study on the antifungal properties of pine phenolics showed that lignin possessed the greatest individual anti-fungal and fungistatic activity amongst several phenolic compounds produced by *P. nigra*. (Sherwood & Bonello, 2013). However, many types of fungi (for example, the white rot fungi *Trametes versicolor* and *Phanerochaete chrysoporium*) are able to secrete ligninolytic enzymes such as lignin peroxidase, phenol oxidase, and manganese-dependent peroxidase which break down lignin polymers (Novotný *et al.*, 2004).

B: Flavonoids

Flavonoids are secondary metabolites formed from the phenylpropanoid pathway, with C_6-C_3-C_6 chemical structures (Falcone Ferreyra *et al.*, 2012). Flavonoid distribution, composition, and concentration vary among different conifers. For example, the heartwood of members of the *Pinus* genus is typically characterized by a complex mixture of related flavones, flavanones, and flavonols (Erdtman *et al.*, 1966, Niemann, 1988).

Flavonoids are known to play important roles in plant defence against herbivory. Studies have shown that the flavonoids quercetin-3-glucoside and rutin can inhibit the development and increase the mortality of feeding gypsy moths (*Lymantria dispar*) on *P. banksiana* trees (Gould & Lister, 2006, Mierziak *et al.*, 2014). Flavonoids are also active in defence against oomycete pathogens (Gabaston *et al.*, 2017). In a study where the polyphenols isolated from *Pinus pinaster* were tested for their anti-oomycete activity against the oomycete pathogen *Plasmopara viticola,* it was found that the flavonoid pinocembrin showed the greatest ability to inhibit mildew development and block zoospore mobility (Gabaston *et al.*, 2017). Pinocembrin also has antifungal activity against *Candida albicans* and *Penicillium italicum* in similar studies (Peng *et al.*, 2012, Rasul *et al.*, 2013, Gabaston *et al.*, 2017). This flavonoid acts against fungal pathogens such as *P. italicum* through the inhibition of respiration and subsequent disruption of energy homeostasis, leading to cell membrane damage and reduced metabolic abilities (Peng *et al.*, 2012). The flavanone naringenin has also been studied for its antifungal properties. In *Pinus halepensis* provenances that were tested for their resistance against the fungal pathogen *Gremmeniella abietina,* the concentration of naringenin in the tissue of resistant trees was significantly higher than in the tissue of susceptible trees (Romeralo *et al.*, 2016). The authors suggested that naringenin could potentially be used as a chemical marker for disease-resistant *P. halepensis* (Romeralo *et al.*, 2016).

It has been shown that the anti-pathogenic activities of flavonoids depend on the structure of the flavonoid. The strongest antifungal activity has been demonstrated by unsubstituted- flavanones and flavones; it seems that the antifungal properties of these compounds are in most cases reduced by the presence of methoxy and hydroxyl groups (Weidenbörner & Jha, 1993, Christensen *et al.*, 1998, Mierziak *et al.*, 2014).

Proanthocyanidins (commonly referred to as condensed tannins) are polymeric flavonoids consisting of two or more flavan-3-ol monomers joined together (Dixon *et al.*, 2005, Hammerbacher *et al.*, 2014). It is believed that the primary biological role of proanthocyanidins in plants is defence against pathogens and herbivores (Dixon *et al.*, 2005). In Norway spruce the levels of proanthocyanidins, flavan-3-ol monomers, and an enzyme responsible for the production of flavan-3-ol monomers increased in response to infection by the fungal pathogen *E. polonica* (Hammerbacher *et al.*, 2014). When the antifungal activities of these compounds were tested *in vitro,* it was found that growth of *E. polonica* was significantly reduced when treated with mixtures of proanthocyanidins and (2S, 3R)-*trans*-flavan-3-ol equivalent to the concentrations found in induced spruce sapling bark (Hammerbacher *et al.*, 2014). In pines, research on the specific antifungal and defensive roles of flavonoids in general and proanthocyanidins in particular is limited, although their presence in pine stems, bark, seeds, and needles have been well documented (Packer *et al.*, 1999, Touriño *et al.*, 2005, Lantto *et al.*, 2009, Karapandzova *et al.*, 2015)

C: Stilbenes

Stilbenes, with chemical structure C_6-C_2-C_6, are abundant phenolic compounds in the needles, bark, wood, and roots of many conifer species. Among the stilbenes, pinosylvin and its monomethyl ether (first extracted from the heartwood of *P. sylvestris* in the 1930's) have been shown to play important roles in conifer defence (Erdtman, 1939, Jorgensen, 1961, Lee *et al.*, 2005, Hammerbacher *et al.*, 2011). In a study on the chemicals found in the woody tissues of *P. resinosa* trees, pinosylvin and pinosylvin monomethyl were detected in the sapwood when their production was induced upon mechanical damage of the trees' bark and cambium, and upon fungal penetration of the sapwood of the stems and roots (Jorgensen, 1961). In assays on the properties of pinosylvin, it was found that this stilbene had antibacterial properties as well as antifungal properties, and that it showed particularly high activity against *Saccharomyces cerevisiae* and *C. albicans* (Lee *et al.*, 2005). Higher concentrations of pinosylvin and its monomethyl ether in the heartwood of *P. sylvestris* also influences the tree's resistance or susceptibility to the actions of wood-decaying fungi (Venäläinen *et al.*, 2004). In an assay where ten decay-resistant and ten decay-susceptible *P. sylvestris* trees were analysed, it was found that the average concentration of the stilbenes was significantly higher in the heartwood of resistant trees. The concentration of total phenolics was also significantly higher in resistant trees, underlining the importance and synergistic modes of action of phenolics in fungal resistance in pines (Venäläinen *et al.*, 2004).

Some studies have shown, however, that certain stilbenes only have antifungal properties against pathogens such as *D. pinea* when applied at higher concentrations than those naturally occurring in pines (Sherwood & Bonello, 2013). It has been suggested that certain stilbenes might not be involved in defence, but that they rather act as chemical markers for disease or that they contribute to symptom development through autotoxic effects on the host (Bonello *et al.*, 1993, Bonello & Blodgett, 2003). A study on the contribution of phenolics to SIR in *P. nigra* found that while a mixture of phenolics (including pinosylvin and pinosylvin monomethyl ether) showed higher fungistatic activity against *D. pinea*, fungistasis was still achieved with mixtures where the stilbenes were omitted (Sherwood & Bonello, 2013). It is therefore possible that these stilbenes contribute to pine defence against fungal pathogens, but are not required (Sherwood & Bonello, 2013).

1.4 Other factors which influence disease and pest resistance in pines

Although the inherent genetic traits of a tree play a large role in determining its health and durability, several other factors influence its resistance against pests and diseases. Drought, nutrient and carbon availability, soil quality, the altitude at which the tree is growing, sun exposure, and the age or developmental stage of the tree are all important factors contributing to its overall health. Because primary and secondary metabolism is intricately linked, the production of defensive metabolites such as terpenes and phenolics is also controlled by a combination of environmental and genetic influences.

1.4.1 Abiotic stress

The intensity and timing of chemical defence responses in conifers are in part determined by the presence or absence of abiotic stresses in the tree's environment. A lack of adequate sunlight, low levels key nutrients in the soil, and periods of reduced rainfall can influence a tree's ability to allocate resources to defence. Models have been formulated to explain how plants allocate resources during environmental stress such as low nutrient availability. One such model is the optimum defence theory (ODT), which suggests that plants growing in resource-limited environments invest more resources into the production of phytochemicals and other constitutive defences (Rhoades, 1979, Herms & Mattson, 1992, Sampedro *et al.*, 2011). This theory posits that resource-limited plants would have a greater need to protect themselves constitutively, as the cost of regrowth after damage would be greater in resource-limited environments (McKey, 1974, Marquis, 1992, Sampedro *et al.*, 2011, Tsunoda *et al.*, 2017, Huang *et al.*, 2019). Another model, the growth-differentiation balance hypothesis (GDBH), proposes that growth limitations brought about by abiotic stresses such as nutrient limitation will result in an increase of internal resources that are no longer allocated to growth (Herms & Mattson, 1992). These accumulated carbohydrate resources can then be allocated to differentiation-related processes such as secondary metabolism, the thickening of leaf cuticles, and trichome production with little additional cost to the plant (Herms & Mattson, 1992, Stamp, 2004). Both models can explain the phenomenon of pines showing a general increase in constitutive defences under certain resource-limited and abiotic stress conditions (Herms & Mattson, 1992, Sampedro *et al.*, 2011).

1.4.1.1 Drought stress and light availability

Drought stress can influence the nutrient uptake ratios of trees as well as alter the levels of important osmolytes such as amino acids, inorganic ions and sugars in their tissues (Schulze, 1991, Turtola *et al.*, 2003). Moreover, drought conditions can also limit a tree's photosynthetic capabilities and change its carbon allocation patterns (Teskey *et al.*, 1987). Carbon is an essential element in plant metabolism, due to its ability to easily form bonds with oxygen, hydrogen, and numerous other elements to produce the majority of primary and secondary metabolites (Huang *et al.*, 2019). The allocation of carbon throughout the plant is controlled by the balance between carbon fixation via photosynthesis and carbon demand for storage, defence, reproduction, and growth (Huang *et al.*, 2019).

When carbon is only available in low concentrations due to drought-induced stomatal closure or reduced photosynthetic assimilation due to shading, plant growth is limited. Research on carbon allocation patterns in Norway spruce growing in carbon-limited environments suggests that the trees downregulate growth and respiration processes in order to use the majority of newly-acquired carbon for the production of secondary metabolites (Huang *et al.*, 2019). The trees only maintain low concentrations of non-structural carbohydrates such as sugars and starch, while prioritising the investment of carbon into the production of secondary metabolites (Huang *et al.*, 2019). Prioritising the production of phenolics and terpenes over processes such as growth and respiration could mean that spruce trees prioritize maintaining their defence systems – thus supporting both the ODT and GDBH models for resource allocation during stressful environmental conditions (Huang *et al.*, 2019). This prioritization of resource

allocation towards phytochemicals ensures that the above-ground plant tissues, which are responsible for carbon assimilation and therefore vital under limited carbon conditions, are still protected against pathogen and insect attack (Tsunoda *et al.*, 2017, Huang *et al.*, 2019). Long-term survival is thus prioritised over growth (Huang *et al.*, 2019).

Prioritization of constitutive defensive metabolite production under drought-stress has also been noted in other conifers. In a study on the oleoresin composition in the woody tissues of *P. taeda* and the needles of *P. ponderosa*, *P. halepensis*, and Norway spruce, drought stress led to an increase in the concentration of individual monoterpenes (Hodges & Lorio Jr, 1975, Kainulainen *et al.*, 1992, Johnson *et al.*, 1997, Llusià & Peñuelas, 1998, Turtola *et al.*, 2003). Likewise, *P. sylvestris* and Norway spruce seedlings grown under water stress for two growing seasons, contained increased levels of total monoterpene and diterpene resin acid concentrations in their woody tissues (Turtola *et al.*, 2003). In that study, the higher concentration of abietane-type resin acids was specifically noted. These resin acids are known to have strong activity against certain types of fungi (Micales *et al.*, 1994, Turtola *et al.*, 2003). Studies have also shown that the concentration of α-pinene increased in *P. taeda* under drought stress; in contrast, the concentrations of limonene, myrcene and β-pinene decreased (Gilmore, 1977). It is thought that conifer seedlings that naturally grow in dryer climates or during periods of drought will compensate for these stressful conditions by producing a higher concentration of constitutive oleoresin, leading to reduced growth and a potential increase in their ability to resist herbivore and pathogen attack in accordance with the ODT and GDBH models (Turtola *et al.*, 2003). The flow and overall concentration of constitutively produced resin has also been found to increase in drought-stressed pines (Coley *et al.*, 1985, Wagner, 1986, Turtola *et al.*, 2003).

Pine trees have also been studied to determine whether drought stress affects their production of fungus-induced chemical defences. In one such study, *P. banksiana* seedlings subjected to drought stress were inoculated with the fungal pathogen *G. clavigera* (Klutsch *et al.*, 2017). It was found that lower water availability did not have an effect on the accumulation of defensive monoterpenes (Raffa & Berryman, 1983, Klutsch *et al.*, 2017). These findings suggest that drought-stressed *P. banksiana* seedlings reserve their resources for the production of constitutive chemicals, but do not respond to attack by producing newly-formed phytochemicals. The flow of induced resin has also been reported to decrease under drought-stress conditions in *P. taeda*, *Pinus edulis*, and *P. sylvestris* (Croisé & Lieutier, 1993, Cobb *et al.*, 1997, Lombardero *et al.*, 2000, Turtola *et al.*, 2003). As constitutive resin flow increases under drought stress, it is likely that drought-stressed conifers may not have enough readily available resources to increase their induced resin-based defences by the same proportion as non-stressed trees (Turtola *et al.*, 2003).

Studies on the effect of drought conditions on the accumulation and production of phenolic compounds in pines are limited. In a study on the resistance of young *P. sylvestris* trees to the bark beetle-associated fungus, *Ophiostoma ips,* it was found that water stress had very little impact on the phenolic compounds produced by the trees (Croisé *et al.*, 1998). In this study, drought-stress did not have a significant impact on the concentrations of monophenols in either the healthy phloem or in the phloem inoculated with *O. ips.* The authors concluded that these results support the hypothesis that short-term drought stress does not significantly impact on the defensive abilities

of pine (Croisé *et al.*, 1998). However, drought-stress conditions that last for several years, could have a more profound impact on the accumulation of the tree's secondary metabolites (Croisé *et al.*, 1998).

Studies on the effects of drought-stress on SIR in pines indicated that the trees' ability to activate systemic defences against a pathogen is reduced when water availability is inadequate. Under normal watering conditions, previously infected *P. banksiana* seedlings treated with phytohormones could fight off a subsequent *G. clavigera* infection (Klutsch *et al.*, 2017). However, previously infected *P. banksiana* seedlings grown under drought-stress and treated with phytohormones were susceptible to subsequent *G. clavigera* infection (Klutsch *et al.*, 2017). Drought-stressed seedlings likely allocated their resources to an initial constitutive defence response and were therefore unable to provide resources for a SIR defence response against subsequent infection (Klutsch *et al.*, 2017).

The consensus idea that drought-stressed pines can defend themselves successfully against pathogenic microbes, is not unanimous. In some cases drought stress can lower a tree's defences against insect and pathogen attack. The reduced defence capacity of a tree may result from reduced carbon uptake due to limited photosynthesis and stomatal closure. Severe carbon starvation may lead to a lower rate of defence metabolite production and reduced compensatory growth or callus production around wounded tissue due to the complete depletion of stored carbon reserves (Christiansen *et al.*, 1987, Turtola *et al.*, 2003, Gaylord *et al.*, 2013, Klutsch *et al.*, 2017).

1.4.1.2 Soil nutrients

Studies on the terpene content of plants growing in soil with different nutrient compositions indicate that the soil type can have significant consequences for secondary metabolite accumulation in conifers (Ormeño *et al.*, 2008). For example, the total content of monoterpenes such as Δ-terpinene, Δ3-carene, and sabinene in *P. halepensis* was higher in trees growing in calcareous soils with a higher nutrient content compared to siliceous soils (Ormeño *et al.*, 2008). However, similar studies with other pine species did not show conclusive results (Ormeño *et al.*, 2008). For example, multiple studies showed that varying levels of nitrogen in the soil can result in either enhancement, reduction, or no change in the terpene concentration in the needles of mature *P. sylvestris* trees (Kainulainen *et al.*, 1996, Heyworth *et al.*, 1998, Kainulainen *et al.*, 2000, Ormeño *et al.*, 2008).

The effect of soil nutrient content on the production of phenolics has also been studied. Under phosphorus-limited conditions, young *P. pinaster* trees had a higher concentration of constitutive phenolics and diterpenes in their needles and stems, respectively (Sampedro *et al.*, 2011). Phosphorus-limited conditions also lead to an increase in inducible phenolics in the needles but did not affect the production of inducible phenolics in the stem. The *P. pinaster* trees all showed either reduced growth rates, unaffected concentrations of non-structural carbohydrates, or a reduction in phosphorous concentrations in the needles (Sampedro *et al.*, 2011). Similar studies have shown that an increase in nutrient-availability can result in a higher concentration of phenolic compounds in the phloem, and was compensated for by concentrations of phenolics in needles and shoots (Holopainen *et al.*, 1995, Björkman *et al.*, 1998, Sampedro *et al.*, 2011, Wallis *et al.*, 2011).

1.4.2 Effects of plant ontogeny on defence

Plant ontogeny is defined as the growth and development of a plant throughout its lifetime. As trees age, an increase in storage capacity and access to water and nutrients generally occurs. On the other hand, metabolic activity and photosynthesis, root-to-shoot ratio and growth rate tend to decrease with age. Morphological differences in shoot orientation, leaf morphology or adventitious roots also exist between juvenile and mature trees of the same species. The patterns of resource allocation also typically shift throughout the tree's development. As the tree experiences changes throughout its development, its ability to protect itself against pathogen, herbivore, and mechanical damage changes as well (Kozlowski, 1971, Marquis, 1984, Poethig, 1990, Trumble *et al.*, 1993, Gedroc *et al.*, 1996, Atwell *et al.*, 1999, Boege, 2005).

Age-related resistance, or ontogenetic disease resistance, usually increases with age (Boege & Marquis, 2005). For example, in a study on the effect of MeJA application on long-term resistance mechanisms in pine seedlings, it was found that young seedlings did not develop resistance to *F. circinatum* and *F. oxysporum* infection (Vivas *et al.*, 2012). It was thought that these results were due to the fact that the physiological mechanisms needed for effective resistance had not yet developed in young seedlings (Vivas *et al.*, 2012). Resin production was much lower in pines that are one or two years old relative to the resin production of older trees (Wainhouse *et al.*, 2009, Vivas *et al.*, 2012). Exogenous MeJA application is also known to induce traumatic resin duct formation in older pines, although traumatic resin ducts have never been reported in seedlings younger than one year of age (Martin *et al.*, 2002, Hudgins *et al.*, 2004, Huber *et al.*, 2005). Therefore, the development of long-term resistance of *P. pinaster* against *F. circinatum* infection could depend on the host tree's age (Vivas *et al.*, 2012).

The terpene composition profiles of pines also differ with the age of the tree. In a study on phloem and needle monoterpenes of *P. banksiana*, it was found that juvenile and fully grown *P. banksiana* trees have differences in both their induced- and constitutive monoterpene profiles (Erbilgin & Colgan, 2012). Interestingly, the study found that constitutive monoterpene concentrations were consistently higher in the phloem of juveniles, while induced monoterpene concentration was higher in the phloem of older trees (Erbilgin & Colgan, 2012). Juveniles typically have poorly developed physical defences compared to fully grown pines, and therefore may have to rely more heavily on preformed chemical defences (Franceschi *et al.*, 2005, Barton & Koricheva, 2010, Erbilgin & Colgan, 2012). Older trees also have higher storage capacity and higher capacity to produce photosynthetic products, and therefore have more resources for an effective induced chemical response than juveniles (Lerdau & Gershenzon, 1997, Erbilgin & Colgan, 2012). The effects of plant age on the phenolic content of pine bark and needles have not been studied yet.

1.5 *Fusarium circinatum*

The causal agent of pitch canker, *Fusarium circinatum,* is a destructive fungal pathogen of pines in many parts of the world (Hepting & Roth, 1953, McCain *et al.*, 1987, Muramoto & Dwinell, 1990, Nirenberg & O'Donnell, 1998, Wingfield *et al.*, 2002, Landeras *et al.*, 2005, Coutinho *et al.*, 2007). Currently, it is thought that *F. circinatum* is

exclusively pathogenic to conifers, specifically of species belonging to the *Pinus* genus (Swett *et al.*, 2014). To date, 67 susceptible pine species or hybrid species have been found, with species such as *P. radiata, P. patula,* and *P. sylvestris* being among the most susceptible to this disease (Drenkhan *et al.*, 2020). *Fusarium circinatum* spores typically infect their hosts by entering through wounds in the plant tissue, with dispersal of infective spores occurring primarily via wind, water, and insect vectors (McNee *et al.*, 2002, Wingfield *et al.*, 2008, Santana *et al.*, 2016, Drenkhan *et al.*, 2020). Long-distance dispersal of this pathogen and its introduction into new areas occurs through human activities such as the transport of contaminated wood and timber products, plant materials, seed, and soil (Zamora-Ballesteros *et al.*, 2019, Drenkhan *et al.*, 2020). *Fusarium circinatum* can also colonize various grasses, maize, and dicot species asymptomatically, often resulting in the pathogen infecting pines growing in close proximity. This ability, along with this pathogen's proficiency to survive in soil for at least 3 years, renders *F. circinatum* a formidable threat to pine plantations across the world (Wingfield *et al.*, 2008, Swett *et al.*, 2014, Santana *et al.*, 2016).

In pine trees, *F. circinatum* infection results in the formation of cankerous lesions on the woody tissues. Cankers forming at the sites of initial infection are soaked with viscous, pitch-like resin – hence the common name 'pitch canker disease' (Wingfield *et al.*, 2008). Cankers often girdle the branches and trunk of the diseased tree, which obstructs the flow of water and nutrients through the tree (Gordon *et al.*, 2001, Wingfield *et al.*, 2008). As a consequence of branch girdling, die-back occurs in the area between the canker and the branch-tip (Gordon *et al.*, 2001, Wingfield *et al.*, 2008). As branch die-back occurs, needles will begin to wilt and discolour, eventually dying and falling to the ground (Gordon *et al.*, 2001, Wingfield *et al.*, 2008). Individual branch infections and die-back will not lead to tree mortality, but multiple infections on the stems and trunk of the tree will lead to widespread girdling and dieback in the tree canopy. Girdling on a large scale will result in tree mortality (Blakeslee & Oak, 1979, Wingfield *et al.*, 2008). Reproductive structures of mature pines are also subject to *F. circinatum* infection, which leads to mortality of female flowers and mature cones, misshapen cones, the abortion of cones before maturity is reached, and seed deterioration (Barrows-Broaddus, 1990, Correll *et al.*, 1991).

Due to the fact that disease symptoms differ depending on the age of the pine tree, the term 'pitch canker' is only applied to infected trees (Wingfield, 1999). In pine seedlings, *F. circinatum* infection results in root and collar rot as well as purple discolouration and wilting at the growing tip (Mitchell *et al.*, 2011). Seedling roots are also typically underdeveloped and show discoloured lesions and cortex necrosis (Viljoen *et al.*, 1994). Infection at this stage has a high mortality rate (Barnard & Blakeslee, 1980, Mitchell *et al.*, 2011). Infections occurring in nursey seedlings are attributed to contaminated irrigation water, seedling trays, and transmission from other infected seedlings (Coutinho *et al.*, 2007, Wingfield *et al.*, 2008, Mitchell *et al.*, 2011). Wounding caused by insects or other damage leads in some cases to *F. circinatum* infection, but not all cases of infection can be attributed to this – there is some uncertainty regarding the infection process in nursery seedlings due to a lack of evidence suggesting that infection arises only after wounding (Storer *et al.*, 1998, Mitchell *et al.*, 2011).

Fusarium circinatum can also infect developing pine seeds, which can lead to the infections of nursery seedlings (Dwinell & Fraedrich, Storer *et al.*, 1998). Typically, infected pine seeds visibly deteriorate and infection

reduces seedling emergence, but asymptomatic infected seeds can still germinate and lead to similarly asymptomatic seedlings from which *F. circinatum* can be isolated (Storer *et al.*, 1998, Wingfield *et al.*, 2008). Asymptomatic seedlings in nurseries are thought to be healthy, and are then used for the establishment of new plantations. When these seedlings are planted, they can grow into asymptomatic pines which harbour *F. circinatum* as an endophyte (Storer *et al.*, 1998). These trees have a great impact on the success of the establishment of new pine tree plantations in South Africa, as the post-planting mortality rate of these asymptomatic seedlings is severe (Wingfield *et al.*, 2008, Mitchell *et al.*, 2011). Mortality usually commences 3 to 6 months after new plantations are established, and disease symptoms during this period are similar to those seen in nursery seedlings (Crous, 2005). Between the years 2002 and 2016, high post-planting mortality rates in South African *P. patula* trees lead to a decline in the cultivation of this species by ca. 14% (Crous, 2005, Mitchell *et al.*, 2011, Visser *et al.*, 2019).

This pathogen made its first recorded appearance in the south-eastern United States in 1946. Since then, the disease has spread to many other parts of America, and subsequently the rest of the world (Hepting & Roth, 1946, Dwinell *et al.*, 1985, Ridley & Dick, 2000, Wingfield *et al.*, 2008). In 1953, *F. circinatum* was discovered on pines in Haiti, and in the 1980's on the Japanese islands of Okinawa and Amami Ōshima (Hepting & Roth, 1953, Kobayashi, 1989). In Europe*, F. circinatum* was found to be responsible for root disease in nursery seedlings in northern Spain, and pitch canker has been reported in Italy and Portugal since the early 2000s (Landeras *et al.*, 2005, Pérez-Sierra *et al.*, 2007). In native Mexico and Chile, *F. circinatum* is causing disease in natural pine stands and nursery seedlings, respectively (Santos & Tovar, 1991, Guerra-Santos, 1999, Wingfield *et al.*, 2002). *Fusarium circinatum* began appearing in South Africa in the 1980s where it was first discovered causing root disease on *P. patula* seedlings (Viljoen *et al.*, 1994). Initially only found in a single nursery, *F. circinatum* has slowly spread to other parts of the country, now appearing in most pine-growing nurseries in South Africa (Britz *et al.*, 2005, Wingfield *et al.*, 2008). In 2005, cankers on the stems of *P. radiata* trees in established plantations in the Western Cape province were discovered, and once it was confirmed that *F. circinatum* was responsible, it became clear that this pathogen was no longer limited to nurseries and newly established plantations (Coutinho *et al.*, 2007, Wingfield *et al.*, 2008). More recently, the disease has been reported in the Western Cape on 12- to 15-year-old *P. radiata* trees , as well as in Kwa-Zulu Natal and the Eastern Cape on *Pinus greggii* (Steenkamp *et al.*, 2014, Santana *et al.*, 2016).

Fusarium circinatum is known to be an introduced pathogen to South Africa. Population genetic data shows that *F. circinatum* in SA has low genotypic diversity compared to *F. circinatum* populations in Mexico, where it is thought to have originated from (Wingfield *et al.*, 2008, Mitchell *et al.*, 2011). It is also thought that this pathogen was not introduced to SA only once, but multiple times. Analysis of pitch canker outbreaks in *P. radiata* and *P. greggii* plantations in the Western- and Eastern Cape provinces as well as KwaZulu-Natal show evidence of multiple distinct introduction events (Steenkamp *et al.*, 2014, Santana *et al.*, 2016, Fru *et al.*, 2017).

1.6 *Fusarium circinatum* and pine defence

Much of our knowledge on the physical and chemical defence systems of conifers is derived from studies that focus on defence against bark beetles and their fungal symbionts (Croisé & Lieutier, 1993, Franceschi *et al.*, 2005, Krokene, 2015). Fewer studies focused on how these trees respond to fungal pathogens that do not rely on insect vectors and use other infection strategies (Luchi *et al.*, 2005, Bonello *et al.*, 2006, Wallis *et al.*, 2008). Furthermore, only a small subset of these studies has explored pine defences to *F. circinatum* infection. These studies focused mainly on the transcriptional response of pine inoculated with *F. circinatum* and SIR against pitch canker disease (Fitza *et al.*, 2011, Amaral *et al.*, 2019, Visser *et al.*, 2019, Hernandez-Escribano *et al.*, 2020). Research on specific physical and chemical defence responses against this pathogen is thus currently very limited. For example, the formation of wound periderms and traumatic resin ducts has been documented in 2 year-old seedlings responding to *F. circinatum* infection, but other anatomical defences against this pathogen in older trees are not known (Kim *et al.*, 2009). The chemical defences of pine against this pathogen have also not been studied (Kim *et al.*, 2009, Kim *et al.*, 2010). As *F. circinatum* is an economically and ecologically important pathogen of pines, broadening our understanding of how this pathogen interacts with its host will allow us to improve genetic breeding strategies and select more successful clonal stock and hybrids for establishing plantations.

1.6.1 Gene regulation and phytohormone signalling

Studies on the regulation of gene expression in pines in response to infection by *F. circinatum* has shed light on the genetic basis of its defence against this fungal pathogen. In a study on *P. radiata* seedlings infected with *F. circinatum,* the role of differentially expressed genes (DEGs) in the host-pathogen interaction was investigated (Carrasco *et al.*, 2017). The most noteworthy upregulated DEGs identified in this study were the genes involved in primary and secondary metabolism (Carrasco *et al.*, 2017). The upregulated genes included those involved in phenylpropanoid metabolism, phytohormone signalling, cell wall biosynthesis, and proteolysis (Carrasco *et al.*, 2017). In other words, these DEGs form part of the plant's mechanisms regulating the formation of physical and chemical barriers that restrict pathogen invasion (Franceschi *et al.*, 2005, Veluthakkal & Dasgupta, 2010, Carrasco *et al.*, 2017). In addition to these noteworthy DEGs, several transcription factors (TF) and TF families that were associated with key resistance processes were also identified in this study (Carrasco *et al.*, 2017). These included TF families that are known to be involved in the regulation of secondary metabolism and in plant-pathogen interactions, by regulating cell death and disease resistance (Mead, 2000, Udvardi *et al.*, 2007, Carrasco *et al.*, 2017). The authors hypothesized that these highly-expressed TFs have overlapping functions, and may repress or activate certain genes through *cis*-acting sequence elements which respond to *F. circinatum* infection (Carrasco *et al.*, 2017).

Other genes involved in pine defence are also often found to be upregulated in response to *F. circinatum* infection, notably those involved in the production of pathogenesis-related (PR) proteins (Donoso *et al.*, 2015). PR proteins make up the largest group of defence-related proteins in plants, and include antimicrobial peptides, signalling molecules, and enzymes involved in secondary metabolism (Donoso *et al.*, 2015). The genes responsible for the

production of thaumatin-like protein (PR5) are among the genes found to be commonly upregulated upon *F. circinatum* infection (Donoso *et al.*, 2015, Amaral *et al.*, 2019). PR5 has been identified in numerous plant species as having antifungal properties, potentially by permeabilizing fungal membranes through the creation of transmembrane pores or through damage to the cell wall (Roberts & Selitrennikoff, 1990, Li & Asiegbu, 2004, Donoso *et al.*, 2015). Studies on this protein found that wounded and inoculated *P. pinaster* seedlings showed a significant increase of PR5 compared to wounded seedlings alone, indicating that it is produced specifically in response to the presence of the pathogen (Amaral *et al.*, 2019).

Genes encoding PR9 class proteins that function as peroxidases, and PR3 class proteins that function as chitinases, are also commonly upregulated (Davis *et al.*, 2002, Donoso *et al.*, 2015, Amaral *et al.*, 2019). Peroxidases play an important role in cell wall synthesis and reinforcement through the polymerization of lignin and are also involved in defence signalling (Koutaniemi *et al.*, 2007, Donoso *et al.*, 2015). Chitinases are responsible for hydrolysing chitin in fungal cell walls thereby preventing the growth of hyphae into the intercellular spaces (Mauch *et al.*, 1988, Collinge *et al.*, 1993, Donoso *et al.*, 2015). Other genes involved in pathogen defence upregulated in pines infected by *F. circinatum* include pinosylvin synthase (PST), phenylalanine ammonia lyase (PAL), and phenylcoumaranbenzylic ether reductase (PCBER) (Donoso *et al.*, 2015). PST is responsible for the synthesis of the stilbene pinosylvin, a compound produced early in response to fungal and environmental triggers (Donoso *et al.*, 2015). The upregulation of genes involved in pinosylvin production was higher in pines that were classified as resistant, indicating its potential role in pathogen resistance (Schwekendiek *et al.*, 1992, Donoso *et al.*, 2015). PAL is a key enzyme in the biosynthesis of monolignols, which polymerize to form reinforcing lignin in cell walls, and PCBER belongs to a family of reductases responsible for the biosynthesis of antimicrobial lignans (Dixon & Paiva, 1995, Turley, 2008, Donoso *et al.*, 2015). It must be noted that the transcriptional responses triggered by *F. circinatum* are part of a quantitative defence response where a large number of genes are expressed in concert to contribute together to defence and resistance in the tree (Donoso *et al.*, 2015).

Studies on the roles of phytohormones produced by pines in response to *F. circinatum* infection have elucidated the defence signalling pathway triggering a resistance response to this pathogen. A study in which *P. pinaster* seedlings were infected with *F. circinatum* demonstrated that pine resistance relies on the early induction of defence related genes and the actions of hormones such as salicylic acid, JA and ethylene (Hernandez-Escribano *et al.*, 2020). It was found that genes involved in ethylene biosynthesis, as well as genes involved in JA biosynthesis, were induced in response to *F. circinatum* inoculation (Hernandez-Escribano *et al.*, 2020). Exogenous application of MeJA has been shown to play a role in the induction of chalcone synthase, which plays a key role in the flavonoid biosynthesis pathway and is known to be involved in plant defence against pathogens (Creelman *et al.*, 1992, Richard *et al.*, 2000, Hernandez-Escribano *et al.*, 2020). Ethylene and jasmonates also play roles in the induction of certain PR proteins involved in the degradation of glucans in the cell walls of invading pathogens. This renders the fungal cell more susceptible to lysis and open to attack by other defence molecules (Hématy *et al.*, 2009, Durai & Schulz, 2016, Hernandez-Escribano *et al.*, 2020). An upregulation of PR5 was detected three days post-inoculation, before fungal

penetration, which indicates quick activation of defence responses elicited by phytohormones released in response to infection (Hernandez-Escribano *et al.*, 2020). These studies demonstrated the importance of phytohormones in the pine-*F. circinatum* pathosystem, and the potential of exogenous hormone application to induce plant resistance against impending infection by fungal pathogens (Hernandez-Escribano *et al.*, 2020).

1.6.2 Physical and chemical defence responses against **F. circinatum** *infection in pines*

The direct physical and chemical defence responses against *F. circinatum* infection in pines have not been studied extensively. However, certain aspects of the interaction between this pathogen and its host are known. For example, studies on wound periderm formation of pines in response to *F. circinatum* infection indicate that pines counteract attack by this pathogen by blocking its growth or the spread of fungal toxins, and preventing water and nutrients from reaching infected cells (Vance *et al.*, 1980, Rittinger *et al.*, 1987, Smith *et al.*, 2007, Kim *et al.*, 2009). Upon investigation of wound periderms using transmission electron microscopy, it was found that cell walls became impregnated with lignin and suberin polymers, in a process referred to as ligno-suberization (Kim *et al.*, 2009). In that study, pine species with higher levels of ligno-suberization in their wound periderms were more resistant to *F. circinatum* infection (Kim *et al.*, 2009). In these resistant pines, fungal hyphae isolated from the plant tissue showed higher levels of hyphal vacuolation, a symptom consistent with fungi growing under nutrient-deprived conditions and a factor that weakens the rigidity, tensile strength and impairs the metabolism of hyphae (Paul *et al.*, 1994, Li *et al.*, 2002, Green *et al.*, 2005, Kim *et al.*, 2009). Vacuolated hyphae are also more susceptible to physical and chemical damage imposed by other defence mechanisms of the tree (Green *et al.*, 2005, Kim *et al.*, 2009). It is likely that the nutrient-deficient conditions within the highly ligno-suberized cells of resistant pines allowed the tree to restrict the growth and proliferation of the invading fungus (Kim *et al.*, 2009).

The flow of resin in a pine tree increases in response to wounding and infection with *F. circinatum*. This has been studied in mechanically wounded and *F. circinatum*-inoculated *P. rigida* and *P. densiflora* seedlings (Kim *et al.*, 2010). Wounded and inoculated seedlings produced twice as much resin 10 days post-inoculation than trees that were only mechanically wounded. Induced resin flow was also observed in *P. nigra* and *P. taeda* trees infected with other pathogenic fungi (Luchi *et al.*, 2005, Knebel *et al.*, 2008, Kim *et al.*, 2010). Traumatic resin ducts form upon detection of wounding and fungal infection (Franceschi *et al.*, 2005). The resin formed in these ducts is often more fungistatic than constitutively produced resin, due to different terpene and phenolic composition in induced resin (Nagy *et al.*, 2000, Franceschi *et al.*, 2005). This is potentially due to the appearance of PP cells in the xylem in the vicinity of newly-formed traumatic resin ducts, increasing the phenolic content and toxicity of the secreted resin (Nagy *et al.*, 2000). In a study focussing on traumatic resin duct formation in *P. radiata* seedlings in response to *F. circinatum* inoculation, the number of resin ducts in the xylem were found to increase significantly (Martín-Rodrigues *et al.*, 2013). The epithelial cells lining the resin ducts in the xylem of infected tissue were larger compared to those in uninfected tissue (Martín-Rodrigues *et al.*, 2013). It was also noted that newly-formed resin ducts were attacked and damaged by the infecting pathogen. However, the total number of new resin ducts still increased in inoculated seedlings, whereas wounded seedlings did not show an increase in resin duct formation (Martín-Rodrigues *et al.*, 2013).

Interestingly, traumatic resin duct formation and increased resin flow in pines do not always appear to be effective against *F. circinatum* infection (Barrows-Broaddus & Dwinell, 1984, Martín-Rodrigues *et al.*, 2013). This pathogen has been noted to exploit resin ducts by proliferating through the ducts into deeper parts of the phloem, where there are more abundant and nutrient-rich resources available (Martín-Rodrigues *et al.*, 2013). Tree death due to water restriction was also shown to occur as a side effect of enhanced resin production (Barrows-Broaddus & Dwinell, 1984, Gordon *et al.*, 2011, Martín-Rodrigues *et al.*, 2013). Pine trees store starch in epithelial cells lining resin ducts as well as in the surrounding parenchyma cells. These resources close to the site of penetration provide additional nutrients to the infecting *F. circinatum* (Hudgins *et al.*, 2005, Nagy *et al.*, 2006, Martín-Rodrigues *et al.*, 2013). Studies on other conifers with different fungal pathogens found similar results. For example, Norway spruce clones that are susceptible to *H. annosum*-infection were found to produce more traumatic resin ducts in response to this pathogen compared to resistant clones (Krekling *et al.*, 2004, Martín-Rodrigues *et al.*, 2013). The authors propose that the pathogen likely proliferates more extensively in the tissues of susceptible clones, thereby increasing the amount of damage and eliciting a larger resin-duct formation response (Krekling *et al.*, 2004). It is possible that traumatic resin ducts and increased resin flow could be a more effective defence strategy against threats such as invading insects, compared to threats such as fungal infection. However, current knowledge on the defence responses of pine to infection by *F. circinatum* is sparse and should be a major focus of future studies (Kim *et al.*, 2009, Kim *et al.*, 2010, Martín-Rodrigues *et al.*, 2013).

1.6.3 Systemic induced resistance (SIR), and the relative resistance of pines against **F. circinatum** *infection*

The effect of pine SIR in response to infection by *F. circinatum* has been investigated in numerous studies. In young *P. radiata* trees infected with *F. circinatum,* a significant reduction in lesion length was observed in trees that had suffered previous infections by the same pathogen (Bonello *et al.*, 2001). In an experiment where the young trees were subjected to four inoculation events several weeks apart, the lesion lengths measured after the final inoculation were on average 68% shorter than the lesion measurements taken after the first inoculation, with lesion lengths declining consistently after each inoculation event (Bonello *et al.*, 2001). This indicates that the young trees were acquiring resistance to the pathogen over time, and responding to infection more effectively with each passing inoculation. Similar studies on *P. radiata* trees in the field has shown that trees that partially recovered from natural *F. circinatum* infections, respond more successfully to artificial inoculations with the fungus (Gordon *et al.*, 2011). In that study, lesions that developed upon inoculation of trees that were previously naturally infected by *F. circinatum* were significantly shorter than those that developed on trees that had never been infected. The same study also showed that trees in areas where *F. circinatum* was recently introduced became more resistant to infection over time. These findings show that SIR occurs in *P. radiata* under natural conditions, and that it contributes to the resistance to *F. circinatum* infection in the field (Gordon *et al.*, 2011). In a study where the roots of *P. radiata* seedlings were exposed to *F. circinatum,* it was found that symptomless root infection can induce SIR in seedlings (Swett & Gordon, 2017). Enhanced resistance to shoot infection following stem inoculation was observed, indicating that colonization of seedlings roots by *F. circinatum* made them less vulnerable to mortality associated with stem infections. This effect

was determined to be a type of SIR, as resistance to the infection occurred a distance away from the original colonization site, i.e. resistance was systemic (Swett & Gordon, 2017).

Although most pine species are hosts to *Fusarium circinatum*, it is notable that some species are more susceptible than others, with quantitative differences having been observed when comparing susceptible and resistant species (Rockwood *et al.*, 1988, Viljoen *et al.*, 1995, Hodge & Dvorak, 2007). Pine host susceptibility to *F. circinatum* infection has been ranked comprehensively by Drenkhan *et al* in a 2020 review. Grown under greenhouse conditions, young pines belonging to species such as *P. patula, P. radiata, P. sylvestris,* and *P. taeda* are classified as highly susceptible, while *P. tecunumanii, P. thunbergii, P. resinosa,* and *P. nigra* were classified as tolerant under similar conditions (Drenkhan *et al.*, 2020). Some pines can be classified as moderately susceptible, such as *P. banksiana* and *P. elliottii,* while *P. koraiensis* was classified as resistant (Drenkhan *et al.*, 2020). However, these classifications are highly variable. Some pines, such as *P. lambertiana* and *P. jeffreyi*, only show resistance to *F. circinatum* in greenhouse experiments but not in the field (Storer *et al.*, 1994, Gordon *et al.*, 1998, Hodge & Dvorak, 2000, Mead, 2000). Pines such as *P. nigra* has been classified as tolerant when grown in greenhouse conditions, but highly susceptible when grown in growth chambers (Drenkhan *et al.*, 2020). Often, species that have been classified as relatively resistant can succumb to *F. circinatum* infection when under high inoculum- and adverse environmental pressures. The relative resistance observed in some pine species thus relies not only on inherent genetic factors, but also on environmental factors (Blakeslee & Rockwood, 1999, Lopez-Zamora *et al.*, 2007, Wingfield *et al.*, 2008). Similarly, varying levels of resistance can be observed between pines belonging to different age classes, i.e. between mature trees (older than *ca.* 11 years) and young or newly emerged trees of the same species (Drenkhan *et al.*, 2020). Within-species genetic variation has also been observed in *Pinus* species from California, the south-eastern United States, and countries in Central America, including Mexico (Dwinell & Barrows-Broaddus, 1979, Bronson *et al.*, 1992, Blakeslee & Rockwood, 1999, Storer *et al.*, 1999, Hodge & Dvorak, 2007).

1.7 Implications for the pine industry and future research directions

Pine trees are exceptionally valuable to the economies of numerous countries around the world. In South Africa alone, the commercial forestry sector provides employment for an estimated 165 000 people and contributes roughly R69 billion to the economy yearly (Forestry South Africa, 2020, Department of Forestry Fisheries and the Environment, 2021). One of the most significant challenges to the industry is the wide range of forest pests and pathogens that threaten the health of pine trees in plantations. The actions of fungal pathogens such as *F. circinatum* and *D. pinea* and pests such as the Sirex woodwasp and Deodar weevil lead to major profit losses due to unsuccessful plantation establishment, insufficient timber and pulp quality, and tree and seedling death on a large scale (Gebeyehu & Wingfield, 2003, Wingfield *et al.*, 2008, Bihon *et al.*, 2011, Mitchell *et al.*, 2011, Hurley *et al.*, 2012). Many of the pests and pathogens that pose the biggest threat to the South African pine industry are introduced to the country through unregulated trade of plant material and the introduction of contaminated timber products, water, and soil

(Wingfield *et al.*, 2008, Fisher *et al.*, 2012, Stenlid & Oliva, 2016). Exacerbating the negative effects of the introduction of these pests and pathogens into the country is climate change, as changing climatic conditions can expand the host- and geographic range of a pest or pathogen and decrease the pine tree's defensive abilities (Agrios, 1988, Anderson *et al.*, 2004).

Based on the ever-changing biotic and abiotic pressures, the South African pine industry is motivated to invest in the development of new pine varieties and hybrids with specific desirable traits such as drought tolerance and enhanced disease resistance, in addition to optimal wood quality and fast growth rates. This is done by selective breeding and cross-breeding of pine species with desirable traits to create hybrids that have the desirable traits of both parent species. In the case of breeding for *F. circinatum* resistance, for example, hybrid species such as *P. patula* x *P. tecunumanii* have the combined traits of *P. patula's* superior wood quality and *P. tecunumannii's* higher levels of disease resistance (Visser *et al.*, 2019). Disease resistance in pines relies on an interconnected system of constitutive and induced defences, many of which have been studied in depth in pines and in other conifers and many of which do not feature prominently in the current literature (Franceschi *et al.*, 2005). For example, the formation of traumatic resin ducts and an increase in resin production in response to insect wounding and fungal infection in conifers is well understood, but the effects of infection on resin terpene concentration and profiles remains uncertain (Franceschi *et al.*, 2005). Similarly, it is known that PP cells accumulate phenolics in response to insect wounding and fungal infection in some conifer species, and it is known that PP cells are present in pines, but the specific phenolic responses in pines are unknown (Hudgins *et al.*, 2003, Franceschi *et al.*, 2005, Nagy *et al.*, 2006). Almost nothing is known about pine's specific response to *F. circinatum* infection in terms of defensive metabolite production. A broader understanding of the defence response that this pathogen elicits in its pine tree host could lead to improved breeding strategies and the selection of more robust and disease-resistant trees for plantation establishment.

1.8 Aims and Objectives of this study

The aim of this research project was to investigate the production of terpenes and phenolics in resistant and susceptible *P. patula* X *P. tecunumanii* hybrid clones in response to *F. circinatum* infection. Although it is well understood that pines produce secondary defence metabolites in response to abiotic and biotic stresses, the underlying mechanisms of a successful defence response against a threat like an invading fungal pathogen is unknown. There is significant variation among different species and provenances of pines in terms of their relative susceptibility to *F. circinatum* infection. I therefore explored this variation by studying the production of defensive phytochemicals in multiple hybrid genotypes of *P. patula* X *P. tecunumanii* with different levels of susceptibility. The pine forestry industry makes use of *P. patula* X *P. tecunumanii* hybrids for the superior wood quality contributed by the *P. patula* parent, and the higher *F. circinatum*-resistance of the *P. tecunumanii* parent. Due to the economic importance of pines to this industry, knowledge of the factors contributing to the resistance against *F. circinatum* could result in the identification of new resistance markers for breeding more tolerant hybrid varieties.

The objectives of my study were to identify and analyse the different terpenes and phenolics produced by several low- and high-elevation genotypes (hereafter referred to as LE and HE genotypes, respectively) of *P. patula* X *P. tecunumanii* hybrids in response to *F. circinatum* inoculation (*P. patula* crossed either with frost-tolerant *P. tecunumanii* originating from altitudes above 1 500 m (HE) or with low elevation frost-intolerant provenances (LE)). The specific research questions for this study were: (1) how does phytochemical production in response to *F. circinatum* infection change over three different time points post-inoculation? Additionally, (2) does phytochemical production differ between LE and HE genotypes and if so, (3) how does this affect the susceptibility of these genotypes to *F. circinatum* infection? Finally, (4) how is phytochemical production and susceptibility to *F. circinatum* infection impacted by an increase in growth temperature for the *P. patula* X *P. tecunumanii* hybrid clones? Taking my review of the literature into consideration, I hypothesize that higher defensive phytochemical production in *P. patula* X *P. tecunumanii* hybrids is associated with less severe *F. circinatum* infection.

CHAPTER 2:

MATERIALS AND METHODS

2.1 Inoculation trials and Experimental design

2.1.1 Trial 1: Inoculation of LE and HE P. patula X P. tecunumanii *hybrid genotypes with* F. circinatum *for phytochemical analysis seven weeks post-inoculation*

The *Fusarium circinatum* strain CMWF 24, provided by the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI; University of Pretoria, South Africa) was grown on potato dextrose agar (PDA; 20 g potato dextrose and 15 g agar (Merck, Germany) made up to 1 L with distilled water) for 8 days at 22 °C. Eight different one-year old *P. patula* X *P. tecunumanii* clones (high-elevation genotypes P1TH2; P5TH2; P7TH4; P10TH2; P10TH3; and low-elevation genotypes P5L1; P7TL3; P9TL1) obtained from SAPPI Ltd were used in this inoculation trial (Table 6). Six months prior to the experiment the clones were transplanted from seedling plugs into 750 mL pots containing pine bark potting mix (Leroy-Merlin, South Africa) and grown in a greenhouse at approximately 25 °C.

Inoculations were performed on 9 February 2021. Using a scalpel, a 10 mm X 6 mm wound was made in the outer bark of each sapling not entirely removing the detached piece of bark. A small circular plug of *F. circinatum* mycelium (8 mm in diameter) was placed into the slit with the mycelium facing towards the sapwood. The half-detached piece of bark was folded back to cover the wound and the inoculated section of the stem was wrapped with Parafilm (Bemis Company, USA). Control saplings were treated with sterile PDA instead of fungal mycelium. The saplings were grown in a greenhouse at 23 °C ± 4 °C (Table 6). Seven weeks post-inoculation on 30 March 2021, stem tissue samples of approximately 5 cm in length were harvested from the stem surrounding the inoculation site. Stem tissue samples were frozen in liquid nitrogen immediately upon collection, and stored at -40 °C. Between five and ten replicate samples were used for each hybrid genotype (Table 6). The lesion that formed around the resin-soaked inoculation point was measured with a caliper before tissue collection. Each lesion or dead plant was assigned a disease score as a metric of relative susceptibility to the infection (lesions of 0 – 19 mm = disease score of 0; 20 – 29 mm = disease score of 1; 30 – 59 mm = disease score of 2; 60 mm – DEAD = disease score of 3) (Table 9). Disease scores were used to compare the susceptibility of a genotype to its production of defensive phytochemicals .

Table 6. Number of *Pinus patula* X *P. tecunumanii* clones inoculated with *Fusarium circinatum* strain CMWF 24, and grown in a greenhouse at 23 °C for the duration of the trial. Lesion measurements taken and stem tissue samples were harvested at 7 weeks post-inoculation.

1st Inoculation Trial – 23 °C			
P. patula* x *P. tecunumanii **Genotype**	**Treatment**		**Total Number of Saplings**
	Inoculated	**Control**	
P1TH2	5	7	12
P5TH2	5	9	14
P7TH4	8	9	17
P10TH2	9	10	19

P10TH3	9	9	18
P5TL1	8	6	14
P7TL3	9	8	17
P9TL1	8	7	15

2.1.2 Trial 2: Inoculation of two LE **P. patula X P. tecunumanii** *hybrid genotypes with two* **F. circinatum** *strains to monitor phytochemical responses over a time course of 28 days*

Two *Fusarium circinatum* strains (CMWF 24 and CMWF 2615) provided by the culture collection of FABI (University of Pretoria, South Africa) were cultured on PDA for 8 days at 22 °C. One-year old LE genotypes (P9TL9 and P7TL1) of *Pinus patula* X *P. tecunumanii* hybrids were inoculated on 30 July 2020 with either sterile PDA, CMWF 24, or CMWF 2615 using the same procedures as previously described. The saplings were grown in a greenhouse at 23 °C ± 4 °C.

Stem tissue samples were collected at the leading edge of the lesion formed around the inoculation point three timepoints (5 days-, 14 days-, and 28 days) post inoculation (therefore on the 4th, 13th, and 27th of August 2020. Harvested tissue was approximately 5 cm in length. At the 28-day timepoint post-inoculation, lesions were measured using a ruler. Stem tissue samples were immediately frozen in liquid nitrogen and stored at -40 °C. Between five and seven replicate saplings were used for each treatment per time point (Table 7).

Table 7. Number of *Pinus patula* X *P. tecunumanii* clones inoculated with two strains of *Fusarium circinatum*. At each of the three timepoints (5 days-, 14 days-, and 28 days) post-inoculation, roughly a third of each group of samples were selected for stem tissue sample collection, and lesion measurements were taken at the final timepoint post-inoculation.

2nd Inoculation Trial			
***Pinus patula* x *P. tecunumanii* Genotype**	***Fusarium circinatum* strain used for inoculation**	**Total number of inoculated saplings**	**Total number of control saplings**
P7TL1	CMWF 24	20	21
	CMWF 2615	20	
P9TL9	CMWF 24	22	22
	CMWF 2615	21	

2.1.3 Trial 3: Inoculation of three **P. patula X P. tecunumanii** *hybrid genotypes with* **F. circinatum** *to study the effect of high temperature on disease development and phytochemical accumulation*

On 9 February 2021, *F. circinatum* strain CMWF 24 was used to inoculate *P. patula* x *P. tecunumanii* HE genotypes P5TH2 and P10TH2, and LE genotype P5TL1 using the same procedures as described above (Table 8). Half of all inoculated and control saplings were placed in a greenhouse at 23 °C ± 4 °C, and half were placed in a greenhouse at 28 °C ± 4 °C (Table 8). Seven weeks post-inoculation on 30 March 2021, lesion measurements were taken with a

caliper and stem tissue samples of approximately 5 cm in length were harvested from the stem surrounding the inoculation site, frozen in liquid nitrogen immediately upon collection, and stored at -40 °C.

Table 8. Number of *Pinus patula x P. tecunumanii* clones inoculated with *Fusarium circinatum strain* CMWF 24, and grown at either 23 °C or 28 °C for the duration of the trial. Lesion measurements were taken and stem tissue samples were harvested at 7 weeks post-inoculation.

3rd Inoculation Trial						
***P. patula* x *P. tecunumanii* Genotype**	**28 °C**			**23 °C**		
	Treatment		**Total Number per Clone**	**Treatment**		**Total Number per Clone**
	Inoculated	**Control**		**Inoculated**	**Control**	
P5TH2	7	9	16	5	9	14
P10TH2	5	9	14	9	10	19
P5TL1	9	9	18	8	6	14

2.2 Liquid-chromatography mass-spectrometry (LC-MS)

Individual frozen stem tissue samples were added along with liquid nitrogen to a mortar, and manually ground to a fine powder using a pestle. For LC-MS analysis specifically, subsamples of the ground material were freeze-dried in a Virtis AdVantage Plus EL-85 bench-top freeze drier (SP Scientific, USA) at 20 mbar for 24 hours. Individual freeze-dried stem tissue samples were then weighed to 30 mg. Phytochemicals were extracted by adding 1.5 ml absolute methanol (Merck, Germany) to the sample placed in a 2 ml tube for 2 hours at room temperature. Samples were shaken intermittently during the 2-hour duration. Samples were then centrifuged at a maximum g-force of 10 000 x *g* for 30 minutes, after which 1 ml of the supernatant was transferred to a glass vial.

Compound separation and quantification were done through liquid chromatography-mass spectrometry (LC-MS) using an Agilent 1100 HPLC system (Agilent Technologies, USA) coupled to a Bruker Daltronics Esquire 3000 electrospray ion trap mass spectrometer (Bruker Daltonics, Germany). The compounds were then separated on a Nucleodur Sphinx column (dimensions: 250 x 4.6 mm diameter and particle size of 5 µm; Macherey-Nagel, Germany) with a solvent gradient from 95% water with formic acid (0.1% v/v; Sigma, USA) to 95% acetonitrile (Merck, USA) over 35 min at a flow rate of 0.8 ml/min.

Column flow was diverted in a 4:1 ratio into the mass spectrometer, where compounds were analysed in negative mode scanning a m/z ratio between 50 and 1 600, with an optimal target mass of 400 Da. The mass spectrometer was operated under the following conditions: skimmer voltage of 60 V; capillary voltage of 4 200 V; nebulizer pressure of 35 psi; drying gas of 11 L/min; gas temperature of 330 °C; and capillary exit potential of - 121 V.

Data acquisition and preliminary analysis were carried out with Bruker HyStar software (Bruker, Germany). Data files generated by the program were exported and converted from .d format to .mzXML format using the Proteowizard MS-convert software (www.proteowizard.sourceforge.net/)(Chambers *et al.*, 2012). Data in .mzXML

format were uploaded onto the XCMS-online website (www.xcmsonline.scripps.edu/)(Tautenhahn *et al.*, 2012) grouped by clone and treatment and analysed using the parameters for single quadrupole mass spectrometers (261). The analysis method was modified using the orbiwrap retention time correction and 300 ppm feature correction tolerance. The results table from XCMS-online was curated as follows: Peak areas with an intensity lower than 5 000 and peaks eluting from the column between 0 and 4 minutes as well as between 28 and 35 minutes were discarded. This curation was conducted to reduce artifact peaks in the dataset. The curated XCMS data table was subsequently uploaded into MetaboAnalyst (V 3.0) (www.metaboanalyst.ca/)(Xia *et al.*, 2015), a platform which allows high-throughput multivariate statistical analysis and interpretation of large sets of metabolomic data. The data was transformed by log transformation and analysed for significant differences in metabolite concentration in different clones and treatments.

2.3 Gas-chromatography mass-spectrometry (GC-MS)

2.3.1 Mono- and sesquiterpene analysis

Subsamples of the finely-ground stem tissue samples were weighed to 50 mg. Phytochemical extraction was performed by adding 1 ml hexane to each sample and shaking continuously for 1 hour at room temperature. Samples were then centrifuged at a maximum g-force of 10 000 x *g* for 20 minutes, after which 1 ml of supernatant was transferred to glass vials.

Mono- and sesquiterpenes present in the samples were separated and quantified using an Agilent 7890 (Agilent, USA) gas chromatograph-mass spectrometer equipped with an HP-5ms capillary column (Agilent, USA). Splitless injections (1 µl extract) were made with the Agilent 7890 autosampler, using helium as a carrier gas at a flow rate of 24 ml/min and an inlet temperature of 250 °C. The temperature program was initiated at 40 °C, which increased at 5 °C per minute until a maximum temperature of 200 °C was reached and then held for 2 minutes, with an initial solvent delay of 3.5 minutes. The mass spectrometer operated in scan mode with a mass range of 40-450 *m/z*, with the ion source maintained at 70 eV.

2.3.2 Diterpene analysis

Subsamples of the hexane extracts (as described above) were taken and prepared for derivatization, a process which volatilizes polar compounds such as diterpenes for easier GC detection. To derivatize the diterpene analytes, 12 µl of trimethylsulfonium hydroxide (TMSH) (Merck, Germany) was placed into a small glass insert within a GC-MS vial. To the TMSH, 100 µl of the hexane extract was added. The lid of each derivatized sample was wrapped in Parafilm to prevent evaporation of the highly volatile samples, and left for the reaction to occur overnight at room temperature.

The diterpene resin acids present in the sample were analysed and identified with an Agilent 7890 (Agilent, USA) GCMS equipped with an HP5ms capillary column. Split injections (1 µl extract) with a split ratio of 10:1 were made. Helium was used as carrier gas with a flow rate of 16 ml/min and an inlet temperature of 250 °C. The

temperature program was initiated at 40 °C, which increased at 20 °C per minute until a maximum temperature of 300°C was reached and held for 8 minutes, with an initial solvent delay of 3.5 minutes. The mass spectrometer was operated in scan mode with a mass range of 40-450 *m/z*, with the ion source maintained at 70 eV.

2.3.3 Terpene identification

Chromatograms produced by GC-MS analyses were processed using the Agilent MassHunter Unknowns Analysis software (Agilent, USA). This software deconvolutes co-eluted peaks in the chromatograms and identifies compounds present in the sample through comparison of mass spectra to reference data in the NIST (National Institute of Standards and Technology, 2017) library. Deconvolution parameters were as follows: left *m/z* delta: 0.3; right *m/z* delta: 0.7; sharpness threshold: 25 %. The NIST library was searched using a minimum match factor of 30 and a maximum number of 75 peaks were searched to limit peak detection. The name, peak area, mass, and retention time were obtained for each relevant compound present in each separate sample.

2.4 Statistical analysis

For all inoculation trials, data obtained from GCMS and LCMS analyses was further analysed using R (www.r-project.org) to determine if statistically significant trends were present in the data. For trial 1, the dataset was analysed for statistical significance between the relative abundance of each compound produced by the different genotypes in response to infection and in the mock-inoculated controls. The data were also analysed for correlations between the compounds produced and the disease score of the different genotypes. In trial 2, the dataset was analysed for statistically significant differences between the relative abundance of compounds produced at three different timepoints post-inoculation. In trial 3, the dataset was analysed for statistically significant differences between compounds produced by trees which were grown under different temperature conditions for the duration of the infection.

Statistically significant differences in the data was determined using one-way analysis of variance (ANOVA) on log-transformed data, and Tukey's posthoc pairwise comparison test at a 95% confidence level using the LAERCIO package. For trial 1 data, significant correlations between the disease scores assigned to each genotype and mean relative abundance of compounds produced by each genotype were determined through linear regression and ANOVA. Graphs were generated in Excel (Microsoft, USA) and data are presented as means ± standard error (SE).

Data obtained from GCMS and LCMS analyses were also transformed by log transformation and reformatted for analysis using the MetaboAnalyst software, where the data was analysed for significant differences in metabolite concentration in the different clones and when inoculated with different strains of *F. circinatum*. MetaboAnalyst was used for the generation of principal component analysis (PCA) plots (Supplemental figure 2).

CHAPTER 3:

RESULTS

3.1 Trial 1: Effects of *F. circinatum* infection on LE and HE genotypes of *P. patula X P. tecunumanii* hybrids

3.1.1 Stem lesions induced by F. circinatum *infection on LE and HE genotypes* of P. patula X P. tecunumanii *hybrids*

In trial 1, *F. circinatum*-induced stem lesions in the *P. patula* X *P. tecunumanii* hybrids ranged from 1 to ≥60 mm. Multiple saplings also died throughout the course of the trial. On average, the disease severity of LE genotypes was lower compared to HE genotypes. This resulted in shorter lesions and subsequently lower disease scores (Table 9; Figure 2). A more severe disease response was noted for HE genotypes, with longer lesions and a higher mortality rate contributing to higher disease scores (Table 9; Figure 2).

Table 9. Disease scores and lesion lengths for each LE and HE genotype of the *P. patula* x *P. tecunumanii* hybrids. A disease score was assigned to each inoculated and control sample according to the length of the lesion that developed seven weeks post-inoculation with *F. circinatum* strain CMWF 24 or mechanical wounding (control samples), and a mean disease score was subsequently determined. SE = Standard error. Lesion lengths of 0 – 19mm = disease score of 0; 20 – 29mm = 1; 30 – 59mm = 2; 60mm – DEAD = 4.

Genotype	Average disease score			
	Control	Control SE	Inoculated	Inoculated SE
P1TH2	0,333	0,125	2,333	0,103
P5TH2	0	0	2,222	0,121
P7TH4	0	0	1,875	0,092
P10TH2	0,111	0,042	1,889	0,042
P10TH3	0	0	1,889	0,042
P5TL1	0,167	0,063	0,556	0,066
P7TL3	0,125	0,051	1,111	0,075
P9TL1	0	0	1,143	0,115

Average disease scores were used as a metric for susceptibility because a large range of lesion lengths were recorded for the different plants belonging to each genotype. Many plants also died as a result of the infection, necessitating a metric which could include plants that had no observable lesions (due to deterioration and overall discoloring of the dead stem) but were still clearly affected by the presence of the pathogen. Additionally, many control plants showed no observable lesion development. The disease scores therefore serve as a way to group the plants more broadly into four categories ranging from ‘the least diseased’ to ‘the most diseased’ rather than simply comparing the average length of the lesions.

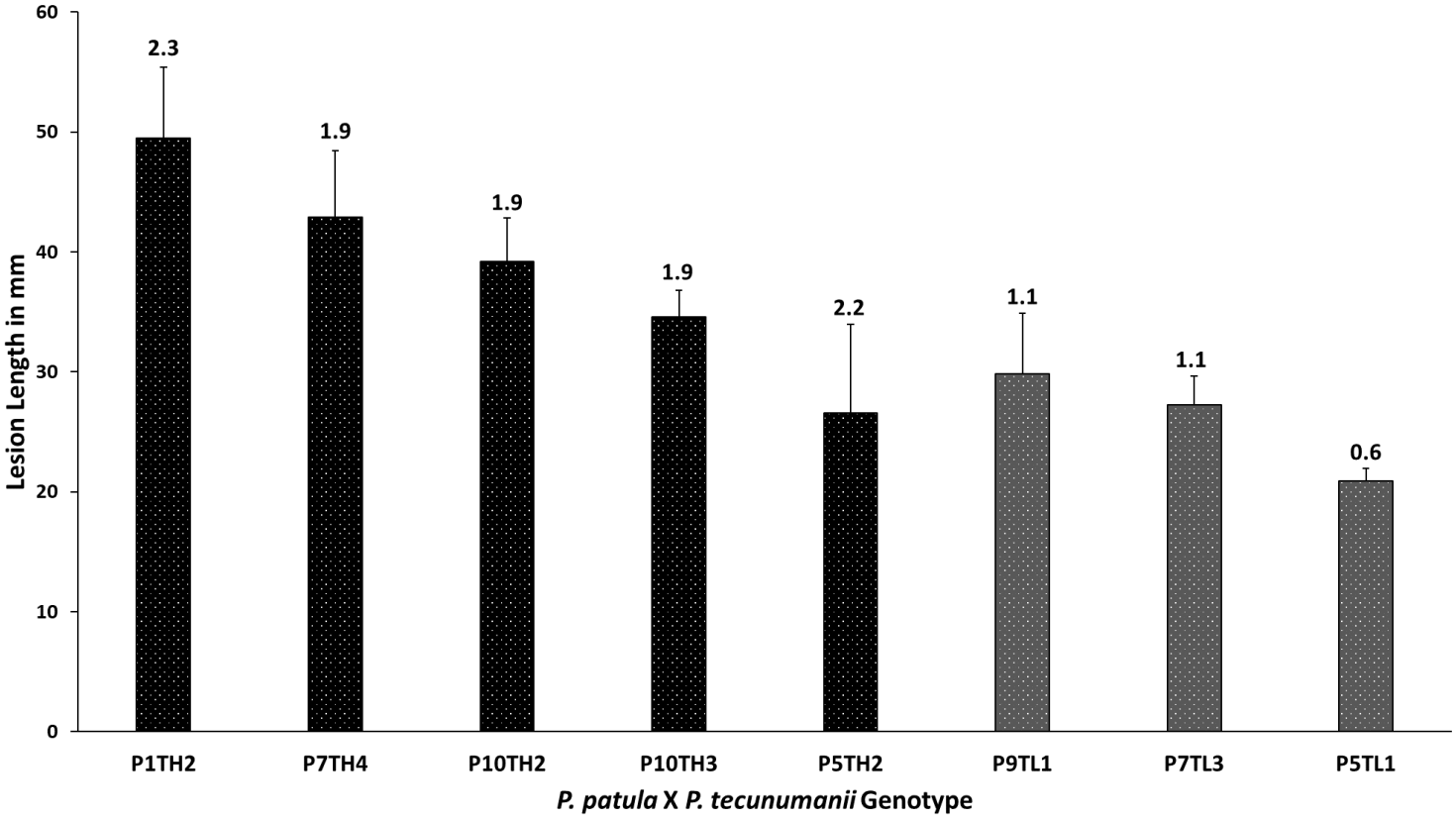


Figure 2. Mean lesion lengths and disease scores for the LE and HE genotypes of *P. patula* X *P. tecunumanii* hybrids (five HE genotypes and three LE genotypes) inoculated with *F. circinatum* strain CMWF 24. Lesion lengths were measured seven weeks after inoculation. Data presented in graphs are mean lesion lengths in cm + SE (*n* = refer to Table 6). Disease scores assigned to each genotype are displayed above the bars.

3.1.2 Relative abundances of phenolics and mono-, di- and sesquiterpenes in P. patula X P. tecunumanii *hybrids*

In trial 1, I identified and quantified the most abundant monoterpenes, sesquiterpenes, and diterpene resin acids in the hybrid genotypes. Amongst the monoterpenes, only α-pinene, β-phellandrene, and β-pinene were produced in large concentrations. α-Pinene was produced in far greater concentrations in the LE hybrids compared to the HE hybrids. The monoterpenes myrcene, camphene, and α-terpinene and sesquiterpenes longifolene, caryophyllene, and germacrene-D were produced in similar, comparatively small concentrations (Table 10). Amongst the diterpene resin acids, neoabietic- and dehydroabietic acid were most abundant. The remaining diterpene resin acids were produced in similar concentrations in both LE and HE genotypes.

Amongst the most abundant polar compounds produced, ten phenolic compounds were selected for further analysis. These phenolics include the stilbene pinosylvin, the flavonoids laricitrin 3-glucoside, dimethylstrobochrysin, catechin, and proanthocyanidin B1 (dimer of catechin), and the lignans (-)-nortrachelogenin and matairesinol. Three unknown phenolics (unknown with a molecular weight of 336 Da, 364 Da, and 378 Da) were also identified. Catechin was by far the most abundantly produced phenolic among both LE and HE hybrids, followed by the 336 Da and 364 Da unknowns. The remaining phenolics were produced in comparable concentrations (Table 10). For all the compound

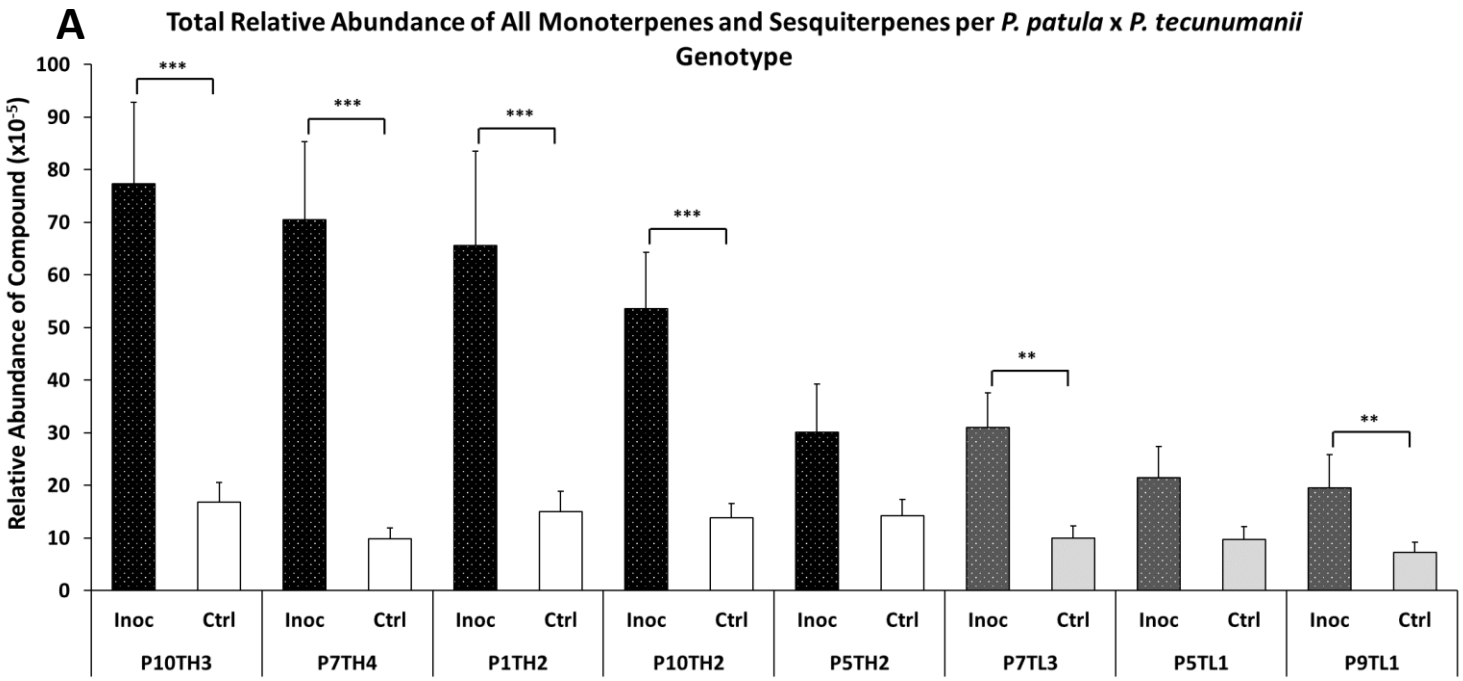
groups, control samples produced a higher proportion of the most abundant compound compared to the proportion of that compound produced by inoculated samples (Table 10).

Table 10. Proportions and retention times of each compound for identified monoterpenes and sesquiterpenes, diterpene resin acids, and phenolics for all HE and all LE genotypes of the *P. patula* x *P. tecunumanii* hybrids. Data displayed only includes that of samples inoculated with *F. circinatum* strain CMWF 24 and control (mechanically wounded) samples.

Mono- (M) and Sesquiterpenes (S)	Retention time (min)	High-elevation Genotypes		Low-elevation Genotypes	
		Inoculated	Control	Inoculated	Control
		Proportion of total identified mono- and sesquiterpenes			
α-Pinene (M)	8,19	49%	49%	58%	61%
β-Phellandrene (M)	11,05	38%	37%	28%	26%
β -Pinene (M)	9,45	8%	5%	9%	6%
β-Myrcene (M)	9,94	1%	2%	1%	1%
Camphene (M)	8,61	1%	1%	1%	1%
α-Terpinene (M)	12,87	1%	1%	1%	1%
Caryophyllene (S)	22,12	1%	2%	1%	2%
Longifolene (S)	21,77	1%	2%	1%	1%
Germacrene-D (S)	23,66	<1%	2%	<1%	1%
Diterpene resin acids		**Proportion of total identified diterpene resin acids**			
Dehydroabietic acid	13,16	35%	39%	34%	36%
Neoabietic acid	13,61	26%	30%	29%	30%
Levopimaric acid	13,05	10%	13%	15%	14%
Abietic acid	13,37	12%	7%	10%	8%
Isodextropimaric acid	12,82	10%	4%	6%	4%
Isopimaric acid	13,01	7%	7%	6%	8%
Phenolics		**Proportion of total identified phenolics**			
Catechin	16	45%	53%	47%	55%
Unknown 336	24	17%	11%	10%	8%
Matairesinol	22	6%	8%	8%	6%
Pinosylvin	24	2%	2%	8%	9%
Unknown 364	26	10%	7%	7%	5%
Laricitrin 3-glucoside	19	5%	8%	7%	8%
Dimethylstrobochrysin	24	7%	1%	5%	5%
Unknown 378	18	3%	4%	3%	2%
Proanthocyanidin B1	15	3%	5%	3%	5%
- (-)-Nortrachelogenin	21	2%	1%	2%	2%

3.1.3 Between-genotype comparison of total relative abundance of compounds produced

In trial 1, HE genotypes produced larger relative concentrations of phytochemicals (total abundance of monoterpenes and sesquiterpenes, diterpene resin acids, and phenolics), with genotypes P7TH4, P10TH3, P1TH2, and P10TH2 producing the highest levels in each compound category (Figures 3 A-C). LE genotype P9TL1 produced the lowest relative concentrations of mono- and sesquiterpenes, diterpene resin acids, and phenolics, followed by the LE genotype P5TL1 (Figures 3 A-C). LE genotype P7TL3 was also among those with the lowest levels of production, with the exception of phenolics, for which this genotype ranked third highest in relative abundance (Figures 3 A-C). In each compound group, significant differences were found between the total relative abundance of compounds produced by the inoculated saplings compared to the mock inoculated control (ANOVA; $p < 0.05$; Figures 3 A-C). Nearly all compounds were produced in significantly higher concentrations by the inoculated trees compared to the control trees. Among the mono- and sesquiterpenes, genotypes P5TH2 and P5TL1 showed no significant differences between inoculated trees and control trees (Figures 3 A-C). Among the phenolics, genotypes P10TH2, P10TH3, and P9TL1 showed no significant differences between inoculated trees and control trees (Figures 3 A-C).



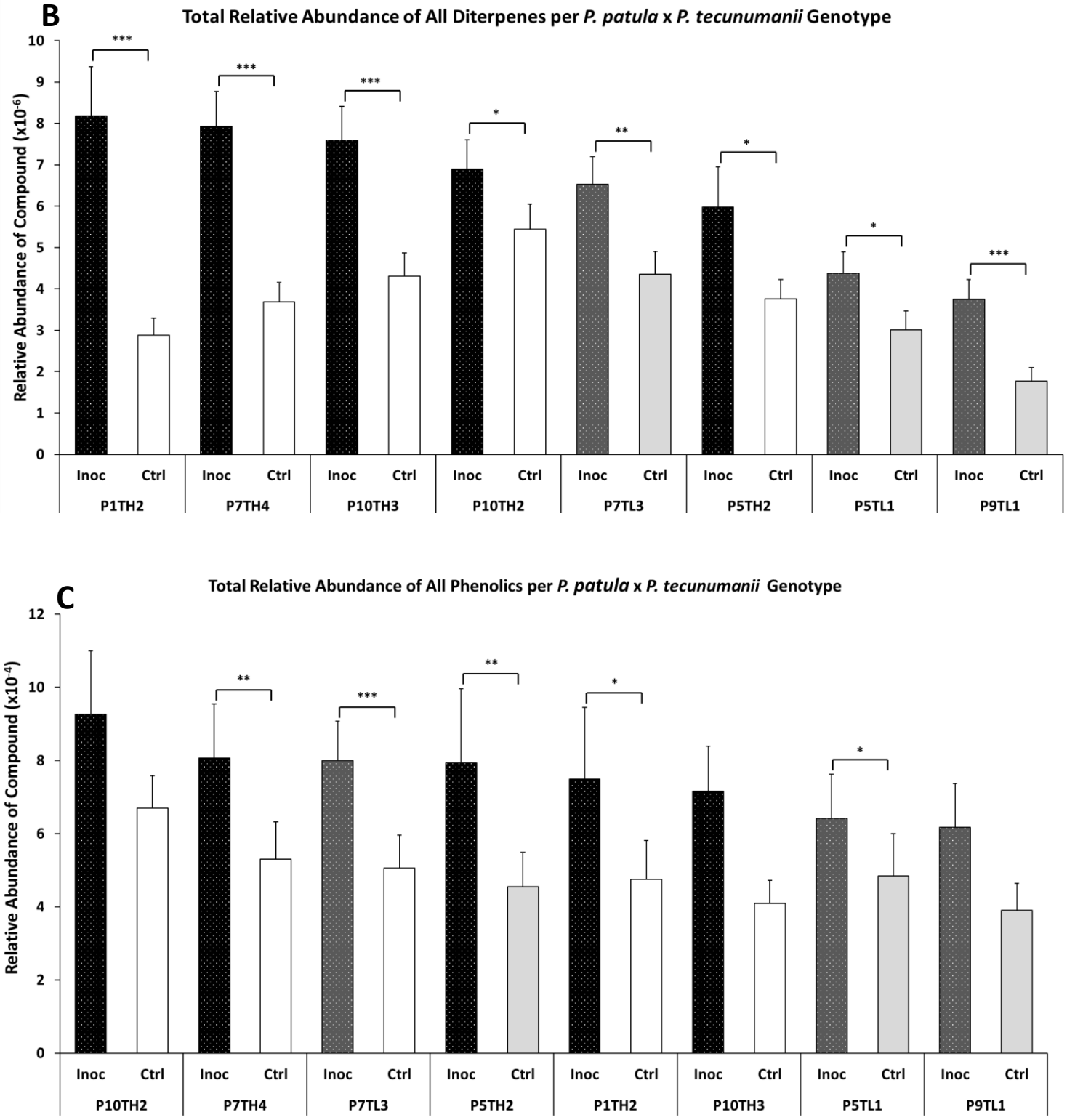


Figure 3. Total combined relative abundance of all monoterpenes and sesquiterpenes (A), all diterpene resin acids (B), and all identified phenolics (C) in *F. circinatum*-inoculated and uninoculated controls of LE and HE genotypes of *P. patula* x *P. tecunumanii* hybrids. Five HE genotypes and three LE genotypes were inoculated with *F. circinatum* strain CMWF 24; control samples were mechanically wounded. Stem tissue samples were harvested seven weeks after inoculation and analysed using GCMS and LCMS. Data was analysed using one-way ANOVA, and significant differences in the total relative abundance of compounds produced by the inoculated and control saplings are indicated above the bars (ANOVA; $p < 0.05$ *; $p < 0.01$ **; $p < 0.005$ ***). Data presented in all graphs are chromatogram base peak area means + SE (n = refer to Table 6). Inoc, Samples inoculated with *F. circinatum*; Ctrl, Mechanically wounded control samples; P10TH2, P10TH3, P7TH4, P5TH2, P1TH2, High-elevation *P. patula* x *P. tecunumanii* hybrids; P9TL1, P7TL3, P5TL1, Low-elevation *P. patula* x *P. tecunumanii* hybrids.

3.1.4 Correlation between disease score and relative abundance of individual compounds produced

A correlation analysis was conducted to determine if individual disease scores correlated with the concentration of phytochemicals produced by inoculated *P. patula* X *P. tecunumanii* hybrids. Generally, a higher disease score was associated with a higher abundance of phytochemicals produced by the pines. Amongst the nine most abundantly-produced mono- and sesquiterpenes, β-phellandrene, myrcene, α-terpinene, camphene, caryophyllene, and germacrene-D correlated positively with the disease score (ANOVA; $p < 0.05$; Figures 4 and 5). On the other hand, the monoterpenes α- and β-pinene and sesquiterpene longifolene did not correlate with the disease score (ANOVA; $p > 0.05$; Figures 4 and 5).

Amongst the six most abundantly-produced diterpene resin acids, the concentrations of dehydroabietic acid in the *P. patula x P. tecunumanii* genotypes correlated positively with an increase in the disease score (ANOVA; $p < 0.05$; Figure 6). For the other diterpene acids identified, namely abietic-, neoabietic-, isopimaric-, levopimaric-, and isodextropimaric acid, an increase in production was not correlated with the disease score (ANOVA; $p > 0.05$; Figures 6). Levopimaric acid showed a slight, but not significant, negative correlation with the with an increase in disease score (ANOVA; $p > 0.05$; Figure 6).

Amongst all 10 of the most abundant phenolics, no significant correlations with the disease scores could be found. Similar to the terpenes, however, the majority of identified phenolics (catechin, Unknown compounds 336, 364, and 378, dimethylstrobochrysin, and proanthocyanidin B1) showed slight, but insignificant positive correlations with the disease scores (ANOVA; $p > 0.05$; Figure 7). For the phenolics matairesinol, laricitrin-3-glucoside, pinosylvin, and -(-)- nortrachelogenin, there was a slight, but insignificant negative correlation with the disease scores (ANOVA; $p > 0.05$; Figure 7).

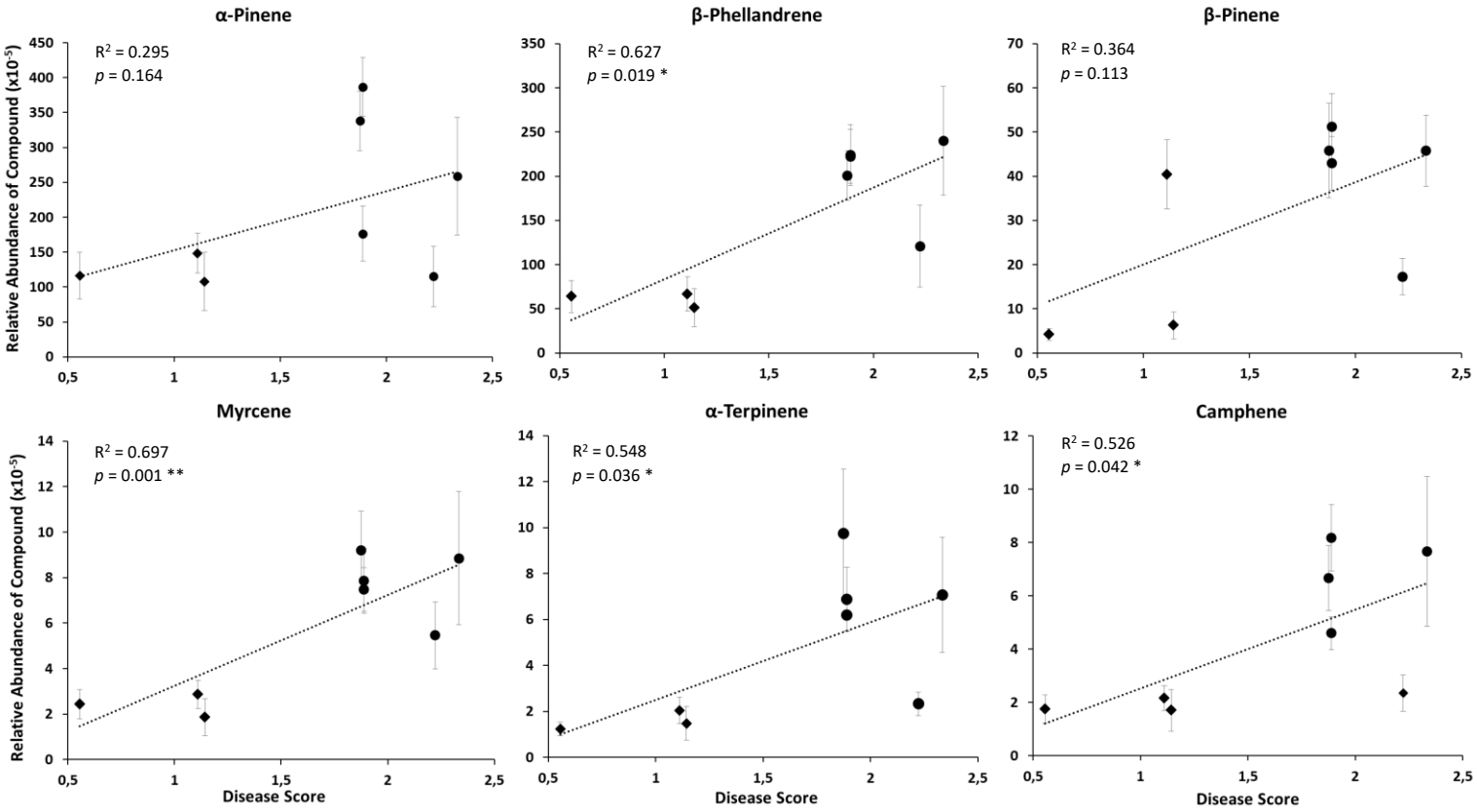


Figure 4. Analysis of the correlation between mean relative abundance of monoterpenes produced by and the disease score assigned to eight *P. patula* x *P. tecunumanii* genotypes indicates a general positive linear correlation between monoterpene production and disease score. *P. patula* x *P. tecunumanii* genotypes were inoculated with *F. circinatum* strain CMWF 24 and analysed by GCMS seven weeks post-inoculation. The determination coefficient (R^2) and probability of a significant relationship between the abundance of each compound and the disease score (*p*) are displayed on each graph (ANOVA; $p < 0.05$ *; $p < 0.01$ **; $p < 0.005$ ***). Data presented in all graphs are chromatogram base peak area means ± SE (*n* = refer to Table 6). HE, High-elevation; LE, Low-elevation. Key: ◆ = Low-elevation *P. patula* x *P. tecunumanii* hybrids ● = High elevation *P. patula* x *P. tecunumanii* hybrids

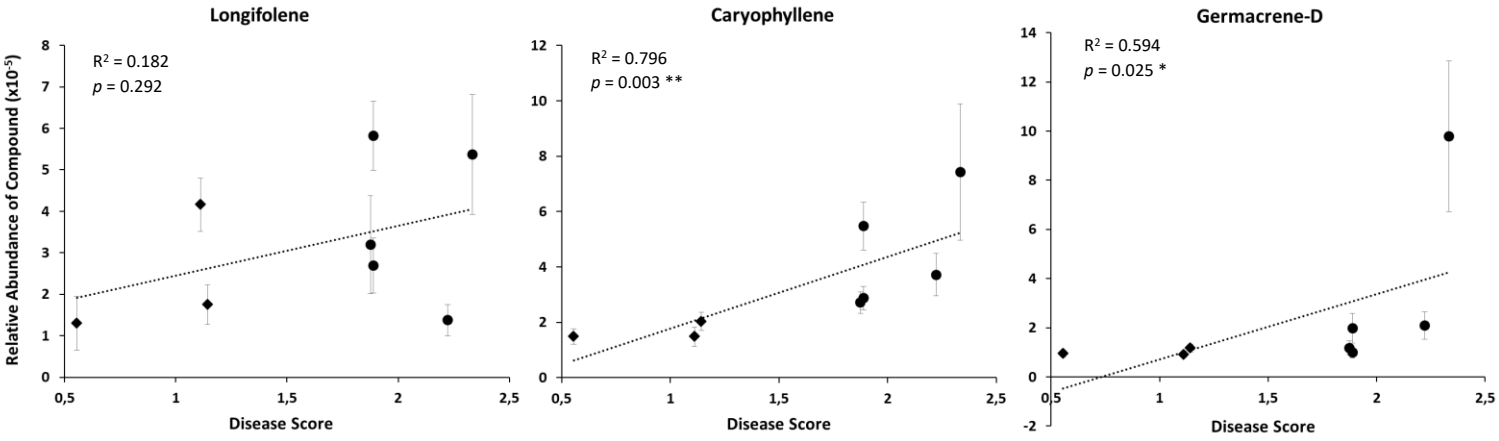


Figure 5. Analysis of the correlation between mean relative abundance of sesquiterpenes produced by and the disease score assigned to eight *P. patula* x *P. tecunumanii* genotypes indicates a general positive linear correlation between sesquiterpene production and disease score. *P. patula* x *P. tecunumanii* genotypes were inoculated with *F. circinatum* strain CMWF 24 and analysed by GCMS seven weeks post-inoculation. The determination coefficient (R^2) and probability of a significant relationship between the abundance of each compound and the disease score (*p*) are displayed on each graph (ANOVA; $p < 0.05$ *; $p < 0.01$ **; $p < 0.005$ ***). Data presented in all graphs are chromatogram base peak area means ± SE (*n* = refer to Table 6). HE, High-elevation; LE, Low-elevation. Key: ◆ = Low-elevation *P. patula* x *P. tecunumanii* hybrids ● = High elevation *P. patula* x *P. tecunumanii* hybrids

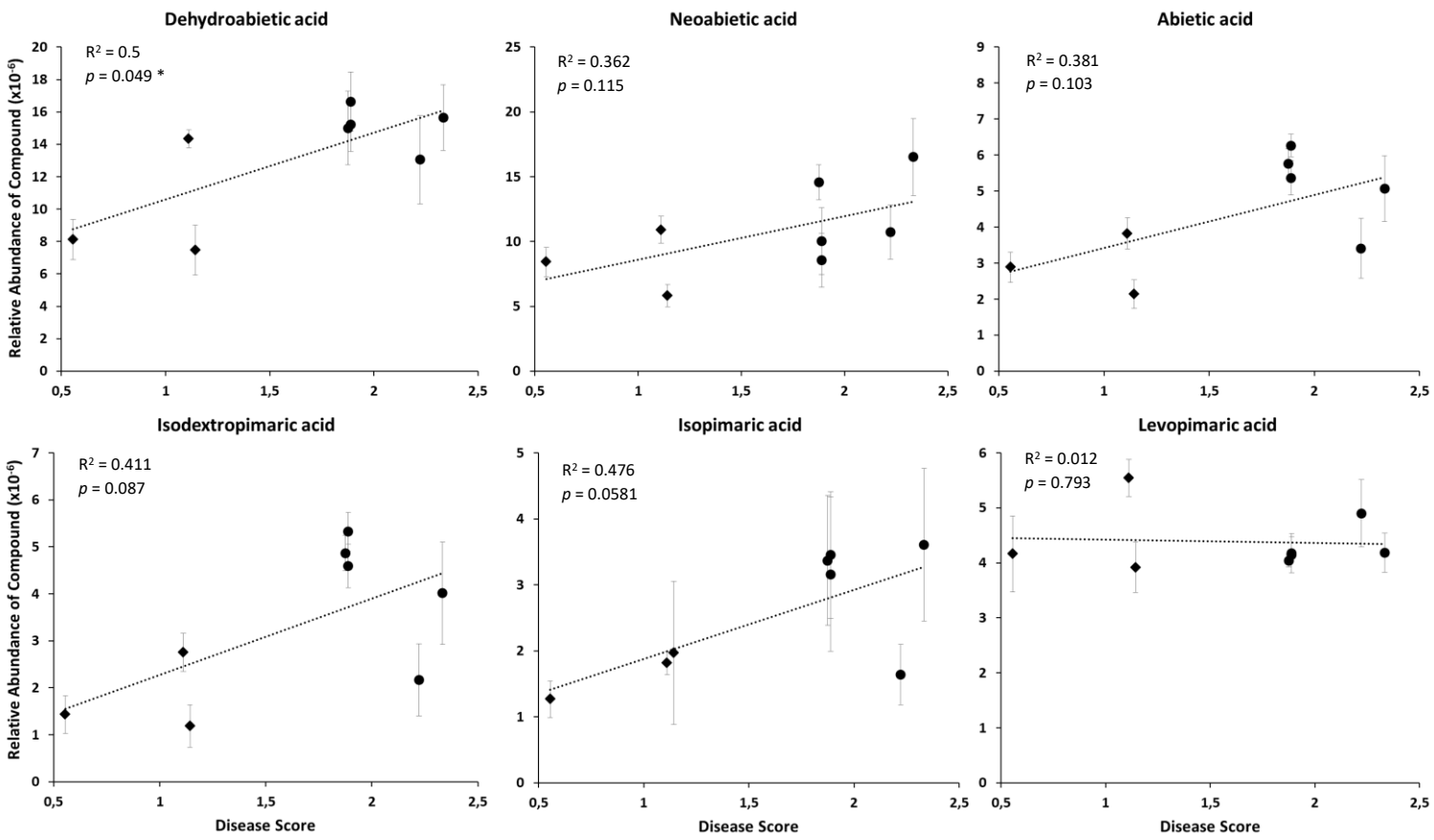


Figure 6. Analysis of the correlation between mean relative abundance of diterpene resin acids produced by and the disease score assigned to eight *P. patula* x *P. tecunumanii* genotypes indicates a general positive linear correlation between diterpene resin acid production and disease score. *P. patula* x *P. tecunumanii* genotypes were inoculated with *F. circinatum* strain CMWF 24 and analysed by GCMS seven weeks post-inoculation. The determination coefficient (R^2) and probability of a significant relationship between the abundance of each compound and the disease score (*p*) are displayed on each graph (ANOVA; $p < 0.05$ *; $p < 0.01$ **; $p < 0.005$ ***). Data presented in all graphs are chromatogram base peak area means ± SE (*n* = refer to Table 6). HE, High-elevation; LE, Low-elevation. Key: ◆ = Low-elevation *P. patula* x *P. tecunumanii* hybrids ● = High elevation *P. patula* x *P. tecunumanii* hybrids

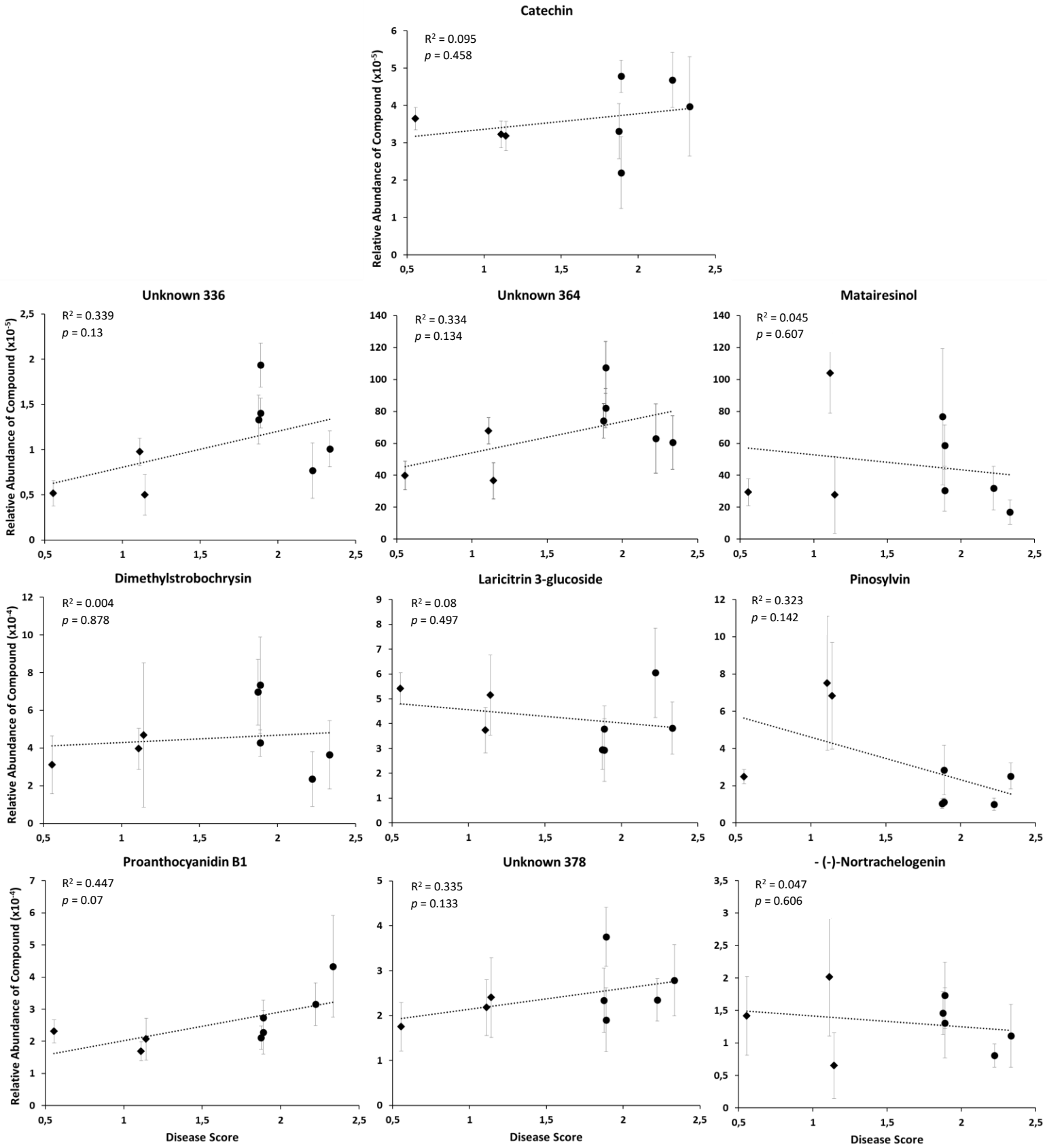


Figure 7. Analysis of the correlation between mean relative abundance of phenolics produced by and the disease score assigned to eight *P. patula* x *P. tecunumanii* genotypes indicates a general positive linear correlation between phenolic production and disease score. *P. patula* x *P. tecunumanii* genotypes were inoculated with *F. circinatum* strain CMWF 24 and analysed by LCMS seven weeks post-inoculation. The determination coefficient (R^2) and probability of a significant relationship between the abundance of each compound and the disease score (*p*) are displayed on each graph (ANOVA; $p < 0.05$ *; $p < 0.01$ **; $p < 0.005$ ***). Data presented in all graphs are chromatogram base peak area means ± SE (*n* = refer to Table 6). HE, High-elevation; LE, Low-elevation. Key: ◆ = Low-elevation *P. patula* x *P. tecunumanii* hybrids ● = High elevation *P. patula* x *P. tecunumanii* hybrids

3.2 Trial 2: Inoculation of LE genotypes of *P. patula X P. tecunumanii* hybrids with *F. circinatum*

3.2.1 Production of compounds by LE genotypes at three timepoints post-inoculation

To assess the manner in which production of the identified terpenes and phenolics changes during disease progression, two LE genotypes (P9TL9 and P7TL1) of *P. patula* x *P. tecunumanii* hybrids were inoculated with *F. circinatum* (strains CMWF 24 and CMWF 2615) (Trial 2, Table 7). GC-MS and LC-MS analyses were performed on the stem tissue samples collected at three timepoints post-inoculation (TPI), namely 5 days- , 14 days-, and 28 days post-inoculation (DPI).

PCA (principal component analysis, a statistical procedure which clusters data points based on their similarity to one another as a means to compare different variables) revealed no significant difference in the relative concentrations of phytochemicals produced by the two different genotypes (Supplemental figure 2: Pine genotypes). Similarly, PCA revealed no significant differences in production of phytochemicals between the trees inoculated with the two different strains of *F. circinatum* (Supplemental figure 2: Fungal isolates). As neither the LE genotype nor *F. circinatum* strain used for inoculation significantly impacted the production of phytochemicals over time, I analysed the data obtained from trial 2 as a single data set.

Among the monoterpenes (Figure 8) and sesquiterpenes (Figure 9), a clear trend in production could be observed, where compounds were produced in significantly smaller concentrations at the first TPI (5 DPI) compared to the second and third TPI (14 DPI and 28 DPI). For the majority of compounds, the second and third TPI did not differ significantly in terms of relative concentration of each compound produced (ANOVA; $p > 0.05$; Figures 8 and 9). The exceptions were the sesquiterpenes caryophyllene and germacrene-D, which decreased significantly in concentration between the first- and third TPI (ANOVA; $p < 0.05$; Figure 9). For all monoterpenes and the sesquiterpenes, there were no significant differences between production in inoculated and control samples at the first TPI. For the monoterpenes and the sesquiterpene longifolene, the significant differences between inoculated and control samples were only evident at the second- and third TPI (ANOVA; $p < 0.05$; Figures 8 and 9).

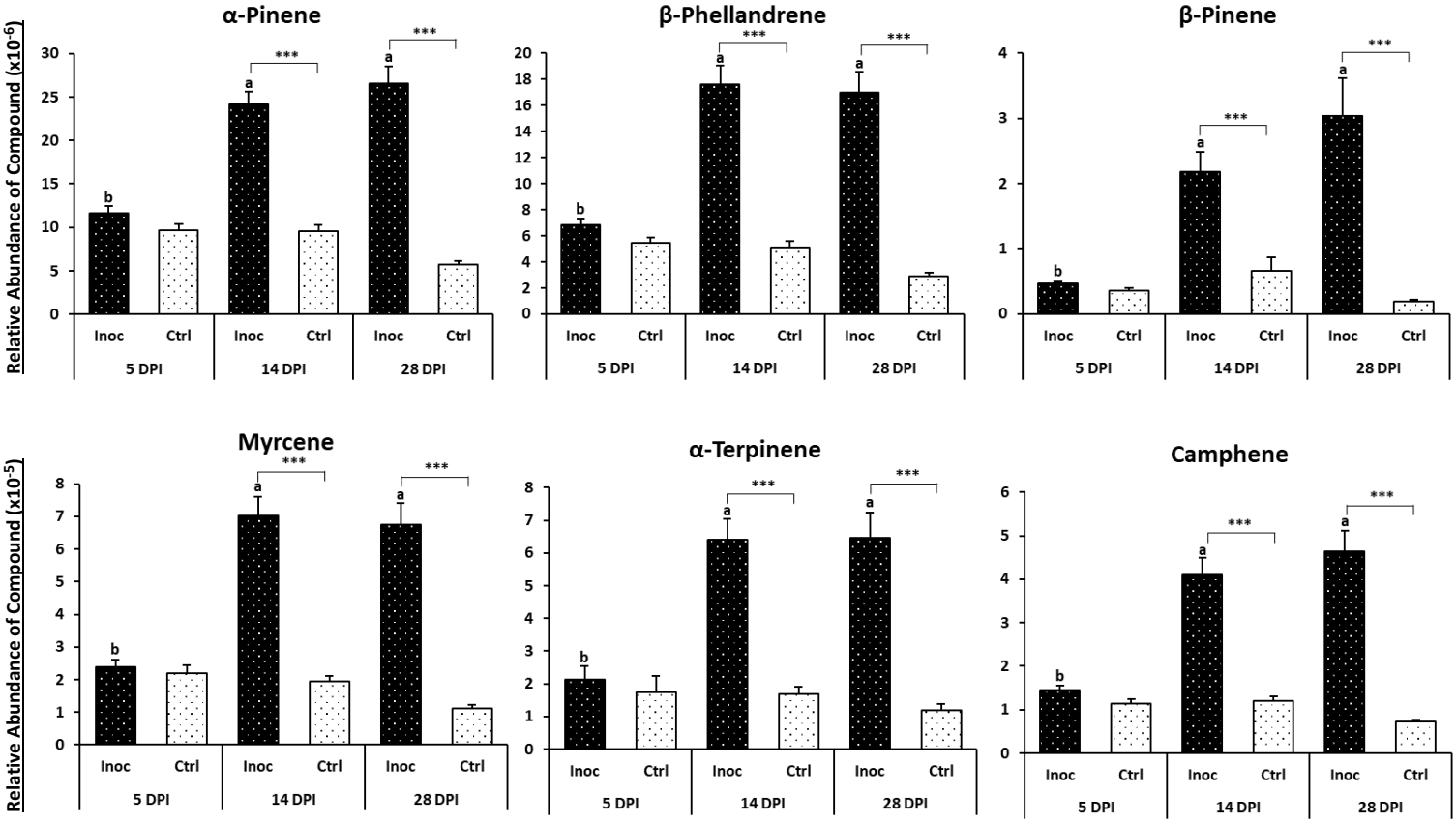


Figure 8. Relative abundance of the most abundant monoterpenes produced by LE *P. patula* x *P. tecunumanii* genotypes over a time course of 28 days after inoculation. Compounds are in the order of most- to least abundant. *Pinus* hybrid genotypes P9TL9 and P7TL1 were inoculated with *F. circinatum* strains CMWF 24 and CMWF 2615; control samples were mechanically wounded. At each timepoint post-inoculation (5 days, 14 days, and 28 days) stem tissue samples were harvested and analysed using GCMS. Bars with different letters indicate a significant difference between the relative phytochemical abundance between the inoculated samples at the three timepoints post-inoculation (Tukey's post-hoc test; $p < 0.05$ at 95 % confidence). Differences between inoculated and control samples at each timepoint post-inoculation are indicated on each graph (ANOVA; $p < 0.05$ *; $p < 0.01$ **; $p < 0.005$ ***). Data presented in all graphs are chromatogram normalized base peak area means + SE (n = refer to Table 7). DPI, Days post-inoculation; Inoc, Samples inoculated with *F. circinatum*; Ctrl, Mechanically wounded control samples.

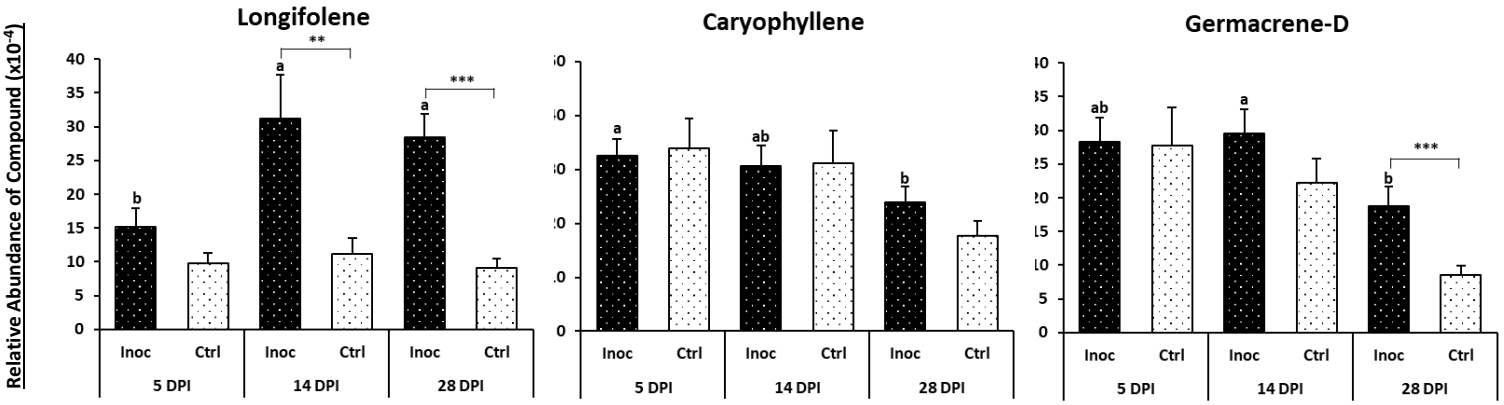


Figure 9. Relative abundance of the most abundant sesquiterpenes produced by LE *P. patula* x *P. tecunumanii* genotypes over a time course of 28 days after inoculation. Compounds are in the order of most- to least abundant. *Pinus* hybrid genotypes P9TL9 and P7TL1 were inoculated with *F. circinatum* strains CMWF 24 and CMWF 2615; control samples were mechanically wounded. At each timepoint post-inoculation (5 days, 14 days, and 28 days) stem tissue samples were harvested and analysed using GCMS. Bars with different letters indicate a significant difference between the relative phytochemical abundance between the inoculated samples at the three timepoints post-inoculation (Tukey's post-hoc test; $p < 0.05$ at 95 % confidence). Differences between inoculated and control samples at each timepoint post-inoculation are indicated on each graph (ANOVA; $p < 0.05$ *; $p < 0.01$ **; $p < 0.005$ ***). Data presented in all graphs are chromatogram normalized base peak area means + SE (n = refer to Table 7). DPI, Days post-inoculation; Inoc, Samples inoculated with *F. circinatum*; Ctrl, Mechanically wounded control samples.

Among the diterpene resin acids, all compounds except for isopimaric acid showed a similar trend in production – a significant increase only after the first TPI ($p < 0.05$), after which no significant difference could be found between production at the second and third TPI (ANOVA; $p > 0.05$; Figure 10). Neoabietic acid, the most abundantly produced resin acid, was produced in significantly higher concentrations at the third TPI compared to the first and second TPI (ANOVA; $p < 0.05$; Figure 10). There was a significant difference between controls and inoculated samples at all three TPI (ANOVA; $p < 0.05$; Figure 10).

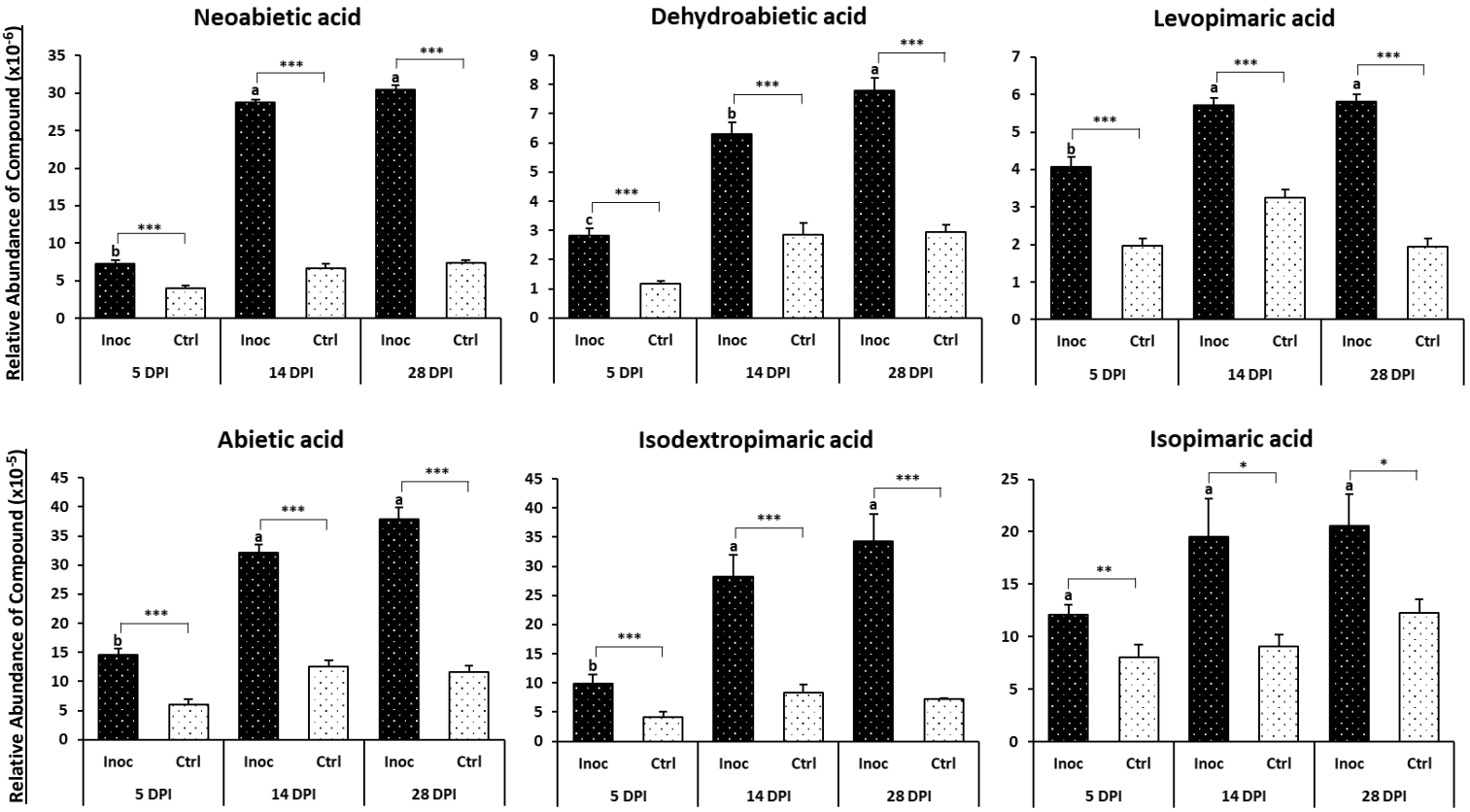


Figure 10. Relative abundance of the most abundant diterpene resin acids produced by LE *P. patula* x *P. tecunumanii* genotypes over a time course of 28 days after inoculation. Compounds are in the order of most- to least abundant. *Pinus* hybrid genotypes P9TL9 and P7TL1 were inoculated with *F. circinatum* strains CMWF 24 and CMWF 2615; control samples were mechanically wounded. At each timepoint post-inoculation (5 days, 14 days, and 28 days) stem tissue samples were harvested and analysed using GCMS. Bars with different letters indicate a significant difference between the relative phytochemical abundance between the inoculated samples at the three timepoints post-inoculation (Tukey's post-hoc test; $p < 0.05$ at 95 % confidence). Differences between inoculated and control samples at each timepoint post-inoculation are indicated on each graph (ANOVA; $p < 0.05$ *; $p < 0.01$ **; $p < 0.005$ ***). Data presented in all graphs are chromatogram normalized base peak area means + SE (n = refer to Table 7). DPI, Days post-inoculation; Inoc, Samples inoculated with *F. circinatum*; Ctrl, Mechanically wounded control samples.

Among the phenolics, the same compounds were identified as in trial 1, with the addition of epicatechin. No clear general trend in the production of phenolics could be observed. A minority of compounds, namely the 364 Da unknown, matairesinol, dimethylstrobochrysin, and -(-)-nortrachelogenin followed the same trend in production as the terpenes, with an increase in production after the first TPI and no change between the second and third TPI (Tukey's post-hoc test; $p < 0.05$; Figure 11). Catechin, epicatechin, and proanthocyanidin B1 did not significantly change in their relative concentrations across the three TPI. The 336 Da unknown, pinosylvin, and the 378 Da unknown showed an increase in production at the second TPI followed by a decrease in production at the third TPI (Tukey's post-hoc test; $p < 0.05$; Figure 11). Laricitrin-3-glucoside production increased significantly at the third TPI (Tukey's post-hoc test; $p < 0.05$; Figure 11). Most phenolics were produced in significantly higher concentrations in the inoculated and control samples at the third TPI, and a minority were produced in significantly higher concentrations at the second TPI (ANOVA; Figure 11).

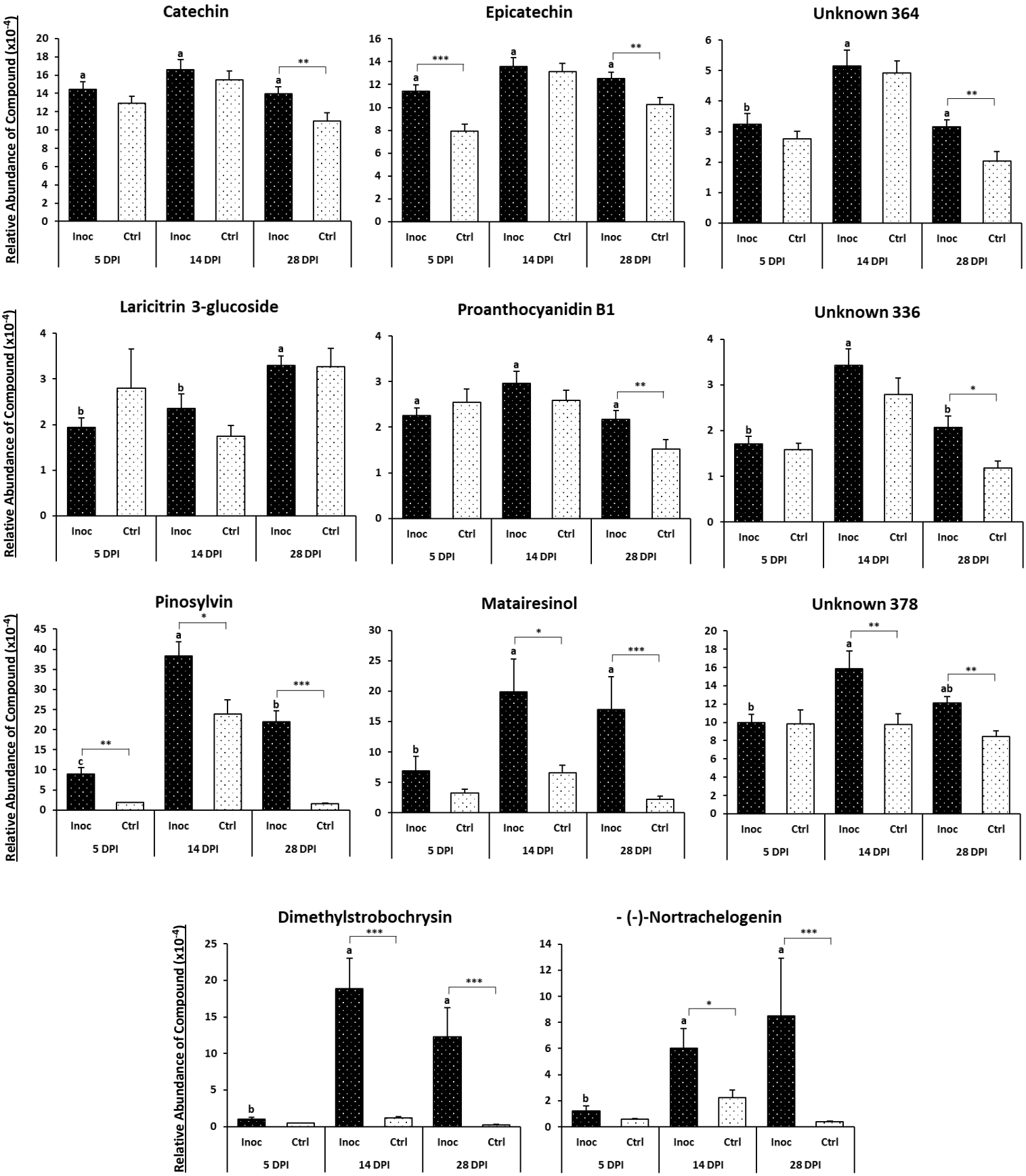

Catechin
Epicatechin
Unknown 364
Laricitrin 3-glucoside
Proanthocyanidin B1
Unknown 336
Pinosylvin
Matairesinol
Unknown 378
Dimethylstrobochrysin
- (-)-Nortrachelogenin
Relative Abundance of Compound (x10-4)
Inoc
Ctrl
5 DPI
14 DPI
28 DPI

Figure 11. Relative abundance of the most abundant phenolics produced by LE *P. patula* x *P. tecunumanii* genotypes over a time course of 28 days after inoculation. Compounds are in the order of most- to least abundant. *Pinus* hybrid genotypes P9TL9 and P7TL1 were inoculated with *F. circinatum* strains CMWF 24 and CMWF 2615; control samples were mechanically wounded. At each timepoint post-inoculation (5 days, 14 days, and 28 days) stem tissue samples were harvested and analysed using LCMS. Bars with different letters indicate a significant difference between the relative phytochemical abundance between the inoculated samples at the three timepoints post-inoculation (Tukey's post-hoc test; $p < 0.05$ at 95 % confidence). Differences between inoculated and control samples at each timepoint post-inoculation are indicated on each graph (ANOVA; $p < 0.05$ *; $p < 0.01$ **; $p < 0.005$ ***). Data presented in all graphs are chromatogram normalized base peak area means + SE (n = refer to Table 7). DPI, Days post-inoculation; Inoc, Samples inoculated with *F. circinatum*; Ctrl, Mechanically wounded control samples.

3.3 Trial 3: Comparison between *F. circinatum*-inoculated LE and HE genotypes of *P. patula X P. tecunumanii* hybrids grown under different greenhouse conditions

3.3.1 Relationship between genotype disease score and relative abundance of compounds produced under different temperature conditions

To investigate the connection between the disease score, growth temperature and relative production of phytochemicals, I conducted trial 3 in which *F. circinatum* inoculated *P. patula* x *P. tecunumanii* hybrid genotypes were subjected to different greenhouse conditions for a 7-week period post-inoculation. HE genotypes P10TH2 and P5TH2, as well as LE genotype P5TL1 (genotypes with relatively high, medium, and low levels of production of secondary compounds; Figure 12A – C) were inoculated with *F. circinatum* strain CMWF 24, with half of the saplings were grown at 23 °C and half at 28 °C (Table8; Table 11).

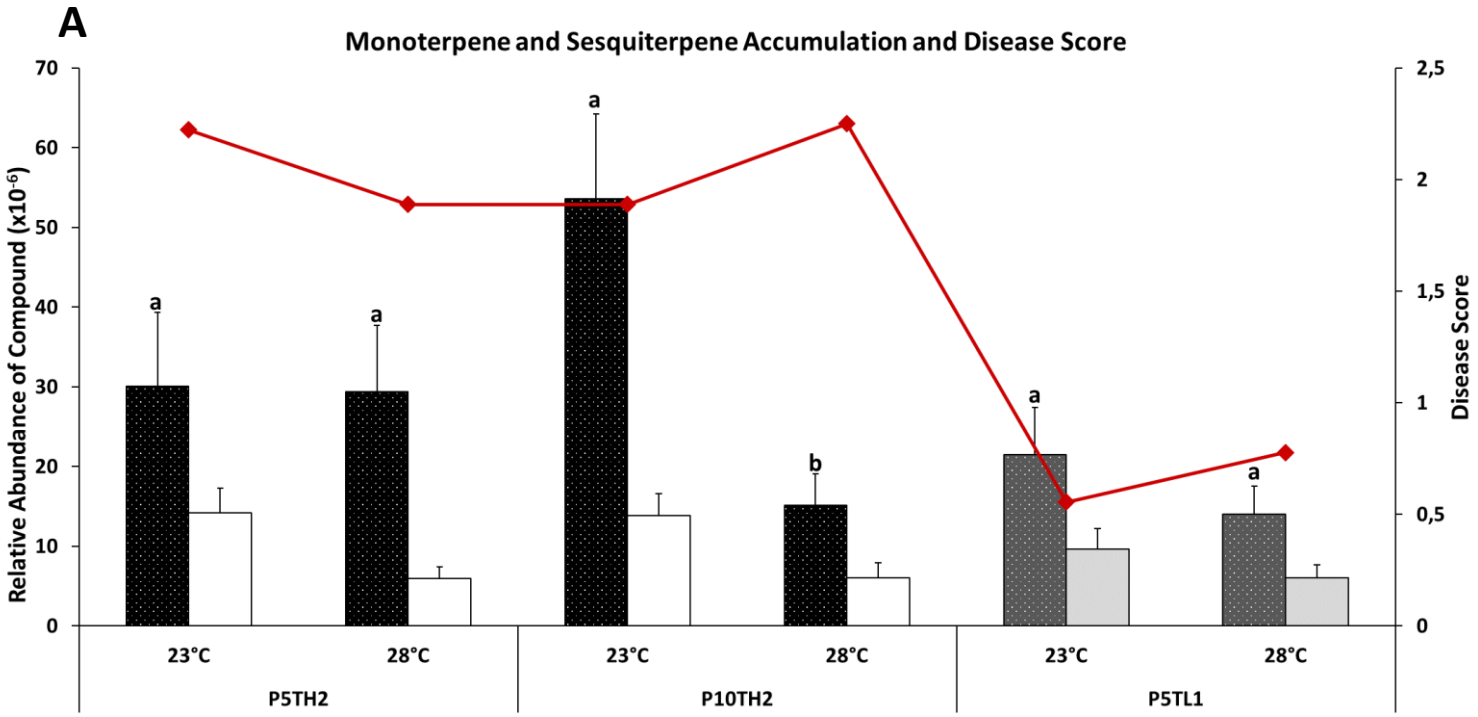
Table 11. Disease score for each LE and HE genotype of the *P. patula X P. tecunumanii* hybrids at two different growth temperatures (23 °C and 28 °C). A disease score was assigned to each inoculated and control sample according to the length of the lesion that developed seven weeks post-inoculation with *F. circinatum* strain CMWF 24 or mechanical wounding (control samples). SE = Standard error. Lesion lengths of 0 – 19mm = disease score of 0; 20 – 29mm = 1; 30 – 59mm = 2; 60mm – DEAD = 4.

Growth Temperature	Genotype	Average disease score			
		Control	Control SE	Inoculated	Inoculated SE
23°C	P10TH2	0,111	0,042	1,889	0,042
	P5TH2	0	0	2,222	0,121
	P5TL1	0,167	0,063	0,556	0,066
28°C	P10TH2	0	0	2,25	0,101
	P5TH2	0,111	0,042	1,889	0,042
	P5TL1	0	0	0,778	0,055

The relative concentration of phytochemicals produced under the two different temperature conditions only differed significantly between members of genotype P10TH2, where inoculated samples grown at 28 °C produced significantly lower concentrations of monoterpenes and sesquiterpenes and diterpene resin acids, but not phenolics (ANOVA; $p < 0.05$; Figure 12A – C). For members of the genotypes P5TH2 and P5TL1, there was no significant difference

between the relative concentration of phytochemicals produced under the different temperature conditions (ANOVA; $p > 0.05$; Figure 12A – C). However, although not statistically significant, slightly lower concentrations of phytochemicals were produced by the saplings grown at 28 °C compared to 23 °C in every case, a trend which can be observed even among the controls (Figure 12A – C).

For the genotype P5TH2, the disease score for inoculated samples decreased along with the decrease in production of phytochemicals under the higher temperature conditions. This trend can be observed for monoterpenes and sesquiterpenes, diterpene resin acids, and phenolics (Figure 12A – C). This trend is in accordance with the results displayed in section 3.1.4. Overall, the LE genotypes produced lower relative amounts of secondary compounds had similarly low disease scores (Figure 12A – C, and Figures 3 – 6). However, genotypes P10TH2 and P5TL1 showed a higher disease score for samples grown at 28°C, along with a decrease in relative abundance of compounds produced (Figure 12A – C). This trend can also be observed for mono- and sesquiterpenes, diterpene resin acids, and phenolics (Figure 12A – C). This result is different to the results seen in the correlation analysis and for the members of genotype P5TH2 in this analysis, lower levels of compound production at higher temperatures appear to lead to more severe disease symptoms.



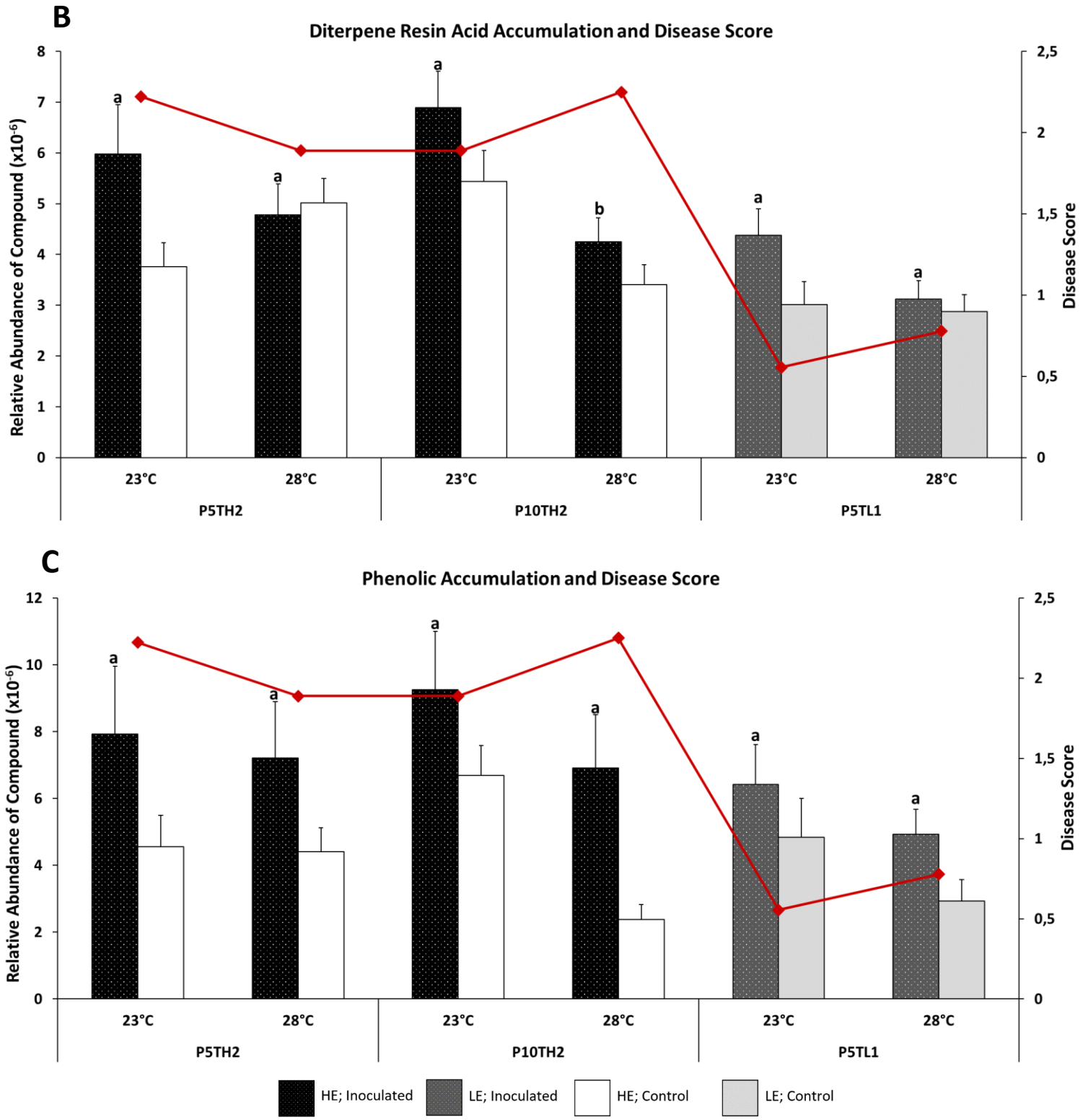


Figure 12. Comparison between the production of mono- and sesquiterpenes (A), diterpene resin acids (B), and phenolics (C) and disease score in three *P. patula* x *P. tecunumanii* genotypes grown at two different growth temperatures post-inoculation with *F. circinatum* strain CMWF 24. Saplings were either grown in a greenhouse maintained at 23 °C, or in a greenhouse maintained at 28 °C. After seven weeks, lesion measurements were taken and stem tissue samples were harvested and analysed using GCMS (terpenes) and LCMS techniques (phenolics). Data presented in all graphs are chromatogram base peak area means + SE (*n* = refer to Table 8) and disease scores. For each individual genotypes, bars with different letters indicate significant differences between the relative abundance of compounds produced by the trees grown at 23 °C vs those grown at 28 °C (Tukey's post-hoc test; $p < 0.05$ at 95 % confidence). Trendline shows trend in disease score between samples incubated at different temperatures. Inoc, Samples inoculated with *F. circinatum*; Ctrl, Mechanically wounded control samples; HE, High-elevation; LE, Low-

CHAPTER 4:
DISCUSSION

4.1 Phytochemical production in LE and HE genotypes of *P. patula* X *P. tecunumanii* hybrids

Few studies have been conducted on the composition of terpenes in Southern pine species such as *P. patula* and *P. tecunumanii,* and none on the phytochemical composition of the hybrids of these two species. In this study, I identified a range of terpenes and phenolics in *P. patula* X *P. tecunumanii* stem tissue samples seven weeks after mechanical wounding or inoculation with *F. circinatum.* Overall, inoculated saplings produced significantly higher concentrations of individual phytochemicals compared to the mechanically wounded controls. However, the ratios between the concentrations of different phytochemical were comparable between the control and the inoculated saplings, indicating that individual phytochemicals were upregulated by a similar order of magnitude upon induction (Table 10). The terpene profile obtained for the *P. patula* X *P. tecunumanii* hybrids was comparable to that observed in other *Pinus* species (Marpeau *et al.*, 1989). For example, similar to previous reports, both wounded and inoculated saplings produced the monoterpene α-pinene at a significantly higher concentration than the other monoterpenes and sesquiterpenes (Klepzig *et al.*, 1995, Tognetti *et al.*, 1997, Manninen *et al.*, 2002). The sesquiterpenes identified in our study were produced at significantly lower concentrations. This is consistent with previous inoculation studies that also find that sesquiterpenes were produced in far lower concentrations compared to monoterpenes and diterpenes in other *Pinus* species (Marpeau *et al.*, 1989, Manninen *et al.*, 2002).

Pine diterpene resin acids such as levopimaric-, abietic-, and neoabietic acid are typically produced in higher concentrations constitutively, relative to the lower constitutive concentrations of dehydroabietic acid (Anderson *et al.*, 1969, Lewinsohn *et al.*, 1993, Lange *et al.*, 1994, Manninen *et al.*, 2002, Keeling & Bohlmann, 2006). However, I found that dehydroabietic- and neoabietic acid production was the highest, and levopimaric acid production was significantly lower. Evidence suggests that resin acids such as levopimaric acid are unstable and oxidize into abietic acid, which then oxidizes into dehydroabietic acid in the presence of air (Enoki, 1976). Considering that our study was based on mechanical wounding of the outer stem of the saplings, it is thus possible that the resin acids oxidized to form dehydroabietic acid upon exposure to the air, which could result in the higher concentration of this compound in my samples.

The diterpene resin acids were produced more abundantly by both control and inoculated saplings in my study. The higher production of diterpene resin acids can be attributed to the fact that I analyzed stem tissue in our study. Certain diterpene resin acids are produced more abundantly in the stems of pines compared to other tissues such as the needles, which is likely due to the higher concentration of resin found in the stems (Manninen *et al.*, 2002). Compared to monoterpenes that are produced in similar amounts in the needles and stems of, for example, *P. sylvestris,* diterpene resin acids are thought to play a more important role as defence compounds against organisms such as bark boring insects that mechanically wound the stem (Manninen *et al.*, 2002).

Studies on the composition of pine phenolics are limited, and no study has been conducted in either *P. patula* or *P. tecunumanii*. Here, I identified 11 phenolic compounds, including stilbenes, flavonoids, lignans, and unknown phenolics in *P. patula* X *P. tecunumanii* hybrids, which I identified through LCMS analysis and cross reference to studies done on other conifer species. The most abundant phenolic in our study was the flavan-3-ol catechin. Catechin has also been reported in Norway spruce (*Picea abies*) as an abundant, bioactive defence metabolite with anti-fungal activity (Hammerbacher *et al.*, 2014). However, in Norway spruce the stilbene, astringin, is significantly more abundant than catechin (Hammerbacher *et al.*, 2011). I also detected the stilbene pinosylvin in the stems of the *P. patula* X *P. tecunumanii* hybrids, but this phytochemical was significantly less abundant than the other phenolics identified in our study.

4.2 Changes in phytochemical production after inoculation over time in LE genotypes of *P. patula* X *P. tecunumanii* hybrids

Phytochemicals of wounded control saplings in our study did not significantly increase throughout the 28-day trial period. Similarly, monoterpene and diterpene resin acid production in *Pinus contorta* saplings remained constant between 4 and 12-days post-wounding (Croteau, 1987). Pan *et al.* (2018) also observed no increase in monoterpene concentration in wounded *Pinus yunnanensis* trees, between 10-, 20-, and 30 days post-wounding. In contrast, in mechanically wounded *P. pinaster* trees, α- and β-pinene production increased 24-fold and 10-fold respectively over 54 days (Marpeau et al., 1989). However, Marpeau *et al*. (1989) studied twigs of mature pine trees, while our study and that of Croteau (1987) focused on young pine saplings wounded on the leader stem, while Pan et al. (2018) inoculated the leader stems of 30-year old pines. Differences observed in the wound response might therefore be attributed to plant age, as well as the position of the wound (leader stem vs twigs) or the time interval between wounding and analysis (4-30 days vs 54 days).

At 5 days post-inoculation with *F. circinatum*, the phytochemical concentrations in our control and inoculated saplings did not significantly differ. However, at 14 days post-inoculation, significant differences were found between wounded and inoculated samples. Similar to our findings, significant differences in phytochemical accumulation in wounded and fungus-inoculated trees were observed at the earlier stages of infection in multiple previous studies on pines (Cheniclet, 1987, Croteau, 1987, Pan *et al.*, 2018). For example, in wounded *P. yunnanensis* trees and trees infected by blue-stain fungi, significant differences in the production of certain monoterpenes were already observed at 10 days post-inoculation (Pan *et al.*, 2018). Furthermore, significant differences in monoterpene and diterpene resin acid production between wounded and *Grosmannia clavigera*-inoculated *P. contorta* saplings could be observed at 8 days post-inoculation (Croteau, 1987). Interestingly, fungus-derived pathogen-associated molecular patterns (PAMPs) (such as proteinase inhibitor inducing factor and chitosan) application induced significantly higher terpene production compared to fungal inoculation alone, also between 4 and 12 days post-application (McFarland & Ryan, 1974, Croteau, 1987). Therefore, it is evident that the time-lag between fungal inoculation of trees and a chemical defence response

is typically less than 14 days in pine. Further research should be done to understand the early onset of the response in resistant and susceptible varieties of pine to determine if an earlier response is more effective in defending against *F. circinatum* infection.

In inoculated saplings, overall phytochemical production increased significantly between 5 days and 14 days post-inoculation, but between 14 days and 28 days post-inoculation, there was no significant increase in the production of most phytochemicals. Similarly, in *P. yunnanensis* trees inoculated with three different species of blue-stain fungi, monoterpene concentration did not increase significantly between 10 days- and 30 days post-inoculation (Pan *et al.*, 2018). This apparent plateau or partial decline in phytochemical concentration could potentially be attributed to the pathogen that degrades defence chemicals, or metabolizes them as a carbon source. Fungal pathogens such as *E. polonica* and *G. clavigera* have been observed to produce enzymes that enables them to utilize phenolics and terpenes produced by their plant hosts as substrates for energy metabolism (Hammerbacher *et al.*, 2013, Haridas *et al.*, 2013, Wadke *et al.*, 2016). Phenolic degradation has the added advantage of serving as a detoxifying mechanism, thereby allowing the pathogen to further colonize the woody tissues of the tree (El Hadrami *et al.*, 2015, Wadke *et al.*, 2016).

4.3 Differences in disease response between LE and HE genotypes of *P. patula* X *P. tecunumanii* hybrids

The LE *P. patula* x *P. tecunumanii* genotypes analysed in this study developed shorter lesions, had lower mortality rates, and were more resistant to *F. circinatum* infection compared to HE genotypes. LE *P. tecunumanii* and its LE hybrid crosses are known to be more resistant to *F. circinatum* infection and to develop shorter lesions (Mitchell *et al.*, 2012, Mitchell *et al.*, 2013). Studies focused on *P. tecunumanii* LE and HE seedlings and hybrid crosses with *P. patula* found very little variation in the severity of the disease response to *F. circinatum* among LE provenances or hybrids. Significant variation was, however, found among HE provenances and hybrids (Mitchell *et al.*, 2012, Mitchell *et al.*, 2013). This suggests that the level of *F. circinatum*-tolerance of a HE *P. patula* X *P. tecunumanii* hybrid cross, could be increased by selecting a more resistant HE *P. tecunumanii* as a parent.

The overall concentration of phytochemicals produced by LE genotypes in our study was significantly lower compared to HE genotypes. Unexpectedly, the more susceptible HE genotypes with higher disease scores also produced higher relative amounts of phytochemicals in response to *F. circinatum* infection. Interestingly, pines that are susceptible to *F. circinatum* infection have been observed to respond with similar increases in phytochemical concentration, resin production, and traumatic resin duct formation (Martín-Rodrigues *et al.*, 2013). In measuring the effect of *F. circinatum* infection in *P. radiata* seedlings, the volume of exuded resin and the density of traumatic resin ducts in the xylem increased significantly over the course of 56 days (Martín-Rodrigues *et al.*, 2013). Overcoming this defence response, the infecting *F. circinatum* exploited the newly-formed traumatic resin ducts for vertical colonization through the area around the inoculation point (Martín-Rodrigues *et al.*, 2013). Similar studies have also concluded that pine resin ducts are utilized by *F. circinatum* for vertical proliferation, and studies on the closely-related

Norway spruce found that a higher number of traumatic resin ducts are produced by susceptible clones in response to *H. annosum* inoculation (Barrows-Broaddus & Dwinell, 1984, Krekling *et al.*, 2004, Martín-Rodrigues *et al.*, 2013). The production of resin in response to *F. circinatum* infection could therefore be proportional to the degree of susceptibility of the tree to the infection (Enebak & Stanosz, 2003, Kim *et al.*, 2010, Martín-Rodrigues *et al.*, 2013). This would explain the negative correlation between low phytochemical production and disease severity seen in the current study – that is, a more susceptible tree will produce a larger quantity of resin and therefore more phytochemicals. It is therefore possible that the more resistant LE *P. patula* X *P. tecunumanii* hybrids in our study produced fewer resin ducts, less resin, and subsequently a lower concentration of phytochemicals than the more susceptible HE hybrids, explaining their reduced susceptibility.

The direct effects of individual phytochemicals on the growth and proliferation of *F. circinatum* was not evaluated in our study. However, related research showed that the effectiveness of defensive phytochemicals could depend on the virulence of the pathogen, and that a greater tolerance to pine resin could potentially contribute to the pathogenic nature of *F. circinatum* (Friel *et al.*, 2007, Slinski *et al.*, 2015). For example, avirulent *Fusarium temperatum* is more severely inhibited by *P. radiata* resin and isolated monoterpenes than the virulent *F. circinatum* in terms of radial growth, spore germination and survival, and dry weight accumulation (Slinski *et al.*, 2015). The authors of that study theorized that *F. temperatum'*s inability to cause disease in pines could be its significantly lower rate of spore survival upon resin exposure (Eckhardt *et al.*, 2009, Slinski *et al.*, 2015). This difference in spore survival rate was less notable when exposed to volatile monoterpenes, indicating that diterpene-rich oleoresin could have a bigger inhibitory effect than volatile monoterpenes alone (Slinski *et al.*, 2015). Among individual monoterpenes tested, α-pinene had the weakest inhibitory effect on the two *Fusarium* species. Limonene and camphene had the strongest inhibitory effects. In our study, camphene comprised roughly 1% of the identified monoterpenes and sesquiterpenes among inoculated and control samples, while limonene was not detected at all.

The differences between the resistant LE and susceptible HE genotypes observed in this study are likely a result of differences on a genetic scale. In 2021, a study utilizing dual RNA-seq found that resistant *P. pinea* and susceptible *P. radiata* had different gene regulatory responses to *F. circinatum* infection (Zamora-Ballesteros *et al.*, 2021). A quicker defence response through elaborate transcriptional reprogramming in the early stages of infection benefitted *P. pinea.* Furthermore, resistant *P. pinea* upregulated significantly more genes than susceptible *P. radiata,* including those involved in terpene biosynthesis, regulation of the phenylpropanoid pathway, phytohormone signaling, and R protein production. A delayed response in the early stages of infection and the expression of fewer defence-related genes in *P. radiata* likely led to weaker defence responses at later stages of infection, such as less-effective phytohormone biosynthesis and cell-reinforcement through lignification (Zamora-Ballesteros *et al.*, 2021). However, although Zamora-Ballesteros *et al.* (2021) found an upregulation of the genes responsible for phytochemical production in more resistant trees, our study clearly showed that the biosynthesis of phytochemicals may not be important for an effective defence response. On the other hand, many other defence-related processes are also set into motion when *F. circinatum* infection is initiated. These responses, such as rapid phytohormone signaling and early-

onset of lignification, might be more effective than the phytochemical response when it comes to preventing fungal proliferation and future research in this field would be beneficial (Paniagua *et al.*, 2017, Zamora-Ballesteros *et al.*, 2021).

4.4 Effect of growth temperature on the phytochemical production of *P. patula* X *P. tecunumanii* hybrids

It is not known how climate change will affect the production of phytochemicals in pines. In our study, an overall decrease in phytochemical production and disease severity was observed at higher growth temperatures, although this decrease was only significant for terpene production in a single HE *P. patula* X *P. tecunumanii* hybrid. Interestingly, constitutive concentrations of phytohormones in *P. sylvestris* seedlings decreased significantly in response to a 1 °C increase in growth temperature, but similar to our results, a significant decrease in concentrations of phenolics such as lignin, pinosylvin, and astringin was observed in the current-year stems of these seedlings (Ghimire *et al.*, 2019). The authors speculated that an increase in growth and reduced carbon allocation to phenolic production could be responsible for this phenomenon (Kivimäenpää *et al.*, 2017, Ghimire *et al.*, 2019). Similar elevated-temperature conditions resulted in the decrease in concentrations of flavonoids in the bark and needles of Norway spruce seedlings, as well as in proanthocyanidins and other phenolics in the needles of *P. sylvestris* trees (Räisänen *et al.*, 2008, Virjamo *et al.*, 2014, Ghimire *et al.*, 2019). However, contrasting results on the effect of higher temperatures on the production of phytochemicals have also been reported. For example, at warmer temperatures, constitutive phenolics such as proanthocyanidins and flavonoids in the previous-years' wood of *P. sylvestris* increased (Ghimire *et al.*, 2019). Furthermore, in seedlings of *P. sylvestris* and Norway spruce, a higher terpene concentration in the current-year needles and stems were observed at 23 °C compared to 16 °C (Sallas *et al.*, 2003). Additionally, significant increases in the production of certain terpenes were observed in the needles of *P. sylvestris* seedlings, but not in the stems (Sallas *et al.*, 2003). The authors proposed that elevated temperature conditions causes an increase in monoterpene production in the needles, which facilitates elevated terpene emission into the atmosphere that occurs in trees grown at higher temperatures (Guenther *et al.*, 1991, Sallas *et al.*, 2003). It is thought that elevated terpene emissions from needles protect the plant from heat stress by scavenging reactive oxygen species that potentially interfere with photosynthesis (Guenther *et al.*, 1991, Singsaas *et al.*, 1997, Tian *et al.*, 2020).

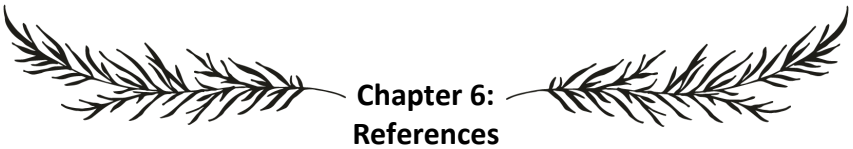
No clear deductions could be made on the effect of higher temperatures on the susceptibility of *P. patula* X *P. tecunumanii* hybrids to *F. circinatum* in our study. However, it is known that HE varieties had better *F. circinatum* tolerance on cooler, high-elevation planting sites. In contrast, LE varieties were more tolerant on warmer, low-elevation sites (Kanzler *et al.*, 2014). Climate change may thus increase the susceptibility of HE hybrids while not affecting the resistance of LE hybrids, but further research is required to verify these predictions.

Chapter 5:

Conclusion

The fungal pathogen *F. circinatum* is one of the most devastating threats to South African pine-based forestry and is responsible for significant annual economic losses. The development of *Pinus* hybrids that are tolerant to infection by this fungus, in addition to having desirable wood quality and growth properties, is an ongoing goal of this industry. Significant variation is known to exists among different species and provenances of pine in their relative susceptibility to *F. circinatum* infection. However, the underlying mechanisms contributing to pine's ability to successfully evade or overcome infection is not well understood. Pine trees respond to *F. circinatum* infection through increase in resin production and the formation of traumatic resin ducts, as well as an upregulation in the production of phytochemicals with known fungicidal and fungistatic properties such as terpenes and phenolics. However, evidence suggests that this pathogen can successfully resist and overcome the effects of these physical and chemical defences. For example, *F. circinatum* has been observed to utilize preformed and traumatic resin ducts for proliferation throughout the tree and to gain access to nutrients. In our study, most phytochemicals produced in response to *F. circinatum* infection were produced less abundantly by the more resistant LE *P. patula* X *P. tecunumanii* hybrids. For a number of the phytochemicals, specifically terpenes found predominantly in the resin, a higher concentration significantly correlated with an increase in disease severity. My initial hypothesis that higher defence compound production is associated with increased *F. circinatum* resistance in pines must therefore be rejected. Additionally, although the connection between a warmer growth environment and disease severity as determined by lesion formation was inconclusive, I found an overall lower concentration of phytochemicals in pines in response to a 5 °C increase in growth temperature.

Future research on the seemingly contradictory disease response of these more resistant pines would shed more light on the underlying mechanisms of the pine-*F. circinatum* interaction. Potential avenues to explore include investigating whether *F. circinatum* degrades pine-produced terpenes and phenolics to gain further access to woody tissues, or whether it utilizes these phytochemicals as carbon sources. If this would be the case, the link between successful fungal degradation of these phytochemicals and how it affects tree health should also be considered. Potential differences in the early phytochemical defence response between resistant and susceptible *P. patula* X *P. tecunumanii* hybrids should also be investigated, and whether an earlier defence response could potentially be responsible for pines successfully overcoming *F. circinatum* infection.



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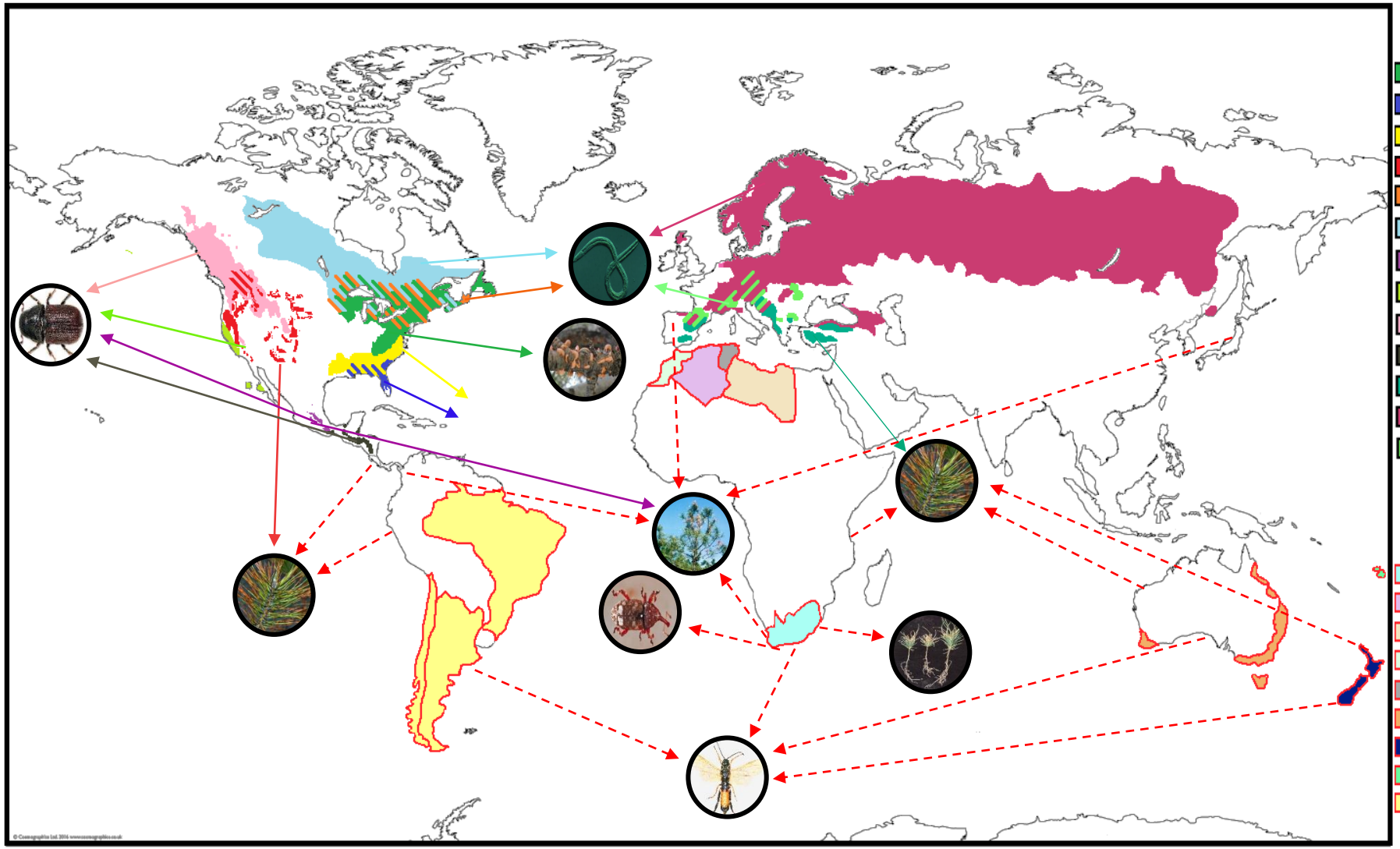
Chapter 7:
Supplemental Figures



Key: Native ranges of *Pinus sp.*

- *Pinus strobus*
- *Pinus elliottii*
- *Pinus taeda*
- *Pinus ponderosa*
- *Pinus resinosa*
- *Pinus banksiana*
- *Pinus patula*
- *Pinus radiata*
- *Pinus contorta*
- *Pinus tecunumanii*
- *Pinus nigra*
- *Pinus sylvestris*
- *Pinus mugo*

Key: Locations of non-native *Pinus sp.* plantations

- South Africa: *Pinus patula, P. elliottii, P. radiata*
- Algeria: *P. halepensis, P. brutia*
- Libya: *P. halepensis, P. brutia*
- Morocco: *P. halepensis, P. brutia*
- Tunisia: *Pinus halepensis, P. brutia*
- Australia: *Pinus radiata, P. caribaea, P. oocarpa*
- New Zealand: *Pinus radiata*
- Fiji: *Pinus caribaea*
- South America: *Pinus radiata, P. elliottii, P. taeda*

Key: Fungal diseases and pathogens of *Pinus sp.*


White pine blister rust (*Cronartium ribicola*)


Diplodia blight (*Diplodia pinea*)


Pitch canker (*Fusarium circinatum*)


Dothistroma needle blight (*Dothistroma pini* and *D. septosporum*)


Fusiform rust (*Cronartium quercuum* f. sp. *fusiforme*)

Key: Insect and nematode pests of *Pinus sp.*


Sirex woodwasp (*Sirex noctilio*)

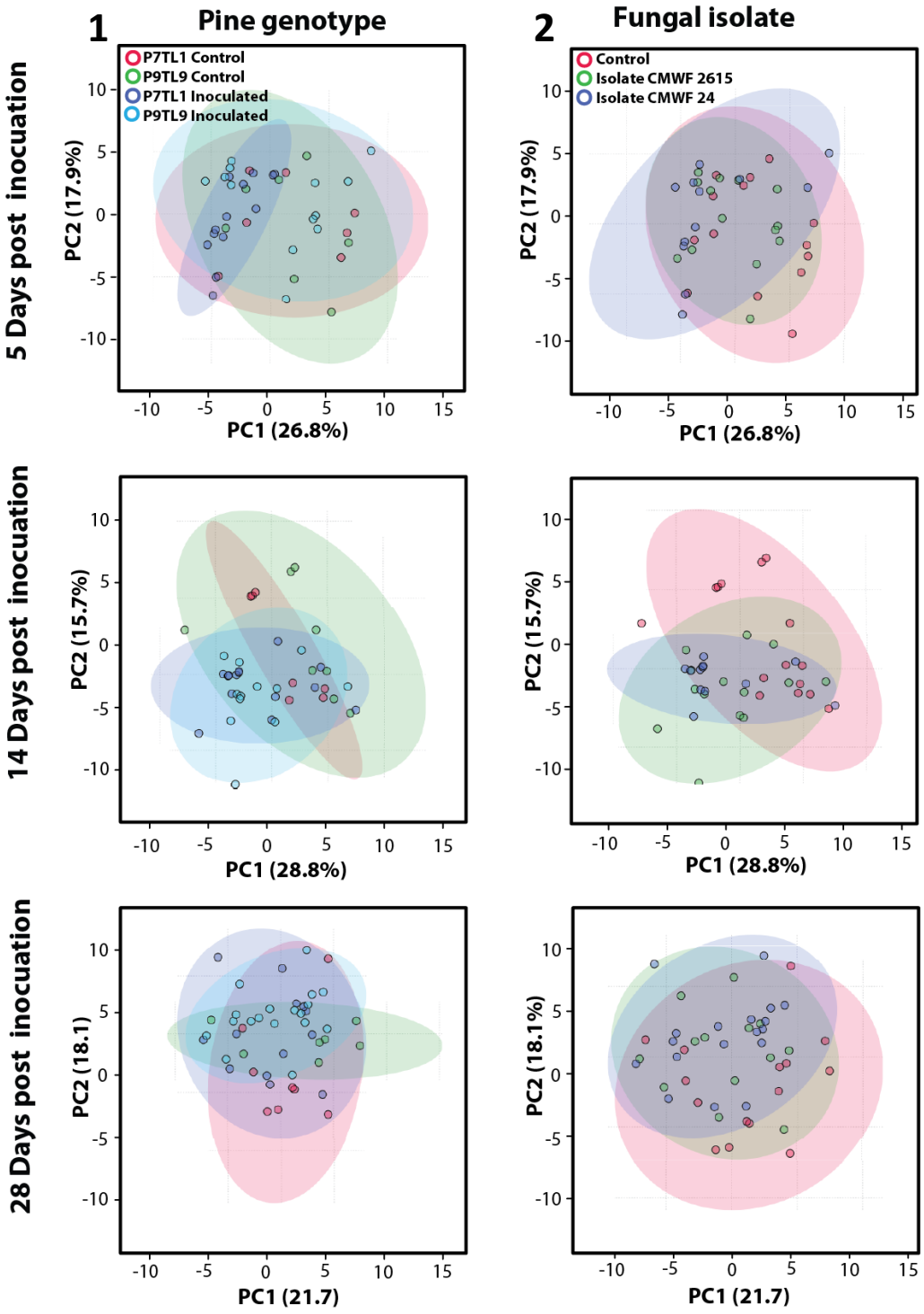

Deodar weevil (*Pissodes nemorensis*)


Mountain pine beetle (*Dendroctonus ponderosae*)

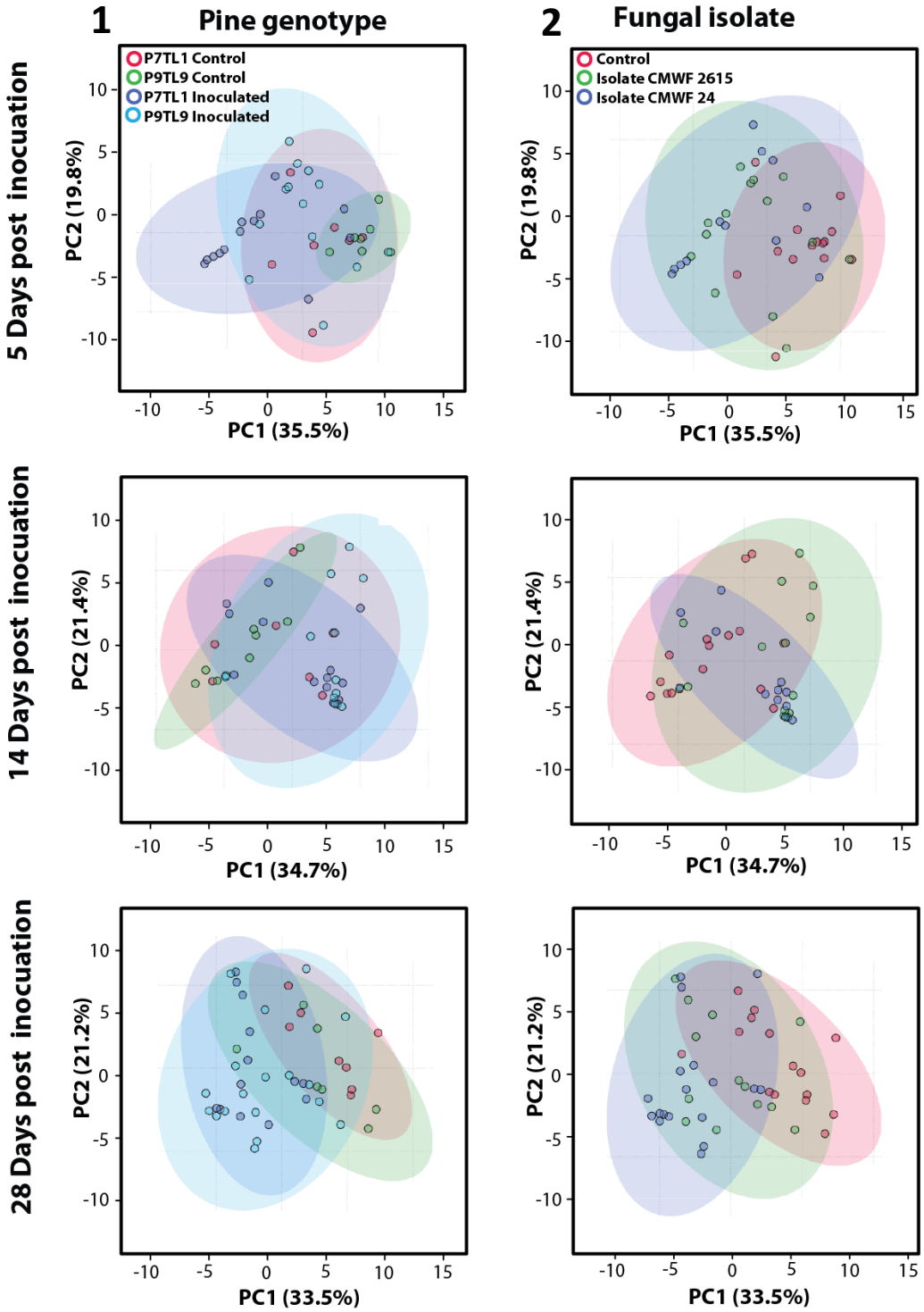

Pine wilt disease (*Bursaphelenchus xylophilus*)

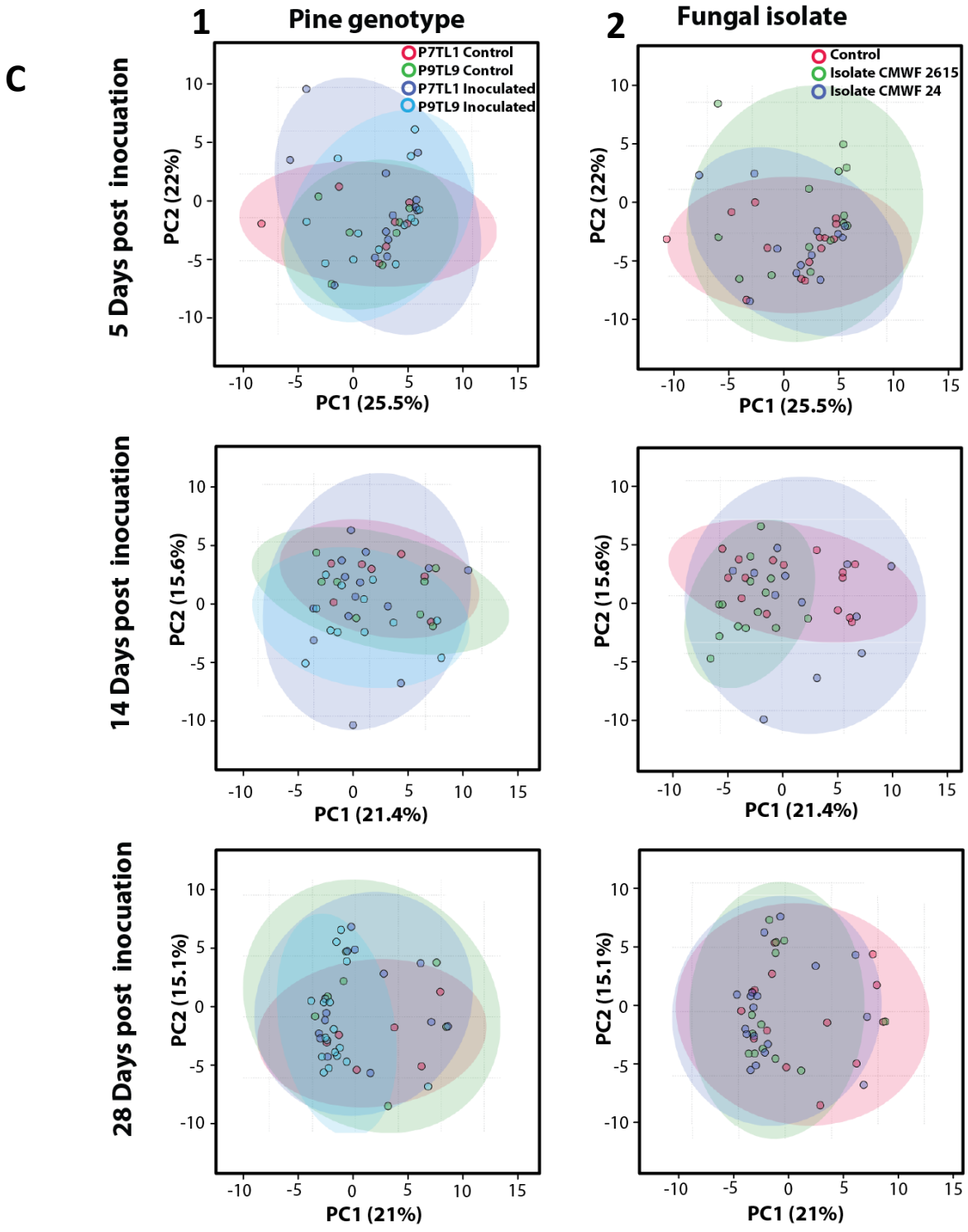
Supplemental figure 1. Map detailing the native ranges and locations of non-native *Pinus sp.* plantations and the causal agents of pine diseases prevalent at each location. Colored map locations outlines in red indicate non-native pine plantations. Map locations are a rough estimate and are not drawn to scale.

A


1
Pine genotype
2
Fungal isolate
P7TL1 Control
P9TL9 Control
P7TL1 Inoculated
P9TL9 Inoculated
Control
Isolate CMWF 2615
Isolate CMWF 24
5 Days post inocuation
14 Days post inocuation
28 Days post inocuation
PC1 (26.8%)
PC2 (17.9%)
PC1 (28.8%)
PC2 (15.7%)
PC1 (21.7)
PC2 (18.1)
PC2 (18.1%)

B


1
Pine genotype
2
Fungal isolate
P7TL1 Control
P9TL9 Control
P7TL1 Inoculated
P9TL9 Inoculated
Control
Isolate CMWF 2615
Isolate CMWF 24
5 Days post inocuation
14 Days post inocuation
28 Days post inocuation
PC1 (35.5%)
PC2 (19.8%)
PC1 (34.7%)
PC2 (21.4%)
PC1 (33.5%)
PC2 (21.2%)



Supplemental figure 2. Trial 3: Principal component analysis (PCA) plots indicating the relationship between different *P. patula* x *P. tecunumanii* genotypes (A_1, B_1, and C_1) and between samples inoculated with different strains of *F. circinatum* (A_2, B_2, C_2) through their mean mono- and sesquiterpene (A), diterpene resin acid (B), and phenolic (C) production. Overlap of clusters in each plot show that the different variables (genotype, and inoculant) could be distinguished from the controls, but not from one another (ANOVA; $p < 0.05$). *Pinus* hybrid genotypes P9TL9 and P7TL1 were inoculated with *F. circinatum* strains CMWF 24 and CMWF 2615; control samples were mechanically wounded. At each timepoint post-inoculation (5 days, 14 days, and 28 days) between 5-7 replicates of stem tissue samples were harvested and analysed using GCMS and LCMS. Metabolic data was analysed and PCA plots were created using MetaboAnalyst software.