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The partial characterisation of the terpene synthase genes implicated in the defence response by *Pinus patula* and *Pinus tecunumanii* to *Fusarium circinatum*

by

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Declaration

I, Robyn Leigh Smith, declare that this dissertation, which I hereby submit for the degree *Magister Scientiae* 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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Abbreviations

aa	Amino acid
ANOVA	Analysis of variance
BLAST	Basic local alignment search tool
bp	Base pairs
BP	Biological process
BR	Biological replicate
BUSCO	Benchmarking Universal Single-Copy Orthologs
CK	Cytokinin
CPM	Counts Per Million
CPS	Copalyl Synthase
DE	Differentially Expressed/ Differential Expression
Di-TPS	Diterpene synthase
DMAPP	Dimethylallyl diphosphate
DNA	Deoxyribonucleic acid
dpi	days post inoculation
DXOP	Deoxyxylulose-5-phosphate
et al.	*et alia*
ETI	Effector triggered immunity
f. sp.	*forma specialis*
FABI	Forestry and Agricultural Biotechnology Institute
FAO	Food and Agriculture Organization
FES	Forestry Economics Services
FMG	Forest Molecular Genetics
FPKM	Fragments per kilobase of transcript per million fragments mapped
FSA	Forestry South Africa
Gb	Gigabase
GDP	Gross Domestic Product
GO	Gene ontology

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GRI	Genomics Research Institute
HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme A
HR	Hypersensitive response
HSD	Honestly significant difference
I / Inoc	Inoculated
IPP	Isopentenyl diphosphate
IspS	Isoprene synthase
KEGG	Kyoto encyclopaedia of genes and genomes
km	kilometers
KO	Kegg orthology
KS	Kaurene synthase
LE	Low elevation
LRR	Leucine-rich repeat
MAMP	Microbe-associated molecular pattern
MANOVA	Multivariate analysis of variance
MEP	Methylerythritol-4-phosphate
M / Mock	Mock-inoculated
MonoTPS	Monoterpene synthase
mRNA	Messenger RNA
MVA	Mevalonic-acid
NCBI	National centre for biotechnology information
NGS	Next-Generation sequencing
NIFA	National Institute of Food and Agriculture
NO	Nitric oxide
NRF	National Research Foundation
nt	Nucleotide(s)
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PE	Paired-end
Pipt	*Pinus patula*
Pnte	*Pinus tecunumanii*
PR	Pathogenesis-related
PRR	Pattern recognition receptor
PTI	Pattern triggered immunity
RT-PCR	Real-time reverse transcription PCR

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R gene/protein	Resistance gene/protein
RAPD	Random amplification of polymorphic DNA
RBH	Reciprocal best hit
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
RQI	RNA quality index
rRNA	Ribosomal RNA
Sesqui-TPS	Sesquiterpene synthase
SNP	Single nucleotide polymorphism
spp.	species
TAIR	The Arabidopsis Information Resource
TPM	Transcripts per million
TPS	Terpene synthase
UniProtKB	Universal protein resource knowledge base
USD	US dollars
var.	Variety

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Preface

Pines are of substantial economic importance. Pitch canker disease caused by *Fusarium circinatum* represents a significant threat to many parts of the world. Research has shown that certain *Pinus spp.* are particularly susceptible to this phytopathogen, predominantly as seedlings. Unfortunately, the commercial pine species grown most extensively in South Africa, *Pinus patula*, is highly susceptible. Post-planting survival of *Pinus patula* seedlings has been severely reduced due to the seedling form of this disease; *Fusarium*-wilt. Presently, there is no effective way to control infections by *F. circinatum*, but hybridisation of *P. patula* with other relatively *Fusarium*-tolerant species, such as *Pinus tecunumanii*, has shown promise. However, the mechanisms, which underlie *P. tecunumanii's* relative resistance, remains uncertain.

Chapter 1 is a literature review, putting the research chapter into the context of existing knowledge. The review includes a description of the importance of *Pinus spp*., the products supplied by these species, and their defensive mechanisms. The defence induced synthesis of secondary metabolites in *Pinus spp*. is discussed, focusing on the productions of terpenes by terpene synthase enzymes. This chapter outlines the issues faced by commercial *Pinus patula* plantations in Southern Africa as a result of the phytopathogen *Fusarium circinatum.*

Chapter 2 is a research chapter, which describes the differences observed in induced transcriptional responses to infection by two *Pinus* species with contradistinctive susceptibility to infection by *Fusarium circinatum*. The partial characterisation of the terpene synthase genes which are differentially regulated in *Pinus patula* and *Pinus tecunumanii*, are assessed for their possible contribution toward *F. circinatum* susceptibility.

Chapter 3 is the concluding remarks, a review of what was observed during this research, as well as describing the future research prospects which could augment the findings of this study.

The outcomes of this research were presented as the following conference poster:

Smith, R., Visser, E., Coetzee, M., and Naidoo, S., 2018. The terpene synthase genes implicated in the defence response by *Pinus patula* and *Pinus tecunumanii* to *Fusarium circinatum.* Joint South African Society for Bioinformatics (SASBi) South African Society for Genetics (SAGS) Congress 2018. 16 – 18 October. Golden Gate Hotel, Golden Gate Highlands National Park, South Africa.

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Chapter 1

Literature review

The putative role of terpenes in defence against a fungal pathogen in pine seedlings

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Pitch canker disease, caused by *Fusarium circinatum* (teleomorph *Gibberella circinata*) Nirenberg and O'Donnell 1998, is regarded as one of the most significant species affecting commercial *Pinus* plantations worldwide (Wingfield *et al.* 2008; Reynolds and Gordon 2019). Herein we review the ecological and agricultural importance of pines, and consider the conjecture surrounding *Pine spp*. with varying levels of susceptibility to *F. circinatum.* Particularly, we consider the impact of this on South African agriculture, where *Pinus patula* has been the predominant commercial *Pine* spp. for many decades due to its propensity to grow in a cooler climate (Hodge and Dvorak 2007; Mitchell *et al.* 2011). Unfortunately, *P. patula* is particularly susceptible to *F. circinatum,* and this disease has resulted in major seedling losses since its introduction in the 1990s (Wingfield *et al.* 1999; Coutinho *et al.* 2007).

Research has implicated quantitative and qualitative characteristics of the terpene profile as a determinants of the susceptibility of *Pinus spp.* to *Fusarium* infection (Bohlmann and Way 2012; Bullington *et al.* 2018; Neis *et al.* 2018). A complex combination of terpene molecules form the predominant constituents of conifer volatile emissions, as well as serving as antimicrobial and antiherbivory components in oleoresin (Abbas *et al.* 2017; Celedon and Bohlmann 2019). Terpene biosynthesis has been shown to be induced by herbivore damage, pathogen recognition, and environmental stimuli (Nagegowda 2010; Zulak and Bohlmann 2010).

While the production of terpenes is regulated by many factors, it is principally dependant on the transcriptional regulation of terpene synthase genes (Fäldt *et al.* 2003; Nagegowda 2010; Trindade *et al.* 2016). The involvement of terpenes in various physiological and ecological functions requires the regulation of expression of numerous genes, though the terpene synthases are the focus of this study. The comparison of gene expression between two *Pinus spp.* with contradistinctive susceptibility to *F. circinatum* infection should elucidate whether there is evidence to believe that the hosts terpene profile are valuable as a contributing factor to susceptibility.

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1.1 *Pinus* spp. and the significance of *Fusarium circinatum*

Pines are conifers of substantial global economic importance because of their wood properties, and compatibility to grow within large-scale plantations (Burgess and Wingfield 2001). Several species of conifers have lifespans of up to a few thousand years, which suggests that the success of these trees is supported by considerable phenotypic plasticity and robustness (Loehle 1987; Hammerschmidt 2006). This is because, during their exceptionally long lifetime, these trees are exposed to numerous pests and pathogens. These pathogens have far shorter generation times than their long-established hosts, and are theoretically at an advantage over the host in the evolutionary arms-race of defence (Achotegui-Castells *et al.* 2016; Slinski *et al.* 2016). In response, conifers have evolved a unique collection of diverse physical and chemical defences to tolerate threats (Trapp and Croteau 2001b; Celedon and Bohlmann 2019).

1.1.1 Global consequences of *Fusarium circinatum* infection

Pitch canker represents a significant threat to pines in many parts of the world, two recent articles cover this in detail; Lombardero *et al.* (2019) and Reynolds and Gordon (2019). As is clear, *Fusarium* infections are a particular issue in regions where natural forests and plantations are composed of susceptible *Pinus spp*., such as in Australia and New Zealand (Barnard and Blakeslee 1987; Wingfield *et al.* 2008; Summerell *et al.* 2011). Additionally, *F. circinatum* has been reported in Mexico, Japan, Chile, Spain, as well as in the west of the USA (Viljoen 1994; Pfenning *et al.* 2014; Reynolds and Gordon 2019; Yang *et al.* 2019).

Characteristically, mature trees respond to infection by this pathogen by producing large quantities of terpene-rich oleoresin, or so-called ‘pitch’, as well as by forming cankerous lesions in the woody tissue (Dwinell 1985; Barnard and Blakeslee 1987; Bezos *et al.* 2017). Consequently, a common symptom of this disease in plantations is canopy dieback. Occurring as a result of obstruction of the vascular tissue, by large girdling lesions in the tissue adjoining the branch tip (Dwinell 1985). As the disease progresses lesions merge, cutting entire branches off from the vascular tissue, and in some cases, lesions within the main vascular bundle cause mortality of mature trees (Hodge and Dvorak 2000; Bezos *et al.* 2017).

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Crucially, the degree of susceptibility to *F. circinatum* varies considerably among *Pinus* species (Dwinell 1985; Hodge and Dvorak 2000). Species such as *P. patula* and *P. radiata* are acutely susceptible to this phytopathogen, whereas other species, such as *P. tecunumanii*, are much less vulnerable (Eguiluz *et al.* 1996; Hodge and Dvorak 2000; Hongwane *et al.* 2018). Susceptibility to *F. circinatum* also differs within species, for instance, *P. tecunumanii* from high elevations is significantly less resistant than *P. tecunumanii* from lower elevations (Hodge and Dvorak 2000, 2007; Mitchell *et al.* 2014). However, symptoms differ depending on the *F. circinatum* genotype, climatic conditions, as well as being influenced by regional insects (Nel *et al.* 2014; Gordon and Reynolds 2017; Fru *et al.* 2018; Quesada *et al.* 2019).

1.1.2 *Pinus patula* in South African forestry

First introduced into South Africa in 1907, subsequent selections have resulted in *P. patula* having been developed more than any other *Pinus* species, optimising growth and wood quality (King 1938; Burgess and Wingfield 2001). Previously, this species was the most prolifically grown in the country, with roughly 50% of the softwood forestry area in South Africa composed of *P. patula* (Wingfield *et al.* 1999; Mitchell *et al.* 2012). Recently, the productivity of this species is increasinly being negatively affected by fungal pathogens, with significant financial consequences (Mitchell *et al.* 2011, 2012; Forestry 2017). Although still a principal plantation species, there is mounting reluctance to use pure *P. patula* (Mitchell *et al.* 2011; Forestry 2017; Hongwane *et al.* 2018). Other species grown commercially in South Africa include those which are relatively frost tolerant; *Pinus radiata*, *Pinus taeda* and *Pinus elliottii*, though these species are also susceptible to Pitch canker disease (Dvorak 2001; Mabaso *et al.* 2019).

A possible alternative species, pure *P. tecunumanii* low elevation (LE), does not perform well in Southern Africa due to its poor cold tolerance, it is grown throughout Colombia and northern Mozambique (Eguiluz *et al.* 1996; Hodge and Dvorak 2000; Hongwane *et al.* 2018). Importantly, members of this species are particularly resistant to infection by *F. circinatum*, and are being used in South Africa as hybrids, combined with frost-tolerant *P. patula* (Kanzler *et al.* 2012, 2014; Mitchell *et al.* 2013; Hongwane *et al.* 2018). While *P. patula* x *P. tecunumanii (LE)* hybrids exhibit higher levels of *F. circinatum* resistance (Hodge and Dvorak 2000; Roux *et al.* 2007; Mitchell *et al.* 2013; Ford *et al.* 2014), *P. patula* x *P. tecunumanii*

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(HE) are significantly less susceptible to the cold (Granados *et al.* 2013; Kanzler *et al.* 2014; Hongwane *et al.* 2018).

Presently, there is no effective way to control infections by *F. circinatum* (Martínez-Álvarez *et al.* 2016; Amaral *et al.* 2019). However, the implementation of an integrated management approach has been shown to lessen the economic impact of the disease. This involves quarantine, as well as the selection for clones and hybrids that are more resistant (Gordon *et al.* 2015; Iturritxa *et al.* 2017; Lombardero *et al.* 2019). Improving *P. patula's* tolerance to this phytopathogen will be a major consideration when selecting future clones (Mitchell *et al.* 2012, 2013; Gordon *et al.* 2015; Mabaso *et al.* 2019).

1.1.3 *Fusarium circinatum* as a seedling wilt disease

The seedling form of this disease is known as *F. circinatum*-wilt, and was first discovered in South Africa in 1990 (Viljoen 1994; Wingfield *et al.* 1999). Currently, *P. patula's* survival within nurseries and after field establishment has been drastically reduced due to the young trees increased susceptibility to *F. circinatum* (Crous 2005; Jones *et al.* 2014; Swett *et al.* 2015). This form of the disease is of concern as it causes wilting, chlorosis, and ultimately extensive losses of *P. patula* seedlings (Wingfield *et al.* 1999; Aegerter and Gordon 2006; Mitchell *et al.* 2011, 2012).

Fusarium circinatum has been shown to be hemibiotrophic, and not strictly necrotrophic as previously thought, and accordingly can colonise hosts without causing any visible symptoms (Swett *et al.* 2015, 2018). This fungal pathogen can cryptically infect the roots of seedlings, often only becoming symptomatic post infection by the stress associated with transferral of young trees into plantations (Swett *et al.* 2015, 2018). These losses could be mitigated by selecting seedlings with greater resistance to infections, as well as the development of improved detection methods (Morris *et al.* 2014; Gordon *et al.* 2015; Hongwane *et al.* 2018).

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1.2 Plant defence

When a plant is confronted by an antagonist, be it insect or pathogen, there is a continuum of possible outcomes, from acute susceptibility to comprehensive resistance (Eyles *et al.* 2010). An important determinant of the interaction between plants and their challengers is that these organisms must be capable of overcoming the plants diverse defence strategies (Pearce 1996; Hammerschmidt 2009). This includes multiple chemical and physical defences utilised by the plant to inhibit infection by pathogens (Pearce 1996; Fu and Dong 2013; Pusztahelyi *et al.* 2015). Constitutive defences represent the first line of protection, incorporating sequestered metabolites such as phenolics and terpenoids, and lignified tissues which form physical barriers (Shestibratov; Witzell and Martín 2008; Pusztahelyi *et al.* 2015). When a microbe is able to overcome these defences and cause infection, the tree's induced defences are prompted (Agrawal 1999; Eyles *et al.* 2010).

Ideally, the pathogen is recognised by cells of a host plant, allowing the induction of a highly coordinated defence response to prevent colonisation and disease progression (Kovalchuk *et al.* 2013; Liang *et al.* 2014; Arango-Velez *et al.* 2016). Rapid pathogen recognition by the host, which triggers a defence response early on, increases the chances that the host will successfully subdual it (Liang *et al.* 2014; Arango-Velez *et al.* 2016; Amaral *et al.* 2019). This would be an "incompatible" interaction, as the host is able to resist the establishment of infection. Conversely, a "compatible" interaction occurs if the host is susceptible, and either does not recognise, or responds inadequately to the intruder, allowing the infection to spread (Grewal *et al.* 2012; Oliveira-Garcia and Valent 2015).

1.2.1 Induced resistance

Induced defence involves various compounds, the major ones being; terpenoids, phenolics, alkaloids, pheromones, phytoalexins, phytohormones, Anti-Microbial Peptides (AMPs), Pathogenesis-Related (PR) proteins, as well as those which contribute to the Hypersensitive (HR) and Hypersensitive-like Responses (Pearce 1996; Conrath *et al.* 2002; Eyles *et al.* 2010). PR proteins are either antimicrobial or act to strengthen the host's cell walls through cross-linking reactions and lignification (van Loon *et al.* 2006; Visser *et al.* 2018). Certain hydrolytic enzymes which degrade fungal cell walls, such as chitinases and B-1,3-glucanases, are classic

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examples of PR proteins utility in defence (Liu *et al.* 2005; Naidoo *et al.* 2013). Alternatively, recognition of a pathogen can lead to HR, which is the rapid death of affected cells; this is most effective in the containment of biotrophic pathogens (Kinloch and Dupper 2002; Glazebrook 2005). However, the most diverse range of defences are inevitably offered by the most heterogenous molecules produced, the terpenes (Bohlmann *et al.* 1998a; Chen *et al.* 2011).

Numerous metabolic pathways contribute to defence within *Pinus spp.*, and their activation is highly coordinated with the development of disease (Hammerschmidt 2006; Fraser *et al.* 2015). Orchestrating a successful defence requires the induction, and simultaneous repression of pathways, which contribute to resistance qualitatively or quantitatively (Bonello *et al.* 2006; Hammerschmidt 2006; Witzell and Martín 2008; Kovalchuk *et al.* 2019). To achieve this, each pathway is controlled by various mechanisms of transcriptional, translational and post-translational modification (Grewal *et al.* 2012).

1.2.2 Plant secondary metabolites

Plant secondary metabolites do not directly participate in the primary processes of life, such as photosynthesis, respiration, solute transport, or in the formation of primary metabolites (Fraenkel 1959). However, research has shown that plant secondary metabolites play vital ecological functions, and consequently, plant evolutionary fitness is greatly influenced by the ecological range of functions of their secondary metabolites (Lichtenthaler 1999; Moore *et al.* 2014; Singh and Sharma 2015). Therefore, contradictory to the idea of these compounds as by-products, the selective pressures on plants for greater reproductive fitness has contributed to the evolution of this array of metabolic pathways, through which plants manufacture chemicals that can be detrimental or beneficial to other organisms (Fraenkel 1959; Padovan *et al.* 2014). This divergent part of plant evolution can also be regarded as “specialised” metabolism because it is an instance of phenotypic adaptations to particular environments, and thus not necessarily found throughout all plant lineages (Lichtenthaler 1999; Chen *et al.* 2011). So, in addition to being vital to plant defence and signalling, this attribute of secondary metabolite distribution and evolution makes them of phylogenetic relevance too (Moore *et al.* 2014).

While all spore-bearing and seed plants are able to produce terpenoids, polyphenols have such a broad distribution that they are thought to have been produced by ancient multicellular algae (Ashour *et al.*; Lichtenthaler 1999; Wink 2014). As secondary metabolism is so fundamental

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to plant defence, it is thought that these pathways must be at least as old as land plants are (Lichtenthaler 1999; Wink 2014). The terpene synthase gene family is thought to have originated from an ancestral bifunctional Copalyl-Diphosphate/ Kaurene synthase enzyme (Yahyaa *et al.* 2015). This would suggest the basal pathways leading to phenolics and terpenoids must be at least 450 million years old (Padovan *et al.* 2014; Wink 2014; Celedon and Bohlmann 2019).

1.2.3 Plant terpenes

Terpenes form the most abundant group of plant secondary metabolites, and are immensely structurally diverse, at present around 50,000 individual compounds have been identified (Vranová *et al.* 2012; Padovan *et al.* 2014; Muhlemann *et al.* 2014). Terpenes are hydrocarbons with the general formula $(C_5H_8)n$, while terpenoids can be described as "modified" terpenes, to which a functional group that typically contain oxygen atoms added (Yadav *et al.* 2014; Ludwiczuk *et al.* 2016). However, in practice, the term "terpenes" is used to include terpenes as well as their derivatives.

The value of plant terpenes in nature, and to humans, is difficult to overstate. Terpenes contribute to the responses prompted by both biotic and abiotic stresses, as well as forming volatile signals to attract or deter other organisms (Dorman and Deans 2000; Moore *et al.* 2014). Over and above the physiological, and ecological functions terpenes play in plants, there is an enormous application of these compounds in the pharmaceutical, agricultural, and cosmetic industries, particularly for their aromatic qualities (Singh and Sharma 2015; Abbas *et al.* 2017). The polyterpene we use most extensively is rubber, but some other common terpene-based products include camphor, methanol, detergents, solvents, limonene, antiallergenic agents, as well as the nepetalactone in catnip (Croteau *et al.* 2000; Thimmappa *et al.* 2014; Ludwiczuk *et al.* 2016).

Plant terpenes are used extensively for their antiseptic properties, as being reactive open-chain or cyclic unsaturated compounds (Cowan 1999; Yadav *et al.* 2014). The antimicrobial activity of terpenes is of particular interest as a potential alternative to antibiotics, as we face a global increase in bacterial resistance (Singh and Sharma 2015; Abbas *et al.* 2017). Terpenes have been used in traditional remedies for centuries, and yet are being applied in novel

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pharmaceutical instances, such as the treatment of cancer with antineoplastic terpenes; paclitaxel, and ingenol mebutate (Thormar 2010; Mafu and Zerbe 2018; Seca and Pinto 2018).

There are terpenes that can be found in all vascular plants. These are those which perform crucial functions, such as sterols in membrane structures, carotenoid pigments, and phytohormones such as abscisic acid and gibberellins (Gershenzon and Dudareva 2007; Abbas *et al.* 2017). Additional research has revealed many mono-, sesqui- and diterpenes that are fundamental to the environmental interactions of plants, as well as the interactions that take place between plants (Gershenzon and Dudareva 2007; Yu and Utsumi 2009; Abbas *et al.* 2017) .

Each plant species has had to evolve the ability to produce a particular complement of terpenes that are most advantageous in its unique ecological niche (Tholl 2006; Gershenzon and Dudareva 2007; Chen *et al.* 2011). Accordingly, terpene diversity is enhanced by the process of adaptive selection; as other organisms evolve responses to plant metabolites, selection pressures lead to the acquisition of novel compounds (Fraenkel 1959; Moore *et al.* 2014; Pusztahelyi *et al.* 2015; Achotegui-Castells *et al.* 2016). As a result, plants are known to be able to synthesise thousands of distinct metabolites, and that number continues to grow (Hartmann 2007; Wink 2010). However, each individual plant species is able to produce only a subset of these compounds (Hartmann 2007; Chen *et al.* 2011; Moore *et al.* 2014; Wink 2014).

As defence is vital to evolutionary success, it is possible that each individual interaction between the host and its aggressor has driven the diversification of terpene profiles within, and between species (Padovan *et al.* 2010; Naidoo *et al.* 2014). This would be consistent with the significant quantitative and qualitative variations observed between, and even within species of *Eucalyptus* (Padovan *et al.* 2014) and *Pinus* (Ro and Bohlmann 2006; Zerbe and Bohlmann 2014; Trindade *et al.* 2016). The intraspecific diversity of terpenes implicates this metabolic pathway as an adaptive response, optimising fitness in diverse ecological instances (Moore *et al.* 2014).

Plants interact with their environment through the emission of volatile terpenoid compounds (Das *et al.* 2013; Zerbe and Bohlmann 2014). A low-level of volatiles are continually released by all plants, however, large volumes are released in response to damage or infection (Gershenzon and Dudareva 2007; Das *et al.* 2013). Due to their low molecular weight, mono- and sesquiterpenes vaporise at relatively low temperatures, ideally suited for conveying

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information over as wide as possible a range (Yadav *et al.* 2014; Muhlemann *et al.* 2014; Ludwiczuk *et al.* 2016). Another advantage of using these lipophilic molecules to communicate is their enormous structural diversity, which allows messages to be fairly specific (Das *et al.* 2013; Abbas *et al.* 2017). However, the complexity of these combinations is such that it is difficult to associate any particular attribute to any one species of terpene (Huber *et al.* 2004; Degenhardt *et al.* 2009; Boutanaev *et al.* 2015; Green *et al.* 2017).

Terpene biosynthesis and diversity

Although this class of compounds includes many which are extremely variable from one another in chemical structure, all of them originate from two biosynthetic pathways, as is outlined in Figure 1.1 (Dudareva *et al.* 2013; Abbas *et al.* 2017). In vascular plants, the plastidial Methyl-Erythritol-Phosphate (MEP) and classical cytosolic Mevalonic-Acid (MVA) pathways generate distinct collections of terpenes (Figure 1.1, (Zulak and Bohlmann 2010; Abbas *et al.* 2017). These two pathways occur in distinct subcellular compartments and typically function independently from one another, although interaction between them has been observed (Hemmerlin *et al.* 2003; Dudareva *et al.* 2005). These two related, but distinct biosynthetic pathways result in the production of two inter-convertible C_5 monomers (Figure 1.1), which are the universal precursors of all isoprenoid compounds, including terpenes. These monomers are isopentenyl-diphosphate (IPP), and its allelic isomer dimethylallyl-diphosphate (DMAPP) (Yadav *et al.* 2014).

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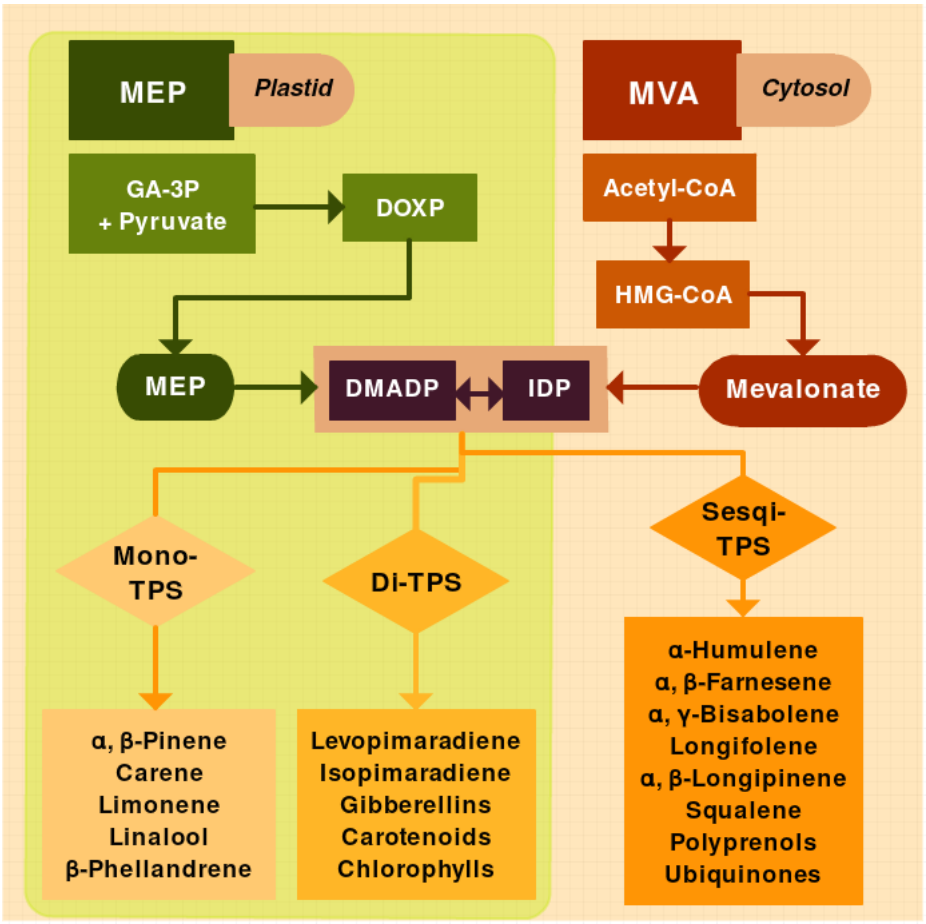

Figure 1.1: The iso-, mono-, sesqui-, and diterpene biosynthetic pathways in conifers. The plastidial MEP pathway starts with the condensation of pyruvate and GA-3P, while the MVA pathway spans the cytosol, peroxisomes and endoplasmic reticulum and starts with the condensation of acetyl-CoA. The result is various terpenoid molecules; listed are examples formed by Monoterpene Synthases (Mono-TPS), Sesquiterpene Synthases (Sesqui-TPS), and Diterpene Synthases (Di-TPS) in conifers. 3-Hydroxy-3-methylglutaryl (HMG-CoA), isopentenyl diphosphate (IPP), dimethylallyl diphosphate (DMAPP), deoxyxylulose-5-phosphate (DXOP), and methylerythritol-4-phosphate (MEP).

The MVA pathway operates primarily in the cytosol and mitochondria (Luskey and Stevens 1985; Basson *et al.* 1988; Igual *et al.* 1992; Rodwell *et al.* 2000), beginning with the condensation of Acetyl-CoA to form 3-Hydroxy-3-MethylGlutaryl-CoA (HMG-CoA). This is converted to mevalonate, which through sequential phosphorylation and decarboxylation events, results in the production of IPP (Tholl *et al.* 2005; Chen *et al.* 2011). The IPP molecules derived from the MVA pathway (Figure 1.1) can then be converted, by the action of enzyme isopentenyl diphosphate isomerase, to DMAPP (Chappell 1995; McGarvey 1995). The predominant products of the MVA pathway are sterols, sesquiterpenes, and ubiquinones (Singh and Sharma 2015).

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In the plastid, the MEP pathway (Figure 1.1), also referred to as the mevalonate-independent pathway, biosynthesises both IPP and DMAPP (Hemmerlin *et al.* 2003). The products of this pathway go on to form monoterpenes, diterpenes, certain phytohormones, carotenoids, as well modifications to photosynthesis-related compounds, tocopherols, chlorophyll, phylloquinones, and plastoquinones (Singh and Sharma 2015). All the enzymes of the MEP pathway are contained within the plastids (Suire *et al.* 2000; Hsieh *et al.* 2008).

The MEP pathway is initiated by the condensation of pyruvic acid and glyceraldehyde-3-phosphate (GAP), synthesising 1-deoxy-d-xylulose-5-phosphate (DOXP), which is converted to MEP. Two MEP molecules fuse to form hydroxy-methyl-butenyl-4-diphosphate (HMBPP), and with additional modifications, both DMAPP and IPP are produced, though not in equal proportions (Baker 1992; Cunningham *et al.* 2000; Rohdich *et al.* 2003; Tritsch *et al.* 2010; Thimmappa *et al.* 2014). Once formed, DMAPP and IPP monomers are condensed to form the precursors to all terpenes (Figure 1.1; Nagegowda 2010; Singh and Sharma 2015). Terpenes are grouped according to the monomers they are made up of. Though, it can be difficult to discern the original five-carbon residues due to extensive metabolic modifications. Terpenes containing two C_5 units are monoterpenes (C_{10}), while terpenes with three monomers are sesquiterpenes (C_{15}), and diterpenes (C_{20}) contain four monomers. Accordingly, we have triterpenes (C_{30}) and tetraterpenes (C_{40}), while anything larger is broadly classified as a polyterpenoid (Nagegowda 2010; Chen *et al.* 2011).

This diversity can be attributed to two classes of enzymes belonging to multiple large gene families; the cytochrome P450-dependent mono-oxygenases (CyP450s), and the terpene synthases (Zulak and Bohlmann 2010). Several of the CyP450s enzymes are known to be promiscuous, often responsible for adding the extremely variable modifications observed within an entire group of functionally or structurally related diterpenoids (Zulak and Bohlmann 2010; Wen *et al.* 2018). Terpene synthases are a large family of enzymes that synthesise thousands of terpene products from very few substrates (Nagegowda 2010; Zulak and Bohlmann 2010; Padovan *et al.* 2014). While some terpene synthases are highly restricted in their product profiles, many of them catalyse the reactions for multiple products (Steele *et al.* 1998; Fäldt *et al.* 2003; Martin 2004). These enzymes are categorised according to their substrate specificity and phylogeny (Bohlmann *et al.* 1998a).

All plant terpene synthase enzymes belong to a family with diverged but related functions and are thought to have arisen from a common evolutionary origin, the duplication of an ancestral

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gene (Hayashi *et al.* 2006; Degenhardt *et al.* 2009). This is consistent with the fact that the moss, *Physcomitrella patens*, only includes one functional terpene synthase gene, a bifunctional kaurene synthase, which is homologous with both angiosperm and gymnosperm terpene synthase genes (Trapp and Croteau 2001a; Keeling *et al.* 2010).

Plant terpene synthases fall into one of two classes; I; kaurene synthase-type, and II; copalyl-diphosphate synthase-type, according to the reactions in which they participate (Degenhardt *et al.* 2009; Chen *et al.* 2011). Mono- and sesquiterpene synthases typically have class I domains (Dudareva 1996), while diterpene synthases can exist as either monofunctional or bifunctional enzymes, retaining either, or both class I and II functional domains (Peters *et al.* 2000). All terpene synthases contain an aspartate-rich, metal-binding domain, allows substrate binding by the coordination of divalent metal ions (Lesburg 1997). The functional promiscuity of these enzymes is due to the stochastic bond rearrangements that occur within their active sites, forming unusual combinations of carbocation intermediates (Steele *et al.* 1998; Degenhardt *et al.* 2009).

Terpene synthases are further grouped into clades (Bohlmann *et al.* 1998a; Dudareva 2003). Previous phylogenetic analyses have defined representative clades of terpene synthase sequences from gymnosperms and angiosperms (Chen *et al.* 2011). Currently, seven terpene synthase clades are recognised, as seen in Table 1.1, adapted from Chen *et al.* (2011). However, more recent phylogenetic analysis has suggested that the conifer-specific clade TPS-d (Table 1.1) is polyphyletic and could be adapted to accommodate more subsections in future (Hall *et al.* 2013a). This clade contains a significant proportion of the enzymes of conifer terpene synthesis (Bohlmann *et al.* 1998b; Pazouki and Niinemets 2016). Therefore, it is imperative to gain insight into the diversity within this clade. The rest of the conifer terpene synthases fall into the TPS-c and TPS-e/f subfamilies (Table 1.1), which have corresponding orthologs within angiosperms (Keeling *et al.* 2010; Chen *et al.* 2011; Boutanaev *et al.* 2015). However, while terpene synthase genes fall into seven distinct clades, plant lineages have the majority of their terpene synthases in one or two clades, having been derived through lineage-specific expansion (Trapp and Croteau 2001a; Degenhardt *et al.* 2009; Chen *et al.* 2011).

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Table 1.1: Function and taxonomic distribution of terpene synthase clades in plants, as defined by Chen et al. (2011):

Subfamily	Groups	Functions	Distribution
	TPS-a-1	Sesqui-TPS	Dicots
TPS-a	TPS-a-2	Sesqui-TPS	Monocots
TPS-b		Mono-TPS, IspS	Angiosperms
TPS-c		CPS/KS, CPS, Di-TPS	Land plants
	TPS-d-1	Mono-TPS, Sesqui-TPS	Gymnosperms
	TPS-d-2	Sesqui-TPS	Gymnosperms
TPS-d	TPS-d-3	Di-TPS, Sesqui-TPS	Gymnosperms
TPS-e/f		KS, Di-TPS, Mono-TPS, Sesqui-TPS	Vascular plants
TPS-g		Mono-TPS, Sesqui-TPS, Di-TPS	Angiosperms
TPS-h		Putative bifunctional Di-TPS	*Selaginella moellendorffii*

** SesquiTPS, sesquiterpene synthase; MonoTPS, monoterpene synthase; IspS, isoprene synthase; CPS, copalyl synthase; KS, kaurene synthase; DiTPS, diterpene synthase.*

In silico annotation of terpene synthases is problematic, because of the reactions catalysed by these enzymes and their primary structures are not exhibitive of their product profiles. An example of this is the extensive, yet distinct products produced by two terpene synthases with similar primary structures; γ -selinene synthase and γ-humulene synthase (Steele *et al.* 1998; Little and Croteau 2002). Although the mechanisms behind the extraordinary product plasticity of these enzymes are not predictable, the production of specific terpenes has been shown to be associated with particular amino acids sequences at sites of catalytic activity (Katoh *et al.* 2004).

Terpene synthase genes are well characterised in a wide variety of plants. This includes *Arabidopsis thaliana*, *Cucumis sativus*, *Nicotiana attenuate*, *Eucalyptus grandis*, and *Santalum album* (Facchini and Chappell 1992; Chen 2003; Mercke 2004; Jones *et al.* 2008; Degenhardt *et al.* 2009; Külheim *et al.* 2015). Most of our knowledge of terpene synthases in conifers comes from studies on *Picea abies*, *Picea sitchensis*, *Picea glauca*, *Abies grandis*, *Pinus taeda*, *Pseudotsuga menziesii*, and *Taxus media* (Byun-McKay *et al.* 2006; Ralph *et al.* 2006, 2008; Ro and Bohlmann 2006; Zulak *et al.* 2009; Zulak and Bohlmann 2010; Keeling *et al.* 2010, 2011; Bohlmann and Way 2012; Hall *et al.* 2013a).

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Terpenes in conifers

Oleoresin is a viscose blend of volatile terpenes (terpenoids) and non-volatile diterpene resin acids, and is an important constituent for defence in conifers (Bohlmann *et al.* 1998a; Fäldt *et al.* 2003; Witzell and Martín 2008; Abbott *et al.* 2010; Zulak and Bohlmann 2010). The antimicrobial and antiherbivory component of conifer oleoresin is the volatile turpentine fraction, which includes a variable range of structurally diverse volatile mono- and sesquiterpenes (Zulak and Bohlmann 2010). In addition to being toxic to predators, the turpentine fraction acts as a solvent, carrying non-volatile diterpenes to where they are needed to form mechanical barriers or seal wounds (Bohlmann *et al.* 1998a).

Traumatic ducts, which are formed in *Picea* and other conifers, are specialised anatomical structures which accumulate and store oleoresin (Zulak and Bohlmann 2010; Singh and Sharma 2015). The epithelial cells lining the interiors of these structures are understood to be where terpene biosynthesis occurs (Franceschi *et al.* 2005; Zulak and Bohlmann 2010; Kshatriya *et al.* 2018). Although these resin ducts exist constitutively, recognition of a potential threat or treatment with methyl jasmonate prompts their *de novo* formation (Krokene *et al.* 2008a; Zulak and Bohlmann 2010; Keeling *et al.* 2011; Bohlmann and Way 2012).

Methyl jasmonate is a lipophilic phytohormone which is implicated in defensive signalling (Creelman and Mullet 1997; Abbas *et al.* 2017). Topical application of this phytohormone induces the biosynthesis of terpene rich oleoresin and volatile terpenoids, similar to that typically initiated by pathogen recognition, thereby enabling the examination of the traumatic resin response in conifers (Barnard and Blakeslee 1987; Krokene *et al.* 2008b; Zulak and Bohlmann 2010; Reglinski *et al.* 2017).

In conifers, the production of defence-related terpenes is dynamic, allowing it to be adaptable in fluctuating environmental conditions, and to evolving biotic threats (Ro and Bohlmann 2006; Achotegui-Castells *et al.* 2016). In addition, terpene profiles have been shown to vary extensively between species (Bohlmann *et al.* 1998b; Trapp and Croteau 2001b; Martin and Bohlmann 2005). The efficiency with which the trees chemical defence can guard against pathogens is largely dependent on the composition and quantity of oleoresin terpenes (Köpke *et al.* 2010; Keeling *et al.* 2011; Buschiazzo *et al.* 2012).

Importantly, the emission of certain volatile monoterpenoids, such as pinenes and limonenes, has been shown to be under such strong genetic control that it can be used to distinguish

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different genotypes (Forrest 1980; Kinloch *et al.* 1986). In particular, volatile terpenoids make reliable chemo-taxonomic markers because their biosynthesis is primarily under the control of gene expression, and therefore not significantly influenced by environmental factors (Erbilgin and Colgan 2012; Mitić *et al.* 2017a, 2018).

Many of the conifer terpene synthases that have been functionally characterised are described in an inclusive review by Keeling and Bohlmann (2006a). Despite their enormous size, the genomes of *Picea glauca*, *Pinus lambertiana*, and *Pinus taeda* have been sequenced (Birol *et al.* 2013; De La Torre *et al.* 2014; Neale *et al.* 2014; Zimin *et al.* 2014; Wegrzyn *et al.* 2014; Warren *et al.* 2015; Stevens *et al.* 2016). However, the identification of these genes within economically important conifer species has been augmented by transcriptome sequencing projects, such as that performed in *Pinus monticola*, *Pinus flexilis*, and *Pinus albicaulis* (Lorenz *et al.* 2012; Celedon and Bohlmann 2017; Cai *et al.* 2018; Shalev *et al.* 2018).

Non-volatile terpenes of conifer oleoresin are not known to be further biochemically modified by CyP450 enzymes, the diterpene compounds can be further oxidised into resin acids by multi-substrate CyP450s (McGarvey 1995; Keeling and Bohlmann 2006b). Genes from within the CYP720B superfamily have been shown to be responsible for at least two of the three successive oxidation steps required to modify diterpenoids (Ro and Bohlmann 2006; Hamberger and Bohlmann 2006). Therefore, it is possible that relatively few diterpene synthases and CyP450s are able to produce the extensive biochemical variability and plasticity of the host's terpene profile (Martin 2004; Zulak and Bohlmann 2010; Bathe and Tissier 2019; Celedon and Bohlmann 2019).

Hamberger and Bohlmann (2006) revealed that terpene synthase genes belong to a large a conifer-specific gene family, CYP720B, which is comparable in size to that of the terpene synthase gene families in conifers. The first CyP450 gene to be functionally characterised in conifers, CYP720B1, was identified from loblolly pine and implicated in the synthesis of diterpenoids (Ro *et al.* 2005; Ro and Bohlmann 2006). Since then, many more CyP450 enzymes have been described, as has been comprehensively reviewed by Bathe and Tissier (2019).

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Prospects for metabolic engineering of terpenes

The importance of terpenes *in planta*, as well as commercially, makes their potential for manipulation through metabolic engineering attractive. The synthesis of terpenes is regulated at multiple levels, therefore any form of perturbation, particularly early on in the pathway, can lead to broad-spectrum changes in observed phenotype (Capell and Christou 2004). Previously, the transcription of monoterpene, sesquiterpene, and carotenoid pathway genes have been enhanced by overexpression of the rate-limiting enzymes responsible for producing precursor molecules (Figure 1.1) such as DXR, DXS, and HMGR (Dalla Costa *et al.* 2018; Henke *et al.* 2018; Zhang *et al.* 2018). However, the modulation of principal regulatory enzymes results in the indiscriminate alteration of metabolite concentrations (Zerbe and Bohlmann 2015; Abdallah and Quax 2017; Bian *et al.* 2017; Mewalal *et al.* 2017).

Metabolic engineering could augment plant resistance on multiple fronts including altering the timing, composition, or magnitude of the defence response mounted against a pest or pathogen (Tholl 2015). It has been shown that the overexpression of certain terpene synthase genes is a promising technique of terpene profile manipulation when addressing the abovementioned issues in transgenic plants (Krasnyanski *et al.* 1999; Aharoni *et al.* 2005; Zerbe and Bohlmann 2015; Bian *et al.* 2017; Neis *et al.* 2018). Recently, it was shown that the downregulation of d-limonene synthase increases the resistance of citrus crops to the phytopathogenic fungus *Phyllosticta citricarpa* (Rodríguez *et al.* 2018).

Research in *Pinus* species has revealed that in mature trees, as in seedlings, it is possible to induce alterations in the concentration and composition of monoterpenes and that this response is consistent within genotypes regardless of the trees age (Erbilgin and Colgan 2012). Although an observed increase in total monoterpene concentrations can provide an indication of the trees defence response to pathogen infection, this does not necessarily correspond to increased resistance (Heijari *et al.* 2005; Wen *et al.* 2018). It has been suggested that individual compounds within the terpenome contribute more strongly to a successful response than others, making their specific concentrations more indicative of observed resistance than the total terpene concentration (Zhao *et al.* 2011; Erbilgin and Colgan 2012; Schiebe *et al.* 2012; Trindade *et al.* 2016; Green *et al.* 2017; Reglinski *et al.* 2017; Mitić *et al.* 2018).

In the future, research should also be done to assess the correlation between terpene synthase gene translation and the corresponding terpenome of conifers (Zhang *et al.* 2018). Furthermore,

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detailed knowledge of the particular expression profiles associated with certain diseases, infections, or stresses could eventually be used for diagnostic and taxonomic purposes (Abbas *et al.* 2017; Mitić *et al.* 2017b). It is worth noting that the positive results achieved by the overexpression of genes of terpene biosynthesis, which prompted the ample supply of isoprenoid precursors, can impose a cost on plant growth and fitness (Aharoni *et al.* 2005). This is due to the reduced supply of precursors to pathways of primary metabolism, as well as the potential toxicity of the compounds produced in excess within plant cells (Capell and Christou 2004; Bian *et al.* 2017; Owen *et al.* 2017; Dowd *et al.* 2018; Zhang *et al.* 2018). Therefore, before the true environmental and economic potential can be recognised, a more holistic approach is necessary to elucidate the complex genetic circuitry which underlies the terpenome (Mewalal *et al.* 2017).

Finally, endophyte research shows potential as a biological control against phytopathogenic fungi (Wingfield *et al.* 2008; Naik 2018). In *Pinus spp.*, the application of beneficial microorganisms is being investigated to reduce the severity of *F. circinatum* infection (Martínez-Álvarez *et al.* 2016; Iturritxa *et al.* 2017; Bullington *et al.* 2018), further corroborating that sustainable resistance will require a wide-ranging understanding of the multicomponent defence of *Pinus spp.* by numerous interconnected mechanisms (Amaral *et al.* 2019).

1.3 Conclusions

The fitness of forest and plantation tree populations is expected to decline rapidly as a result of the observed average increase in global temperatures. Studies modelling future conditions, show that approximately 60% of tree species adapted to temperate regions will be disadvantaged by the warmer climates forecast, and conifers are predicted to be amongst the most negatively affected (Hamann and Wang 2006; Alberto *et al.* 2013; Prunier *et al.* 2016; Simon and Adamczyk 2019; Celedon and Bohlmann 2019).

To address these issues, it is necessary to better understand the phenotypic plasticity within conifer terpenomes. Conifers produce a large array of phenolic and other chemical defence compounds, a considerable portion of the molecules within their defensive repertoire are terpenes (Trapp and Croteau 2001b; Krokene *et al.* 2008a). The sheer quantity and diversity

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of terpene molecules hints at their importance; they are known to facilitate numerous plant interactions, as well as being vital to defence (Mitić *et al.* 2017b; Celedon and Bohlmann 2019).

Pitch canker on mature trees manifests as resinous lesions, further implying the involvement of terpenes in this defensive response (Dwinell 1985; Ploetz 2006; Hodge and Dvorak 2007; Martín-Rodrigues *et al.* 2013). Previous research by Seeve (2010) on the putative defence-related genes undergoing differential expression in *Pinus taeda* infected with *Fusarium circinatum* has highlighted the potential involvement of terpene synthase genes in this interaction. This is consistent with the known capability of particular terpenes to inhibit phytopathogenic fungi growth *in vitro* (Cowan 1999; Keeling and Bohlmann 2006b; Bakkali *et al.* 2008; Slinski *et al.* 2015).

The differences in induced responses to infection between the two *Pinus* species, with contradistinctive susceptibility, could help to elucidate the mechanisms which underlie the relative resistance of *P. tecunumanii* (Trindade *et al.* 2016; Arango-velez *et al.* 2018). The aim of this study is the identification and comparative expression analysis of various terpene synthase genes in *P. patula* and *P. tecunumanii* (Visser *et al.* 2018). Although our focus is on the transcriptional regulation, we believe that this is a sufficient indication of terpene production, as multiple studies have shown that transcriptional levels are a good proxy for the level of terpene biosynthesis (Wang *et al.* 2010; Kovalchuk *et al.* 2019).

Above all, insight into the terpenome could help mitigate fungal infections in long-living conifer species, which are predicted to become more prevalent as a consequence of climate change (Lindner *et al.* 2010; Dudareva *et al.* 2013; Garrett *et al.* 2015; Iturritxa *et al.* 2017; Singh *et al.* 2018).

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Chapter 2

In silico characterisation of the terpene synthase gene families in *Pinus patula* and *Pinus tecunumanii*

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This chapter is written as a manuscript for submission to a research journal (BMC Genomics).

RLS carried out all experimental work, data generation and analysis, and prepared this manuscript. SN conceived this study and provided supervision, advice and direction at all stages of the project. MC provided support for the phylogenetic aspects of the project. EV provided transcriptome data, supervision, technical expertise and advice throughout the project.

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2.1 Abstract

Pitch canker, caused by *Fusarium circinatum* (teleomorph *Gibberella circinata*), is regarded as one of the most severe phytopathogenic diseases affecting commercial *Pinus* plantations worldwide, and is anticipated to be aggravated by the increase in temperature and humidity associated with climate change. In South Africa, the preferred commercial pine species has been *Pinus patula*. The fungus causes *Fusarium*-wilt on seedlings and has drastically decreased the post-planting survival of seedlings. Hybridisation of *P. patula* with *Fusarium*-tolerant species such as *Pinus tecunumanii*, has led to the development of an alternative planting stock. However, the mechanisms underlying *P. tecunumanii*'s relative resistance remains inconclusive. Previous research has highlighted the putative involvement of terpene synthases in defence against *Fusarium* spp., as their regulation is significantly altered early in response to this pathogen. RNA-seq reference transcriptomes for *P. patula* and *P. tecunumanii* have provided the much-needed genetic resources necessary to interrogate the putative defence related genes undergoing differential expression due to *F. circinatum* infection. The aim of this study was to determine whether observed differences in resistance between these two hosts, is correlated with induced terpene synthase gene expression profiles, and to resolve the implicated orthologs. The differential expression patterns of genes identified as terpene synthase orthologs was assessed for their potential to contribute to resistance. While the two host species, *P. patula* and *P. tecunumanii,* did appear to respond dissimilarly, this is not indicative of terpene synthesis playing a causal role in defence.

Keywords: transcriptomics, defence response, host–pathogen interaction, pitch canker, *Fusarium*-wilt, differential gene expression, *Pinus patula*, *Pinus tecunumanii*, terpene synthases

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2.2 Introduction

Forest trees, particularly conifers, are vulnerable to climate change because their long lifespans do not allow for quick adaptation to changes in environmental conditions (Lindner *et al.* 2010; Raitelaitytė *et al.* 2016). This is exacerbated by the fact that phytopathogenic fungal infections are characteristically dependent on humidity and temperature (Garrett *et al.* 2015). In particular, the potential impact of climate change on *F. circinatum* could increase the damage caused by pitch canker to plantations of susceptible *Pinus spp.* (Watt *et al.* 2011; Nier and Dobrza 2018; Quesada *et al.* 2019). Therefore, analysis of defensive genes in conifer species could reveal sequence variants contributing to susceptibility. This should help to elucidate the characteristics of a successful defence response, as a potential focus for genetic improvement through genetic engineering and breeding.

Fusarium circinatum (Nirenberg and O'Donnell 1998) poses one of the most significant and costly threats to *Pinus* and other conifer species, in both commercial and natural pine forests (Barnard and Blakeslee 1987; Wingfield *et al.* 2008; Raitelaitytė *et al.* 2016; Gordon and Reynolds 2017). The pathogen causes pitch canker on mature trees and *Fusarium*-wilt on seedlings (Iturritxa *et al.* 2011; Mitchell *et al.* 2014). *Fusarium* infection reduces the growth of the tree and increases its susceptibility to biotic and abiotic stresses (Seeve 2010; Jones *et al.* 2014; Mitchell *et al.* 2014). Infected seedlings have a high mortality rate, especially in nurseries, where cryptic root infections can be extensive (Martín-Rodrigues *et al.* 2015; Swett *et al.* 2015, 2018).

The typical defensive response to infection by *F. circinatum* in mature trees is the accumulation of oleoresin, or "pitch", at the site of infection (Dwinell 1985; Wingfield *et al.* 1999). Oleoresin is produced, and stored in dedicated anatomical structures; the cortical and xylem trauma-associated resin ducts (Trapp and Croteau 2001; Martin 2002; Krokene *et al.* 2008). When a tree's defences are triggered, the accumulated oleoresin "pitches out" of these structures to inundate the antagonist (Zulak and Bohlmann 2010). In addition, volatile constituents within the resin act as airborne signalling molecules, alerting neighbouring trees or predators of the attack (Abbas *et al.* 2017). Terpenes within the oleoresin have known antimicrobial properties for instance, α-pinene disrupts the integrity of fungal cell membranes, while other monoterpenes have been shown to inhibit vital fungal enzymes (Uribe *et al.* 1985; Marei *et al.* 2012; Slinski *et al.* 2015; Iturritxa *et al.* 2017).

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The efficiency with which a host tree activates its terpene defensive responses has been proposed as a reliable indication of its resistance (Zhao *et al.* 2011; Schiebe *et al.* 2012; Flø *et al.* 2018; Wen *et al.* 2018). Constitutive and induced terpene profiles are under strong genetic control and can differ greatly in both their composition and quantity, depending on tree provenance, population, or variety (Keeling *et al.* 2010; Bohlmann and Way 2012; Pham *et al.* 2014; Wen *et al.* 2018). However, mediating a suitable response requires a substantial expense of energy to coordinate modifications to numerous metabolic and signalling processes, therefore it is detrimental to the growth and development of the host (Hu *et al.* 2018).

Fusarium circinatum is thought to have originated in Mexico, and was first recorded in North Carolina (USA) in the 1940s (Barnard and Blakeslee 1987; Gordon *et al.* 2001; Porter 2010). The pathogen has also been reported in Haiti, South Africa, Japan, Korea, Mexico, Chile, Uruguay, Spain, France, Italy, and Portugal (Barnard and Blakeslee 1987; Wingfield *et al.* 2008; Hodge and Jetton 2010; Mead 2013; Bezos *et al.* 2017; Iturritxa *et al.* 2017). Further spread poses a significant threat to many countries were *Pinus* spp. are found naturally or grown commercially. In South Africa, *Pinus patula* has been the most prolifically grown commercial pine species (Mitchell *et al.* 2011; Fru *et al.* 2017). However, in the last few decades, cultivation and establishment of this species in plantations has been severely hampered by *Fusarium* wilt. In this country, *F. circinatum* was first reported in the 1990s, followed by several publications dealing with its biology, population structure and genomics (Viljoen 1994; Wingfield *et al.* 1999, 2008; Crous 2005; Fru *et al.* 2017; Gordon and Reynolds 2017; Van Wyk *et al.* 2018; Hongwane *et al.* 2018). Due to the importance of *F. circinatum* to South African commercial forestry, it was the first fungal pathogen genome to be sequenced (Wingfield *et al.* 2012).

Hybrids between *P. patula* and *Fusarium*-tolerant pine species have been suggested as alternative planting stock (Hodge and Dvorak 2007; Mitchell *et al.* 2011, 2012). Hybridisation of resistant *P. tecunumanii* low elevation (LE) with *P. patula* has shown promise for producing less susceptible hybrids, while maintaining wood properties (Roux *et al.* 2007; Mitchell *et al.* 2011, 2013; Ford *et al.* 2014; Kanzler *et al.* 2014; Fru *et al.* 2017; Hongwane *et al.* 2017). Unfortunately, pure *P. tecunumanii* is not a viable replacement for *P. patula* because it is prone to stem breakage in South Africa's climate; particularly the low elevation ecotypes (Hodge and Dvorak 2000; Granados *et al.* 2013; Leibing *et al.* 2013).

This study aimed to elucidate the diversity and differential expression of terpene synthase genes in conifers, with the intention to determine whether the observed genotypic differences in

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resistance correlate with induced terpene synthase gene expression profiles. The quantitative and qualitative characteristics of a host's terpene profile are hypothesised to be an important determinant of its susceptibility to infection. The differences in induced responses to infection between *P. patula* and *P. tecunumanii*, with contradistinctive susceptibility could elucidate the contribution of these genes. As there are no reference genomes currently available for these two *Pinus* spp., assembled RNA-sequence reference transcriptomes for *P. patula* and *P. tecunumanii* (Visser *et al.* 2018), allowed transcriptomic analysis of these non-model organisms. Identification of orthologous groups of protein sequences between related *Pinus* spp. enabled the clustering of genes of related structure, and thereby function which lead to the putative annotation of genes *via* their homology with characterised ones. In addition, differential expression was assessed from RNA isolated from a similar inoculation trial (Visser 2015; Visser *et al.* 2018). Finally, an *in-silico* analysis was done with the aim to identify terpene synthase diversity in these two *Pinus* spp. in order to elucidate whether genotypic differences in resistance correlate with differences in the induced terpene synthase gene expression profiles.

2.3 Materials and methods

2.3.1 Plant material and *Fusarium circinatum* infection trial

Six-month-old *P. patula* (4 individual families), *P. tecunumanii* (mixed families), as well as multiple open-pollinated families of *P. patula x P. tecunumanii* hybrid seedlings were obtained from Dr. Nicky Jones (Sappi Forests, Shaw Research Centre, Howick, KZN, Table 2.1). Before the inoculation, seedlings were allowed to acclimatise for two weeks. At the end of the acclimatisation period, the total number of surviving seedlings was 1708. Table 2.1, Table S1.
The total number of pure family *Pinus patula* seedlings was 748, and *Pinus tecunumanii* 746, which allowed a statistically significant number of seedlings to be allocated to each biological replicate (BR), within both mock-inoculated and inoculated groups. This allowed for >50 seedlings per BR at each of the four time points, from which tissue was harvested, and the plant discarded. (Table S1)

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The experimental design had to be adapted due to constraints introduced by the limited number of *Pinus patula* x *Pinus tecunumanii* hybrid seedlings (Table 2.1 and Table S1). While there was a total of 148 seedlings, they were dispersed over 15 hybrid open-pollinated families. The high level of variation within the hybrid group required the families to be grouped, preventing formation of balanced BR. To account for this, hybrid seedlings were inoculated (I), but no control group was kept for comparison, as in Figure 2.1. Statistical inference allowed for the estimation of mock-inoculated *Pinus patula* x *Pinus tecunumanii* hybrid control groups at each time point (Table S1).

Inoculations were conducted following the protocol of Porter (2010) and Nel *et al.* (2014). This involved removing the tip of the apical bud and placing 10µl of either the fungal inoculum, or 15% (v/v) sterile glycerol, on the wound, depending on whether the seedling is to be inoculated or mock-inoculated (Figure 2.1). *Fusarium circinatum* (isolate CMWF1217) was grown on ½ potato dextrose agar (PDA; Merck 1938) at 25°C for ten days. Spores were collected by surface washing with 15% (v/v) sterile glycerol. Spore concentration was quantified using a haemocytometer and adjusted to 5×10^4 spores/mL using 15% (v/v) sterile glycerol.

Table 2.1: Six-month-old *Pinus* seedlings used in the *F. circinatum* inoculation trial

***P. patula* (Individual families)**	***P. patula x P. tecunumanii***		***P. tecunumanii* (Seed mix)**
P9	P9 x P. tec118 LE	P1 x P. tec23 LE	P. tec 8 LE
P17	P17 x P. tec133 LE	P16 x P. tec323 LE	P. tec 33 LE
P38	P32 x P. tec133 LE	P40 x P. tec233 LE	P. tec 323 LE
P42	P38 x P. tec323 LE	P41 x P. tec33 LE	
	P38 x P. tec133 LE	P42 x P. tec233 LE	
	P25 x P. tec23 LE	P5 x P. tec33 LE	
	P8 x P. tec23 LE	P5 x P. tec233 LE	
	P8 x P. tec33 LE		

Tissue was harvested at 1, 3, 5, and 7 days post inoculation (dpi) for three BRs per group (Figure 2.1), flash frozen using liquid nitrogen and stored at -80°C until use. For each BR the top 1 cm of the shoot tissue (stem and needles) of at least three seedlings were harvested. Infection by *F. circinatum* was confirmed based on culture morphology on ½ PDA by re-

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isolation using tissue harvested from inoculated plants at 14 dpi (Figure 2.1). Lesion lengths were recorded weekly, from a minimum of three plants per group, from one to six weeks post inoculation as a measure of disease progression (Figure 2.3). The length of clearly necrotic tissue was compared across species, by comparison of the lesioned tissue to percent of live seedling stem remaining as in Hodge and Dvorak (2007).

To test the significance of lesion development at each timepoint, a multivariate analysis of variance was carried out. Factors were imbalanced due to the experimental design, there were no mock-inoculated samples to compare to inoculated hybrid measurements (Figure 2.1 and Figure 2.3). This was necessary due to the limited number of hybrid seedlings available (Table S1). To account for the imbalance in analysis of lesion development, intra-subject models were created by collapsing unbalanced factors into group variables, specifically treatment (mock-inoculated or inoculated) and species (*P. patula*, hybrid, or *P. tecunumanii*) at each time point (Figure 2.3). Analysis was conducted using the ‘‘MANOVA’’ function from the car package (Fox *et al.* 2007) in R 3.5.1 (R Core Team 2017), accounting for the intra-subject design. Tukey test of interactions was performed using a linear mixed effects model and Benjamini-Hochsberg false discovery rate correction ($p < 0.10$). The results of the Tukey are summarised in a compact letter table, in which shared letters indicate no significant difference between groups (Figure 2.3).

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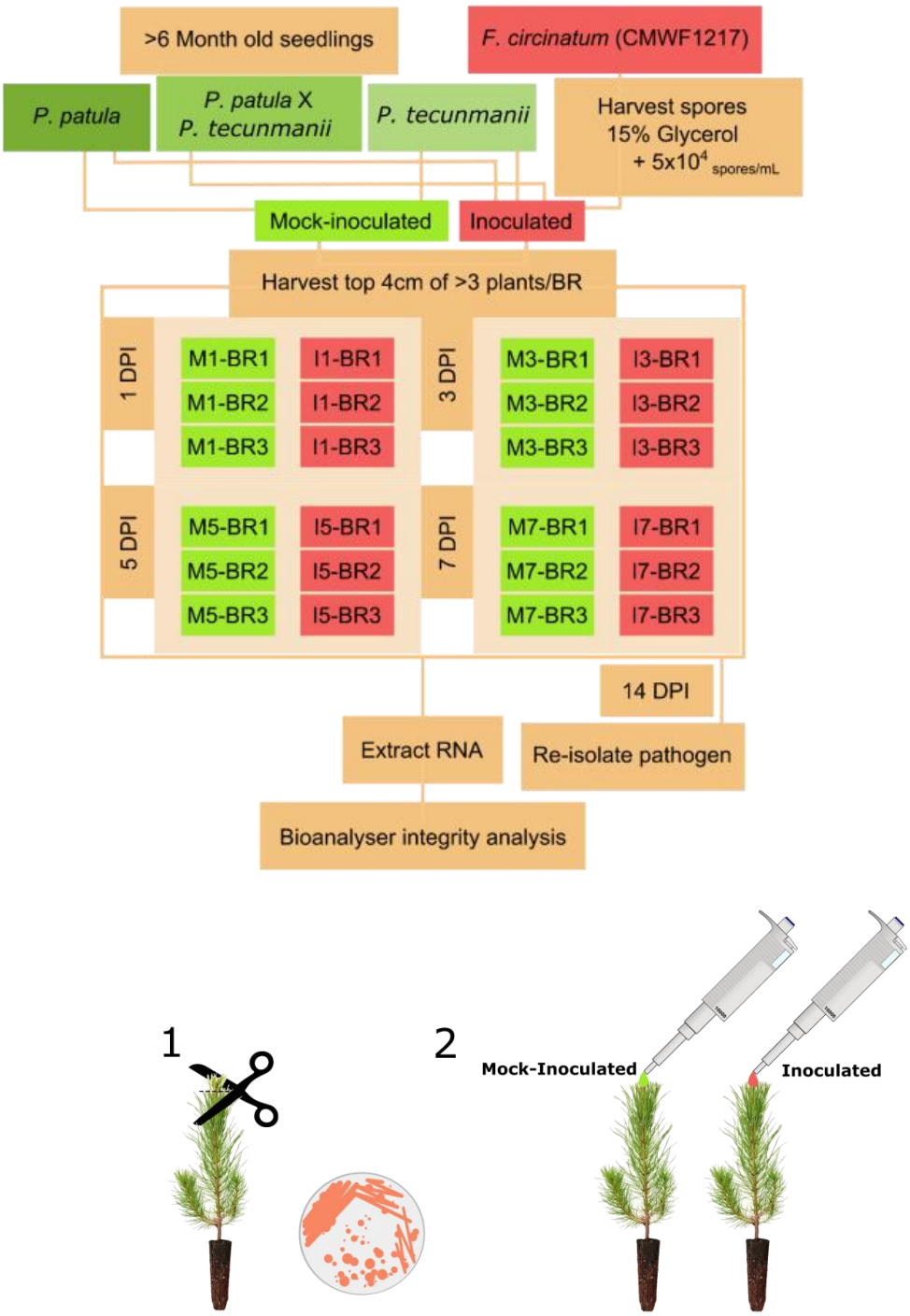

Figure 2.1: Outline of infection trial layout. Top: Six-month-old *Pinus patula* and *Pinus tecunumanii* seedlings were mock-inoculated (M) with sterile glycerol or inoculated (I) with *Fusarium circinatum* spores. Due to technical constraints, *Pinus patula* X *Pinus tecunumanii* hybrid seedlings were inoculated (I), but no control group was kept for comparison. Tissue was harvested at 1, 3, 5, and 7 dpi for 3 biological replicates (BR) per group. Disease progression monitored as lesion development within a separate group of seedlings for 6 weeks post-inoculation. *Fusarium circinatum* was re-isolated to confirm the presence of this fungus at 14 days post inoculation (dpi). Bottom 1: *Fusarium circinatum* spores are harvested and the apical meristem removed from the seedling. Bottom 2: Seedlings either mock-inoculated or inoculated in the same procedure.

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2.3.2 RNA extraction and sequencing

Tissue harvested from mock-inoculated and inoculated samples (Figure 2.1) was ground in liquid nitrogen using mortar and pestles. Total RNA was extracted using Norgen's Plant/Fungi RNA Purification Kit (Norgen Biotek Corp., Thorold, ON, Canada). Modifications to the standard protocol were as follows; 100mg of frozen tissue was added to 1ml of lysis buffer preheated to 55°C. To account for additional tissue, samples were centrifuged at 14,000 x g for 2 minutes, and the resulting supernatant was transferred into filter column, RNA was stored in the elution buffer at -80°C. RNA concentration and integrity were analysed using an Agilent 2100 Bioanalyzer (Figure 2.4). Samples with an RNA Quality Index (RQI/RIN) value below 7.0 were discarded and re-isolated where possible.

Although RNA was successfully isolated from all the samples, the -80^{o}C freezer malfunctioned prior to processing the RNA and all these samples were lost. Instead, RNA-seq data generated by Dr Erik Visser (Visser et al. 2018) was available for *P. patula* and *P. tecunumanii* isolated at 3 and 7dpi from a similar inoculation trial. With the availability of this data, the investigation of the terpene synthase gene families in these pine species could still proceed (Figure 2.2).

In silico gene expression analysis was determined by Kallisto transcripts per million (TPM) abundance values. TPM values for putative terpene synthase genes were imported to R 3.6.1. Significant effect of factors was calculated using a two-way analysis of variance (ANOVA, $p < 0.05$) with inclusion of an interaction term between timepoint and treatment, followed by a Tukey Honestly Significant Difference (HSD) post hoc test to identify significant differences between sample sets (adjusted $p < 0.05$). The results are shown in Figure S2.

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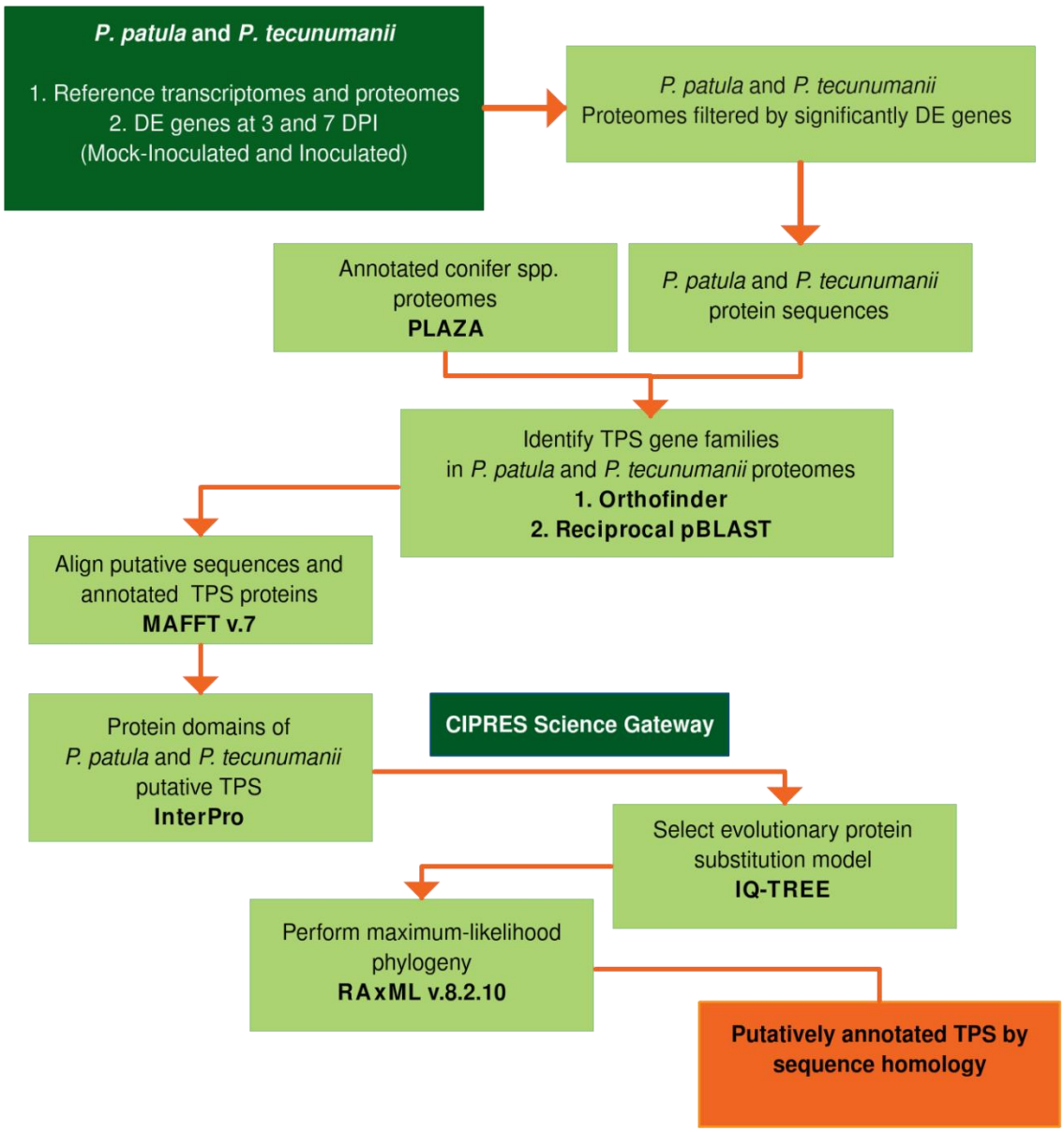

Figure 2.2: Summary of *in silico* annotation process of terpene synthase genes. DE genes and reference proteome were obtained from Visser (2015). The differential expression patterns of genes identified as putative terpene synthase orthologs was assessed for their potential to contribute to resistance. Maximum-likelihood phylogenetic analysis was used to resolve the implicated ortholog.

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2.3.3 Identification of terpene synthase genes

To elucidate the potential defensive contribution based on the pattern of expression of these genes, they were putatively identified. Before performing phylogenetic analysis, the subset of transcript sequences belonging to terpene synthase orthogroups in *P. patula* and *P. tecunumanii* were identified (Figure 2.2). The proteomes of several species were obtained from PLAZA, as in Shalev *et al.* (2018). This included *Arabidopsis thaliana, Populus trichocarpa, Oryza sativa japonica,* various *Pinus* spp., *Picea* spp., *Gnetum montanum,* and *Ginkgo biloba*. (Figure 2.2 and Table S1). Along with proteomes of *P. patula* and *P. tecunumanii* (Visser *et al.* 2015), orthogroups across all proteomes were inferred by Orthofinder 1.1.4 (Emms and Kelly 2015). Gene families annotated as terpene synthases were confirmed by a reciprocal pBLAST approach. Both techniques enabled identification of putative terpene synthase genes within *P. patula* and *P. tecunumanii*, based on sequence similarity with previously annotated terpene synthase proteome sequence data (Aubourg *et al.* 2002; Keeling and Bohlmann 2006b; Keeling *et al.* 2011; Külheim *et al.* 2015).

Through phylogenetic analysis, a total of 156 putative terpene synthase protein sequences, including those of *P. patula* and *P. tecunumanii*, were putatively identified as belonging to gene families of interest and prepared for phylogenetic analysis (Figure 2.2). Sequences were aligned using MAFFT v.7 (Katoh *et al.* 2018). Transcripts were removed if they lacked certain conserved protein domains known to be important to the catalytic activity of terpene synthases (Figure 2.6) as identified by domains inferred by InterPro (Hunter *et al.* 2009).

Sequence alignments were uploaded to the CIPRES Science Gateway (Miller *et al.* 2010) and IQ-TREE (Trifinopoulos *et al.* 2016) was used to select the most appropriate evolutionary protein substitution model (Figure 2.2). RAxML v 8.2.10 (Stamatakis 2014) was used to generate a maximum-likelihood phylogeny, implementing the Jones-Taylor-Thornton (JTT) + GAMMA amino acid evolution model, with empirical base frequencies determined directly from the alignment (+F). Support for the clustering of sequences were obtained using rapid bootstrapping (Figure S1). The tree was rooted to midpoint (Figure 2.2 and Figure S1).

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2.3.4 Differential terpene synthase gene expression

Inoculated samples from each of the two hosts, *P. tecunumanii* and *P. patula*, were compared against their respective mock-inoculated samples with DESeq2 1.18.1 (Love *et al.* 2014), to identify differentially expressed genes (Figure 2.2; Visser *et al.* 2018). Genes that were significantly upregulated, relative to the control transcriptome of each of the species, were identified using a Wald test with the Benjamini & Hochberg false discovery rate correction ($p < 0.10$). For *P. patula* 323 and 7 453 significantly differentially expressed genes (Inoculated vs. Mock-Inoculated) were identified at 3 and 7 dpi respectively, while 735 and 2 499 significantly differentially expressed genes were identified for *P. tecunumanii* (Visser *et al.* 2018).

2.4 Results

2.4.1 *Fusarium circinatum* infection trial

Inoculation with *F. circinatum* produced significantly longer necrotic lesions in *P. patula* as compared to *P. tecunumanii* and the hybrid seedlings (Figure 2.3). There were no significant differences in necrosis lengths between *P. tecunumanii* and the hybrid seedlings (Figure 2.3).

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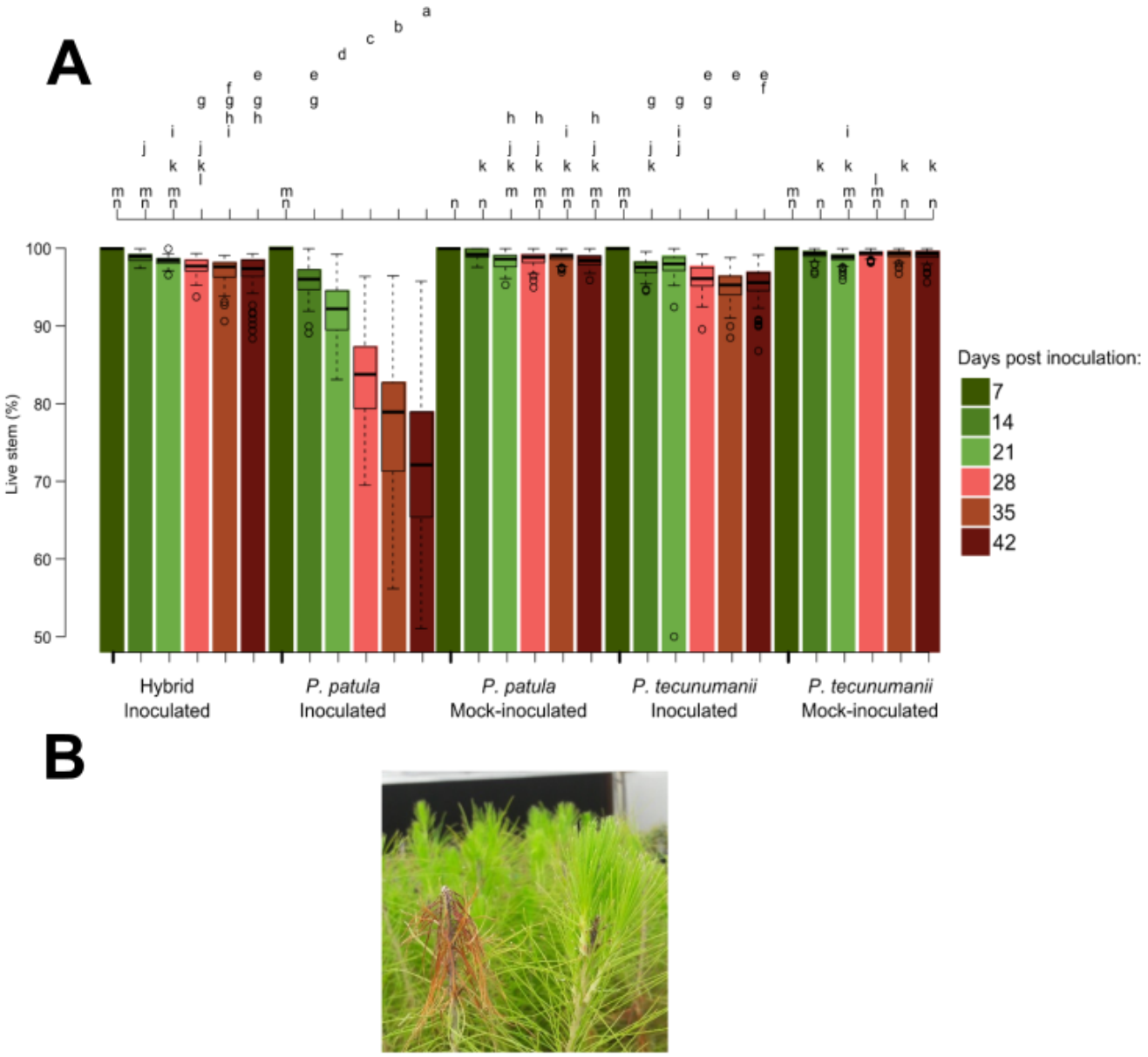

Figure 2.3: Comparative necrotic lesion lengths induced by *Fusarium circinatum* on inoculated *Pinus* seedlings. A: Lesion development as indicated as percentage live stem, over time for seedlings. Error bars represent the standard errors of the means. Compact letter table indicates associations between groups based on Tukey results, where shared letters indicate no significant difference between groups. **B:** Representative *P. patula* (left) and *P. tecunumanii* (right) inoculated seedlings 42dpi (days post inoculation).

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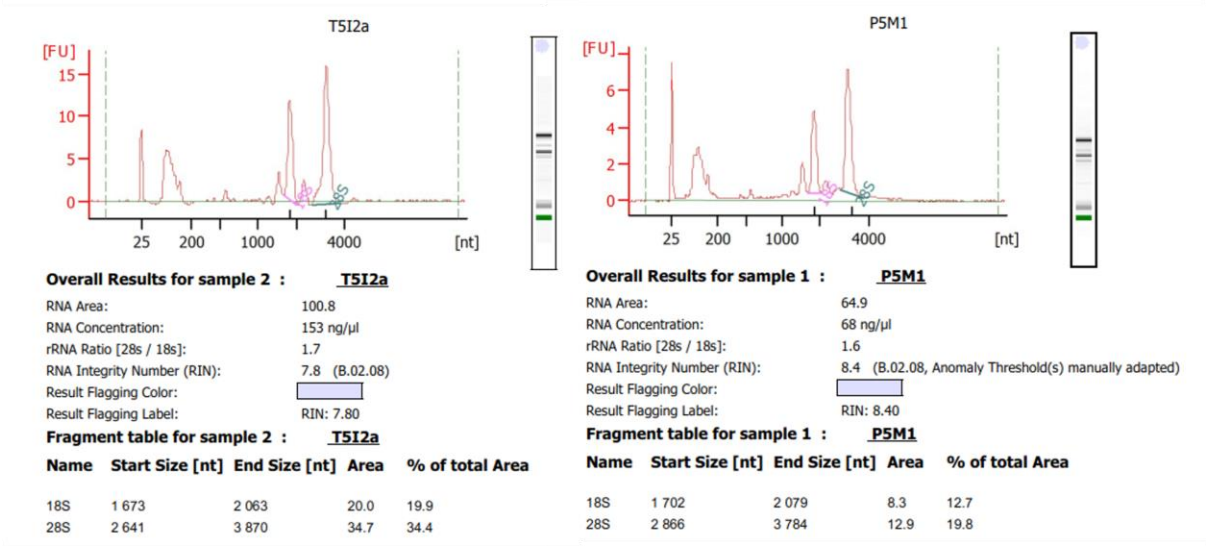

Figure 2.4: Representative results of RNA integrity assessment by Agilent 2100 Bioanalyzer of samples from *P. tecunumanii* (left) and *P. patula* (right) harvested at 5dpi. Both samples were of satisfactory integrity, having RQI/RIN values >7.0.

2.4.2 Putative terpene synthase gene identification

The alteration of terpene synthase gene expression by each host in response to the pathogen is evidently distinct. Figure 2.5 shows a subset of the phylogenetic tree constructed to further classify and annotate these transcripts according to characterised conifer terpene synthase transcripts. The number of putative terpene synthases responding to *F. circinatum* infection was nine in *P. patula*, compared to six in *P. tecunumanii* (Figure 2.5). Most of the genes expressed in *P. tecunumanii* had corresponding orthologs in *P. patula*. The only gene identified as being upregulated in *P. tecunumanii* for which a corresponding ortholog was not differentially regulated in *P. patula* was transcript 'Pnte25LSn_DN87317_c0_g2', annotated as a gamma-bisabolene synthase (Figure 2.5). *Pinus tecunumanii* orthologs were not identified for four of the genes differentially expressed as part of the defence response in *P. patula*. This included the downregulation in *P. patula* of two transcripts 'Pipt31HSn_DN242281_c0_g', a cineole synthase, and 'Pipt31HS_DN235878_c0_g1', a monofunctional pimaradiene synthase. Interestingly, this putative pimaradiene synthase is the only diterpene synthase identified as being involved in the defence response (Figure 2.5).Two other genes identified exclusively in

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P. patula, 'Pipt31HSn_DN207510_c1_g1' and 'Pipt25HSn _DN220074_c0_g4', both being putative sesquiterpene synthases, were downregulated at 7- days post inoculation.

All sets of orthologous transcripts were identified as being differentially regulated in both hosts as a result of *F. circinatum* infection (Figure 2.5). Including, transcripts 'Pipt25LSn _DN279416_c1_g3' and 'Pnte31HSn_ DN77088_c0_g5', which putatively encode orthologous alpha-humulene synthases in *P. patula* and *P. tecunumanii*, respectively. It is worth noting that while *P. tecunumanii* responded by strongly upregulating the transcription of alpha-humulene synthase at both 3- and 7-days post infection, *P. patula* responded in the reverse, downregulating this transcripts expression at the later of the two time points (Figure 2.5). Although less strongly regulated, another instance of contradistinctive response in *P. patula* and *P. tecunumanii* is the regulation of another pair of orthologous genes. These genes encoded alpha-pinene synthases and represented by the transcripts 'Pipt31HSn_DN236119_c0_g2' and 'Pnte25LS_DN112020_c3_g1' respectively. In this study, alpha-pinene synthase gene expression was upregulated in *P. tecunumanii* but downregulated in *P. patula* (Figure 2.5).

There were six transcripts identified as encoding putative farnesene synthases, three alpha-farnesene, and three beta-farnesene synthases (Figure 2.5). This included a pair of alpha-farnesene synthases within *P. patula*; 'Pipt25LS_DN334024_c3_g4', and 'Pipt31HS_DN265815_c0_g3', a single alpha-farnesene synthase 'Pnte31LSn_DN97191_c0_g1' is identified as being upregulated in *P. tecunumanii*. The inferred primary structure of two of these putative alpha-farnesene synthases are shown in more detail in Figure 2.6. The other triplet of orthologous genes putatively annotated as encoding beta-farnesene synthases, included a single transcript, 'Pipt31HS_DN278168_c4_g1' in *P. patula*, and a paralogous pair in *P. tecunumanii*, 'Pnte25LSncg_GG_4275_c0_g1' and 'Pnte31HSn_DN82496_c0_g3' (Figure 2.5 and Figure S1).

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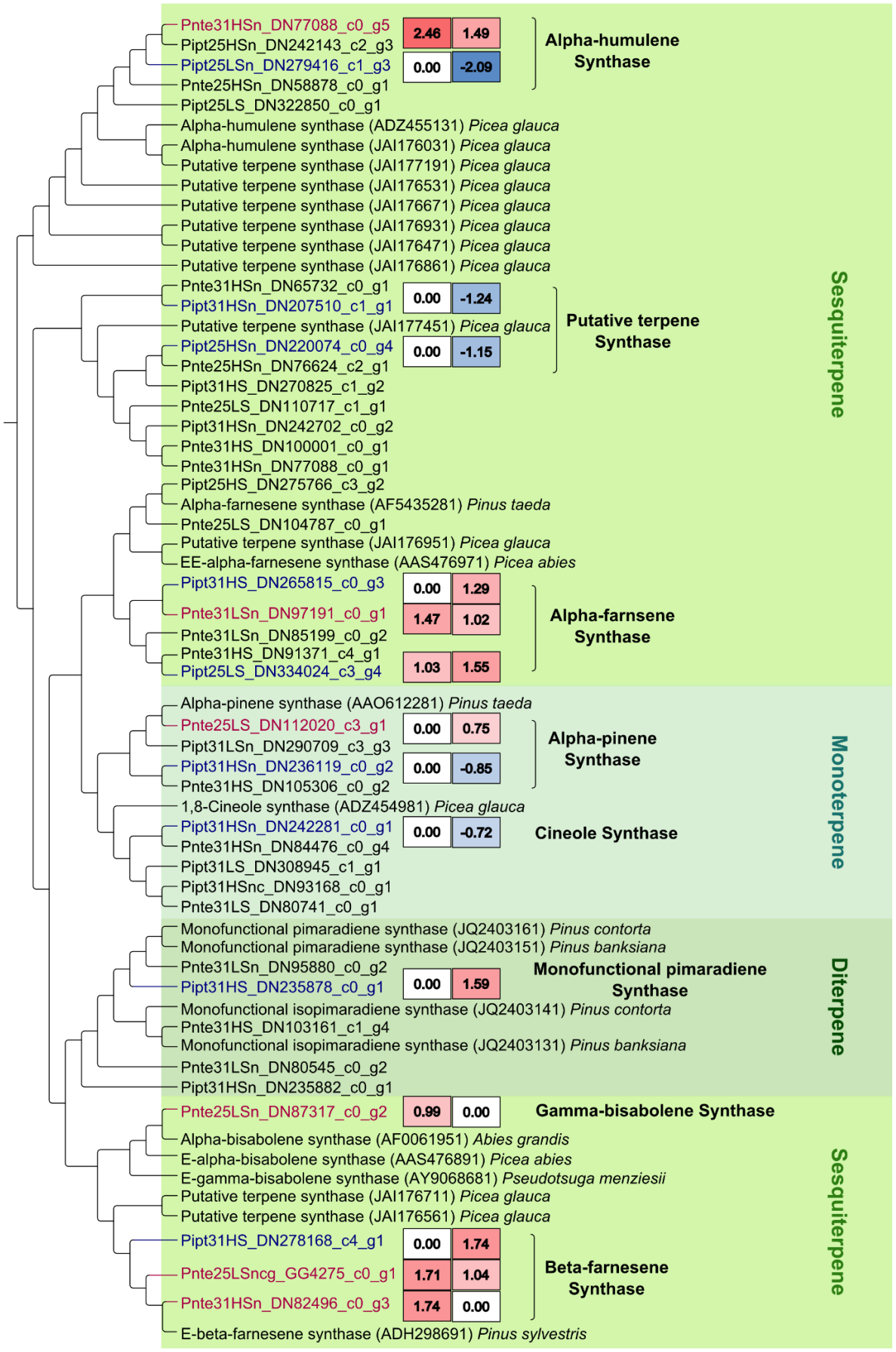

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Figure 2.5: Phylogenetic analysis of putative terpene synthases response in *P. patula* and *P. tecunumanii* to allow their partial characterisation. The putative terpene synthase genes significantly differentially expressed in the inoculated seedlings at least one of the two timepoints of interest as shown as $\log_2$(Fold Change) values in boxes next to highlighted terminal nodes (left and right boxes showing 3-, and 7 days post inoculation respectively). All internal nodes are supported by >80% bootstrapping.

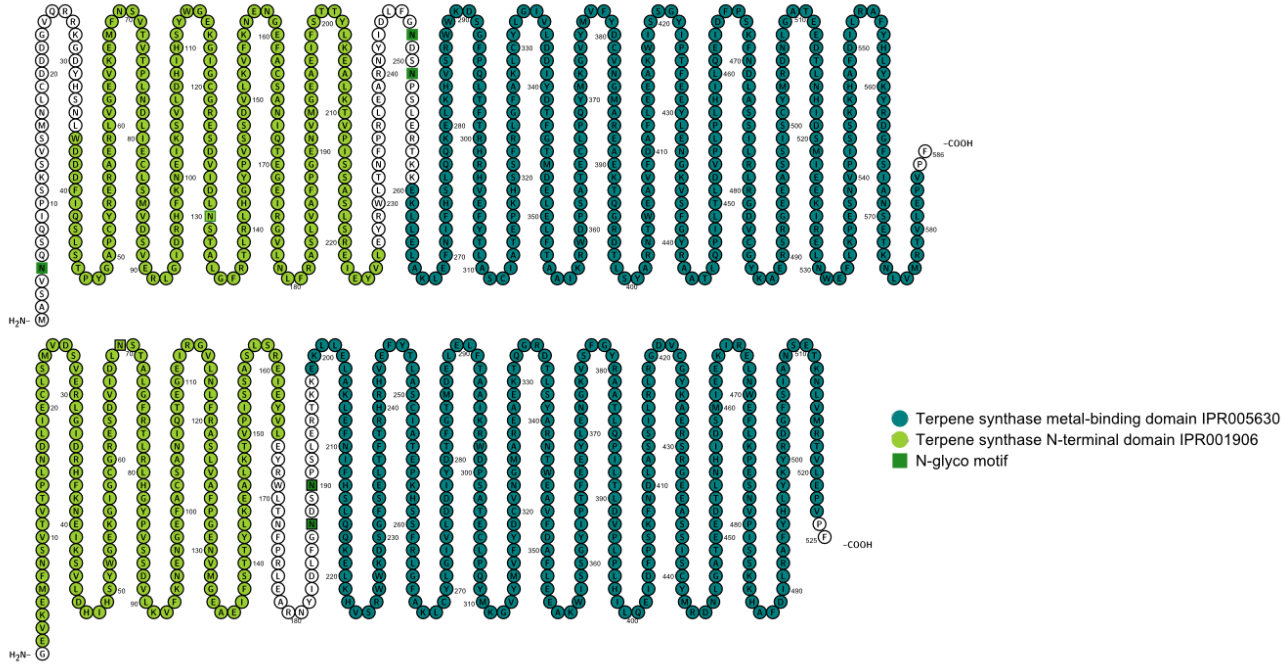

Figure 2.6: Primary structures of putative alpha-farnesene synthase orthologs Pipt31HS_DN265815_c0_g3 in *P. patula* and Pnte31LSn_DN97191_c0_g1in *P. tecunumanii* (top and bottom respectively). These orthologs are regulated in opposite directions by each host responding to the same pathogen. Conserved protein domains known to be important to the catalytic activity of terpene synthases identified by InterPro are shown.

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2.4.3 Defensive gene expression in *P. patula* and *P. tecunumanii*

The variance in expression of putative terpene synthase genes, by the two host species, was compared to quantify their defence response when challenged by *F. circinatum*. Gene expression recognised as being incongruent with the respective control expression was attributed to the defensive response and was compared between the two hosts (Figure 2.2). These genes were those which displayed at least a 0.7 $\log_2$(Fold Change) alteration in expression, in either direction, when compared to the mock inoculated control group ($-0.70 < \log_2(\text{Fold Change}) > 0.70$). This study found eight putative terpene synthase gene orthogroups to be significantly differentially expressed by either host, at 3- and/or 7- dpi with *F. circinatum* (Figure 2.5).

2.5 Discussion

The production of a diverse suite of terpene molecules is a prominent chemical and physical defence system of *Pinaceae* (Keeling and Bohlmann 2006a; Zulak and Bohlmann 2010; Zhao *et al.* 2011). Earlier research has shown that *Pinus* spp. differ in terpene defensive properties that could influence their relative susceptibility, such as constitutive terpenoid composition or lower induced terpene production (Arango-Velez *et al.* 2016). There has been increasing research focussing on the transcriptional and phenotypical terpene response by predominant *Pinus* spp. against phytopathogenic fungi colonisation (Storer *et al.* 1998; Erbilgin and Colgan 2012; Mead 2013; Wang *et al.* 2014; Raitelaitytė *et al.* 2016; Arango-velez *et al.* 2018; Hammerbacher *et al.* 2019; Woranoot *et al.* 2019). The defence strategies, which have diversified within each lineage, differ in their relative efficacy to resist infection by *F. circinatum*. Specifically, the qualitative and quantitative characteristics of the host's terpene profile is an important determinant of the susceptibility of *Pinus* spp. to *F. circinatum* (Dvorak *et al.* 2009; Seeve 2010; Kanzler *et al.* 2012; Swett *et al.* 2018, 2014; Carrasco *et al.* 2017; Gordon and Reynolds 2017; Amaral *et al.* 2019; Chen *et al.* 2019; Lombardero *et al.* 2019; Reynolds and Gordon 2019).

In this study, we focused on the transcriptional consequences to terpene synthase gene expression in two *Pinus* spp. initiated by the fungal pathogen *F. circinatum* (Figure 2.2). The

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more susceptible of the two species, *P. patula*, is still of considerable importance to local agriculture, although often at huge economic expense due to its acute susceptibility to *Fusarium*-wilt (Figure 2.3). (Mitchell *et al.* 2011; Wingfield *et al.* 2012; Fru *et al.* 2018; Hongwane *et al.* 2018; Mabaso *et al.* 2019). This pathogen is making commercial reliance on this species difficult, so research into resistance breeding is increasingly becoming important. The second species that we have included, *P. tecunumanii* (LE), is currently of little agricultural significance in South Africa, but is relatively resistant to *F. circinatum* (Hodge and Dvorak 2000; Mitchell *et al.* 2012; Hongwane *et al.* 2018). Differences can be observed by comparative lesion development between *P. patula* and *P. tecunumanii* seedlings that have been colonised by *F. circinatum* (Figure 2.3). The aim of this study was then to determine whether these difference in relative susceptibility, is correlated with discrepancies in the transcriptional regulation of putative terpene synthase genes.

It must be noted that terpene synthase product profiles cannot be accurately predicted based on sequence analysis alone. Correlation of enzymes with similar structures previously characterised, have allowed annotation for of the genes identified in this study. However, there is always the possibility that two highly dissimilar terpene synthases share a similar product profile. An instance of this was observed by Bohlmann *et al.* (1997), when comparing the limonene synthase of *Mentha spicata* (spearmint) to that of *Abies grandis*. The two enzymes produced the same terpene products, although they share less than a third of their sequence identities with one another (Bohlmann *et al.* 1997). Nevertheless, we can gain insight into the characteristic response of each host by assessing characterised functions of orthologous enzymes.

2.5.1 Comparative necrotic lesion development

The relative seedling tolerances of *P. patula* and *P. tecunumanii*, as well as the predicted intermediate tolerance of *P. patula* x *P. tecunumanii* hybrid families, was initially assessed by measuring development of lesions (Figure 2.3). The proportionate progression of the lesioned stem was compared statistically to gain insights into each species' defence responses. Significantly longer necrotic lesions were observed in *P. patula*, when compared to *P. tecunumanii* were observed, as was expected in accordance with previous research (Hodge and Dvorak 2000; Kanzler *et al.* 2014; Visser *et al.* 2015). Interestingly, while the lesions of *P.*

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patula continued to develop throughout the observation period, by 42dpi, *P. tecunumanii* had largely recovered and was beginning to establish new growth (Figure 2.3).

Instead of the hybrid seedlings having an intermediate susceptibility to *F. circinatum*, as could have been expected (Kanzler *et al.* 2014), no significant increase in hybrid necrotic lesion lengths were observed compared to those in *P. tecunumanii* (Figure 2.3). This is likely due to the hybrid seedlings having been a few months older than the pure parent species. Hybrid seedlings were therefore slightly larger, and presumably had more established defence systems. However, it is not unusual for the rate of lesion development in hybrid genotypes to be equivalent to *P. tecunumanii*. The severe rate of lesion development in *P. patula* seedlings is seldom observed among hybrid species (Kanzler *et al.* 2014).

2.5.2 Defensive contribution of terpene synthase gene expression

Two sets of annotated homologs, a pair of alpha-farnesene synthases in *P. patula*, and beta-farnesene synthases in *P. tecunumanii*, were found to contain similar sequences to each other. This would suggest that paralogous pairs represent nearly identical allelic variants, or recently duplicated genes within each of the host genomes. Interestingly, the alpha-farnesene synthase paralogs annotated in *P. tecunumanii* appear to be more closely related to one of the alpha-farnesene synthases in *P. patula*, than the two *P. patula* paralogs are to each other. The beta-farnesene genes identified within *P. patula* were also sequentially very similar to their respective orthologs in *P. tecunumanii*.

The monoterpene alpha-pinene synthase has been fully annotated in *Pinus taeda*, and its product profile is understood to predominantly compromise of alpha-pinene (~80%), with small amounts of beta-pinene (Phillips *et al.* 2003). *Pinus taeda* is relatively closely-related to the two species considered in this study (De La Torre *et al.* 2014; Visser 2015). It can thus be assumed that the increased upregulation of alpha-pinene synthase in *P. tecunumanii* results in an increased concentration of alpha-pinene. The other monoterpene, namely cineole synthase, has been functionally characterised in spruce (*Picea sitchensis* and *Picea glauca*). This enzyme has been determined being a multi-product enzyme (Keeling *et al.* 2011). The importance of this enzyme to the contribution of resistance is likely minimal as it was not significantly differentially regulated in *P. tecunumanii*, and only slightly decreased expression in *P. patula* (-0.72 $\log_2$(Fold Change)).

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The potential for significant contribution by the sesquiterpene fraction in pathogen defence is interesting because these molecules constitute less than 10% of the oleoresin (Steele *et al.* 1998). However, sesquiterpene volatiles are known as allelochemicals (Wedge *et al.* 2000), and contribute to both indirect (Schnee *et al.* 2006), and direct defences against plant pathogens (Bohlmann *et al.* 1998a; Park *et al.* 2014; Pham *et al.* 2014). The inducible production of sesquiterpenes, via transcriptional upregulation of sesquiterpene synthase, has been shown to be significant toward phytopathogenic defence (Piesik *et al.* 2011; Park *et al.* 2014; da Luz *et al.* 2017; Woranoot *et al.* 2019). Sesquiterpenes include photosensitizers, which once activated, enzymatically or by light, produce highly reactive free radicals (Ashour *et al.*; Burden and Kemp 1984; Pearce 1996). In particular, many sesquiterpene lactones have been shown to exhibit antifungal activity when introduced to phytopathogenic *Fusarium* spp. (Wedge *et al.* 2000).

In an earlier study, analysis of alpha-humulene synthase orthologs in *Pinus sylvestris* and *P. sitchensis* has shown similar product profiles, with the two major products being alpha-humulene and beta-caryophyllene (Keeling *et al.* 2011). The alpha-humulene synthase gene is seemingly highly significant in the relative susceptibility of *P. patula* to *F. circinatum* as these orthologs show the most dissimilar expression patterns. Farnesene synthases have been characterised in *P. sitchensis* and *P. sylvestris*, and their products have been shown to be predominantly alpha- and beta-farnesene (Ralph *et al.* 2006; Köpke *et al.* 2010). The final sesquiterpene that was observed, gamma-bisabolene synthase, has been characterised in *Abies grandis*, and is associated with the synthesis of gamma-bisabolene, as well as the monoterpene limonene (Bohlmann *et al.* 1998b; Steele *et al.* 1998). These enzymatic product profiles are, by reference, relatively similar to those of annotated genes identified within *P. patula* and *P. tecunumanii*. Monofunctional pimaradiene and iso-pimaradiene synthases, characterised in *Pinus banksiana* and *Pinus contorta*, were earlier shown to contribute marginally to the diterpene fraction of oleoresin (Hall *et al.* 2013b). Therefore, in accordance with previous research (Byun-McKay *et al.* 2006), the relative susceptibility of *P. patula* appears to be primarily dependent on the expression of mono- and sesquiterpene genes.

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2.5.3 Differential gene expression 3- and 7-days post infection (dpi)

Visser *et al.* (2018) observed greatest defensive transcriptional variation between *P. tecunumanii* and *P. patula* during the early stages of colonisation by *F. circinatum*. The defence response induced by *F. circinatum* was distinguished from other influences on gene expression, such as by mechanical wounding during inoculation, by comparison with mock-inoculated controls of each species (Figure 2.1). Differentially expressed terpene synthase genes that were identified in *P. patula and P. tecunumanii* were split across three orthogroups (Figure 2.5). The largest group contained sequences putatively annotated as sesquiterpene synthases. These terpene synthase genes grouped into the major TPS-d1, TPS-d2 and, TPS-d3 clades according to their likely orthologs (Table 1.1, Chapter 1). Identified mono- and sesquiterpenes belonged to TPS-d1 clade, which includes alpha-pinene synthases, and farnesene synthases. The TPS-d2 clade is composed of enzymes such as longifolene synthases; none of these were identified in this study. Finally, the single pimaradiene synthase belongs to the TPS-d3 clade (Figure 2.5 and Figure S1).

The observation is that *P. patula* differentially regulated nine putative terpene synthase genes in comparison to only six in *P. tecunumanii*. However, it should be considered that while all six genes identified in *P. tecunumanii* are upregulated, only three of the genes in *P. patula*'s nine are upregulated (Figure 2.5 and Figure S2). Therefore, we could hypothesise that while *P. patula* responds by altering the expression of a wider range of terpene synthases, it is not necessarily investing greater resources than *P. tecunumanii*. Comparison of their constituent, and induced terpene concentrations will be important to determine how the altered expression builds on the chemistry of the oleoresin (Keeling *et al.* 2010; Zulak and Bohlmann 2010; Hall *et al.* 2013a; Trindade *et al.* 2016; Mitić *et al.* 2017; Bullington *et al.* 2018; Kshatriya *et al.* 2018; Lombardero *et al.* 2019).

The putative monoterpene synthase alpha-pinene, was upregulated at 7dpi in inoculated *P. tecunumanii* seedlings (Figure 2.5 and Figure S2). At 7dpi, *P. patula* responded more substantially, altering the expression of all nine putatively annotated genes (Figure 2.5). Starting with the upregulation of a single alpha-farnesene synthase gene at 3dpi and augmenting this response at 7dpi. This includes downregulating a pair of putative monoterpene synthases, alpha-pinene and cineole synthase, altering the expression of numerous sesquiterpene synthases, and remarkably, upregulating a putative diterpene, mono-functional pimaradiene

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synthase. At 7dpi the same genes in *P. tecunumanii* continued to respond as at 3dpi, an additional transcript observed at 7dpi was upregulation of alpha-pinene synthase (Figure 2.5). The first two of *P. tecunumanii*'s four sesquiterpene synthase transcripts could only be annotated as putative sesquiterpene synthases, and no orthologous transcripts were identified as being altered in *P. tecunumanii*. The third and final sesquiterpene gene observed to have been downregulated by *P. patula* at 7dpi, alpha-humulene synthase, is of the most interest. This alpha-humulene synthase is the most differently transcriptionally regulated gene by both *P. patula* and *P. tecunumanii*, though, the two hosts differ in the direction in which they regulate its expression (Figure 2.5 and Figure S2).

2.6 Conclusions

This study has allowed the partial characterisation, based on functional genomics, of the terpene synthase genes implicated in defence against *F. circinatum* in two *Pinus* spp. that are important to the local forestry. In conclusion, *P. tecunumanii* was observed to have upregulated the transcription of five putative sesquiterpene synthase transcripts (Figure 2.5), which could suggest increased sesquiterpene concentrations (Lombardero *et al.* 2019). By comparison, it can be argued that no significant increase in sesquiterpene concentrations was observed in *P. patula* (Figure 2.5). In fact, *P. patula* was observed to upregulate three sesquiterpene synthases, the same number as downregulated (Figure 2.5). Yet, no direct correlation has been found between the terpene constituents of host oleoresin and its susceptibility to *F. circinatum* (Marei *et al.* 2012; Slinski *et al.* 2015; Iturritxa *et al.* 2017; Roth *et al.* 2018).

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Chapter 3

Concluding Remarks

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3.1 Concluding remarks

Comparison of orthologous terpene synthase gene expression induced by infection in *P. patula* and *P. tecunumanii* indicate that qualitative and/or quantitative variations in terpene profiles could contribute to *F. circinatum* susceptibility. The potential link between defensive terpene regulation and *F. circinatum* susceptibility has been more thoroughly studied in *Pinus radiata*, as this species is of global agricultural importance and is especially susceptible to the pitch canker and *Fusarium*-wilt (Wingfield *et al.* 2008; Carrasco *et al.* 2017).

Pinus patula's terpene defensive regulation appears initially inefficient at 3dpi, compared to that of *P. tecunumanii*. Of the nine putative terpene synthase genes identified as differentially expressed by *P. patula* in response to *F. circinatum*, only one shows altered expression at 3dpi (Figure 2.5). This gene, 'Pipt31HSn_DN236119_c0_g2', was putatively annotated as an alpha-farnesene synthase, shows the most upregulation ($\log_2$(Fold Change) > 1.0). Inversely, *P. tecunumanii* has responded to *F. circinatum* at 3dpi by upregulating the expression of four genes. Interestingly, all genes identified were upregulated at 3dpi were sesquiterpene synthases (Figure 2.5). None of the annotated genes were observed to have been down-regulated ($\log_2$(Fold Change) <−1) at 3dpi. In disaccord with *P. tecunumanii*, *P. patula*'s defensive terpene synthase regulation is noticeably latent at 3dpi. Therefore, we hypothesise that the terpene metabolomic response by *P. patula* may be inadequate during the early stages of infection, underpinning its susceptibility.

In a study similar to this one, Lombardero *et al.* (2019) compared terpene compositions of *P. radiata* to those of relatively resistant *Pinus pinaster*, to identify potential differences that could underly susceptibility. They observed these two species to share similar constituent concentrations of monoterpenes and diterpenes (Flø *et al.* 2018; Lombardero *et al.* 2019). However, the concentrations of mono- and diterpenes induced by *F. circinatum* were shown to be significantly higher in *P. radiata* than in *P. pinaster* (Lombardero *et al.* 2019). Interestingly, both constituent and induced sesquiterpene concentrations were notably higher in *P. pinaster* than in *P. radiata*, identifying sesquiterpene concentrations as a potential marker of susceptibility (Lombardero *et al.* 2019). Sesquiterpenes observed in significantly higher concentrations in *P. pinaster* were beta-myrcene, camphene, and most remarkably, alpha-humulene (Lombardero *et al.* 2019).

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Each host-pathogen interaction has its own set of complicating variables to consider. For instance, it has been observed that downregulation of terpenoid biosynthesis could contribute to host resistance, as the non-volatile components of oleoresin could serve as a carbon source for the pathogen (Martín-Rodrigues *et al.* 2013; Gordon *et al.* 2015; Lo Presti *et al.* 2015) Studies found that quantitative differences in terpenes do not appear to correlate with host resistance either, in fact, *F. circinatum* colonises resin ducts (Marei *et al.* 2012; Martín-Rodrigues *et al.* 2013; Wen *et al.* 2018). However, even if the regulation of terpene synthesis is not the principal determinant of resistance, the association may indirectly assist with the selection of resistant genotypes in the future. This is particularly true as the focus of this study has been exclusively the *in silico* analysis of the terpene synthase genes which are differentially regulated in response to infection with *F. circinatum*, and only the induced defensive response prompted by this phytopathogen was observed.

3.2 Future work and significance

In addition to assessing terpene concentrations of *P. patula* and *P. tecunumanii*, research into the contribution by broader biochemical defences to susceptibility is required (Gordon *et al.* 2015; Reglinski *et al.* 2017; Celedon and Bohlmann 2019). It is important to build on our observations at a transcriptional level by performing metabolic analysis, to determine to what extent the biochemistry of these two species differ. The *in vivo* characterisation of annotated terpene synthases identified herein will advance our understanding of their products, and ultimately, their contribution to defence against *F. circinatum.*

The initiation and regulation of defensive biochemical pathways is complex, and their efficacy can affect host susceptibility to colonisation by pathogens (Hu *et al.* 2018; Neis *et al.* 2018). Understanding this will involve the functional characterisation of numerous upstream enzymes, such as cytochrome P450-dependent monooxygenases (Trindade *et al.* 2016; Wen *et al.* 2018). Further comparative analysis of other important defence pathways in *Pinus* spp. could reveal important transcriptional divergences between *P. patula* and *P. tecunumanii*. Visser *et al.* (2018) assessed the contribution of pathogenesis-related genes that are differentially expressed by *P. patula* and *P. tecunumanii* in response to infection by *F. circinatum.*

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Knowledge of terpene-based defence mechanisms that effectively limit *F. circinatum* infection could contribute to the reduction of the impact on *P. patula*. Genetic engineering and targeted breeding have already shown the potential to increase the levels of constitutively produced terpenes, yet at a cost to carbon efficiency (Peter 2018). Therefore, it would be preferential to target the increased expression of particular terpene synthases, minimising the overall energetic investment (Huber *et al.* 2004; Zerbe and Bohlmann 2014, 2015; Paknikar and Fondekar 2018; Celedon and Bohlmann 2019).

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Supplementary Material

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Supplementary Table

Table S1: Extended list of six-month-old *Pinus* seedlings, obtained from Dr. Nicky Jones (Sappi Forests, Shaw Research Centre, Howick, KZN). Counts post two-week acclimatisation period.

Seedling	**Total Count:** 1708
***Pinus patula* pure families**	
P9- *P. patula*	98
P17-*P. patula*	192
P38- *P. patula*	146
P42- *P. patula*	200
Total *P. patula:* 746	
Pinus tecunumanii **Low-Elevation (LE) seed mix**	
PEC 0076- P. *tecunumanii*	814
Total *P. tecunumanii*: 814	
P. patula x P. tecunumanii **hybrid open-pollinated families**	
PPTL024.3-Hybrid	10
PPTL017.4-Hybrid	10
PPTL021.1-Hybrid	10
PPTL027.3-Hybrid	9
PPTL004.3-Hybrid	10
PPTL015.2-Hybrid	10
PPTL026.3-Hybrid	10
PPTL010.3-Hybrid	10
PPTL027.2-Hybrid	9
PPTL025.3-Hybrid	10
PPTL010.4-Hybrid	10
PPTL008.2-Hybrid	10
PPTL022.2-Hybrid	10
PPTL001.1-Hybrid	10
PPTL017.3-Hybrid	10
Total h*ybrid* seedlings:148	

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Supplementary Figures

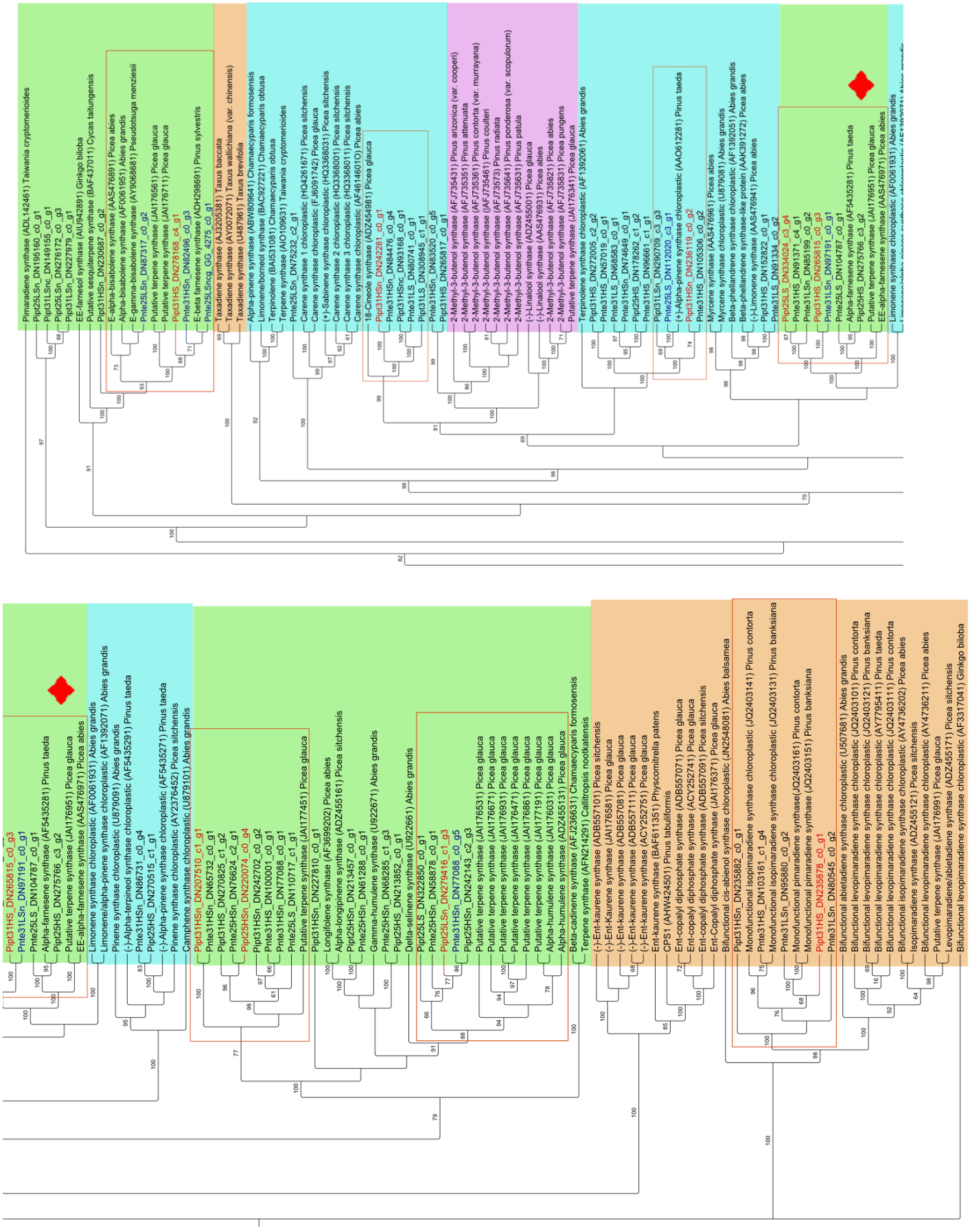

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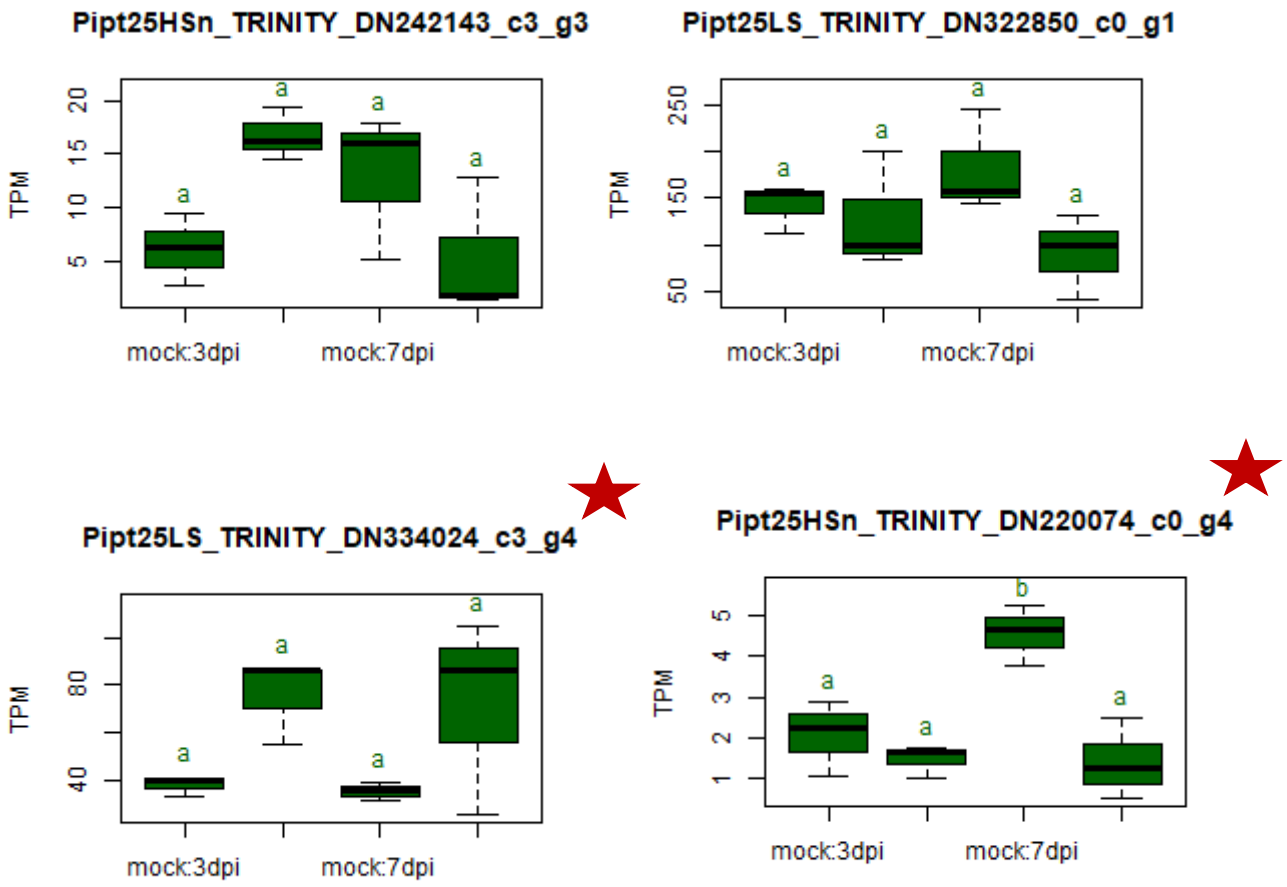
Figure S1: Phylogenetic analysis of putative terpene synthases in numerous Conifer spp. The putative terpene synthase genes are significantly differentially expressed in the inoculated seedlings at least one of the two timepoints of interest are coloured (red: *P. patula*, blue; *P. tecunumanii*). These were those displayed altered expression, compared to the mock inoculated control group ($-0.70 < \log_2(\text{Fold Change}) > 0.70$). Red blocks indicate subset of phylogeny used in final annotation of differentially expressed genes. Other conifer genes were previously annotated as terpene synthase genes (Shalev et al. 2018). All internal nodes are supported by >80% bootstrapping. Red diamonds indicate the continuum of the phylogeny across the divide.

Sesquiterpenes:



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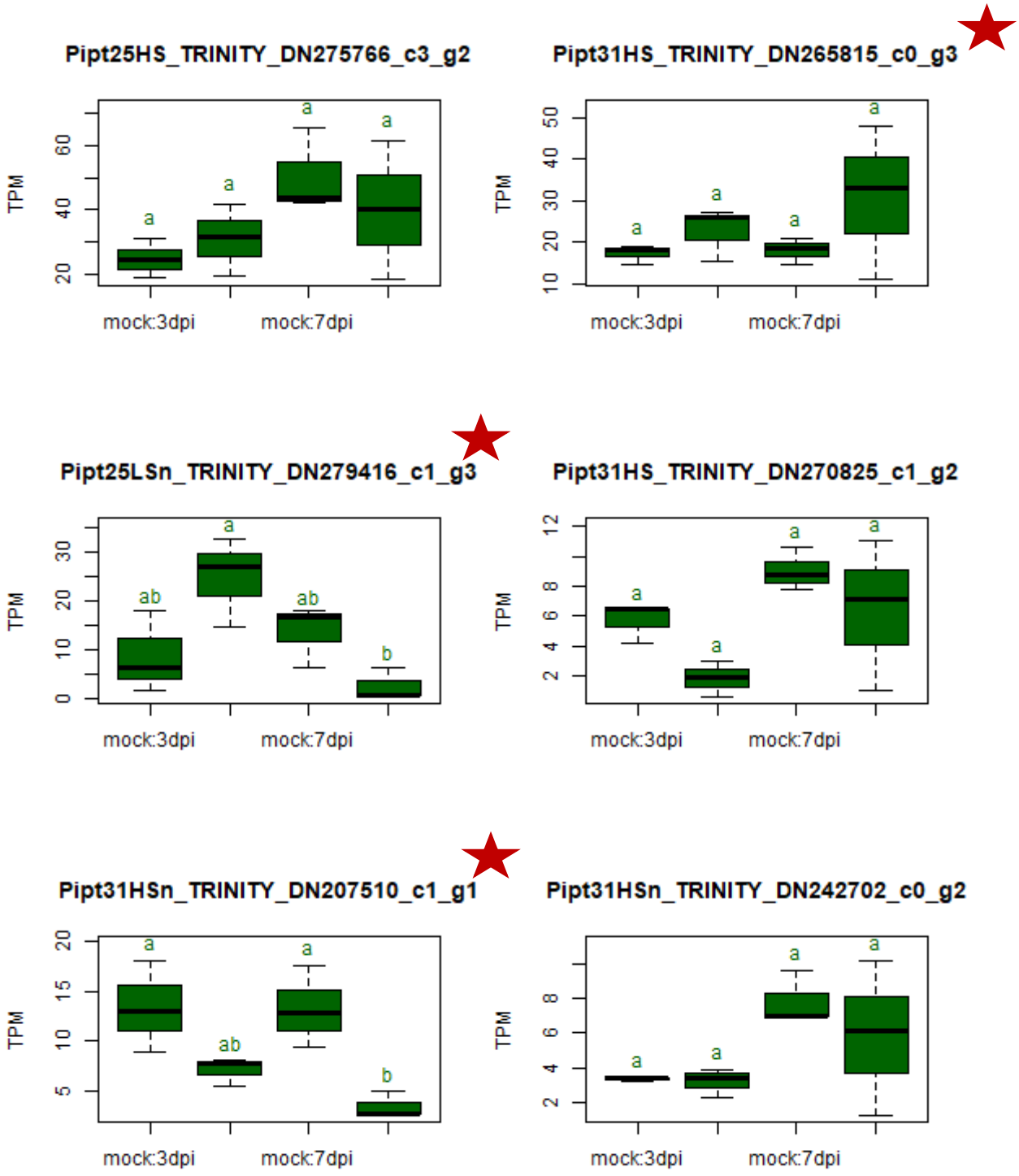

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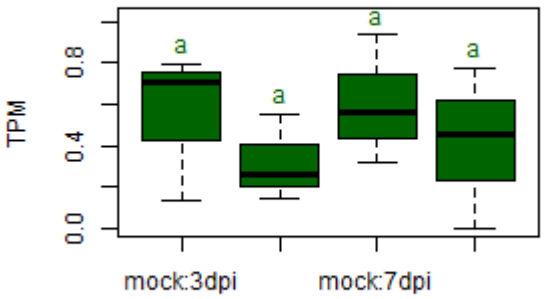
Pnte25HSn_TRINITY_DN58878_c0_g1



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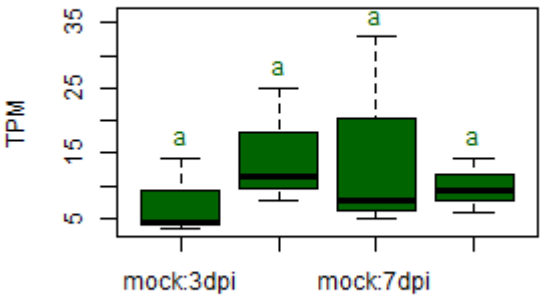

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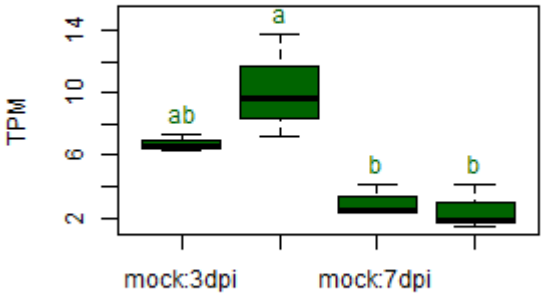

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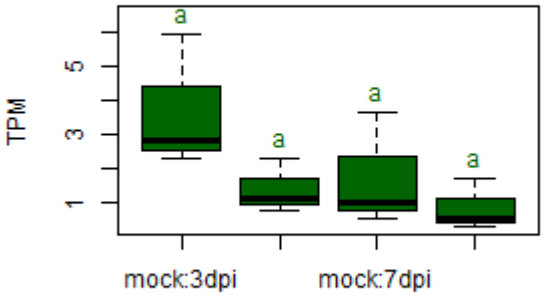

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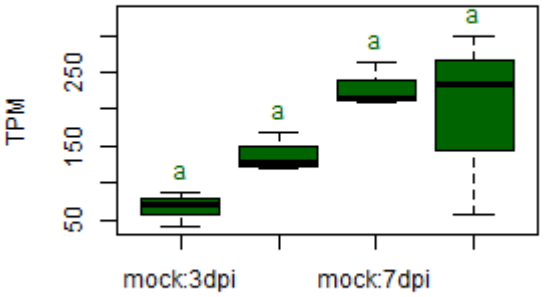

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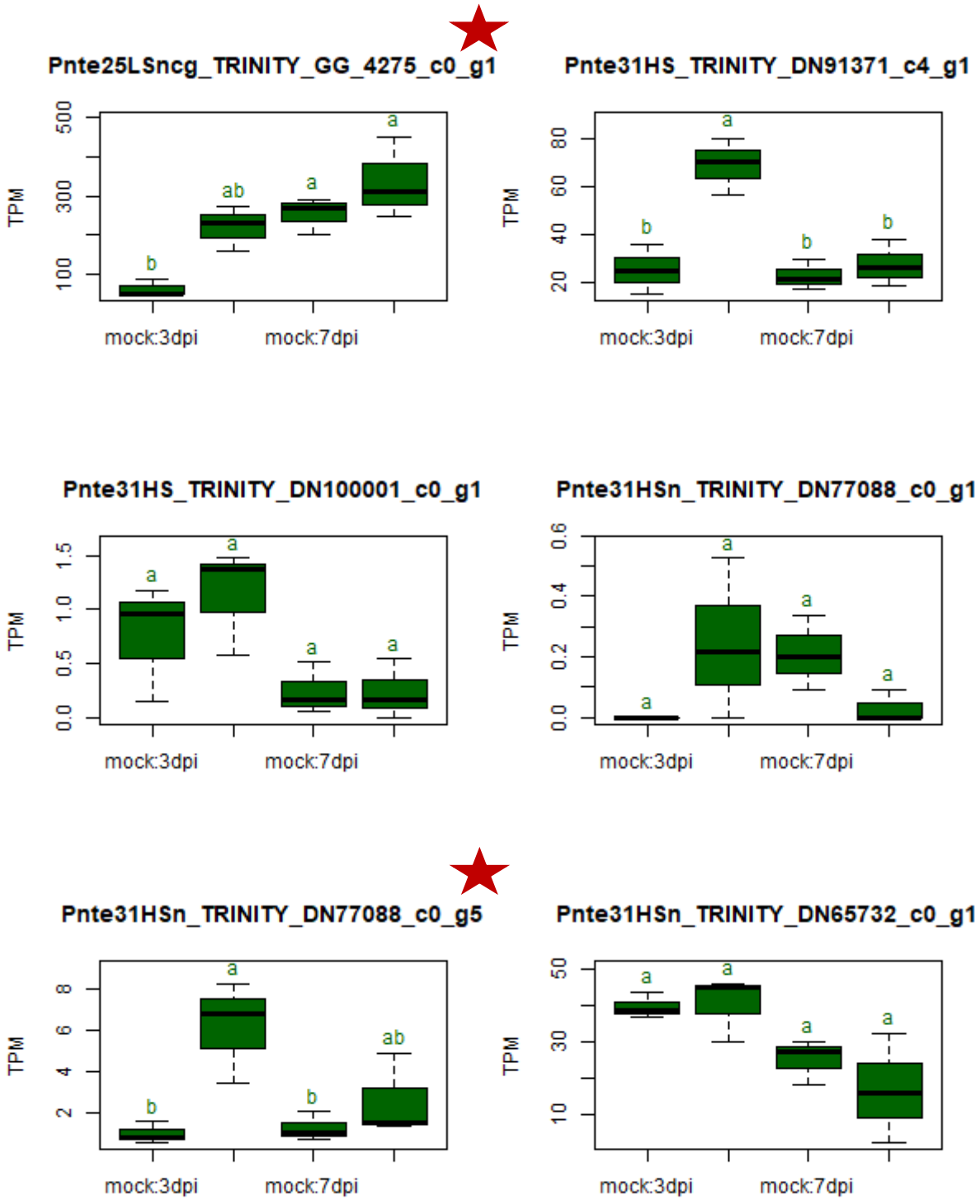

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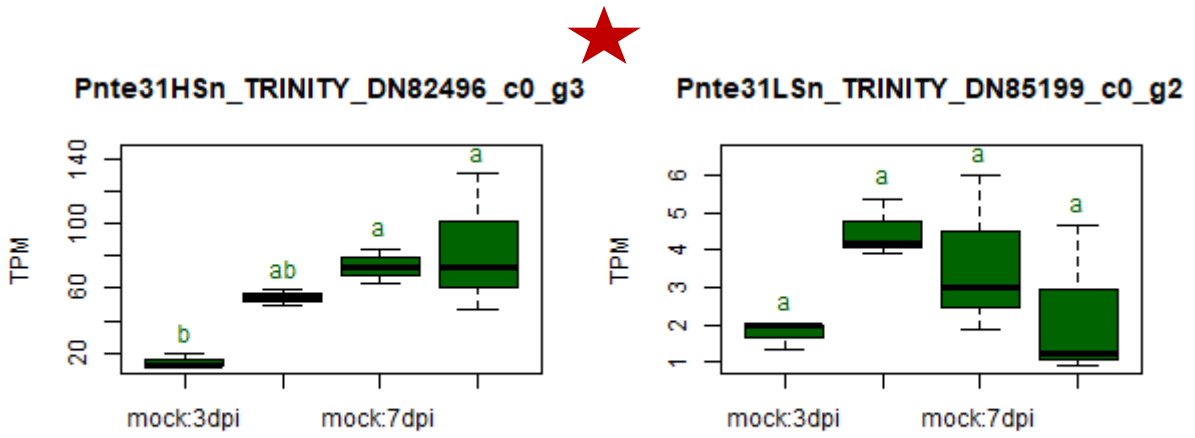

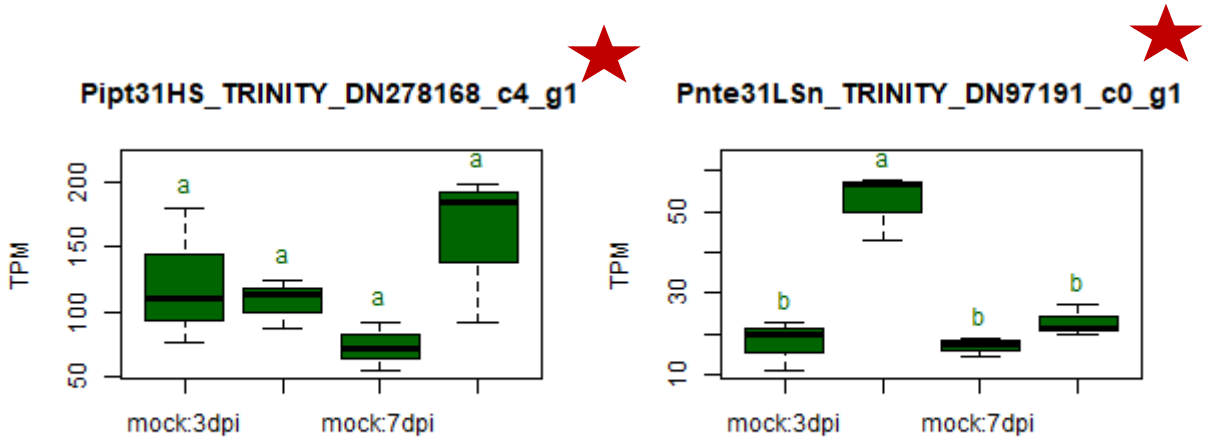

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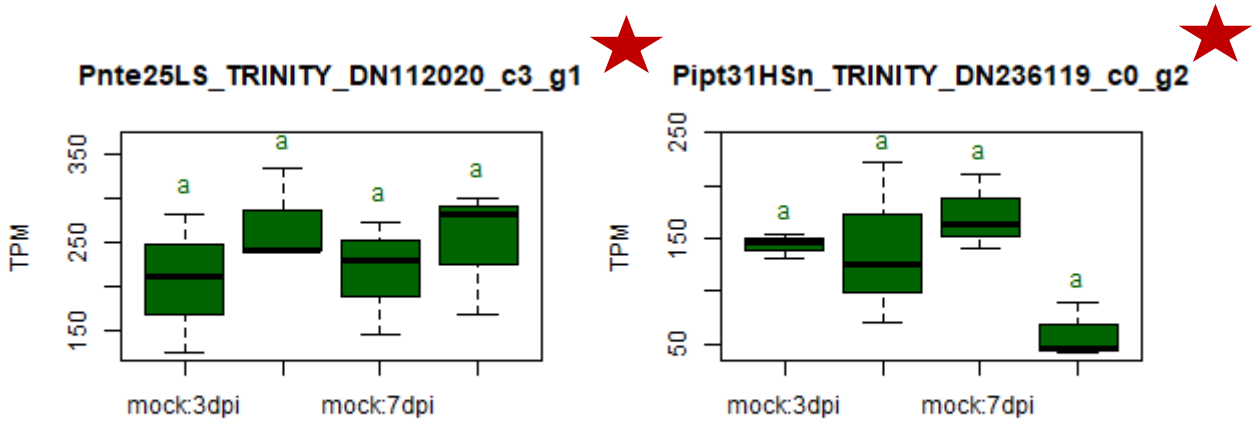

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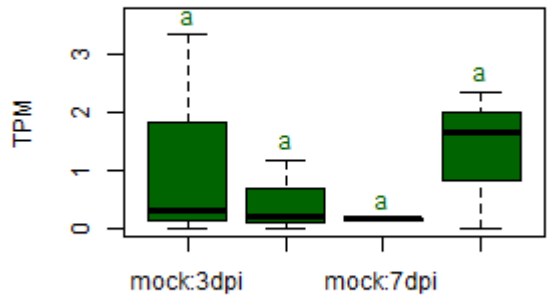

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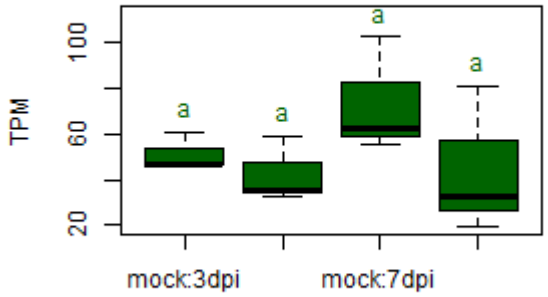

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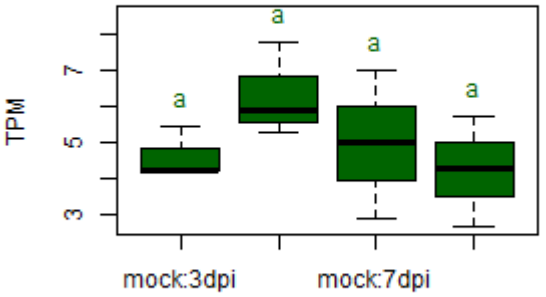

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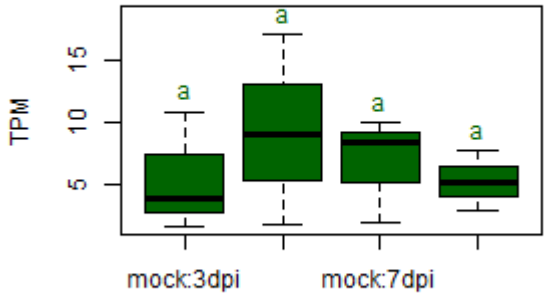

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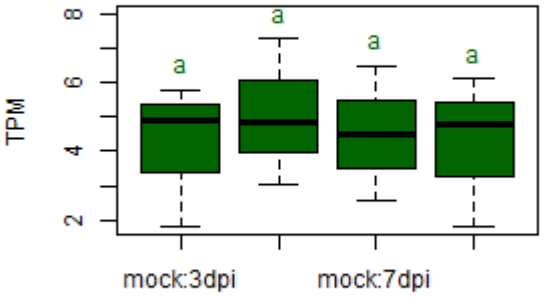

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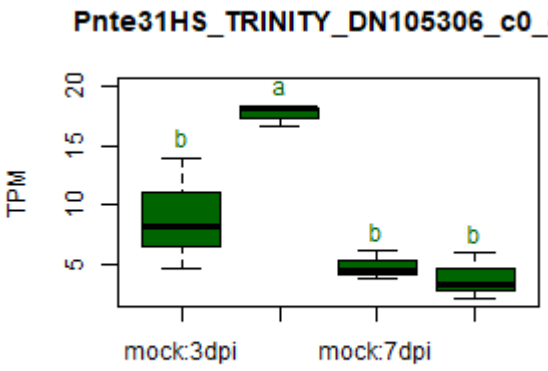

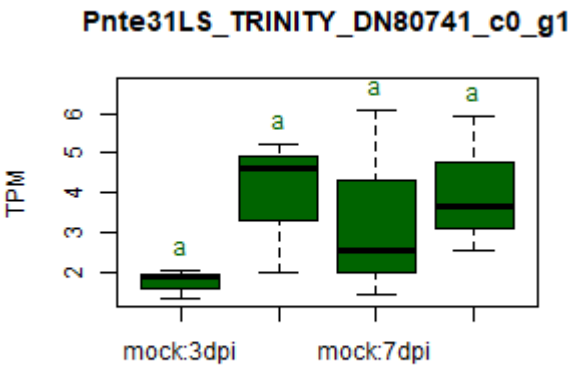

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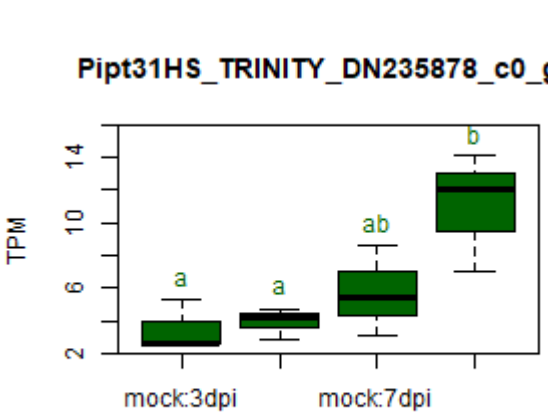

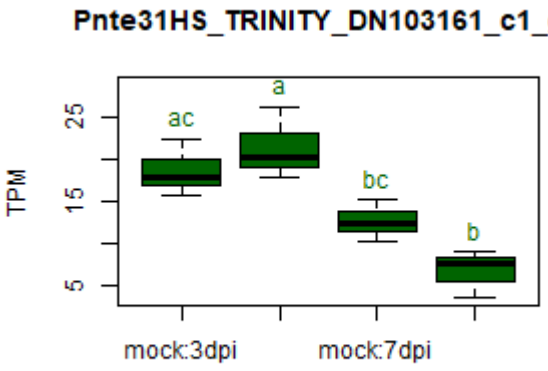

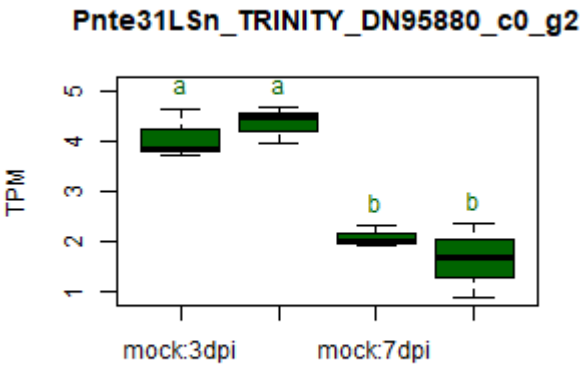

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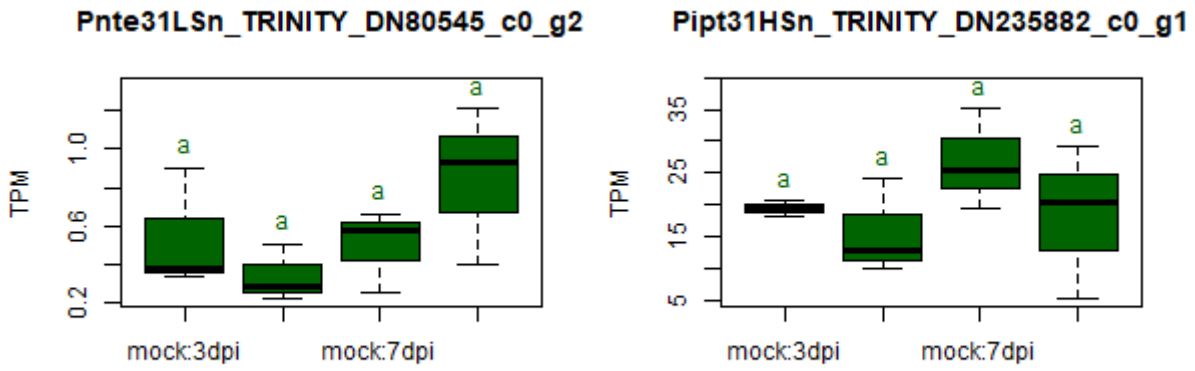

Figure S2: Boxplots of expression for putative terpene synthase genes in each sample set. Y-axes represent transcripts per million (TPM), X-axes indicate mock-inoculated (mock), and omission represent inoculated (inoc) samples at 3- and 7-days post inoculation (dpi). Genes are arranged to correspond to the phylogenetic tree in Figure 2.5. Letters represent adjusted p-values from a Tukey HSD test, with shared letters representing adjusted $p > 0.95$. Stars represent significantly differentially regulated in the inoculated samples when compared to the mock inoculated. (Pnte, *P. tecunumanii*: Pipt, *P. patula).*

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