University Library

A gateway to Melbourne's research publications

Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:
Yong, Wilson Thau Lym

Title:
Genetics of myrtle rust resistance in Eucalyptus

Date:
2018

Persistent Link:
https://hdl.handle.net/11343/219352

Terms and Conditions:
Terms and Conditions: Copyright in works deposited in Minerva Access is retained by the copyright owner. The work may not be altered without permission from the copyright owner. Readers may only download, print and save electronic copies of whole works for their own personal non-commercial use. Any use that exceeds these limits requires permission from the copyright owner. Attribution is essential when quoting or paraphrasing from these works.

Genetics of myrtle rust resistance in *Eucalyptus*

Wilson Thau Lym Yong

ORCID ID: 0000-0001-8431-8486

Doctor of Philosophy

May 2018

School of Ecosystem and Forest Sciences

Faculty of Science

The University of Melbourne

Submitted in total fulfilment of the requirements of the degree

"Produced on archival quality paper"

This page intentionally left blank

Abstract

Myrtle rust, also known as eucalyptus rust or guava rust, caused by the pathogenic fungus *Austropuccinia psidii* Beenken (formerly *Puccinia psidii* Winter), is a major threat to Australia's natural vegetation and to *Eucalyptus* dependent industries worldwide. However, the presence of specific resistance to this introduced pathogen in Australian myrtaceous species raises intriguing questions relating to the underlying mechanisms of resistance and the evolutionary trajectories resulting in the observed variations of susceptibility. This thesis aims to identify rust resistance mechanisms in two key *Eucalyptus* species i.e. *Eucalyptus globulus* Labill. ssp. *globulus* and *Eucalyptus obliqua* L'Hér. that predate contemporary contact between the host and pathogen, using phenotypic and molecular analyses. Rust screening was carried out to examine the genetic variation of susceptibility to *A. psidii* in geographic races (and sub-races) of *E. globulus* ssp. *globulus* and regional populations of *E. obliqua*. Analysis revealed genetic variation between and within geographic populations of both species that will allow natural adaptation to the new disease threat, and divergence between the two species representative of the two most species-rich *Eucalyptus* subgenera i.e. *Symphyomyrtus* and *Eucalyptus*. Detailed observations of the rust infection process by scanning electron microscopy did not indicate any distinct differences in either ontogeny or morphology of *A. psidii* infection structures between different rust response phenotypes i.e. completely resistant, hypersensitive and highly susceptible in both *Eucalyptus* species. Further histological examination revealed direct (non-stomatal) penetration of germinating urediniospores through the leaf cuticle in susceptible phenotypes while the infection was stopped at the leaf surface, without host penetration, in completely resistant phenotypes. Non-host/non-pathogen interactions, i.e. the growth of variable length germ tubes in random directions and reduced probability of germ tubes in locating stomata in *Eucalyptus* and *A. psidii* may be due to the lack of basic compatibility or specific recognition events between the plant and the pathogen as has been observed in other non-host pathosystems. Foliar terpenes were analysed as possible preformed resistance compounds associated with rust resistance in both *Eucalyptus* species. While terpene components were found to be significantly associated with susceptibility differences between rust response phenotypes, the components were

not consistent between *E. globulus* and *E. obliqua* and quite different to the previously identified resistance biomarkers in *Eucalyptus* hybrids. Less specificity between terpene composition and rust resistance may be due to the complexity of terpene defensive chemistry between *Eucalyptus* species and the synergistic effects of terpene compounds in combination with other metabolites in producing similar defensive responses against the same pathogen. Finally, a genome wide association study (GWAS) of rust resistance in *E. obliqua* identified genomic regions including those corresponding to the reported major QTLs, i.e. *Ppr1* to *Ppr5*, for rust resistance in *E. grandis* and *E. globulus* from subgenus *Symphyomyrtus*. Functional annotations of positional candidate genes also revealed some hypothetical proteins related to defences against pathogens such as an enzymatic class of *R* genes, and genes annotated as nucleotide-binding (NB) proteins, leucine-rich repeat (NB-LRR) domains and transcription factors. The co-location of rust resistance loci between subgenera *Eucalyptus* and *Symphyomyrtus* is consistent with the hypothesis that the host and pathogen had an ancient evolutionary connection dating perhaps to 30 million years ago, and the presence of specific recognition to *A. psidii* in modern Australian *Eucalyptus* may be due to the retention of ancient *R* genes. Alternatively, the resistance genes may be effective against and maintained by the current native pathogens but happen to be partly effective against *A. psidii* between resistant myrtaceous plants across the Australian landmass. The present study contributes to our understanding of the complex *Eucalyptus* – *A. psidii* pathosystem and the underlying mechanisms of rust resistance at both a fundamental and applied level for sustainable disease management in *Eucalyptus* forests and commercial breeding for rust resistance.

Declaration

This is to certify that:

i. the thesis comprises only my original work towards the PhD except where indicated in the Preface,

ii. due acknowledgement has been made in the text to all other material used,

iii. the thesis is fewer than 100,000 words in length, exclusive of tables, bibliographies and appendices.

Wilson Thau Lym Yong

Preface

This thesis is submitted for the degree of Doctor of Philosophy at the University of Melbourne. The research conducted for this thesis forms part of an ARC-linkage project (LP13010045) funded by the Australian Research Council. The project represents a major collaborative effort led by the University of Melbourne with support and significant intellectual input from industrial partners including Southern Tree Breeding Association (STBA), Hancock Victorian Plantations (HVP), VicForests, Australian Bluegum Plantations (ABP), Victorian Department of Economic Development, Jobs, Transport and Resources (DEDJTR), Gydle Inc. and New South Wales Department of Primary Industries (NSW DPI). Most of the research was undertaken at the School of Ecosystem and Forest Sciences, the University of Melbourne in Creswick, Victoria, and the Victorian DEDJTR AgriBiosciences Centre in Bundoora, Victoria.

The underlying work would have been impossible to complete without receiving support and help in different ways from many people. My research questions were formulated together with my supervisor, Dr. Peter Ades, and co-supervisors, Professor Gerd Bossinger and Dr. Josquin Tibbits. The rust screening trials including sample preparation (*Eucalyptus* seeds sorting, imbibition and sowing) in Chapter 2 were organised with the assistance of Alice Gower to accommodate the present and other collaborative studies. The rust inoculation and phenotypic scoring were carried out by Dr. Karanjeet Sandhu at the Plant Breeding Institute of the University of Sydney in Cobbitty, New South Wales. The experiments in Chapter 3 (microscopic study) and Chapter 4 (terpene analyses) were designed through consultation with Professor Paul Taylor and Dr. Jason Goodger, respectively. The scanning electron microscopy was carried out under the guidance of Roger Curtain at the Bio21 Advanced Microscopy Facility, the University of Melbourne. The genome assembly for Chapter 5 was performed by Dr. Josquin Tibbits in collaboration with Philippe Rigault at Gydle, Quebec, Canada.

Acknowledgements

First and foremost, I thank God for the blessings He has bestowed upon me and for giving me the physical and mental strength to undertake my research study up to the completion. Without His blessings, this achievement would not have been possible. I am also grateful to the University of Melbourne for offering me a PhD position under the School of Ecosystem and Forest Sciences (formerly the Department of Forest and Ecosystem Science) based in Creswick.

I would like to extend my sincerest thanks to my main supervisor Dr. Peter Ades for his extraordinary scientific guidance, enthusiasm, and incredible motivational capabilities. He has been a constant source of support that has proven to be truly invaluable over the past three years when my study has seemed so overwhelming. I also want to thank my co-supervisor Professor Gerd Bossinger and Dr. Josquin Tibbits for their continued friendship, endless support, inspiration, and for showing perpetual confidence in my research and laboratory skills.

I would like to express my deepest gratitude to Professor Paul Taylor for his valuable advice and interesting discussions as the chairman of my advisory committee and for the opportunity to work with him in his esteemed research laboratories. I have learned a lot under his guidance and greatly appreciate his professional approach and constructive suggestions throughout my academic quest. My appreciation also goes to Dr. Karanjeet Sandhu, Dr. Jason Goodger, Dr. Mike Bayly, Dr. Angus Carnegie, Dr. Simon Crawford, and Roger Curtain for their intellectual input and access to research facilities.

I acknowledge the valuable contribution from Alice Gower, Md. Abdullahil Baki Bhuiyan, Hossein Valipour Kahrood, Agnieszka Wujeska-Klause and Fatima Akter Runa in completion of my study, especially in technical assistance during sample preparation for my research activities and insightful sharing of their valuable research experiences. I also wish to thank all the industrial partners and participating universities for collaboration in ARC-linkage project (LP13010045) to ensure that the present research remains focused and relevant.

To the staff and students at the University of Melbourne, Creswick campus, I extend a heartfelt thank-you for those who have provided me support in the last few years and through your encouragement I have been able to excel in my research study. I am also indebted to all my friends, too numerous to mention, who have travelled this journey with me, thank you for your continuous moral support and inspiration throughout my life. Also, financial assistance from the Malaysian government via Academic Training Scheme of Public Higher Education Institutions (Skim Latihan Akademik Institut Pengajian Tinggi Awam, SLAI), and studentship from the Faculty of Science, the University of Melbourne are gratefully acknowledged.

Finally, I want to thank, with all my heart, my family for unconditionally supporting me in any possible way and keeping my life balanced and healthy. This includes my exceptionally understanding wife Chai Hoon Sam, whose unfailing support and encouragement have accompanied me throughout this exciting journey. Without love and patience from you and our sons – Calvert, Bernard and Alfred, I would have never gone this far.

Table of Contents

Abstract …… i
Declaration …… iii
Preface …… iv
Acknowledgements …… v
Table of contents …… vii
List of figures …… ix
List of tables …… xiii

Chapter 1 Introduction: Genetic rust resistance in *Eucalyptus* species and potential implication of molecular approaches in disease management …… 1
Eucalyptus species and their importance …… 1
Geographic variation in *Eucalyptus* …… 3
Molecular genetic variation in *Eucalyptus* …… 6
Myrtle rust and its potential threat on *Eucalyptus* …… 8
Genetic variation of rust resistance in *Eucalyptus* …… 11
Major gene and minor quantitative loci for myrtle rust resistance … 17
Potential genetic markers and the *Eucalyptus* reference genome …. 21
Implication of molecular approaches in disease management …… 24
Thesis structure and aims …… 27

Chapter 2 Geographical patterns of variation in susceptibility of *Eucalyptus globulus* Labill. ssp. *globulus* and *Eucalyptus obliqua* L'Hér. to myrtle rust disease …… 31
Abstract …… 31
Introduction …… 32
Materials and methods …… 36
Results …… 44
Discussion …… 78
Conclusion …… 82

Chapter 3 **Infection process of *Austropuccinia psidii* on *Eucalyptus globulus* and *Eucalyptus obliqua* leaves of different rust response phenotypes** 83
Abstract 83
Introduction 84
Materials and methods 88
Results 92
Discussion 103
Conclusion 110

Chapter 4 **Phytochemical analysis of the essential oils from *Eucalyptus globulus* and *Eucalyptus obliqua* in association with myrtle rust resistance** 111
Abstract 111
Introduction 112
Materials and methods 116
Results 119
Discussion 127
Conclusion 131

Chapter 5 **Genome wide association study of myrtle rust (*Austropuccinia psidii*) resistance in *Eucalyptus obliqua* (subgenus *Eucalyptus*)** .. 133
Abstract 133
Introduction 134
Materials and methods 140
Results 144
Discussion 154
Conclusion 167

Chapter 6 **Concluding remarks and future perspectives** 169

References 175

List of Figures

Figure 1-1	Distribution of *Eucalyptus obliqua* in Australia …………………	4
Figure 1-2	Distribution of *Eucalyptus globulus* ssp. *globulus* in Australia ..	4
Figure 1-3	Schematic life cycle of *Austropuccinia psidii* …………………	10
Figure 1-4	Distribution of myrtle rust along the east coast of Australia and the time of spread …………………………………………….	12
Figure 2-1	Forest district, inventory area and forest block boundaries in Tasmania ………………………………………………………..	40
Figure 2-2	Distribution of seedlings in *Eucalyptus* species according to the percentage of rust severity response categories (numeric scores) …………………………………………………….	45
Figure 2-3	Least-squares means for rust severity between *Eucalyptus globulus* races ……………………………………………….	55
Figure 2-4	Binary symptomatic trait frequency between *Eucalyptus globulus* races ……………………………………………….	56
Figure 2-5	Binary hypersensitivity trait frequency between *Eucalyptus globulus* races ……………………………………………….	57
Figure 2-6	Binary pustulation trait frequency between *Eucalyptus globulus* races …………………………………………………….	58
Figure 2-7	Pearson's correlations between rust disease severity of *Eucalyptus globulus* races and their respective elevation and climatic conditions ………………………………………..	59
Figure 2-8	Pearson's correlations between binary rust symptomatic trait frequency of *Eucalyptus globulus* races and their respective elevation and climatic conditions ………………………………	60
Figure 2-9	Pearson's correlations between binary rust hypersensitivity trait frequency of *Eucalyptus globulus* races and their respective elevation and climatic conditions ………………………………	61
Figure 2-10	Pearson's correlations between binary rust pustulation trait frequency of *Eucalyptus globulus* races and their respective elevation and climatic conditions ………………………………	62

Figure 2-11	Least-squares means for rust severity between *Eucalyptus obliqua* forest districts ……………………………………………	70
Figure 2-12	Binary symptomatic trait frequency between *Eucalyptus obliqua* forest districts ……………………………………………	71
Figure 2-13	Binary hypersensitivity trait frequency between *Eucalyptus obliqua* forest districts ……………………………………………	72
Figure 2-14	Binary pustulation trait frequency between *Eucalyptus obliqua* forest districts ………………………………………………..	73
Figure 2-15	Pearson's correlations between rust disease severity of *Eucalyptus obliqua* forest districts and their respective elevation and climatic conditions ………………………………………..	74
Figure 2-16	Pearson's correlations between binary rust symptomatic trait frequency of *Eucalyptus obliqua* forest districts and their respective elevation and climatic conditions ……………………	75
Figure 2-17	Pearson's correlations between binary rust hypersensitivity trait frequency of *Eucalyptus obliqua* forest districts and their respective elevation and climatic conditions ………………..…	76
Figure 2-18	Pearson's correlations between binary rust pustulation trait frequency of *Eucalyptus obliqua* forest districts and their respective elevation and climatic conditions ……………………	77
Figure 3-1	Percentages of urediniospore germination and appressorium formation between 6 and 120 h.a.i. in different rust response phenotypes of *Eucalyptus globulus* ……………………………	94
Figure 3-2	Percentages of urediniospore germination and appressorium formation between 6 and 120 h.a.i. in different rust response phenotypes of *Eucalyptus obliqua* …………………………...	95
Figure 3-3	Percentages of different length and average length of germ tube between 6 and 120 h.a.i. on different rust response phenotypes of *Eucalyptus globulus* ………………………………………	96
Figure 3-4	Percentages of different length and average length of germ tube between 6 and 120 h.a.i. on different rust response phenotypes of *Eucalyptus obliqua* …………………………………...…..	97

Figure 3-5	Scanning electron micrographs of the infection structure formation by *Austropuccinia psidii* on *Eucalyptus* …………….	98
Figure 3-6	Density of urediniospores between 6 and 120 h.a.i. on different rust response phenotypes of *Eucalyptus* species ……………….	99
Figure 3-7	Light micrographs of the pathogenesis of *Austropuccinia psidii* at 120 h.a.i. in *Eucalyptus obliqua* …………………………….	101
Figure 3-8	Anatomical difference with irregular surface topography and crystalline-like structures in the leaf of 6-month-old and 12-month-old *Eucalyptus obliqua* in contrast to *E. globulus*. Hyphal colonisation and formation of uredinial primordia by *Austropuccinia psidii* at 8 d.a.i. before emergent of fully developed uredinia containing urediniospores at 10 d.a.i. on the leaf surfaces of *E. globulus* susceptible phenotype …………….	102
Figure 3-9	*Austropuccinia psidii* infection process on different rust response phenotypes of *Eucalyptus* species …………………….	104
Figure 4-1	Significant compounds identified in *Eucalyptus globulus* in association with myrtle rust resistance ………………………….	122
Figure 4-2	Significant compounds identified in *Eucalyptus obliqua* in association with myrtle rust resistance ………………………….	123
Figure 4-3	Tentative biomarkers for myrtle rust resistance in *E. grandis* × *E. urophylla* hybrids …………………………………………….	123
Figure 4-4	Scatterplot of canonical discriminant functions between rust response phenotypes of *Eucalyptus globulus* with 95% confidence circle and 70% confidence ellipse plotted around the canonical mean of each phenotype …………………………….	125
Figure 4-5	Scatterplot of canonical discriminant functions between rust response phenotypes of *Eucalyptus obliqua* with 95% confidence circle and 70% confidence ellipse plotted around the canonical mean of each phenotype …………………………….	126
Figure 5-1	Genome-wide pattern of linkage disequilibrium (LD) decay for *Eucalyptus obliqua* up to 1000 bp pairwise SNP distances estimated by r^2 along physical distance in bases ……………….	146

Figure 5-2 Manhattan plot of the genome wide association study (GWAS) results for the binary symptomatic response of *Eucalyptus obliqua* to *Austropuccinia psidii* based on the $–\log_{10} P$ value along the 11 *Eucalyptus* chromosomes ………………………… 149

Figure 5-3 Manhattan plot of the genome wide association study (GWAS) results for the binary hypersensitive response of *Eucalyptus obliqua* to *Austropuccinia psidii* based on the $–\log_{10} P$ value along the 11 *Eucalyptus* chromosomes ………………………… 150

Figure 5-4 Manhattan plot of the genome wide association study (GWAS) results for the binary pustulation response of *Eucalyptus obliqua* to *Austropuccinia psidii* based on the $–\log_{10} P$ value along the 11 *Eucalyptus* chromosomes ………………………… 151

Figure 5-5 Manhattan plot of the genome wide association study (GWAS) results for the numeric severity response of *Eucalyptus obliqua* to *Austropuccinia psidii* based on the $–\log_{10} P$ value along the 11 *Eucalyptus* chromosomes ………………………………….. 152

Figure 5-6 Quantile-quantile (Q-Q) plots for genome wide association study (GWAS) results for binary symptomatic, hypersensitive, pustulation, and numeric severity responses of *Eucalyptus obliqua* to *Austropuccinia psidii* …………………..................... 153

Figure 5-7 The position of previously reported *Ppr* QTLs (*Ppr1* to *Ppr5*) and myrtle rust resistance SNPs ($–\log_{10} P > 6.0$) identified in the present study based on the physical map of *Eucalyptus* …… 162

List of Tables

Table 1-1 Scoring system used to categorise visible symptoms observed on 3 weeks after inoculation of whole plants with myrtle rust … 14

Table 1-2 Greenhouse scale developed for scoring host response against *Austropuccinia psidii* ………………………………………... 15

Table 2-1 Number of *Eucalyptus globulus* ssp. *globulus* families used in the mixed effect analysis of myrtle rust susceptibility and their respective racial and subracial classifications …………………. 42

Table 2-2 Number of *Eucalyptus obliqua* seedlots according to their family types and forest districts included in the mixed effect analysis of myrtle rust susceptibility …………………………... 43

Table 2-3 Results of mixed models fitted for quantitative myrtle rust disease severity between *Eucalyptus globulus* and *Eucalyptus obliqua* ………………………………………………………. 46

Table 2-4 Results of mixed models fitted for binary symptomatic response to myrtle rust between *Eucalyptus globulus* and *Eucalyptus obliqua* ………………………………………………………. 46

Table 2-5 Results of mixed models fitted for binary hypersensitive response to myrtle rust between *Eucalyptus globulus* and *Eucalyptus obliqua* …………………………………………..….. 47

Table 2-6 Results of mixed models fitted for binary pustulation response to myrtle rust between *Eucalyptus globulus* and *Eucalyptus obliqua* ………………………………………………………. 47

Table 2-7 Results of mixed models fitted for quantitative myrtle rust disease severity between races and sub-races in *Eucalyptus globulus* ……………………………………………………... 48

Table 2-8 Results of mixed models fitted for binary symptomatic response to myrtle rust between races and sub-races in *Eucalyptus globulus* ……………………………………………………... 48

Table 2-9	Results of mixed models fitted for binary hypersensitive response to myrtle rust between races and sub-races in *Eucalyptus globulus* ……………………………………………….	49
Table 2-10	Results of mixed models fitted for binary pustulation response to myrtle rust between races and sub-races in *Eucalyptus globulus* ……………………………………………………...	49
Table 2-11	Least-squares means (LSM) and standard errors (SE) for rust response in numeric score recorded from 13 *Eucalyptus globulus* races ………………………………………………	51
Table 2-12	Binary symptomatic trait for rust response in trait frequency recorded from 13 *Eucalyptus globulus* races …………………...	52
Table 2-13	Binary hypersensitivity trait for rust response in trait frequency recorded from 13 *Eucalyptus globulus* races …………………...	53
Table 2-14	Binary pustulation trait for rust response in trait frequency recorded from 13 *Eucalyptus globulus* races …………………...	54
Table 2-15	Results of mixed models fitted for quantitative myrtle rust disease severity between forest districts in *Eucalyptus obliqua* ..	64
Table 2-16	Results of mixed models fitted for binary symptomatic response to myrtle rust between forest districts in *Eucalyptus obliqua* …..	64
Table 2-17	Results of mixed models fitted for binary hypersensitive response to myrtle rust between forest districts in *Eucalyptus obliqua* ………………………………………………………	65
Table 2-18	Results of mixed models fitted for binary pustulation response to myrtle rust between forest districts in *Eucalyptus obliqua* …..	65
Table 2-19	Least-squares means (LSM) and standard errors (SE) for rust response in numeric score recorded from 19 *Eucalyptus obliqua* forest districts ……………………...……………………...	66
Table 2-20	Binary symptomatic trait for rust response in trait frequency recorded from 19 *Eucalyptus obliqua* forest districts …...……...	67
Table 2-21	Binary hypersensitivity trait for rust response in trait frequency recorded from 19 *Eucalyptus obliqua* forest districts …...……...	68

Table 2-22	Binary pustulation trait for rust response in trait frequency recorded from 19 *Eucalyptus obliqua* forest districts …..……...	69
Table 4-1	Terpene compounds identified in *Eucalyptus globulus* and *Eucalyptus obliqua* leaf samples by GC-FID and GC-MS ……...	120
Table 4-2	Terpene compounds selected by stepwise forward variable selection using Wilks' Lambda criterion ($P < 0.10$) for differentiation of rust response phenotypes in *Eucalyptus globulus* and *Eucalyptus obliqua* ………………………………	122
Table 4-3	Summary of the multivariate analyses of stepwise model selected terpene compounds in response to myrtle rust resistance in *Eucalyptus globulus* and *Eucalyptus obliqua* with variance components estimated through the Wilks' Lambda statistical test …………………………………………………	124
Table 4-4	Prediction capacity of rust response discriminant model by cross validation according to response phenotype in *Eucalyptus globulus* and *Eucalyptus obliqua* ………………………………	124
Table 4-5	Prediction capacity of rust response discriminant model by cross validation between *Eucalyptus globulus* and *Eucalyptus obliqua* …………………………………………………………	128
Table 5-1	Significant SNP markers ($-\log_{10} P > 6.0$) associated with binary and numeric responses to myrtle rust in *Eucalyptus obliqua*	147
Table 5-2	Results of the putative candidate genes associated with binary symptomatic response of *Eucalyptus obliqua* to *Austropuccinia psidii* identified by *in-silico* searching of the significant SNP markers of 10 kbp physical nucleotide region with *Eucalyptus grandis* reference genome using Phytozome …………………...	155
Table 5-3	Results of the putative candidate genes associated with binary hypersensitive response of *Eucalyptus obliqua* to *Austropuccinia psidii* identified by *in-silico* searching of the significant SNP markers of 10 kbp physical nucleotide region with *Eucalyptus grandis* reference genome using Phytozome …	159

Table 5-4 Results of the putative candidate genes associated with binary pustulation response of *Eucalyptus obliqua* to *Austropuccinia psidii* identified by *in-silico* searching of the significant SNP markers of 10 kbp physical nucleotide region with *Eucalyptus grandis* reference genome using Phytozome ………………….... 160

Table 5-5 Results of the putative candidate genes associated with numeric severity response of *Eucalyptus obliqua* to *Austropuccinia psidii* identified by *in-silico* searching of the significant SNP markers of 10 kbp physical nucleotide region with *Eucalyptus grandis* reference genome using Phytozome ………………….... 161

Chapter 1

Introduction: Genetic rust resistance in *Eucalyptus* species and potential implication of molecular approaches in disease management

***Eucalyptus* species and their importance**

Eucalyptus (Myrtaceae) is a large genus of evergreen trees and shrubs consists of about 839 species (Booth et al. 2015). The genus first came to the attention of the scientific world in the late eighteenth century, being introduced by the taxonomic description by Charles Louis L'Héritier de Brutelle (L'Héritier de Brutelle 1963). *Eucalyptus* species are now a major source of forest products in many countries around the world, with great economic, cultural and environmental significance. Most *Eucalyptus* species have their origin in Australia but some occur naturally in New Guinea and as far as Mindanao in the Philippines and Flores in Indonesia (Chippendale 1988; Paine et al. 2011). *Eucalyptus* is reported to be the most valuable and widely planted hardwood genus in the twenty-first century with more than 20 million hectares of plantation worldwide and growth rates of wood volume exceeding 35 m^3 ha^{-1} $year^{-1}$ (Albaugh et al. 2013; Forrester et al. 2010; Naidoo et al. 2014). Many species have been tested for adaptability and utility around the world but only a relatively small number of species are globally cultivated since the 1990s (Booth et al. 2015).

According to Stanturf et al. (2013), worldwide the nine dominant commercial *Eucalyptus* species are *Eucalyptus camaldulensis* Dehnh., *Eucalyptus grandis* W. Hill ex Maiden, *Eucalyptus tereticornis* Smith, *Eucalyptus globulus* Labill., *Eucalyptus nitens* H. Deane & Maiden, *Eucalyptus urophylla* S.T. Blake, *Eucalyptus saligna* Smith, *Eucalyptus dunnii* Maiden and *Eucalyptus pellita* F. Muell, and their hybrids, which account for more than 90% of *Eucalyptus* plantation forests. Among these species, *E. camaldulensis* is the most widely distributed of all *Eucalyptus* in its native range, with occurrence in mainland Australia from subtropical environments in the Northern Territory to temperate climates of southern Victoria (Butcher et al. 2002). The wood

harvested from the commercial *Eucalyptus* species is used primarily as raw material for the pulp and paper industry (Pirralho et al. 2014).

Most of Australia's native forests have *Eucalyptus* species forming the dominant canopy (Pink 2012). These species occur in a wide range of physical forms from shrubs to trees and occupy a broad range of climatic and ecological niches across the country (Booth et al. 2015). *Eucalyptus* species were used for fuelwood, fencing and construction in the late eighteenth century apart from the medicinal use of their essential oils extracted from the leaves of some species. Their favourable characteristics, including rapid growth rates, high quality wood fibre and wide adaptability to environmental conditions, have contributed to the great interest of the genus as valuable forest products (Stanturf et al. 2013; Strauss 2001). In addition to their economic value, native *Eucalyptus* forests are also important as repositories for great genetic diversity and maintenance of ecological balance (Turnbull 1995). Their continental scales of dominance have provided abundant food resources for a large diversity of animal taxa including insects, birds and mammals (Strauss 2001).

The genus *Eucalyptus* has been divided into ten subgenera (Bayly 2016; Noble 1989), the largest of which are *Symphyomyrtus* and *Eucalyptus* (previously informal *Monocalyptus*; Pryor and Johnson 1971) with about 450 and 110 species respectively (Boland et al. 2006). The main commercially important native *Eucalyptus* species in south-eastern Australia include *Eucalyptus delegatensis* R.T. Baker, *Eucalyptus obliqua* L'Hér. and *Eucalyptus regnans* F. Muell. (Nevill et al. 2014). Among these species, *E. obliqua* is the most widespread of the monocalypts (subgenus *Eucalyptus*), with populations found in Victoria, South Australia, New South Wales and Tasmania (Figure 1-1; Bloomfield et al. 2011). This species is also reported to be the most widespread *Eucalyptus* in Tasmania (Williams and Potts 1996) with extensive occurrence in wet sclerophyll forest designated for wood production purposes (Neyland et al. 2009). However, the demand for *Eucalyptus* wood products from native forests has generally exceeded supply in Australia and there has been great effort of domestication of forest species for sustainable wood production in plantations by the Australian government and private investors.

Among the domesticated *Eucalyptus*, *E. globulus* ssp. *globulus* from the subgenus *Symphyomyrtus* is one of the most commonly planted species comprising over half of the hardwood plantations by area in Australia (Pink 2012). This species is native to south-eastern Australia (Figure 1-2; Williams and Potts 1996) with its main occurrence in coastal and lowland areas of eastern and south-eastern Tasmania and the Bass Strait islands but extending to western Tasmania and southern Victoria. Its fast growth rate, wide adaptability to soils and climates, ease of management and valuable wood properties have made *E. globulus* a prime candidate for planting for the pulp industry and various other purposes worldwide (Turnbull 1999). The Australian national *E. globulus* breeding program commenced in 1995 by the Southern Tree Breeding Association (STBA) with a founder population of more than 600 collections from throughout the species' geographic range (McRae et al. 2004). Both native and plantation *Eucalyptus* forests in Australia are important to provide the majority of the timber and a significant proportion of paper products to the country as well as the minority products such as natural oils, firewood and craft wood (Pink 2012).

Geographic variation in *Eucalyptus*

The study of geographic variation within the native ranges of forest trees is important for understanding the genetic structure of the species and the significance of evolutionary forces that caused the observed patterns of variation. The knowledge of spatial distribution of genetic variation is essential for the implementation of strategies for exploitation and conservation of genetic resources in forest management and tree improvement programs (Potts and Jordan 1994b). The distinct patterns of geographic variation in tree species are associated with their evolutionary histories and ongoing adaptive processes in their current environments. The key evolutionary forces which drive patterns of genetic differentiation in forest trees are revealed to be genetic drift, natural selection and hybridisation (Jones et al. 2013). Based on previous morphological research and provenance trials, Eldridge et al. (1993) reported geographic variation of economically important traits such as growth rate, oil content, wood properties, extreme environmental tolerance and disease resistance in various *Eucalyptus* species, including the major plantation species: *E. camaldulensis*, *E. globulus*, *E. grandis* and *E.*

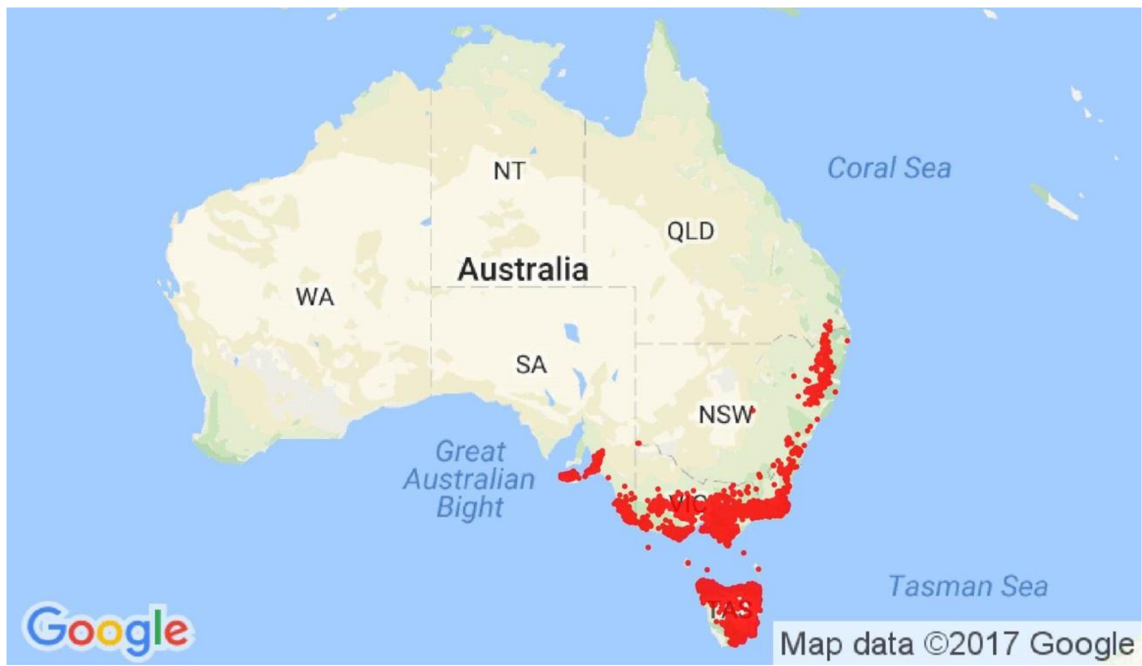

Figure 1-1: Distribution of *Eucalyptus obliqua* in Australia (source: The Australasian Virtual Herbarium, https://avh.chah.org.au).

Figure 1-2: Distribution of *Eucalyptus globulus* ssp. *globulus* in Australia (source: The Australasian Virtual Herbarium, https://avh.chah.org.au).

tereticornis, and other wood-producing species of south-eastern Australia: *E. obliqua*, *E. delegatensis*, *E. nitens* and *E. regnans*.

Racial classification has been used to distinguish groups of individuals (or subdivision of a species) based on obvious morphological differences or clear geographical boundaries (Jordan et al. 1994). According to the dictionary of genetics by King et al. (2006), a race is defined as a phenotypically and/or geographically distinctive subspecific group, composed of individuals inhabiting a defined geographical and/or ecological region, and possessing characteristic phenotypic and gene frequencies that distinguish it from other such groups. Earlier field trials carried out by Green (1971) and Brown et al. (1976) have demonstrated significant variations of several growth parameters such as height, diameter at breast height (dbh) and tree volume, and survival rate in *E. obliqua* between geographically different localities (provenances) and between parent trees (families) within localities. However, subsequently not much research was directed at *E. obliqua* as the species is not planted but mainly regenerated naturally across its distributional range, and therefore attracting less attention from the research community in contrast to the widely planted species around the world.

Due to the global importance of *E. globulus* as a pulpwood species with favourable pulping quality, this species is one of the best studied species in *Eucalyptus* for potential tree improvement and selective breeding in the plantation forest industry. Spatial variation has been reported in *E. globulus* for various morphological traits including growth performance (Jordan et al. 1994), juvenile foliage morphology (Potts and Jordan 1994a), seedling abnormalities (Potts and Jordan 1994b), Mycosphaerella disease resistance (Carnegie and Ades 2005; Carnegie et al. 1994), drought resistance (Dutkowski 1995), frost resistance (Zang et al. 1995), longhorn borer resistance (Soria and Borralho 1997), and sawfly resistance (Jordan et al. 2002). *E. globulus* has primarily been classified into 12 geographic races based on the similarities in growth response (Jordan et al. 1994) but the respective classification has subsequently been revised into 13 races and 20 sub-races based on wider morphological traits including bark thickness, wood basic density, flowering precocity, survival and other growth parameters (Dutkowski and Potts 1999). The revised racial classification relied less on

geographic discontinuities as required for growth data analysis and has led to better identification of race boundaries and new divisions within races. The sub-race identification of Dutkowski and Potts (1999) has further been used to define genetic groups of *E. globulus* in the evaluation of the Australian *Eucalyptus* breeding program (McRae et al. 2004) and the study of genetic wood architecture of *E. globulus* for potential wood property improvement (Stackpole et al. 2011).

Molecular genetic variation in *Eucalyptus*

The use of morphological traits for racial classification in species complexes is challenging due to the methodological difficulty in distinguishing between homology (similarity due to common ancestry) and homoplasy (similarity due to convergence) within species (Jones et al. 2013). In the last decades, molecular studies have often been used as a complement to evaluate the genetic differentiation and evolutionary histories of the species complexes. Data generated from molecular analyses have been fundamental to various disciplines of genetics and genomics research including phylogeny, phylogeography and taxonomy (Grattapaglia et al. 2012). The discrepancy between molecular and morphological classifications may be attributable to resolution with finer morphological structures undetectable at molecular levels or real differences between methodologies with molecular approaches accessing neutral variation while morphological analyses capture patterns of both selective and neutral variations.

Molecular analyses have been used to investigate the geographical structure of *E. globulus* native populations (Jones et al. 2002; Steane et al. 2006). Based on microsatellite variation analysis, morphologically similar *E. globulus* from Tasmanian and Victorian localities have appeared to be molecularly distinct (Jones et al. 2002). This observation has affirmed the previous report of molecular distinction between Tasmanian and Victorian *E. globulus* based on random amplified polymorphic DNA (RAPD) markers (Nesbitt et al. 1995). Besides, variation patterns in *E. globulus* identified by Steane et al. (2006) using nuclear microsatellite markers and quantitative genetic traits has revealed the similarities between geographically neighbouring races using molecular markers but contrasting spatial differentiation between some of the nearby geographical races based on morphological traits. This has further anticipated

that genetic variation between geographical races may reflect the adaptive differences that evolved in response to the selection difference in distinctive natural environments. Chloroplast markers have also been used to examine genetic variation in *E. globulus* and previous studies revealed that patterns of variability in chloroplast DNA did not conform to subspecies boundaries but correlated more with geographic distribution within the species (Freeman et al. 2001; Jackson et al. 1999). The same observations were reported in other *Eucalyptus* species in which chloroplasts appeared to be geographically structured and shared between morphological species within a spatial region (McKinnon et al. 2004; McKinnon et al. 1999; McKinnon et al. 2001; Nevill et al. 2010; Nevill et al. 2014; Rathbone et al. 2007; Steane et al. 1998).

Genetic differentiation has likewise been analysed in *E. obliqua* across environmental gradients in fine local scales for a range of quantitative traits including growth rate i.e. height and volume, foliage disease susceptibility and browsing damage, frost resistance and bark thickness (Wilkinson 2008). Bloomfield et al. (2011) reported no significant nuclear microsatellite differentiation but distinct geographic partitioning of chloroplast DNA haplotypes in *E. obliqua* across several steep environmental gradients. This observation has suggested the maintenance of quantitative trait variations in *E. obliqua* by natural selection and other environmental forces, and population differentiation by pollen-mediated gene flow (greater than 150 times the level of seed-mediated gene flow) in the species along the local environmental gradients. The implications of haplotype diversity with regard to hybridisation between south-east Australian *Eucalyptus* species viz. *E. delegatensis*, *E. obliqua* and *E. regnans* of subgenus *Eucalyptus* (Nevill et al. 2014), and several species of subgenus *Symphyomyrtus* including *E. globulus* (McKinnon et al. 2004) in glacial refugia have also been discussed. The extensive inter-specific sharing of chloroplast haplotype in these *Eucalyptus* species with different ecological tolerances has suggested the ability of natural hybridisation in *Eucalyptus* as a response to rapid environmental changes during the Last Glacial Maximum (LGM). Long-term persistence and recolonisation of *E. regnans* in many regions as revealed by microsatellite analysis has also suggested the capability of the species to maintain a diverse genetic structure during the harsh LGM (Nevill et al. 2010). Hence, molecular genetic studies have been proposed to assist

various genetic applications including sampling strategies for breeding programmes, biotechnology tools for tree improvement, and formation of conservation management strategies for *Eucalyptus* forest (Butcher et al. 2002; Jones et al. 2006; Myburg et al. 2008; Shepherd et al. 2010).

Myrtle rust and its potential threat on *Eucalyptus*

Since the past century, a number of pathogenic fungi of the order Pucciniales (previously known as Uredinales) have been reported to infect members of Myrtaceae (Coutinho et al. 1998). *Austropuccinia psidii* Beenken (Beenken 2017; syn. *Puccinia psidii* Winter or *Uredo psidii* Simpson, Thomas & Grgurinovic, Sphaerophragmiaceae, Pucciniales), also known as eucalyptus rust, guava rust or myrtle rust, is currently a major disease threat to *Eucalyptus* related industry in many parts of the world and particularly in Australia where Myrtaceae dominate the majority of ecosystems (Coutinho et al. 1998; Glen et al. 2007; Grgurinovic et al. 2006). The pathogen was first described in 1884 on common guava (*Psidium guajava* L.) in Brazil (Winter 1884). It is believed to be native to South and Central America (Glen et al. 2007; Morin et al. 2014; Simpson et al. 2006) but has spread to Florida (Marlatt and Kimbrough 1979), Hawaii (Uchida et al. 2006) and California (Zambino and Nolan 2011) in the USA, and more recently to Japan (Kawanishi et al. 2009), Australia (Carnegie et al. 2010), China (Zhuang and Wei 2011), South Africa (Roux et al. 2013) and New Caledonia (Giblin 2013), before being discovered in Indonesia (McTaggart et al. 2016a), Singapore (du Plessis et al. 2017) and New Zealand (Cavill 2017). In the Australian incursion, myrtle rust was originally identified as *Uredo rangelii* J.A. Simpson, K. Thomas & C.A. Grgurinovic based on the presence of a tonsure on the urediniospores (Carnegie et al. 2010). *U. rangelii* was first reported on *Myrtus communis* L. in Argentina and *Syzygium jambos* L. in Jamaica as a species distinct from *U. psidii* (Simpson et al. 2006). However, following the discovery of the *Puccinia*-like teliospores in the introduced rust in Australia, *U. rangelii* has later been regarded as a member of the *P. psidii* sensu lato complex belong to the family Pucciniaceae (Carnegie and Lidbetter 2012; Carnegie et al. 2010) until recently being redefined as *A. psidii* with a new genus name in the newly circumscribed family Sphaerophragmiaceae (Beenken 2017). To date, there has been

only one *A. psidii* strain reported present in Australia (Carnegie and Lidbetter 2012; Sandhu et al. 2016).

A schematic life cycle of *A. psidii* (reported as *P. psidii*; Figure 1-3) was proposed by Glen et al. (2007), despite aeciospores not having been observed or recognised in nature due to their morphological similarity to urediniospores (Figueiredo 2001), and the roles of teliospores and basidiospores in the life cycle being controversial (McTaggart et al. 2017; Morin et al. 2014). *A. psidii* is generally considered an autoecious rust (McTaggart et al. 2016b), with an incomplete lifecycle in myrtaceous hosts (Glen et al. 2007; Morin et al. 2014) but it has also been suggested that the rust may be heteroecious with an unknown aecial host (Simpson et al. 2006). Some authors have referred to *A. psidii* as a hemicyclic rust with loss of spermogonia and aecia stages in its lifecycle (Helfer 2014; Petersen 1974) and continuous maintenance of its contemporary populations via asexual reproduction (urediniospores) on its primarily myrtaceous hosts (Morin et al. 2014). Previous microscopic examination of basidiospore inoculations on susceptible *Agonis flexuosa* (Willd.) Sweet and *S. jambos* by Morin et al. (2014) revealed no spermogonia development or signs of cell penetration on the leaf surface, suggesting *A. psidii* basidiospores have lost their infectious ability. However, more recent inoculations showed that basidiospores of *A. psidii* could cause infection on myrtaceous species, and the following analysis using microsatellite markers found that the resulting sori were the progeny of recombination in teliospores (McTaggart et al. 2017). The finding suggests that *A. psidii* may complete its sexual life cycle on a species of Myrtaceae, and rejects the hypothesis that *A. psidii* is heteroecious with an unknown aecial host as the basidiospores are infecting the same hosts on which telia and uredinia are formed. The details of the life cycle, spore types and germination conditions, infection process and epidemiology, as well as host range and symptomology of *A. psidii* have been thoroughly reviewed by Coutinho et al. (1998) and Glen et al. (2007).

For many years, *A. psidii* has been considered a significant threat to Australian biodiversity and the *Eucalyptus* forest industry worldwide. The rust has an extremely wide host range between genera and species of Myrtaceae which makes it a tree pathogen with considerable global importance (Coutinho et al. 1998). *A. psidii* has the

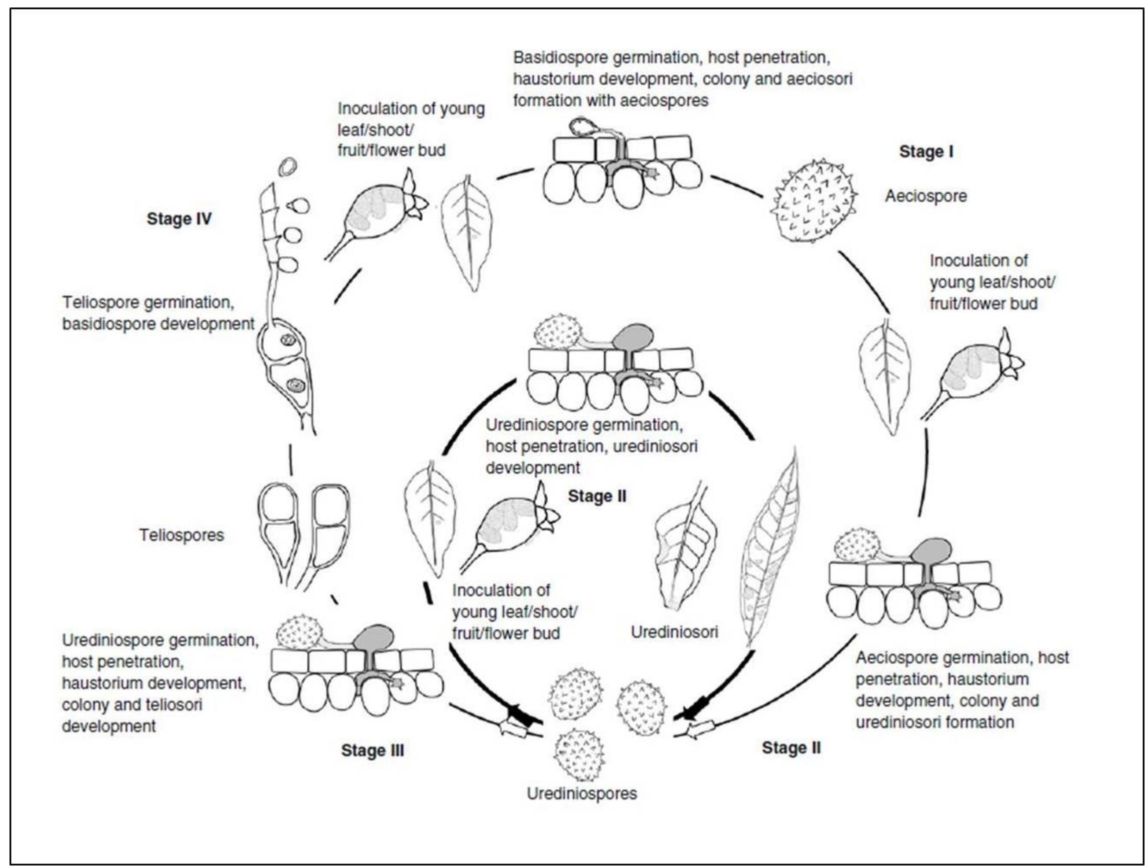

Figure 1-3: Schematic life cycle of *Austropuccinia psidii* (Glen et al. 2007); aeciospores are morphologically similar to urediniospores and their presence is not observed or recognised in nature (Figueiredo 2001); while the actual roles of teliospores and basidiospores in the life cycle are controversial (McTaggart et al. 2017; Morin et al. 2014).

potential to cause devastating foliar disease and major damage to natural ecosystems dominated by Myrtaceae and commercial plantings in many countries around the world (Grgurinovic et al. 2006; Simpson et al. 2006), including almost 1 million hectares of eucalypt plantations in Australia and over 20 million hectares worldwide (Carnegie 2015; Naidoo et al. 2014). Since its first detection in April 2010 on the central coast of New South Wales (Carnegie et al. 2010), the rust has spread rapidly along the east coast of Australia (Figure 1-4) and is now found from temperate regions of Victoria to tropical regions of north Queensland (Pegg et al. 2014a). The disease was also reported for the first time in January 2015 infecting *Eucalyptus* plantations which were surrounded by native forest stands in New South Wales (Carnegie 2015).

A. psidii infects young and actively growing foliage of myrtaceous plants, as well as floral buds and young fruits in some hosts (Glen et al. 2007; Morin et al. 2012). Symptoms of infection by *A. psidii* on different host species range from relatively mild, with minor leaf spots or rust sori, to extreme, with severe foliage and stem dieback (Pegg et al. 2014b). The infection results in reduced growth, defoliation, malformations of the affected organs and death of young trees (Coutinho et al. 1998; Glen et al. 2007). *A. psidii* was reported to achieve successful germination and infection under high humidity or leaf wetness and sufficient exposure to darkness (Piza and Ribeiro 1988; Silva et al. 2013). Human assisted mechanisms including movement of infected nursery plants as well as favourable climatic conditions have both contributed to the rapid spread of *A. psidii* with disease outbreaks in different regions of Australia (Pegg et al. 2014b). To date, the known impact of myrtle rust on *Eucalyptus* has remained limited with disease reported mainly in young seedlings. There is serious concern regarding the potential impact of *A. psidii* on native forest and commercial *Eucalyptus* and screening for resistant genotype across species under glasshouse and field conditions has been ongoing in Australia since the detection of the rust in 2010.

Genetic variation of rust resistance in *Eucalyptus*

About 1646 species from 70 genera of Myrtaceae (out of 3600 species from ~155 genera) are reported native to the Australian continent, the majority in the ‘eucalypt group’ and ‘melaleuca group’ (Zauza et al. 2010). *A. psidii* is one of the biggest threats

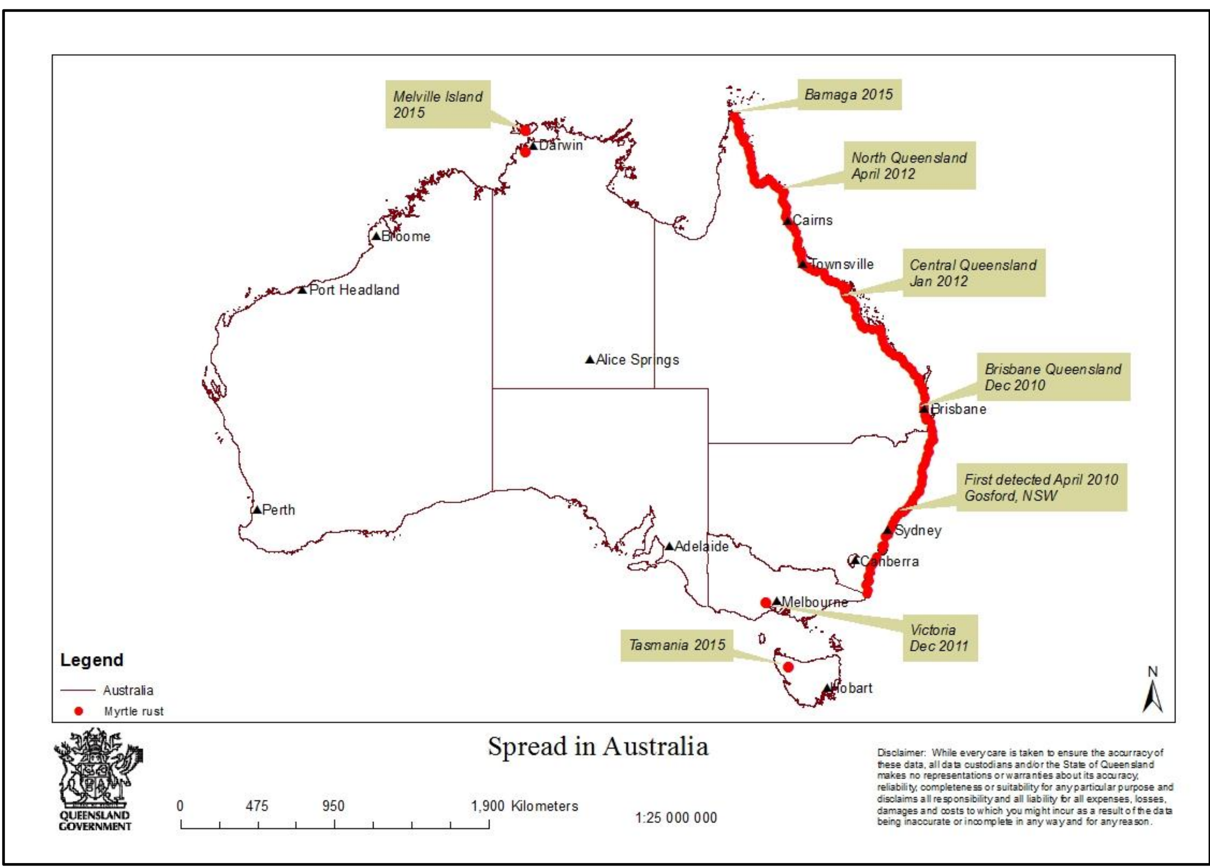

Figure 1-4: Distribution of myrtle rust along the east coast of Australia and the time of spread (source: Queensland Government, Australia).

to natural and planted myrtaceous species with the areas of species origin being the most vulnerable to the disease (Coutinho et al. 1998; Lee et al. 2015). In Australia, *A. psidii* was found on 107 host species from 30 genera of Myrtaceae based on the observations of natural infection in 2012 (Carnegie and Lidbetter 2012) but the record has since rapidly increased to more than 200 species from 37 genera by 2015 (Lee et al. 2015). This observation has not included approximately 116 host species known only from host screening to bring the total known host range in Australia to 346 species from 56 genera to date (Carnegie and Giblin 2016). Meanwhile, the current global host list for *A. psidii* has been reported to attain 450 species from 73 genera of Myrtaceae based on both the observations of natural infection and host screening (Carnegie et al. 2016). A range of scoring systems have been developed to categorise visible infection symptoms for analysis of disease severity following natural infection or artificial inoculation in a glasshouse (Table 1-1 & 1-2; Morin et al. 2011; Sandhu and Park 2013). The scoring systems are generally based on the evaluation by Junghans et al. (2003b) where the inoculated host with lowest rating in severity is considered to be completely resistant while the host with the highest rating is extremely susceptible.

The implications of *A. psidii* incursion are wide-ranging in Australia where myrtaceous genera including *Eucalyptus*, *Corymbia*, *Leptospermum*, *Melaleuca* and *Syzygium* (Wilson et al. 2005) constitute the majority of natural plant communities. Morin et al. (2012) reported a broad range of infection symptoms developed in 83 out of 122 taxa of Myrtaceae following artificial rust inoculation, with the majority of the taxa within *Eucalyptus* and *Corymbia*. For instance, no visible symptoms were observed on *Eucalyptus grandis* × *camaldulensis* hybrids after artificial rust inoculation but a range of symptoms were discovered across replicates of *E. grandis* and *E. camaldulensis* (Carnegie and Lidbetter 2012; Morin et al. 2012; Zauza et al. 2010). Variation in resistance to the rust was also found between different accessions of the same species as highlighted by Morin et al. (2012) in *Lophostemon suaveolens* (Sol. ex Gaertn.) Peter G. Wilson & J.T. Waterh. where the artificially inoculated accession did not develop any rust infection but the infection symptoms were discovered on the field accession, reflecting phenotypic and/or genotypic differences between individual plants within the same taxon. Furthermore, the commonly cultivated myrtaceous species in Australian

Table 1-1: Scoring system used to categorise visible symptoms observed on 3 weeks after inoculation of whole plants with myrtle rust (Morin et al. 2011)

Disease score	Symptoms	Examples
1	No visible symptoms or some discolouration or chlorosis present that cannot categorically be attributed to the rust.	a
2	Chlorotic, purplish or necrotic spots or blotches only.	b
3	Purplish or necrotic flecks, spots or blotches with underdeveloped uredinia. Pin size uredinia. Limited sporulation.	c
4	Fully-developed, normal size uredinia with or without purplish halos that cover less than 25% of the leaf surface. Abundant sporulation and uredinia may be present on stems.	d
5	Fully-developed, normal size uredinia with or without purplish halos that cover more than 25% of the leaf surface. Abundant sporulation and uredinia may be present on stems.	e

a = *Eucalyptus agglomerate*
b = *Lophostemon suaveolens*
c = *Eucalyptus regnans*
d = *Eucalyptus pelita*
e = *Syzygium jambos*

Table 1-2: Greenhouse scale developed for scoring host response against *Austropuccinia psidii* (Sandhu and Park 2013)

Infection Type	Scale	Host Response
No visible sign of infection	0	Highly resistant (HR)
Mild hypersensitivity/flecks/dark flecks/necrosis	;/;+/N/1	Resistant (R)
Restricted pustule/dark gray surrounding/chlorosis/necrosis	1+ to 2+/CN	Moderately Resistant (MR)
Small to medium sized pustules low in frequency and may be with some chlorosis present	3 to 3C	Moderately Susceptible (MS)
Fully developed pustules on leaves and medium to high in frequency	3+	Susceptible (S)
Abundance of fully developed pustules on leaves, twigs and buds	4	Very susceptible (VS)

; = Light brown flecking
;+ = Dark & bigger sized flecks
N = Necrosis
C = Chlorosis

nurseries and gardens including *A. flexuosa*, *S. jambos*, *Gossia inophloia* (J.F. Bailey & C.T. White) N. Snow & Guymer and *Syzygium anisatum* (Vickery) Craven & Biffin were reported to be severely affected by *A. psidii* in the field (Carnegie and Cooper 2011; Carnegie and Lidbetter 2012).

Existing literature reports great variation in the susceptibility to *A. psidii* infection not only between different *Eucalyptus* species but also between provenances within species and between families of the same provenance (Carnegie and Lidbetter 2012; Lee et al. 2015; Morin et al. 2012; Zauza et al. 2010). Zauza et al. (2010) indicated that between *Eucalyptus* tested, *E. cloeziana*, *E. grandis*, *E. regnans* and *Eucalyptus diversicolor* F. Muell ranked as the most susceptible to *A. psidii* with frequency of resistant plants < 50% regardless of their provenance. Large differences in rust resistance between provenances within species were also detected in their observation where *E. regnans* collected from Victoria showed only 7% of resistant samples as compared to 86% of resistant in samples collected from Tasmania. *E. globulus* collected from the above provenances also exhibited significant differences with 65% of rust resistant samples observed in Victoria as compared to 75% in Tasmanian samples. This variation in resistance between geographic races might be due to genetic drift, hybridisation and/or selection as part of the evolutionary process over generations (Hamilton et al. 2013).

Until now, little is known about the quantitative variation and genotype by environment interaction (G × E) of the rust resistance in *Eucalyptus* species as the hosts have unknown evolutionary connections with the pathogen (Tobias et al. 2016). Miranda et al. (2013) have investigated genetic parameters viz. genetic variation, heritability and G × E interaction for rust resistance in an open-pollinated progeny trial of *E. grandis*. Their results demonstrated that the expression of resistance variation in *E. grandis* is different between studied sites and subsequently indicated the differences in pathogenic behaviour of the *A. psidii* populations in different areas. On the other hand, Silva et al. (2013) reported the stable behaviour of open-pollinated families of *E. grandis* to rust susceptibility with simple G × E interaction where eight of the 20 progenies showed the best rust tolerance across the nine different sites in São Paulo State. The contradiction between these studies may explain the complexity of the *Eucalyptus* – rust pathosystem

in response to abiotic variables e.g. local and regional variations in climate (Carnegie et al. 2016; Helfer 2014), while low G × E interaction and high genetic correlation between different sites by Silva et al. (2013) may suggest good stability of the *E. grandis* progenies and the conceivable selection for resistance between the studied sites. Moreover, recent work by Lee et al. (2015) reporting *A. psidii* resistance in *E. cloeziana* indicated that inland provenances were more resistant to rust infection as compared to coastal provenances in Queensland. The high heritability of rust resistance in *E. cloeziana* has also suggested the significant level of additive genetic variance within their studied provenances.

Higher variations in micro-environmental conditions such as climate, temperature and humidity reduce the heritability of rust resistance in *Eucalyptus* whereas more uniform environmental conditions increase the heritability as evident in progeny trials of several species (Balmelli et al. 2014; Miranda et al. 2013; Pinto et al. 2014; Santos et al. 2014), indicating the influence of environmental factors on rust resistance trait expression in *Eucalyptus*. There are many factors required for the disease to be expressed in the field, including the presence of actively-growing young shoots, conducive climatic conditions and availability of inoculum (Morin et al. 2012). Native populations with highly susceptible species in the areas of greatest climatic potential for rust epidemics, particularly coastal areas of New South Wales, Queensland and the Northern Territory in Australia are projected to be vulnerable to infection if the pathogen is introduced (Booth et al. 2000; Glen et al. 2007). Up to the present, the natural variation and underlying mechanism of *A. psidii* resistance in *Eucalyptus* species and populations remain unclear with limited reports on the natural patterns of resistance variation in the species.

Major gene and minor quantitative loci for myrtle rust resistance

Investigation into the genetic basis of rust resistance in *Eucalyptus* was initiated over a decade ago with preliminary identification of a major gene, *Ppr1* (*P. psidii* resistance gene 1), associated with *A. psidii* resistance in *E. grandis* (Junghans et al. 2003a). The mode of resistance inheritance in the *E. grandis* – *A. psidii* pathosystem was initially proposed to be regulated by one major, dominant gene in the relationship of gene-for-

gene interaction (Flor 1971), although the hypothesis has been challenged by the co-evolution paradigm (Jones and Dangl 2006) as both *Eucalyptus* and *A. psidii* are from different centers of origin and the resistance gene did not co-evolve with the pathogen (Potts et al. 2016; Tobias et al. 2016). The major *Ppr1* locus was subsequently positioned on linkage group 3 of the *Eucalyptus* microsatellite reference map and the position has been validated in genetically unrelated pedigrees of hybrids between *E. grandis* resistant trees and *E. tereticornis* and *E. camaldulensis* (Mamani et al. 2010). The segregation of the dominant allele in variable genetic background families as observed by Mamani et al. (2010) has further suggested that large proportion of rust resistance variation in *E. grandis* to be controlled by a single major *Ppr1* gene.

Plant resistance (*R*) genes are a crucial component in plant genomes that confer disease resistance against pathogens by producing R proteins. Several classes of *R* genes have been studied and characterised with a majority containing a nucleotide-binding site and leucine-rich repeat domains (Dodds and Rathjen 2010; Gururani et al. 2012; Hammond-Kosack and Jones 1997). R protein-triggered resistance is normally a race-specific defense response in plants which is effective against the pathogen strains producing the avirulence (Avr) effector protein recognised by that R protein (Gururani et al. 2012). This effector-triggered immunity response is frequently associated with an oxidative burst and hypersensitive response or programmed cell death (Naidoo et al. 2014). Many of the rust resistant *Eucalyptus* identified from host screening were discovered to exhibit a hypersensitive reaction manifested by rapid death of the invaded cells and necrosis of the adjacent cells in some cases (Alves et al. 2012; Moon et al. 2007; Xavier et al. 2001). As reported by Yamamoto (1995), the hypersensitive response involved in pathogen resistance may be controlled by the combined effect of multiple dominant genes.

According to the described pattern of major gene inheritance in *Eucalyptus*, minor changes of the pathogen in their genetic structure may result in the defeat of the resistance mechanism. As reported in Brazil, an earlier classified resistant clone of *E. grandis* hybrid harboring the *Ppr1* gene was found infected by a newly discovered *A. psidii* race in their later cultivation stage (Graça et al. 2011). Physiological

specialisation of *A. psidii* has been examined by Coelho et al. (2001) under cross-inoculation on *E. grandis*, *P. guajava* and *S. jambos* where three biotypes were detected based on their pathogenicity and host compatibility as follows: the first compatible with *E. grandis* and *S. jambos*; the second with *E. grandis* and *P. guajava*; and the third only with *P. guajava*. Glen et al. (2007) have also reviewed and described several strains or biotypes of *A. psidii*, each with different host range and sporulation proficiency, e.g. two strains in Jamaica infected *Pimento* spp. and *Syzygium* spp., respectively, but neither strain infected *P. guajava* (MacLachlan 1938); and strains from *Melaleuca quinquenervia* (Cav.) S.T. Blake and *Pimenta dioica* (L.) Merr. in Florida did not infect *S. jambos* (Rayachhetry et al. 2001). Furthermore, a unique strain of *A. psidii*, different to other pandemic strains which has spread to Pacific countries such as Australia, Hawaii and Indonesia, has been detected in South Africa with broad distribution yet an unknown source of introduction (Roux et al. 2016).

Although it was reported until recently that there is only one of the numerous *A. psidii* biotypes present in Australia (Carnegie and Lidbetter 2012; Sandhu et al. 2016), the capability of long distance dispersal of rust urediniospores across a wide geographic range may contribute to the forming of large effective populations of pathogen, and subsequently result in their high genetic variability and evolutionary potential (Brown and Hovmøller 2002; Graça et al. 2011; Wingen et al. 2013), as apparently occurred with the disease outbreak or worldwide distribution of wheat rusts viz. *Puccinia striiformis* Westend. (stripe or yellow rust; Hovmøller et al. 2011; Hovmøller et al. 2010; Hovmøller et al. 2008) and *Puccinia graminis* Pers. (stem or black rust; Singh et al. 2011; Singh et al. 2008). Under these circumstances, the resistant *Eucalyptus* genotype tested against a single *A. psidii* biotype may not be vigorous to the challenges of other biotypes of the pathogen (Glen et al. 2007).

Despite major gene resistance to *A. psidii* being supported by a preliminary study (Junghans et al. 2003a), more recent analyses of inter-specific *Eucalyptus* hybrids have discovered different segregation patterns of rust resistance in *Eucalyptus – A. psidii* interaction, suggesting the pathosystem involves multiple quantitative resistance loci (Alfenas et al. 2012). Phenotypic variation as observed by Alves et al. (2012) in rust

resistance has pointed to the existence of minor effect loci within each of the different phenotypic classes where the resistant plants were classified into S0 (immune), S0HR (hypersensitive reaction) and S1 (small pustules) while the susceptible plants were classified into S2 (medium-size pustules) and S3 (large pustules). Although most of the hypersensitive reactions found were associated with a single or multiple dominant genes, the presence of recessive genes controlling hypersensitive reactions has been reported in other pathosystems (Saini et al. 2002; Wang et al. 1994). Junghans et al. (2003a) have suggested that the expression of the major rust resistance gene in *Eucalyptus* is largely dependent on the genetic background such as the presence of suppressor or modifier genes as secondary effects to the resistance gene. Besides, both additive and non-additive (epistasis) genetic variations were reported as equally important to the contribution of rust resistance in *Eucalyptus* (Alfenas et al. 2012). As proposed by Alves et al. (2012), the effects of epistatically interacting secondary loci may have the similar contribution to the genetic rust resistance in *Eucalyptus* as the effect of favourable allele on the *Ppr1* locus. More recently, four major additive effect loci, two influencing symptomatic responses i.e. whether a plant exhibited disease symptoms (*Ppr2* and *Ppr3*), and two influencing hypersensitive reactions (*Ppr4* and *Ppr5*), were identified in *E. globulus* in response to *A. psidii* (Butler et al. 2016). The loci were then located on four different linkage groups of the *E. globulus* linkage map (Hudson et al. 2012), i.e. linkage groups 3 and 7 for *Ppr2* and *Ppr3*, and linkage groups 6 and 9 for *Ppr4* and *Ppr5*, respectively, without overlapping the previously identified major *Pp1* locus.

In order to understand the resistance mechanisms underlying the *Eucalyptus – A. psidii* pathosystem, Moon et al. (2007) constructed two contrasting SAGE (Serial Analysis of Gene Expression) libraries for rust susceptible and resistant populations of *E. grandis* and observed their distinct differences by comparing the type of genes and their expression levels. Their study has identified 471 differentially expressed genes (unique tags) between susceptible and resistant libraries with 239 preferentially expressed in the susceptible and 232 in the resistant. Susceptible plants showed gene expression linked to generalised stress responses and detoxification without inducing competent host defense response to rust infection. Conversely, resistant plants demonstrated up-

regulation of genes contributing to two resistant phenotypes: 1. Cellular polarisation with massive cytoskeleton rearrangement, cytoplasm and nucleus translocation, and strengthening of cell wall against fungal penetration. 2. Anti-pathogen mechanisms including systemic resistance, antioxidative and hypersensitive responses. While most of the recent genetic studies have not contradicted the report by Junghans et al. (2003a) on the presence of major gene (*Ppr1*) resistance to *A. psidii* in *Eucalyptus*, they also collectively support phenotypic variability for rust resistance in *Eucalyptus* being determined by multiple interacting loci of small effects (Alfenas et al. 2012; Alves et al. 2012; Butler et al. 2016; Naidoo et al. 2014; Thumma et al. 2013). Detailed association analyses highlighted a large family of nucleotide-binding leucine-rich repeat (NB-LRR) genes in the vicinity of *Ppr1* locus and few were significantly associated with rust resistance variation in *E. grandis* (Thumma et al. 2013).

Potential genetic markers and the *Eucalyptus* reference genome

Despite their worldwide commercial importance, little effort has been dedicated to investigate *Eucalyptus* species at their molecular level for genetic improvement as relative to research in agricultural crops. The earliest molecular studies in these forest species are supposed to begin in 1990s with phylogenetic analysis of chloroplast DNA by using restriction fragment length polymorphism (RFLP) in six *Eucalyptus* species (Steane et al. 1991), development and screening of RFLP probe libraries for *E. grandis* and *E. urophylla* (Wolff et al. 1993), molecular cloning of genes encoding lignin biosynthesis enzyme in *Eucalyptus gunnii* Hook.f. (Grima-Pettenati et al. 1993), and construction of genetic linkage maps for *E. grandis* and *E. urophylla* using random amplified polymorphic DNA (RAPD) markers and pseudo-test cross configuration (Grattapaglia and Sederoff 1994). The comparatively small genome of *Eucalyptus* species and other characteristics such as ease of vegetative propagation and tissue culture, and availability of stable transformation and transient expression methods have contributed to the attractiveness of *Eucalyptus* species as candidate model systems for woody plant molecular genetics (Chauhan et al. 2014; de Oliveira et al. 2017; Girijashankar 2011; Grattapaglia and Bradshaw Jr. 1994; Le Roux and Van Staden 1991).

Grattapaglia et al. (2012) have reviewed the development and applications of various types of DNA markers including RFLP, RAPD, amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), inter simple sequence repeat (ISSR), diversity arrays technology (DArT), single nucleotide polymorphism (SNP), chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) markers in *Eucalyptus* and other myrtaceous genera. Between the reported DNA markers, microsatellites or SSRs are the most broadly used markers in *Eucalyptus* for various molecular applications including species identification or germplasm characterisation, phylogeny and phylogeography, genetic and functional diversity studies, quantitative trait loci (QTL) identification, and ecological and breeding strategies (Brondani et al. 1998; Brondani et al. 2006; Faria et al. 2011; Grattapaglia et al. 2012; He et al. 2012; Ottewell et al. 2005; Sumathi and Yasodha 2014; Zhou et al. 2014). In an earlier study, Byrne et al. (1996) identified four nuclear microsatellite loci from *E. nitens* and confirmed that they were amplifiable and polymorphic in several other species in the same genus, indicating the potential transferability of microsatellites between *Eucalyptus* species. Brondani et al. (1998) revealed the abundance of SSRs in *Eucalyptus* genome and high information content of these markers for individual identification via genetic characterisation and linkage mapping of twenty microsatellite loci in *E. grandis* and *E. urophylla*. Bundock et al. (2000) have later incorporated microsatellite markers originated from *E. grandis* / *E. urophylla* into the RAPD genomic maps of *E. globulus* for the identification of homologous linkage groups between these species. Moreover, numerous sets of novel microsatellites have been developed from the existing *Eucalyptus* expressed sequence tag (EST) resources to provide an increasingly larger set of interspecific markers and consensus mapping information for potential genomics and breeding applications across variable genetic backgrounds in the genus (Brondani et al. 2006; He et al. 2012; Zhou et al. 2014). The characteristics of microsatellites such as universal distribution in genome, high mutation rate, heterozygousity, locus specificity, co-dominance, multi allelism, association with gene expression and transferability across species have enabled the construction of high-resolution genetic maps for *Eucalyptus* and other plant species linking between their phenotypic and genotypic variation (Grattapaglia et al. 2015; Sumathi and Yasodha 2014).

In contrast to microsatellites, SNP markers are typically bi-allelic (although tri-allele SNPs are also known) with limited polymorphisms per marker (Rafalski 2002; Telfer et al. 2015; Thavamanikumar et al. 2011). Unlike microsatellites or other DNA markers i.e. RFLPs and AFLPs, SNPs have relatively low mutation rates and their bi-allelic quality (having two alleles as opposed to all possible forms of four nucleotides) has simplified the interpretation of marker variation and facilitated high-throughput genotyping (Brumfield et al. 2003; Thavamanikumar et al. 2011). While multiple mutations at a single site are very unlikely, observed variation in SNP frequency may be due to a combination of selection, random genetic drift and/or variation in mutation rates (Novaes et al. 2008; Thavamanikumar et al. 2011). SNPs also occur in high density and represent a useful resource for genetic association mapping linking candidate gene-associated SNPs to variation in complex phenotypic traits (Thavamanikumar et al. 2013). A set of SNPs linked to the lignin biosynthesis genes in *E. globulus*, cinnamoyl CoA reductase (*CCR*) and cinnamyl alcohol dehydrogenase 2 (*CAD2*), indicated polymorphism caused by missense mutation affecting highly conserved amino acids may alter enzyme function and therefore cause variation in lignin profiles (Poke et al. 2003).

Notwithstanding the frequency and position of polymorphisms, and the presence of haplotypes and linkage disequilibrium (LD) are some of the factors influencing SNPs selection for genotyping (Tabor et al. 2002), SNPs generated by non-synonymous substitution in coding regions (Nei 2005) are generally targeted for association studies as they are more likely to impact the phenotypes than synonymous substitution which do not change the amino acid sequence (Thavamanikumar et al. 2013). Thumma et al. (2012) have identified not only candidate genes but also functional SNPs involved in differential expression in *E. camaldulensis* seedlings subjected to different water stress treatments via transcriptome sequencing. Nine stable marker-trait associations have been reported arising from seven SNPs in six candidate genes controlling a series of wood quality and growth traits in *E. globulus* (Thavamanikumar et al. 2014). SNP markers were reported to be prominently superior to microsatellite markers in data accuracy, reproducibility and robustness (Telfer et al. 2015), and a preferable choice of marker system nowadays due to their abundance and high level of polymorphism,

especially for species with large genome size and low microsatellite diversity (Kadirvel et al. 2015; Unterseer et al. 2014). Nevertheless, the comparative genotyping cost per sample has restricted the widespread use of whole-genome level SNP assays in non-model species but limited complex trait dissection to the candidate gene approach (Thavamanikumar et al. 2014).

In the efforts of genomic research for sustainable improvement of *Eucalyptus* species, a large number of *Eucalyptus* ESTs has been identified and deposited in the private and semi-private international databases as a resource for various genetic analyses including discovery of informative polymorphisms and development of molecular markers in genetic linkage mapping projects (Hirakawa et al. 2011). Following the release of the whole genome sequence of black cottonwood tree, *Populus trichocarpa* Torr. & A. Gray ex. Hook. (Tuskan et al. 2006), whole genome sequencing for *E. camaldulensis* and *E. grandis* have been completed and released by the Kazusa DNA Research Institute in Japan and the US Department of Energy Joint Genome Institute (DOE-JGI) in collaboration with members of the *Eucalyptus* Genome Network (EUCAGEN) respectively (Hirakawa et al. 2011; Myburg et al. 2014). The *Eucalyptus* genome sequence is the first reference genome in the Rosid order Myrtales, an important sister lineage to the Eurosids, and has permitted not only basic and applied biological research in Myrtaceae but also comparative genomic studies within the Rosids and the Eudicots (Grattapaglia et al. 2012). Based on the availability of this high quality reference genome, an integrative framework involving large scale genotypic and phenotypic data sets will be conceivable to dissect the complex associations between individual genomic and the observed phenotypic variation in *Eucalyptus* (Myburg et al. 2014).

Implication of molecular approaches in disease management

Interactions of forest disturbances such as climate change and outbreaks of pathogens have caused progressive impacts on the growth, geographic distribution and sustainability of forest tree species in their natural ecosystems (Helfer 2014; Sturrock et al. 2011). The effects of this new disease – myrtle rust on naïve hosts in native *Eucalyptus* forest are far more unpredictable than they would be on hosts that have had a long association with the pathogen (Tobias et al. 2016). Most recent genetic studies

have strongly indicated that disease resistance is a complex trait controlled by the combined effects of a number of major and minor genes (Alfenas et al. 2012; Alves et al. 2012; Santos et al. 2014). However, present molecular studies of resistance loci provide little information on the ecological patterns of functional variation (de Meaux and Mitchell-Olds 2003) as the geographic heterogeneity may cause unique evolutionary dynamics at different locations (Thompson 1999). Gene flow, genetic drift and various forms of natural selection have been reported to influence the evolution of plant host-pathogen interactions and resultant patterns of resistance variation (Burdon and Thrall 1999; Nuismer et al. 1999; Potts et al. 2016). Therefore, simultaneous analysis of molecular variation at both neutral and ecologically important, viz. defense-related loci, is recommended for elucidating the causes of selection and observed patterns of resistance diversity in *Eucalyptus* species (de Meaux and Mitchell-Olds 2003; Steane et al. 2006).

Deciphering the genetic architecture of ecologically important traits including quantitative disease resistance is crucial for forest tree improvement and management (Plomion et al. 2016). Tree pathologists and forest managers should explore the genetic and molecular basis of quantitative resistance for durable disease management in their forests. Previous deployment of a single resistance marker (AT9/917) based on *Ppr1* in *E. grandis* breeding programs for plantation areas prone to *A. psidii* (Labate et al. 2009) has failed following the infection of trees or resistant clones carrying the favourable alleles at that locus by a new rust strain (Graça et al. 2011). In view of the detrimental effect of myrtle rust on *Eucalyptus* wood production and tree population sustainability, genetic dissection for identification of genome-wide molecular disease resistance markers has become an urgent need to mitigate the risk posed by *A. psidii* on *Eucalyptus* related industries. Since the host and pathogen had no recent evolutionary connections, further understanding of the pre-adaptive evolution, i.e. evolution of novel adaptive traits or the presence of pre-adaptive mechanisms to other evolutionary selection pressure (Lee 2002; Tobias et al. 2016), in rust resistance and the causes of allele maintenance in nature will be of advantage for sustainable disease management in *Eucalyptus* forests.

The coupling of the *Eucalyptus* reference genome and expression QTL (e-QTL) mapping approach has warranted gene-level investigations of rust resistance (Alves et al. 2012; Mamani et al. 2010; Thumma et al. 2013), including searching for specific *R* genes and development of allele-specific SNP based assays for marker assisted selection (MAS). The relatively small genome size of *Eucalyptus* (Grattapaglia and Bradshaw Jr. 1994; Grattapaglia et al. 2012) and continuous cost reduction of next generation sequencing (NGS) have enabled genome wide association studies (GWAS) for identifying molecular markers associated with numerous complex traits including quantitative disease resistance across the entire genome of *Eucalyptus* (Plomion et al. 2016; Thavamanikumar et al. 2013). A genomic selection (GS) approach has been recently proposed to exploit the effects of high density markers (large numbers of random markers or small numbers of associated markers) for prediction of an individual's phenotype based on its genotype (Grattapaglia and Resende 2011; Resende et al. 2012a; Thavamanikumar et al. 2013). Resende et al. (2012a) have demonstrated accuracies of GS between 0.55 and 0.88 for pulp yield, wood quality and growth traits in *Eucalyptus* based on more than 3000 DArT markers.

Despite high accuracies, predictions of genomic breeding values for complex traits across different studied sites and/or populations with GS models have been limited by variation in LD between populations and significant G × E interaction (Thavamanikumar et al. 2013). One of the major requirements for effective application of GS in forest tree management is the development of small effective populations with high LD (Grattapaglia and Resende 2011). However, most forest tree populations with large numbers of open pollinated families have low LD and consequently low accuracy of GS prediction models. In order to improve the application, Thavamanikumar et al. (2013) have suggested the use of stable markers (with similar allelic effects across different populations) from association studies to develop the prediction models in low LD populations. The proposed models based on a modest number of markers i.e. 90 SNPs were then able to result in 0.20 to 0.47 accuracies in prediction of several wood quality traits in different populations of *E. nitens*, and the predictive abilities were similar to those reported for wood quality traits in a highly structured and high LD

population of loblolly pine (Resende et al. 2012b), as well as pulp yield and other growth traits in *Eucalyptus* hybrids (Resende et al. 2012a).

With the advent of high throughput technologies and efficient genomic approaches, accumulation of genomic resources including reference genomes and epigenome sequences will provide insights into the unique biology, evolutionary history and adaptive divergence of *Eucalyptus* species (Myburg et al. 2014; Plomion et al. 2016). Identification of additive and non-additive and epistasis genetic variation in the determination of rust resistance and other traits of interest would allow substantial genetic effects to be captured for successful genotype selection (Alves et al. 2012; Resende et al. 2017a). Fundamental studies in comparative genome evolution and population genomics based on environmental association analysis in *Eucalyptus* may enable discovery of correlations between environmental variables and potentially useful sequence variants at the genome-wide level across a wide range of populations (Grattapaglia et al. 2012), as recently reported in widespread *Eucalyptus* species spanning strong environmental gradients for adaptation to aridity i.e. rainfall and temperature (Steane et al. 2017). Moreover, advances in genomic technologies coupled with new bioinformatics tools would permit the improvement of current SNP discovery, genotyping and sequencing methods, and subsequently help to reveal the roles of molecular players in their underlying mechanisms of complex developmental processes, natural adaptations and responses to external disturbances including pathogen invasion in *Eucalyptus* species (Grattapaglia and Kirst 2008; Plomion et al. 2016).

Thesis structure and aims

The present study aims to analyse the quantitative and molecular genetic basis of *A. psidii* rust resistance in natural populations of *E. globulus* ssp. *globulus* and *E. obliqua*, and to investigate the pathogen infection process and host defense mechanisms underlying the *Eucalyptus* – *A. psidii* pathosystem. Australian *Eucalyptus* spp. are a major source of forest products in many countries around the world and of major economic significance. *E. globulus* is the most important *Eucalyptus* species in Australian plantations while *E. obliqua* is ecologically and commercially important in native forests across a wide region of south-eastern Australia. These species represent

the two largest and most important subgenera of *Eucalyptus* viz. *Symphyomyrtus* and *Eucalyptus*, respectively, for potentially wider application of common findings across the genus.

With its detrimental impact to a huge number of myrtaceous hosts, myrtle rust is a major threat to *Eucalyptus* dependent industry worldwide and to Australia with its ecosystems dominated by Myrtaceae. Globally more than 400 myrtaceous species including members of *Eucalyptus* have been reported susceptible to *A. psidii* to date, with a range of symptoms from relatively mild infection to severe defoliation and death of young trees. The increasing numbers of known susceptible hosts has prioritised research investment to minimise the damaging effects of this pathogen in native ecosystems and *Eucalyptus* plantations. As Australian *Eucalyptus* species have evolved in the absence of the myrtle rust pathogen questions that arise include: "what is the underlying mechanism of resistance to this disease?" and "how has variation in resistance evolved in the absence of the pathogen?" This thesis aims to answer these questions by identifying some of the underlying molecular mechanisms associated with rust resistance in both *E. globulus* ssp. *globulus* and *E. obliqua* using phenotypic and molecular analyses. Emerging applications of high throughput technologies and efficient genomic approaches including SNP discovery and genotyping via next generation sequencing have been suggested to decipher the resistance mechanisms underlying *Eucalyptus* – *A. psidii* pathosystem and pre-adaptive evolution in rust resistance.

The specific aims of this study are:

1. to examine the genetic variation of *A. psidii* rust susceptibility in geographic races (and sub-races) of *E. globulus* ssp. *globulus* and regional populations of *E. obliqua* for understanding of pathosystems involving native *Eucalyptus* species and *A. psidii*, and prediction of the main evolutionary forces which have shaped the observed patterns of resistance variation (Chapter 2);
2. to elucidate the infection process of *A. psidii* on *Eucalyptus* leaves, and identify stages at which pathogen invasion is stopped or constrained in resistant phenotypes in order to understand modes of defense responses (Chapter 3);

3. to compare the chemical composition of foliar essential oils in *Eucalyptus* between rust resistant and susceptible phenotypes, and investigate the relationship between phytochemical properties in *Eucalyptus* foliage and rust resistance (Chapter 4);
4. to dissect the complex resistance trait in *E. obliqua* for exploration of potential genetic markers correlated with rust resistance phenotypes, and to compare the genome-wide resistance QTL generated from *E. obliqua* with the *A. psidii* resistance markers reported from *Symphyomyrtus* species i.e. *E. globulus* and *E. grandis* (Chapter 5).

Each chapter has been written in the format of a journal article or article in development; hence repetition of backgrounds, concepts and ideas in between chapters is unavoidable. Chapter 1 presents the general introduction and background of the study including thesis structure and overall aim. Chapters 2 to 5 are experimental chapters addressing the specific aims and respective methodology involved in the study. Chapter 6 summarises the key findings of the thesis and their potential implications in *Eucalyptus* forest industries. Advanced knowledge generated from the study will be of valuable in development of durable rust resistance and sustainable disease management in *Eucalyptus* forests. The outcome of this study will benefit managers of native and plantation forests and should enable the selection of resistant candidates. The study will also aim to shed light on the evolution of pre-adaptive resistance in plants.

This page intentionally left blank

Chapter 2

Geographical patterns of variation in susceptibility of *Eucalyptus globulus* Labill. ssp. *globulus* and *Eucalyptus obliqua* L'Hér. to myrtle rust disease

Abstract

Myrtle rust, caused by the pathogen *Austropuccinia psidii* Beenken, is a worldwide disease threat to Myrtaceae plant species, communities. Many Australian ecosystems have a dominant flora that is Myrtaceae rich and these, along with the industries that rely on them are particularly vulnerable to this disease. *Eucalyptus* is a major genus within Myrtaceae in Australia with over 800 endemic species. The existing literature reports wide variation in response to *A. psidii* infection between *Eucalyptus* species, from full susceptibility to resistance. This study aims to define the geographical patterns of rust susceptibility variation across the natural ranges of *Eucalyptus globulus* Labill. and *Eucalyptus obliqua* L'Hér., two commercially and ecologically important species from different *Eucalyptus* subgenera. Rust phenotypic screening of seedlings of *E. globulus* races and *E. obliqua* forest districts revealed highly significant differences in disease severity and symptomatic trait expression between the two species in response to *A. psidii* infection. Geographical patterns of rust susceptibility in *E. globulus* indicated a trend for decreased susceptibility and increased hypersensitivity to *A. psidii* northward from south- to north-eastern Tasmania, eastward along Otway Ranges and southward from Strzelecki Ranges to Wilson Promontory Lighthouse, but no distinct geographical patterns were displayed between provenances of *E. obliqua*. Furthermore, few significant correlations were obtained between climatic conditions (e.g. annual rainfall, maximum and minimum temperatures and elevation) and rust susceptibility at the provenance level for both species. The outcomes of this study will enhance our understanding of the *Eucalyptus* – *A. psidii* pathosystem and the potential drivers of rust resistance in *Eucalyptus*.

Introduction

Most forest tree species demonstrate geographical patterns of genetic variation in ecologically important traits as a result of exposure to numerous evolutionary forces viz. genetic drift, hybridisation and local selection across their distributional range (Hamrick 2004). Understanding spatially structured genetic variation can provide insights into the evolutionary histories of forest tree and the role of ecological factors in shaping the current patterns of geographic variation (Shepherd et al. 2010). In Australia, most native forests are dominated by *Eucalyptus* species ranging from small shrubs to tall trees, occupying broad range of climatic and ecological niches across the country (Booth et al. 2015). Assessment of the pattern of adaptive variation within a *Eucalyptus* species' range is important in conserving the locally adaptive genotypes and contributing subsequently to the effective ecological restoration (O'Brien et al. 2007) and disease management (Potts et al. 2016).

Eucalyptus is the largest genus of the eucalypt group containing ten subgenera (Bayly 2016) with *Symphyomyrtus* and *Eucalyptus* (previously informal *Monocalyptus*; Pryor and Johnson 1971) being the two major subgenera consist of about 450 and 110 species respectively (Boland et al. 2006). *Eucalyptus globulus* Labill. from subgenus *Symphyomyrtus* and *Eucalyptus obliqua* L'Hér. from subgenus *Eucalyptus* (Brooker 2000) are two commercially and ecologically important species with natural ranges in south-eastern Australia from southern New South Wales to southern Tasmania, and extension of *E. obliqua* northward to northern New South Wales and westward to Kangaroo Island, South Australia (Eldridge et al. 1993). *E. globulus* is internationally one of the most widely cultivated tree species, mainly for pulpwood production (Larcombe et al. 2016) and analysis of morphological variation across its native range has clustered the species into four geographical-morphological subspecies viz. *globulus*, *bicostata*, *maidenii* and *pseudoglobulus* (Kirkpatrick 1974). While *E. obliqua* is also an important timber species it is widespread and ecologically important being found as a major floristic component of most wet sclerophyll forest systems (Moore 2015; Neyland et al. 2009). The occurrence of different ecotypic populations in the species and the pattern of genetic diversity in their native range are little studied (Bloomfield et al. 2011).

E. globulus ssp. *globulus* is the most economically important of the *E. globulus* species complex, being the main species planted internationally. Even within this subspecies there is considerable variation with three main geographic groups recognised by Potts and Jordan (1994b): (1) continuous coastal areas in eastern Tasmania; (2) Bass Strait islands integrated with mainland; and (3) isolated regions in western Tasmania. Subdivisions of a species (or subspecies) with genetically similar individuals occupying a particular provenance are classified as geographic races (Zobel and Talbert 1984). For *E. globulus* ssp. *globulus* a geographical race classifications have been proposed based on numerous phenotypic traits including growth (Jordan et al. 1994; Potts and Jordan 1994b), fungal resistance (Carnegie et al. 1994), and extreme environmental resistance, e.g. frost (Almeida et al. 1995; Zang et al. 1995) and drought (Dutkowski 1995). Based on the geographic consistency and similarities in growth response Jordan et al. (1994) identified 12 geographic races of *E. globulus* ssp. *globulus*. This classification was revised by Dutkowski and Potts (1999) based on a wider variety of traits including bark thickness, wood basic density via pilodyn penetration, survival and flowering precocity in field trials. This revised classification relied less on geographic discontinuities in the distribution than on demonstrated genetic similarity in field trials and is proposed to better distinguish sub-races with races. Overall it distinguishes 13 races and 20 sub-races and is now the basis of race and sub-race classification used in breeding within Australia.

Spatial distribution of genetic variation as a response to natural selection and environmental change has almost always been found in different populations of *Eucalyptus* species with most of the economically important traits vary geographically within species. Previous reports indicate significant variation in growth parameters such as height and volume between provenances of *E. obliqua* (Brown et al. 1976; Green 1971). Kirkpatrick (1976) also discovered geographic variation in seedling morphology and characteristics of reproductive organs (fruit and seed) between disjunct populations of *E. glaucescens* Maiden et Blakely. Different rainfall populations of *E. viminalis* Labill. were found significant different in drought tolerance (Ladiges 1974). Survival, growth and stem diameter traits were also reported to be significant different between

provenances of *E. camaldulensis* Dehnh. (Bush et al. 2013; Mahmood et al. 2003) and *E. marginata* Donn ex Smith (O'Brien et al. 2007). Moreover, analysis of foliar defense chemical in both *E. melliodora* A. Cunn. ex Schauer and *E. tricarpa* (L.A.S. Johnson) L.A.S. Johnson & K.D. Hill revealed fine-scale spatial correlation of plant secondary metabolites and biochemical diversity in *Eucalyptus* species (Andrew et al. 2007a; Andrew et al. 2010).

Molecular analyses have also been used to investigate the geographical structure of *Eucalyptus* species in native populations, as described in *E. globulus* ssp. *globulus* (Jones et al. 2002; Steane et al. 2006), *E. obliqua* (Bloomfield et al. 2011), *E. camaldulensis* (Dillon et al. 2015) and *E. cladocalyx* F. Muell (Ballesta et al. 2015). Variation patterns in *E. globulus* as compared by Steane et al. (2006) using microsatellite markers and quantitative traits have revealed the similarities between geographically neighbouring races with molecular data analysis but contrasting spatial differentiation between some of the nearby geographical races with analysis of quantitative data. The disparity between the molecular and morphological classifications may be partially due to resolution with finer structures likely present but undetectable in the molecular data sets used to date although they may also represent real differences between methodology with the molecular approaches accessing what is almost certainly neutral variation and the morphological analyses capturing selective as well as neutral patterns. If this is the case the genetic variation between different geographic races might indeed reflect the adaptive differences evolved in response to selection discrepancy in distinctive natural environments with respect to climate or edaphic conditions.

In recent years, an emerging disease threat to the eucalypt related industry in Australia is the discovery and spread of myrtle rust caused by *Austropuccinia psidii* Beenken (Beenken 2017; previously reported as *Puccinia psidii* Winter sensu lato or *Uredo rangelii* J.A.Simpson, K.Thomas & C.A.Grgurinovic) which was first detected in April 2010 on the central coast of New South Wales (Carnegie et al. 2010). The rust rapidly spread along the east coast of Australia from temperate regions of Victoria to tropical regions of north Queensland (Pegg et al. 2014a), with natural infected host range from

107 species and 30 genera of Myrtaceae in 2012 (Carnegie and Lidbetter 2012) increased to more than 200 species and 37 genera in 2015 (Lee et al. 2015). The disease was reported for the first time in January 2015 in *Eucalyptus* plantations in Australia which were surrounded by native forest stands based on surveys conducted in New South Wales (Carnegie 2015). There is also significant concern regarding the potential impact *A. psidii* infection will have on native forest species and screening in glasshouse and field conditions has been ongoing since the detection of the rust in 2010. Globally reported hosts of *A. psidii* have risen over 450 species from 73 genera, recorded from both natural infection and artificial inoculation (Carnegie et al. 2016; Giblin and Carnegie 2014). In screening trials, wide variation of myrtle rust resistance levels has been observed between and within many *Eucalyptus* and *Corymbia* spp. (Pegg et al. 2014a; Zauza et al. 2010), with infection responses ranging from highly susceptible to completely resistant i.e. no observable disease (Morin et al. 2012; Pegg et al. 2014b; Sandhu and Park 2013; Zauza et al. 2010).

While these studies indicate considerable resistance may be present naturally, detailed investigation of geographic distribution of rust resistance within species is necessary before prediction of likely rust impact can be made for native stands. Also, understanding the sources and reasons for resistance segregating at reasonable frequencies in populations and species that have unknown exposure to the pathogen in evolutionary time will also require such detailed studies (Tobias et al. 2016). The heterogeneous distribution of pathogen resistance between geographic populations or races might be due to genetic drift, hybridisation and/or selection over the evolutionary time scales (Hamilton et al. 2013; Mitchell-Olds et al. 2007). Direct pathogen-imposed selection pressure (Burdon 2001), biotic and abiotic environmental factors such as climatic variables and genotype-by-environment interaction (Bryner and Rigling 2011) are contributing to the natural patterns of genetic variation in pathogen resistance within the host species and the overall disease risk variation across the larger spatial scales. The expanding host range for a single genotype of *A. psidii* found in Australia (Machado et al. 2015) and the presence of specific resistance to the newly encountered pathogen in several myrtaceous species (Morin et al. 2012; Potts et al. 2016) have prompted speculation on how these complex interaction can occur in the myrtaceae –

myrtle rust pathosystem in which both host and pathogen had no recent evolutionary connections (Tobias et al. 2016).

The present study aims to examine the genetic variation of *A. psidii* rust susceptibility in quantitative (disease severity according to numeric score) and binary traits (symptomatic, hypersensitivity and pustulation) between geographic races (and sub-races) of *E. globulus* ssp. *globulus* (hereafter referred to as *E. globulus*) and regional populations (in forest district levels) of *E. obliqua*, two important species representing the two main *Eucalyptus* subgenera *Symphyomyrtus* and *Eucalyptus* respectively, for the potential application of common findings more widely across the genus. The specific aims are to understand the pathosystems involving native *Eucalyptus* species and *A. psidii*, and to potentially predict the main evolutionary forces which have shaped the observed patterns of resistance variation. The outcome of this study will be of benefit to the management of rust disease in native forest and the selection of resistant races for maximises production in plantations as well as for understanding the evolution of pre-adaptive resistance.

Materials and Methods

Experimental design

A total of 1447 *E. globulus* seedlots comprising 1251 open pollinated families (genotypes) obtained from broad based native and multigenerational breeding populations with accurate pedigree information were sampled for a major screening trial to estimate the heritability of myrtle rust resistance in the species. A subset of this data was then extracted from the original screening dataset for only the seedlots collected directly from the wild mother trees to examine the geographical patterns of rust susceptibility in their native populations. *E. globulus* seeds were collected from ranges of geographical provenance and imbibed in potassium nitrate (KNO_3) for 14 days before sown in 40-celled Hiko Seedling trays (Arborgreen, Australia) with potting mixtures for regeneration. The hyco tray is 352 × 216 × 87 mm in dimensions and contains 40 (8 × 5) cells with 93 cc of internal cell volume. A single tray was designated to accommodate eight screening plots with five cells per plot in the *E. globulus* trial. Between five to fifteen seeds per seedlot were initially sown in a plot with one to three

seeds per cell to obtain as possible, depending on their germination capacity and growth, the final seedlings after thinning of one seedling per cell or five seedlings per plot for myrtle rust screening. The trial for *E. globulus* was carried out as randomised incomplete block design in seven batches (Batch 1 to 7) to include 680 to 3615 potential individuals and 170 to 723 plots in a batch with randomly repetition of individuals in seedlots and highly unbalanced replication of some seedlots across batches. Fifty-five seedlots with their provenances across the native range of *E. globulus* were selected as controls and included in all the batches. From the total of 20055 cells sown, total of 11350 regenerated individuals from 1237 seedlots and 1053 families were inoculated with *A. psidii* and screened for phenotypic response. Out of the 11350 individuals, 5912 from 820 seedlots and 748 families belonged to native populations and used for geographical patterns of rust susceptibility analysis in *E. globulus*.

Screening of *E. obliqua* was carried out in a single batch (Batch 8) with 818 seedlots including single mother-parent open pollinated families and multiple-parent bulks sown in the trial. The same 55 *E. globulus* control seedlots were also included in the trial and resulted in total of 873 seedlots. *E. obliqua* seeds collected from across the geographic ranges were imbibed in clean water for 28 days prior to sowing in 40-celled hyco trays with potting mixtures while the *E. globulus* seeds were imbibed as described above. A single hyco tray was designated to accommodate eight screening plots with five cells per plot or twelve plots, ten with three cells and two with five cells per plot, in this particular batch of screening trial. Between three to fifteen seeds per *E. obliqua* bulk seedlot were sown in a three-celled plot, and five to twenty-five seeds per both *E. obliqua* single mother-parent family and *E. globulus* control seedlot were sown in a five-celled plot to obtain as possible, depending on their germination capacity and growth, one seedling per cell after thinning. From the 3449 cells sown, total of 1479 regenerated individuals from 691 seedlots were inoculated with *A. psidii* and screened for phenotypic response. Out of the 1479 individuals, 1073 from 527 seedlots belonged to *E. obliqua* and used for geographical patterns analysis of rust susceptibility in the species. All the sown trays in eight bathes of screening trial were covered with a layer of vermiculite to retain moisture and avoid seed loss during irrigation. All the seedlings were irrigated twice a day and acclimated in glasshouse conditions in HVP Plantations

in Gippsland, Victoria for 10 to 12 weeks prior to dispatch to Plant Breeding Institute (PBI) in University of Sydney, Cobbitty, for rust inoculation.

Rust inoculation

E. globulus and *E. obliqua* seedlings in their respective screening batches were inoculated with urediniospores derived from a single *A. psidii* uredinium multiplied on *Syzygium jambos* in an accredited inoculation chamber in PBI using a fine mist spray on adaxial and abaxial leaf surfaces (Sandhu and Park 2013). The rust inoculum was prepared from PBI rust collection culture no. 622 with concentration of 2.0 mg urediniospores suspended in 1.0 ml of light mineral oil (Univar Solvent L Naphtha 100, Univar Australia Pty Ltd). The fine mist sprayer was attached to a motorised compressor to atomise the rust suspension during inoculation. A highly susceptible *S. jambos* with young and actively growing leaves was used as an infection control in all the trials. After inoculation, the chamber door was kept closed for 5 min to allow completely settlement of urediniospores on leaf surfaces before inoculated seedlings were transferred to a dark room for incubation under 20 °C and greater than 95% relative humidity for 24 h. After 24 h, seedlings were moved to naturally lit microclimate rooms maintained at 22 ± 2 °C for 13 days of incubation prior to disease assessment.

Phenotypic assessments

Disease severity assessment was carried out by the rust scoring method according to Sandhu and Park (2013). A disease scale was developed to measure the rust response of the inoculated plants with the following rating scale: 0 = no visible sign of infection (highly resistant); 1 = presence of mild hypersensitive reaction with highly restricted pustule, fleck and necrosis (resistant); 2 = hypersensitive reaction with restricted pustule, chlorosis and necrosis (moderately resistant); 3 = small to medium sized pustules in low frequency and may be presence of chlorosis (moderately susceptible); 4 = fully developed pustules on leaves in medium to high frequency (susceptible); 5 = abundance of fully developed pustules on leaves, twigs and buds (very susceptible). Above rating resulted in six categories of rust severity response with the respective

numeric scores from 0 to 2 considered as rust resistance and 3 to 5 considered as rust susceptible.

Data analysis

Apart from disease severity analysed as quantitative trait (based on numeric score), rust susceptibility in *E. globulus* and *E. obliqua* were also categorised in various binary traits i.e. symptomatic, hypersensitivity and pustulation for analysis. The geographic variation of rust susceptibility for *E. globulus* was examined at the pre-classified race and sub-race levels (Dutkowski and Potts 1999) with modification (Dutkowski and Potts 2012; Lopez et al. 2001) while the geographical analysis for *E. obliqua* was carried out at the forest district level. Forest districts are the major forest regions each administered by a single management group and comprise areas between 3,500 and 100,000 hectares in size (averaging 30,000 ha), typically bounded by major rivers, roads, or ridge-lines (Figure 2-1; Forestry Tasmania 2010). Statistical analysis and model fitting was carried out using available packages in R statistical software version 3.3.0 (R Core Team 2016). Packages lme4 (Bates et al. 2015) and lmerTest (Kuznetsova et al. 2016) were used to perform a linear mixed effects or generalised linear mixed effects analyses of the relationship between rust susceptibility (quantitative and binary traits) and species races (and sub-races) or forest districts and families or seedlots fitted using restricted maximum likelihood (REML) estimation. Package lsmeans (Lenth 2016) was used to calculate the least-squares means and standard errors for rust severity response in the respective locality level and Tukey-Kramer multiple range test was performed in the same package to compare the significance of the difference in rust susceptibility between races of *E. globulus* and forest districts of *E. obliqua*. Package ggmap (Kahle and Wickham 2013) was used to produce the rust severity map according to the least-squares means of numeric score and frequency of binary traits for each locality level (race or forest district) of seed origin in *E. globulus* and *E. obliqua*. Analysis of rust susceptibility in *E. globulus* was carried out by using phenotypic data generated from Batch 1 to 7 screening trials with mixed model formulated as:

$y = \mu$ + Race + Sub-race(Race) + *Family(Race*Sub-race)* + *Batch* + *Plot(Batch)* + Residuals

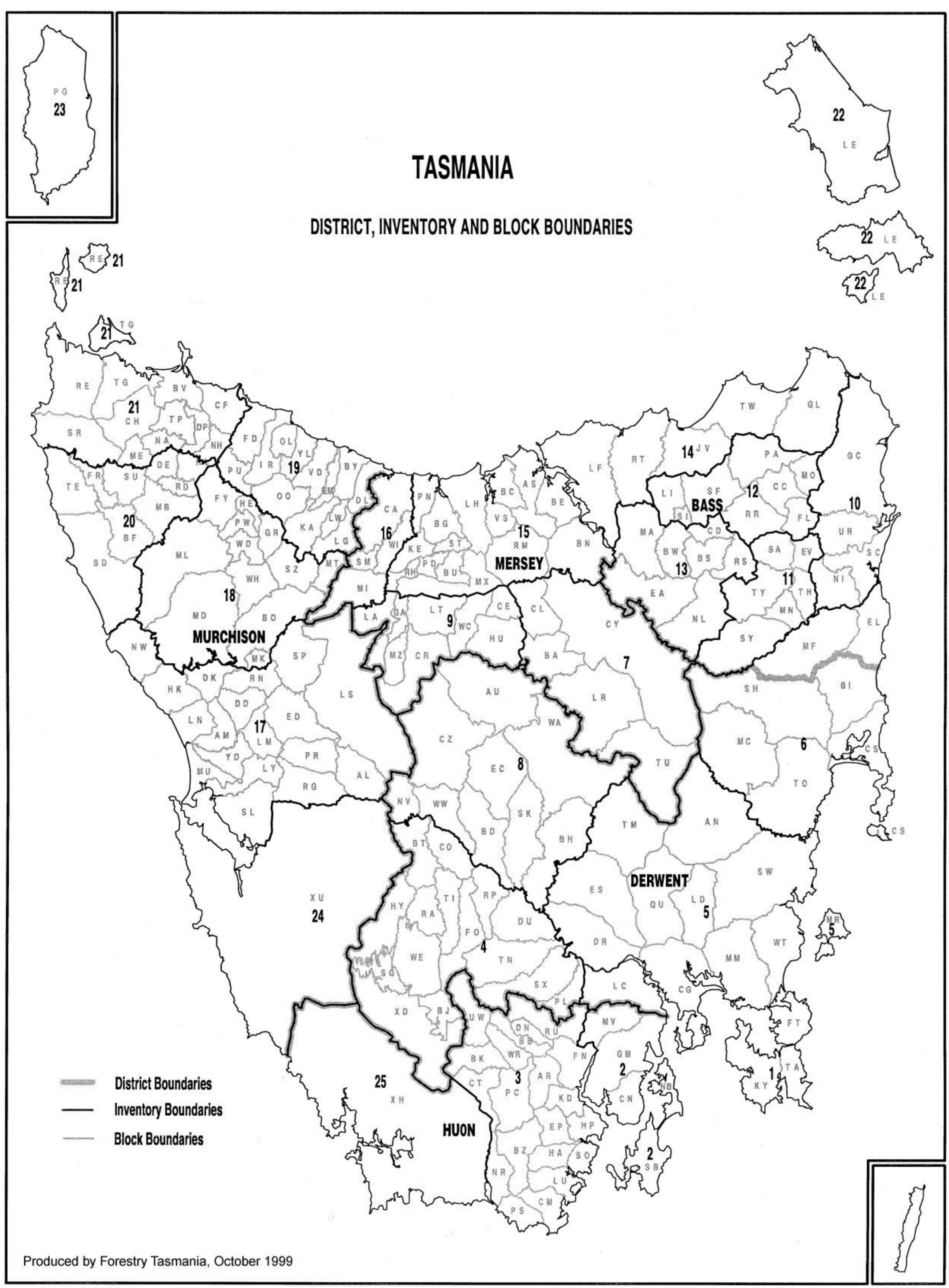

Figure 2-1: Forest district, inventory area and forest block boundaries in Tasmania (Whiteley 1999). The codes follow Forestry Tasmania (2010).

where y is the observation of rust susceptibility in numeric score or trait frequency for the seedling and μ is the overall mean. The model was fitted with race, and sub-race nested in race treated as fixed effects, intercepts for family nested in sub-race nested in race, batch, and plot nested in batch applied as random effects. The number of open pollinated families included in the analysis and their respective racial and sub-racial classifications are as listed in Table 2-1. Analysis of *E. obliqua* for rust susceptibility was performed using phenotypic data obtained from only Batch 8 screening trial with the following model:

$y = \mu + \text{District} + \textit{Seedlot(District)} + \textit{Plot} + \text{Residuals}$

with forest district treated as a fixed effect, intercepts for seedlot nested in forest district, and plot applied as random effects. The number of seedlots used in the analysis according to their family types (single mother-parent family or multiple-parent bulk) and respective forest districts is as enumerated in Table 2-2. For comparison of rust susceptibility between species, phenotypic data from Batch 8 trial consisting of 527 *E. obliqua* and 55 control *E. globulus* seedlots was analysed with the subsequent model:

$y = \mu + \text{Species} + \textit{Seedlot(Species)} + \textit{Plot} + \text{Residuals}$

with species treated as a fixed effect, intercepts for seedlot nested in species, and plot applied as random effects. *P* values were obtained by likelihood ratio tests of the full model with the effect in question against model without the respective effects for significance analyses. The effect of altitude (elevation) and climatic variables i.e. mean annual rainfall, mean maximum and minimum temperatures on rust susceptibility in both *Eucalyptus* species at their respective site of seed origin were examined using Pairwise Pearson’s correlation test in R. The climatic data for accumulated years were obtained from the Australian Bureau of Meteorology (http://www.bom.gov.au) with respect to each population locality.

Table 2-1: Number of *Eucalyptus globulus* ssp. *globulus* families used in the mixed effect analysis of myrtle rust susceptibility and their respective racial and subracial classifications according to Dutkowski and Potts (1999) with modification (Dutkowski and Potts 2012; Lopez et al. 2001)

Code [a]	Race	Sub-race	State of Country	Number of Families
1	Western Otways	Far West Otways	Victoria	10
		Western Otways	Victoria	102
2	Eastern Otways	Cape Patton	Victoria	18
		Eastern Otways	Victoria	21
		Lorne PO	Victoria	8
3	Strzelecki Ranges	Strzelecki Foothills	Victoria	4
		Strzelecki Ranges	Victoria	114
4	Southern Gippsland	Coastal Plain	Victoria	4
		Foothills	Victoria	2
5	Wilsons Promontory Lighthouse	Wilsons Promontory Lighthouse	Victoria	39
6	Furneaux	Flinders Island	Tasmania	80
		Southern Furneaux	Tasmania	75
7	North-eastern Tasmania	Inland North-eastern Tasmania	Tasmania	8
		North-eastern Tasmania	Tasmania	38
		St Helens	Tasmania	5
8	Dromedary	Dromedary	Tasmania	3
9	South-eastern Tasmania	South-eastern Tasmania	Tasmania	136
10	Southern Tasmania	Southern Tasmania	Tasmania	29
		Tasman Peninsula	Tasmania	8
11	Recherche Bay	Recherche Bay	Tasmania	4
12	Western Tasmania	Western Tasmania	Tasmania	13
13	King Island	King Island	Tasmania	27

[a] The codes follow Dutkowski and Potts (1999)

Table 2-2: Number of *Eucalyptus obliqua* seedlots according to their family types and forest districts included in the mixed effect analysis of myrtle rust susceptibility

Code	Forest District	State of Country	Number of Seedlots	
			Single Parent	Bulk
1	Bass	Tasmania	17	82
2	Huon	Tasmania	0	1
3	Derwent	Tasmania	5	72
4	Mersey	Tasmania	11	83
5	Murchison	Tasmania	3	116
6	Dandenong	Victoria	5	2
7	Broadford	Victoria	0	15
8	Benalla	Victoria	0	4
9	Mansfield	Victoria	0	2
10	Alexandra	Victoria	0	3
11	Noojee	Victoria	1	19
12	Erica	Victoria	0	22
13	Bairnsdale	Victoria	0	44
14	Nowa Nowa	Victoria	0	1
15	Orbost	Victoria	6	2
16	Cann River	Victoria	1	0
17	Bendoc	Victoria	2	2
18	South Coast	New South Wales	4	0
19	North Coast	New South Wales	0	2

Results

Response variation between E. globulus *and* E. obliqua

Responses of *E. globulus* and *E. obliqua* to *A. psidii* in the trials were highly variable and ranged from seedlings with no observable disease symptom (completely resistant) to abundance of fully developed pustules (highly susceptible) as reported in previous literatures for many other *Eucalyptus* species (Morin et al. 2011; Morin et al. 2012; Pegg et al. 2014b; Zauza et al. 2010). Distribution of individuals in both *Eucalyptus* species from different subgenera demonstrated bimodal pattern of disease severity with higher percentage of seedlings exhibiting the extreme phenotypes than the intermediate disease states (Figure 2-2). Analyses of linear mixed models for Batch 8 data fitted with the fixed species effect showed highly significant differences ($P < 0.001$) between *E. globulus* and *E. obliqua* in quantitative disease severity (Table 2-3) and binary symptomatic trait (Table 2-4) in response to *A. psidii* infection but no significant differences in binary hypersensitivity (Table 2-5) and pustulation (Table 2-6) traits between the species. Due to the earlier report of resistant traits (completely resistant or immune and hypersensitive) underlain by different genetic mechanisms (Butler et al. 2016) and preliminary finding of distinctive binary traits based on heritability estimations (unpublished), analyses of hypersensitivity and pustulation traits in both *Eucalyptus* species were carried out in exclusion of asymptomatic seedlings (completely resistant to *A. psidii*). The rust susceptibility difference between species was due to *E. obliqua* from subgenus *Eucalyptus* being more susceptible to rust infection with least-squares mean severity (2.50 ± 0.05) significantly higher than the mean (1.52 ± 0.15) of *E. globulus* from subgenus *Symphyomyrtus*. The unbalanced seedlot numbers included in the trial have also produced significant random effect ($P < 0.05$) on the analysis of rust susceptibility (quantitative and binary traits) between the *Eucalyptus* species.

Response variation between races (and sub-races) of E. globulus

There were significant difference ($P < 0.05$) in quantitative disease severity (Table 2-7) and highly significant differences ($P < 0.001$) in all the binary (symptomatic, hypersensitivity and pustulation) traits (Table 2-8 to 2-10) between *E. globulus* races in mixed model analysis of rust susceptibility with race and sub-race within race fitted as fixed effects. Western Otways was the most susceptible to rust infection with mean

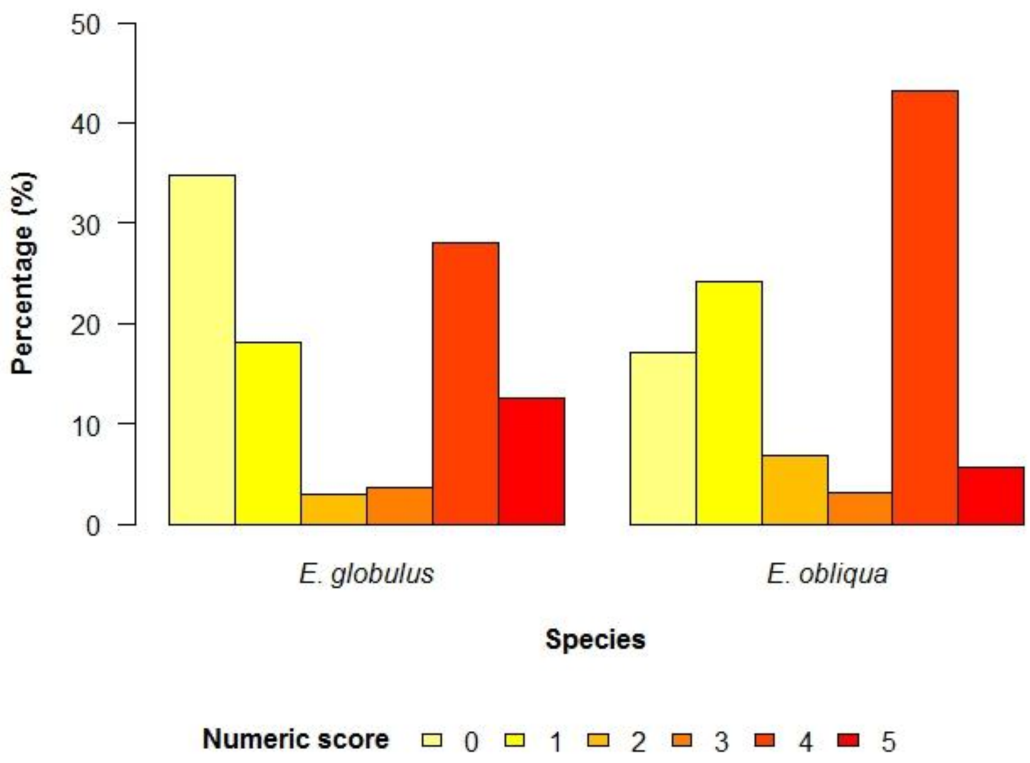

Figure 2-2: Distribution of seedlings in *Eucalyptus* species according to the percentage of rust severity response categories (numeric scores).

Table 2-3: Results of mixed models fitted for quantitative myrtle rust disease severity between *Eucalyptus globulus* and *Eucalyptus obliqua*

EFFECT	df	Mean square	F	Prob [a]
Fixed				
Species	1	85.867	36.908	< 0.001 ***
Error	1474	1.956		
			Chisq.	Prob
Random				
Seedlot			5.069	0.024 *
Plot			0.110	0.741

[a] Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table 2-4: Results of mixed models fitted for binary symptomatic response to myrtle rust between *Eucalyptus globulus* and *Eucalyptus obliqua*

EFFECT	df	Mean square	Chisq.	Prob [a]
Fixed				
Species	1	48.243	39.437	< 0.001 ***
Error	1475	0.680		
Random				
Seedlot			3.871	0.049 *
Plot			0	0.995

[a] Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table 2-5: Results of mixed models fitted for binary hypersensitive response to myrtle rust between *Eucalyptus globulus* and *Eucalyptus obliqua*

EFFECT	df	Mean square	Chisq.	Prob [a]
Fixed				
Species	1	0.157	0.149	0.700
Error	1167	1.073		
Random				
Seedlot			6.200	0.013 *
Plot			0	0.998

[a] Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table 2-6: Results of mixed models fitted for binary pustulation response to myrtle rust between *Eucalyptus globulus* and *Eucalyptus obliqua*

EFFECT	df	Mean square	Chisq.	Prob [a]
Fixed				
Species	1	1.162	1.169	0.280
Error	1167	0.769		
Random				
Seedlot			6.352	0.012 *
Plot			0	0.999

[a] Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table 2-7: Results of mixed models fitted for quantitative myrtle rust disease severity between races and sub-races in *Eucalyptus globulus*

EFFECT	df	Mean square	F	Prob [a]
Fixed				
Race	12	5.330	2.157	0.012 *
Sub-race(Race)	9	1.740	0.704	0.706
Error	5886	2.160		
			Chisq.	Prob
Random				
Family(Race*Sub-race)			302.110	< 0.001 ***
Batch			156.700	< 0.001 ***
Plot(Batch)			41.795	< 0.001 ***

[a] Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Table 2-8: Results of mixed models fitted for binary symptomatic response to myrtle rust between races and sub-races in *Eucalyptus globulus*

EFFECT	df	Mean square	Chisq.	Prob [a]
Fixed				
Race	12	4.890	55.209	< 0.001 ***
Sub-race(Race)	9	0.371	5.694	0.770
Error	5887	0.794		
Random				
Family(Race*Sub-race)			112.280	< 0.001 ***
Batch			100.230	< 0.001 ***
Plot(Batch)			41.138	< 0.001 ***

[a] Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Table 2-9: Results of mixed models fitted for binary hypersensitive response to myrtle rust between races and sub-races in *Eucalyptus globulus*

EFFECT	df	Mean square	Chisq.	Prob [a]
Fixed				
Race	12	5.529	66.344	< 0.001 ***
Sub-race(Race)	9	0.663	5.971	0.743
Error	4198	0.811		
Random				
Family(Race*Sub-race)			200.370	< 0.001 ***
Batch			50.237	< 0.001 ***
Plot(Batch)			17.713	< 0.001 ***

[a] Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table 2-10: Results of mixed models fitted for binary pustulation response to myrtle rust between races and sub-races in *Eucalyptus globulus*

EFFECT	df	Mean square	Chisq.	Prob [a]
Fixed				
Race	12	5.304	60.868	< 0.001 ***
Sub-race(Race)	9	0.753	8.030	0.531
Error	4198	0.711		
Random				
Family(Race*Sub-race)			201.040	< 0.001 ***
Batch			8.086	0.004 **
Plot(Batch)			12.460	< 0.001 ***

[a] Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

disease severity significantly greater than six other races namely Wilsons Promontory Lighthouse, Furneaux, North-eastern Tasmania, South-eastern Tasmania, Southern Tasmania and King Island (Table 2-11). Western Otways also exhibited significant difference from north- and south-eastern Tasmania in symptomatic trait (Table 2-12), and Wilsons Promontory Lighthouse and Furneaux in hypersensitivity (Table 2-13) and pustulation (Table 2-14) traits. Meanwhile, Dromedary in the present trials showed 100% of pustule development and no hypersensitive response to *A. psidii* infection, in contrast to Wilsons Promontory Lighthouse with highest hypersensitivity and lowest pustulation frequencies.

The race effects demonstrated a trend for decreased susceptibility to *A. psidii* northward from south- to north-eastern Tasmania, eastward from west to east in the Otway Ranges, and southward from Strzelecki Ranges to Wilsons Promontory Lighthouse in quantitative disease severity (Figure 2-3) and binary traits with increased hypersensitivity following the trend (Figure 2-4 to 2-6). Bass Straits islands i.e. Furneaux and King Island had susceptibility below the grand mean of total observations (2.37 ± 0.03) in contrast to Western Tasmania, Recherche Bay and Dromedary in Tasmania with mean severity greater than the grand mean in the trials. Sub-racial classification displayed no significant effect on seedling susceptibility to *A. psidii*, indicating the variation of rust response has substantially captured in the race effects. Family nested in sub-race nested in race, and other random effects produced highly significant differences ($P < 0.001$) on rust susceptibility due to highly unbalanced designed of the screening trials. Moreover, elevation had no significant effect on rust susceptibility in *E. globulus* races albeit races at higher elevation were found less susceptible to rust with reduced pustulation (Figure 2-7 to 2-10). The correlations between rust susceptibility of races and other climatic variables viz. mean annual rainfall, mean maximum and minimum temperatures of their respective site of origin were low (r = -0.1 to 0.38) and insignificant with $P > 0.1$ (Figure 2-7 to 2-10).

Response variation between forest districts of E. obliqua

Difference in quantitative disease severity between *E. obliqua* forest districts were only weakly significant ($P < 0.10$) according to mixed model analysis with district fitted as a

Table 2-11: Least-squares means (LSM) and standard errors (SE) for rust response in numeric score recorded from 13 *Eucalyptus globulus* races

Race	LSM	SE	Tukey's grouping [a]
North-eastern Tasmania	1.59	0.30	a
Southern Tasmania	1.63	0.31	a
Wilsons Promontory Lighthouse	1.75	0.31	a
King Island	1.81	0.32	a
South-eastern Tasmania	1.96	0.24	a
Furneaux	2.12	0.23	a
Southern Gippsland	2.22	0.59	ab
Eastern Otways	2.33	0.29	ab
Recherche Bay	2.42	0.56	ab
Dromedary	2.42	0.83	ab
Strzelecki Ranges	2.43	0.23	ab
Western Tasmania	2.51	0.41	ab
Western Otways	2.74	0.24	b

[a] Means with the same letter are not significantly different according to Tukey-Kramer multiple range test ($P < 0.05$)

Table 2-12: Binary symptomatic trait for rust response in trait frequency recorded from 13 *Eucalyptus globulus* races

Race	Frequency	Tukey's grouping [a]
North-eastern Tasmania	0.451	a
South-eastern Tasmania	0.461	a
King Island	0.556	abc
Southern Tasmania	0.570	ab
Dromedary	0.600	abc
Furneaux	0.666	ab
Western Tasmania	0.677	abc
Wilsons Promontory Lighthouse	0.690	abc
Southern Gippsland	0.700	abc
Recherche Bay	0.730	abc
Eastern Otways	0.787	bc
Western Otways	0.818	c
Strzelecki Ranges	0.829	bc

[a] Means with the same letter are not significantly different according to Tukey-Kramer multiple range test ($P < 0.05$)

Table 2-13: Binary hypersensitivity trait for rust response in trait frequency recorded from 13 *Eucalyptus globulus* races

Race	Frequency	Tukey's grouping [a]
Dromedary	0.000	abcd
Western Tasmania	0.138	abc
South-eastern Tasmania	0.178	ab
Western Otways	0.211	a
Recherche Bay	0.259	abcd
Strzelecki Ranges	0.279	abc
Eastern Otways	0.294	abc
North-eastern Tasmania	0.333	abcd
Southern Gippsland	0.381	abcd
Furneaux	0.404	bc
King Island	0.433	cd
Southern Tasmania	0.471	cd
Wilsons Promontory Lighthouse	0.575	d

[a] Means with the same letter are not significantly different according to Tukey-Kramer multiple range test ($P < 0.05$)

Table 2-14: Binary pustulation trait for rust response in trait frequency recorded from 13 *Eucalyptus globulus* races

Race	Frequency	Tukey's grouping [a]
Wilsons Promontory Lighthouse	0.448	a
Southern Tasmania	0.638	abc
Southern Gippsland	0.667	abc
King Island	0.683	abc
Furneaux	0.692	b
North-eastern Tasmania	0.755	abc
Eastern Otways	0.771	bc
Strzelecki Ranges	0.773	bc
Recherche Bay	0.833	abc
South-eastern Tasmania	0.846	bc
Western Otways	0.873	c
Western Tasmania	0.892	bc
Dromedary	1.000	abc

[a] Means with the same letter are not significantly different according to Tukey-Kramer multiple range test ($P < 0.05$)

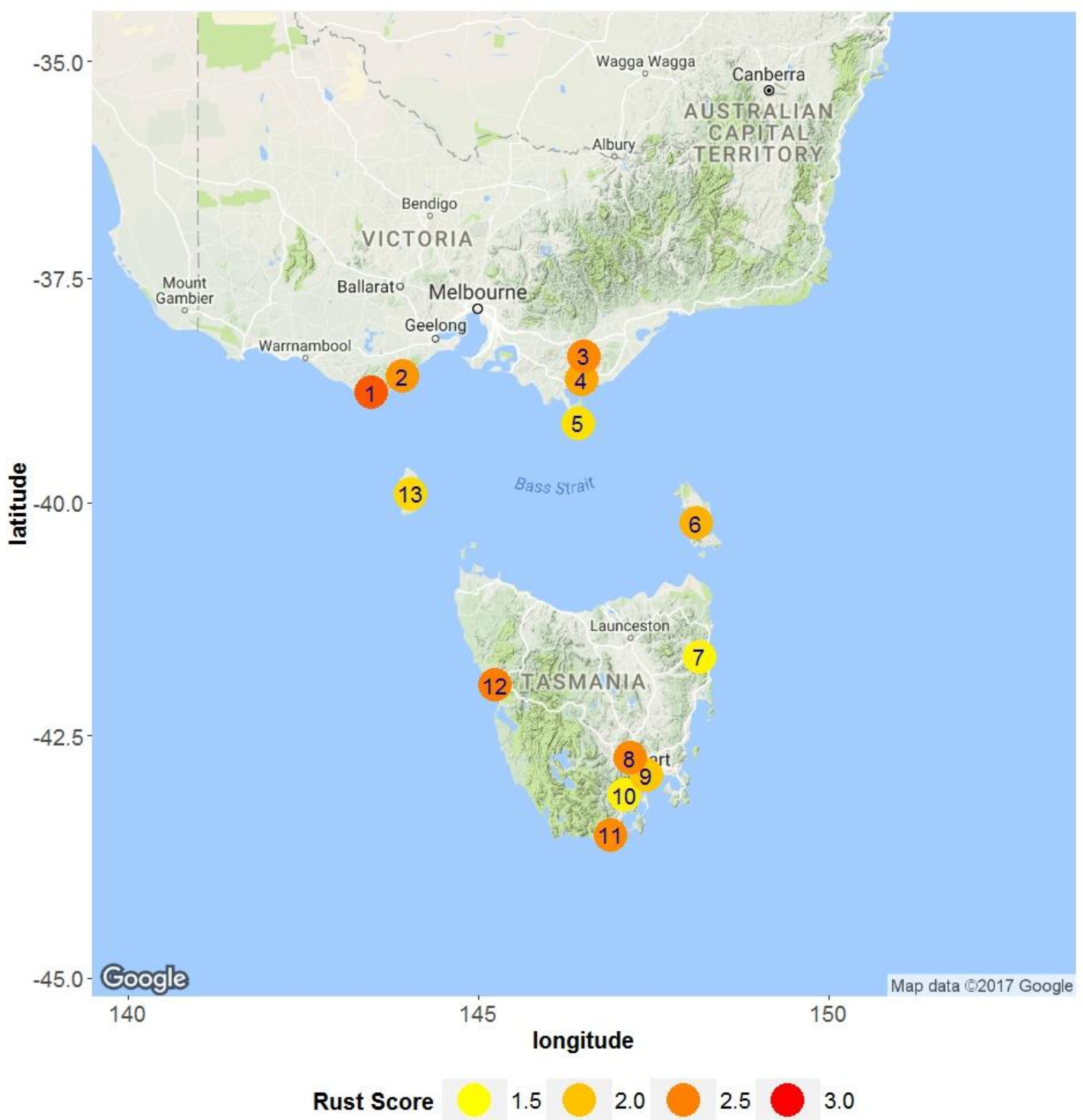

Figure 2-3: Least-squares means for rust severity between *Eucalyptus globulus* races. Numbers according to the race codes in Table 2-1.

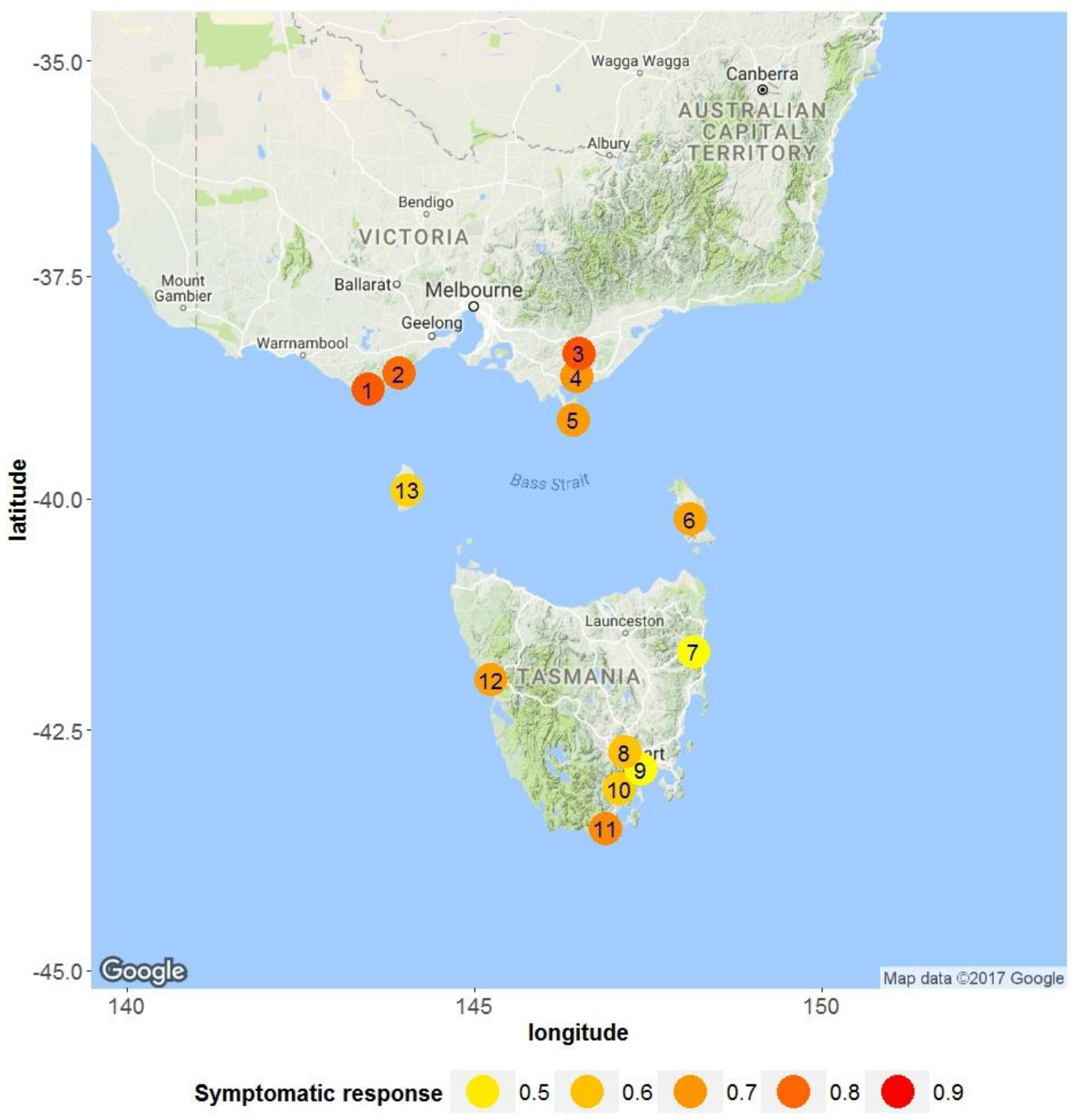

Figure 2-4: Binary symptomatic trait frequency between *Eucalyptus globulus* races. Numbers according to the race codes in Table 2-1.

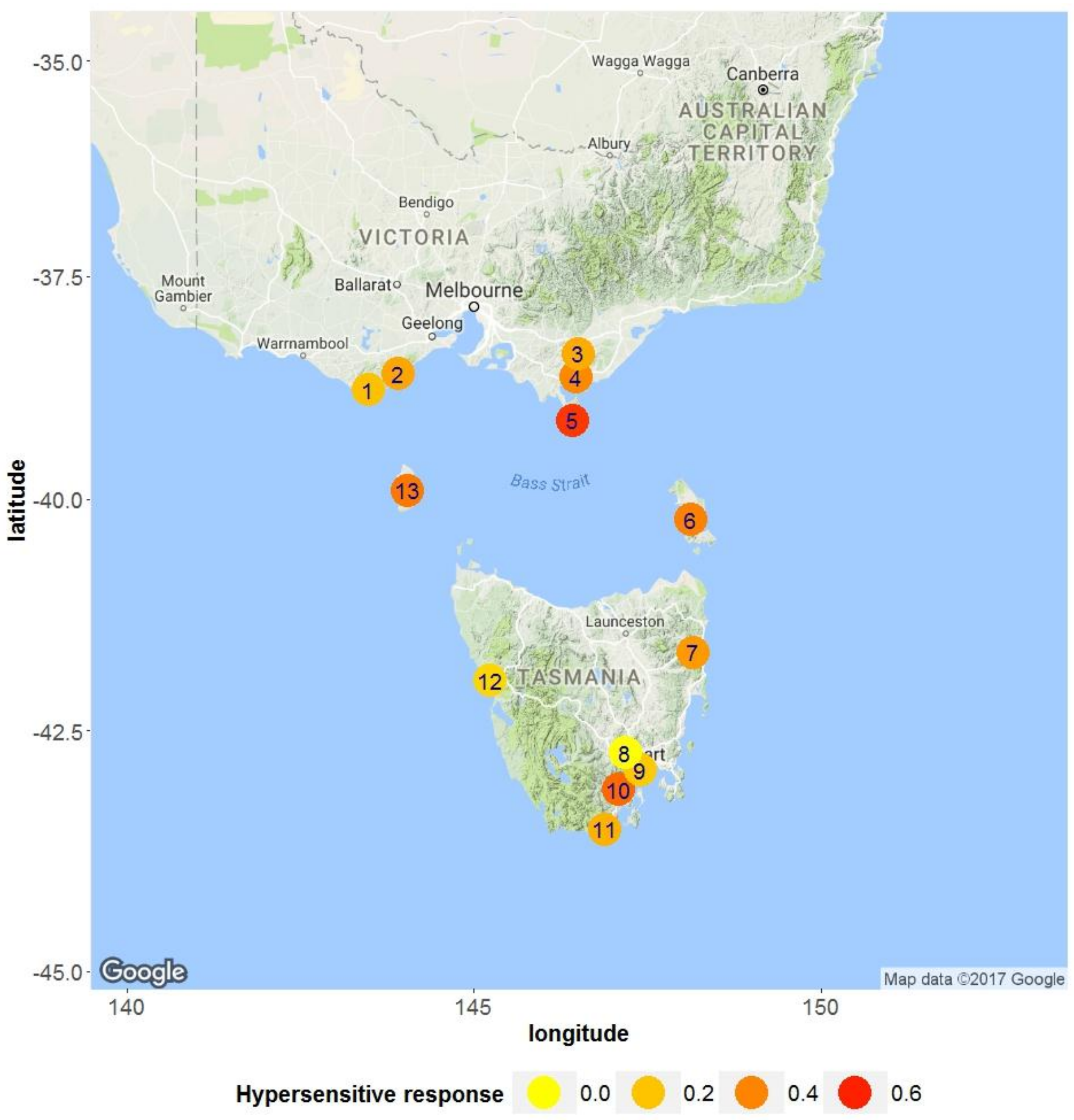

Figure 2-5: Binary hypersensitivity trait frequency between *Eucalyptus globulus* races. Numbers according to the race codes in Table 2-1.

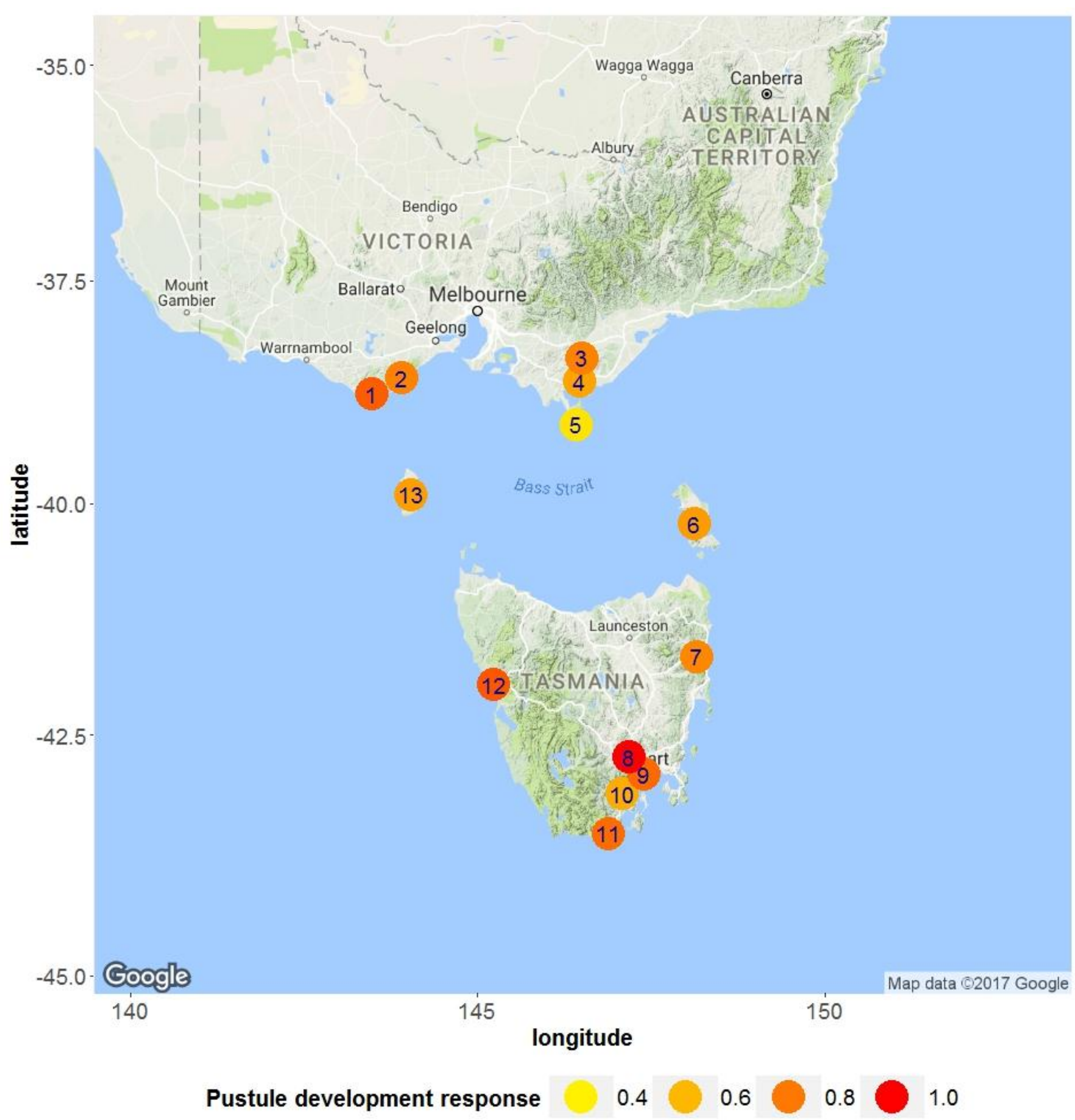

Figure 2-6: Binary pustulation trait frequency between *Eucalyptus globulus* races. Numbers according to the race codes in Table 2-1.

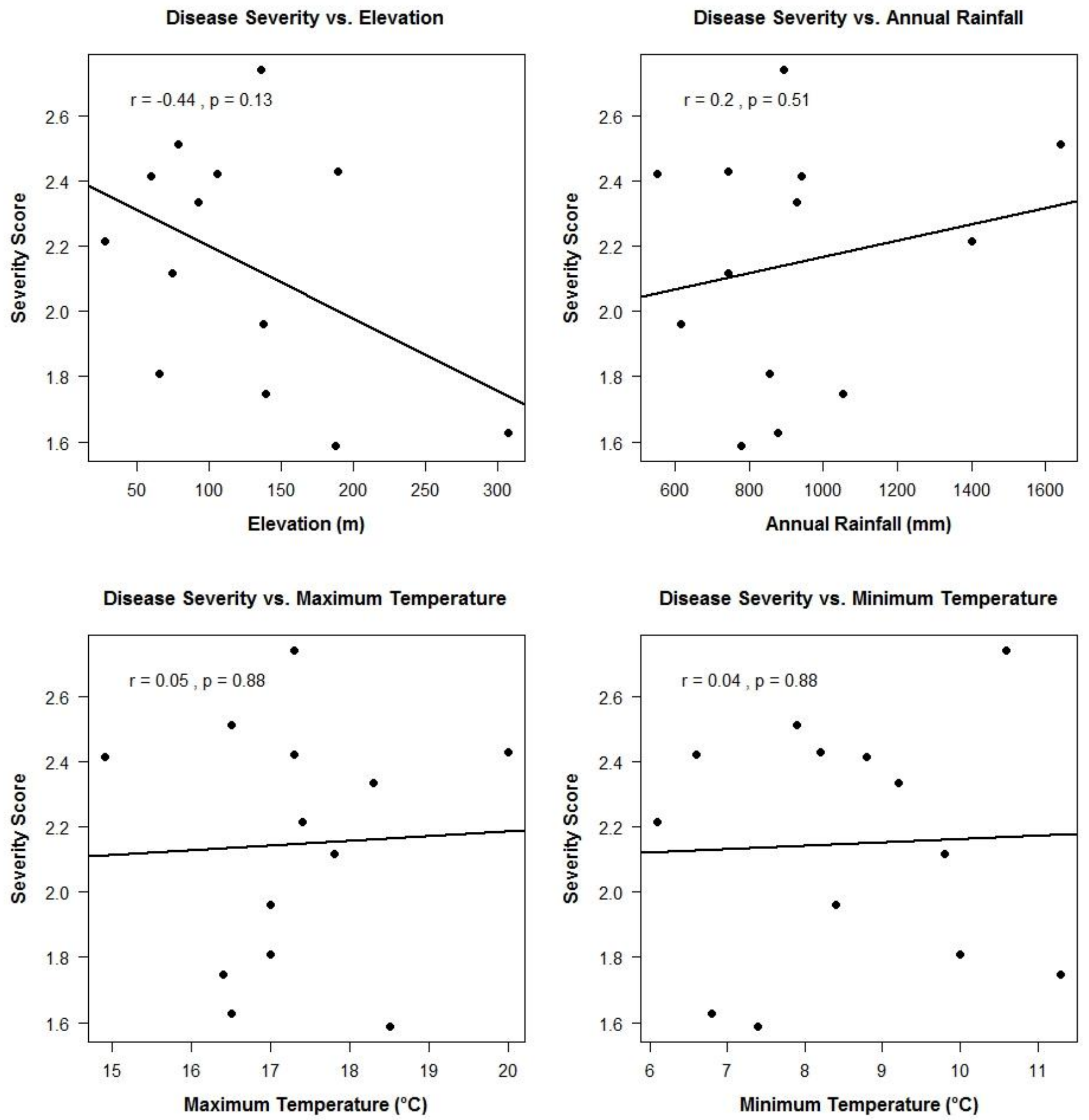

Figure 2-7: Pearson's correlations between rust disease severity of *Eucalyptus globulus* races and their respective elevation and climatic conditions.

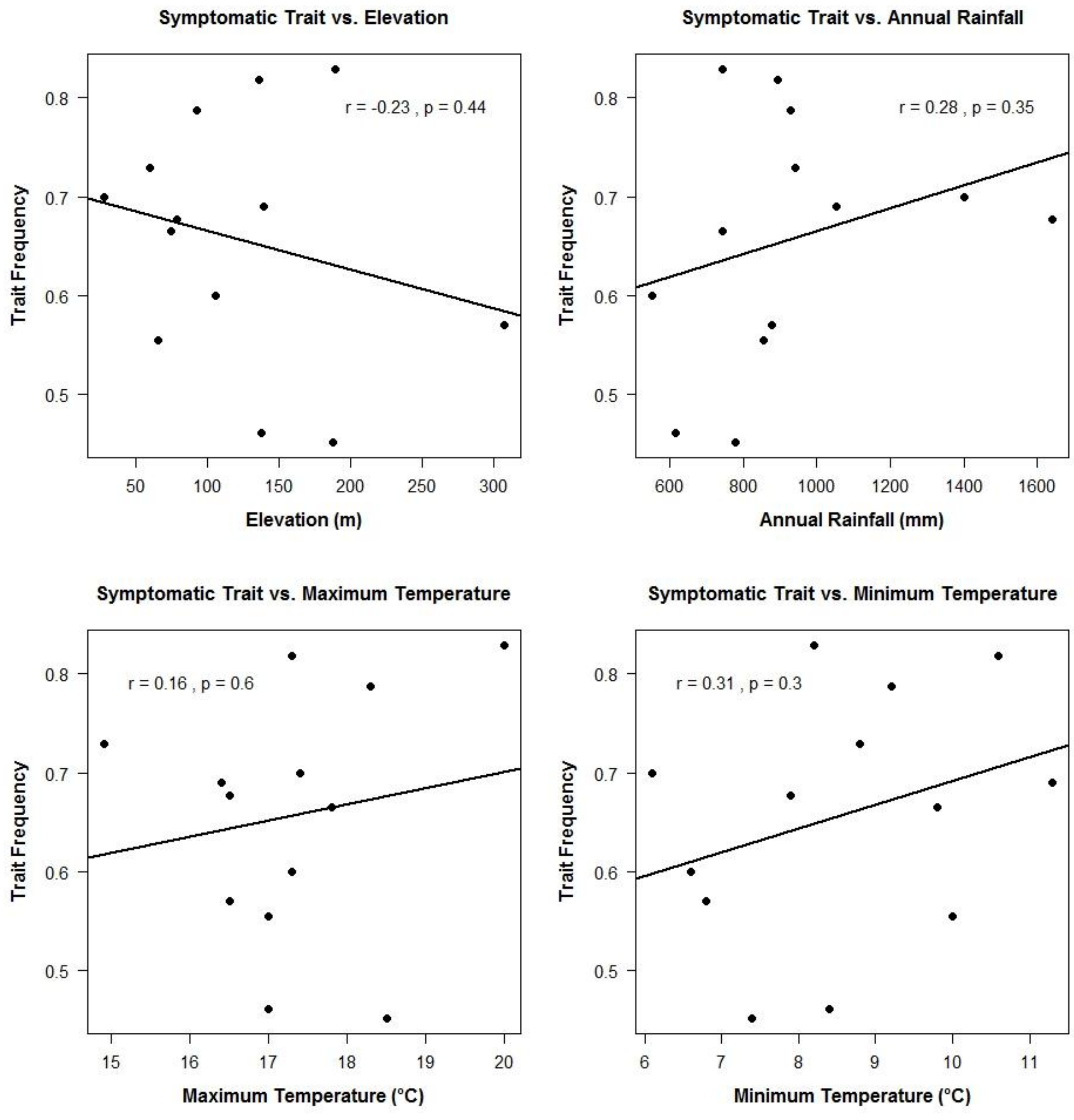

Figure 2-8: Pearson's correlations between binary rust symptomatic trait frequency of *Eucalyptus globulus* races and their respective elevation and climatic conditions.

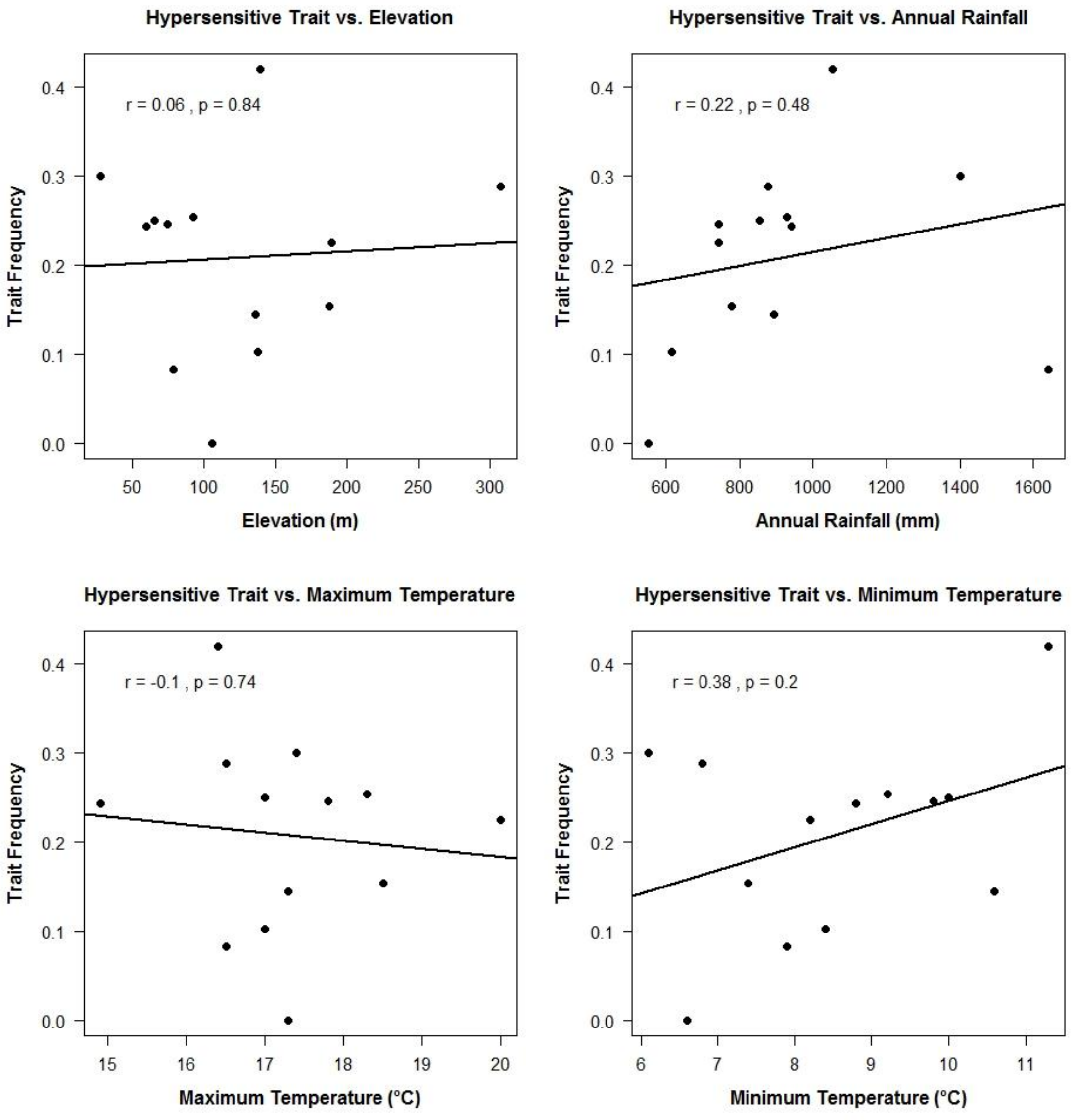

Figure 2-9: Pearson's correlations between binary rust hypersensitivity trait frequency of *Eucalyptus globulus* races and their respective elevation and climatic conditions.

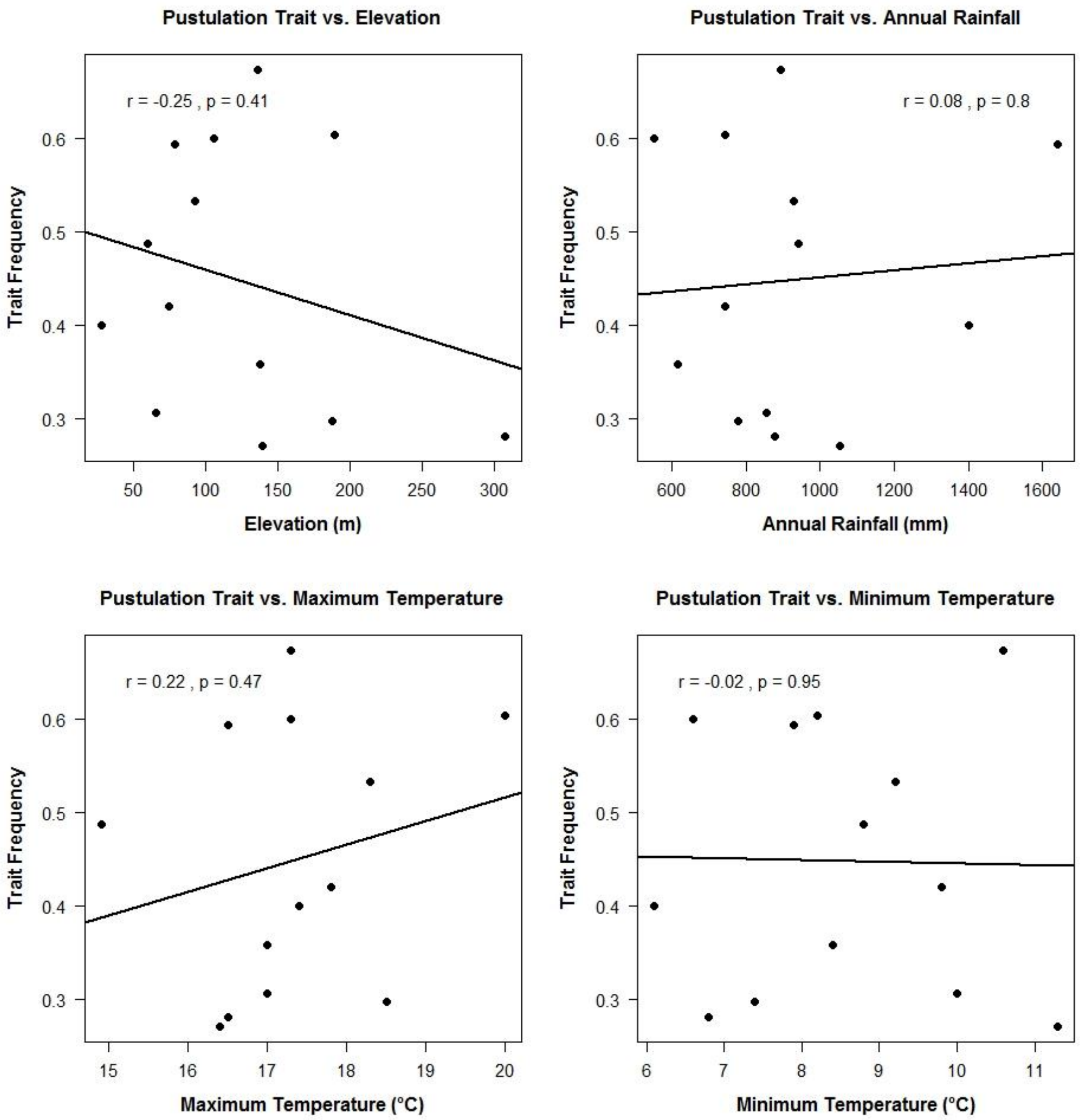

Figure 2-10: Pearson's correlations between binary rust pustulation trait frequency of *Eucalyptus globulus* races and their respective elevation and climatic conditions.

fixed effect (Table 2-15). However, analyses of binary rust susceptibility traits resulted in significant district difference ($P < 0.05$) in hypersensitivity to *A. psidii* but no significant differences in symptomatic and pustulation traits between *E. obliqua* districts (Table 2-16 to 2-18). The unbalanced seedlot numbers have produced significant random effect ($P < 0.05$) on the rust susceptibility analysis in the trial except hypersensitivity trait with reduced significance ($P < 0.1$). Despite wide variation of rust response, Tukey-Kramer multiple comparison test showed no significant different in quantitative disease severity (Table 2-19) and binary symptomatic trait (Table 2-20) between the examined districts. Differences between *E. obliqua* districts in binary hypersensitivity and pustulation traits were mainly due to the provenance effects between Murchison and Orbost (Table 2-21), and Murchison and Broadford (Table 2-22), respectively.

Analysis of least-squares mean disease severity and binary trait frequency displayed indistinct geographical pattern of rust susceptibility in *E. obliqua* districts with neighbouring provenances exhibited greater differences as evident in Nowa Nowa and Bairnsdale in contrast to other distanced provenances (Figure 2-11 to 2-14). Mansfield was the most susceptible to rust infection followed by Alexandra, Bairnsdale and Erica with increased hypersensitive response to *A. pdisii*. However, no significant correlation was observed between altitude variation and rust susceptibility in ranges of *E. obliqua* forest district apart from the binary rust symptomatic trait ($P = 0.1$) as demonstrated in Figure 2-15 to 2-18. Bairnsdale is located at low elevation (about 20 m) but had mean disease severity close to Alexandra and Erica at higher elevation (about 250 and 420 m respectively). Similarly, North Coast at relatively highest elevation (about 1150 m) had nearly lowest rust severity in contrast to Mansfield (about 320 m) and Alexandra. Based on the present analysis, *E. obliqua* forests in Tasmania had moderate rust susceptibility with least-squares mean close to the grand mean of total observations (2.47 ± 0.05). Pearson's correlation test also resulted in low (r = -0.31 to 0.37) and insignificant correlations ($P > 0.1$) between *E. obliqua* rust susceptibility and the climatic parameters viz. mean annual rainfall, mean maximum and minimum temperatures of their respective site of origin (Figure 2-15 to 2-18) except for maximum temperature effect on rust symptomatic trait ($P < 0.05$).

Table 2-15: Results of mixed models fitted for quantitative myrtle rust disease severity between forest districts in *Eucalyptus obliqua*

EFFECT	df	Mean square	F	Prob [a]
Fixed				
District	18	3.886	1.600	0.057 .
Error	1051	2.073		
			Chisq.	Prob
Random				
Seedlot			5.118	0.024 *
Plot			0	1

[a] Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Table 2-16: Results of mixed models fitted for binary symptomatic response to myrtle rust between forest districts in *Eucalyptus obliqua*

EFFECT	df	Mean square	Chisq.	Prob [a]
Fixed				
District	18	1.025	18.446	0.427
Error	1052	0.661		
Random				
Seedlot			4.274	0.039 *
Plot			0	0.998

[a] Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Table 2-17: Results of mixed models fitted for binary hypersensitive response to myrtle rust between forest districts in *Eucalyptus obliqua*

EFFECT	df	Mean square	Chisq.	Prob [a]
Fixed				
District	18	1.744	31.389	0.026 *
Error	869	1.094		
Random				
Seedlot			3.030	0.082 .
Plot			0	0.999

[a] Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Table 2-18: Results of mixed models fitted for binary pustulation response to myrtle rust between forest districts in *Eucalyptus obliqua*

EFFECT	df	Mean square	Chisq.	Prob [a]
Fixed				
District	18	1.433	25.800	0.104
Error	869	0.817		
Random				
Seedlot			4.569	0.033 *
Plot			0	0.999

[a] Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Table 2-19: Least-squares means (LSM) and standard errors (SE) for rust response in numeric score recorded from 19 *Eucalyptus obliqua* forest districts

Forest District	LSM [a]	SE
Nowa Nowa	1.00	1.71
North Coast	1.67	0.81
Broadford	1.75	0.36
Orbost	1.87	0.44
Noojee	2.16	0.28
Benalla	2.18	0.79
Mersey	2.24	0.14
Derwent	2.41	0.14
South Coast	2.46	0.54
Murchison	2.52	0.12
Bendoc	2.61	0.59
Dandenong	2.61	0.51
Huon	2.67	1.14
Cann River	2.71	0.91
Bass	2.76	0.13
Erica	2.83	0.32
Bairnsdale	3.00	0.22
Alexandra	3.77	0.89
Mansfield	4.50	1.21

[a] No significant different according to Tukey-Kramer multiple range test ($P < 0.05$)

Table 2-20: Binary symptomatic trait for rust response in trait frequency recorded from 19 *Eucalyptus obliqua* forest districts

Forest District	Frequency [a]
Huon	0.667
North Coast	0.667
Noojee	0.755
Broadford	0.769
Dandenong	0.769
Murchison	0.775
South Coast	0.800
Mersey	0.810
Derwent	0.816
Cann River	0.857
Bass	0.876
Bendoc	0.917
Bairnsdale	0.925
Orbost	0.952
Erica	0.968
Alexandra	1.000
Benalla	1.000
Mansfield	1.000
Nowa Nowa	1.000

[a] No significant different according to Tukey-Kramer multiple range test ($P < 0.05$)

Table 2-21: Binary hypersensitivity trait for rust response in trait frequency recorded from 19 *Eucalyptus obliqua* forest districts

Forest District	Frequency	Tukey's grouping [a]
Huon	0.000	ab
Mansfield	0.000	ab
Cann River	0.167	ab
Dandenong	0.200	ab
Alexandra	0.250	ab
Murchison	0.273	a
Bairnsdale	0.306	ab
Bass	0.337	ab
Bendoc	0.364	ab
Erica	0.400	ab
South Coast	0.417	ab
Derwent	0.419	ab
Noojee	0.432	ab
Mersey	0.456	ab
North Coast	0.500	ab
Benalla	0.600	ab
Broadford	0.600	ab
Orbost	0.750	b
Nowa Nowa	1.000	ab

[a] Means with the same letter are not significantly different according to Tukey-Kramer multiple range test ($P < 0.05$)

Table 2-22: Binary pustulation trait for rust response in trait frequency recorded from 19 *Eucalyptus obliqua* forest districts

Forest District	Frequency	Tukey's grouping [a]
Nowa Nowa	0.000	ab
Benalla	0.400	ab
Broadford	0.450	a
North Coast	0.500	ab
Orbost	0.600	ab
Mersey	0.698	ab
Noojee	0.730	ab
Erica	0.767	ab
Derwent	0.768	ab
Bass	0.788	ab
Dandenong	0.800	ab
Bairnsdale	0.806	ab
Bendoc	0.818	ab
Cann River	0.833	ab
South Coast	0.833	ab
Murchison	0.847	b
Alexandra	1.000	ab
Huon	1.000	ab
Mansfield	1.000	ab

[a] Means with the same letter are not significantly different according to Tukey-Kramer multiple range test ($P < 0.05$)

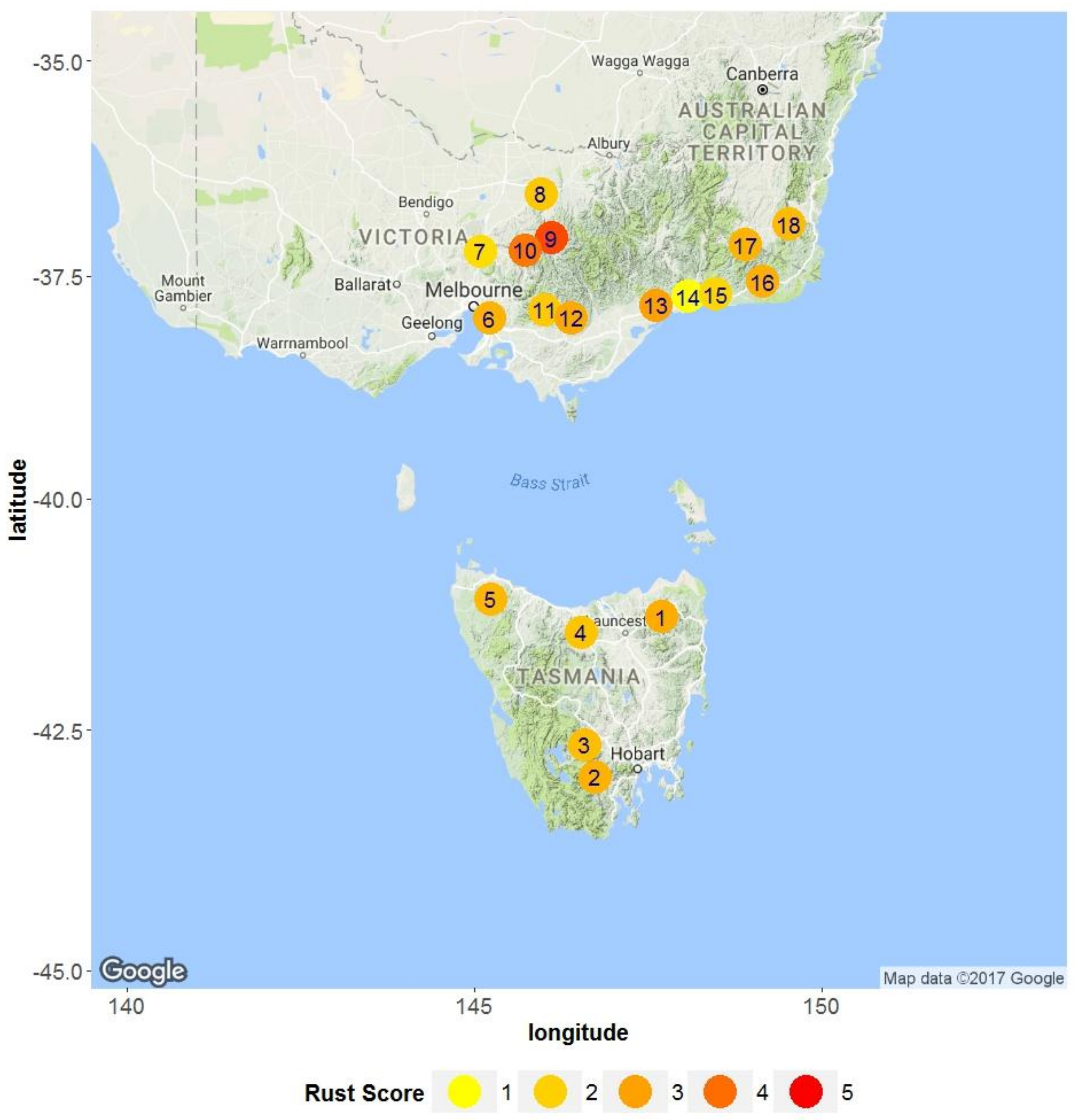

Figure 2-11: Least-squares means for rust severity between *Eucalyptus obliqua* forest districts. Numbers according to the district codes in Table 2-2 with North Coast not shown in the Figure.

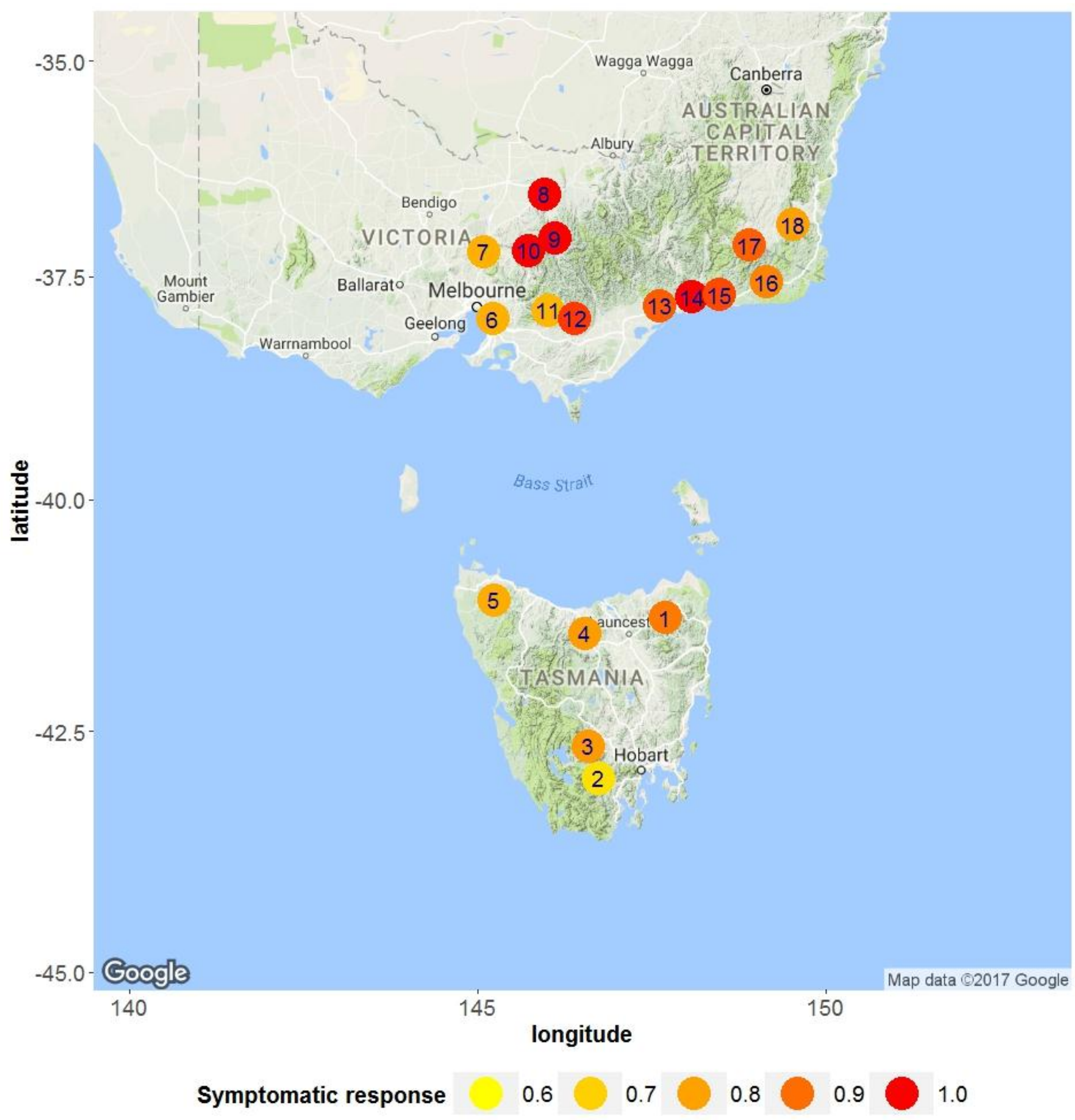

Figure 2-12: Binary symptomatic trait frequency between *Eucalyptus obliqua* forest districts. Numbers according to the district codes in Table 2-2 with North Coast not shown in the Figure.

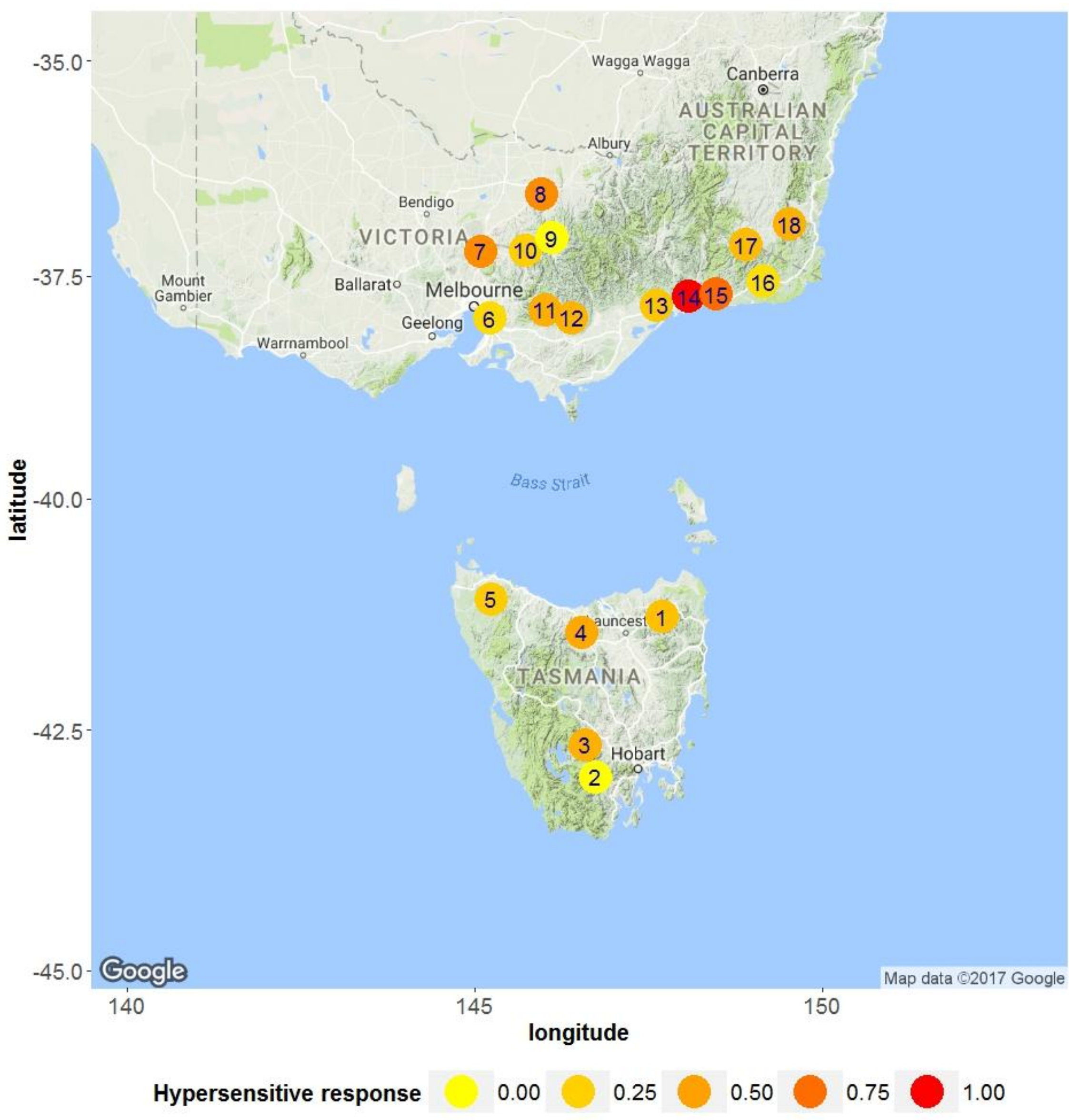

Figure 2-13: Binary hypersensitivity trait frequency between *Eucalyptus obliqua* forest districts. Numbers according to the district codes in Table 2-2 with North Coast not shown in the Figure.

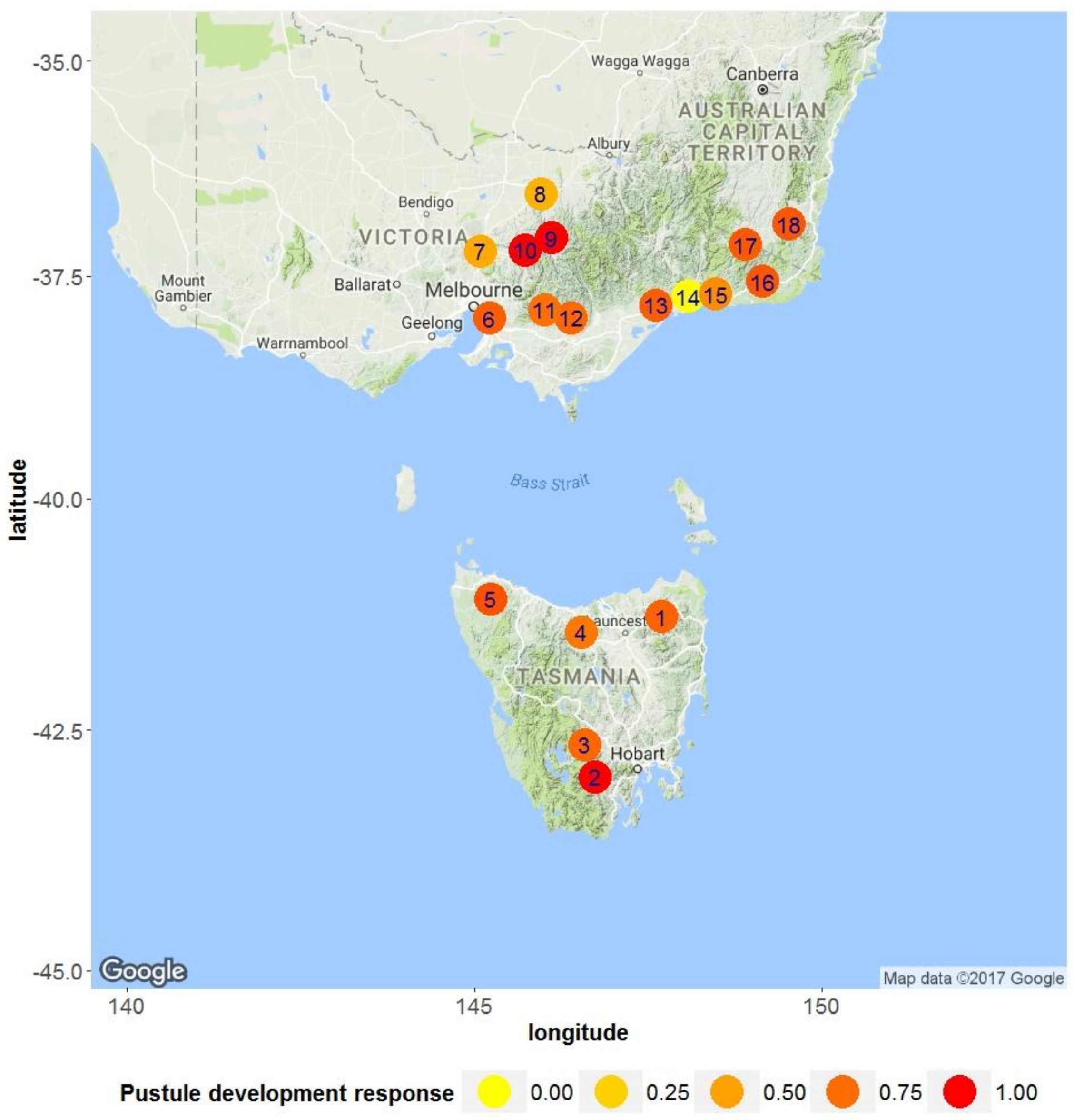

Figure 2-14: Binary pustulation trait frequency between *Eucalyptus obliqua* forest districts. Numbers according to the district codes in Table 2-2 with North Coast not shown in the Figure.

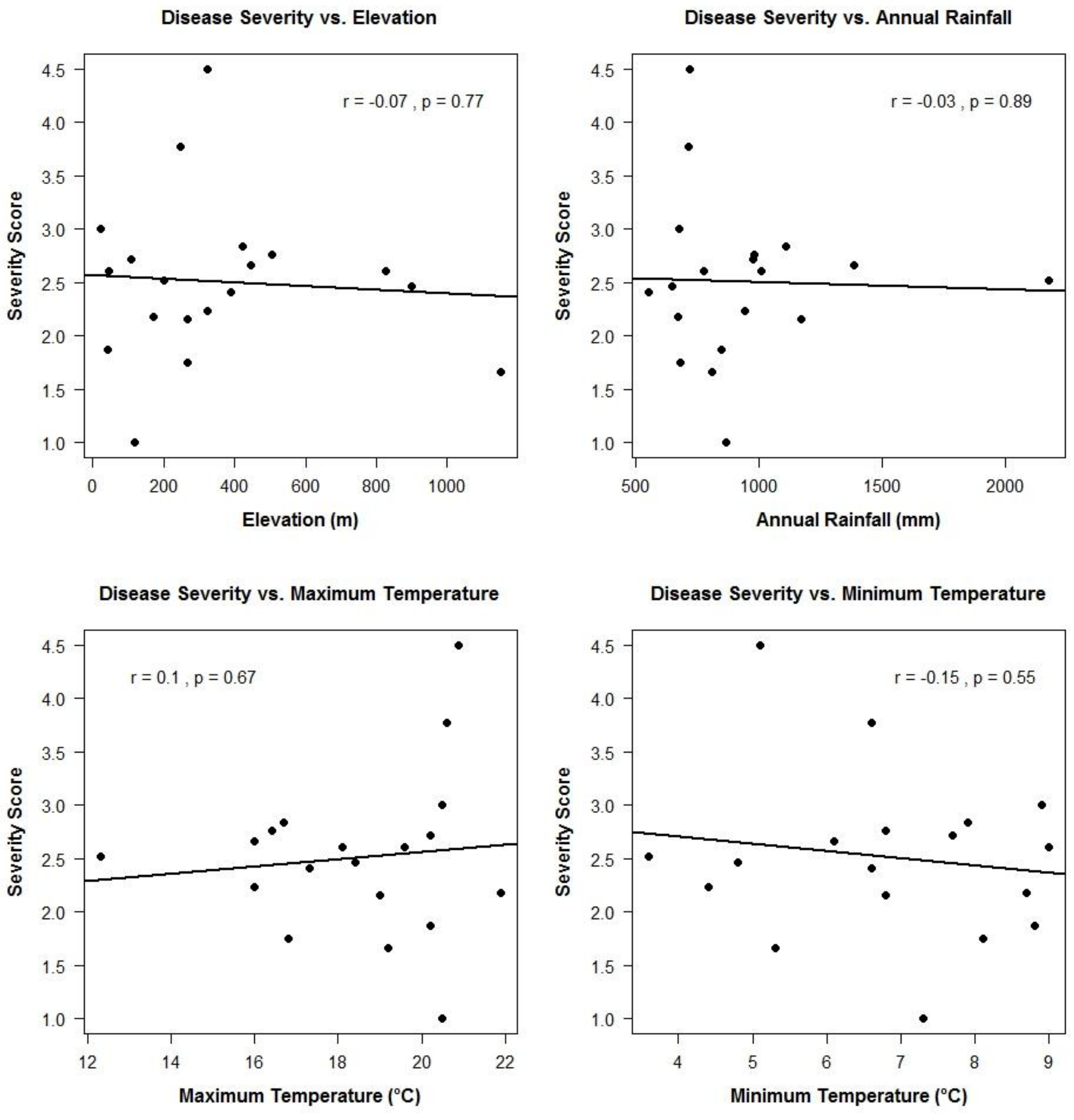

Figure 2-15: Pearson's correlations between rust disease severity of *Eucalyptus obliqua* forest districts and their respective elevation and climatic conditions.

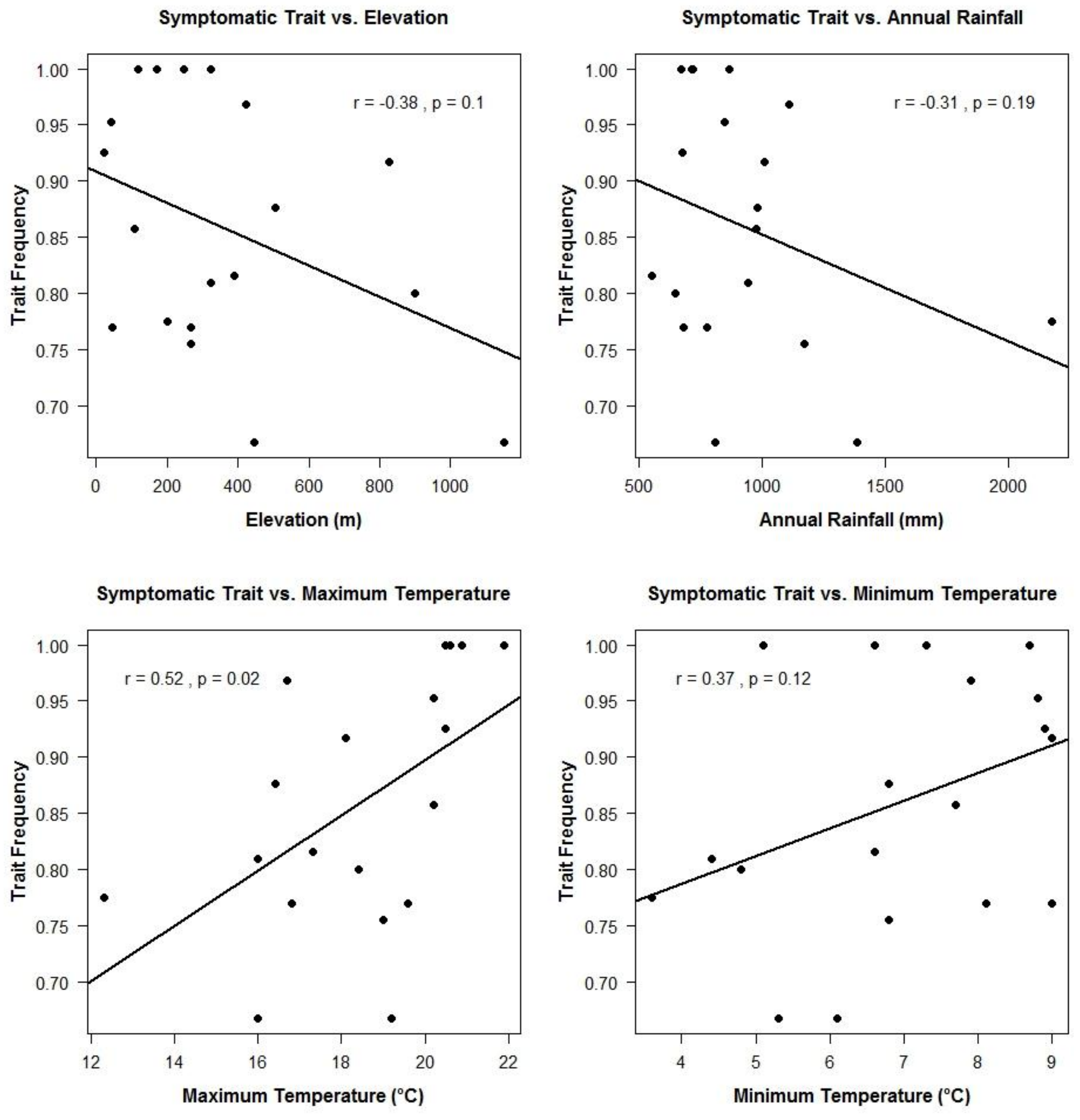

Figure 2-16: Pearson's correlations between binary rust symptomatic trait frequency of *Eucalyptus obliqua* forest districts and their respective elevation and climatic conditions.

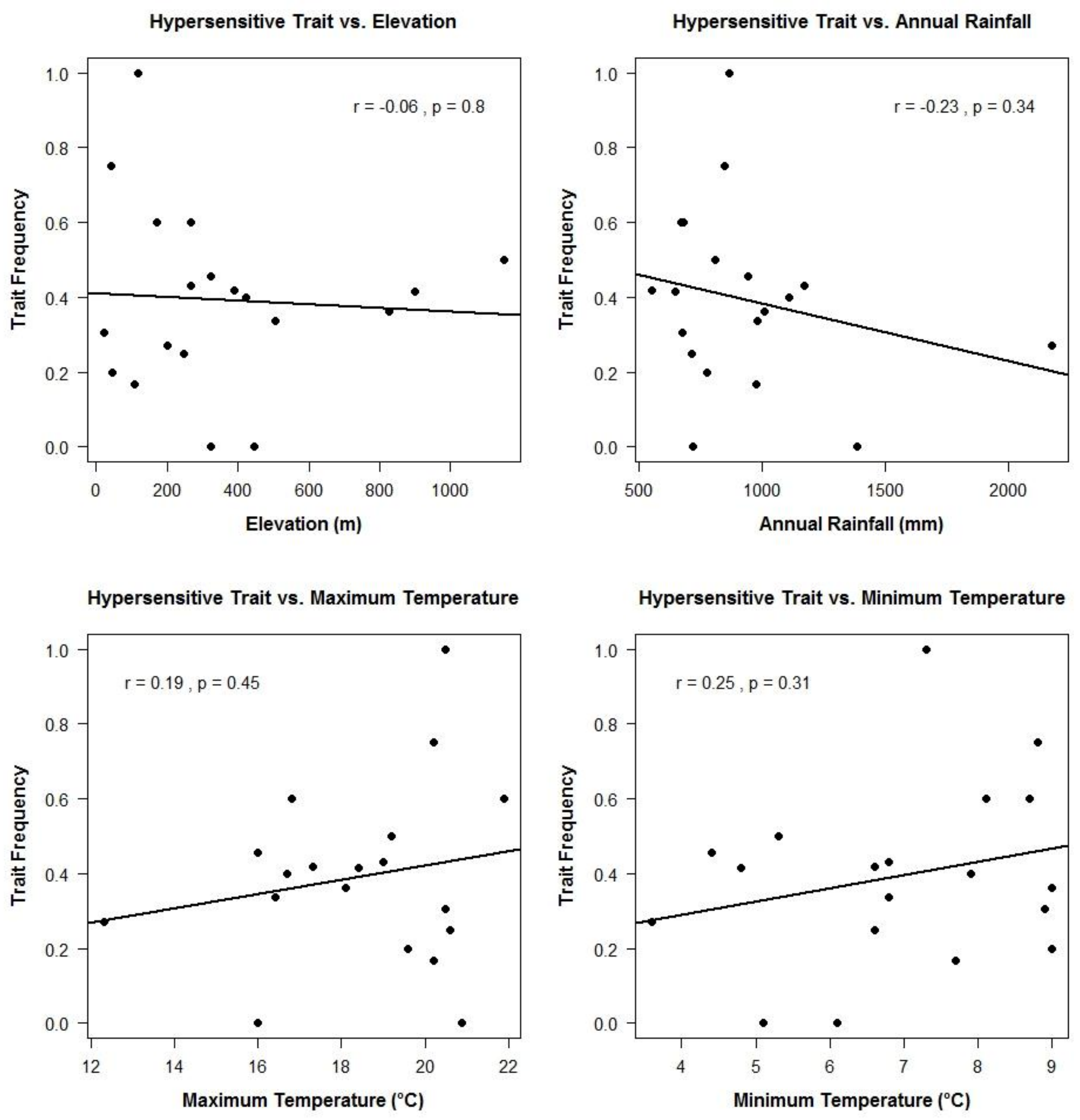

Figure 2-17: Pearson's correlations between binary rust hypersensitivity trait frequency of *Eucalyptus obliqua* forest districts and their respective elevation and climatic conditions.

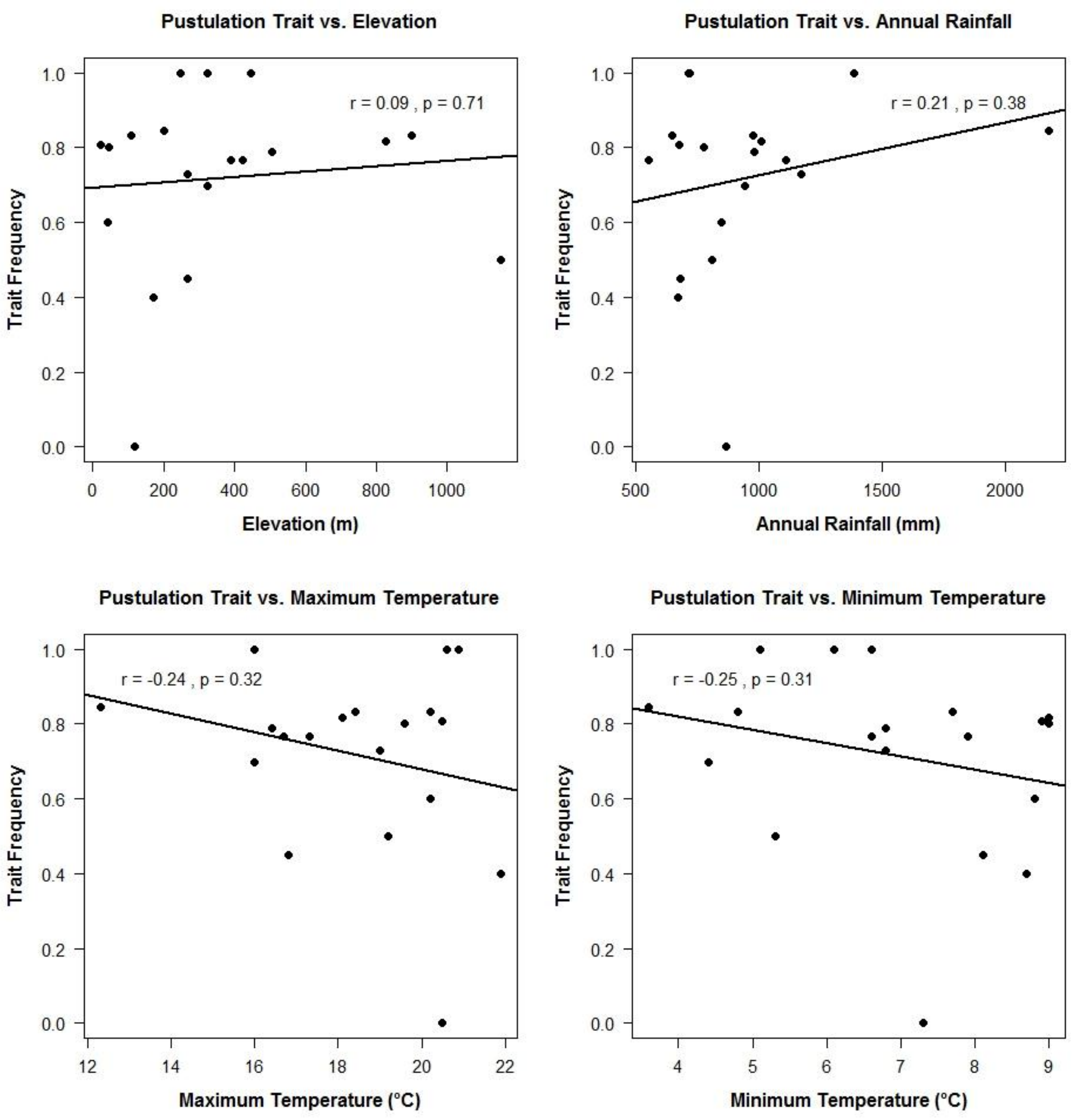

Figure 2-18: Pearson's correlations between binary rust pustulation trait frequency of *Eucalyptus obliqua* forest districts and their respective elevation and climatic conditions.

Discussion

Present analysis revealed highly significant difference in myrtle rust susceptibility (quantitative disease severity and binary symptomatic trait) between *E. globulus* and *E. obliqua* sampled over a large scale across their natural ranges (Table 2-3 & 2-4). This finding has strengthened the hypothesis of evolutionary subgeneric divergence in modelling the susceptibility difference between the two represented and most species-rich *Eucalyptus* subgenera (*Symphyomyrtus* and *Eucalyptus*) to pathogen *A. psidii* (Potts et al. 2016). The subgeneric differences between *Symphyomyrtus* and *Eucalyptus* were also reported in numerous economically and ecologically important traits including plant growth and productivity (Davidson and Reid 1980; Turnbull et al. 1993), responses to enemies and pathogens (Carnegie et al. 1998; Nichol et al. 1992; Tippett et al. 1985), adaptation to exotic environments (Anekonda et al. 1999, 2000), and foliar nutrients and chemistry (Millner and Kemp 2012; Wallis et al. 2010a). While species in *Symphyomyrtus* demonstrated faster early growth rates (Davidson and Reid 1980) and greater tolerance to climatic and edaphic conditions (Florence 1996), *Eucalyptus* species were less damaged by herbivorous insects and pathogenic fungi (Noble 1989; Stone et al. 1998). Gilbert et al. (2012) described the applicability of phylogenetic signal as an evolutionary tool for prediction of plant pathogen-host range in ecological and conservation management, in consonance with Potts et al. (2016) and present finding in proposing the *Eucalyptus* phylogenetic history as rust vulnerability predictor for biosecurity risk assessment and sustainable breeding strategies. Host specialisation by pathogens has also been delineated in previous literatures with significant implications on various disciplines viz. epidemiology, biodiversity, ecology and agroforestry (Altizer et al. 2003; Gilbert and Webb 2007; Schroth et al. 2000).

Rust susceptibility difference observed between *E. globulus* races (Table 2-7 to 2-10) and *E. obliqua* districts (Table 2-15 & 2-17) may due to genetic drift and local adaptation as the key drivers of genetic variation across their geographic locations and scales. Based on the aggregation of species occurrences, species within *Eucalyptus* display more clustered distributions in contrast to *Symphyomyrtus* species with more disjunct distributions (Williams and Potts 1996). Spatially structured variation in quantitative traits between populations may result from genetic effects diverged by local

adaptation or drift and environmental effects mediated by phenotypic plasticity, i.e. differential expression in response to environmental variation (Andrew et al. 2010). Genetic drift and natural selection are the two main evolutionary processes occurring at multiple geographic scales of *E. globulus* and give rise to differential adaptation and variations of quantitative traits in the species complex (Jones et al. 2013). Wilkinson (2008) also revealed significant genetic differentiation in quantitative traits affecting fitness of *E. obliqua* such as growth rate and disease susceptibility maintained by differential selection forces along their geographical gradients. While *Eucalyptus* species were reported less susceptible to fungal pathogens such as *Phaeoseptoria eucalypti* (Nichol et al. 1992) and *Mycosphaerella* spp. (Carnegie et al. 1998), present analysis revealed *E. obliqua* to be more vulnerable to *A. psidii* with most of the genetic variation was between individuals within the examined districts. The weak genetic structuring between *E. obliqua* districts in response to *A. psidii* (Table 2-19 & 2-20) may result from large interpopulation gene flow as described by Pegg et al. (2014a) in the variation of rust susceptibility in *Corymbia* (sister lineage) species. Varying magnitude of gene flows caused by pollinator groups such as birds and insects or physical geographic barriers such as Bass Straits and Great Dividing Range are also predicted to drive the genetic differentiation patterns in *E. globulus* (Jones et al. 2013; Yeoh et al. 2012), as hypothesised for significant rust susceptibility difference between the subspecies races. In addition, Potts et al. (2016) reported significant provenance differences in susceptibility to *A. psidii* in the rare and threatened *E. morrisbyi* species, suggesting possibly loss of resistance genes from the isolated and highly genetically differentiated population through drift.

Despite responses within populations and provenances of *E. globulus* and *E. obliqua* to rust varied from completely resistant to highly susceptible, no significant correlation between climatic conditions at the provenance origin and disease severity was detected in the present study with the exception of elevation and mean maximum temperature effects on binary rust symptomatic trait in *E. obliqua* (Figure 2-7 to 2-10 & 2-15 to 2-18). This finding is congruent with the similar pattern observed in *Corymbia* species where both the most resistant and susceptible provenances were ranked from the similar dry inland areas (Pegg et al. 2014a). On the other hands, Silva et al. (2013) reported

significant relationship between climatic conditions i.e. annual average temperature and rust development in *E. grandis* field trials, and highly genetic correlation between experimental sites with similar climatic classifications. Preliminary assessment of the high risk areas for *A. psidii* in Australia had been carried out before the arrival of pathogen and identified northern parts of the Northern Territory and eastern coastal areas of northern New South Wales and Queensland as the highest vulnerable regions subjected to the event of rust introduction (Booth et al. 2000; Glen et al. 2007). The initial spread of *A. psidii* along the east coast of Australia since April 2010 (Carnegie and Lidbetter 2012; Carnegie et al. 2010) has further confirmed the preliminary risk map produced according to Booth and Jovanovic (2012). Recent climatic niche modelling has predicted the extension of potential risk range for persistence of *A. psidii* into higher altitudes and latitudes i.e. mesic midlands of Victoria and northern extremes of Tasmania based on new data on the distribution and biology of *A. psidii* (Kriticos et al. 2013), indicating parts of the natural populations of *E. globulus* and *E. obliqua* may be at risk. Nevertheless, successful development of disease in the field required not only the conducive climatic conditions for rust infection but also many other factors such as presence of actively-growing young shoots and availability of abundant inoculum (Morin et al. 2012). Urediniospore germination and subsequent epidemiology of the disease are also affected by temperature, leaf wetness duration, relative humidity, light intensity and photoperiod (Glen et al. 2007; Silva et al. 2013) as optimised in the artificial rust inoculation protocol. Moreover, presence of different biotypes or evolution of new virulent strains of *A. psidii* may result in pathogenicity shift of the pathogen in native (Sandhu et al. 2016).

The presence of specific responses within *Eucalyptus* species and other myrtaceous naïve host to the newly encounter *A. psidii* pathogen has complicated the ‘zig-zag’ model (Jones and Dangl 2006) of plant-pathogen interactions. Tobias et al. (2016) have proposed the ‘trench warfare’ concept (Stahl et al. 1999), the recognition potential of complexed *R* gene products (Eitas and Dangl 2010) and the ‘guard’ and ‘decoy’ models (van der Hoorn and Kamoun 2008) to explain the emerging cases of specific rust resistance observed in Australian Myrtaceae. The eucalypt-like fossils found in South America (Gandolfo et al. 2011) complemented with the results of dated molecular

phylogeny (Thornhill et al. 2015) indicated possibly co-existence of eucalypt ancestors and rust pathogen or its ancestors during Eocene or Palaeocene on the ancient southern supercontinent of Gondwana. The ancient exposure between host and pathogen, and persistence of ancient *R* genes may therefore give rise to the scenario of specific resistant across and within the modern Australian myrtaceous species and their provenances (Potts et al. 2016; Tobias et al. 2016). The subgeneric differences between *Symphyomyrtus* and *Eucalyptus* in many ecologically important traits including susceptibility to rust may as well result from pre-adaptation to various biotic or abiotic stressors since the beginning of divergence (Potts et al. 2016). Alternatively, indirect pathogen recognition through similar 'invasion patterns' produced by the co-evolved pathogens and host decoy receptor proteins as the targets of effectors (van der Hoorn and Kamoun 2008) may also explain the observed levels of rust resistance in Australia. However, the constituents of common 'invasion patterns' between newly encounter and co-evolved pathogens and the common effector target across multiple (about 73 known infected) genera of Myrtaceae in the proposed model remain unknown (Tobias et al. 2016).

Studies on genetic rust resistance in *Eucalyptus* species revealed the complexity of resistance traits involving both major- and minor-effect genes (Alves et al. 2012; Junghans et al. 2003a). A major locus associated with *A. psidii* rust resistance (*Ppr1*) was preliminary identified in *E. grandis* (Junghans et al. 2003a) and positioned on the linkage group 3 of the *Eucalyptus* microsatellite reference map (Mamani et al. 2010). Bulk segregant analysis based on 40 nucleotide-binding leucine-rich repeat (NB-LRR) candidate genes on *Ppr1* locus has predicted 13 single nucleotide polymorphisms (SNPs) potentially involved in *A. psidii* rust resistance and accounted for 70% of genetic variation in resistance (Thumma et al. 2013). Further analysis of quantitative trait loci (QTLs) in *E. globulus* revealed additional four QTLs influencing multiple mechanisms for resistance to *A. psidii* (Butler et al. 2016). Two putative QTLs identified for presence or absence of disease symptoms were located on linkage group 3 (*Ppr2*) and 7 (*Ppr3*) and explained 32% of the phenotypic variance, while another two putative QTLs identified for presence or absence of a hypersensitive reaction were located on linkage group 6 (*Ppr4*) and 9 (*Ppr5*) on *E. globulus* linkage map and

accounted for 25% of the phenotypic variance. Various host defences including preformed physical and chemical barriers and induced defence mechanisms were reported underlying rust resistance in *Eucalyptus* and controlled by many loci of different effects (Butler et al. 2016; Naidoo et al. 2014; Potts et al. 2016). A study of gene expression in *E. grandis* has demonstrated up-regulation of genes involved in cellular polarisation and systematic resistance mechanisms in the resistant plants (Moon et al. 2007). Detailed investigation on *A. psidii* infection process and differential responses between resistant and susceptible hosts i.e. where does the resistance occur will be of value for further understanding of *Eucalyptus – A. psidii* pathosystem and underlying molecular mechanisms of resistance.

Conclusion

The present study reported highly significant differences in quantitative disease severity and binary symptomatic trait in response to *A. psidii* infection between *E. globulus* and *E. obliqua* representing the two important and most species-rich *Eucalyptus* subgenera. Mixed effects model analyses also revealed significant differences in rust susceptibility between provenances (races or forest districts) of both *Eucalyptus* species except for binary rust symptomatic and pustulation traits in *E. obliqua*. However, there were no significant correlations between rust susceptibility and climatic conditions at the provenance origin of both *Eucalyptus* species apart from binary rust symptomatic trait in *E. obliqua*. The findings have predicted evolutionary subgeneric divergence and multiple evolutionary forces viz. genetic drift and local adaptation as well as varying magnitude of gene flows in shaping the observed geographical patterns of susceptibility variation in native *E. globulus* and *E. obliqua* populations. Further to this, the combined effects of a number of major and minor genes and underlying molecular mechanisms for the complex *A. psidii* rust resistance trait in *Eucalyptus* will require further exploration.

Chapter 3

Infection process of *Austropuccinia psidii* on *Eucalyptus globulus* and *Eucalyptus obliqua* leaves of different rust response phenotypes

Abstract

The myrtle rust incursion has posed a serious biosecurity threat to Australia with potential damaging impacts and devastating consequences to its native flora and plant industries. The present study aims to investigate the infection process of *Austropuccinia psidii* Beenken, the myrtle rust pathogen, on *Eucalyptus globulus* Labill. and *Eucalyptus obliqua* L'Hér., the two commercially and ecologically important *Eucalyptus* species from different subgenera. The ontogeny and morphology of infection structure development of *A. psidii* on different rust response phenotypes i.e. completely resistant, hypersensitive and highly susceptible of both *Eucalyptus* species inoculated with single-uredinium-derived urediniospores were examined by scanning electron microscopy. No differences were observed in urediniospore germination, appressorium formation and germ tube length between rust response phenotypes and the growth of germ tubes had no affinity towards stomata in both *Eucalyptus* species. Histological observations indicated direct penetration of infection pegs through the leaf cuticle and no penetration beyond epidermis on rust resistant phenotype of *E. obliqua*. Similar pattern of *A. psidii* infection process between rust response phenotypes and unusual mode of host penetration suggest that *Eucalyptus* defence responses against *A. psidii* might result from non-host resistance. Moreover, no observable disease after repeated rust inoculation in previously known rust susceptible phenotype of *E. obliqua* might due to early acquisition of adult plant resistance to *A. psidii* in the 12-month-old plants. The findings of this study provide valuable insights into the interactions between hosts and pathogen in *Eucalyptus* – *A. psidii* pathosystem, which might be useful for the development of effective rust control strategies across *Eucalyptus* subgenera.

Introduction

Rust diseases caused by pathogenic fungi of the order Pucciniales (equivalent to Uredinales) are amongst the most common and notorious fungal diseases afflicting a wide range of vascular plants from ferns to advanced monocots and dicots (Helfer 2014; Kolmer et al. 2009). The rust fungi are a monophyletic group composing more than 120 genera and 6000 species in the highly diverse phylum of Basidiomycota (Aime et al. 2006; Duplessis et al. 2011; Hibbett et al. 2007). They are globally distributed and cause diseases in many economically important plant species including cereals, legumes, ornamentals, and fruit trees, and pose a serious threat to cropping systems and global food security (Garnica et al. 2014; Kolmer et al. 2009). Rust fungi are highly specialised, obligate biotrophs, obtaining nutrients from living plant tissues and dependent on their hosts for reproduction and completion of life cycles (Duplessis et al. 2011). The common pattern of a rust life cycle involves five different spore stages on two different unrelated hosts (Van Alfen 2001). This life cycle is the most complex for macrocyclic heteroecious rusts (as illustrated in Figure 1-3) for in which there is a complete suite of five distinct spore types and it requires two hosts for completion of the cycle (Kolmer et al. 2009). Rust fungi that do not produce urediniospores are referred to as demicyclic and those that do not produce urediniospores and aeciospores or with shorter life cycles composed of three or fewer spore stages are microcyclic (Carris et al. 2012; Quilliam and Shattock 2003). The life cycles that remain on a single host are called autoecious in which the rusts pass through their entire life stages on the same species or group of host plants (Kolmer et al. 2009).

The rust diseases of woody plants of economic importance include white pine blister rust caused by *Cronartium ribicola* J.C.Fisch. ex Rabenh. affecting five-needle pines (Jurgens et al. 2003; Maloy 2003), western gall rust caused by *Endocronartium harknessii* (J.P.Moore) Hirats. (or *Peridermium harknessii* for asexual name) afflicting two- and three-needle pines e.g. *Pinus radiata* D.Don (Allen and Hiratsuka 1985; Kaitera and Nuorteva 2008; Old 1981; Peterson 1971), fusiform rust resulted from *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* infection on slash and loblolly pines (Goddard et al. 1975; Kinloch and Stonecypher 1969; Schmidt 2003), poplar rust caused by *Melampsora medusae* Thüm., *M. larici-populina* Kleb. and their

interspecific hybrid *M. medusae-populina* sp. nov. on poplars and conifers (Bourassa et al. 2007; Newcombe and Chastagner 1993; Spiers and Hopcroft 1994), destructive coffee rust resulted from *Hemileia vastatrix* Berk. & Broome infection (Arneson 2000; Silva et al. 2006), soybean rust caused by *Phakopsora pachyrhizi* Syd. & P.Syd. (aggressive pathogen) and *P. meibomiae* (Arthur) Arthur (mild pathogen) affecting soybean and legumes including woody shrub *Erythrina herbacea* L. (Gevens et al. 2008; Goellner et al. 2010; Rupe and Sconyers 2008), and devastating cedar-apple rust caused by *Gymnosporangium juniperi-virginianae* Schwein. on juniper and rosaceous species (Aldwinckle 1975; Biggs et al. 2009; Korban et al. 1987).

The current understandings of rusts are, however, mostly derived from studies of species infecting cereals such as the three rust diseases i.e. stem rust caused by *Puccinia graminis* f. sp. *tritici* Pers. (Schumann and Leonard 2000; Wanyera et al. 2006), leaf rust caused by *P. triticina* Eriks. (Bolton et al. 2008) and stripe rust caused by *P. striiformis* f. sp. *tritici* Eriks. on wheat (Chen et al. 2014) for the obvious reason of their critical importance for food supply. The concept of *forma specialis* (f. sp.) was first introduced by Eriksson (1894) and its biological level is used to differentiate the genetically distinguishable (physiologically distinct but morphologically indistinguishable) race or biotype of a species of pathogen that can infect primarily a particular host or a host of a defined genotype/phenotype (Bettgenhaeuser et al. 2014; Rédei 2008).

Some of these rust fungi cause premature defoliation which reduces the photosynthetic capacity of the plants (Arneson 2000; Rupe and Sconyers 2008), while the others cause decreased productivity (Bolton et al. 2008; Chen et al. 2014), fruit infection (Biggs et al. 2009), formation of swollen canker (Maloy 2003) and death of infected tissues (Kolmer et al. 2009), leading to significant economic losses and potential food security threats.

In the recent years, incursion of myrtle rust caused by *Austropuccinia psidii* Beenken (Beenken 2017), which reported previously as *Puccinia psidii* Winter sensu lato (Winter 1884) or *Uredo rangelii* J.A.Simpson, K.Thomas & C.A.Grgurinovic (Simpson et al. 2006), in Australia (Carnegie and Cooper 2011; Carnegie et al. 2010) has posed a

serious biosecurity threat to the country with its biodiversity and industries (e.g. forestry, essential oils, nursery and cut flower) reliance on Myrtaceae (Carnegie et al. 2016). The pathogen was first described from common guava (*Psidium guajava* L.) in Brazil (Winter 1884) and is believed to be native to South and Central America (Simpson et al. 2006). It gained notoriety and became particularly prominent in scientific literature following the severe damage on exotic *Eucalyptus* species in Brazil (Ferreira 1989; Joffily 1944). *A. psidii* has rapidly expanded its global distributional range over the past decades from Florida (Marlatt and Kimbrough 1979), Hawaii (Uchida et al. 2006) and California (Zambino and Nolan 2011) in the United States to Japan (Kawanishi et al. 2009), Australia (Carnegie et al. 2010), China (Zhuang and Wei 2011), South Africa (Roux et al. 2013), New Caledonia (Giblin 2013), and lately to Indonesia (McTaggart et al. 2016a), Singapore (du Plessis et al. 2017) and New Zealand (Cavill 2017).

The pathogen was identified for the first time in Australia in April 2010 on Myrtaceae of three different genera viz. *Agonis flexuosa* (Willd.) Sweet cv. 'Afterdark', *Callistemon viminalis* (Sol. ex Gaertn.) G.Don and *Syncarpia glomulifera* (Sm.) Nied. in a cut flower property on the central coast of New South Wales (Carnegie et al. 2010). Since its first detection, the rust has spread rapidly throughout natural ecosystems along the east coast from New South Wales to north Queensland (Carnegie and Lidbetter 2012; Pegg et al. 2014b) and became established, although to a limited extent, in Victoria, Tasmania and the Northern Territory (Carnegie et al. 2016).

The host range of a rust fungus is the collection of plant species that can be colonised by the pathogen for completion of its life cycle in natural habitat (Bettgenhaeuser et al. 2014; Schulze-Lefert and Panstruga 2011). Some rust species are highly specialised and can infect only a few host species in one or two genera (narrow host range) while the others are able to parasitise considerable number of hosts which can belong to different plant genera (broad host range). *A. psidii* has a wide host range with reports globally from over 450 species in 73 genera of Myrtaceae infected both natural infection and artificial inoculation (Carnegie and Lidbetter 2012; Giblin and Carnegie 2014; Morin et al. 2012; Pegg et al. 2014b), being one of the limited rust fungi (such as *P. graminis* and

P. pachyrhizi) that parasitise on numerous host genera (Bettgenhaeuser et al. 2014; McTaggart et al. 2016a).

The pathogen is referred to as hemicyclic fungi or hemiform (Petersen 1974) for which only three spore stages viz. urediniospores, teliospores and basidiospores are known. The rust may be heteroecious with an undiscovered alternate aecial host or their basidiospores are infrequently functional with loss of infection capability (Morin et al. 2014). The arrival of *A. psidii* in Australia with its enormous diversity of Myrtaceae has resulted in exposure of a large number of susceptible, naïve hosts to the pathogen, and rapid expansion of its host range (Carnegie et al. 2016). The pathogen had been reported earlier to infect and complete its life cycle on *Eucalyptus agglomerata* Maiden, *E. cloeziana* F.Muell., *E. grandis* W.Hill ex Maiden and *E. pilularis* Smith, the key wood production species in eastern Australia (Carnegie and Lidbetter 2012). There are now 232 including 18 exotic species of known *A. psidii* hosts due to natural infection and 115 hosts recorded from artificial inoculation in Australia since its first detection (Carnegie et al. 2016; Giblin and Carnegie 2014), with rust responses within host species ranging from completely resistant to highly susceptible (Morin et al. 2012; Pegg et al. 2014b; Sandhu and Park 2013). The wide and expanding host range of this invasive pathogen has resulted in significant impacts on commercial production of plantation forestry globally and potential threats to native plant communities and associated ecosystems in Australia dominated by Myrtaceae (Carnegie et al. 2016; McTaggart et al. 2016a; Roux et al. 2013).

The aim of this study is to determine at which point is resistance expressed in the pre- and post-penetration phases of the infection between *A. psidii* and two commercially and ecologically important *Eucalyptus* species viz. *Eucalyptus globulus* Labill. ssp. *globulus* (hereafter referred to as *E. globulus*) and *Eucalyptus obliqua* L'Hér. via scanning electron microscopy (SEM) and histopathology. The specific aims are to describe the ontogeny and compare the morphology of infection structures on both host genotypes with known different rust response phenotypes i.e. completely resistant, hypersensitive and highly susceptible, and to identify the modes of resistance and/or constraints to disease initiation in resistant phenotypes. The outcome of this study will

enhance our fundamental understanding of the interactions between host and pathogen in the *Eucalyptus* – *A. psidii* pathosystem, and the results generated might be useful for the development of new and more effective disease control strategies in *Eucalyptus*-related industries and provide more precisely defined phenotypic classes for genetic association studies.

Materials and Methods

Plant material

One hundred and twenty-two *E. globulus* and 87 *E. obliqua* seedlings comprising known *A. psidii* resistant, hypersensitive and susceptible phenotypic classes were selected from the Batch 8 rust phenotypic screening trial (as described in Chapter 2) and reserved for microscopic study and phytochemical analysis (as reported in Chapter 4). Seedlings from the previous rust inoculation studies were thoroughly flushed to remove any possible rust spores on their leaves, and severely infected twigs and branches cut to allow for regeneration and new shoot growth. The treated seedlings were kept in naturally lit microclimate rooms maintained at 22 ± 2 °C in the Plant Breeding Institute (PBI) of the University of Sydney, Cobbitty for 8 weeks prior to second inoculation and leaf sampling. From the total reserved seedlings, 38 *E. globulus* consisting of 16 rust resistant, 11 hypersensitive (with fleck, necrosis or chlorosis) and 11 susceptible phenotypes, and 30 *E. obliqua* consisting of 11 resistant, 9 hypersensitive and 10 susceptible phenotypes were randomly sampled for microscopic study. Carborundum powder (silicon carbide, 400 mesh particle size, Sigma) was sprinkled uniformly and thinly on one of the leaves (adaxial and abaxial surfaces) of all the known resistant seedlings and rubbed gently according to Dijkstra and de Jager (1998) to create artificial wounds on their cuticle prior to rust inoculation to enable fungal passage through the physical barrier into the plant cells.

Fungal isolate and preparation of inoculum

A standard culture of *A. psidii* from single pustule increased isolate (Au_3) with accession number 115012 was used for all the germplasm testing in PBI (Sandhu and Park 2013). The isolate was collected from infected *A. flexuosa* in a street of Leonay, New South Wales in 2011 and multiplied on highly susceptible host plant *Syzygium*

jambos L. (Alston) for increasing rust inoculum. The increased isolates were preserved in liquid nitrogen and maintained as standard culture (PBI collection rust culture no. 622) for all the artificial rust inoculation in PBI. The rust inoculum was prepared with concentration of 2.0 mg urediniospores suspended in 1.0 ml of light mineral oil (Univar Solvent L Naphtha 100, Univar Australia Pty Ltd) and 0.05% of Tween 20 (Sigma) as a surfactant prior to inoculation.

Rust inoculation and incubation

E. globulus and *E. obliqua* seedlings were inoculated by spraying with a fine mist of prepared inoculum containing fresh urediniospores of *A. psidii* on their adaxial and abaxial leaf surfaces (Sandhu and Park 2013). The fine mist sprayer was attached to a motorised compressor to atomise the rust suspension in an accredited inoculation chamber. A highly susceptible *S. jambos* with young and actively growing leaves was used as susceptible control in the inoculation. The chamber door was kept closed after inoculation for 5 min to allow completely settlement of urediniospores on the leaf surfaces. The inoculated seedlings were then transferred to a dark room for incubation under 20 °C and greater than 95% relative humidity. Following 24 h of incubation, seedlings were returned to the microclimate rooms for time serial leaf sampling.

Time series sampling and fixation

The inoculated seedlings were sampled according to their respective known phenotypes (obtained from rust phenotypic assessment as described in Chapter 2) over defined time intervals at 6, 12, 24, 48, 72, 96 and 120 hours after inoculation (h.a.i.). Three replicates of a young whole leaf (any but not all of the first expanded four leaves from the tip, and not the new leaves that expanded after rust inoculation) were collected randomly from each phenotype but different seedlings at each time point and fixed immediately in FAA fixative composed of 10% formalin, 5% acetic acid, 50% ethanol and 35% sterile water. The collected samples were then transported to the University of Melbourne, Parkville for microscopic study. The inoculated seedlings were retained in the microclimate rooms until 14 days after inoculation (d.a.i.) for a second disease assessment to confirm their phenotypes and only leaf samples collected from the confirmed rust response phenotypes were used in microscopic study. Carborundum powder-dusted leaves were

examined for the potential role of leaf cuticle as preformed barrier in *A. psidii* resistance.

Scanning electron microscopy

Leaf pieces 36-mm^2 (6 × 6 mm) in size were excised from the FAA fixed samples (two pieces per sample for observation of adaxial and abaxial surfaces) and rinsed three times in water for 30 min each, before being dehydrated in increasing concentrations (10, 30, 50, 70, 90 and twice of 100%) of anhydrous ethanol for 30 min each. The dehydrated leaf samples were dried in a Balzers CPD 010 critical point dryer and mounted onto 12.5 mm aluminium stubs with double-sided carbon tabs. The dried samples were then gold-coated in a Dynavac Xenosput sputter coater and observed using a Fei Quanta cryo SEM operating at 10.0 kV and working distance of 10.0 mm. SEM images were captured under an Everhart Thornley detector (ETD) controlled by xT microscope Server/Control software (version 2.4; Fei Company, Hillsboro, Oregon, U.S.A.).

Rust re-inoculation and sampling for histopathology

The previously sampled 38 *E. globulus* and 30 *E. obliqua* were reserved for a third rust inoculation after the seedlings were thoroughly flushed and heavily pruned to remove any possible rust, and maintained in the microclimate rooms for regrowth. Following 6 months of cultivation this last rust inoculation was performed on the regrown plants according to the procedures described earlier and three replicates of actively growing young leaf were collected from resistant and susceptible phenotypes at 8, 9 and 10 d.a.i. for both *Eucalyptus* species. The collected samples were then fixed in FAA fixative prior to transport from PBI to the University of Melbourne for histopathology study. Again the inoculated plants were kept in the same microclimate rooms until 14 d.a.i. for repeated disease assessment and phenotypes confirmation.

Tissue processing for histopathology

Sample preparation for histopathology was carried out according to the procedures described by Bhuiyan et al. (2015). Leaf tissues of 1-cm^2 were cut from FAA fixed samples and dehydrated in an ethanol series (20, 40, 60, 80 and twice of 100% for 5 min each) before being immersed in xylene and paraffin (1:1) at 60 °C for 6 h followed by

immersion in 100% paraffin at 60 °C for overnight. The tissues were embedded in paraffin blocks (one tissue per block) on a Leica EG1150 H paraffin embedding station by using Leica Parafree stainless steel base mold (25 × 25 mm) fitted with Leica white bulk MC base multi-cassette. The tissue-embedded blocks were allowed for solidification on a cold stage (-5 °C) of Leica EG1150 C for 20 min prior to block trimming and sectioning with a Leica RM2125 RTS rotary microtome. Multiple ribbons of 7-8 μm thickness were sectioned and floated on water bath before placed on Leica Surgipath Snowcoat microscope slides (2.5 × 7.0 × 1.0 cm) and labelled with a pencil. The slides were incubated on hot plate at 60 °C for 1 h followed by immersion in absolute xylene and two ethanol treatments (100% and 80% concentrations respectively) for 5 min each.

Differential staining and light microscopy

The prepared leaf sections were stained using a quadruple stain modified from Johansen (1940) to better differentiate between different structures or cellular components of the specimens. The slides were immersed in Safranin O composed of 100 ml of methyl cello solve, 50 ml each of absolute ethanol and deionised water, 4 ml of 37% formalin, and 2 g each of sodium acetate and Safranin O powder (Sigma) for 2 h to overnight before being rinsed in distilled water 2-3 times. The slides were then immersed in crystal violet composed of 1 g Crystal violet powder (Sigma) in 100 ml of sterile distilled water for 5 min before washed with distilled water twice. Extra stain was removed from the specimens using EMT containing 95% ethanol, methyl cello solve and tert-butanol (1:1:1) followed by 5 min stained in Fast Green FCF solution composed of 0.25 g Fast Green powder (Sigma) in 50 ml of methyl cello solve and clove oil (1:1), 150 ml of each absolute ethanol and tert-butanol, and 3.5 ml of glacial acetic acid. Again the slides were washed with EMT for removal of extra stain before final stained with Orange G containing 1 g Orange G powder (Sigma) in 200 ml of methyl cello solve and 100 ml of absolute ethanol for 3 min. Extra stain was then removed using two changes of CME (clove oil: methyl cell solve: 95% ethanol in 1:1:1) and CMX (clove oil: methyl cello solve: xylene in 1:1:1) for 15 s each before immersion of slides in xylene for 5 min. The specimen slides were mounted with Leica Surgipath Micromount and covered with glass cover slips prior to examining with

bright field (BF) imaging mode under a Leica DM6000 B light microscope controlled by Leica Application Suite (LAS version 4.8.0; Leica Microsystems, Switzerland).

Image processing and data analysis

SEM micrographs were used to investigate the urediniospore germination and formation of infection structures by *A. psidii* on *Eucalyptus* leaves. Twenty random images 375 × 320 μm in size, covering 2.4 mm^2 leaf areas for each adaxial and abaxial surface of one phenotype (total of 4.8 mm^2 per phenotype) were captured for each post-inoculation time point between 6 and 120 h.a.i. per *Eucalyptus* species. Urediniospore density on leaf specimens and percentages of urediniospore germination, appressorium formation and germ tube length in three categories (< 10 μm, 10 – 100 μm and > 100 μm) on the different rust response phenotypes were analysed using ImageJ (National Institute of Health, Maryland, U.S.A.) and R statistical software version 3.3.0 (R Core Team 2016). Fifteen specimen slides, each containing 10-20 leaf sections, were produced from resistant and susceptible phenotypes of *Eucalyptus* species collected at 120 h.a.i. and between 8 and 10 d.a.i. (about 3-4 slides for each time point and total of 30 slides per species) for histopathology. Micrographs generated from the histological observation were used to compare the mechanisms of penetration and colonisation in *Eucalyptus* leaf tissues of rust susceptible and resistant phenotypes.

Results

Disease assessment and phenotypes confirmation

Disease assessment after repeated rust inoculation revealed identical score (according to the rust scoring method as described in Chapter 2) for all the inoculated seedlings except for the known susceptible plants of *E. obliqua* following third inoculation. Carborundum powder-dusted leaves on resistant phenotypes of both *Eucalyptus* species also exhibited no symptoms of infection, confirming their consistency in response to *A. psidii* even with wounded cuticle. Abrasive carborundum powder (400-600 mesh) is commonly used to create micro-wounds on the leaf surface without causing excessive damage to the plant tissues during mechanical inoculation, typically for active transmission of virus particles into the plant cells, to increase the efficiency of infection (Albrechtsen 2006; Hull 2005; Kalmus and Kassanis 1945).

Urediniospore germination and appressorium formation

The present SEM observations showed no differences in ontogeny and morphology of the infection structures formed by *A. psidii* between rust resistant, hypersensitive and susceptible phenotypes of either species. Germination of urediniospores started earlier than 6 h.a.i. resulting in 76.5 – 100.0 and 68.0 – 84.9% of urediniospores germinated at 24 h.a.i. on *E. globulus* (Figure 3-1) and *E. obliqua* (Figure 3-2) respectively. At 48 h.a.i., more than 70.0% of the germinated urediniospores had formed appressoria with variable length germ tubes growing across different phenotypes of both *Eucalyptus* species (Figure 3-3 & 3-4). Long germ tubes (> 100 µm; Figure 3-5a) were observed as early as 12 h.a.i. in both resistant and susceptible phenotypes of *E. obliqua* while the medium (10 – 100 µm; Figure 3-5b) and short germ tubes (< 10 µm; Figure 3-5c) were formed randomly over time post-inoculation in all the phenotypes of both *Eucalyptus* species. Despite the trends of urediniospore germination and appressorium formation were consistent between all the phenotypes of *E. globulus* (Figure 3-1), the respective events were varied in *E. obliqua* (Figure 3-2) which may possibly due to the loss of urediniospores or appressoria during sample preparation for microscopy. The results obtained from examining different rust response phenotypes for urediniospore density were similar in both *Eucalyptus* species (Figure 3-6), yet the distribution of the urediniospores was more on the adaxial (less stomatal aperture) than the abaxial leaf surfaces in all the phenotypes of *E. globulus*.

Host penetration

The growth of *A. psidii* germ tubes appeared to have no affinity towards the natural openings i.e. stomata (Figure 3-5d & e) in both *E. globulus* and *E. obliqua* suggesting an unusual rust penetration mechanism (non-stomatal penetration) on the respective hosts. Occasional appressoria over the stomatal aperture were intermittently observed (Figure 3-5f), but they were a small proportion of the total appressorial penetration. Attempts of penetration were typically directly through the leaf cuticle and epidermis via the formation of appressoria on either leaf surface in both *Eucalyptus* species. The use of quadruple stain in histological observations has successfully aided visualisation of rust infection structures and could differentiate between urediniospores and

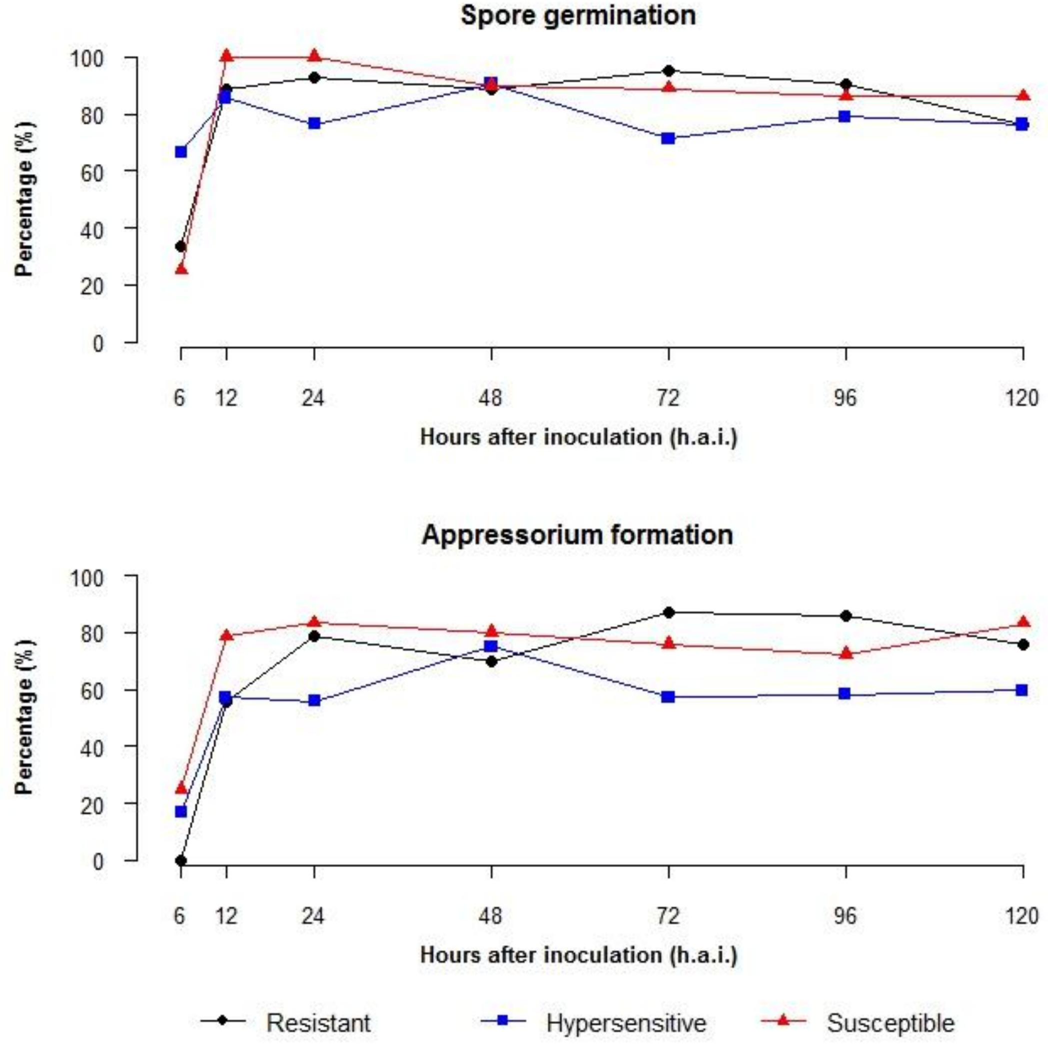

Figure 3-1: Percentages of urediniospore germination and appressorium formation between 6 and 120 h.a.i. in different rust response phenotypes of *Eucalyptus globulus*.

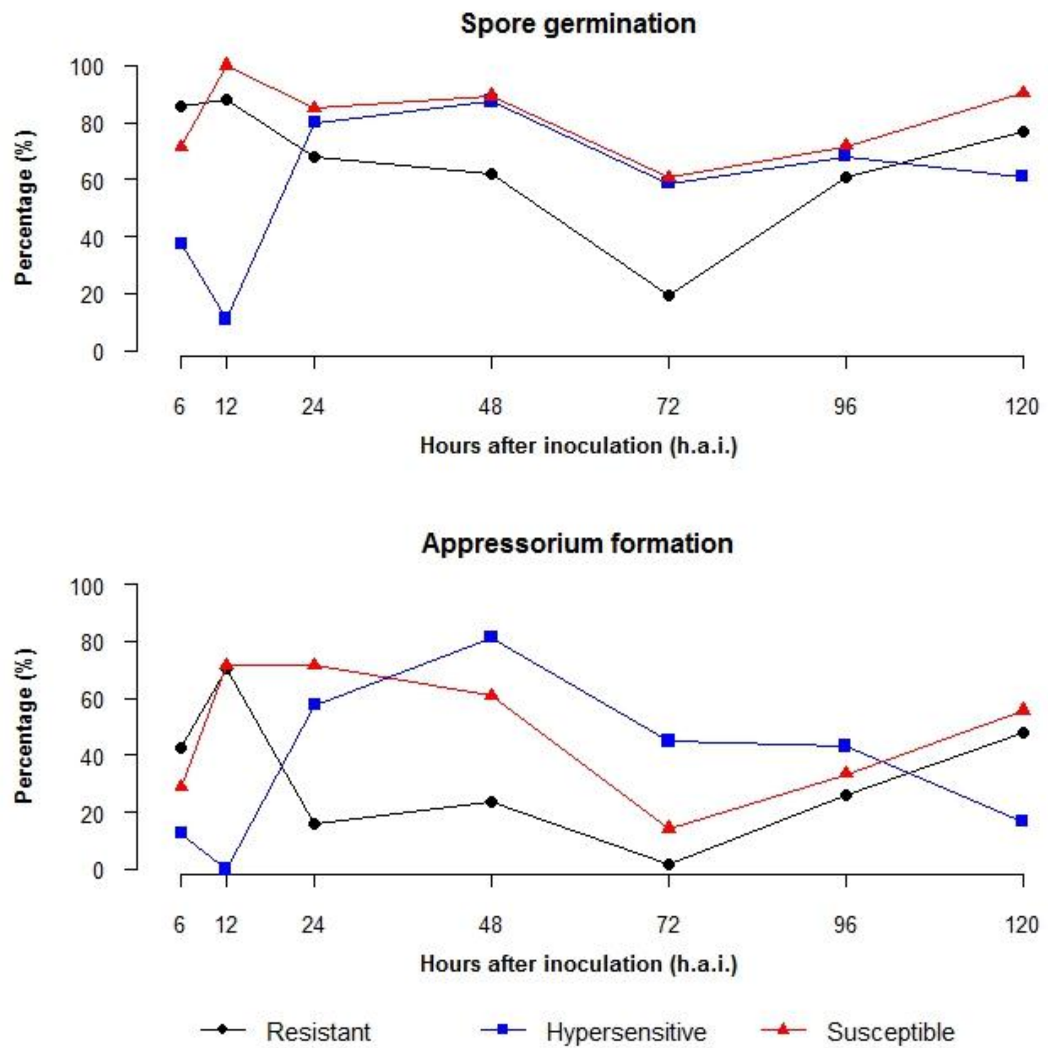

Figure 3-2: Percentages of urediniospore germination and appressorium formation between 6 and 120 h.a.i. in different rust response phenotypes of *Eucalyptus obliqua*.

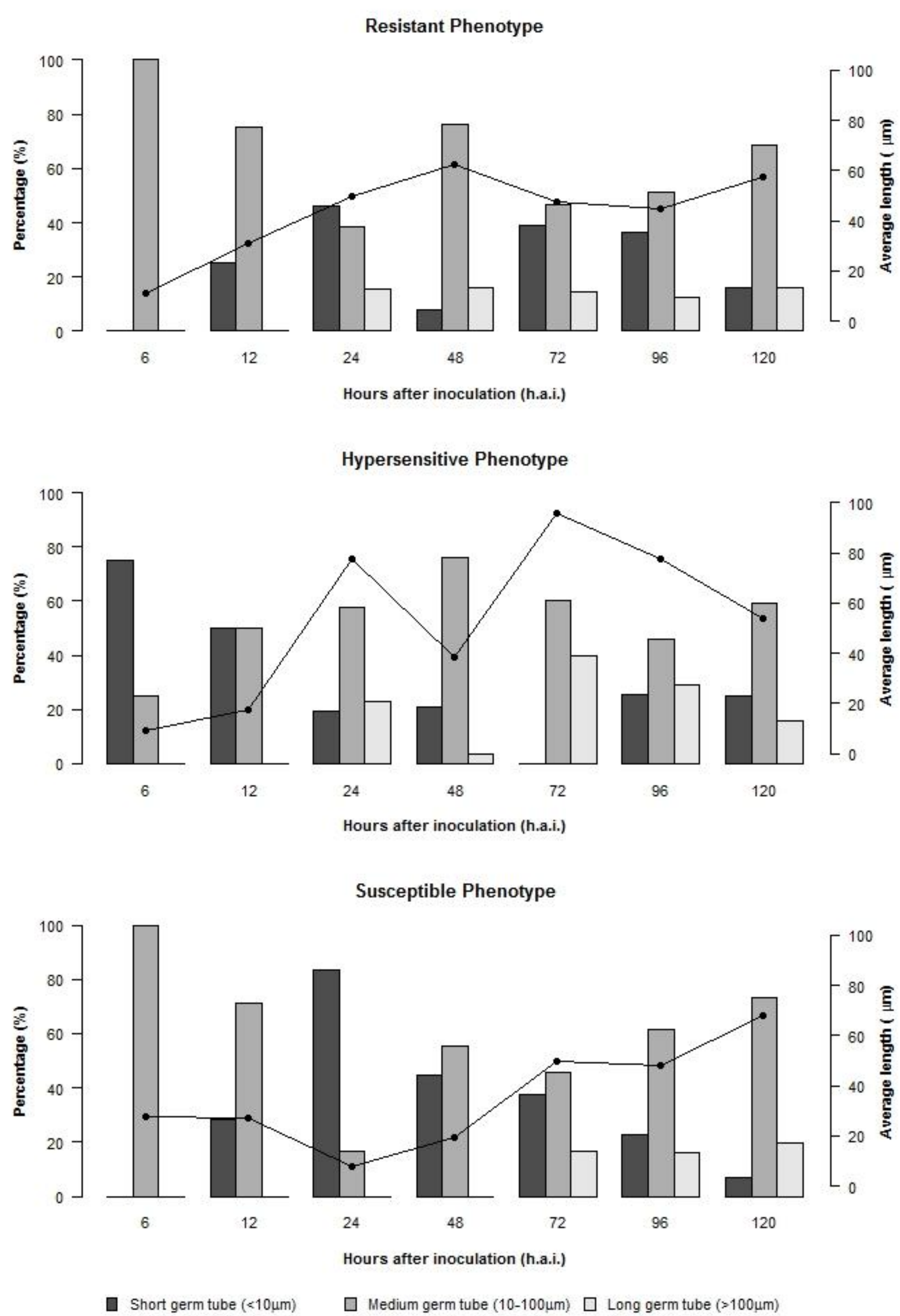

Figure 3-3: Percentages of different length and average length of germ tube between 6 and 120 h.a.i. on different rust response phenotypes of *Eucalyptus globulus*.

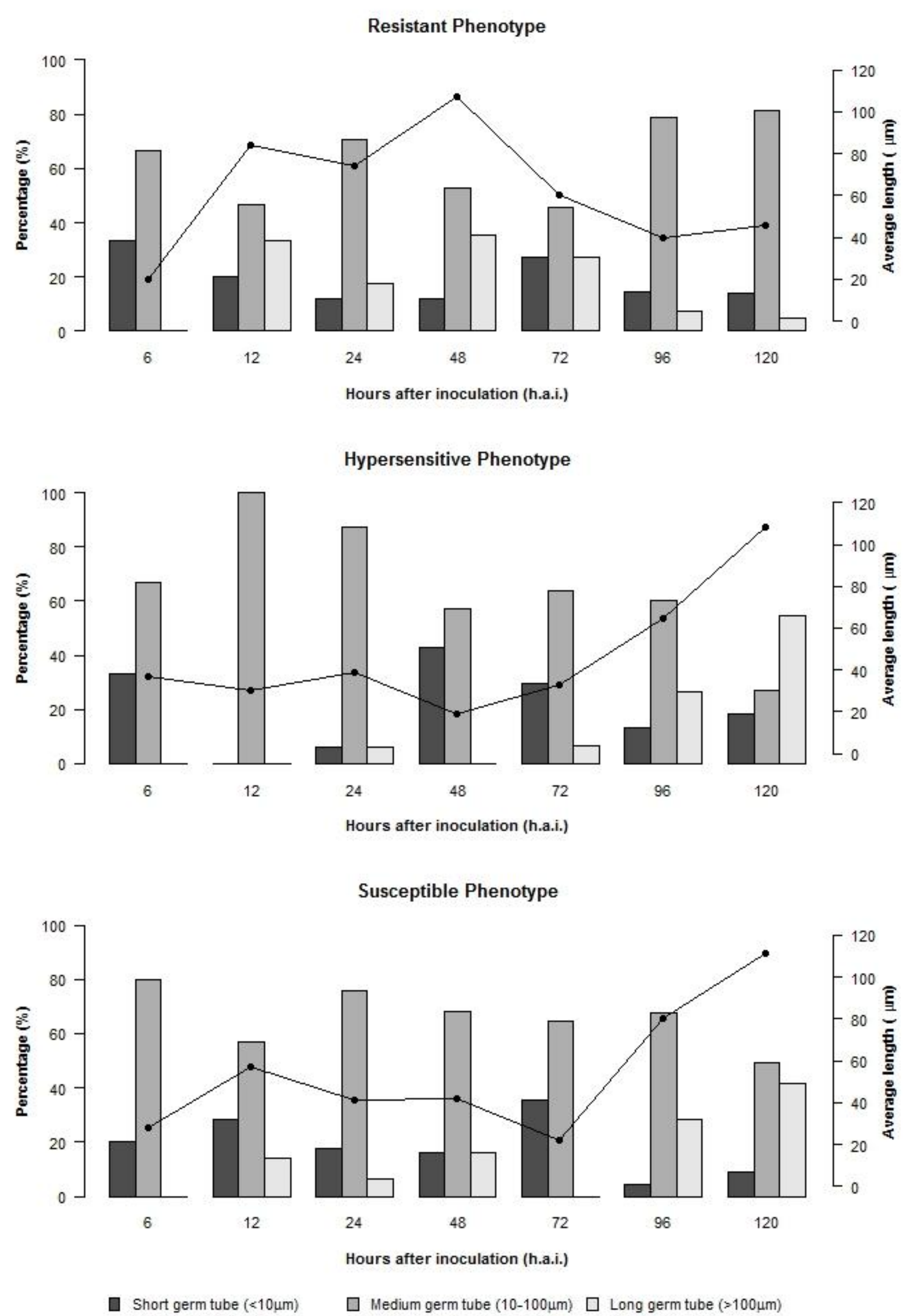

Figure 3-4: Percentages of different length and average length of germ tube between 6 and 120 h.a.i. on different rust response phenotypes of *Eucalyptus obliqua*.

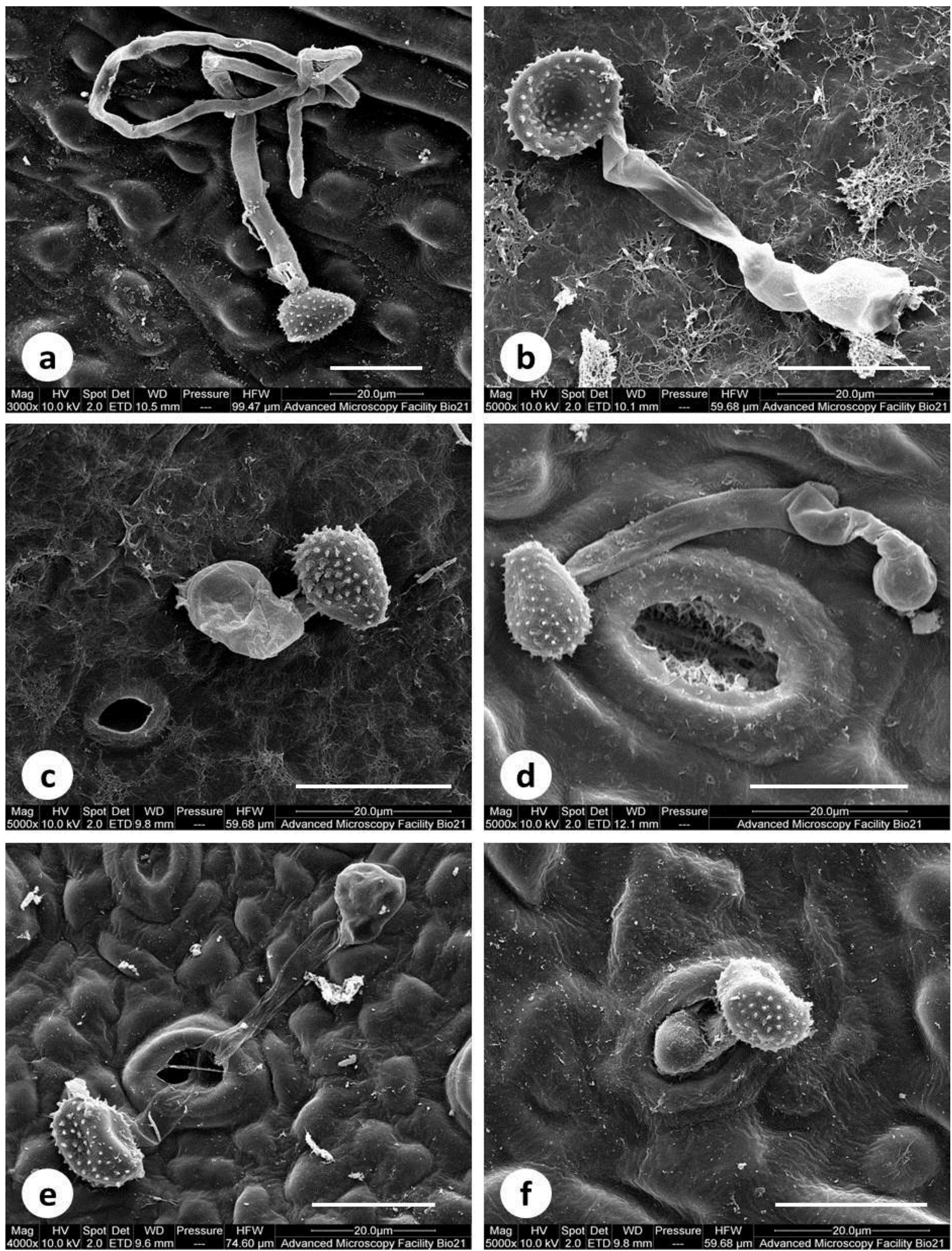

Figure 3-5: Scanning electron micrographs of the infection structure formation by *Austropuccinia psidii* on *Eucalyptus*. Germination of long **(a)**, medium **(b)** and short **(c)** germ tubes on *Eucalyptus* leaves with no affinity towards stomata **(d and e)**. Stomatal penetration following appressorium formation occasionally occurred **(f)**. Figures **a**, **d**, **e** and **f** are *E. obliqua*, and figure **b** and **c** are *E. globulus*. Scale bars = 20 µm.

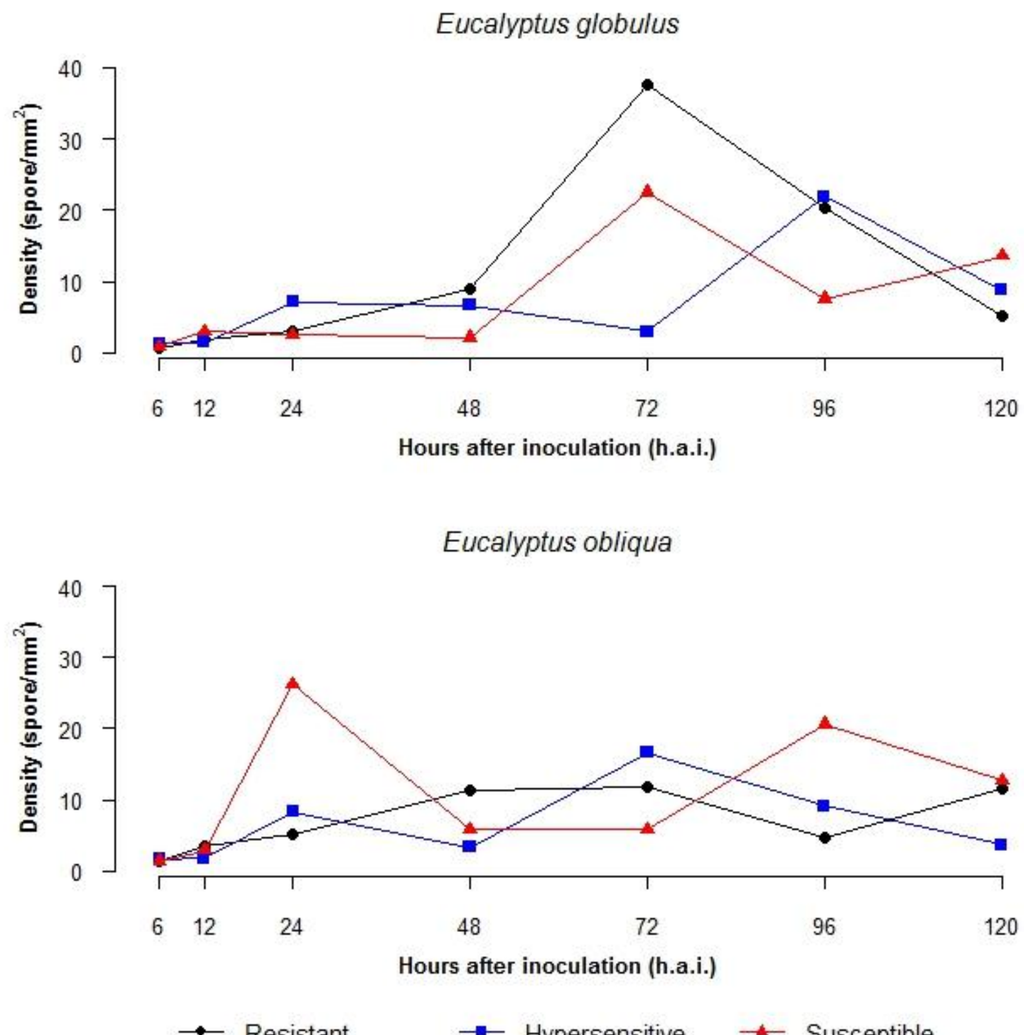

Figure 3-6: Density of urediniospores between 6 and 120 h.a.i. on different rust response phenotypes of *Eucalyptus* species.

appressoria by differential staining (Figure 3-7a). Comparison between rust resistant and susceptible phenotypes showed no penetration of infection peg through the leaf epidermis and looser attachment of appressoria on the rust resistant phenotypes of *E. obliqua* (Figure 3-7b & c), while appressoria were firmly attached to the leaf surfaces of rust susceptible phenotypes and penetration had occured at 120 h.a.i. (Figure 3-7d, e & f). The continuous leaf sections were examined to ensure penetration has actually stopped at the cuticle level and no extension of the infection peg in any direction underneath the cuticle of *E. obliqua* rust resistant phenotypes. However, no urediniospores or appressoria were observed at 120 h.a.i. in the histological examination of any *E. globulus* rust response phenotype. It is likely that the urediniospores and appressoria were washed off during tissue processing due to weak attachment of the infection structures to the leaves. A more irregular surface topography was evident on the leaves of *E. obliqua* (Figure 3-8a) suggesting physical trapping of the urediniospores and appressoria on the uneven leaf surfaces may have helped them during sample preparation for microscopy. In addition, anatomical difference and the presence of crystalline-like structures, may be the composition of lignins or cutins or similar structural compounds stained red by safranin, were discovered in the leaves of *E. obliqua* (Figure 3-8a & b) in contrast to *E. globulus* (Figure 3-8c).

Colonisation and uredinia formation

Hyphal colonisation (Figure 3-8d) and formation of uredinial primordia (Figure 3-8e) were observed at 8 d.a.i. within the leaves of *E. globulus* rust susceptible phenotype. Further development of uredinia caused the eruption of leaf epidermis and emergence of uredinial sori on the leaf surfaces of *E. globulus* at 10 d.a.i. (Figure 3-8f). Sporulation by *A. psidii* was visible macroscopically at 8 d.a.i. on both adaxial and abaxial leaf surfaces of the rust susceptible *E. globulus* plants but by 6 d.a.i. on the highly susceptible *S. jambos* control plants.

However, no lesions observed up to 14 d.a.i. on any of the previously known rust susceptible *E. obliqua* plants after the third rust inoculation, and further histological examinations also indicated no hyphal colonisation or uredinia formation in the respective specimens. This phenomenon may due to the early vegetative phase change

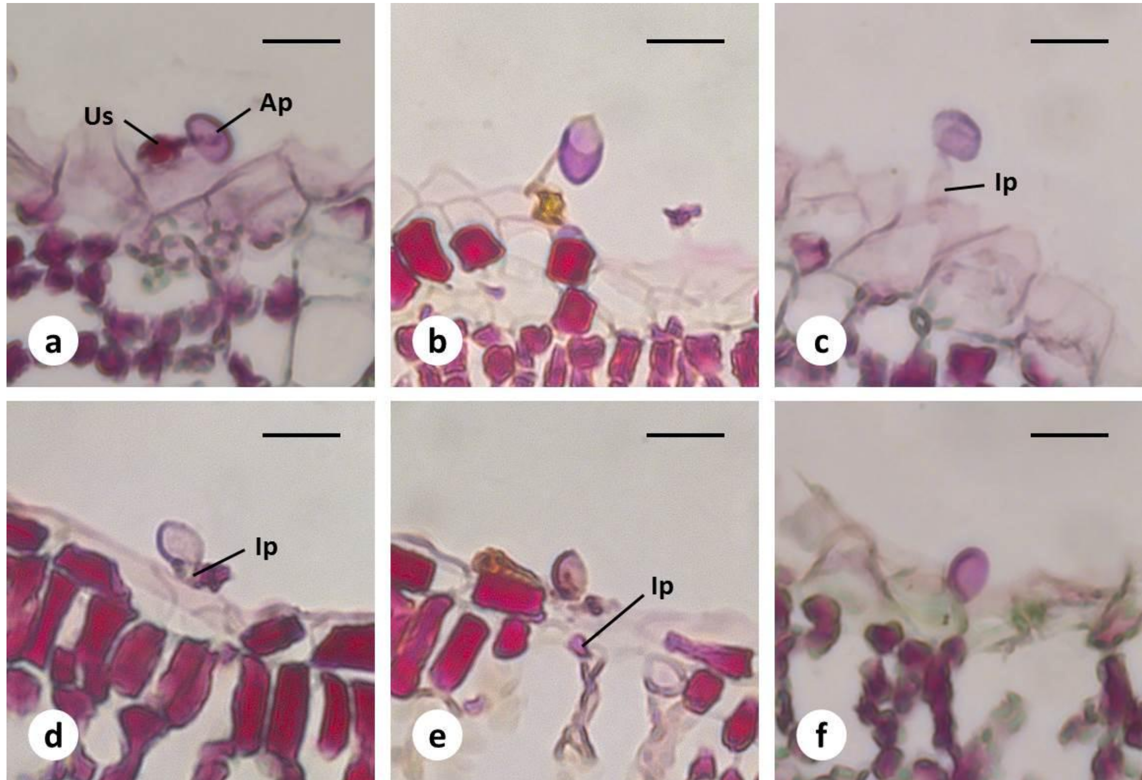

Figure 3-7: Light micrographs of the pathogenesis of *Austropuccinia psidii* at 120 h.a.i. in *Eucalyptus obliqua*. Urediniospore germination and appressorium formation **(a)** with no penetration **(b)** or penetration stopped after cuticle on epidermis level **(c)** in rust resistant phenotype. Penetration of infection peg **(d and e)** and firm attachment of appressorium **(f)** occurred on rust susceptible phenotype. Us = urediniospore; Ap = appressorium; Ip = infection peg. Scale bars = 20 µm.

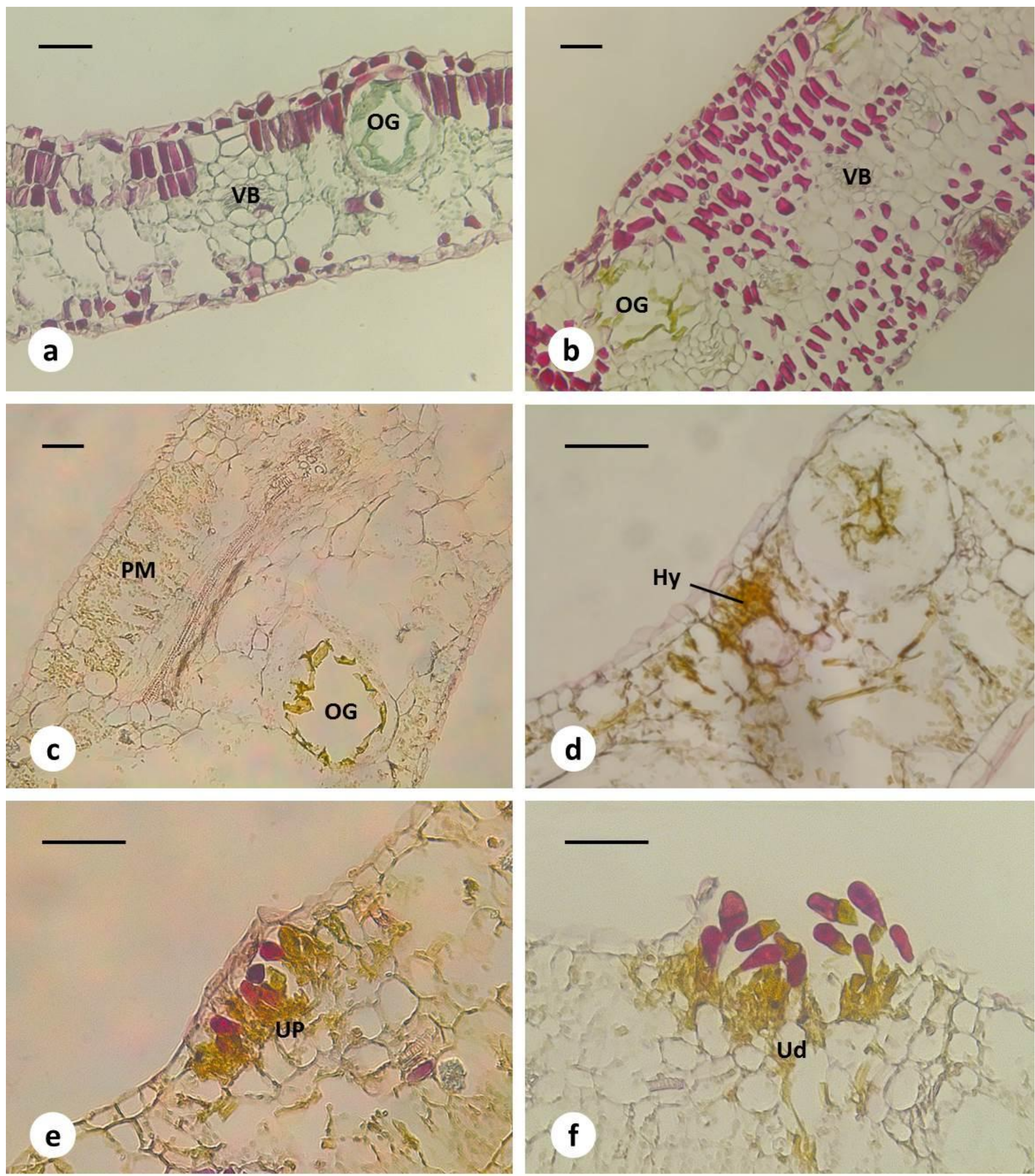

Figure 3-8: Anatomical difference with irregular surface topography and crystalline-like structures in the leaf of 6-month-old **(a)** and 12-month-old *Eucalyptus obliqua* **(b)** in contrast to *E. globulus* **(c)**. Hyphal colonisation **(d)** and formation of uredinial primordia by *Austropuccinia psidii* at 8 d.a.i. **(e)** before emergent of fully developed uredinia containing urediniospores at 10 d.a.i. **(f)** on the leaf surfaces of *E. globulus* susceptible phenotype. OG = oil gland; VB = vascular bundles; PM = palisade mesophyll layer; Hy = hyphae; UP = uredinial primordia; Ud = uredinia. Scale bars = 50 μm.

which results in the development of adult plant resistance in *E. obliqua* against *A. psidii* infection. Deposits of the lignin-like structural compounds were also noticed higher in the leaves collected from 12-month-old known susceptible *E. obliqua* plants (Figure 3-8b) as compared to the younger (6-month-old) seedlings obtained from earlier sampling (Figure 3-8a). Plants were visually assessed again at 28 d.a.i. and no further infection symptoms had developed on the previously known rust susceptible *E. obliqua*, confirming the shift of rust susceptible to resistant phenotypes in the species. Nevertheless, early acquisition of adult plant resistance and transition of rust response phenotypes did not occur in *E. globulus*.

Discussion

This study provides, to the extent of found knowledge, the first microscopic examination of the infection process of *A. psidii* in the leaves of *E. globulus* and *E. obliqua* representing the two major *Eucalyptus* subgenera *Symphyomyrtus* and *Eucalytpus* respectively, albeit *A. psidii* infection process in another *Symphyomyrtus* species *E. grandis* has previously been reported (Xavier et al. 2001; Xavier et al. 2015). Based on the analysis of more than 1600 SEM micrographs and histological observation of more than 600 leaf sections, Figure 3-9 summarises the infection process of *A. psidii* pathogen on different rust response phenotypes of *Eucalyptus* species. High percentages of urediniospore germination and appressorium formation were observed in the first 24 h.a.i. as similarly occurred in *E. grandis*, on both the rust resistant and susceptible phenotypes (Xavier et al. 2001). The initial infection process of *A. psidii* was followed by penetration of infection pegs directly through the leaf cuticle and epidermal cell wall into the mesophyll, occurred before 120 h.a.i. as observed in the present study, or as early as 24 h.a.i. as described in *E. grandis* (Xavier et al. 2001) and highly susceptible *S. jambos* (Hunt 1968).

Non-stomatal penetration is unusual for dikaryotic rust spores (e.g. urediniospores) and has only been reported from *P. pachyrhizi* on soybean (Bonde et al. 1976; Edwards and Bonde 2011; Marchetti et al. 1975), *Phakopsora apoda* (Har. & Pat.) Mains on kikuyu grass *Pennisetum clandestinum* Hochst. ex Chiov. (Adendorff and Rijkenberg 2000), *Physopella zeae* (Mains) Cumm. & Ramachar on maize (Bonde et al. 1982) and

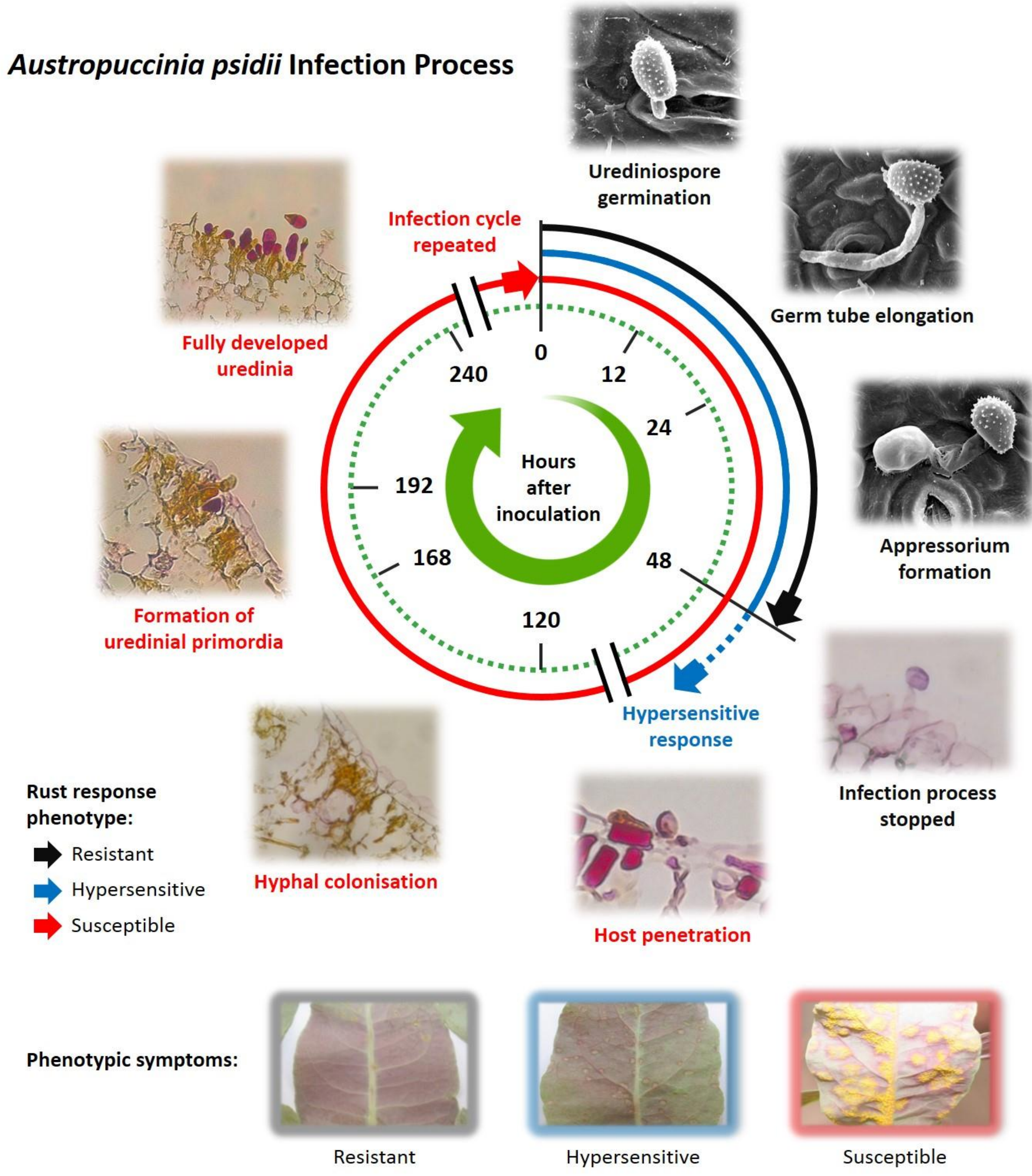

Figure 3-9: *Austropuccinia psidii* infection process on different rust response phenotypes of *Eucalyptus* species. Initial infection events from urediniospore germination to appressorium formation occur within the first 48 h.a.i. on all the phenotypes. The infection process then stopped on the resistant plants at the cuticle or epidermis levels without further penetration. However, host penetration must occur on the plants showing hypersensitive response prior to pathogen recognition in the host and localised cell death. The pathogens are able to complete the infection cycle in 10 days or shorter on the susceptible plants by formation of all the necessary cellular components for colonisation and successful reproduction.

Ravenelia humphreyana P.Henn. on poinciana *Caesalpinia pulcherrima* (L.) Sw. (Hunt 1968). The unusual mode of penetration and non-specific induction of appressoria in the infection process of *A. psidii* may explain its broad host range. In *P. pachyrhizi* successful penetration occurs not only on the host (i.e. soybean) but also on non-host plants such as barley (Hoefle et al. 2009), *Medicago truncatula* Gaertn. (Uppalapati et al. 2012) and *Arabidopsis thaliana* (L.) Heynh. (Loehrer et al. 2008).

Prior to appressorium formation, germ tube extension and differentiation can occur in response to a number of signals on host leaves including surface hardness and topography, hydrophobicity, and plant signalling compounds (Tucker and Talbot 2001). Hunt (1968) suggested that the length of urediniospore germ tubes may be related to the mode of penetration with germ tubes much shorter or even absent in direct or cuticularly penetrating species as compare to indirect or stomatally penetrating rusts. This speculation may due to the direct penetrators are less specific about their penetration site selection than indirect penetrators as described in *P. apoda* where the pathogen was capable of forming appressoria anywhere on the leaf surface which reduced the energy spent on exploratory germ tube growth (Adendorff and Rijkenberg 2000). The growth of variable length germ tubes (Figure 3-3 & 3-4) in random directions and reduced probability of germ tubes in locating stomata (Figure 3-5) has proposed a non-host interaction (Bettgenhaeuser et al. 2014; Nürnberger and Lipka 2005) between *Eucalyptus* and *A. psidii*, as similarly observed in wheat stripe rust pathogen on non-host broad bean (Cheng et al. 2012).

Defence responses to invading pathogens may involve preformed (physical or chemical) barriers or induced resistances based on complex sensory, signalling and executive mechanisms in plant-pathogen interactions (Naidoo et al. 2014). The constitutive defensive barriers such as wax layers, rigid cell walls or secondary metabolites on the plant surface may initially limit the establishment of pathogen infection structures (Nürnberger and Lipka 2005), but if the external barriers are breached, the active defence mechanisms consisting of pathogen-associated molecular pattern (PAMP) triggered immunity (PTI) and effector-triggered immunity (ETI) involving specific disease resistance (*R*) genes (Bettgenhaeuser et al. 2014; Jones and Dangl 2006) may be

activated in response to pathogen invasion. Leaf cuticle is the first barrier to be overcome by cuticularly penetrating fungi in which basic compatibility is required for successful pathogen adhesion and penetration (Bettgenhaeuser et al. 2014; Serrano et al. 2014). Formation of a fibril-like matrix (usually referred to as an extracellular matrix) is reported in most appressorium-cuticle interactions as a prerequisite for appressorium adhesion to plant leaf surface (Adendorff and Rijkenberg 2000; Edwards and Bonde 2011), has been observed in *A. psidii* attachment on *E. grandis* as well (Xavier et al. 2015). The failure of *A. psidii* appressoria to firmly attach on the leaf surface (Figure 3-7b & c), however, may explain the role of cuticle in preventing pathogen adhesion on rust resistant phenotypes. In the event of successful pathogen adhesion, the cuticle and epidermal cell walls may be breached by penetration structures secreting cuticle-dissolving enzymes (cutinases) and cell-wall-digesting enzymes (cellulases and hemicellulases) or mechanical force generated from high appressorial turgor pressure as demonstrated in *P. pachyrhizi* (Edwards and Bonde 2011; Loehrer et al. 2014). Martin (1964) suggested that cuticle wax quantity may determine the fungal behaviour in the infection process viz. spore deposition and germination, while Heather (1967a) and (1967b) discovered the role of leaf waxes in preventing spore germination of *Phaeoseptoria eucalypti* (Hansf.) Walker on *Eucalyptus bicostata* Maiden, Blakely & Simmons. Vaz Patto and Niks (2001) also showed the presence of leaf waxes i.e. distribution of epicuticular wax crystals (Lewis and Day 1972) over the stomata in restricting the appressorium formation of rust fungus *Puccinia hordei* G.H. Otth on wild barley *Hordeum chilense* Roem. & Schult. leaves. In addition, there is a gradual reduction in fungal germination, appressorium formation, host penetration and sporulation with increased in leaf physiological age in *E. grandis*, with higher cuticle thickness and more waxes in the older leaves (Xavier et al. 2015). Nevertheless, compositional variation in wax chemistry between *Eucalyptus* species (Hallam and Chambers 1970; Li et al. 1997) may significantly affect the susceptibility to *A. psidii*, mainly with increasing levels of β-diketone (C33), and decreasing levels of two alkanals (C24 and C26) and triterpenoid methyl moronate in the direction of more disease resistant in subgenus *Symphyomyrtus*, as compared to subgenus *Eucalyptus* (Potts et al. 2016).

Ranges of susceptibility from completely resistant or fully immune (no visible disease symptoms) to intermediate resistance (hypersensitive and formation of small uredinia) and highly susceptible (development of mature uredinia pustules) observed in *Eucalyptus* species may indicate the continuum of *A. psidii* infection outcomes in non-host interaction with *Eucalyptus* as similarly occurred in barley with *P. triticina* isolates (Neu et al. 2003) and other non-host pathosystems as reviewed by Bettgenhaeuser et al. (2014). Non-host resistance is the most common form of plant defence mechanism that provides broad-spectrum immunity to a plant species against vast majority of potential pathogens including bacteria, fungi and oomycetes (Heath 2000b; Mysore and Ryu 2004; Niks and Marcel 2009; Senthil-Kumar and Mysore 2013). Mysore and Ryu (2004) classified non-host resistance into two types: type I which does not produce any visible symptoms, and type II which results in a rapid hypersensitive response with localised cell death. A rust pathogen that is capable of parasitising a plant species is known to be an adapted pathogen where it can form all the necessary cellular components for colonisation and successful reproduction. Previous studies reported the effectiveness of non-host resistance against various rust pathogens and a number of defence mechanisms range from pre-haustorial (i.e. basic incompatibility, topographical interruption, degradation of infection structure and inhibition of haustorium formation) to post-haustorial resistance (i.e. hypersensitive reaction and inducible defence signalling) possessed by rust resistant plants/cultivars (Bettgenhaeuser et al. 2014; Heath 1981; Nürnberger and Lipka 2005). Plant cell wall and epidermis constitute formidable defences to a wide range of non-host pathogens such as syntheses of essential components i.e. syntaxin vesicle targeting protein, glucosyltransferase, and ATP-binding cassette transporter encoded by penetration resistance (*PEN*) genes in *A. thaliana* for basal penetration resistance against non-adapted mildew pathogens species (Collins et al. 2003; Lipka et al. 2010; Lipka et al. 2005; Stein et al. 2006; Underwood and Somerville 2013). The *PEN* genes (or homolog *ROR* genes) have also been reported to play a crucial role in penetration resistance to *P. pachyrhizi* on non-host barley (Hoefle et al. 2009) and *A. thaliana* (Langenbach et al. 2013; Loehrer et al. 2008), suggesting common epidermal defence mechanisms against diverse pathogen species in non-host pathosystems.

Previous study reported hypersensitive reactions in rust resistant phenotypes of *E. grandis* after 48 h of *A. psidii* inoculation, in contrast with susceptible phenotype where hypertrophy of infected tissues and macroscopic disease symptoms were observed 6 d.a.i., and uredinia formation at 9 d.a.i. (Xavier et al. 2001). In many pathosystems, pre-haustorial non-host resistance is frequently backed up by post-haustorial hypersensitive response against successful cell wall penetration by any pathogen infection units (Heath 2000b, 2001; Lipka et al. 2005), as revealed in certain plant-rust combinations such as wheat and rye with barley leaf rust (*P. hordei*; Niks and Dekens 1991), and broad bean with wheat stripe rust (*P. striiformis* f. sp. *tritici*; Cheng et al. 2012). The process of cell death in non-host plants, however, may vary from the hypersensitive resistance of the host species (Christopher-Kozjan and Heath 2003), and regularly governed by the joint effect of multiple quantitative trait loci (QTLs) and occasionally a major *R* gene (Heath 1997; Niks 2014). Pathogen recognition in non-host plants may be triggered by general or exogenous elicitors (Gómez-Gómez and Boller 2002; Montesano et al. 2003; Nürnberger et al. 2004), such as chitin and ergosterol in fungi (Felix et al. 1993; Granado et al. 1995), for subsequent activation of PAMP-induced non-host defences. Endogenous elicitors such as breakdown products of the plant cell wall released by glucohydrolytic activities from attacking pathogen may also activate the innate defence mechanisms in non-host species in response to microbial invasion (Vorwerk et al. 2004). Multiple *R* genes may be involved in simultaneous recognition of their corresponding *avr* gene-encoded products in non-host resistance for activation of their complex plant surveillance system (Mysore and Ryu 2004; Nürnberger and Lipka 2005). Butler et al. (2016) also reported different set of QTLs responsible for different modes of defence responses (immune or completely resistant and hypersensitive reaction) in *E. globulus* against *A. psidii*. The presence of specific recognition to this newly encountered pathogen and intermediate proportions of resistant and susceptible plants in populations have raised question about common selection pressure to other pathogen in determining *A. psidii* resistance across so many Australian myrtaceous species where the host and pathogen had no recent evolutionary connections. Tobias et al. (2016) have subsequently proposed the retention of ancient *R* genes through prolonged ‘trench warfare’ (Stahl et al. 1999), pairing of resistance gene products (Eitas and Dangl 2010) and guarding of host integrity (van der Hoorn and Kamoun 2008) to

explain the specific rust resistance in myrtaceous hosts, and suggest that common pathogen 'invasion pattern' and effector target may be associated with the complex *Eucalyptus – A. psidii* interactions.

Moreover, the absence of observable disease or even evident of infection in previously known rust susceptible plants of *E. obliqua* after the third rust inoculation in the present study is intriguing and has prompted speculation about early acquisition of adult plant resistance in the species (about 12-month-old) due to ontogenetic changes in leaf characteristics such as cuticle chemistry or physical structure, albeit *E. obliqua* leaves still look very juvenile at this stage. *Eucalyptus* are heteroblastic with progression vegetative phase changes in morphology between juvenile and adult forms which give rise to markedly differences in shape, size, colour, orientation, glaucousness, anatomy, physiology, chemistry and resistance to herbivores and pathogens between juvenile and adult leaves (Cameron 1970; Dungey et al. 1997; James and Bell 2001; Johnson 1926; Lawrence et al. 2003; Li et al. 1995, 1996; Pederick 1979). Previous studies revealed variation in *Eucalyptus* species in susceptibility to *Mycosphaerella* leaf diseases as a result of heteroblastic transition (phase change from juvenile to adult leaves) in which both juvenile and adult foliage of over 50 species of *Symphyomyrtus* and *Eucalyptus* subgenera were found susceptible to *Mycosphaerella cryptica* (Cooke) Hansford but only juvenile foliage of a restricted range of species in the series *Viminales* of *Symphyomyrtus* (closely allied with *E. globulus* Labill.) were infected by *Teratosphaeria nubilosa* (Cooke) Crous & U.Braun (Carnegie et al. 1998; Carnegie et al. 1994; Dick 1982; Dungey et al. 1997; Park and Keane 1982). Sánchez Márquez et al. (2011) also discovered greater diversity of foliar fungal species and higher susceptibility to a wide range of pathogens and fungi in *E. globulus* juvenile leaves due to less effective or undeveloped mechanisms of general defence (non-specific structural or biochemical defence mechanisms) against fungal invasion in juvenile than in adult leaves.

Despite the reported damaging effect of *A. psidii* on Australia's biodiversity, especially to the key species such as *Rhodamnia rubescens* (Benth.) Miq. (brush turpentine) and *Rhodomyrtus psidioides* (G.Don) Benth. (native guava) in natural ecosystems (Carnegie

et al. 2016), the early onset of adult plant resistance to *A. psidii* as observed in *E. obliqua* in the present study may suggest the minimal effects of this invasive pathogen to the sclerophyll forests dominated by native *E. obliqua* and related *Eucalyptus* species with early heteroblastic transition capability. Nevertheless, major ecological disturbances such as fires, insect outbreaks or clearcutting in the forest and *Eucalyptus* plantation may result in regeneration of epicormic shoots or coppices which are very susceptible to *A. psidii* pathogen as evident in new growth of *Melaleuca quinquenervia* (Cav.) S.T.Blake (Rayachhetry et al. 1997), *R. rubescens* and *R. psidioides* (Carnegie et al. 2016), and thereby precautionary steps are required for effective disease management in *Eucalyptus* forests.

Conclusion

The results of this study indicate that resistance to *A. psidii* in *E. globulus* and *E. obliqua* may due to non-host resistance involving pre-haustorial and post-haustorial defence mechanisms underlain by joint effect of multiple quantitative genes. The present findings contribute to better understanding of general defence responses in *Eucalyptus* including early acquisition of adult plant resistance in *E. obliqua* against *A. psidii*, which might be useful in development of effective strategies for invasive rust control. Further investigation of foliar chemistry and gene expression responses to *A. psidii* infection across *Eucalyptus* subgenera with resistant and susceptible phenotypes may provide valuable comprehensions into *Eucalyptus* – *A. psidii* pathosystem as well as the potential drivers for rust resistance trait.

Chapter 4

Phytochemical analysis of the essential oils from *Eucalyptus globulus* and *Eucalyptus obliqua* in association with myrtle rust resistance

Abstract

Eucalyptus foliar terpenes have been reported widely to function as preformed chemical defences against mammalian herbivores and defoliating insects, via direct toxicity or indirect priming of systemic defences. Following the potential identification of plant-derived metabolites as disease resistance biomarkers, this present study compares the chemical composition of foliar essential oils in *Eucalyptus globulus* Labill. and *Eucalyptus obliqua* L'Hér. between distinct myrtle rust (*Austropuccinia psidii* Beenken) response phenotypes i.e. completely resistant, hypersensitive and highly susceptible, using canonical discriminant analysis. Stepwise forward variable selection identified six terpene compounds (bicyclogermacrene, globulul, geraniol, β-pinene, *cis-p*-menth-2-en-1-ol and δ-terpinene) in *E. globulus* and four terpenes (δ-cadinene, caryophyllene oxide, longifolenaldehyde and α-caryophyllene) in *E. obliqua* significantly discriminating between rust response phenotypes. The selected compounds varied between *E. globulus* and *E. obliqua* and were different from the rust resistance biomarkers published from a previous study of *E. grandis* × *E. urophylla* hybrids (1,8-cineole and α-terpinyl acetate). While the discriminant functions were significant within species, cross validation between *Eucalyptus* species was not significant. These findings indicate the possible complexity and variability of terpene defensive chemistry between *Eucalyptus* species and the combined effects or synergistic effects of terpenes in combination with other plant metabolites which have barely been evaluated. However, the effectiveness of discriminant functions in phenotype classification and the underlying defensive mechanisms by which the identified compounds impact rust resistance remain to be elucidated.

Introduction

Many plant secondary metabolites have been reported to play an important role in plant fitness (Bustos-Segura et al. 2015; Steppuhn et al. 2004) and exhibited physiological and ecological functions associated with adaptive evolution and diversification (Hartmann 1996; Kursar et al. 2009; Moore et al. 2014; Poelman et al. 2008). While primary metabolism supports growth, development and reproduction, plant secondary metabolism has been known to facilitate interactions with the biotic and abiotic environments, including defences against herbivores and pathogens (Neilson et al. 2013; Seigler 2012). Chemical defences involving secondary metabolites in terrestrial plants, including forest trees, can be constitutive, or induced by injury or exposure to invading agents (Franceschi et al. 2005; Kovalchuk et al. 2013; Naidoo et al. 2014; Pearce 1996). The synthesis of low-molecular-weight (LMW) compounds such as terpenoids (Keeling and Bohlmann 2006; Schwab and Wüst 2015), phenolic compounds (Bi and Felton 1995; Moreira et al. 2012) and alkaloids (Facchini 2001; Ziegler and Facchini 2008) as classified according by their biosynthetic pathways can provide advantages to the producing species in resistance to physical attack by predators, parasites and pathogens, and successful adaptation to different types of abiotic environmental stresses (Hadacek 2002; Kovalchuk et al. 2013; Pritchard and Birch 2011). Defensive LMW secondary metabolites with antimicrobial, antinutritive, antioxidant or antidigestive properties that are synthesised *de novo* during plant-pathogen interactions are described as phytoalexins (Eyles et al. 2010; Hammerschmidt 1999), while toxic LMW antimicrobial compounds constitutively present in plant tissues are referred to as phytoanticipins (VanEtten et al. 1994).

In *Eucalyptus* leaves, essential oils comprising a complex mixture of monoterpenes, sesquiterpenes and formylated phloroglucinol compounds (Keszei et al. 2008) are typically produced and stored in the sub-dermal secretory cavities or “oil glands”. The stored oils in *Eucalyptus* species have been widely reported to function as preformed chemical defences against mammalian herbivores and defoliating animals (Lawler et al. 1999; McArthur et al. 2010; Moore et al. 2004; O'Reilly-Wapstra et al. 2004; Wiggins et al. 2006) such as *Pseudocheirus peregrinus* Boddaert (common ringtail possums), *Trichosurus vulpecula* Kerr (common brush-tailed possum), *Thylogale billardierii*

Desmarest (red-bellied pademelon) and *Phascolarctos cinereus* Goldfuss (koala). Essential oils contained in trichomes and other glandular structures in *Eucalyptus* have also been demonstrated to be an effective physicochemical defence mechanism against various leaf-chewing insects (Andrew et al. 2007b; Bowers et al. 2000; Rapley et al. 2007; Troncoso et al. 2011) including *Anoplognathus montanus* Macleay (Christmas beetle), *Ctenarytaina eucalypti* Maskell (blue gum psyllid) and the larvae of *Mnesampela privata* Guenée (autumn gum moth). Secretory cells and glands that produce and transport defensive substances can also occur in other parts of the plant such as bark, phloem, medulla, roots and petioles (Naidoo et al. 2014), and their existence and abundance in the respective tissues are widely different between *Eucalyptus* species (Carr and Carr 1970). The defensive action of essential oils can be direct toxicity via allelopathic or antimicrobial activity (Eyles et al. 2003a; Eyles et al. 2003b; McLean et al. 1993), or indirect priming of systemic defences in both the host and neighbouring plants (Eyles et al. 2010; Naidoo et al. 2014). Furthermore, Goodger et al. (2009) showed the foliar secretory cavities of several *Eucalyptus* species contain not only volatile essential oils but also non-volatile, resinous compounds of unknown function, indicating the complexity of terpene biosynthesis and their functionality in *Eucalyptus* natural defences.

Qualitative analysis revealed over 100 different terpenes may be present in the leaves of an individual *Eucalyptus* species and the combined effects of different terpenes along with the quantitative effects of individual compounds have hardly been evaluated (Naidoo et al. 2014; Padovan et al. 2014). Külheim et al. (2015) identified 113 and 106 putative functional terpene synthase genes in the genome sequences of *Eucalyptus grandis* W.Hill ex Maiden (Myburg et al. 2014) and *Eucalyptus globulus* Labill. (Department of Energy-Joint Genome Institute), respectively, and discovered the largest proportion of the genes were expressed in green tissues such as mature and young leaves and floral buds, and significant group of genes were also expressed in non-green tissues including roots, xylem and phloem. Terpene diversity in *Eucalyptus* species was reported to be driven by variation in terpene synthase enzymes (Keszei et al. 2010; Padovan et al. 2014), which catalyse the synthesis of ranges of terpene products from a relatively small set of common substrates (Wise et al. 1998). These multi-product

enzymes have variable catalytic pockets or different areas in the active site for distinct sequential steps in the reaction cascade to facilitate the production of multiple terpene skeletons (Köllner et al. 2006). Terpene products are formed from C_5 precursors and their qualitative profile in producing species are determined at two key points in the terpene biosynthetic pathway viz. the synthesis of geranyl pyrophosphate (GPP – C_{10}) and farnesyl pyrophosphate (FPP – C_{15}), precursors for monoterpenes and sesquiterpenes, respectively (Külheim et al. 2015; Padovan et al. 2014). The efficiency of unique terpene synthases involved in the respective biosynthetic pathways can influence the monoterpene:sesquiterpene ratio in different cellular compartments (Lichtenthaler 1999; McCaskill and Croteau 1995) and subsequently resulted in multitude of terpene carbon skeletons in the producing species (Degenhardt et al. 2009).

Significant qualitative and quantitative variation in foliar terpenes has been reported widely at individual, population and taxonomic levels in the family Myrtaceae, including *Eucalyptus* species (Keszei et al. 2010; Keszei et al. 2008). The considerable differences in yield and composition of volatile leaf oils between species indicates a strong genetic basis for these traits in both *Symphyomyrtus* and *Eucalyptus* subgenera (Doran and Matheson 1994; Li and Madden 1995; Li et al. 1995, 1996; O'Reilly-Wapstra et al. 2011; O'Reilly-Wapstra et al. 2005). Previous studies have also shown strong ontogenetic variation in foliar terpene expression across distinct life stages e.g. seedlings, juveniles and adults (Goodger and Woodrow 2009; O'Reilly-Wapstra et al. 2007), and within a single life stage (Borzak et al. 2015; Goodger et al. 2013a; Goodger et al. 2013b; McArthur et al. 2010; Padovan et al. 2013) of various *Eucalyptus* species. In addition to the genetic effects, environmental factors including nutrient availability and water supply (Gleadow and Woodrow 2002), along with genotype × environment interactions (Andrew et al. 2010) also found to be involved in the divergence of foliar chemicals in *Eucalyptus*, which may then influence their interactions with the encountered herbivores and pathogens in their native community. Wallis et al. (2010b) found no correlation between juvenile and maturing ramets of *E. grandis* in their foliar chemistry, implying the contributions of gene × environment interactions and age effects in the deployment of foliar chemicals in the species. Further understanding of terpene variation and causes of variation in *Eucalyptus* may improve the knowledge of

evolution of terpene diversity within the species-rich genus and their respective ecological interactions (Keszei et al. 2008; Padovan et al. 2014), i.e. explaining how the complex terpene mixtures are assembled in *Eucalyptus* foliage and how genetic variation interacts with environmental factors to produce various types and quantities of terpenes.

Despite terpenes being one of the most important groups of secondary metabolites across the plant kingdom and known to play many roles in natural systems (Padovan et al. 2014), *Eucalyptus* terpenes, to date, have mostly been implicated in the ecological interactions with herbivores (Edwards et al. 1993; Edwards et al. 1990; Hume and Esson 1993; Lawler et al. 1999; Morrow and Fox 1980; Stone and Bacon 1994) and pathogens (Eyles et al. 2003b; Hantao et al. 2013a; Potts et al. 2016). Hantao et al. (2013a) have tentatively identified more than 40 volatile organic compounds using modern analytical methods as potential disease biomarkers for diagnosis of *Teratosphaeria nubilosa* (Cooke) Crous & U.Braun infection in *E. globulus*. In a more recent study, Potts et al. (2016) discovered *Eucalyptus* species from subgenus *Symphyomyrtus* harbouring a significant composition, in both quantity and diversity, of foliar oil compounds tended to be more resistant to myrtle rust than species from subgenus *Eucalyptus*. Myrtle rust is a fungal disease caused by the exotic pathogen *Austropuccinia psidii* Beenken (Beenken 2017; initially identified as *Puccinia psidii* Winter sensu lato or *Uredo rangelii* J.A.Simpson, K.Thomas & C.A.Grgurinovic) which infects plants in the Myrtaceae family (Carnegie and Lidbetter 2012). The virulent pathogen has recently been introduced into Australia (Carnegie 2015) which can cause severe damage to native *Eucalyptus* by reducing biomass accumulation and vigour of the trees, and lead to major negative economic impact (Morin et al. 2012). Following the potential of metabolomics or analyses of plant-derived metabolites as efficient diagnostic assays for plant diseases (Hantao et al. 2013a; Schwab et al. 2008), two terpene compounds i.e. 1,8-cineole or eucalyptol and α-terpinyl acetate were detected in the hybrids of *E. grandis* and *Eucalyptus urophylla* S.T.Blake as potential biomarkers for myrtle rust resistance (Hantao et al. 2013b). This study aims to examine the chemical composition of foliar essential oils in *E. globulus* ssp. *globulus* (hereafter reported as *E. globulus*) and *Eucalyptus obliqua* L'Hér., the two commercially and

ecologically important species from *Symphyomyrtus* and *Eucalyptus*, respectively, and compare the phytochemical properties between their myrtle rust response phenotypes (completely resistant, hypersensitive and highly susceptible) for understanding of the relationship between *Eucalyptus* foliar terpenes and *A. psidii* rust resistance traits.

Materials and Methods

Plant material

A total of 122 *E. globulus* and 87 *E. obliqua* seedlings comprising known *A. psidii* resistant, hypersensitive and susceptible phenotypes were selected from Batch 8 of the rust phenotypic screening trial (as described in Chapter 2) and reserved for phytochemical and microscopic studies. Infected seedlings from the previous rust inoculation were thoroughly flushed to remove any possible rust spores on their leaves, and severely infected twigs and branches were cut to allow for regeneration and new shoot growth. The treated seedlings were kept in naturally lit microclimate rooms maintained at 22 ± 2 °C in the Plant Breeding Institute (PBI) in the University of Sydney, Cobbitty for 8 weeks prior to phytochemical and microscopy sampling.

Leaf sampling

Two replicates of young whole leaf (third or fourth pairs of leaves from the tip) were sampled from all the reserved *E. globulus* and *E. obliqua* seedlings and stored at -80 °C prior to transport (on dry ice) to the University of Melbourne, Parkville for phytochemical analyses. The leaf-sampled seedlings were then re-inoculated with *A. psidii* inoculum (PBI rust collection culture no. 622) according to Sandhu and Park (2013) for the designed microscopic study (as reported in Chapter 3) and phenotype confirmation. The inoculated seedlings were remained in the microclimate rooms until 14 days after inoculation (d.a.i.) for disease reassessment and only leaf samples collected from the confirmed rust response phenotypes were used in the subsequent phytochemical analyses.

Biomass determination and terpene extraction

From the collected leaf samples, leaves of 60 different plants comprising 20 samples of each pre-defined rust response phenotype: completely resistant (asymptomatic),

hypersensitive (presence of fleck, necrosis and chlorosis) and highly susceptible (abundance of fully developed pustules) were randomly selected from each *E. globulus* and *E. obliqua* for leaf chemical analyses. The selected leaves were scanned using scanner with scale included for leaf area determinations prior to grinding to a fine powder in liquid nitrogen with pre-chilled mortar and pestle. The individual ground leaf tissues were weighed for fresh weight calculations before terpene extraction following method described in Goodger et al. (2008). Terpenes were extracted from ground leaves in hexane containing 100 mg l^{-1} tridecane as an internal standard and incubated at 50 °C with agitation at 100 rpm for 7 days. Extracts were then dehydrated with anhydrous Na_2SO_4 and stored at -20 °C until analysed. After terpene extraction, the remaining ground leaf materials were oven-dried at 65 °C to constant weight for dry weight calculations.

Terpene quantification by GC-FID

Hexane extracts were analysed by Gas Chromatography with Flame Ionisation Detection (GC-FID) using a Perkin Elmer Autosystem XC (Perkin Elmer, Melbourne, Australia) fitted with a Zebron ZB-5 low polarity column (30 m × 0.25 mm i.d., Phenomenex) according to Goodger et al. (2016). Helium was used as the carrier gas at a flow rate of 1 ml min^{-1}. The injector temperature was 250 °C and detector temperature was 220 °C. The column temperature was held at 120 °C for 1 min following injection, then ramped at 7 °C min^{-1} to 180 °C and held at that temperature for a further 10 min. The minimum detectable concentration for terpene compounds using a 1 µl injection was 0.01 µg ml^{-1} of hexane extracts. GC-FID constituent identification and quantification was based on standard series of commercially available monoterpenes and sesquiterpenes (Sigma-Aldrich, St. Louis, USA) as described in Goodger et al. (2007) and GC-MS analyses.

Terpene constituent identification by GC-MS

Gas Chromatography Mass Spectrometry (GC-MS) was performed on hexane extracts of bulk leaves from each *Eucalyptus* species using an Agilent 7890A GC and 5975C MS (Agilent Technologies, Santa Clara, USA) following Goodger et al. (2016). Samples (1 µl) were injected in splitless mode into a GC-MS system comprising a

Gerstel 2.5.2 autosampler, a 7890A Agilent gas chromatograph and a 5975C Agilent quadrupole MS (Agilent, Santa Clara, USA). The MS was adjusted according to the manufacturer's recommendations using tris-(perfluorobutyl)-amine (FC-43). The following MS source conditions were used: injection temperature 250 °C, transfer line 280 °C, ion source 230 °C, quadrupole 150 °C, 70 eV (EI mode), 2.66 scans s^{-1}, and scanning range *m/z* 50 – 600. The GC fractionation was performed on a 30 m VF-5MS column with 0.2 µm film thickness and a 10 m Integra guard column (J & W, Agilent). The carrier gas and temperature program were the same as for the GC-FID. Mass spectra were evaluated using Agilent MSD ChemStation E.02.02.1431 for GC-MS, and mono- and sesquiterpenes identified using either the NIST 11 or Adams 2012 mass spectra libraries.

Statistical analyses

All the clearly identified terpene compounds in rust response phenotypes of *E. globulus* and *E. obliqua* were used for statistical analyses in R statistical software version 3.3.0 (R Core Team 2016). Stepwise linear or generalised canonical discriminant analysis was performed using packages MASS (Venables and Ripley 2002) and candisc (Friendly and Fox 2016) in R. The analyses were applied to the terpene amounts adjusted to mg g^{-1} dry weight of leaf tissues, log-transformed to minimise the domination of compounds with relatively large concentrations, and normalised to a common standard deviation. The variables included in the formation of discrimination functions for the classification of correct group within *Eucalyptus* species were determined with a stepwise forward variable selection using a Wilks' Lambda as a criterion and an *F* statistic factor to establish the significance of the changes in Lambda when a new variable was tested in package klaR (Weihs et al. 2005) in R. Multivariate analysis of variance (MANOVA) was performed to test the significance of the fitted discriminant models by consideration of the selected variables simultaneously. The prediction capacity of the discriminant models within species was studied by leave-one-out cross validation in order to determine the stability of the model. Cross species validations were also carried out by using *E. globulus* and *E. obliqua* discriminant models fitted with the terpene variables common in both, and in comparison with the significant compounds identified for myrtle rust resistance in *E. grandis* × *E. urophylla*

hybrids (Hantao et al. 2013b). Differences in total oil content, total mono- and sesquiterpene content per leaf area and per tissue dry weight were examined between different rust response phenotypes of *E. globulus* and *E. obliqua*.

Results

A range of terpene compounds including monoterpenes and sesquiterpenes (Table 4-1) were identified in the leaves of *E. globulus* and *E. obliqua*. Visual inspection of their respective chromatograms revealed no obvious correlations between chromatographic profiles and resistance or susceptibility of the analysed samples to the myrtle rust disease. In the stepwise forward variable selection, a total of six and four terpene variables were identified as significant at $P < 0.10$ by an approximate F-test decision using Wilks' Lambda criterion (Table 4-2) in *E. globulus* and *E. obliqua*, respectively for differentiation of rust response phenotypes i.e. completely resistant, hypersensitive and highly susceptible. The selected compounds in *E. globulus* (Figure 4-1) are four monoterpenes (β-pinene, δ-terpinene, *cis*-*p*-menth-2-en-1-ol and geraniol) and two sesquiterpenes (bicyclogermacrene and globulol), while those in *E. obliqua* (Figure 4-2) are all sesquiterpenes (α-caryophyllene, δ-cadinene, caryophyllene oxide and longifolenaldehyde). The identified compounds were all different between *Eucalyptus* species and different from the tentative rust resistance biomarkers viz. 1,8-cineole and α-terpinyl acetate (Figure 4-3) reported in *E. grandis* × *E. urophylla* hybrids (Hantao et al. 2013b).

Generalised canonical discriminant analyses based on stepwise model selected terpene variables resulted in highly significant ($P < 0.001$) and significant ($P < 0.01$) separation between rust response phenotypes in *E. globulus* and *E. obliqua*, respectively (Table 4-3). The phenotypes separation in *E. globulus* (Figure 4-4) was explained by 65.8% of first discriminant function (x-axis) and 34.2% of second discriminant function (y-axis), while the separation in *E. obliqua* (Figure 4-5) was achieved by 80.6% and 19.4% of first and second discriminant functions, respectively. During cross validation within species, the classification model for each *Eucalyptus* species was rebuilt and the cases removed were classified in a new model with a classification matrix as summarised in Table 4-4. Prediction capacity of discriminant models fitted for *E. globulus* showed

Table 4-1: Terpene compounds identified in *Eucalyptus globulus* and *Eucalyptus obliqua* leaf samples by GC-FID and GC-MS

Compound name	Formula	Terpene class	Retention time (min) [a]	CAS [b]
α-Thujene	$C_{10}H_{16}$	Monoterpene	6.42	2867-05-2
α-Pinene	$C_{10}H_{16}$	Monoterpene	6.65	80-56-8
Sabinene	$C_{10}H_{16}$	Monoterpene	7.68	3387-41-5
β-Pinene	$C_{10}H_{16}$	Monoterpene	7.83	127-91-3
Myrcene	$C_{10}H_{16}$	Monoterpene	8.06	123-35-3
α-Phellandrene	$C_{10}H_{16}$	Monoterpene	8.52	99-83-2
δ-3-Carene	$C_{10}H_{16}$	Monoterpene	8.77	13466-78-9
p-Cymene	$C_{10}H_{14}$	Monoterpene	8.96	99-87-6
Limonene	$C_{10}H_{16}$	Monoterpene	9.08	138-86-3
β-Phellandrene *	$C_{10}H_{16}$	Monoterpene	9.12	555-10-2
1,8-Cineole	$C_{10}H_{18}O$	Monoterpene	9.14	470-82-6
γ-Terpinene	$C_{10}H_{16}$	Monoterpene	9.71	99-85-4
δ-Terpinene	$C_{10}H_{16}$	Monoterpene	10.29	586-62-9
Linalool	$C_{10}H_{18}O$	Monoterpene	10.55	78-70-6
cis-p-Menth-2-en-1-ol	$C_{10}H_{18}O$	Monoterpene	11.08	29803-82-5
trans-p-Menth-2-en-1-ol	$C_{10}H_{18}O$	Monoterpene	11.42	29803-81-4
Terpinen-4-ol	$C_{10}H_{18}O$	Monoterpene	12.12	562-74-3
Cryptone	$C_{9}H_{14}O$	Monoterpene	12.23	500-02-7
α-Terpineol	$C_{10}H_{18}O$	Monoterpene	12.37	98-55-5
cis-piperitol *	$C_{10}H_{18}O$	Monoterpene	12.41	16721-38-3
trans-Piperitol	$C_{10}H_{18}O$	Monoterpene	12.60	16721-39-4
cis-Carveol	$C_{10}H_{16}O$	Monoterpene	13.05	1197-06-4
Geraniol	$C_{10}H_{18}O$	Monoterpene	13.21	106-24-1
Piperitone	$C_{10}H_{16}O$	Monoterpene	13.33	89-81-6
α-Terpinyl acetate	$C_{12}H_{20}O_2$	Monoterpene	14.66	80-26-2
α-Terpinene	$C_{10}H_{16}$	Monoterpene	14.77	99-86-5
β-Geranyl acetate	$C_{12}H_{20}O_2$	Monoterpene	15.09	105-87-3
Copaene	$C_{15}H_{24}$	Sesquiterpene	15.17	3856-25-5
β-Elemene	$C_{15}H_{24}$	Sesquiterpene	15.35	515-13-9
α-Gurjunene	$C_{15}H_{24}$	Sesquiterpene	15.61	489-40-7

Table 4-1 (*Continued*)

trans-Caryophyllene	$C_{15}H_{24}$	Sesquiterpene	15.70	87-44-5
Calarene	$C_{15}H_{24}$	Sesquiterpene	15.81	17334-55-3
Aromadendrene	$C_{15}H_{24}$	Sesquiterpene	16.05	109119-91-7
α-Caryophyllene *	$C_{15}H_{24}$	Sesquiterpene	16.07	6753-98-6
allo-Aromadendrene	$C_{15}H_{24}$	Sesquiterpene	16.13	25246-27-9
α-Selinine	$C_{15}H_{24}$	Sesquiterpene	16.15	473-13-2
9-epi-*E*-Caryophyllene	$C_{15}H_{24}$	Sesquiterpene	16.34	68832-35-9
Viridiflorene	$C_{15}H_{24}$	Sesquiterpene	16.71	21747-46-6
Bicyclogermacrene	$C_{15}H_{24}$	Sesquiterpene	16.78	24703-35-3
δ-Cadinene	$C_{15}H_{24}$	Sesquiterpene	17.02	483-76-1
α-Elemol	$C_{15}H_{26}O$	Sesquiterpene	17.54	639-99-6
Unidentified compound 1	NA	Sesquiterpene	17.72	NA
Spathulenol	$C_{15}H_{24}O$	Sesquiterpene	17.79	6750-60-3
Caryophyllene oxide	$C_{15}H_{24}O$	Sesquiterpene	17.90	1139-30-6
Viridiflorol	$C_{15}H_{26}O$	Sesquiterpene	18.02	552-02-3
Globulol **	$C_{15}H_{26}O$	Sesquiterpene	18.11	51371-47-2
Unidentified compound 2	NA	Sesquiterpene	18.15	NA
γ-Eudesmol	$C_{15}H_{26}O$	Sesquiterpene	18.35	1209-71-8
α-Eudesmol	$C_{15}H_{26}O$	Sesquiterpene	18.72	473-16-5
β-Eudesmol	$C_{15}H_{26}O$	Sesquiterpene	18.75	473-15-4
Longifolenaldehyde	$C_{15}H_{24}O$	Sesquiterpene	19.81	19890-84-7

[a] Retention time was based on standard series of commercially available terpene compounds using Zebron ZB-5 low polarity column (30 m × 0.25 mm i.d., Phenomenex)

[b] CAS Registry Numbers follow Chemical Abstracts Service (CAS) Registry Database, American Chemical Society (ACS)

* Compounds only present in *E. obliqua*

** Compounds only present in *E. globulus*

NA = not applicable for unidentified compounds

Table 4-2: Terpene compounds selected by stepwise forward variable selection using Wilks' Lambda criterion ($P < 0.10$) for differentiation of rust response phenotypes in *Eucalyptus globulus* and *Eucalyptus obliqua*

Species	Selected variable	Variable code	Wilks' Lambda	*F* statistic	*P* value
E. globulus	Bicyclogermacrene	v1	0.833	5.695	0.0056
	Globulol	v2	0.752	3.023	0.0565
	Geraniol	v3	0.663	3.716	0.0305
	β-Pinene	v4	0.603	2.661	0.0788
	cis-p-Menth-2-en-1-ol	v5	0.549	2.595	0.0839
	δ-Terpinene	v6	0.501	2.530	0.0893
E. obliqua	δ-Cadinene	v7	0.898	3.237	0.0466
	Caryophyllene oxide	v8	0.827	2.419	0.0981
	Longifolenaldehyde	v9	0.740	3.238	0.0467
	α-Caryophyllene	v10	0.655	3.506	0.0369

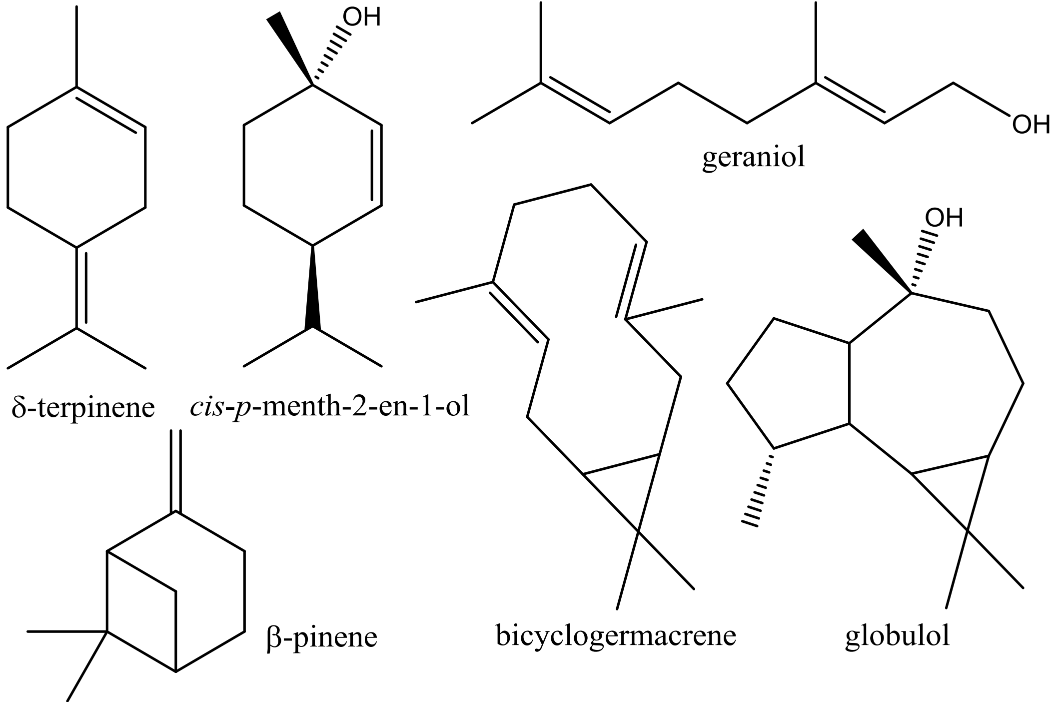

Figure 4-1: Significant compounds identified in *Eucalyptus globulus* in association with myrtle rust resistance.

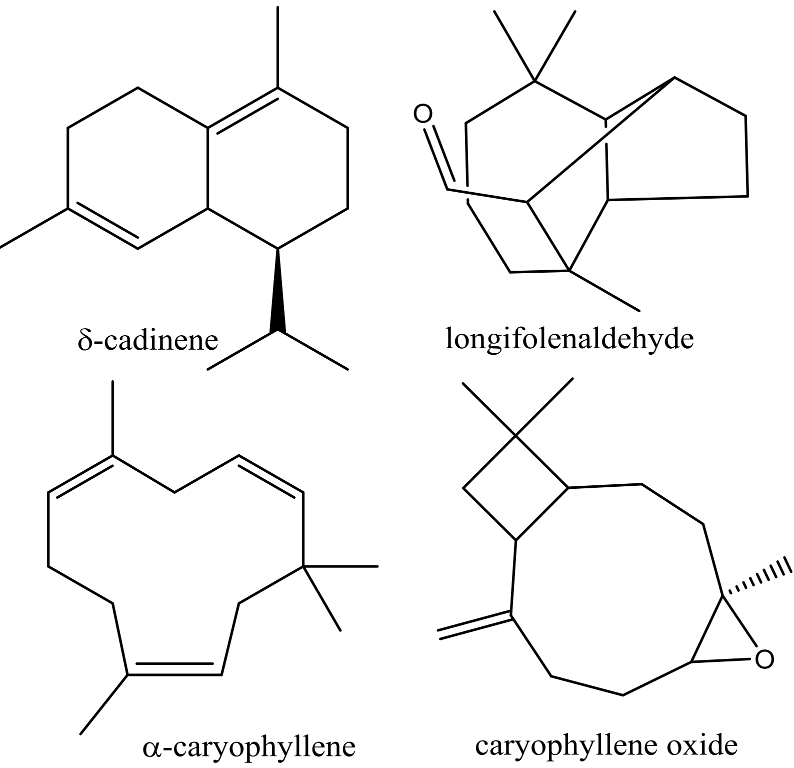

Figure 4-2: Significant compounds identified in *Eucalyptus obliqua* in association with myrtle rust resistance.

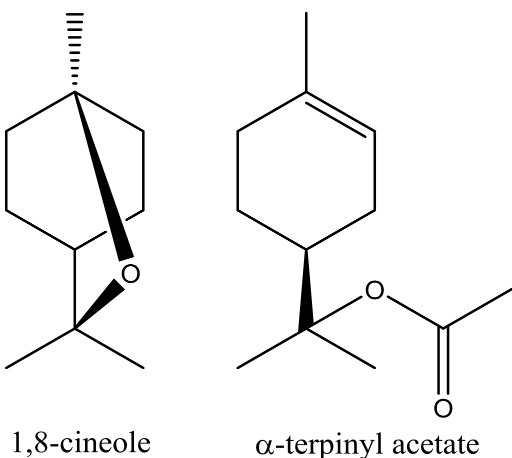

Figure 4-3: Tentative biomarkers for myrtle rust resistance in *Eucalyptus grandis* × *Eucalyptus urophylla* hybrids (Hantao et al. 2013b).

Table 4-3: Summary of the multivariate analyses of stepwise model selected terpene compounds in response to myrtle rust resistance in *Eucalyptus globulus* and *Eucalyptus obliqua* with variance components estimated through the Wilks' Lambda statistical test

Species	df	Wilks' Lambda	Num df	Den df	*F* statistic	*P* value [a]
E. globulus	2	0.501	12	104	3.581	< 0.001 ***
E. obliqua	2	0.655	8	108	3.187	0.003 **

[a] Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

df = degrees of freedom

Num df = numerator degrees of freedom

Den df = denominator degrees of freedom

Table 4-4: Prediction capacity of rust response discriminant model by cross validation according to response phenotype in *Eucalyptus globulus* and *Eucalyptus obliqua*

Species	Phenotype	Predicted group membership [a]			Total
		Resistant	Hypersensitive	Susceptible	
E. globulus	Resistant	12	2	6	20
	Hypersensitive	5	13	2	20
	Susceptible	7	3	10	20
E. obliqua	Resistant	8	10	2	20
	Hypersensitive	2	17	1	20
	Susceptible	2	8	10	20

[a] Predicted group based on number of cases correctly classified in cross validation within species

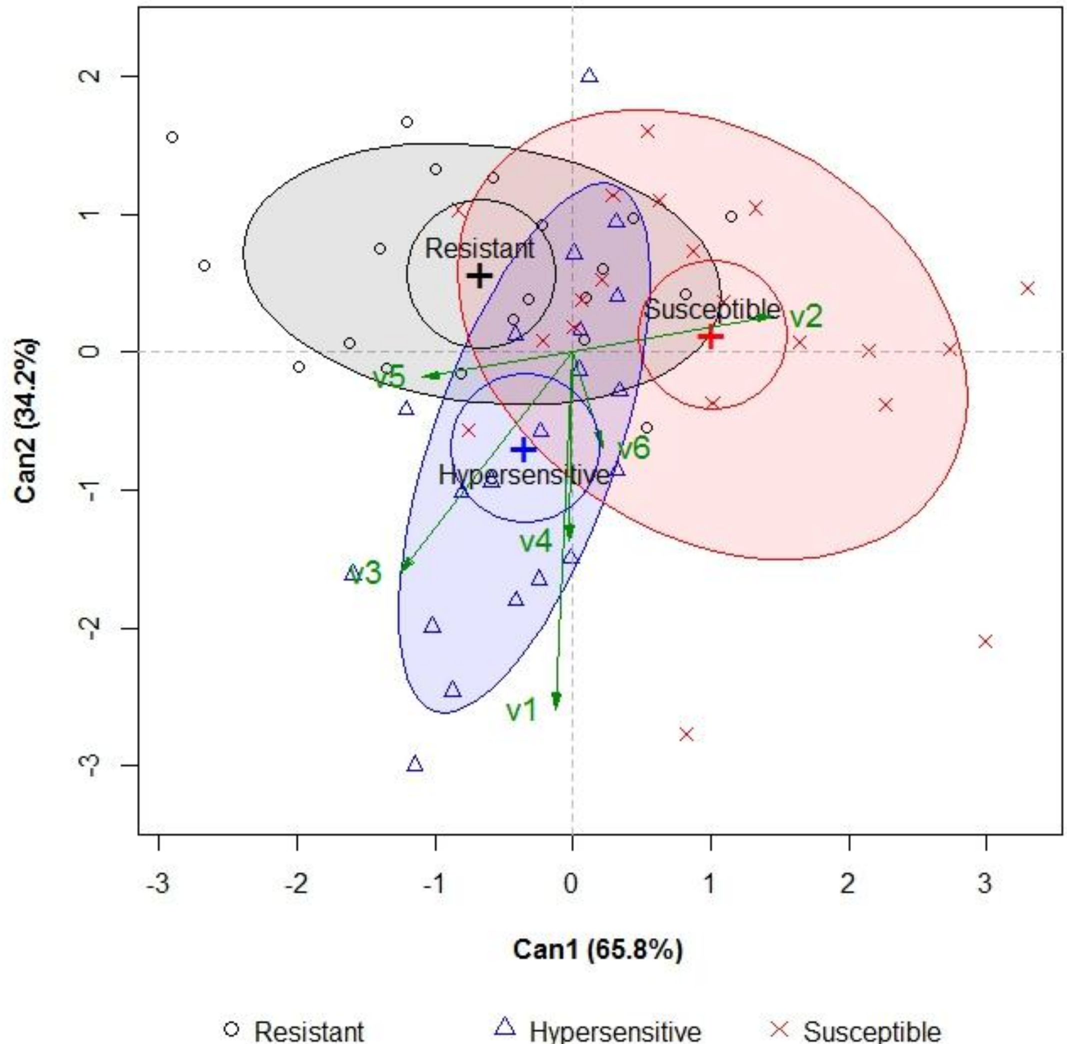

Figure 4-4: Scatterplot of canonical discriminant functions between rust response phenotypes of *Eucalyptus globulus* with 95% confidence circle and 70% confidence ellipse plotted around the canonical mean of each phenotype. Variable vector labels according to the variable codes in Table 4-2.

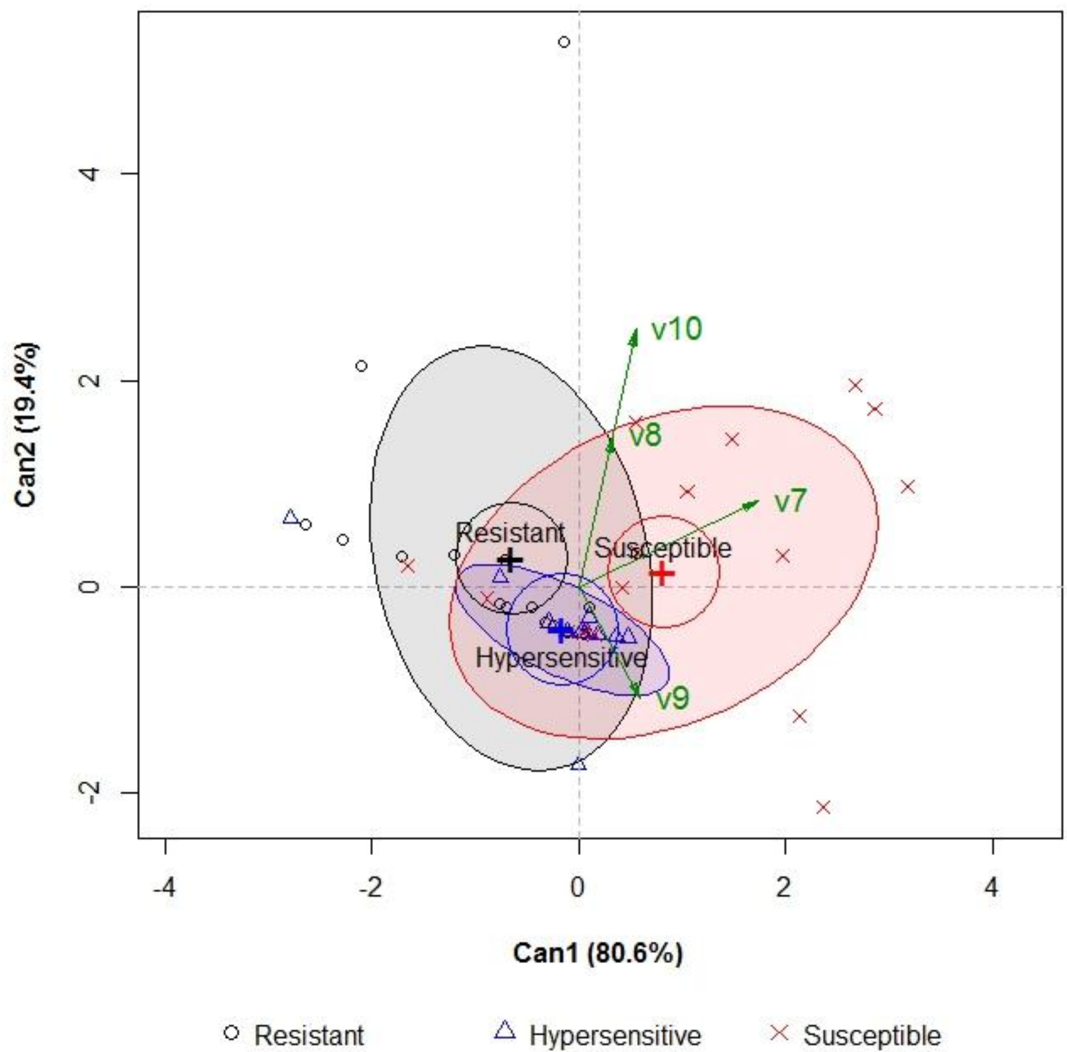

Figure 4-5: Scatterplot of canonical discriminant functions between rust response phenotypes of *Eucalyptus obliqua* with 95% confidence circle and 70% confidence ellipse plotted around the canonical mean of each phenotype. Variable vector labels according to the variable codes in Table 4-2.

66.7% of correctly classified cases and 58.3% in the case of *E. obliqua*. However, no predictive power was observed in cross species validation which resulted in 63.3 to 66.7% of error rates for *E. globulus* models applied to *E. obliqua* and vice versa (Table 4-5).

When canonical discriminant analysis was carried out on just 1,8-cineole and α-terpinyl acetate, the compounds for myrtle rust resistance identified in *E. grandis* × *E. urophylla* hybrids, there was no significant separation of the phenotypes in either *E. globulus* (P = 0.334) or *E. obliqua* (P = 0.391), further suggesting the ineffectiveness of the transferring biomarkers for rust response phenotypes between *Eucalyptus* species. Analysis of variance also indicated no significant difference in total oil content, total mono- and sesquiterpene contents between different rust response phenotypes of studied *Eucalyptus* species except total oil (P = 0.092) and total sesquiterpene (P = 0.029) per tissue dry weight of *E. globulus*.

Discussion

The key finding of the present work is the identification of unique sets of terpene constituents in each *Eucalyptus* species associated with myrtle rust phenotype. While there is significant discrimination using sets of compounds within species there is no consistency between species and a discriminant function for one species is ineffective when applied to the other.

Differences in terpene profile between *Eucalyptus* species are well known to be driven by variation in terpene synthase enzymes which catalyse the conversion of a small set of substrates into ranges of terpene products (Keszei et al. 2008; Külheim et al. 2015). Albeit no terpene compounds are found exclusively in *Eucalyptus*, a considerable literature has reported chemotypic variations in foliar terpenes at individual, population and species levels (Boland et al. 1991; Brophy and Southwell 2002; Keszei et al. 2008; Padovan et al. 2012), resulted in identification of large number of chemotypes in *Eucalyptus* species (Padovan et al. 2014). Chemotypes are defined as discontinuous chemical differences between individuals within a population or a plant species that

Table 4-5: Prediction capacity of rust response discriminant model by cross validation between *Eucalyptus globulus* and *Eucalyptus obliqua*

Species	Phenotype	Predicted group membership in other species [a]			Total
		Resistant	Hypersensitive	Susceptible	
E. globulus	Resistant	20	0	0	20
	Hypersensitive	20	0	0	20
	Susceptible	20	0	0	20
E. obliqua	Resistant	14	0	6	20
	Hypersensitive	17	0	3	20
	Susceptible	12	0	8	20

[a] Predicted group based on number of cases correctly classified in cross validation between species, i.e. *E. globulus* fitted model validated by *E. obliqua* objects and vice versa

cannot be distinguished from each other by morphometric properties (Keszei et al. 2008; Penfold and Willis 1953). Padovan et al. (2014) discovered 175 myrtaceous species with more than one chemical form or chemotype, emphasizing the presence of foliar terpene diversity within the family and their complexity in responses to local environments. Different plant chemotypes of myrtaceous species were revealed to establish different levels of ecological interactions with biological agents or insect herbivores as evident in *Melaleuca quinquenervia* (Cav) S.T.Blake (Padovan et al. 2010) and *Melaleuca alternifolia* (Maiden & Betche) Cheel (Bustos-Segura et al. 2015). *M. quinquenervia* chemotype dominated by viridiflorol enhanced the oviposition preference of psyllid *Boreioglycaspis melaleucae* Moore (Wheeler and Ordung 2005), while *E*-nerolidol chemotype increased the performance of weevil *Oxyops vitiosa* Pascoe (Wheeler 2006). Larval growth rate of *Faex* sp. (Coleoptera, Chrysomelidae) beetles was found higher in *M. alternifolia* chemotypes with high concentration of 1,8-cineole, and chemotypes with high concentration of terpinolene were found less damage by *Paropsisterna tigrina* Chapuis (Bustos-Segura et al. 2015).

Genetic mosaicism (Edwards et al. 1990; Gill 1986; Whitham and Slobodchikoff 1981) in *Eucalyptus* where several chemotypes exist within a single plant (Edwards et al. 1993; Penfold and Morrison 1936) result from accumulation of somatic mutations. Within mosaic trees leaves with much higher concentrations of 1,8-cineole were found to be more resistant to several species of Christmas beetles (*Anoplognathus* spp.) than low cineole leaves in *Eucalyptus melliodora* A.Cunn. ex Schauer and *Eucalyptus sideroxylon* A.Cunn. ex Woolls (Padovan et al. 2012). The monoterpenes 1,8-cineole and α-pinene appear to be the most common and abundant terpenoids across the majority of species in the Myrtaceae (Boland et al. 1991; Brophy et al. 2006; Brophy and Southwell 2002; Keszei et al. 2010) except species from the tribes Myrteae, Kanieae, Syzygieae, Xanthostemoneae, Syncarpieae and Lindsayomyrteae with 1,8-cineole replaced by sesquiterpene β-caryophyllene, and species from Lindsayomyrteae, Metrosidereae and Backhousieae with α-pinene substituted by sesquiterpene *allo*-aromadendrene as the most abundant terpenes (Padovan et al. 2014). Both 1,8-cineole and α-pinene have been shown to exhibit antibacterial, antifungal and antiviral activities *in vitro*, and contribute to the bio-activity of foliar essential oil in *Eucalyptus*-related

pharmaceuticals (Elaissi et al. 2012a; Elaissi et al. 2012b; Sebei et al. 2015). Despite 1,8-cineole being reported to play an important role in defences against insect herbivores (Padovan et al. 2012) and browsing marsupials (Lawler et al. 1999; O'Reilly-Wapstra et al. 2004), Bustos-Segura et al. (2015) discovered that *M. alternifolia* plants with a high concentration of 1,8-cineole were more likely to be infected by *A. psidii* pathogen under controlled environmental conditions. Despite the identification of 1,8-cineole, along with α-terpinyl acetate, as biomarkers for rust-resistant clones in *E. grandis* × *E. urophylla* hybrids (Hantao et al. 2013b), neither compound was found to be associated with resistance in *E. globulus* and *E. obliqua*.

Intra- and interspecific variations of plant secondary metabolites can be influenced by both genetic and environmental factors (Andrew et al. 2005; Bustos-Segura et al. 2017; Külheim et al. 2011; O'Reilly-Wapstra et al. 2014; Potts et al. 2016; Senior et al. 2016) which result in the present geographical structure of their distribution. Although several studies have shown that *E. globulus* (ssp. *globulus*) displays geographically structured genetic variation in plant growth and fitness such as wood density and survival (Dutkowski and Potts 1999; Potts and Jordan 1994a; Steane et al. 2006), Wallis et al. (2011) reported that variation between trees within populations contributed most variation in leaf chemistry, followed in importance by variation between geographic regions. While concentrations of 1,8-cineole and α-pinene were found to exhibit variability associated with *Eucalyptus* subgeneric affiliation and/or geographic location (Boland et al. 1991; Steinbauer 2010), Potts et al. (2016) found increasing levels of 1,8-cineole and α-pinene, as well as limonene and pinocarvone in subgenus *Symphyomyrtus* to be associated with greater resistance to myrtle rust relative to subgenus *Eucalyptus*. The existence of a co-evolutionary arms race between *Eucalyptus* and their local browsing herbivores or virulent pathogens may explain the diversification of defensive terpene profiles between and within species (Padovan et al. 2014). While natural selection may favour reduction or elimination of secondary metabolites considering their costs of production (Neilson et al. 2013; Stamp 2003), *Eucalyptus* species are maintaining significant qualitative and quantitative variation in their terpene profiles as a viable evolutionary response for survival in a large range of environmental conditions (Keszei et al. 2008; Padovan et al. 2014). Nevertheless, no consensus terpene

compounds were identified between *E. globulus* and *E. obliqua* against the same invasive pathogen (*A. psidii*) in the present study in which both host and pathogen had no recent evolutionary connections (Tobias et al. 2016). This finding may due to the combined effects of different terpenes or synergistic effects of terpenes in combination with other plant metabolites in different *Eucalyptus* species in producing equivalent defensive responses against the pathogen, or the canonical discriminant function technique in the present analysis may result in spurious conclusions despite their statistical significance. Cross-validation is an extreme test of a fitted model on an independent data set and it is difficult to separate the two possible causes. Further to this, molecular analyses of genetic differences between rust resistant and susceptible phenotypes in *Eucalyptus* may be of significant in deciphering the resistance mechanisms underlying *Eucalyptus – A. psidii* pathosystem and to provide valuable insights into the drivers for resistance.

Conclusion

The current study has identified six and four unique terpene compounds in *E. globulus* and *E. obliqua*, respectively, that significantly differentiated between their rust response. Despite their significance within species these discriminant functions and reported rust resistance biomarkers are ineffective in predicting the rust responses between *Eucalyptus* species. These findings indicate the complexity of *Eucalyptus* defensive chemistry in response to pathogen invasion. Further investigation of the molecular mechanisms differing between rust resistant and susceptible phenotypes may elucidate the possible role of *Eucalyptus* foliar terpenes in myrtle rust resistance.

This page intentionally left blank

Chapter 5

Genome wide association study of myrtle rust (*Austropuccinia psidii*) resistance in *Eucalyptus obliqua* (subgenus *Eucalyptus*)

Abstract

Myrtle rust (*Austropuccinia psidii* Beenken) is exotic to Australia yet specific resistance is present in a range of naïve host myrtaceous plants. Resistance to myrtle rust is quantitative in nature and controlled by variation in many genes or multiple interacting loci. This study aims to discover relationships between genomic sequence variation and multiple resistance traits i.e. numeric severity, binary symptomatic, hypersensitive and pustulation response to *A. psidii* in *Eucalyptus obliqua* L'Hér., a representative species from subgenus *Eucalyptus*. This dissection will use a genome wide association study (GWAS) to identify novel genomic loci associated with *A. psidii* resistance in *E. obliqua*, and to gain insight into the role of the previously reported rust resistance loci (*Ppr1-5*) in species from subgenus *Symphyomyrtus*. A total of 1.13 million single nucleotide polymorphisms (SNPs) were genotyped and tested for association with rust resistance in 624 phenotyped *E. obliqua* individuals from different, single mother tree seedlots. The analysis detected 33 highly significant SNP-trait associations, of which 26 associated with the binary trait symptomatic/asymptomatic response to *A. psidii*. This study provides an integrated view of *A. psidii* resistance resources across *Eucalyptus* subgenera as well as information of trait-associated putative candidate genomic regions. These merit further examination towards the discovery of resistance genes and the development of marker based resistance breeding programs.

Introduction

Myrtle rust caused by the fungus *Austropuccinia psidii* Beenken (Beenken 2017; previously reported as *Puccinia psidii* Winter sensu lato or *Uredo rangelii* J.A.Simpson, K.Thomas & C.A.Grgurinovic) poses a major threat to global biodiversity and *Eucalyptus* based forest industries worldwide (Coutinho et al. 1998; Grgurinovic et al. 2006). The pathogen was first described by Winter in 1884 on infected common guava (*Psidium guajava* L., reported as *Psidium pomiferum* L.) of the plant family Myrtaceae in Brazil (Winter 1884). It is believed to be South American origin (Simpson et al. 2006) but gained international notoriety by causing severe damage to commercially grown allspice (*Pimenta dioica* (L.) Merr.) in Jamaica and Florida (MacLachlan 1938; Marlatt and Kimbrough 1979), guava (*Psidium guajava* L.) plantations in Brazil (Ferrari et al. 1997; Ribeiro and Pommer 2004) and exotic *Eucalyptus* species in South America (Ferreira 1983, 1989; Joffily 1944). *A. psidii* has a wide host range in the Myrtaceae family (Carnegie and Lidbetter 2012; Morin et al. 2012), enabling it, over the past decades, to spread rapidly across different continents from South America (Simpson et al. 2006) to North America (Marlatt and Kimbrough 1979; Zambino and Nolan 2011), Asia (du Plessis et al. 2017; Kawanishi et al. 2009; McTaggart et al. 2016a; Zhuang and Wei 2011), Africa (Roux et al. 2013), and Oceania i.e. Australia (Carnegie et al. 2010) and New Zealand (Cavill 2017).

Incursion of myrtle rust in Australia with its natural ecosystems dominated by Myrtaceae has raised concerns about potentially devastating impacts this exotic pathogen may have on the Australian landscape and flora (Carnegie and Cooper 2011). *A. psidii* was first detected in April 2010 at a cut flower growing facility on the central coast of New South Wales (Carnegie et al. 2010) before it spread throughout natural ecosystems along the east coast of Australia (Carnegie and Lidbetter 2012; Pegg et al. 2014b) with limited extensions to Victoria, Tasmania and the Northern Territory (Carnegie et al. 2016). Preliminary bioclimatic modelling predicted most of the eastern coastal areas and northern parts of the Northern Territory are the regions at high risk to *A. psidii* (Booth and Jovanovic 2012; Booth et al. 2000) but a more recent climatic niche model indicated a higher degree of cold tolerance for the pathogen and extension

of its potential risk to mesic midlands of Victoria and northern extremes of Tasmania (Kriticos et al. 2013).

Since the first detection of the rust in Australia, significant research work has been conducted under the National Transitional Management Strategy (Plant Health Australia 2011) to assist in maintaining quarantine protection, improving diagnostic methods and developing resistance breeding strategies (Carnegie and Cooper 2011). Although only one strain or biotype of *A. psidii* has been reported to be present in Australia to date (Carnegie and Lidbetter 2012; Sandhu et al. 2016) it is vital for Australian biosecurity authorities to continue quarantine restrictions to protect against further incursions of other strains into the country because a different pathogen strain could have different virulence or combine with the existing strain to exacerbate the disease and increase the number of species affected (Carnegie and Cooper 2011). As part of the effort for rust management, a standardised method for rust screening was established with the development of the National Myrtle Rust Screening Facility to assess the threat *A. psidii* poses to Australian native flora and commercial *Eucalyptus* species (Sandhu and Park 2013). Disease severity scale and field rating systems (Morin et al. 2012; Pegg et al. 2012; Sandhu and Park 2013) were also developed for germplasm screening in glasshouse and field conditions to evaluate the direct impacts of rust disease on Myrtaceae naïve hosts and potential ecological impacts to Australian ecosystems.

For industries relying on Myrtaceae improved species selection and sustainable breeding strategy, including production nurseries and plantation forests, depends upon continued screening host populations for resistance (Pegg et al. 2014a; Roux et al. 2015). The deployment of resistant genotypes is an effective way of managing rust disease in commercial plantations as highlighted for exotic *Eucalyptus* in Brazil (Miranda et al. 2013; Pinto et al. 2014; Silva et al. 2013). Myrtle rust was found to infect 43 exotic Myrtaceae species in Central and South America of which 36 are native to Australia, but causes disease in only 28 of more than 1000 potential host species in its native range (Simpson et al. 2006). The limited host range of *A. psidii* in the Americas may due to the pathogen having exerted strong selection pressure on the native Myrtaceae for many generations, and the indigenous plants had evolved resistance to

the pathogen during co-evolution (Helfer 2014; Simpson et al. 2006; Thompson and Burdon 1992). While disease control may be achieved by fungicide or chemical treatment (Coutinho et al. 1998; Glen et al. 2007; Martins et al. 2011), selection of resistant species or development of resistant cultivars/clones has been a priority for rust management in *Eucalyptus* plantations across South America (Dianese et al. 1986; Ferreira 1989; Furtado and Marino 2003; Xavier et al. 2001).

After their introduction into new environments pathogens are frequently far more virulent on naïve hosts an observation reported for many non-native tree pathogens, with often devastating effect on native species (Carnegie et al. 2016; Glen et al. 2007). Native pathogens affecting exotic trees or exotic pathogens introduced to new areas often have little apparent impact in the early stages of an epidemic (Bright 1998). The initial low levels of disease may reflect the incubation period of the pathogen prior to multiplication of virulent genotype(s) (Wingfield et al. 2001). While major ecological impacts and large scale tree mortality have not yet been attributed to *A. psidii* in Australia, studies show that the pathogen is severely affecting key species in some natural ecosystems i.e. rainforest understorey species *Rhodamnia rubescens* (Benth.) Miq. (brush turpentine) and *Rhodomyrtus psidioides* (G.Don) Benth. (native guava), and wetland species *Melaleuca quinquenervia* (Cav.) S.T. Blake, and the susceptible host range is expanding as the fungus becomes established in new geographic regions (Carnegie and Cooper 2011; Carnegie et al. 2016; Carnegie and Lidbetter 2012; Morin et al. 2012; Pegg et al. 2014b). Although the *Eucalyptus* forest dependent industries have not yet been severely affected (Carnegie 2015), the essential oil industry, particularly that based on lemon myrtle (*Backhousia citriodora* F. Muell.), has been significantly impacted with infection of immature leaves and shoots causing substantial branch defoliation, shoot dieback and stunted growth of infected plants (Carnegie and Cooper 2011; CRC Plant Biosecurity 2016). Following the first incursion of myrtle rust in 2010, screening for resistant genotypes across Myrtaceae species in glasshouse and field conditions has been ongoing in Australia (Carnegie and Lidbetter 2012; Morin et al. 2012; Pegg et al. 2014a; Pegg et al. 2014b). International forest industries, especially those relying on Australian *Eucalyptus*, may also benefit from adoption of resistant germplasm developed through the screening programs in Australia.

Primarily driven by the aims of reducing losses due to the disease and resistance breeding in plantation forests and to understand the inheritance and genetic architecture of myrtle rust resistance in *Eucalyptus* species a number of quantitative genetic and association studies have been conducted (Alves et al. 2012; Butler et al. 2016; Mamani et al. 2010; Thumma et al. 2013). The first quantitative genetic study of myrtle rust resistance was carried out on *Eucalyptus grandis* W. Hill ex Maiden, an Australian species from subgenus *Symphyomyrtus* widely grown for timber, charcoal production and pulp in many parts of the world (Junghans et al. 2003a). With its whole genome sequenced and released (Myburg et al. 2014) and because of its international economic importance, *E. grandis* is currently recognised as a model species in the *Eucalyptus* genus (Neale and Kremer 2011) providing a powerful tool for comparative biology and genetic improvement, e.g. disease resistance, for the genus. A major quantitative trait locus (QTL) for *A. psidii* resistance, named *Ppr1*, was first identified using a large full-sib family of *E. grandis* using bulked segregant analysis (Junghans et al. 2003a). This study suggested a simple genetic architecture with a large proportion of myrtle rust resistance variation might be controlled by a small number of genes with relatively large effects. The *Ppr1* locus was identified on linkage group 3 using a microsatellite reference map for *Eucalyptus* (Brondani et al. 2006), and subsequently validated by association genetics in additional families from independent crosses between *E. grandis* and other *Eucalyptus* species, i.e. *Eucalyptus tereticornis* Sm. and *Eucalyptus camaldulensis* Dehnh. (Mamani et al. 2010). Analysis of gene expression in *E. grandis* indicated two possible responses may be involved in the host defense against *A. psidii*, viz. cellular polarisation and systemic resistance mechanisms, with the genes for the respective mechanisms differentially expressed between rust resistant and susceptible phenotypes (Moon et al. 2007).

E. grandis clones with favourable alleles at the *Ppr1* locus have since been selected and deployed in areas prone to rust disease in Brazil (Labate et al. 2009) and this has been an effective strategy to date in minimizing rust losses. A more recent study has found that previously resistant clones are being infected by a new race of rust pathogen in the later stages of cultivation (Graça et al. 2011) with this failure of *Ppr1* triggering the

need for new investigations and discovery of genes underlying the genetic control of rust resistance in *Eucalyptus*. Alves et al. (2012) discovered additive (major gene) and non-additive (epistatic) effects in hybrids between *E. grandis*, *E. camaldulensis*, *E. tereticornis*, *Eucalyptus globulus* Labill., *Eucalyptus dunnii* Maiden and *Eucalyptus urophylla* S.T. Blake suggesting complexity in *Eucalyptus – A. psidii* interactions. Further analysis indicates rust resistance is quantitative in nature, controlled by multiple genes of variable effect rather than simple as indicated in the early linkage studies (Santos et al. 2014; Thumma et al. 2013). *In silico* searches for potential markers or candidate genes in the *E. grandis* reference genome found a large number of nucleotide-binding leucine-rich repeat (NB-LRR) coding sequences, the major class of plant resistance genes (Dodds and Rathjen 2010; Fluhr 2001), in genomic regions associated with resistance (Thumma et al. 2013; Tobias 2012). Comparison of allele frequencies between rust resistant and susceptible gene pools of *E. globulus* found numerous single nucleotide polymorphism (SNP) markers with modest effects, with some of these located in NB-LRR resistance genes in the vicinity of the *Ppr1* locus (Thumma et al. 2013). Most recently, QTL analysis using a binary response classification in *E. globulus* identified new QTLs underlying different resistance mechanisms against *A. psidii*, with two QTLs (*Ppr2* and *Ppr3*) influencing the symptomatic response (whether a plant exhibited disease symptoms), and two (*Ppr4* and *Ppr5*) influencing the presence or absence of a hypersensitive reaction (Butler et al. 2016).

With the advent of high-throughput genotyping assays and the decreasing cost of sequencing technologies (Elshire et al. 2011; Goodwin et al. 2016; Metzker 2010; Wang et al. 2014), association mapping using high density genome wide markers, or genome wide association study (GWAS), has become a viable method for the dissection of complex trait variation in plants (Huang et al. 2012; Kump et al. 2011; Wen et al. 2015; Zhu et al. 2008). GWAS exploits historical recombination events accumulated over multiple generations in natural populations detecting associations between phenotypes (observable characteristics) of interest and the genotypes (genetic constitution or DNA sequence variants) of studied organisms (Nordborg and Weigel 2008). To avoid discovery of false marker-trait associations such studies require adequate assessment and accuracy of population structure (Kang et al. 2008; Larsson et al. 2013; Stich et al.

2008; Yu et al. 2006). GWAS has been successfully applied in QTL mapping for diverse traits in many plant species of economic importance such as oat, maize, wheat, barley and rice (Klos et al. 2017; Lipka et al. 2013; Maccaferri et al. 2015; Wang et al. 2012; Zhao et al. 2011).

The first GWAS in forest trees investigated wood characteristics (Porth et al. 2013), and other ecological important traits i.e. biomass, ecophysiology and phenology traits (McKown et al. 2014) in *Populus trichocarpa* Torr. & A. Gray ex. Hook. (poplar or black cottonwood), a species of high economic and ecological value in North America and in plantations in temperate regions around the world (Cronk 2005; Rae et al. 2007; Tuskan et al. 2006). These investigations yielded numerous SNPs underlying wood chemistry and ultrastructure traits as well as other ecological trait variation in natural populations of *P. trichocarpa* (McKown et al. 2014; Porth et al. 2013), indicating that complex traits in forest trees are controlled by abundant sequence variants across the genome, each with minor contributions to the phenotypic variance. GWAS in *Eucalyptus* have reported on wood property traits viz. diameter growth and lignin composition in *E. globulus* (Cappa et al. 2013), growth traits in *Eucalyptus benthamii* Maiden & Cambage and *Eucalyptus pellita* F. Muell. (Müller et al. 2017), and multiple productivity traits including disease resistance in *E. grandis* × *E. urophylla* hybrids (Resende et al. 2017b). In candidate gene base studies have also reported associations between candidate gene polymorphisms and wood traits (Denis et al. 2013; Mandrou et al. 2012; Thavamanikumar et al. 2014; Thumma et al. 2009; Thumma et al. 2005). While GWAS have discovered many marker- or SNP-trait associations for potential gene discovery in *Eucalyptus*, recent studies also emphasised regional heritability or genomic relationship mapping to uncover rare genetic variants and identify true QTLs with considerably lower rates of false positives and larger fractions of explained variance (Müller et al. 2017; Resende et al. 2017b).

Although QTL analyses and association mapping for rust resistance have received increasing attention in *Eucalyptus*, the existing studies have mainly focused on species from subgenus *Symphyomyrtus*, as reported lately for *A. psidii* resistance in *E. globulus* (Butler et al. 2016) and *E. grandis* × *E. urophylla* hybrids (Resende et al. 2017b). There

has been no study to date in this regard for any species from next largest subgenus *Eucalyptus*, a lineage which has diverged from their common ancestor with *Symphyomyrtus* at about 33 – 35 million years ago (Grattapaglia et al. 2012; Thornhill et al. 2015) based on the estimation of divergence times using a relaxed molecular clock calibrated with reliable fossil record (Bell et al. 2010; Drummond et al. 2006). The objectives of the present study are to use GWAS to identify genome-wide QTLs underlying resistance responses of *Eucalyptus obliqua* L'Hér. to *A. psidii*. Following this dissection the respective QTL regions will be compared to those reported in other *Symphyomyrtus* species i.e. *E. globulus* and *E. grandis*. These findings will be used to enhance our understanding of the commonalty of underlying rust resistance QTL across *Eucalyptus* subgenera and may shed light on the potential mechanisms that underlie resistance responses to *A. psidii* in *Eucalyptus*.

Materials and Methods

Plant material

A sample of 1073 *E. obliqua* individuals phenotyped as described in Chapter 2. In brief, a total of 818 *E. obliqua* seedlots comprising single mother-parent open pollinated families and multiple-parent bulks were collected from widely spread native populations with regenerated individuals from 624 single mother tree seedlots were assessed for phenotypic rust response. *E. obliqua* seeds were imbibed in clean water for 28 days prior to sowing in 40-celled hyco trays for designated rust screening trial (Batch 8). The regenerated seedlings were irrigated twice daily and grown in glasshouse conditions in HVP Plantations in Gippsland, Victoria for 12 weeks prior to dispatch to Plant Breeding Institute (PBI) in University of Sydney, Cobbitty, for rust inoculation and screening.

Rust inoculation

E. obliqua seedlings were evaluated for phenotypic rust response using artificial inoculation in controlled conditions. In brief, the seedlings were inoculated with *A. psidii* urediniospores derived from a single uredinium multiplied on highly susceptible host plant *Syzygium jambos* L. (Alston) in an accredited inoculation chamber in PBI using a fine mist spray on adaxial and abaxial leaf surfaces. The rust inoculum was

prepared from PBI collection rust culture no. 622 with concentration of 2.0 mg urediniospores suspended in 1.0 ml of light mineral oil (Univar Solvent L Naphtha 100, Univar Australia Pty Ltd) and the inoculation was performed according to the protocol described by Sandhu and Park (2013). The inoculated seedlings were maintained at 22 ± 2 °C in a naturally lit microclimate room prior to disease assessment at 14 days post-inoculation.

Phenotyping and variance component estimation

E. obliqua seedlings were assessed for the severity of *A. psidii* symptoms on new shoots and leaves using a continuous/quantitative disease scale developed by Sandhu and Park (2013) with the following ratings: 0 = no visible sign of infection (highly resistant); 1 = presence of mild hypersensitive reaction with highly restricted pustule, fleck and necrosis (resistant); 2 = hypersensitive reaction with restricted pustule, chlorosis and necrosis (moderately resistant); 3 = small to medium sized pustules in low frequency and may be presence of chlorosis (moderately susceptible); 4 = fully developed pustules on leaves in medium to high frequency (susceptible); 5 = abundance of fully developed pustules on leaves, twigs and buds (very susceptible). The seedlings were also grouped into categories of binary rust response as follows: symptomatic (presence or absence of disease symptoms); hypersensitive (presence or absence of hypersensitive reactions with exclusion of asymptomatic response); and pustulation (presence or absence of developed pustules with exclusion of asymptomatic response). The above disease assessment resulted in four response traits (one numeric and three binary) to *A. psidii* in *E. obliqua* inoculated seedlings, and the genetic variation in individual plant for each trait was estimated as a BLUP (best linear unbiased prediction) to produce a phenotype file using the following mixed model fitted with ASReml version 3 (Gilmour et al. 2009) in R software version 3.3.0 (R Core Team 2016):

$$y = \mu + \text{District} + \textit{ped(Genotype)} + \text{Residuals}$$

where y is the observation of rust response in numeric score or binary trait frequency for the seedling and μ is the overall mean. The model was fitted with forest district treated

as a fixed effect, and individual (genotype) associated with a flat pedigree listing known mother tree applied as a random effect.

Genetic material

Prior to rust inoculation and disease assessment leaf samples from all individuals to be phenotyped were harvested and stored on silica gel (Chem-Supply). Of these, one individual per seedlot was randomly selected from each of the 624 *E. obliqua* seedlots for genotyping and GWAS analysis. Genomic DNA was extracted from the stored leaves of the selected individuals using a CTAB (cetyltrimethylammonium bromide) protocol (Doyle and Doyle 1990) with modification described by Tibbits et al. (2006). The DNA was precipitated by adding isopropanol, followed by washing of the pellet with 70% ethanol, and resuspension in Buffer EB (Qiagen) or 10 mM tris-HCl (pH 8.0) to avoid EDTA (ethylenediaminetetraacetic acid) in the final preparation. Purified DNA was quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific) or NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and assessed for quality by agarose gel electrophoresis. Multiple extractions were performed as necessary to obtain sufficient DNA for genomic library preparation.

Genotyping by whole genome shotgun sequencing

Genomic library preparations were performed using Nextera DNA Library Preparation Kit (Illumina) in 96-well half-skirt PCR microplates (Axygen Scientific). Double-stranded genomic DNA with absorbance ratio values of 1.8 – 2.0 was used as input for the Nextera tagmentation reaction involving DNA fragmentation and tagging. Tagmented DNA was treated with 0.6% SDS (sodium dodecyl sulfate) for dissociation from the Nextera transposome replacing the column cleanup recommended in the manufacturer protocol, followed by library amplification using Nextera Index 1 (i7) and Index 2 (i5) adapters with a limited-cycle PCR program on a Stratagene Mx3005P™ qPCR system (Agilent Technologies). The thermal profile was set as follows: 72 °C for 3 min; 98 °C for 30 sec; and 5 cycles of 98 °C for 10 sec, 63 °C for 30 sec and 72 °C for 3 min. SYBR Green I (Thermo Fisher Scientific) in 1:1000 dilution was added to the PCR mixtures for monitoring the amplification process via detection of fluorescent signal caused by the binding of SYBR Green dye to PCR product (double-stranded

DNA). Amplified libraries were pooled and purified with MinElute PCR Purification Kit (Qiagen) or AMPure XP SPRI (Solid Phase Reversible Immobilisation) paramagnetic beads (Agencourt, Beckman Coulter) according to manufacturers' instructions. Purified library pools were then subjected to size selection using agarose gel electrophoresis by excising the DNA fragments within the desired size range, i.e. 300 – 450 base pairs (bp). The excised libraries were extracted from gel slices with QIAquick Gel Extraction Kit (Qiagen) prior to quality assessed with High Sensitivity D1000 ScreenTape using a 2200 TapeStation system (Agilent Technologies). Prepared libraries were subsequently sequenced using a HiSeq 2000 Sequencing System (Illumina) at the Department of Economic Development, Jobs, Transport and Resources (DEDJTR), Centre for AgriBioscience (AgriBio), Bundoora.

Sequence filtering, annotation and alignment

The data generated from *E. obliqua* whole genome sequencing were called and curated using standard Illumina pipeline software, and the raw sequence reads were quality filtered with nuclear v3.2.6 (Gydle Inc., Montreal, Canada). The filtered reads were aligned using nuclear (Gydle) to a reference resolved *E. obliqua* sequence developed from alignment and resolve v2.6.16 (Gydle) using reads from a previous *Eucalyptus* Genome Project (Bayly et al. 2013) and the *E. grandis* reference genome (Myburg et al. 2014) as the initiating sequence. SNP variants were discovered using find-snp v2.6.16 (Gydle) and custom Perl and awk scripts, followed by genotype calling with custom Perl scripts. Variants were output in a VCF (Variant Call Format) version 4.2 genotype file. SNP calls for all the *E. obliqua* sequenced genotypes were filtered and converted to Hapmap format using a custom Perl scripts incorporating the following criteria: minor allele frequency (MAF) greater than 0.05; missing data less than 20%; and chromosome positions based on previously mapped *E. grandis* reference genome (v2 www.phytozome.net; Myburg et al. 2014).

Linkage disequilibrium (LD) analysis and genome wide association study (GWAS)

Principle component analysis (PCA) used JMP Genomics v.7, PCA for Population Stratification option (SAS Institute 2014) to access the level of genetic structure of the *E. obliqua* genotypes. Linkage disequilibriums (LDs) of all SNP pairs on each

chromosome were computed as the square allele frequency correlation coefficient (r^2) and the genome-wide LD decay was analysed by plotting r^2 from all 11 chromosomes against the corresponding pairwise physical distances (bases) using Trait Analysis by Association, Evolution and Linkage (TASSEL) version 3.0.169 (Bradbury et al. 2007). Rust resistance SNP-marker association analysis was performed using Genome Association and Prediction Integrated Tool (GAPIT; Lipka et al. 2012; Tang et al. 2016) implemented in R statistical software with the input phenotype and genotype files. The P values ($-\log_{10} P$) for each SNP were exported from GAPIT to generate Manhattan plots according to the respective linkage groups for each rust response (numeric and binary) trait of their raw and BLUP phenotypes. GWAS was also performed in TASSEL version 5.2.15 using the mixed linear model (MLM) function to confirm the results obtained with GAPIT. Significance of genome-wide associations between SNP markers and rust responses was analysed at $-\log_{10} P > 6.0$, and the plots and data handling were performed with the open-source statistical programming environment R.

Quantitative trait loci (QTLs) analysis

The significant SNPs for rust resistance in *E. obliqua* were identified by gene accession search and BLAST of nucleotide sequences against the *E. grandis* reference genome (Myburg et al. 2014) for 5 kilobase pairs (kbp) up- and downstream of the lead SNPs (v2 www.phytozome.net), and the positional candidate genes for the putative rust resistance QTL were plotted against reference-sequenced physical map (Hudson et al. 2014; Hudson et al. 2012). For comparison of genetic rust resistance across *Eucalyptus* subgenera (i.e. between *Symphyomyrtus* and *Eucalyptus* with *c.* 33 – 35 million years of estimated divergence time), genes reported to be involved in myrtle rust resistance in *Symphyomyrtus* species viz. *Ppr1* in *E. grandis* (Junghans et al. 2003a), and *Ppr2*, *Ppr3*, *Ppr4* and *Ppr5* in *E. globulus* (Butler et al. 2016), were placed on the physical map for *Eucalyptus* (v2 www.phytozome.net) and thoroughly compared with the putative rust resistance QTL generated from the present study for *E. obliqua* of subgenus *Eucalyptus*.

Results

A total of 1,131,254 SNPs were tested for association in GWAS with the phenotypic traits numeric disease severity and binary symptomatic score, and 1,131,146 SNPs for

binary hypersensitive and pustulation responses to myrtle rust. The average, genome-wide LD for SNPs (MAF > 0.05) spaced with uniform 20 bp physical intervals from 0 to 1000 bp was calculated and a decreasing trend of LD decay to an r^2 below 0.075 within 100 bp was observed (Figure 5-1). Association mapping based on the rust responses revealed 33 significant SNPs ($-\log_{10} P > 6.0$) with most of the markers associated with the binary symptomatic (26 markers) and pustulation (5 markers) traits. The rust response – SNP associations, especially for binary symptomatic response, were distributed across all chromosomes. Of the 26 significant markers associated with the binary symptomatic trait, five markers were located on each of chromosomes 3 and 4, four markers on chromosome 8, and three or fewer on each of the other chromosomes. Furthermore, five markers, all on different chromosomes, viz. 3, 5, 6, 9 and 11, were significantly associated with pustulation response to myrtle rust, but only one significant marker was found associated with hypersensitive and numeric severity trait (Table 5-1).

A visual summary of GWAS results and genomic positions of the QTLs is provided in Manhattan plots for binary symptomatic (Figure 5-2), hypersensitive (Figure 5-3) and pustulation (Figure 5-4), and numeric severity (Figure 5-5) traits. In the MLM analysis, most *P* values were consistent with the expectation along the diagonal in the quantile-quantile (Q-Q) plot (Figure 5-6), indicating suitability of the proposed GWAS model. Sequence similarity searches using BLAST for flanking sequences of 5 kbp up- and downstream of the significant SNP positions revealed 85% of protein accessions hits against the *E. grandis* reference genome. As the completed genome information of *E. obliqua* was not available at the time of writing, 15% of the searched sequences (five significant SNPs) had no hits to any known proteins in the Phytozome database and their proximate regions were further examined for potential candidate genes. The majority of queries identified hypothetical proteins, with predicted functions not known to be related to defences against pathogens but amongst the hits were also a number with putative defence functions, including enzyme kinases or transferases, proteases or hydrolases, nucleotide-binding proteins, and transcription factors. Thus a possible function of the predicted proteins and transcription factors, as well as the uncharacterised proteins, in responses to myrtle rust disease cannot be ruled out.

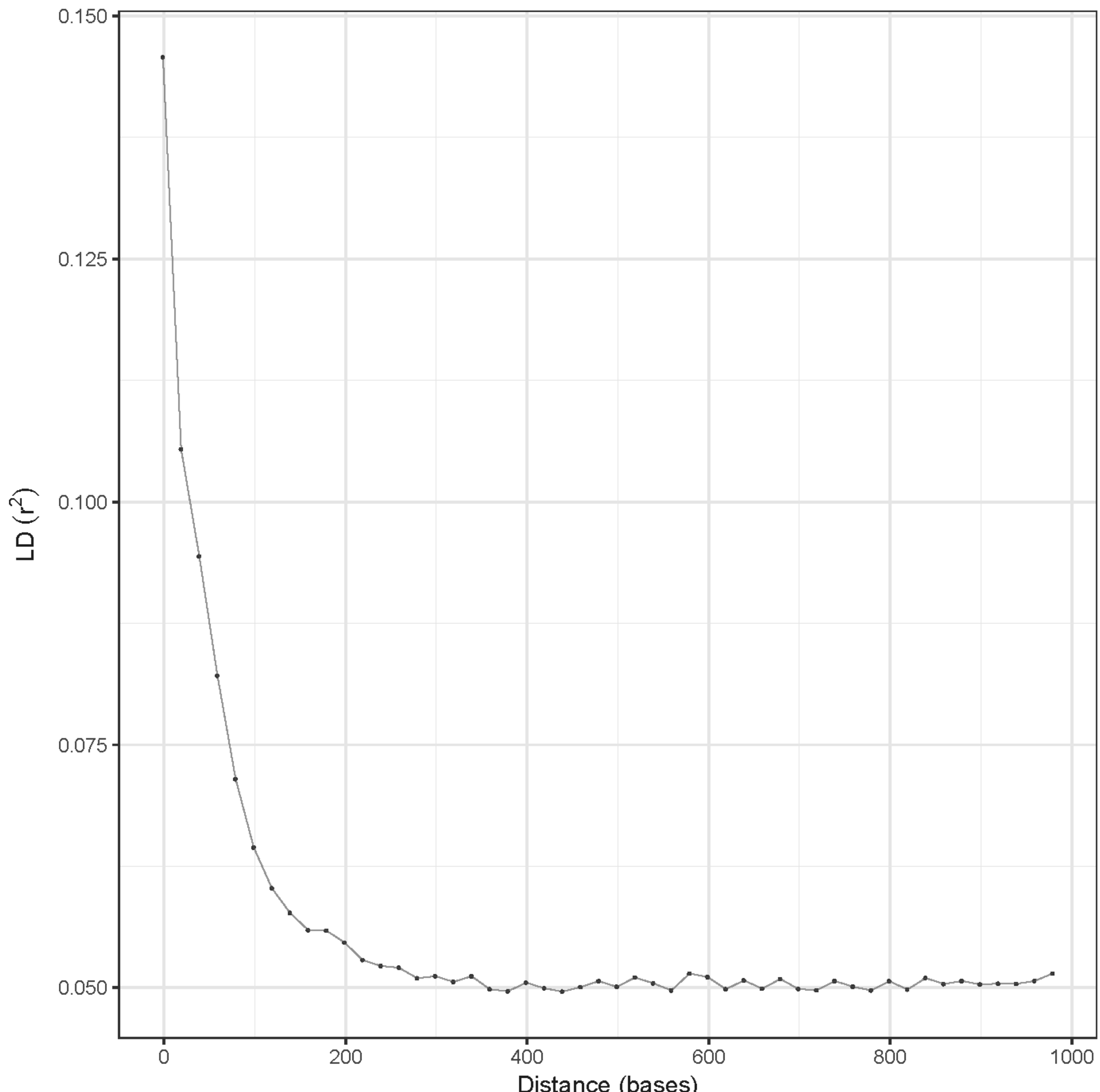

Figure 5-1: Genome-wide pattern of linkage disequilibrium (LD) decay for *Eucalyptus obliqua* up to 1000 bp pairwise SNP distances estimated by r^2 (y-axis) along physical distance in bases (x-axis).

Table 5-1: Significant SNP markers ($-\log_{10} P > 6.0$) associated with binary and numeric responses to myrtle rust in *Eucalyptus obliqua*

Trait	SNP	Chromosome	Position	*P* value
<u>Binary</u>				
Symptomatic	A-0493:16014220-16318932:15_228650	1	16242869	2.99E-07
Symptomatic	B-2072:48515807-48713237:2:1_77578	2	48593384	3.25E-07
Symptomatic	B-2390:57501503-57592459:6:1_87465	2	57588967	3.93E-07
Symptomatic	C-0835:19886015-19910485:1:1_67031	3	19953045	3.69E-07
Symptomatic	C-2263:46421453-46447151:2:2_71497	3	46492949	9.36E-07
Symptomatic	C-2324:47586955-47630737:1_24634	3	47611588	2.53E-08
Symptomatic	C-2542:51401024-51449710:1_19512	3	51420535	5.65E-07
Symptomatic	C-2883:58539653-58549142:1_3711	3	58543363	1.69E-10
Symptomatic	D-0113:7383739-7648802:2_17332	4	7401070	1.84E-07
Symptomatic	D-0113:7383739-7648802:2_17799	4	7401537	4.13E-07
Symptomatic	D-0135:9060248-9248130:01:1_114317	4	9174564	3.20E-07
Symptomatic	D-0167:11236075-11319024:6_261128	4	11497202	7.59E-07
Symptomatic	D-0167:11236075-11319024:6_261405	4	11497479	7.39E-08
Symptomatic	E-0020:1609620-1927068:02_57809	5	1667428	9.77E-07
Symptomatic	E-0186:9136922-9162100:3_41456	5	9178377	9.74E-07
Symptomatic	E-1551:40581074-40639660:2:3_11181	5	40592254	7.28E-07
Symptomatic	F-0858:18322528-18941438:21:1_59705	6	18382232	1.66E-11
Symptomatic	G-0471:19842480-20168842:07:1_56247	7	19898726	2.36E-07

Table 5-1 (*Continued*)

Binary				
Symptomatic	H-1341:33657450-33852066:17_48192	8	33705641	7.44E-07
Symptomatic	H-1341:33657450-33852066:17_156298	8	33813747	2.33E-07
Symptomatic	H-1578:48181307-48617281:2:1_34396	8	48215702	7.74E-07
Symptomatic	H-1989:69881161-70039865:10:1_113033	8	69994193	1.16E-07
Symptomatic	I-1048:25682769-25837187:06:1_43196	9	25725964	7.65E-07
Symptomatic	I-1160:29181065-29251304:1_50051	9	29231115	7.31E-08
Symptomatic	J-0090:6687135-6789860:2:1_24108	10	6711242	8.17E-07
Symptomatic	K-1305:42564204-42648801:1:1_110921	11	42675124	2.69E-08
Hypersensitive	H-1984:69170890-69486670:26_88228	8	69259117	9.48E-07
Pustulation	C-0086:1310624-1357885:1:2_22972	3	1333595	9.78E-07
Pustulation	E-3192:68529069-68573608:4_169478	5	68698546	9.98E-07
Pustulation	F-1419:42202565-42276296:4_214376	6	42416940	1.13E-07
Pustulation	I-1372:36826879-37016685:11_78590	9	36905468	3.50E-07
Pustulation	K-1169:37682931-37903253:3:1_239310	11	37922240	9.27E-08
Numeric				
Severity	G-1052:30959770-30982295:1:1_121518	7	31081287	8.57E-07

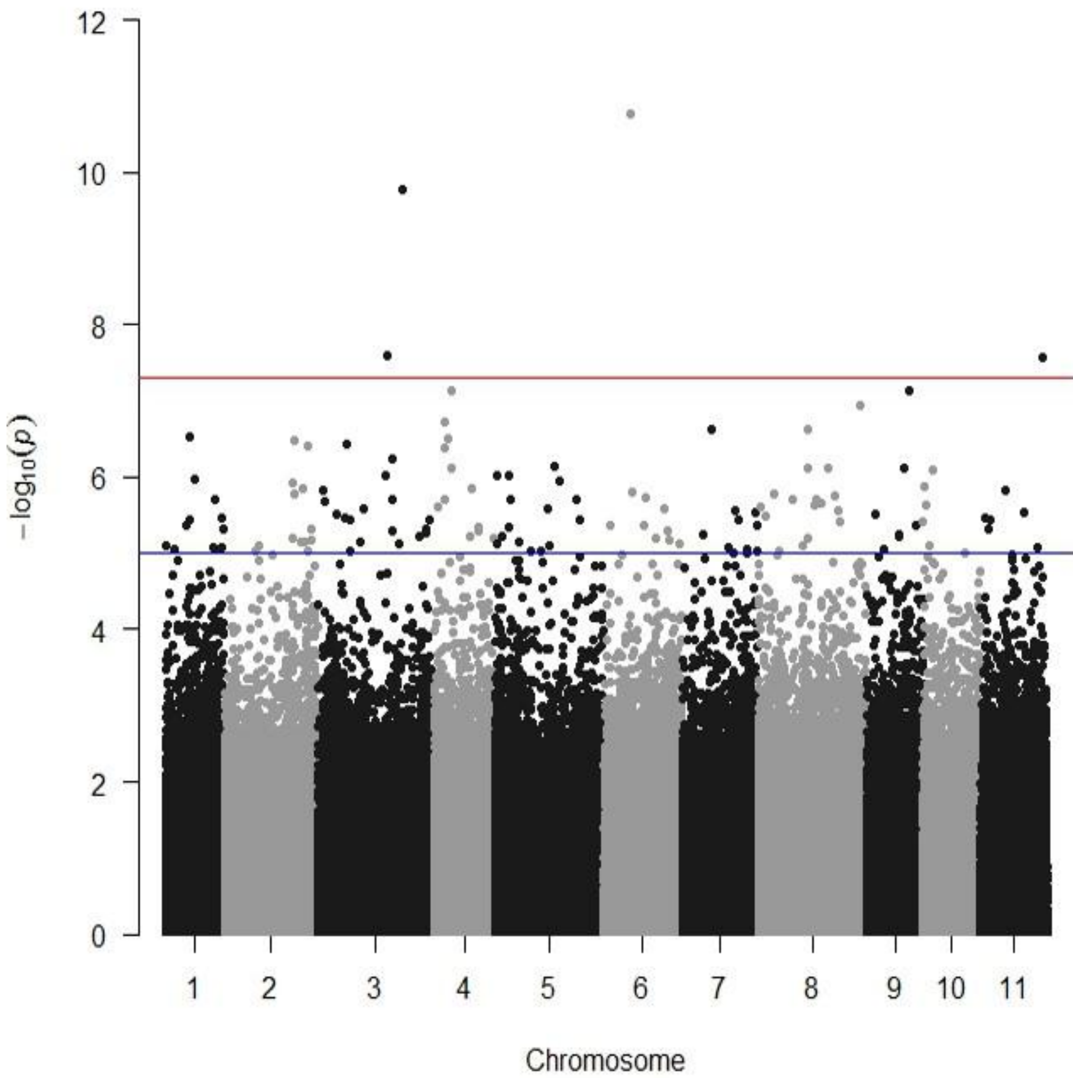

Figure 5-2: Manhattan plot of the genome wide association study (GWAS) results for the binary symptomatic response of *Eucalyptus obliqua* to *Austropuccinia psidii* based on the $–\log_{10}$ *P* value (y-axis) along the 11 *Eucalyptus* chromosomes (x-axis). The blue horizontal line at $–\log_{10}$ (1×10^{-5}) indicates the suggestive association level and the red horizontal line at $–\log_{10}$ (5×10^{-8}) corresponds to the genome wide significant threshold. SNPs at contigs C-2324 and C-2883 on chromosome 3, F-0858 on chromosome 6, and K-1305 on chromosome 11 exceed the predefined level of significant threshold.

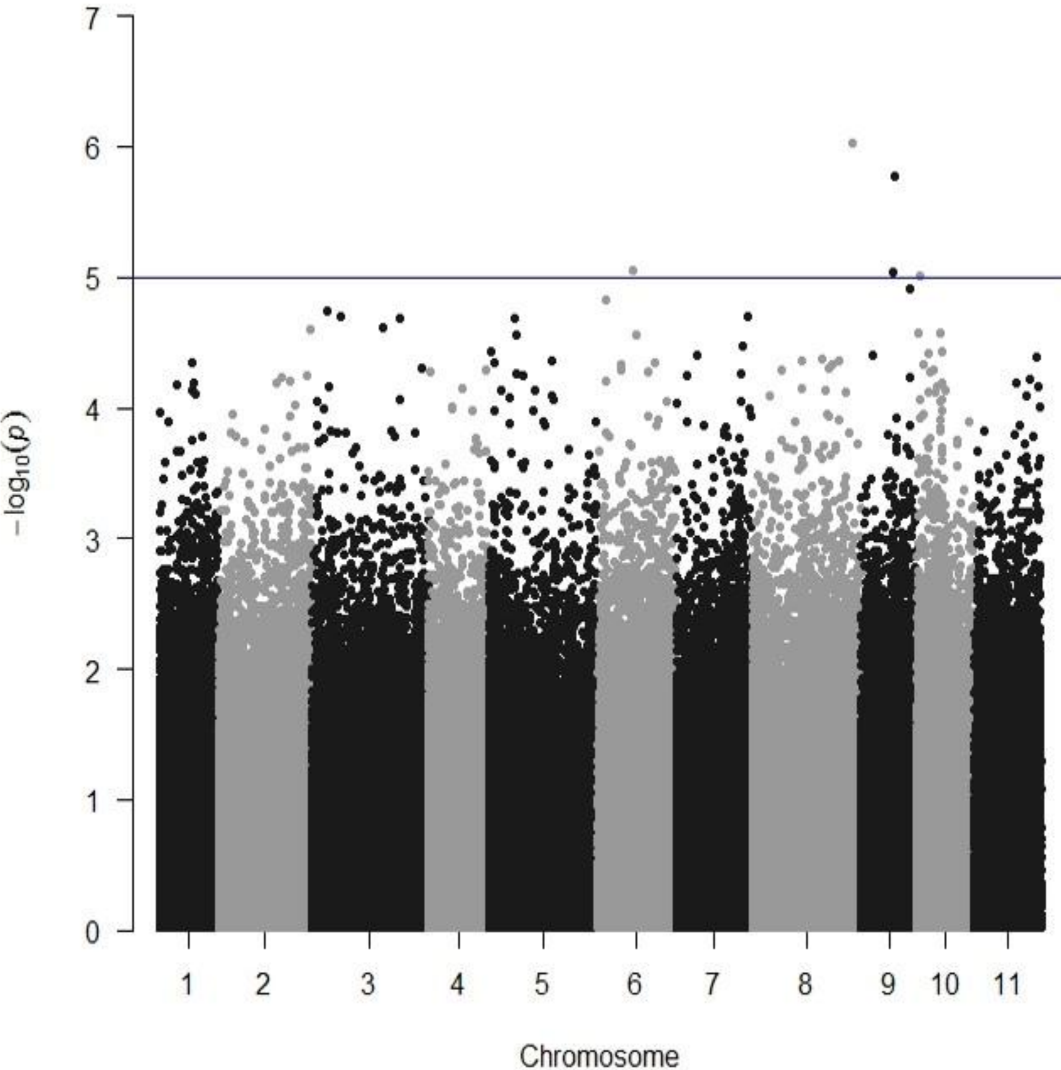

Figure 5-3: Manhattan plot of the genome wide association study (GWAS) results for the binary hypersensitive response of *Eucalyptus obliqua* to *Austropuccinia psidii* based on the $-\log_{10} P$ value (y-axis) along the 11 *Eucalyptus* chromosomes (x-axis). The blue horizontal line at $-\log_{10}(1\times10^{-5})$ indicates the suggestive association level. Only few SNPs on chromosome 6, 8, 9 and 10 exceed the suggestive threshold.

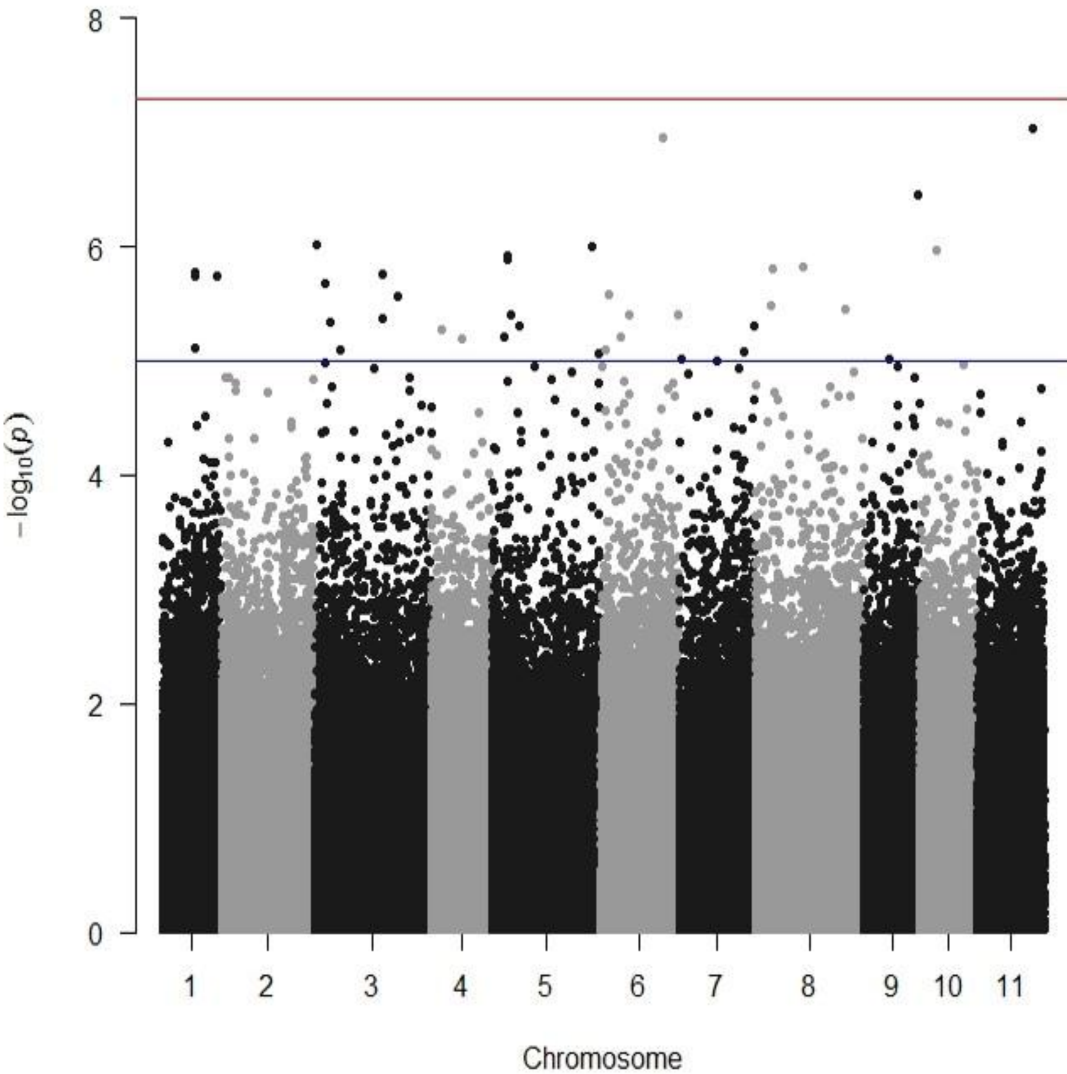

Figure 5-4: Manhattan plot of the genome wide association study (GWAS) results for the binary pustulation response of *Eucalyptus obliqua* to *Austropuccinia psidii* based on the $-\log_{10} P$ value (y-axis) along the 11 *Eucalyptus* chromosomes (x-axis). The blue horizontal line at $-\log_{10} (1\times10^{-5})$ indicates the suggestive association level and the red horizontal line at $-\log_{10} (5\times10^{-8})$ corresponds to the genome wide significant threshold. SNPs at contigs F-1419 on chromosome 6, I-1372 on chromosome 9, and K-1169 on chromosome 11 close to the predefined level of significant threshold.

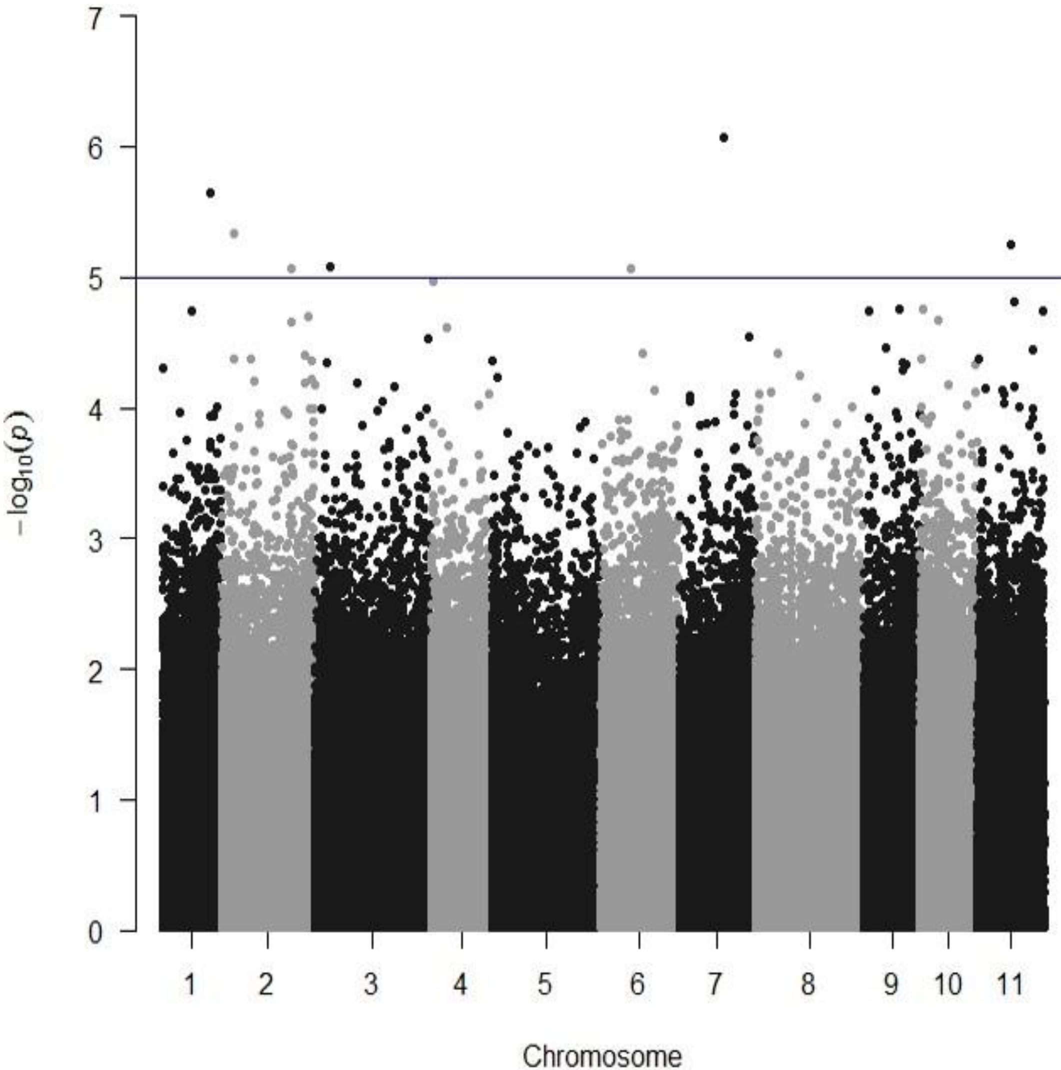

Figure 5-5: Manhattan plot of the genome wide association study (GWAS) results for the numeric severity response of *Eucalyptus obliqua* to *Austropuccinia psidii* based on the $-\log_{10} P$ value (y-axis) along the 11 *Eucalyptus* chromosomes (x-axis). The blue horizontal line at $-\log_{10} (1\times10^{-5})$ indicates the suggestive association level. Only few SNPs on chromosome 1, 2, 3, 6, 7 and 11 exceed the suggestive threshold.

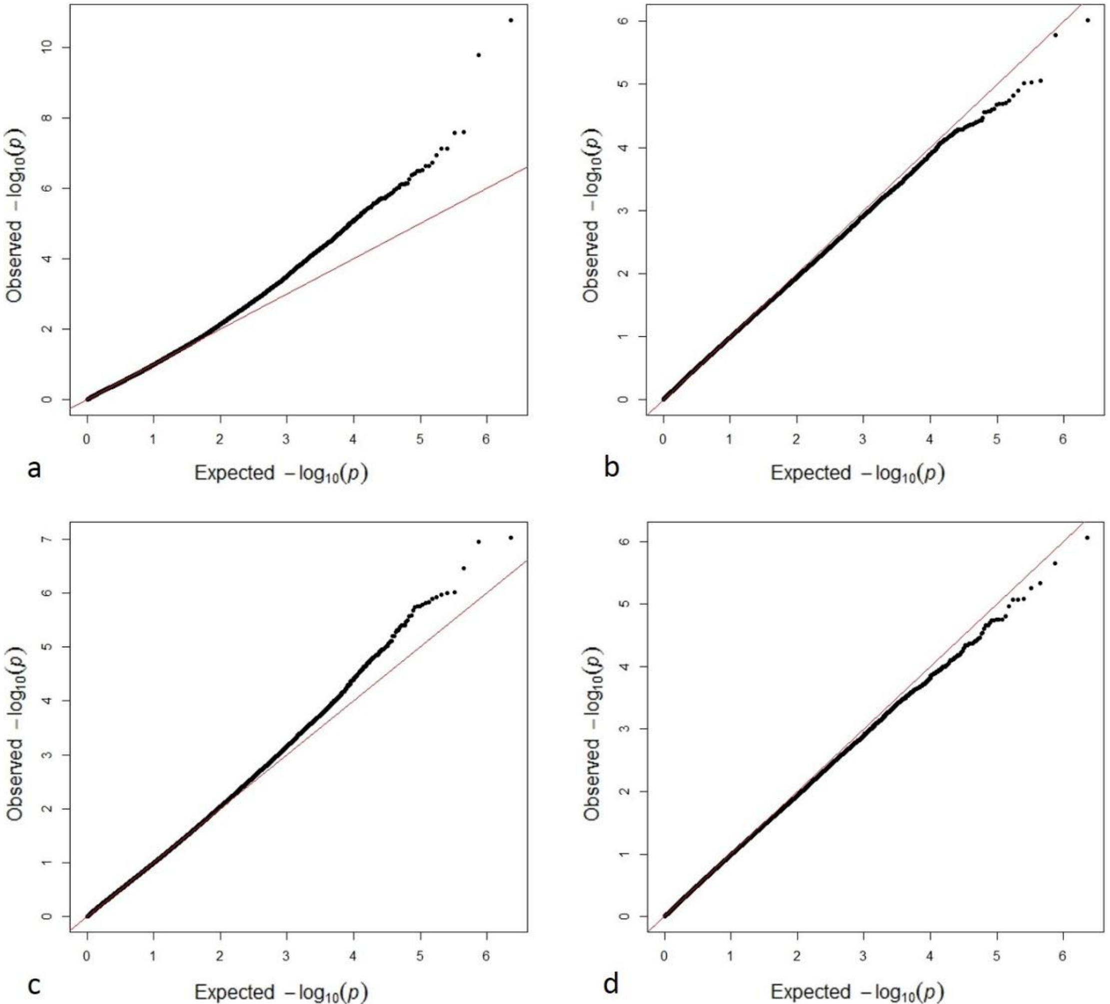

Figure 5-6: Quantile-quantile (Q-Q) plots for genome wide association study (GWAS) results for binary symptomatic **(a)**, hypersensitive **(b)**, pustulation **(c)**, and numeric severity **(d)** responses of *Eucalyptus obliqua* to *Austropuccinia psidii*. The Q-Q plot is used to assess the number and magnitude of observed associations between genotyped SNPs and the trait under study, compared to the association statistics expected under the null hypothesis of no association (Tang et al. 2016). The departure from the 1-1 line in **a** and **c** and the under line in **b** and **d** indicates that background patterns have not been well controlled in these models.

Table 5-2 to 5-5 summarises the potential candidate genes for different response (binary and numeric) traits to *A. psidii* and their physical location on *E. grandis* reference genome and putative function resulting from Phytozome functional annotations.

A number of the annotated genes in symptomatic response to *A. psidii*, i.e. Eucgr.B03282, Eucgr.C02701, Eucgr.D00643, and Eucgr.E00159, were identified as having sequence similarities with classical defence response genes such as protein domains containing leucine rich repeats (LRR) and receptor like protein kinases (RLK) including serine/threonine protein kinase and brassinosteroid (BR)-signaling kinase (Table 5-2). One of the LRR-containing genes (C-2542:51401024-51449710:1_19512) was located within the confidence interval of *Ppr1*, the previously published major locus for *A. psidii* resistance in *E. grandis* (Alves et al. 2012; Junghans et al. 2003a; Mamani et al. 2010). None of the candidate genes for binary rust responses in *E. obliqua* overlap with reported QTL underlying the immune (*Ppr2* and *Ppr3*) and hypersensitive (*Ppr4* and *Ppr5*) responses in *E. globulus* to *A. psidii* (Butler et al. 2016). It is worth noting that the only significant SNP associated with numeric disease severity (G-1052:30959770-30982295:1:1_121518) in the present study was situated within the identified *Ppr3* region. Figure 5-7 illustrates the position of significant SNP markers for rust responses in *E. obliqua* on the reference-sequenced physical map, and the published major QTLs (*Ppr1* to *Ppr5*) were included in the map for basic comparison of genetic architecture in myrtle rust resistance between species from subgenus *Eucalyptus* and *Symphyomyrtus*.

Discussion

This study provides the first report of QTL influencing susceptibility to *A. psidii* in *E. obliqua*, a representative species from subgenus *Eucalyptus*. It follows the earlier identification of myrtle rust resistance QTLs in species or hybrids of subgenus *Symphyomyrtus* i.e. *E. grandis* (Junghans et al. 2003a; Mamani et al. 2010), *E. globulus* (Butler et al. 2016) and interspecific hybrids (Alves et al. 2012; Rosado et al. 2010). The first major effect QTL, *Ppr1* (Junghans et al. 2003a), was identified in *E. grandis* on chromosome 3 and validated in unrelated pedigrees (Alves et al. 2012; Junghans et al. 2003a; Mamani et al. 2010; Rosado et al. 2010). Subsequent, investigations have

Table 5-2: Results of the putative candidate genes associated with binary symptomatic response of *Eucalyptus obliqua* to *Austropuccinia psidii* identified by *in-silico* searching of the significant SNP markers of 10 kbp physical nucleotide region with *Eucalyptus grandis* reference genome using Phytozome

SNP marker [a]	Annotated gene [b]	Physical location [c]	Putative function [d]
A-0493:16014220-16318932:15_228650	Eucgr.A01051	Chr01: 30751860-30756050 forward	Scarecrow-like protein 21
	Eucgr.A01052	Chr01: 30752606-30753109 reverse	Uncharacterised protein
	Eucgr.A01053	Chr01: 30756757-30759031 reverse	WRKY transcription factor 1
B-2072:48515807-48713237:2:1_77578	Eucgr.B02655	Chr02: 46521866-46524220 forward	Tartrate-resistant acid phosphatase type 5
	Eucgr.B02656	Chr02: 46526813-46530499 forward	Tartrate-resistant acid phosphatase type 5
	Eucgr.B02657	Chr02: 46534255-46538214 forward	Tartrate-resistant acid phosphatase type 5
	Eucgr.B02658	Chr02: 46537698-46543765 reverse	Procollagen-proline 3-dioxygenase / Prolyl 3-hydroxylase
B-2390:57501503-57592459:6:1_87465	Eucgr.B03282	Chr02: 54001909-54004975 forward	Plant intracellular Ras-group-related LRR protein 1
	Eucgr.B03283	Chr02: 54000171-54000898 forward	Uncharacterised protein
C-0835:19886015-19910485:1:1_67031	Eucgr.C01270	Chr03: 16743972-16747061 forward	Rac-like GTP-binding protein ARAC7
	Eucgr.C01271	Chr03: 16730686-16738619 forward	WD repeat, SAM and U-box domain-containing protein 1
C-2263:46421453-46447151:2:2_71497	Eucgr.C02440	Chr03: 47773914-47782507 reverse	BNR/Asp-box repeat family protein
C-2324:47586955-47630737:1_24634	Eucgr.C02498	Chr03: 49963703-49971576 forward	Nucleoid-associated protein YbaB

Table 5-2 (*Continued*)

C-2542:51401024-51449710:1_19512	Eucgr.C02699	Chr03: 55311802-55312225 reverse	Uncharacterised protein
	Eucgr.C02701	Chr03: 55322371-55322778 forward	Leucine-rich repeat-containing protein
C-2883:58539653-58549142:1_3711	Eucgr.C03099	Chr03: 62400139-62400981 reverse	Uncharacterised protein
D-0113:7383739-7648802:2_17332	Eucgr.D00405*	Chr04: 7629182-7629604 reverse	Senescence regulator
D-0113:7383739-7648802:2_17799	Eucgr.D00405*	Chr04: 7629182-7629604 reverse	Senescence regulator
D-0135:9060248-9248130:01:1_114317	Eucgr.D00517	Chr04: 9668182-9669366 forward	Cysteine protease
	Eucgr.D00518	Chr04: 9672026-9673606 forward	Peptidase C1-like family
	Eucgr.D00519	Chr04: 9674246-9677556 reverse	50S ribosomal protein L34, chloroplastic
D-0167:11236075-11319024:6_261128	Eucgr.D00643	Chr04: 11913955-11921430 forward	Non-specific serine/threonine protein kinase
	Eucgr.D00644	Chr04: 11922717-11925525 reverse	Alpha/beta hydrolase fold-containing protein
	Eucgr.D00645	Chr04: 11930063-11932890 reverse	Alpha/beta hydrolase fold-containing protein
	Eucgr.D00646	Chr04: 11937893-11940157 reverse	Alpha/beta hydrolase fold-containing protein
	Eucgr.D00647	Chr04: 11944018-11946829 reverse	Alpha/beta hydrolase fold-containing protein
	Eucgr.D00648	Chr04: 11953594-11955751 reverse	Alpha/beta hydrolase fold-containing protein
D-0167:11236075-11319024:6_261405	Eucgr.D00643	Chr04: 11913955-11921430 forward	Non-specific serine/threonine protein kinase

Table 5-2 (*Continued*)

	Eucgr.D00644	Chr04: 11922717-11925525 reverse	Alpha/beta hydrolase fold-containing protein
	Eucgr.D00645	Chr04: 11930063-11932890 reverse	Alpha/beta hydrolase fold-containing protein
	Eucgr.D00646	Chr04: 11937893-11940157 reverse	Alpha/beta hydrolase fold-containing protein
	Eucgr.D00647	Chr04: 11944018-11946829 reverse	Alpha/beta hydrolase fold-containing protein
	Eucgr.D00648	Chr04: 11953594-11955751 reverse	Alpha/beta hydrolase fold-containing protein
E-0020:1609620-1927068:02_57809	Eucgr.E00158	Chr05: 1703771-1705327 forward	Uncharacterised protein
	Eucgr.E00159	Chr05: 1707006-1711455 reverse	BR-signaling kinase 3
	Eucgr.E00160	Chr05: 1713379-1713945 reverse	Uncharacterised protein
	Eucgr.E00161	Chr05: 1719002-1719535 reverse	Uncharacterised protein
E-0186:9136922-9162100:3_41456	Eucgr.E00873	Chr05: 9189214-9196393 forward	Dynamin-related protein 1A
	Eucgr.E00874	Chr05: 9196748-9200726 forward	Triglyceride lipase
	Eucgr.E00875	Chr05: 9202971-9204134 reverse	Calcineurin B subunit (protein phosphatase 2B regulatory subunit)-like protein
E-1551:40581074-40639660:2:3_11181	Eucgr.E04118*	Chr05: 70017737-70018719 forward	Thiamine thiazole synthase
F-0858:18322528-18941438:21:1_59705	Eucgr.F01449	Chr06: 19638241-19642734 reverse	NAC domain-containing protein 21/22
	Eucgr.F01450	Chr06: 19648938-19657161 reverse	rRNA adenine N-6-methyltransferase

Table 5-2 (*Continued*)			
G-0471:19842480-20168842:07:1_56247	Eucgr.G01202	Chr07: 18348644-18354533 forward	Serine carboxypeptidase-like 1
H-1341:33657450-33852066:17_48192	Eucgr.H02473	Chr08: 32377593-32379142 reverse	Pentatricopeptide repeat (PPR) domain
	Eucgr.H02474	Chr08: 32381408-32387953 reverse	Coilin
H-1341:33657450-33852066:17_156298	Eucgr.J01357	Chr10: 15622002-15622939 forward	Calmodulin
	Eucgr.J01358	Chr10: 15625816-15633373 reverse	Polypyrimidine tract-binding protein
H-1578:48181307-48617281:2:1_34396	Eucgr.H03279	Chr08: 63124128-63128106 reverse	Protein dehydration-induced 19
	Eucgr.H03280	Chr08: 63129101-63133077 reverse	Three prime repair exonuclease 1,2
	Eucgr.H03281	Chr08: 63135452-63140219 reverse	Armadillo/beta-catenin-like repeats-containing protein
H-1989:69881161-70039865:10:1_113033	Eucgr.H04940	Chr08: 68786753-68788269 forward	PDDEXK-like family of unknown function
	Eucgr.H04941	Chr08: 68795864-68799627 forward	Large subunit ribosomal protein L17
I-1048:25682769-25837187:06:1_43196	Eucgr.I01588	Chr09: 25808920-25817763 reverse	Alpha/beta hydrolase fold-containing protein / Diacylglycerol acyltransferase (DAGAT)
I-1160:29181065-29251304:1_50051	Eucgr.I01936	Chr09: 29274951-29276869 reverse	Basic helix-loop-helix (bHLH) transcription factor PRE1
J-0090:6687135-6789860:2:1_24108	Eucgr.J00601	Chr10: 6723487-6740161 forward	Chondroitin sulfate proteoglycan 6
	Eucgr.J00602	Chr10: 6735161-6735631 reverse	Uncharacterised protein

Table 5-2 (*Continued*)

K-1305:42564204-42648801:1:1_110921	Eucgr.K03381	Chr11: 42040313-42041554 reverse	Aspartyl protease-like protein
	Eucgr.K03382	Chr11: 42047485-42048916 reverse	Aspartyl protease-like protein
	Eucgr.K03383	Chr11: 42051791-42053940 reverse	Gluconokinase / gluconate kinase
	Eucgr.K03384	Chr11: 42057758-42062542 forward	R3H domain-containing protein 4

[a] Significant marker within 5 kbp up- and downstream of the lead SNP

[b] A plausible biological candidate gene in the locus to the lead SNP

[c] Physical location of annotated gene on *E. grandis* genome

[d] Predicted protein function as identified by Phytozome

* Located outside of the targeted region but < 200 kbps away from the lead SNP

Table 5-3: Results of the putative candidate genes associated with binary hypersensitive response of *Eucalyptus obliqua* to *Austropuccinia psidii* identified by *in-silico* searching of the significant SNP markers of 10 kbp physical nucleotide region with *Eucalyptus grandis* reference genome using Phytozome

SNP marker [a]	Annotated gene [b]	Physical location [c]	Putative function [d]
H-1984:69170890-69486670:26_88228	Eucgr.H04888*	Chr08: 68042546-68043720 forward	GDSL/SGNH-like acyl-esterase family
	Eucgr.H04890*	Chr08: 68063039-68068593 reverse	Ubiquitin carboxyl-terminal hydrolase
	Eucgr.H04891*	Chr08: 68065827-68066287 forward	Uncharacterised protein

[a] Significant marker within 5 kbp up- and downstream of the lead SNP

[b] A plausible biological candidate gene in the locus to the lead SNP

[c] Physical location of annotated gene on *E. grandis* genome

[d] Predicted protein function as identified by Phytozome

* Located outside of the targeted region but < 200 kbps away from the lead SNP

Table 5-4: Results of the putative candidate genes associated with binary pustulation response of *Eucalyptus obliqua* to *Austropuccinia psidii* identified by *in-silico* searching of the significant SNP markers of 10 kbp physical nucleotide region with *Eucalyptus grandis* reference genome using Phytozome

SNP marker [a]	Annotated gene [b]	Physical location [c]	Putative function [d]
C-0086:1310624-1357885:1:2_22972	Eucgr.C00002*	Chr03: 2488634-2489094 reverse	Uncharacterised protein
	Eucgr.C00005*	Chr03: 2801585-2802683 reverse	Uncharacterised protein
	Eucgr.C00006*	Chr03: 2803946-2806814 forward	Uncharacterised protein
E-3192:68529069-68573608:4_169478	Eucgr.E03984	Chr05: 71630563-71637999 reverse	P-loop containing nucleoside triphosphate hydrolase
	Eucgr.E03985	Chr05: 71629968-71631177 forward	Uncharacterised protein
F-1419:42202565-42276296:4_214376	Eucgr.F03363	Chr06: 44974893-44981578 reverse	diacylglycerol kinase 5
	Eucgr.F03364	Chr06: 44982935-44987790 forward	tRNA (guanine(37)-N(1)-)-methyltransferace
	Eucgr.F03365	Chr06: 44984538-44985526 reverse	Uncharacterised protein
I-1372:36826879-37016685:11_78590	Eucgr.I02571	Chr09: 36919556-36919978 forward	No apical meristem (NAM) protein
	Eucgr.I02572	Chr09: 36927884-36929061 forward	Uncharacterised protein
K-1169:37682931-37903253:3:1_239310	Eucgr.K02989	Chr11: 37366853-37368406 forward	Lactosylceramide 4-alpha-galactosyltransferase (A4GALT)
	Eucgr.K02990	Chr11: 37372419-37373272 reverse	Histone H4

Table 5-4 (*Continued*)

[a] Significant marker within 5 kbp up- and downstream of the lead SNP

[b] A plausible biological candidate gene in the locus to the lead SNP

[c] Physical location of annotated gene on *E. grandis* genome

[d] Predicted protein function as identified by Phytozome

* Located outside of the targeted region but < 200 kbps away from the lead SNP

Table 5-5: Results of the putative candidate genes associated with numeric severity response of *Eucalyptus obliqua* to *Austropuccinia psidii* identified by *in-silico* searching of the significant SNP markers of 10 kbp physical nucleotide region with *Eucalyptus grandis* reference genome using Phytozome

SNP marker [a]	Annotated gene [b]	Physical location [c]	Putative function [d]
G-1052:30959770-30982295:1:1_121518	Eucgr.G01704	Chr07: 32601186-32606779 forward	Beta-galactosidase 5
	Eucgr.G01704	Chr07: 32601186-32606779 forward	Beta-galactosidase 5

[a] Significant marker within 5 kbp up- and downstream of the lead SNP

[b] A plausible biological candidate gene in the locus to the lead SNP

[c] Physical location of annotated gene on *E. grandis* genome

[d] Predicted protein function as identified by Phytozome

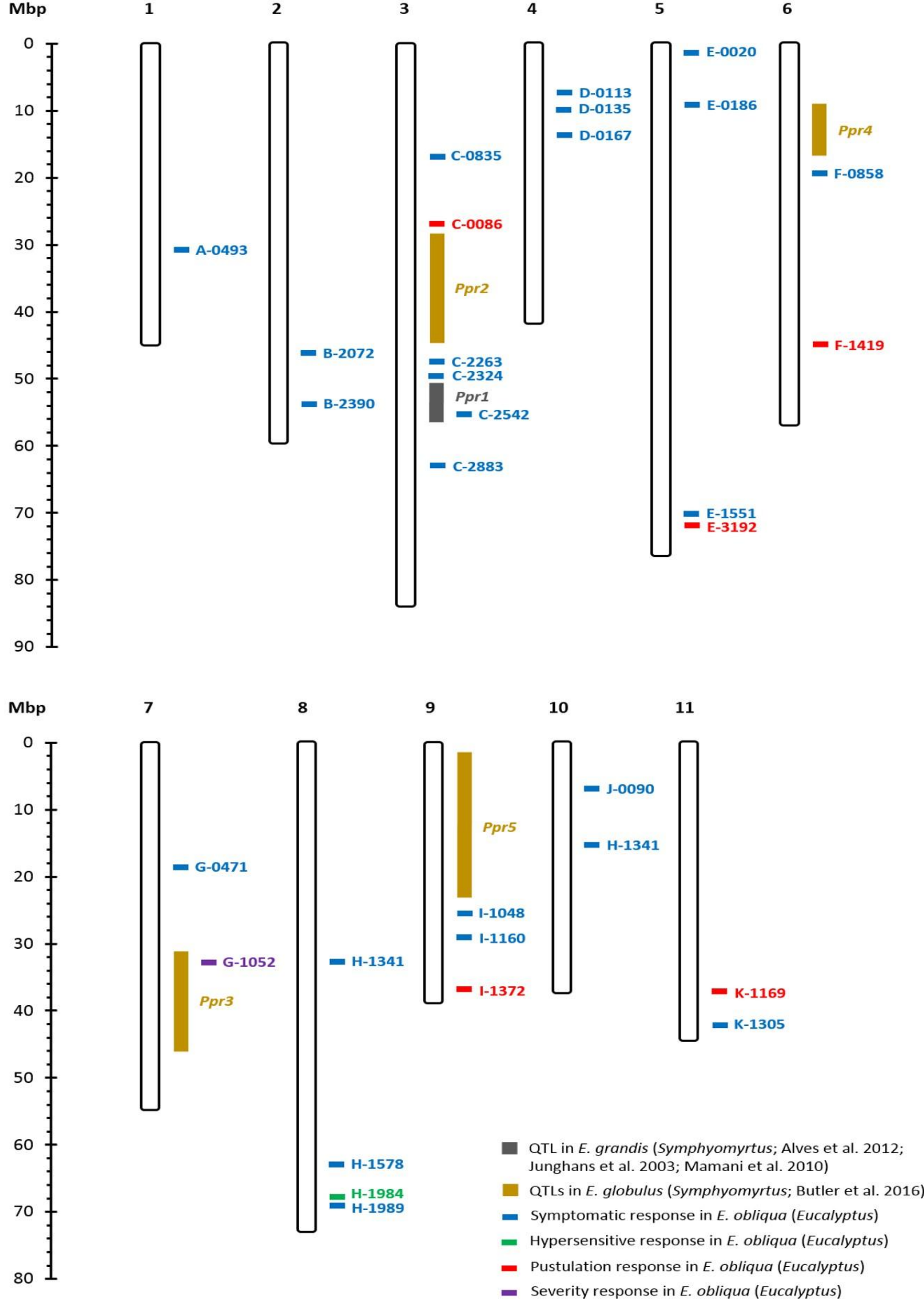

Figure 5-7: The position of previously reported *Ppr* QTLs (*Ppr1* to *Ppr5*) and myrtle rust resistance SNPs ($-\log_{10} P > 6.0$) identified in the present study based on the physical map of *Eucalyptus* (each chromosome is numbered). Mbp = megabase pair.

found other loci and indicate rust resistance in eucalypts to be complex and controlled by multiple interacting loci of variable effect (Alves et al. 2012; Butler et al. 2016; Santos et al. 2014; Thumma et al. 2013). The different host responses to *A. psidii* viz. asymptomatic or immune, hypersensitive and sporulation in *E. obliqua* and other species may reflect diverse defense mechanisms in *Eucalyptus* (Naidoo et al. 2014) underlain by different QTLs involved in the respective resistance mechanisms (Butler et al. 2016).

In general, plant defenses against invading pathogens include preformed physical and chemical barriers (Fahn 1988; Freeman and Beattie 2008; Naidoo et al. 2014; Osbourn 1996), and induced resistances which, based on the different pathogen recognition systems, classify into two main mechanisms: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Chisholm et al. 2006; Dodds and Rathjen 2010; Jones and Dangl 2006). PAMPs or conserved microbial elicitors of whole classes of pathogens (such as fungal chitin and bacterial flagellin) are recognised by plant pattern recognition receptors (PRRs) that trigger numerous responses as the first tier of plant immune system to halt further colonisation of invading organism including non-pathogens (Dodds and Rathjen 2010; Segonzac and Zipfel 2011). PTI or basal resistance is generally an effective and symptomless form of plant resistance that involves extensive molecular changes viz. activation of signaling pathways, transcriptional reprogramming, and production of antimicrobials and other related metabolites (Bigeard et al. 2015; Szatmári et al. 2014). When the first defense system or PTI is defeated and plants become truly exposed to pathogenic threats, specific intracellular recognition mechanisms in plants trigger the second layer of defense, or ETI, mostly mediated by a class of receptor proteins that contain nucleotide-binding (NB) domains and LRRs (Chisholm et al. 2006; Dodds and Rathjen 2010). ETI, also known as gene-for-gene resistance of plants, reinstates and amplifies PTI basal transcriptional programs and antimicrobial defenses via detection of specific pathogen virulence molecules (effectors) by *R* gene products, which eventually leads to the localised plant cell death or hypersensitive response and eradication of that specific pathogen (Cui et al. 2015; Jones and Dangl 2006; Szatmári et al. 2014). In recent QTL analysis of rust resistance, two interacting loci (*Ppr2* and *Ppr3*) were reported to

influence the immune response of *E. globulus* to *A. psidii*, and different and non-overlapping QTLs (*Ppr4* and *Ppr5*) were found to control the hypersensitive reaction against the pathogen. These observations support the hypothesis that different resistance mechanisms, i.e. symptomless basal defenses and modulated ETI responses, operate in resistance to *A. psidii*, and that these are controlled by different QTLs (Butler et al. 2016).

Major effect QTLs for fungal disease resistance in *Eucalyptus* have been repeatedly reported on chromosome 3 including in defense against *Mycospaerella cryptica* leaf disease (Freeman et al. 2008), *Ceratocystis fimbriata* wilt (Rosado et al. 2016) and *Sonderhenia* spp. like symptoms (Butler et al. 2016). The present GWAS also discovered a significant SNP marker (C-2542:51401024-51449710:1_19512) on chromosome 3 at position *c.* 55.3 Mbp for symptomatic response of *E. obliqua* to *A. psidii*. This SNP co-localises with the previously reported locus *Ppr1* in *E. grandis* (Junghans et al. 2003a). A number of other significant SNP for symptomatic and pustulation responses were also found in the vicinity of the other reported chromosome 3 major effect locus *Ppr2* (Butler et al. 2016; Junghans et al. 2003a). Independent studies, again on chromosome 3, discovered a QTL involved in digenic epistasis for *A. psidii* resistance in *E. dunnii* × *E. grandis* hybrids (Alves et al. 2012), and SNP markers linked to rust resistance in *E. globulus* (Thumma et al. 2013). Clustering of QTLs in the position between *c.* 54 and 61 Mbp on *Eucalyptus* chromosome 3 may explain the action of linked genes in this conserved genomic space with the highest densities of disease resistance genes (Christie et al. 2016; Myburg et al. 2014), or the pleiotropic effects of the locus with diverse implication on general disease resistance (Butler et al. 2016; Wang et al. 2001). Recent GWAS and regional heritability mapping (RHM) for rust resistance in *E. grandis* × *E. urophylla* hybrid also detected SNP-trait associations within the same region on chromosome 3. Combined these results suggests a major role for chromosome 3 in the disease resistance responses, including to *A. psidii*, across both species and pathogens (Christie et al. 2016; Resende et al. 2017b), and highlights a number of genomic intervals worthy of further examination towards potential resistance gene discovery.

Typical *R* genes encode proteins that contain NB site and LRR domains in response to the virulence effector molecules of pathogens (Chisholm et al. 2006; Ellis and Jones 1998; Keith and Mitchell-Olds 2013; McDowell and Woffenden 2003). They are often (but not always) associated with hypersensitivity or localised programmed cell death which block the progression of diseases caused by biotrophic pathogens (Caplan et al. 2008; Cui et al. 2015; Heath 2000a; Jones and Dangl 2006; Mur et al. 2008). Other domains involved in R proteins for the detection of broad spectra pathogen effector proteins include Toll/interleukin-1 receptor (TIR), coiled-coil (CC), leucine zipper (LZ), transmembrane (TM) and serine-threonine kinase (STK) domains (Kim et al. 2012; Martin et al. 2003; van Ooijen et al. 2007). Among all known *R* genes, NB-LRR encoding genes are the largest and most variable gene family found in plant kingdom (encompassing more than 80% of the characterised *R* genes), with their copy number varying both between and within species (Shao et al. 2016; Yang et al. 2006; Zhang et al. 2010). Since the first comprehensive study of genes in *Arabidopsis thaliana* (Meyers et al. 2003), NB-LRR gene families have been extensively searched across various plant genomes, e.g. gramineous species (Li et al. 2010; Luo et al. 2012; Monosi et al. 2004; Yang et al. 2006; Zhou et al. 2004), legumes (Ameline-Torregrosa et al. 2008; Shao et al. 2014; Zhang et al. 2011), solanaceous plants (Andolfo et al. 2013; Lozano et al. 2012) and woody species (Kohler et al. 2008; Yang et al. 2008). These have provided opportunities for general study of the structure and function of plant disease resistance-related genes and have supported the breeding of disease-resistant plants (Barbosa-da-Silva et al. 2005; Li et al. 2013; Liu and Ekramoddoullah 2004; Wang et al. 2004).

In *Eucalyptus*, a total of 1487 putative NB-LRR genes were recently identified within the *E. grandis* genome using an iterative search process, including 557 complete gene models incorporating both the NB and LRR domains (Christie et al. 2016). Of these, 423 genes were transcriptionally active and differentially expressed under specific biotic stresses i.e. challenged by a fungal pathogen (*Chrysoporthe austroafricana*) or insect pest (*Leptocybe invasa*). A previous association study of *E. grandis* found half the significant markers for *A. psidii* rust resistance to reside in NB-LRR loci located near the *Ppr1* locus (Thumma et al. 2013). The chromosome 3 genomic region may in fact be composed of high density QTL clusters or superclusters of NB-LRR genes with

different loci potentially underlying the resistance to different pathogens (Barbosa-da-Silva et al. 2005; Butler et al. 2016; Christie et al. 2016). The clustering of *R* genes allows rapid detection and evolutionary change in response to a myriad of diverse pathogen effectors (Fluhr 2001; Gururani et al. 2012; Sinapidou et al. 2004), and may be involved in the duplication or divergence of R proteins that is required for effective recognition of pathogen avirulence factors for standardised and timely defense responses (Eitas and Dangl 2010; Tobias 2012). Apart from NB-LRR genes, the present analysis also identified other candidate genes known to play roles in plant disease resistance, such as enzymatic class of *R* genes (i.e. kinases and hydrolases) and genes annotated as nucleotide-binding proteins and transcription factors. These results are consistent with other association genetic studies for rust resistance in *Eucalyptus* (Butler et al. 2016; Thumma et al. 2013). Similar to Resende et al. (2017b) who also reported suggestive QTL for *A. psidii* resistance in *E. grandis* × *E. urophylla* hybrid clustered on chromosomes 5, 8 and 10, apart from chromosome 3 this study also found significant signals on other chromosomes supporting the hypothesis that rust resistance in *Eucalyptus* is influenced by many loci of variable effects.

The overlapping of rust resistance QTLs between *E. obliqua* and species from subgenus *Symphyomyrtus*, as evidenced by the occurrence of present SNP markers within the *Ppr1* and in the proximity of *Ppr2-5* loci, may indicate the long evolutionary history between *Eucalyptus* (or *Symphyomyrtus*) ancestors and the pathogen or its ancestors dating to the Eocene on the ancient southern supercontinent of Gondwana (Potts et al. 2016; Thornhill et al. 2015). While the presence of specific recognition to newly encountered pathogens is ambiguous for the resistance to *A. psidii* in modern Australian naïve host myrtaceous plants, the presence of eucalypt-like fossils in South America where the pathogen is native (Coutinho et al. 1998; Thornhill et al. 2015) may explain the possibility of ancient exposure between eucalypt host and rust pathogen or their ancestors, and the retention of ancient *R* genes within the host genome as a result of intermittent challenge according to the ‘trench warfare’ rather than ‘arms race’ concept for the evolution of *Eucalyptus* – *A. psidii* interactions (Jones and Dangl 2006; Stahl et al. 1999; Tobias et al. 2016). Trench warfare model was proposed to explain the *R* gene polymorphisms in pathogen recognition and dynamics of disease resistance in

Arabidopsis where the resistant and susceptible alleles are maintained over a long period of time, and the proportions of alleles within populations vary in response to periodic epidemics (Kunkel 1996; Stahl et al. 1999). However, the possibility of the specific resistance genes to *A. psidii* to maintain selection pressure and retain their function for over 30 million years, i.e. dating from a time when ancestors of the host and pathogen were in contact, without intervening pathogen challenges seems implausible as the accumulation of mutations over that period would likely compromise long-term persistence of resistance on the Australian landmass (McDowell and Simon 2006; Tobias et al. 2016). An alternative and more reasonable hypothesis might be that the resistance genes are effective against and are maintained by current native pathogens, and just happen to be at least (partly) effective in defense against *A. psidii*. Tobias et al. (2016) reported the extended modes of plant-pathogen interactions viz. allelic variance in receptor pairs and complexes or additive role of *R* gene products (Eitas and Dangl 2010), and the guard and decoy strategies via recognition of effector modification of common host targets for surveillance mechanisms (van der Biezen and Jones 1998; van der Hoorn and Kamoun 2008), as potential contributors to the observed specific resistance to *A. psidii* across *Eucalyptus* plant family.

Conclusion

In the present GWAS, SNP markers associated with multiple mechanisms (numeric severity, binary symptomatic, hypersensitive and pustulation) for resistance to *A. psidii* were identified in *E. obliqua* and mapped to the *Eucalyptus* reference genome. Several markers were positioned within the major *Ppr1* locus and close to the other *A. psidii* resistance loci, *Ppr2-5*. The co-location of rust resistance loci in *E. obliqua* and *Symphyomyrtus* species may reflect the long evolutionary connection through ancient exposure between *Eucalyptus* host and *A. psidii* pathogen, or the maintenance of resistance genes by common native pathogens which happen to be partly effective against *A. psidii*. This analysis identified putative candidate genes which will require validation in future studies. This study adds to the understanding of the genetic architecture underlying *A. psidii* resistance in *Eucalyptus*. Further identification of novel QTLs and manipulation of loci controlling rust resistance enable molecular based

breeding for resistance and enable studies of population resistance responses in natural systems challenged by this rust pathogen.

Chapter 6

Concluding remarks and future perspectives

Myrtle rust, caused by *Austropuccinia psidii* Beenken, is an exotic pathogen to Australia. Despite this specific resistance has been found across many native myrtaceous species with variability ranging from immune or no observable disease symptoms to hypersensitive, and to full pustule development. Despite significant differences being observed in rust susceptibility between *Eucalyptus* subgenera i.e. *Symphyomyrtus* and *Eucalyptus*, and between and within *Eucalyptus* species, geographic variation of resistance within species was comparatively weak between geographic races of *Eucalyptus globulus* Labill. and between forest districts of *Eucalyptus obliqua* L'Hér. Further, this variation was not explained by environmental characteristics such as home-site elevation, annual rainfall, and maximum and minimum temperatures. The highly significant difference in susceptibility to *A. psidii* between the most species-rich eucalypt subgenera (*Symphyomyrtus* and *Eucalyptus*) also indicates that host phylogenetic history may be a broad indicator of vulnerability to this exotic pathogen, consistent with subgeneric differences in various morphological and ecological traits viz. early growth patterns (Davidson and Reid 1980; Noble 1989; Turnbull et al. 1993), leaf chemistry (Li et al. 1995, 1996, 1997), and susceptibility to other specific enemies such as the foliar pathogen *Phaeoseptoria eucalypti* (Hansf.) Walker (Nichol et al. 1992; Park et al. 2000) and the root pathogen *Phytophthora cinnamomi* Rands (Podger and Batini 1971; Tippett et al. 1985).

Examination of the rust infection process on different rust response phenotypes (completely resistant, hypersensitive and highly susceptible) revealed non-host/non-pathogen interactions, i.e. the growth of variable length urediniospore germ tubes in random directions and reduced probability of germ tubes in locating stomata, which may due to the lack of basic compatibility between *Eucalyptus* and *A. psidii* or lack of pathogenicity factors between the plant and the pathogen as observed in other non-host pathosystems such as wheat stripe rust (*Puccinia striiformis* f. sp. *tritici* Eriks.) in broad

bean (Cheng et al. 2012), barley leaf rust (*Puccinia hordei* G.H. Otth) in wheat (Niks and Dekens 1991; Rodrigues et al. 2004) and soybean rust (*Phakopsora pachyrhizi* Syd. & P.Syd.) in barley (Hoefle et al. 2009). Comprehensive microscopic observation also shown an unusual mode of penetration (non-stomatal penetration) by *A. psidii* on *Eucalyptus* leaves. This mechanism has only been reported from a few dikaryotic rust fungi. It allows penetration success not only on the specific host but also on non-host plants, resulting in a more extensive host range than most rusts (Adendorff and Rijkenberg 2000; Bonde et al. 1982; Edwards and Bonde 2011; Hunt 1968). The initial infection events from urediniospore germination to appressorium formation were found to occur similarly in all the rust response phenotypes within 48 hours after inoculation (h.a.i.) with no obvious differences in ontogeny and morphology of the *A. psidii* infection structures between the phenotypes. The infection process then stopped on the resistant plants at the cuticle or epidermis levels without host penetration while the complete disease cycle was detected on the susceptible plants within 10 days after inoculation (d.a.i.) with the formation of the necessary cellular structures for successful rust colonisation and reproduction. However, some *Eucalyptus* species may develop adult plant resistance to *A. psidii* due to ontogenetic changes in leaf characteristics at as relatively young age as found in 12-month-old *E. obliqua*. This may minimise the detrimental effects of this invasive pathogen in native sclerophyll forests dominated by *E. obliqua* or other *Eucalyptus* species with early heteroblastic transition (phase change from juvenile to adult leaves) capability (James and Bell 2001; Johnson 1926; Lawrence et al. 2003; Li et al. 1995, 1996).

As the rust infection process arrests on leaf surfaces in resistant phenotypes, *Eucalyptus* foliar terpene composition was analysed to test for chemical defence mechanisms against *A. psidii* pathogen. Canonical discriminant analysis found significant associations between particular terpene components and rust response phenotype, but the identified compounds were species-dependent, i.e. six compounds (bicyclogermacrene, globulul, geraniol, β-pinene, *cis-p*-menth-2-en-1-ol and δ-terpinene) were identified from *E. globulus* and four (δ-cadinene, caryophyllene oxide, longifolenaldehyde and α-caryophyllene) from *E. obliqua*. These findings also differed from the reported rust resistance biomarkers found in hybrids of *Eucalyptus grandis*

W.Hill ex Maiden and *Eucalyptus urophylla* S.T.Blake i.e. 1,8-cineole and α-terpinyl acetate. So, while no general chemical terpene defence mechanism is likely operating across *Eucalyptus*, species specific combined effects from different terpenes or synergistic effects of terpene compounds in combination with other plant metabolites in *Eucalyptus* species of great chemotypic diversity (Naidoo et al. 2014; Padovan et al. 2014), may be combining to produce similar defensive responses against the pathogen. The lack of consistency among *Eucalyptus* species in terpene composition associated with rust symptom response contrasts with the specific compounds or chemotypes shown to mediate defenses against insect herbivores (Padovan et al. 2012) and browsing marsupials (Lawler et al. 1999; O'Reilly-Wapstra et al. 2004).

A genome wide association study (GWAS) of rust resistance showed significant associations between SNP markers and multiple responses (numeric severity, binary symptomatic, hypersensitive and pustulation) to *A. psidii* in *E. obliqua*, a representative species from subgenus *Eucalyptus*. When the identified markers were positioned on the reference genome for *Eucalyptus* (v2 www.phytozome.net; Myburg et al. 2014) some SNPs were found to overlap with the region for *Pp1* or be in the proximity of *Ppr2-5*, major QTLs previously reported for rust resistance in *Eucalyptus* species, i.e. *E. grandis* and *E. globulus*, from subgenus *Symphyomyrtus* (Butler et al. 2016; Junghans et al. 2003a). There was a concentration of significant SNP on chromosome 3. Apart from the *Ppr1* and *Ppr2* involved in *A. psidii* resistance, there has been repeated location of fungal disease resistance QTLs on *Eucalyptus* chromosome 3 in other studies of *Mycospaerella cryptica* leaf disease (Freeman et al. 2008), *Ceratocystis fimbriata* wilt (Rosado et al. 2016) and *Sonderhenia* spp. like symptoms (Butler et al. 2016) which points to the major involvement of this chromosome in resistance responses across multiple host species and pathogens (Resende et al. 2017b).

The co-location of rust resistance loci between subgenera *Eucalyptus* and *Symphyomyrtus* may support the hypothesis that the host and pathogen have had an extended evolutionary connection in the past (Potts et al. 2016) with the presence of specific recognition to *A. psidii* in modern Australian *Eucalyptus* due to the retention of ancient *R* genes dating from co-evolution between pathogen effectors and host

resistance genes in *Eucalyptus* common ancestors (Tobias et al. 2016). It has been hypothesised that eucalypts and the rust pathogen, or their ancestors, had a probable historical or geographical link dating back to the Eocene or *c.* 30 million years ago encompassing the breakup of the ancient southern supercontinent of Gondwana when the pathogen and eucalypt host could have co-evolved (Potts et al. 2016; Tobias et al. 2016) but the considerable time between contact makes this hypotheses implausible given the likely accumulation of mutation leading to loss of function in any previous resistance loci in the absence of selection.

The maintenance of rust resistance genes in *Eucalyptus* may alternatively due to the effectiveness in defences against current native pathogens that also happen to be partly effective against *A. psidii*. In this study functional annotation of positional candidate genes near significant SNP in *E. obliqua* were generally hypothetical proteins of unknown function, the analyses also detected several genes known to play a role in plant disease resistance. This included several *R* genes i.e. kinases and hydrolases, and genes annotated as nucleotide-binding (NB) proteins, leucine-rich repeat (NB-LRR) domains and transcription factors. These results are consistent with previous association genetic studies for rust resistance in *Eucalyptus* species from subgenus *Symphyomyrtus* (Butler et al. 2016; Thumma et al. 2013).

The outcomes of this thesis add to previous studies in understanding of the complexity of *Eucalyptus* – *A. psidii* pathosystem and the underlying mechanisms of rust resistance in *Eucalyptus*. The present findings provide insight into the infection process of *A. psidii* on *Eucalyptus* foliage between different rust response phenotypes, and the potential drivers for rust resistance in *E. globulus* and *E. obliqua* representatives of the most important subgenera (*Symphyomyrtus* and *Eucalyptus*). In future, *Eucalyptus* foliar cuticular waxes (amount, composition and distribution pattern) may be analysed for their effects on defences against *A. psidii* pathogen, particularly in resistance to appressorium formation and penetration by germinating urediniospores. Further confirmation and validation of putative candidate genes may also contribute to the dissection of complex molecular nature of rust resistance at the fundamental and applied levels, and exploration of causal resistance mechanisms in the target *Eucalyptus* species

for potential translation into effective disease management in native forests and plantation enterprises.

This page intentionally left blank

References

Adendorff R, Rijkenberg FHJ (2000) Scanning electron microscopy of direct host leaf penetration by urediospore-derived infection structures of *Phakopsora apoda*. Mycological Research 104: 317-324. doi:10.1017/S0953756299001203

Aime MC, Matheny PB, Henk DA, Frieders EM, Nilsson RH, Piepenbring M, McLaughlin DJ, Szabo LJ, Begerow D, Sampaio JP, Bauer R, Weiß M, Oberwinkler F, Hibbett D (2006) An overview of the higher level classification of Pucciniomycotina based on combined analyses of nuclear large and small subunit rDNA sequences. Mycologia 98: 896-905. doi:10.1080/15572536.2006.11832619

Albaugh JM, Dye PJ, King JS (2013) *Eucalyptus* and water use in South Africa. International Journal of Forestry Research 2013, Article 852540: 1-11. doi:10.1155/2013/852540

Albrechtsen SE (2006) Biological assays. In: Testing Methods for Seed-transmitted Viruses: Principles and Protocols. CABI Publishing, Wallingford, pp 47-83

Aldwinckle HS (1975) Pathogenic races of *Gymnosporangium juniperi-virginianae* on apple. Phytopathology 65: 958-961. doi:10.1094/Phyto-65-958

Alfenas AC, Guimarães LMS, de Resende MDV (2012) Genetic basis of resistance in *Eucalyptus* spp. pathosystems. In: Sniezko RA, Yanchuk AD, Kliejunas JT, Palmieri KM, Alexander JM, Frankel SJ (eds) Disease and Insect Resistance in Forest Trees. Proceedings of the Fourth International Workshop on Genetics of Host-Parasite Interactions in Forestry, U. S. Department of Agriculture, Albany, CA, pp 11-15

Allen E, Hiratsuka Y (1985) Artificial inoculation of young seedlings of lodgepole pine with *Endocronartium harknessii*. Canadian Journal of Botany 63: 1168-1170. doi:10.1139/b85-161

Almeida MH, Pereira H, Miranda I, Tome M (1995) Provenance trials of *Eucalyptus globulus* in Portugal. In: Potts BM, Borralho NMG, Reid JB, Cromer RN, Tibbits WN, Raymond CA (eds) Eucalypt Plantations: Improving Fibre Yield and Quality. Proceedings of the CRCTHF-IUFRO Conference, CRC for Temperate Hardwood Forestry, Hobart, pp 195-198

Altizer S, Harvell D, Friedle E (2003) Rapid evolutionary dynamics and disease threats to biodiversity. Trends in Ecology & Evolution 18: 589-596. doi:10.1016/j.tree.2003.08.013

Alves AA, Rosado CCG, Faria DA, Guimaraes LMD, Lau D, Brommonschenkel SH, Grattapaglia D, Alfenas AC (2012) Genetic mapping provides evidence for the role of additive and non-additive QTLs in the response of inter-specific hybrids of *Eucalyptus* to *Puccinia psidii* rust infection. Euphytica 183: 27-38. doi:10.1007/s10681-011-0455-5

Ameline-Torregrosa C, Wang BB, O'Bleness MS, Deshpande S, Zhu H, Roe B, Young ND, Cannon SB (2008) Identification and characterization of nucleotide-binding site-leucine-rich repeat genes in the model plant *Medicago truncatula*. Plant Physiology 146: 5-21. doi:10.1104/pp.107.104588

Andolfo G, Sanseverino W, Rombauts S, Van de Peer Y, Bradeen JM, Carputo D, Frusciante L, Ercolano MR (2013) Overview of tomato (*Solanum lycopersicum*) candidate pathogen recognition genes reveals important *Solanum* R locus dynamics. New Phytologist 197: 223-237. doi:10.1111/j.1469-8137.2012.04380.x

Andrew RL, Peakall R, Wallis IR, Foley WJ (2007a) Spatial distribution of defense chemicals and markers and the maintenance of chemical variation. Ecology 88: 716-728. doi:10.1890/05-1858

Andrew RL, Peakall R, Wallis IR, Wood JT, Knight EJ, Foley WJ (2005) Marker-based quantitative genetics in the wild?: The heritability and genetic correlation of chemical defenses in *Eucalyptus*. Genetics 171: 1989-1998. doi:10.1534/genetics.105.042952

Andrew RL, Wallis IR, Harwood CE, Foley WJ (2010) Genetic and environmental contributions to variation and population divergence in a broad-spectrum foliar defence of *Eucalyptus tricarpa*. Annals of Botany 105: 707-717. doi:10.1093/aob/mcq034

Andrew RL, Wallis IR, Harwood CE, Henson M, Foley WJ (2007b) Heritable variation in the foliar secondary metabolite sideroxylonal in *Eucalyptus* confers cross-resistance to herbivores. Oecologia 153: 891-901. doi:10.1007/s00442-007-0784-1

Anekonda TS, Criddle RS, Bacca M, Hansen LD (1999) Contrasting adaptation of two *Eucalyptus* subgenera is related to differences in respiratory metabolism. Functional Ecology 13: 675-682. doi:10.1046/j.1365-2435.1999.00367.x

Anekonda TS, Criddle RS, Bacca M, Hansen LD (2000) Erratum: Contrasting adaptation of two *Eucalyptus* subgenera is related to differences in respiratory metabolism (vol 13, pg 675, 1999). Functional Ecology 14: 400. doi:10.1046/j.1365-2435.2000.00458.x

Arneson PA (2000) Coffee rust. The Plant Health Instructor. The American Phytopathological Society. doi: 10.1094/PHI-I-2000-0718-02 http://www.apsnet.org/edcenter/intropp/lessons/fungi/basidiomycetes/pages/coffeerust.aspx. Accessed 6 March 2017

Ballesta P, Mora F, Contreras-Soto RI, Ruiz E, Perret S (2015) Analysis of the genetic diversity of *Eucalyptus cladocalyx* (sugar gum) using ISSR markers. Acta Scientiarum - Agronomy 37: 133-140. doi:10.4025/actasciagron.v37i2.19307

Balmelli G, Simeto S, Marroni V, Altier N, Diez JJ (2014) Genetic variation for resistance to *Mycosphaerella* leaf disease and *Eucalyptus* rust on *Eucalyptus globulus* in Uruguay. Australasian Plant Pathology 43: 97-107. doi:10.1007/s13313-013-0254-7

Barbosa-da-Silva A, Wanderley-Nogueira AC, Silva RRM, Berlarmino LC, Soares-Cavalcanti NM, Benko-Iseppon AM (2005) *In silico* survey of resistance (*R*) genes in *Eucalyptus* transcriptome. Genetics and Molecular Biology 28: 562-574. doi:10.1590/S1415-47572005000400011

Bates D, Machler M, Bolker BM, Walker SC (2015) Fitting linear mixed-effects models using lme4. Journal of Statistical Software 67: 1-48. doi:10.18637/jss.v067.i01

Bayly MJ (2016) Phylogenetics studies of eucalypts: Fossils, morphology and genomes. Proceedings of the Royal Society of Victoria 128: 12-24. doi:10.1071/RS16002

Bayly MJ, Rigault P, Spokevicius A, Ladiges PY, Ades PK, Anderson C, Bossinger G, Merchant A, Udovicic F, Woodrow IE, Tibbits J (2013) Chloroplast genome analysis of Australian eucalypts - *Eucalyptus*, *Corymbia*, *Angophora*, *Allosyficarpia* and *Stockwellia* (Myrtaceae). Molecular Phylogenetics and Evolution 69: 704-716. doi:10.1016/j.ympev.2013.07.006

Beenken L (2017) *Austropuccinia*: a new genus name for the myrtle rust *Puccinia psidii* placed within the redefined family Sphaerophragmiaceae (Pucciniales). Phytotaxa 297: 53-61. doi:10.11646/phytotaxa.297.1.5

Bell CD, Soltis DE, Soltis PS (2010) The age and diversification of the angiosperms re-revisited. American Journal of Botany 97: 1296-1303. doi:10.3732/ajb.0900346

Bettgenhaeuser J, Gilbert B, Ayliffe M, Moscou MJ (2014) Nonhost resistance to rust pathogens - a continuation of continua. Frontiers in Plant Science 5, Article 664: 1-15. doi:10.3389/fpls.2014.00664

Bhuiyan MAHB, Groom T, Nicolas ME, Taylor PWJ (2015) Histopathology of *S. tanaceti* infection in pyrethrum leaf lamina. Australasian Plant Pathology 44: 629-636. doi:10.1007/s13313-015-0377-0

Bi JL, Felton GW (1995) Foliar oxidative stress and insect herbivory: primary compounds, secondary metabolites, and reactive oxygen species as components of induced resistance. Journal of Chemical Ecology 21: 1511-1530. doi:10.1007/BF02035149

Bigeard J, Colcombet J, Hirt H (2015) Signaling mechanisms in pattern-triggered immunity (PTI). Molecular Plant 8: 521-539. doi:10.1016/j.molp.2014.12.022

Biggs AR, Rosenberger DA, Yoder KS, Kiyomoto RK, Cooley DR, Sutton TB (2009) Relative susceptibility of selected apple cultivars to cedar apple rust and quince rust. Plant Health Progress. doi:10.1094/PHP-2009-1014-01-RS

Bloomfield JA, Nevill P, Potts BM, Vaillancourt RE, Steane DA (2011) Molecular genetic variation in a widespread forest tree species *Eucalyptus obliqua* (Myrtaceae) on the island of Tasmania. Australian Journal of Botany 59: 226-237. doi:10.1071/BT10315

Boland DJ, Brooker MIH, Chippendale GM, Hall N, Hyland BPM, Johnston RD, Kleinig DA, McDonald MW, Turner JD (2006) Forest Trees of Australia. 5 edn. CSIRO Publishing, Collingwood VIC

Boland DJ, Brophy JJ, House APN (1991) Eucalyptus Leaf Oils: Use, Chemistry, Distillation and Marketing. Inkata Press, Melbourne

Bolton MD, Kolmer JA, Garvin DF (2008) Wheat leaf rust caused by *Puccinia triticina*. Molecular Plant Pathology 9: 563-575. doi:10.1111/j.1364-3703.2008.00487.x

Bonde MR, Bromfield KR, Melching JS (1982) Morphological development of *Physopella zeae* on corn. Phytopathology 72: 1489-1491. doi:10.1094/Phyto-72-1489

Bonde MR, Melching JS, Bromfield KR (1976) Histology of the suscept-pathogen relationship between *Glycine max* and *Phakopsora pachyrhizi*, cause of soybean rust. Phytopathology 66: 1290-1294. doi:10.1094/Phyto-66-1290

Booth TH, Broadhurst LM, Pinkard E, Prober SM, Dillon SK, Bush D, Pinyopusarerk K, Doran JC, Ivkovich M, Young AG (2015) Native forests and climate change: Lessons from eucalypts. Forest Ecology and Management 347: 18-29. doi:10.1016/j.foreco.2015.03.002

Booth TH, Jovanovic T (2012) Assessing vulnerable areas for *Puccinia psidii* (eucalyptus rust) in Australia. Australasian Plant Pathology 41: 425-429. doi:10.1007/s13313-012-0130-x

Booth TH, Old KM, Jovanovic T (2000) A preliminary assessment of high risk areas for *Puccinia psidii* (*Eucalyptus* rust) in the Neotropics and Australia. Agriculture, Ecosystems and Environment 82: 295-301. doi:10.1016/S0167-8809(00)00233-4

Borzak CL, Potts BM, Davies NW, O'Reilly-Wapstra JM (2015) Population divergence in the ontogenetic trajectories of foliar terpenes of a *Eucalyptus* species. Annals of Botany 115: 159-170. doi:10.1093/aob/mcu222

Bourassa M, Bernier L, Hamelin RC (2007) Genetic diversity in poplar leaf rust (*Melampsora medusae* f. sp. *deltoidae*) in the zones of host sympatry and allopatry. Phytopathology 97: 603-610. doi:10.1094/PHYTO-97-5-0603

Bowers WS, Evans PH, Spence SP (2000) Trichomes of *Eucalyptus maculata citriodon* possess a single potent insect repellent. Journal of Herbs, Spices & Medicinal Plants 7: 85-89. doi:10.1300/J044v07n04_10

Bradbury PJ, Zhang Z, Kroon DE, Casstevens TM, Ramdoss Y, Buckler ES (2007) TASSEL: software for association mapping of complex traits in diverse samples. Bioinformatics 23: 2633-2635. doi:10.1093/bioinformatics/btm308

Bright C (1998) Life Out of Bounds: Bioinvasion in a Borderless World. W. W. Norton & Company, New York

Brondani RPV, Brondani C, Tarchini R, Grattapaglia D (1998) Development, characterization and mapping of microsatellite markers in *Eucalyptus grandis* and *E. urophylla*. Theoretical and Applied Genetics 97: 816-827. doi:10.1007/s001220050961

Brondani RPV, Williams ER, Brondani C, Grattapaglia D (2006) A microsatellite-based consensus linkage map for species of *Eucalyptus* and a novel set of 230 microsatellite markers for the genus. BMC Plant Biology 6, Article 20: 1-16. doi:10.1186/1471-2229-6-20

Brooker MIH (2000) A new classification of the genus *Eucalyptus* L'Her. (Myrtaceae). Australian systematic botany 13: 79-148. doi:10.1071/SB98008

Brophy JJ, Goldsack RJ, Craven LA, O'Sullivan W (2006) An investigation of the leaf oils of the Western Australian broombush complex (*Melaleuca uncinata* sens. lat.) (Myrtaceae). Journal of Essential Oil Research 18: 591-599. doi:10.1080/10412905.2006.9699176

Brophy JJ, Southwell IA (2002) *Eucalyptus* chemistry. In: Coppen JJW (ed) *Eucalyptus*: The Genus *Eucalyptus*. Taylor & Francis, New York, pp 102-160

Brown AG, Eldridge KG, Green JW, Matheson AC (1976) Genetic variation of *Eucalyptus obliqua* in field trials. New Phytologist 77: 193-203. doi:10.1111/j.1469-8137.1976.tb01514.x

Brown JKM, Hovmøller MS (2002) Aerial dispersal of pathogens on the global and continental scales and its impact on plant disease. Science 297: 537-541. doi:10.1126/science.1072678

Brumfield RT, Beerli P, Nickerson DA, Edwards SV (2003) The utility of single nucleotide polymorphisms in inferences of population history. Trends in Ecology & Evolution 18: 249-256. doi:10.1016/S0169-5347(03)00018-1

Bryner SF, Rigling D (2011) Temperature-dependent genotype-by-genotype interaction between a pathogenic fungus and its hyperparasitic virus. The American Naturalist 177: 65-74. doi:10.1086/657620

Bundock PC, Hayden M, Vaillancourt RE (2000) Linkage maps of *Eucalyptus globulus* using RAPD and microsatellite markers. Silvae Genetica 49: 223-232

Burdon JJ, Thrall PH (1999) Spatial and temporal patterns in coevolving plant and pathogen associations. The American Naturalist 153: S15-S33. doi:10.1086/303209

Burdon RD (2001) Genetic diversity and disease resistance: some considerations for research, breeding, and deployment. Canadian Journal of Forest Research 31: 596-606. doi:10.1139/cjfr-31-4-596

Bush D, Marcar N, Arnold R, Crawford D (2013) Assessing genetic variation within *Eucalyptus camaldulensis* for survival and growth on two spatially variable saline sites in southern Australia. Forest Ecology and Management 306: 68-78. doi:10.1016/j.foreco.2013.06.008

Bustos-Segura C, Dillon S, Keszei A, Foley WJ, Külheim C (2017) Intraspecific diversity of terpenes of *Eucalyptus camaldulensis* (Myrtaceae) at a continental scale. Australian Journal of Botany 65: 257-269. doi:10.1071/BT16183

Bustos-Segura C, Külheim C, Foley W (2015) Effects of terpene chemotypes of *Melaleuca alternifolia* on two specialist leaf beetles and susceptibility to Myrtle Rust. Journal of Chemical Ecology 41: 937-947. doi:10.1007/s10886-015-0628-0

Butcher PA, Otero A, McDonald MW, Moran GF (2002) Nuclear RFLP variation in *Eucalyptus camaldulensis* Dehnh. from northern Australia. Heredity 88: 402-412. doi:10.1038/sj.hdy.6800074

Butler JB, Freeman JS, Vaillancourt RE, Potts BM, Glen M, Lee DJ, Pegg GS (2016) Evidence for different QTL underlying the immune and hypersensitive responses of *Eucalyptus globulus* to the rust pathogen *Puccinia psidii*. Tree Genetics & Genomes 12, Article 39: 1-13. doi:10.1007/s11295-016-0987-x

Byrne M, Marquezgarcia MI, Uren T, Smith DS, Moran GF (1996) Conservation and genetic diversity of microsatellite loci in the genus *Eucalyptus*. Australian Journal of Botany 44: 331-341. doi:10.1071/BT9960331

Cameron RJ (1970) Light intensity and the growth of *Eucalyptus* seedlings. I. Ontogenetic variation in *E. fastigata*. Australian Journal of Botany 18: 29-43. doi:10.1071/BT9700029

Caplan J, Padmanabhan M, Dinesh-Kumar SP (2008) Plant NB-LRR immune receptors: from recognition to transcriptional reprogramming. Cell Host & Microbe 3: 126-135. doi:10.1016/j.chom.2008.02.010

Cappa EP, El-Kassaby YA, Garcia MN, Acuña C, Borralho NMG, Grattapaglia D, Poltri SNM (2013) Impacts of population structure and analytical models in genome-wide association studies of complex traits in forest trees: a case study in *Eucalyptus globulus*. PloS One 8, e81267: 1-16. doi:10.1371/journal.pone.0081267

Carnegie AJ (2015) First report of *Puccinia psidii* (myrtle rust) in *Eucalyptus* plantations in Australia. Plant Disease 99: 161-161. doi:10.1094/PDIS-09-14-0901-PDN

Carnegie AJ, Ades PK (2005) Variation in *Eucalyptus globulus* Labill. and *E. nitens* Dean and Maiden in susceptibility of adult foliage to disease caused by *Mycosphaerella cryptica* (Cooke) Hansf. Silvae Genetica 54: 174-184

Carnegie AJ, Ades PK, Keane PJ, Smith IW (1998) *Mycosphaerella* diseases of juvenile foliage in a eucalypt species and provenance trial in Victoria, Australia. Australian Forestry 61: 190-194. doi:10.1080/00049158.1998.10674739

Carnegie AJ, Cooper K (2011) Emergency response to the incursion of an exotic myrtaceous rust in Australia. Australasian Plant Pathology 40: 346-359. doi:10.1007/s13313-011-0066-6

Carnegie AJ, Giblin FR (2016) Invasive Species Compendium, Datasheet: *Puccinia psidii* (myrtle rust). Centre for Agriculture and Biosciences International (CABI). http://www.cabi.org/isc/datasheet/45846. Accessed 9 February 2017

Carnegie AJ, Kathuria A, Pegg GS, Entwistle P, Nagel M, Giblin FR (2016) Impact of the invasive rust *Puccinia psidii* (myrtle rust) on native Myrtaceae in natural ecosystems in Australia. Biological Invasions 18: 127-144. doi:10.1007/s10530-015-0996-y

Carnegie AJ, Keane PJ, Ades PK, Smith IW (1994) Variation in susceptibility of *Eucalyptus globulus* provenances to Mycosphaerella leaf disease. Canadian Journal of Forest Research 24: 1751-1757. doi:10.1139/x94-226

Carnegie AJ, Lidbetter JR (2012) Rapidly expanding host range for *Puccinia psidii* sensu lato in Australia. Australasian Plant Pathology 41: 13-29. doi:10.1007/s13313-011-0082-6

Carnegie AJ, Lidbetter JR, Walker J, Horwood MA, Tesoriero L, Glen M, Priest MJ (2010) *Uredo rangelii*, a taxon in the guava rust complex, newly recorded on Myrtaceae in Australia. Australasian Plant Pathology 39: 463-466. doi:10.1071/AP10102

Carr DJ, Carr SGM (1970) Oil glands and ducts in *Eucalyptus* L'Hérit. II. Development and structure of oil glands in the embryo. Australian Journal of Botany 18: 191-212. doi:10.1071/BT9700191

Carris LM, Little CR, Stiles CM (2012) Introduction to Fungi. The Plant Health Instructor. The American Phytopathological Society. doi: 10.1094/PHI-I-2012-0426-01. http://www.apsnet.org/edcenter/intropp/pathogengroups/pages/introfungi.aspx. Accessed 2 March 2017

Cavill G (2017) Myrtle rust found in New Zealand. Department of Conservation, New Zealand. http://www.doc.govt.nz/news/media-releases/2017/myrtle-rust-found-in-new-zealand/. Accessed 3 August 2017

Chauhan RD, Veale A, Cathleen M, Strauss SH, Myburg AA (2014) Genetic transformation of *Eucalyptus* - challenges and future prospects. In: Ramawat KG, Merillon JM, Ahuja MR (eds) Tree Biotechnology. CRC Press, Taylor & Francis Group, Boca Raton, FL, pp 392-445

Chen W, Wellings C, Chen X, Kang Z, Liu T (2014) Wheat stripe (yellow) rust caused by *Puccinia striiformis* f. sp. *tritici*. Molecular Plant Pathology 15: 433-446. doi:10.1111/mpp.12116

Cheng Y, Zhang H, Yao J, Wang X, Xu J, Han Q, Wei G, Huang L, Kang Z (2012) Characterization of non-host resistance in broad bean to the wheat stripe rust pathogen. BMC Plant Biology 12, Article 96: 1-12. doi:10.1186/1471-2229-12-96

Chippendale GM (1988) Flora of Australia, Volume 19: Myrtaceae - *Eucalyptus*, *Angophora*. Australian Government Publishing Service, Canberra

Chisholm ST, Coaker G, Day B, Staskawicz BJ (2006) Host-microbe interactions: shaping the evolution of the plant immune response. Cell 124: 803-814. doi:10.1016/j.cell.2006.02.008

Christie N, Tobias PA, Naidoo S, Külheim C (2016) The *Eucalyptus grandis NBS-LRR* gene family: physical clustering and expression hotspots. Frontiers in Plant Science 6, Article 1238: 1-16. doi:10.3389/fpls.2015.01238

Christopher-Kozjan R, Heath MC (2003) Cytological and pharmacological evidence that biotrophic fungi trigger different cell death execution processes in host and nonhost cells during the hypersensitive response. Physiological and Molecular Plant Pathology 62: 265-275. doi:10.1016/S0885-5765(03)00088-2

Coelho L, Alfenas AC, Ferreira FA (2001) Physiologic variability of *Puccinia psidii* - the rust of *Eucalyptus*. Summa Phytopathologica 27: 295-300

Collins NC, Thordal-Christensen H, Lipka V, Bau S, Kombrink E, Qiu JL, Hückelhoven R, Stein M, Freialdenhoven A, Somerville SC, Schulze-Lefert P (2003) SNARE-protein-mediated disease resistance at the plant cell wall. Nature 425: 973-977. doi:10.1038/nature02076

Coutinho TA, Wingfield MJ, Alfenas AC, Crous PW (1998) Eucalyptus rust: A disease with the potential for serious international implications. Plant Disease 82: 819-825. doi:10.1094/PDIS.1998.82.7.819

CRC Plant Biosecurity (2016) Myrtle rust - a threat to the Australian landscape and plant industries. Plant Biosecurity Cooperative Research Centre (CRC). http://www.pbcrc.com.au/news/2016/pbcrc/myrtle-rust-threat-australian-landscape-and-plant-industries. Accessed 10 August 2017

Cronk QCB (2005) Plant eco-devo: the potential of poplar as a model organism. New Phytologist 166: 39-48. doi:10.1111/j.1469-8137.2005.01369.x

Cui H, Tsuda K, Parker JE (2015) Effector-triggered immunity: from pathogen perception to robust defense. Annual Review of Plant Biology 66: 487-511. doi:10.1146/annurev-arplant-050213-040012

Davidson NJ, Reid JB (1980) Comparison of the early growth characteristics of the *Eucalyptus* subgenera *Monocalyptus* and *Symphyomyrtus*. Australian Journal of Botany 28: 453-461. doi:10.1071/BT9800453

de Meaux J, Mitchell-Olds T (2003) Evolution of plant resistance at the molecular level: ecological context of species interactions. Heredity 91: 345-352. doi:10.1038/sj.hdy.6800342

de Oliveira C, Degenhardt-Goldbach J, Bettencourt GMD, Amano E, Franciscon L, Quoirin M (2017) Micropropagation of *Eucalyptus grandis* x *E. urophylla* AEC 224 clone. Journal of Forestry Research 28: 29-39. doi:10.1007/s11676-016-0282-6

Degenhardt J, Köllner TG, Gershenzon J (2009) Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. Phytochemistry 70: 1621-1637. doi:10.1016/j.phytochem.2009.07.030

Denis M, Favreau B, Ueno S, Camus-Kulandaivelu L, Chaix G, Gion JM, Nourrisier-Mountou S, Polidori J, Bouvet JM (2013) Genetic variation of wood chemical traits and association

with underlying genes in *Eucalyptus urophylla*. Tree Genetics & Genomes 9: 927-942. doi:10.1007/s11295-013-0606-z

Dianese JC, Moraes TSA, Silva AR (1986) Screening *Eucalyptus* species for rust resistance in Bahia, Brazil. Tropical Pest Management 32: 292-295. doi:10.1080/09670878609371082

Dick M (1982) Leaf-inhabiting fungi of eucalypts in New Zealand. New Zealand Journal of Forestry Science 12: 525-537

Dijkstra J, de Jager CP (1998) Mechanical inoculation of plants. In: Practical Plant Virology: Protocols and Exercises. Springer-Verlag, Berlin, Heidelberg, pp 5-13. doi:10.1007/978-3-642-72030-7_1

Dillon S, McEvoy R, Baldwin DS, Southerton S, Campbell C, Parsons Y, Rees GN (2015) Genetic diversity of *Eucalyptus camaldulensis* Dehnh. following population decline in response to drought and altered hydrological regime. Austral Ecology 40: 558-572. doi:10.1111/aec.12223

Dodds PN, Rathjen JP (2010) Plant immunity: towards an integrated view of plant-pathogen interactions. Nature Reviews Genetics 11: 539-548. doi:10.1038/nrg2812

Doran JC, Matheson AC (1994) Genetic parameters and expected gains from selection for monoterpene yields in Petford *Eucalyptus camaldulensis*. New Forests 8: 155-167. doi:10.1007/BF00028191

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12: 13-15

Drummond AJ, Ho SYW, Phillips MJ, Rambaut A (2006) Relaxed phylogenetics and dating with confidence. PLoS Biology 4, e88: 699-710. doi:10.1371/journal.pbio.0040088

du Plessis E, McTaggart AR, Granados GM, Wingfield MJ, Roux J, Ali MIM, Pegg GS, Makinson J, Purcell M (2017) First report of myrtle rust caused by *Austropuccinia psidii* on *Rhodomyrtus tomentosa* (Myrtaceae) from Singapore. Plant Disease. doi:10.1094/PDIS-04-17-0530-PDN

Dungey HS, Potts BM, Carnegie AJ, Ades PK (1997) *Mycosphaerella* leaf disease: genetic variation in damage to *Eucalyptus nitens*, *Eucalyptus globulus*, and their F_1 hybrid. Canadian Journal of Forest Research 27: 750-759. doi:10.1139/x96-210

Duplessis S, Cuomo CA, Lin YC, Aerts A, Tisserant E, Veneault-Fourrey C, Joly DL, Hacquard S, Amselem J, Cantarel BL, Chiu R, Coutinho PM, Feau N, Field M, Frey P, Gelhaye E, Goldberg J, Grabherr MG, Kodira CD, Kohler A, Kües U, Lindquist EA, Lucas SM, Mago R, Mauceli E, Morin E, Murat C, Pangilinan JL, Park R, Pearson M, Quesneville H, Rouhier N, Sakthikumar S, Salamov AA, Schmutz J, Selles B, Shapiro H, Tanguay P, Tuskan GA, Henrissat B, Van de Peer Y, Rouzé P, Ellis JG, Dodds PN, Schein JE, Zhong S, Hamelin RC, Grigoriev IV, Szabo LJ, Martin F (2011) Obligate biotrophy features unraveled by the genomic analysis of rust fungi. Proceedings of the National Academy of Sciences of the United States of America 108: 9166-9171. doi:10.1073/pnas.1019315108

Dutkowski GW (1995) Genetic variation in drought susceptibility of *Eucalyptus globulus* ssp. *globulus* in plantations in Western Australia. In: Potts BM, Borralho NMG, Reid JB, Cromer RN, Tibbits WN, Raymond CA (eds) Eucalypt Plantations: Improving Fibre Yield and Quality. Proceedings of the CRCTHF-IUFRO Conference, CRC for Temperate Hardwood Forestry, Hobart, pp 199-203

Dutkowski GW, Potts BM (1999) Geographic patterns of genetic variation in *Eucalyptus globulus* ssp. *globulus* and a revised racial classification. Australian Journal of Botany 47: 237-263. doi:10.1071/BT97114

Dutkowski GW, Potts BM (2012) Genetic variation in the susceptibility of *Eucalyptus globulus* to drought damage. Tree Genetics & Genomes 8: 757-773. doi:10.1007/s11295-011-0461-8

Edwards HH, Bonde MR (2011) Penetration and establishment of *Phakopsora pachyrhizi* in soybean leaves as observed by transmission electron microscopy. Phytopathology 101: 894-900. doi:10.1094/PHYTO-09-10-0248

Edwards PB, Wanjura WJ, Brown WV (1993) Selective herbivory by Christmas beetles in response to intraspecific variation in *Eucalyptus* terpenoids. Oecologia 95: 551-557. doi:10.1007/BF00317440

Edwards PB, Wanjura WJ, Brown WV, Dearn JM (1990) Mosaic resistance in plants. Nature 347: 434. doi:10.1038/347434a0

Eitas TK, Dangl JL (2010) NB-LRR proteins: pairs, pieces, perception, partners, and pathways. Current Opinion in Plant Biology 13: 472-477. doi:10.1016/j.pbi.2010.04.007

Elaissi A, Rouis Z, Ben Salem NA, Mabrouk S, Ben Salem Y, Salah KBH, Aouni M, Farhat F, Chemli R, Harzallah-Skhiri F, Khouja ML (2012a) Chemical composition of 8 *Eucalyptus* species' essential oils and the evaluation of their antibacterial, antifungal and antiviral activities. BMC Complementary and Alternative Medicine 12, Article 81: 1-15. doi:10.1186/1472-6882-12-81

Elaissi A, Rouis Z, Mabrouk S, Salah KBH, Aouni M, Khouja ML, Farhat F, Chemli R, Harzallah-Skhiri F (2012b) Correlation between chemical composition and antibacterial activity of essential oils from fifteen *Eucalyptus* species growing in the Korbous and Jbel Abderrahman arboreta (North East Tunisia). Molecules 17: 3044-3057. doi:10.3390/molecules17033044

Eldridge K, Davidson J, Harwood C, van Wyk G (1993) Eucalypt Domestication and Breeding. Oxford Science Publications, New York

Ellis J, Jones D (1998) Structure and function of proteins controlling strain-specific pathogen resistance in plants. Current Opinion in Plant Biology 1: 288-293. doi:10.1016/1369-5266(88)80048-7

Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Buckler ES, Mitchell SE (2011) A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PloS One 6, e19379: 1-10. doi:10.1371/journal.pone.0019379

Eriksson J (1894) Ueber die specialisirung des parasitismus bei den getreiderostpilzen. Berichte der Deutschen Botanischen Gesellschaft 12: 292-331. doi:10.1111/j.1438-8677.1894.tb06099.x

Eyles A, Bonello P, Ganley R, Mohammed C (2010) Induced resistance to pests and pathogens in trees. New Phytologist 185: 893-908. doi:10.1111/j.1469-8137.2009.03127.x

Eyles A, Davies NW, Mohammed C (2003a) Novel detection of formylated phloroglucinol compounds (FPCs) in the wound wood of *Eucalyptus globulus* and *E. nitens*. Journal of Chemical Ecology 29: 881-898. doi:10.1023/A:1022979632281

Eyles A, Davies NW, Yuan ZQ, Mohammed C (2003b) Host responses to natural infection by *Cytonaema* sp. in the aerial bark of *Eucalyptus globulus*. Forest Pathology 33: 317-331. doi:10.1046/j.1439-0329.2003.00332.x

Facchini PJ (2001) Alkaloid biosynthesis in plants: biochemistry, cell biology, molecular regulation, and metabolic engineering applications. Annual Review of Plant Physiology and Plant Molecular Biology 52: 29-66. doi:10.1146/annurev.arplant.52.1.29

Fahn A (1988) Secretory tissues in vascular plants. New Phytologist 108: 229-257. doi:10.1111/j.1469-8137.1988.tb04159.x

Faria DA, Mamani EMC, Pappas Jr. GJ, Grattapaglia D (2011) Genotyping systems for *Eucalyptus* based on tetra-, penta-, and hexanucleotide repeat EST microsatellites and

their use for individual fingerprinting and assignment tests. Tree Genetics & Genomes 7: 63-77. doi:10.1007/s11295-010-0315-9

Felix G, Regenass M, Boller T (1993) Specific perception of subnanomolar concentrations of chitin fragments by tomato cells: induction of extracellular alkalinization, changes in protein phosphorylation, and establishment of a refractory state. Plant Journal 4: 307-316. doi:10.1046/j.1365-313X.1993.04020307.x

Ferrari JT, Nogueira EMD, dos Santos AJT (1997) Control of rust (*Puccinia psidii*) in guava (*Psidium guajava*). Acta Horticulturae 452: 55-57. doi:10.17660/ActaHortic.1997.452.8

Ferreira FA (1983) Ferrugem do eucalipto. Revista Árvore 7: 91-109

Ferreira FA (1989) Patologia Florestal: Principais Doenças Florestais no Brazil. Sociedade de Investigações Florestais, Viçosa

Figueiredo MB (2001) Life cycle and ecology of *Puccinia psidii*. O Biologico 63: 69-71

Flor HH (1971) Current status of the gene-for-gene concept. Annual Review of Phytopathology 9: 275-296. doi:10.1146/annurev.py.09.090171.001423

Florence RG (1996) Species and community patterns. In: Ecology and Silviculture of Eucalypt Forests. CSIRO Publishing, Collingwood VIC, pp 15-44

Fluhr R (2001) Sentinels of disease. Plant resistance genes. Plant Physiology 127: 1367-1374. doi:10.1104/pp.010763

Forestry Tasmania (2010) Silvicultural systems for native eucalypt forests. Native Forest Silviculture Technical Bulletin No. 5. Forestry Tasmania, Hobart

Forrester DI, Medhurst JL, Wood M, Beadle CL, Valencia JC (2010) Growth and physiological responses to silviculture for producing solid-wood products from *Eucalyptus* plantations: An Australian perspective. Forest Ecology and Management 259: 1819-1835. doi:10.1016/j.foreco.2009.08.029

Franceschi VR, Krokene P, Christiansen E, Krekling T (2005) Anatomical and chemical defenses of conifer bark against bark beetles and other pests. New Phytologist 167: 353-376. doi:10.1111/j.1469-8137.2005.01436.x

Freeman BC, Beattie GA (2008) An overview of plant defenses against pathogens and herbivores. The Plant Health Instructor. The American Phytopathological Society. doi: 10.1094/PHI-I-2008-0226-01. https://www.apsnet.org/edcenter/intropp/topics/Pages/OverviewOfPlantDiseases.aspx. Accessed 20 December 2017

Freeman JS, Jackson HD, Steane DA, McKinnon GE, Dutkowski GW, Potts BM, Vaillancourt RE (2001) Chloroplast DNA phylogeography of *Eucalyptus globulus*. Australian Journal of Botany 49: 585-596. doi:10.1071/BT00094

Freeman JS, Potts BM, Vaillancourt RE (2008) Few Mendelian genes underlie the quantitative response of a forest tree, *Eucalyptus globulus*, to a natural fungal epidemic. Genetics 178: 563-571. doi:10.1534/genetics.107.081414

Friendly M, Fox J (2016) candisc: Visualizing generalized canonical discriminant and canonical correlation analysis. R package version 0.7-2. URL https://cran.r-project.org/package=candisc

Furtado EL, Marino CL (2003) *Eucalyptus* rust management in Brazil. In: Proceedings of Second IUFRO Rust Forest Trees W.P. Conference. International Union of Forest Research Organizations (IUFRO), Yangling, China, pp 118-124

Gandolfo MA, Hermsen EJ, Zamaloa MC, Nixon KC, Gonzalez CC, Wilf P, Cuneo NR, Johnson KR (2011) Oldest known *Eucalyptus* macrofossils are from South America. PloS One 6, e21084: 1-9. doi:10.1371/journal.pone.0021084

Garnica DP, Nemri A, Upadhyaya NM, Rathjen JP, Dodds PN (2014) The ins and outs of rust haustoria. PLoS Pathogens 10, e1004329: 1-4. doi:10.1371/journal.ppat.1004329

Gevens AJ, Nequi N, Vitoreli A, Marois JJ, Wright DL, Harmon CL, Harmon PF (2008) First report of soybean rust caused by *Phakopsora pachyrhizi* on *Erythrina herbacea* (coral bean). Plant Disease 92: 1472-1472. doi:10.1094/PDIS-92-10-1472C

Giblin F (2013) Myrtle rust report: New Caledonia. University of the Sunshine Coast, Queensland. http://www.davar.gouv.nc/portal/page/portal/davar/librairie/fichiers/23642181.PDF. Accessed 14 March 2017

Giblin F, Carnegie AJ (2014) *Puccinia psidii* (myrtle rust) - global host list. Australian Network for Plant Conservation. http://www.anbg.gov.au/anpc/resources/Myrtle_Rust.html. Accessed 15 March 2017

Gilbert GS, Magarey R, Suiter K, Webb CO (2012) Evolutionary tools for phytosanitary risk analysis: phylogenetic signal as a predictor of host range of plant pests and pathogens. Evolutionary Applications 5: 869-878. doi:10.1111/j.1752-4571.2012.00265.x

Gilbert GS, Webb CO (2007) Phylogenetic signal in plant pathogen-host range. Proceedings of the National Academy of Sciences of the United States of America 104: 4979-4983. doi:10.1073/pnas.0607968104

Gill DE (1986) Individual plants as genetic mosaics: ecological organisms versus evolutionary individuals. In: Crawley J (ed) Plant Ecology. Blackwell Scientific Publications, Oxford, pp 321-343

Gilmour AR, Gogel BJ, Cullis BR, Thompson R (2009) ASReml User Guide Release 3.0. VSN International Ltd, United Kingdom

Girijashankar V (2011) Genetic transformation of eucalyptus. Physiology and Molecular Biology of Plants 17: 9-23. doi:10.1007/s12298-010-0048-0

Gleadow RM, Woodrow IE (2002) Defense chemistry of cyanogenic *Eucalyptus cladocalyx* seedlings is affected by water supply. Tree Physiology 22: 939-945. doi:10.1093/treephys/22.13.939

Glen M, Alfenas AC, Zauza EAV, Wingfield MJ, Mohammed C (2007) *Puccinia psidii*: a threat to the Australian environment and economy - a review. Australasian Plant Pathology 36: 1-16. doi:10.1071/AP06088

Goddard RE, Schmidt RA, Linde FV (1975) Effect of differential selection pressure on fusiform rust resistance in phenotypic selections of slash pine. Phytopathology 65: 336-338. doi:10.1094/Phyto-65-336

Goellner K, Loehrer M, Langenbach C, Conrath U, Koch E, Schaffrath U (2010) *Phakopsora pachyrhizi*, the causal agent of Asian soybean rust. Molecular Plant Pathology 11: 169-177. doi:10.1111/j.1364-3703.2009.00589.x

Gómez-Gómez L, Boller T (2002) Flagellin perception: a paradigm for innate immunity. Trends in Plant Science 7: 251-256. doi:10.1016/S1360-1385(02)02261-6

Goodger JQD, Cao B, Jayadi I, Williams SJ, Woodrow IE (2009) Non-volatile components of the essential oil secretory cavities of *Eucalyptus* leaves: discovery of two glucose monoterpene esters, cuniloside B and froggattiside A. Phytochemistry 70: 1187-1194. doi:10.1016/j.phytochem.2009.06.004

Goodger JQD, Connelly CA, Woodrow IE (2007) Examination of the consistency of plant traits driving oil yield and quality in short-rotation coppice cultivation of *Eucalyptus polybractea*. Forest Ecology and Management 250: 196-205. doi:10.1016/j.foreco.2007.05.015

Goodger JQD, Heskes AM, King DJ, Gleadow RM, Woodrow IE (2008) Micropropagation of *Eucalyptus polybractea* selected for key essential oil traits. Functional Plant Biology 35: 247-251. doi:10.1071/FP07241

Goodger JQD, Heskes AM, Woodrow IE (2013a) Contrasting ontogenetic trajectories for phenolic and terpenoid defences in *Eucalyptus froggattii*. Annals of Botany 112: 651-659. doi:10.1093/aob/mct010

Goodger JQD, Mitchell MC, Woodrow IE (2013b) Differential patterns of mono- and sesquiterpenes with leaf ontogeny influence pharmaceutical oil yield in *Eucalyptus polybractea* R.T. Baker. Trees 27: 511-521. doi:10.1007/s00468-012-0804-6

Goodger JQD, Senaratne SL, Nicolle D, Woodrow IE (2016) Foliar essential oil glands of *Eucalyptus* subgenus *Eucalyptus* (Myrtaceae) are a rich source of flavonoids and related non-volatile constituents. PloS One 11, e0151432: 1-18. doi:10.1371/journal.pone.0151432

Goodger JQD, Woodrow IE (2009) The influence of ontogeny on essential oil traits when micropropagating *Eucalyptus polybractea*. Forest Ecology and Management 258: 650-656. doi:10.1016/j.foreco.2009.04.033

Goodwin S, McPherson JD, McCombie WR (2016) Coming of age: ten years of next-generation sequencing technologies. Nature Reviews Genetics 17: 333-351. doi:10.1038/nrg.2016.49

Graça RN, Aun CP, Guimarães LMS, Rodrigues BVA, Zauza EAV, Alfenas AC (2011) A new race of *Puccinia psidii* defeats rust resistance in eucalypt. Australasian Plant Pathology 40: 442-447. doi:10.1007/s13313-011-0056-8

Granado J, Felix G, Boller T (1995) Perception of fungal sterols in plants (subnanomolar concentrations of ergosterol elicit extracellular alkalinization in tomato cells). Plant Physiology 107: 485-490. doi:10.1104/pp.107.2.485

Grattapaglia D, Bradshaw Jr. HD (1994) Nuclear DNA content of commercially important *Eucalyptus* species and hybrids. Canadian Journal of Forest Research 24: 1074-1078. doi:10.1139/x94-142

Grattapaglia D, Kirst M (2008) *Eucalyptus* applied genomics: from gene sequences to breeding tools. New Phytologist 179: 911-929. doi:10.1111/j.1469-8137.2008.02503.x

Grattapaglia D, Mamani EMC, Silva-Junior OB, Faria DA (2015) A novel genome-wide microsatellite resource for species of *Eucalyptus* with linkage-to-physical correspondence on the reference genome sequence. Molecular Ecology Resources 15: 437-448. doi:10.1111/1755-0998.12317

Grattapaglia D, Resende MDV (2011) Genomic selection in forest tree breeding. Tree Genetics & Genomes 7: 241-255. doi:10.1007/s11295-010-0328-4

Grattapaglia D, Sederoff R (1994) Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross: mapping strategy and RAPD markers. Genetics 137: 1121-1137

Grattapaglia D, Vaillancourt RE, Shepherd M, Thumma BR, Foley W, Külheim C, Potts BM, Myburg AA (2012) Progress in Myrtaceae genetics and genomics: *Eucalyptus* as the pivotal genus. Tree Genetics & Genomes 8: 463-508. doi:10.1007/s11295-012-0491-x

Green JW (1971) Variation in *Eucalyptus obliqua* L'Herit. New Phytologist 70: 897-909. doi:10.1111/j.1469-8137.1971.tb02590.x

Grgurinovic CA, Walsh D, Macbeth F (2006) Eucalyptus rust caused by *Puccinia psidii* and the threat it poses to Australia. EPPO Bulletin 36: 486-489. doi:10.1111/j.1365-2338.2006.01048.x

Grima-Pettenati J, Feuillet C, Goffner D, Borderies G, Boudet AM (1993) Molecular cloning and expression of a *Eucalyptus gunnii* cDNA clone encoding cinnamyl alcohol dehydrogenase. Plant Molecular Biology 21: 1085-1095. doi:10.1007/BF00023605

Gururani MA, Venkatesh J, Upadhyaya CP, Nookaraju A, Pandey SK, Park SW (2012) Plant disease resistance genes: Current status and future directions. Physiological and Molecular Plant Pathology 78: 51-65. doi:10.1016/j.pmpp.2012.01.002

Hadacek F (2002) Secondary metabolites as plant traits: current assessment and future perspectives. Critical Reviews in Plant Sciences 21: 273-322. doi:10.1080/0735-260291044269

Hallam ND, Chambers TC (1970) The leaf waxes of the genus *Eucalyptus* L'Héritier. Australian Journal of Botany 18: 335-386. doi:10.1071/BT9700335

Hamilton MG, Williams DR, Tilyard PA, Pinkard EA, Wardlaw TJ, Glen M, Vaillancourt RE, Potts BM (2013) A latitudinal cline in disease resistance of a host tree. Heredity 110: 372-379. doi:10.1038/hdy.2012.106

Hammerschmidt R (1999) Phytoalexins: What have we learned after 60 years? Annual Review of Phytopathology 37: 285-306. doi:10.1146/annurev.phyto.37.1.285

Hammond-Kosack KE, Jones JDG (1997) Plant disease resistance genes. Annual Review of Plant Physiology and Plant Molecular Biology 48: 575-607. doi:10.1146/annurev.arplant.48.1.575

Hamrick JL (2004) Response of forest trees to global environmental changes. Forest Ecology and Management 197: 323-335. doi:10.1016/j.foreco.2004.05.023

Hantao LW, Aleme HG, Passador MM, Furtado EL, Ribeiro FAL, Poppi RJ, Augusto F (2013a) Determination of disease biomarkers in *Eucalyptus* by comprehensive two-dimensional gas chromatography and multivariate data analysis. Journal of Chromatography A 1279: 86-91. doi:10.1016/j.chroma.2013.01.013

Hantao LW, Toledo BR, Ribeiro FAL, Pizetta M, Pierozzi CG, Furtado EL, Augusto F (2013b) Comprehensive two-dimensional gas chromatography combined to multivariate data analysis for detection of disease-resistant clones of *Eucalyptus*. Talanta 116: 1079-1084. doi:10.1016/j.talanta.2013.08.033

Hartmann T (1996) Diversity and variability of plant secondary metabolism: A mechanistic view. Entomologia Experimentalis et Applicata 80: 177-188. doi:10.1111/j.1570-7458.1996.tb00914.x

He X, Wang Y, Li F, Weng Q, Li M, Xu L, Shi J, Gan S (2012) Development of 198 novel EST-derived microsatellites in *Eucalyptus* (Myrtaceae). American Journal of Botany 99: e134-e148. doi:10.3732/ajb.1100442

Heath MC (1981) Resistance of plants to rust infection. Phytopathology 71: 971-974. doi:0.1094/Phyto-71-971

Heath MC (1997) Signalling between pathogenic rust fungi and resistant or susceptible host plants. Annals of Botany 80: 713-720. doi:10.1006/anbo.1997.0507

Heath MC (2000a) Hypersensitive response-related death. Plant Molecular Biology 44: 321-334. doi:10.1023/A:1026592509060

Heath MC (2000b) Nonhost resistance and nonspecific plant defenses. Current Opinion in Plant Biology 3: 315-319. doi:10.1016/S1369-5266(00)00087-X

Heath MC (2001) Non-host resistance to plant pathogens: nonspecific defense or the result of specific recognition events? Physiological and Molecular Plant Pathology 58: 53-54. doi:10.1006/pmpp.2001/0319

Heather WA (1967a) Leaf characteristics of *Eucalyptus bicostata* Maiden *et al.* seedlings affecting the deposition and germination of spores of *Phaeoseptoria eucalypti* (Hansf.) Walker. Australian Journal of Biological Sciences 20: 1155-1160. doi:10.1071/BI9671155

Heather WA (1967b) Susceptibility of the juvenile leaves of *Eucalyptus bicostata* Maiden *et al.* to infection by *Phaeoseptoria eucalypti* (Hansf.) Walker. Australian Journal of Biological Sciences 20: 769-776. doi:10.1071/BI9670769

Helfer S (2014) Rust fungi and global change. New Phytologist 201: 770-780. doi:10.1111/nph.12570

Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson OE, Huhndorf S, James T, Kirk PM, Lücking R, Thorsten Lumbsch H, Lutzoni F, Matheny PB, McLaughlin DJ, Powell MJ, Redhead S, Schoch CL, Spatafora JW, Stalpers JA, Vilgalys R, Aime MC, Aptroot A, Bauer R, Begerow D, Benny GL, Castlebury LA, Crous PW, Dai YC, Gams W, Geiser DM, Griffith GW, Gueidan C, Hawksworth DL, Hestmark G, Hosaka K, Humber RA, Hyde KD, Ironside JE, Kõljalg U, Kurtzman CP, Larsson KH, Lichtwardt R, Longcore J, Miądlikowska J, Miller A, Moncalvo JM, Mozley-Standridge S, Oberwinkler F, Parmasto E, Reeb V, Rogers JD, Roux C, Ryvarden L, Sampaio JP, Schüßler A, Sugiyama J, Thorn RG, Tibell L, Untereiner WA, Walker C, Wang Z, Weir A, Weiss M, White MM, Winka K, Yao YJ, Zhang N (2007) A higher-level phylogenetic classification of the Fungi. Mycological Research 111: 509-547. doi:10.1016/j.mycres.2007.03.004

Hirakawa H, Nakamura Y, Kaneko T, Isobe S, Sakai H, Kato T, Hibino T, Sasamoto S, Watanabe A, Yamada M, Nakayama S, Fujishiro T, Kishida Y, Kohara M, Tabata S, Sato S (2011) Survey of the genetic information carried in the genome of *Eucalyptus camaldulensis*. Plant Biotechnology 28: 471-480. doi:10.5511/plantbiotechnology.11.1027b

Hoefle C, Loehrer M, Schaffrath U, Frank M, Schultheiss H, Hükelhoven R (2009) Transgenic suppression of cell death limits penetration success of the soybean rust fungus *Phakopsora pachyrhizi* into epidermal cells of barley. Phytopathology 99: 220-226. doi:10.1094/PHYTO-99-3-0220

Hovmøller MS, Sørensen CK, Walter S, Justesen AF (2011) Diversity of *Puccinia striiformis* on cereals and grasses. Annual Review of Phytopathology 49: 197-217. doi:10.1146/annurev-phyto-072910-095230

Hovmøller MS, Walter S, Justesen AF (2010) Escalating threat of wheat rusts. Science 329: 369-369. doi:10.1126/science.1194925

Hovmøller MS, Yahyaoui AH, Milus EA, Justesen AF (2008) Rapid global spread of two aggressive strains of a wheat rust fungus. Molecular Ecology 17: 3818-3826. doi:10.1111/j.136-5-294X.2008.03886.x

Huang X, Zhao Y, Wei X, Li C, Wang A, Zhao Q, Li W, Guo Y, Deng L, Zhu C, Fan D, Lu Y, Weng Q, Liu K, Zhou T, Jing Y, Si L, Dong G, Huang T, Lu T, Feng Q, Qian Q, Li J, Han B (2012) Genome-wide association study of flowering time and grain yield traits in a worldwide collection of rice germplasm. Nature Genetics 44: 32-39. doi:10.1038/ng.1018

Hudson CJ, Freeman JS, Jones RC, Potts BM, Wong MML, Weller JL, Hecht VFG, Poethig RS, Vaillancourt RE (2014) Genetic control of heterochrony in *Eucalyptus globulus*. G3: Genes | Genomes | Genetics 4: 1235-1245. doi:10.1534/g3.114.011916

Hudson CJ, Freeman JS, Kullan ARK, Petroli CD, Sansaloni CP, Kilian A, Detering F, Grattapaglia D, Potts BM, Myburg AA, Vaillancourt RE (2012) A reference linkage map for *Eucalyptus*. BMC Genomics 13, Article 240: 1-11. doi:10.1186/1471-2164-13-240

Hull R (2005) Mechanical inoculation of plant viruses. In: Current Protocols in Microbiology. John Wiley & Sons, Inc., New Jersey. doi:10.1002/9780471729259.mc16b06s13

Hume ID, Esson C (1993) Nutrients, antinutrients and leaf selection by captive koalas (*Phascolarctos cinereus*). Australian Journal of Zoology 41: 379-392. doi:10.1071/ZO9930379

Hunt P (1968) Cuticular penetration by germinating uredospores. Transactions of the British Mycological Society 51: 103-112. doi:10.1016/S0007-1536(68)80126-3

Jackson HD, Steane DA, Potts BM, Vaillancourt RE (1999) Chloroplast DNA evidence for reticulate evolution in *Eucalyptus* (Myrtaceae). Molecular Ecology 8: 739-751. doi:10.1046/j.1365-294X.1999.00614.x

James SA, Bell DT (2001) Leaf morphological and anatomical characteristics of heteroblastic *Eucalyptus globulus* ssp. *globulus* (Myrtaceae). Australian Journal of Botany 49: 259-269. doi:10.1071/BT99044

Joffily J (1944) Ferrugem do eucalipto. Bragantia 4: 475-487. doi:10.1590/S0006-87051944000300001

Johansen DA (1940) Plant Microtechnique. McGraw-Hill, New York

Johnson ED (1926) A comparison of the juvenile and adult leaves of *Eucalyptus globulus*. New Phytologist 25: 202-212. doi:10.1111/j.1469-8137.1926.tb06691.x

Jones JDG, Dangl JL (2006) The plant immune system. Nature 444: 323-329. doi:10.1038/nature05286

Jones ME, Shepherd M, Henry RJ, Delves A (2006) Chloroplast DNA variation and population structure in the widespread forest tree, *Eucalyptus grandis*. Conservation Genetics 7: 691-703. doi:10.1007/s10592-005-9104-7

Jones RC, Steane DA, Lavery M, Vaillancourt RE, Potts BM (2013) Multiple evolutionary processes drive the patterns of genetic differentiation in a forest tree species complex. Ecology and Evolution 3: 1-17. doi:10.1002/ece3.421

Jones RC, Steane DA, Potts BM, Vaillancourt RE (2002) Microsatellite and morphological analysis of *Eucalyptus globulus* populations. Canadian Journal of Forest Research 32: 59-66. doi:10.1139/x01-172

Jordan GJ, Borralho NMG, Tilyard P, Potts BM (1994) Identification of races in *Eucalyptus globulus* ssp *globulus* based on growth traits in Tasmania and geographic distribution. Silvae Genetica 43: 292-298

Jordan GJ, Potts BM, Clarke AR (2002) Susceptibility of *Eucalyptus globulus* ssp. *globulus* to sawfly (*Perga affinis* ssp. *insularis*) attack and its potential impact on plantation productivity. Forest Ecology and Management 160: 189-199. doi:10.1016/S0378-1127(01)00445-5

Junghans DT, Alfenas AC, Brommonschenkel SH, Oda S, Mello EJ, Grattapaglia D (2003a) Resistance to rust (*Puccinia psidii* Winter) in *Eucalyptus*: mode of inheritance and mapping of a major gene with RAPD markers. Theoretical and Applied Genetics 108: 175-180. doi:10.1007/s00122-003-1415-9

Junghans DT, Alfenas AC, Maffia LA (2003b) Rating scale to eucalypts rust severity evaluation. Fitopatologia Brasileira 28: 184-188. doi:10.1590/S0100-41582003000200012

Jurgens JA, Blanchette RA, Zambino PJ, David A (2003) Histology of white pine blister rust in needles of resistant and susceptible eastern white pine. Plant Disease 87: 1026-1030. doi:10.1094/PDIS.2003.87.9.1026

Kadirvel P, Senthilvel S, Geethanjali S, Sujatha M, Varaprasad KS (2015) Genetic markers, trait mapping and marker-assisted selection in plant breeding. In: Bahadur B, Venkat Rajam M, Sahijram L, Krishnamurthy KV (eds) Plant Biology and Biotechnology, Volume II: Plant Genomics and Biotechnology. Springer India, New Delhi, pp 65-88. doi:10.1007/978-81-322-2283-5_4

Kahle D, Wickham H (2013) ggmap: Spatial Visualization with ggplot2. The R Journal 5/1: 144-161. URL http://journal.r-project.org/archive/2013-1/kahle-wickham.pdf

Kaitera J, Nuorteva H (2008) Inoculations of eight *Pinus* species with *Cronartium* and *Peridermium* stem rusts. Forest Ecology and Management 255: 973-981. doi:10.1016/j.foreco.2007.10.005

Kalmus H, Kassanis B (1945) The use of abrasives in the transmission of plant viruses. Annals of Applied Biology 32: 230-234. doi:10.1111/j.1744-7348.1945.tb06240.x

Kang HM, Zaitlen NA, Wade CM, Kirby A, Heckerman D, Daly MJ, Eskin E (2008) Efficient control of population structure in model organism association mapping. Genetics 178: 1709-1723. doi:10.1534/genetics.107.080101

Kawanishi T, Uematsu S, Kakishima M, Kagiwada S, Hamamoto H, Horie H, Namba S (2009) First report of rust disease on ohia and the causal fungus, *Puccinia psidii*, in Japan. Journal of General Plant Pathology 75: 428-431. doi:10.1007/s10327-009-0202-0

Keeling CI, Bohlmann J (2006) Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. New Phytologist 170: 657-675. doi:10.1111/j.1469-8137.2006.01716.x

Keith R, Mitchell-Olds T (2013) Genetic variation for resistance to herbivores and plant pathogens: hypotheses, mechanisms and evolutionary implications. Plant Pathology 62: 122-132. doi:10.1111/ppa.12134

Keszei A, Brubaker CL, Carter R, Köllner T, Degenhardt J, Foley WJ (2010) Functional and evolutionary relationships between terpene synthases from Australian Myrtaceae. Phytochemistry 71: 844-852. doi:10.1016/j.phytochem.2010.03.013

Keszei A, Brubaker CL, Foley WJ (2008) A molecular perspective on terpene variation in Australian Myrtaceae. Australian Journal of Botany 56: 197-213. doi:10.1071/BT07146

Kim J, Lim CJ, Lee BW, Choi JP, Oh SK, Ahmad R, Kwon SY, Ahn J, Hur CG (2012) A genome-wide comparison of NB-LRR type of resistance gene analogs (RGA) in the plant kingdom. Molecules and Cells 33: 385-392. doi:10.1007/s10059-012-0003-8

King RC, Stansfield WD, Mulligan PK (2006) A Dictionary of Genetics. 7 edn. Oxford University Press, New York

Kinloch BB, Stonecypher RW (1969) Genetic variation in susceptibility to fusiform rust in seedlings from a wild population of loblolly pine. Phytopathology 59: 1246-1255

Kirkpatrick JB (1974) The numerical intraspecific taxonomy of *Eucalyptus globulus* Labill. (Myrtaceae). Botanical Journal of the Linnean Society 69: 89-104. doi:10.1111/j.1095-8339.1974.tb01618.x

Kirkpatrick JB (1976) Geographic variation in two disjunctly distributed species of *Eucalyptus*. Journal of Biogeography 3: 151-156. doi:10.2307/3038143

Klos KE, Yimer BA, Babiker EM, Beattie AD, Bonman JM, Carson ML, Chong J, Harrison SA, Ibrahim AMH, Kolb FL, McCartney CA, McMullen M, Fetch JM, Mohammadi M, Murphy JP, Tinker NA (2017) Genome-wide association mapping of crown rust resistance in oat elite germplasm. Plant Genome 10: 1-13. doi:10.3835/plantgenome2016.10.0107

Kohler A, Rinaldi C, Duplessis S, Baucher M, Geelen D, Duchaussoy F, Meyers BC, Boerjan W, Martin F (2008) Genome-wide identification of *NBS* resistance genes in *Populus trichocarpa*. Plant Molecular Biology 66: 619-636. doi:10.1007/s11103-008-9293-9

Köllner TG, O'Maille PE, Gatto N, Boland W, Gershenzon J, Degenhardt J (2006) Two pockets in the active site of maize sesquiterpene synthase TPS4 carry out sequential parts of the reaction scheme resulting in multiple products. Archives of Biochemistry and Biophysics 448: 83-92. doi:10.1016/j.abb.2005.10.011

Kolmer JA, Ordonez ME, Groth JV (2009) The rust fungi. In: Encyclopedia of Life Sciences (eLS). John Wiley & Sons, Ltd, Chichester. doi:10.1002/9780470015902.a0021264

Korban SS, Chen H, Ries SM (1987) Interaction of apple cultivars with populations of cedar-apple rust. Journal of Phytopathology 119: 272-278. doi:10.1111/j.1439-0434.1987.tb04398.x

Kovalchuk A, Keriö S, Oghenekaro AO, Jaber E, Raffaello T, Asiegbu FO (2013) Antimicrobial defenses and resistance in forest trees: challenges and perspectives in a genomic era. Annual Review of Phytopathology 51: 221-244. doi:10.1146/annurev-phyto-082712-102307

Kriticos DJ, Morin L, Leriche A, Anderson RC, Caley P (2013) Combining a climatic niche model of an invasive fungus with its host species distributions to identify risks to natural assets: *Puccinia psidii* sensu lato in Australia. PloS One 8, e64479: 1-13. doi:10.1371/journal.pone.0064479

Külheim C, Padovan A, Hefer C, Krause ST, Köllner TG, Myburg AA, Degenhardt J, Foley WJ (2015) The *Eucalyptus* terpene synthase gene family. BMC Genomics 16, Article 450: 1-18. doi:10.1186/s12864-015-1598-x

Külheim C, Yeoh SH, Wallis IR, Laffan S, Moran GF, Foley WJ (2011) The molecular basis of quantitative variation in foliar secondary metabolites in *Eucalyptus globulus*. New Phytologist 191: 1041-1053. doi:10.1111/j.1469-8137.2011.03769.x

Kump KL, Bradbury PJ, Wisser RJ, Buckler ES, Belcher AR, Oropeza-Rosas MA, Zwonitzer JC, Kresovich S, McMullen MD, Ware D, Balint-Kurti PJ, Holland JB (2011) Genome-wide association study of quantitative resistance to southern leaf blight in the maize nested association mapping population. Nature Genetics 43: 163-168. doi:10.1038/ng.747

Kunkel BN (1996) A useful weed put to work: genetic analysis of disease resistance in *Arabidopsis thaliana*. Trends in Genetics 12: 63-69. doi:10.1016/0168-9525(96)81402-8

Kursar TA, Dexter KG, Lokvam J, Pennington RT, Richardson JE, Weber MG, Murakami ET, Drake C, McGregor R, Coley PD (2009) The evolution of antiherbivore defenses and their contribution to species coexistence in the tropical tree genus *Inga*. Proceedings of the National Academy of Sciences of the United States of America 106: 18073-18078. doi:10.1073/pnas.0904786106

Kuznetsova A, Bruun Brockhoff P, Haubo Bojesen Christensen R (2016) lmerTest: Tests in linear mixed effects model. R package version 2.0-32. URL https://cran.r-project.org/package=lmerTest

L'Héritier de Brutelle CL (1963) Sertum Anglicum 1788: Facsimile with critical studies and a translation. Hunt facsimile series: no. 1. Hunt Botanical Library, Pittsburgh, Pennsylvania

Labate CA, de Assis TF, Oda S, de Mello EJ, González ER, Zauza EAV, Mori ES, de Moraes MLT, Cid LPB, Alfenas AC, Foelkel C, Moon DH, de Carvalho MCCG, Caldas DGG, Carneiro RT, de Andrade A, Salvatierra GR (2009) *Eucalyptus*. In: Compendium of Transgenic Crop Plants, Part 9: Transgenic Forest Tree Species. John Wiley & Sons, Ltd, New Jersey, pp 35-108. doi:10.1002/9781405181099.k0902

Ladiges PY (1974) Variation in drought tolerance in *Eucalyptus viminalis* Labill. Australian Journal of Botany 22: 489-500. doi:10.1071/BT9740489

Langenbach C, Campe R, Schaffrath U, Goellner K, Conrath U (2013) UDP-glucosyltransferase UGT84A2/BRT1 is required for *Arabidopsis* nonhost resistance to the Asian soybean rust pathogen *Phakopsora pachyrhizi*. New Phytologist 198: 536-545. doi:10.1111/nph.12155

Larcombe MJ, Potts BM, Jones RC, Steane DA, Silva JCE, Vaillancourt RE (2016) Managing Australia's eucalypt gene pools: Assessing the risk of exotic gene flow. Proceedings of the Royal Society of Victoria 128: 25-39. doi:10.1071/RS16003

Larsson SJ, Lipka AE, Buckler ES (2013) Lessons from *Dwarf8* on the strengths and weaknesses of structured association mapping. PLoS Genetics 9, e1003246: 1-11. doi:10.1371/journal.pgen.1003246

Lawler IR, Stapley J, Foley WJ, Eschler BM (1999) Ecological example of conditioned flavor aversion in plant-herbivore interactions: effect of terpenes of *Eucalyptus* leaves on feeding by common ringtail and brushtail possums. Journal of Chemical Ecology 25: 401-415. doi:10.1023/A:1020863216892

Lawrence R, Potts BM, Whitham TG (2003) Relative importance of plant ontogeny, host genetic variation, and leaf age for a common herbivore. Ecology 84: 1171-1178. doi:10.1890/0012-9658(2003)084[1171:RIOPOH]2.0.CO;2

Le Roux JJ, Van Staden J (1991) Micropropagation and tissue culture of *Eucalyptus* - a review. Tree Physiology 9: 435-477. doi:10.1093/treephys/9.4.435

Lee CE (2002) Evolutionary genetics of invasive species. Trends in Ecology & Evolution 17: 386-391. doi:10.1016/S0169-5347(02)02554-5

Lee DJ, Brawner JT, Pegg GS (2015) Screening *Eucalyptus cloeziana* and *E. argophloia* populations for resistance to *Puccinia psidii*. Plant Disease 99: 71-79. doi:10.1094/PDIS-04-14-0353-RE

Lenth RV (2016) Least-squares means: the R package lsmeans. Journal of Statistical Software 69: 1-33. doi:10.18637/jss.v069.i01

Lewis BG, Day JR (1972) Behaviour of uredospore germ-tubes of *Puccinia graminis tritici* in relation to the fine structure of wheat leaf surfaces. Transactions of the British Mycological Society 58: 139-145. doi:10.1016/S0007-1536(72)80079-2

Li CJ, Liu Y, Zheng YX, Yan CX, Zhang TT, Shan SH (2013) Cloning and characterization of an NBS-LRR resistance gene from peanuts (*Arachis hypogaea* L.). Physiological and Molecular Plant Pathology 84: 70-75. doi:10.1016/j.pmpp.2013.07.006

Li H, Madden JL (1995) Analysis of leaf oils from a *Eucalyptus* species trial. Biochemical Systematics and Ecology 23: 167-177. doi:10.1016/0305-1978(94)00087-W

Li H, Madden JL, Potts BM (1995) Variation in volatile leaf oils of the Tasmanian *Eucalyptus* species - I. Subgenus *Monocalyptus*. Biochemical Systematics and Ecology 23: 299-318. doi:10.1016/0305-1978(95)97455-6

Li H, Madden JL, Potts BM (1996) Variation in volatile leaf oils of the Tasmanian *Eucalyptus* species - II. Subgenus *Symphyomyrtus*. Biochemical Systematics and Ecology 24: 547-569. doi:10.1016/0305-1978(96)00040-3

Li H, Madden JL, Potts BM (1997) Variation in leaf waxes of the Tasmanian *Eucalyptus* species - I. Subgenus *Symphyomyrtus*. Biochemical Systematics and Ecology 25: 631-657. doi:10.1016/S0305-1978(97)00044-6

Li J, Ding J, Zhang W, Zhang Y, Tang P, Chen JQ, Tian D, Yang S (2010) Unique evolutionary pattern of numbers of gramineous NBS-LRR genes. Molecular Genetics and Genomics 283: 427-438. doi:10.1007/s00438-010-0527-6

Lichtenthaler HK (1999) The 1-deoxy-d-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. Annual Review of Plant Physiology and Plant Molecular Biology 50: 47-65. doi:10.1146/annurev.arplant.50.1.47

Lipka AE, Gore MA, Magallanes-Lundback M, Mesberg A, Lin H, Tiede T, Chen C, Buell CR, Buckler ES, Rocheford T, DellaPenna D (2013) Genome-wide association study and pathway-level analysis of tocochromanol levels in maize grain. G3: Genes | Genomes | Genetics 3: 1287-1299. doi:10.1534/g3.113.006148

Lipka AE, Tian F, Wang Q, Peiffer J, Li M, Bradbury PJ, Gore MA, Buckler ES, Zhang Z (2012) GAPIT: genome association and prediction integrated tool. Bioinformatics 28: 2397-2399. doi:10.1093/bioinformatics/bts444

Lipka U, Fuchs R, Kuhns C, Petutschnig E, Lipka V (2010) Live and let die - *Arabidopsis* nonhost resistance to powdery mildews. European Journal of Cell Biology 89: 194-199. doi:10.1016/j.ejcb.2009.11.011

Lipka V, Dittgen J, Bednarek P, Bhat R, Wiermer M, Stein M, Landtag J, Brandt W, Rosahl S, Scheel D, Llorente F, Molina A, Parker J, Somerville S, Schulze-Lefert P (2005) Pre- and postinvasion defenses both contribute to nonhost resistance in *Arabidopsis*. Science 310: 1180-1183. doi:10.1126/science.1119409

Liu JJ, Ekramoddoullah AKM (2004) Isolation, genetic variation and expression of TIR-NBS-LRR resistance gene analogs from western white pine (*Pinus monticola* Dougl. ex. D. Don.). Molecular Genetics and Genomics 270: 432-441. doi:10.1007/s00438-003-0940-1

Loehrer M, Botterweck J, Jahnke J, Mahlmann DM, Gaetgens J, Oldiges M, Horbach R, Deising H, Schaffrath U (2014) *In vivo* assessment by Mach-Zehnder double-beam interferometry of the invasive force exerted by the Asian soybean rust fungus (*Phakopsora pachyrhizi*). New Phytologist 203: 620-631. doi:10.1111/nph.12784

Loehrer M, Langenbach C, Goellner K, Conrath U, Schaffrath U (2008) Characterization of nonhost resistance of *Arabidopsis* to the Asian soybean rust. Molecular Plant-Microbe Interactions 21: 1421-1430. doi:10.1094/MPMI-21-11-1421

Lopez GA, Potts BM, Dutkowski GW, Traverso JMR (2001) Quantitative genetics of *Eucalyptus globulus*: Affinities of land race and native stand localities. Silvae Genetica 50: 244-252

Lozano R, Ponce O, Ramirez M, Mostajo N, Orjeda G (2012) Genome-wide identification and mapping of NBS-encoding resistance genes in *Solanum tuberosum* group phureja. PloS One 7, e34775: 1-12. doi:10.1371/journal.pone.0034775

Luo S, Zhang Y, Hu Q, Chen J, Li K, Lu C, Liu H, Wang W, Kuang H (2012) Dynamic nucleotide-binding site and leucine-rich repeat-encoding genes in the grass family. Plant Physiology 159: 197-210. doi:10.1104/pp.111.192062

Maccaferri M, Zhang J, Bulli P, Abate Z, Chao S, Cantu D, Bossolini E, Chen X, Pumphrey M, Dubcovsky J (2015) A genome-wide association study of resistance to stripe rust (*Puccinia striiformis* f. sp. *tritici*) in a worldwide collection of hexaploid spring wheat (*Triticum aestivum* L.). G3: Genes | Genomes | Genetics 5: 449-465. doi:10.1534/g3.114.014563

Machado PD, Alfenas AC, Alfenas RF, Mohammed CL, Glen M (2015) Microsatellite analysis indicates that *Puccinia psidii* in Australia is mutating but not recombining. Australasian Plant Pathology 44: 455-462. doi:10.1007/s13313-015-0364-5

MacLachlan JD (1938) A rust of the pimento tree in Jamaica, BWI. Phytopathology 28: 157-170

Mahmood K, Marcar NE, Naqvi MH, Arnold RJ, Crawford DF, Iqbal S, Aken KM (2003) Genetic variation in *Eucalyptus camaldulensis* Dehnh. for growth and stem straightness in a provenance-family trial on saltland in Pakistan. Forest Ecology and Management 176: 405-416. doi:10.1016/S0378-1127(02)00230-X

Maloy OC (2003) White pine blister rust. The Plant Health Instructor. The American Phytopathological Society. doi: 10.1094/PHI-I-2003-0908-01. http://www.apsnet.org/edcenter/intropp/lessons/fungi/Basidiomycetes/Pages/WhitePine.aspx. Accessed 6 March 2017

Mamani EMC, Bueno NW, Faria DA, Guimaraes LMS, Lau D, Alfenas AC, Grattapaglia D (2010) Positioning of the major locus for *Puccinia psidii* rust resistance (*Ppr1*) on the *Eucalyptus* reference map and its validation across unrelated pedigrees. Tree Genetics & Genomes 6: 953-962. doi:10.1007/s11295-010-0304-z

Mandrou E, Hein PRG, Villar E, Vigneron P, Plomion C, Gion JM (2012) A candidate gene for lignin composition in *Eucalyptus*: *cinnamoyl-CoA reductase* (*CCR*). Tree Genetics & Genomes 8: 353-364. doi:10.1007/s11295-011-0446-7

Marchetti MA, Uecker FA, Bromfield KR (1975) Uredial development of *Phakopsora pachyrhizi* in soybeans. Phytopathology 65: 822-823. doi:10.1094/Phyto-65-822

Marlatt RB, Kimbrough JW (1979) *Puccinia psidii* on *Pimenta dioica* in south Florida. Plant Disease Reporter 63: 510-512

Martin GB, Bogdanove AJ, Sessa G (2003) Understanding the functions of plant disease resistance proteins. Annual Review of Plant Biology 54: 23-61. doi:10.1146/annurev.arplant.54.031902.135035

Martin JT (1964) Role of cuticle in the defense against plant disease. Annual Review of Phytopathology 2: 81-100. doi:10.1146/annurev.py.02.090164.000501

Martins MVV, Silveira SF, Maffia LA, Rocabado JMA, Mussi-Dias V (2011) Chemical control of guava rust (*Puccinia psidii*) in the Northern Region of Rio de Janeiro State, Brazil. Australasian Plant Pathology 40: 48-54. doi:10.1007/s13313-010-0012-z

McArthur C, Loney PE, Davies NW, Jordan GJ (2010) Early ontogenetic trajectories vary among defence chemicals in seedlings of a fast-growing eucalypt. Austral Ecology 35: 157-166. doi:10.1111/j.1442-9993.2009.02021.x

McCaskill D, Croteau R (1995) Monoterpene and sesquiterpene biosynthesis in glandular trichomes of peppermint (*Mentha x piperita*) rely exclusively on plastid-derived isopentenyl diphosphate. Planta 197: 49-56

McDowell JM, Simon SA (2006) Recent insights into *R* gene evolution. Molecular Plant Pathology 7: 437-448. doi:10.1111/j.1364-3703.2006.00342.x

McDowell JM, Woffenden BJ (2003) Plant disease resistance genes: recent insights and potential applications. Trends in Biotechnology 21: 178-183. doi:10.1016/S0167-7799(03)00053-2

McKinnon GE, Jordan GJ, Vaillancourt RE, Steane DA, Potts BM (2004) Glacial refugia and reticulate evolution: the case of the Tasmanian eucalypts. Philosophical Transactions of the Royal Society B: Biological Sciences 359: 275-284. doi:10.1098/rstb.2003.1391

McKinnon GE, Steane DA, Potts BM, Vaillancourt RE (1999) Incongruence between chloroplast and species phylogenies in *Eucalyptus* subgenus *Monocalyptus* (Myrtaceae). American Journal of Botany 86: 1038-1046. doi:10.2307/2656621

McKinnon GE, Vaillancourt RE, Jackson HD, Potts BM (2001) Chloroplast sharing in the Tasmanian eucalypts. Evolution 55: 703-711. doi:10.1554/0014-3820(2001)055[0703:CSITTE]2.0.CO;2

McKown AD, Klápště J, Guy RD, Geraldes A, Porth I, Hannemann J, Friedmann M, Muchero W, Tuskan GA, Ehlting J, Cronk QCB, El-Kassaby YA, Mansfield SD, Douglas CJ (2014) Genome-wide association implicates numerous genes underlying ecological trait variation in natural populations of *Populus trichocarpa*. New Phytologist 203: 535-553. doi:10.1111/nph.12815

McLean S, Foley WJ, Davies NW, Brandon S, Li D, Blackman AJ (1993) Metabolic fate of dietary terpenes from *Eucalyptus radiata* in common ringtail possum (*Pseudocheirus peregrinus*). Journal of Chemical Ecology 19: 1625-1643. doi:10.1007/BF00982297

McRae TA, Pilbeam DJ, Powell MB, Dutkowski GW, Joyce K, Tier B (2004) Genetic evaluation in eucalypt breeding programs. Paper presented at the IUFRO Conference, Aveiro, Portugal, 11-15 October 2004

McTaggart AR, Roux J, Granados GM, Gafur A, Tarrigan M, Santhakumar P, Wingfield MJ (2016a) Rust (*Puccinia psidii*) recorded in Indonesia poses a threat to forests and forestry in South-East Asia. Australasian Plant Pathology 45: 83-89. doi:10.1007/s13313-015-0386-z

McTaggart AR, Shivas RG, van der Nest MA, Roux J, Wingfield BD, Wingfield MJ (2016b) Host jumps shaped the diversity of extant rust fungi (Pucciniales). New Phytologist 209: 1149-1158. doi:10.1111/nph.13686

McTaggart AR, Shuey LS, Granados GM, du Plessis E, Fraser S, Barnes I, Naidoo S, Wingfield MJ, Roux J (2017) Evidence that *Austropuccinia psidii* may complete its sexual life cycle on Myrtaceae. Plant Pathology. doi:10.1111/ppa.12763

Metzker ML (2010) Sequencing technologies - the next generation. Nature Reviews Genetics 11: 31-46. doi:10.1038/nrg2626

Meyers BC, Kozik A, Griego A, Kuang H, Michelmore RW (2003) Genome-wide analysis of NBS-LRR-encoding genes in Arabidopsis. Plant Cell 15: 809-834. doi:10.1105/tpc.009308

Millner JP, Kemp PD (2012) Foliar nutrients in *Eucalyptus* species in New Zealand. New Forests 43: 255-266. doi:10.1007/s11056-011-9279-3

Miranda AC, de Moraes MLT, Tambarussi EV, Furtado EL, Mori ES, da Silva PHM, Sebbenn AM (2013) Heritability for resistance to *Puccinia psidii* Winter rust in *Eucalyptus grandis* Hill ex Maiden in Southwestern Brazil. Tree Genetics & Genomes 9: 321-329. doi:10.1007/s11295-012-0572-x

Mitchell-Olds T, Willis JH, Goldstein DB (2007) Which evolutionary processes influence natural genetic variation for phenotypic traits? Nature Reviews Genetics 8: 845-856. doi:10.1038/nrg2207

Monosi B, Wisser RJ, Pennill L, Hulbert SH (2004) Full-genome analysis of resistance gene homologues in rice. Theoretical and Applied Genetics 109: 1434-1447. doi:10.1007/s00122-004-1758-x

Montesano M, Brader G, Palva ET (2003) Pathogen derived elicitors: searching for receptors in plants. Molecular Plant Pathology 4: 73-79. doi:10.1046/j.1364-3703.2003.00150.x

Moon DH, Salvatierra GR, Caldas DGG, de Carvalho MCCG, Carneiro RT, Franceschini LM, Oda S, Labate CA (2007) Comparison of the expression profiles of susceptible and resistant *Eucalyptus grandis* exposed to *Puccinia psidii* Winter using SAGE. Functional Plant Biology 34: 1010-1018. doi:10.1071/FP07094

Moore B, Andrew RL, Külheim C, Foley WJ (2014) Explaining intraspecific diversity in plant secondary metabolites in an ecological context. New Phytologist 201: 733-750. doi:10.1111/nph.12526

Moore BD, Wallis IR, Pála-Paúl J, Brophy JJ, Willis RH, Foley WJ (2004) Antiherbivore chemistry of *Eucalyptus* - cues and deterrents for marsupial folivores. Journal of Chemical Ecology 30: 1743-1769. doi:10.1023/B:JOEC.0000042399.06553.c6

Moore GM (2015) The role of lignotubers (basal burls) in the stress recovery of messmate stringybark, *Eucalyptus obliqua* L'Herit. seedlings and its arboricultural implications. Arboricultural Journal: The International Journal of Urban Forestry 37: 113-125. doi:10.1080/03071375.2015.1066559

Moreira X, Zas R, Sampedro L (2012) Quantitative comparison of chemical, biological and mechanical induction of secondary compounds in *Pinus pinaster* seedlings. Trees 26: 677-683. doi:10.1007/s00468-011-0602-6

Morin L, Aveyard R, Lidbetter J (2011) Myrtle rust: host testing under controlled conditions. Report number C2010/9785. CSIRO, Canberra

Morin L, Aveyard R, Lidbetter JR, Wilson PG (2012) Investigating the host-range of the rust fungus *Puccinia psidii* sensu lato across tribes of the family Myrtaceae present in Australia. PloS One 7, e35434: 1-7. doi:10.1371/journal.pone.0035434

Morin L, Talbot MJ, Glen M (2014) Quest to elucidate the life cycle of *Puccinia psidii* sensu lato. Fungal Biology 118: 253-263. doi:10.1016/j.funbio.2013.12.004

Morrow PA, Fox LR (1980) Effects of variation in *Eucalyptus* essential oil yield on insect growth and grazing damage. Oecologia 45: 209-219. doi:10.1007/BF00346462

Müller BSF, Neves LG, Filho JEA, Resende Jr MFR, Muñoz PR, dos Santos PET, Filho EP, Kirst M, Grattapaglia D (2017) Genomic prediction in contrast to a genome-wide association

study in explaining heritable variation of complex growth traits in breeding populations of *Eucalyptus*. BMC Genomics 18, Article 524: 1-17. doi:10.1186/s12864-017-3920-2

Mur LAJ, Kenton P, Lloyd AJ, Ougham H, Prats E (2008) The hypersensitive response; the centenary is upon us but how much do we know? Journal of Experimental Botany 59: 501-520. doi:10.1093/jxb/erm239

Myburg A, Bradfield J, Cowley E, Creux N, de Castro M, Hatherell TL, Mphahlele M, O'Neill M, Ranik M, Solomon L, Victor M, Zhou H, Galloway G, Horsley T, Jones N, Stanger T, Bayley A, Edwards N, Janse B (2008) Forest and fibre genomics: biotechnology tools for applied tree improvement. Southern Forests 70: 59-68. doi:10.2989/SOUTH.FOR.2008.70.2.1.529

Myburg AA, Grattapaglia D, Tuskan GA, Hellsten U, Hayes RD, Grimwood J, Jenkins J, Lindquist E, Tice H, Bauer D, Goodstein DM, Dubchak I, Poliakov A, Mizrachi E, Kullan ARK, Hussey SG, Pinard D, van der Merwe K, Singh P, van Jaarsveld I, Silva-Junior OB, Togawa RC, Pappas MR, Faria DA, Sansaloni CP, Petroli CD, Yang X, Ranjan P, Tschaplinski TJ, Ye CY, Li T, Sterck L, Vanneste K, Murat F, Soler M, Clemente HS, Saidi N, Cassan-Wang H, Dunand C, Hefer CA, Bornberg-Bauer E, Kersting AR, Vining K, Amarasinghe V, Ranik M, Naithani S, Elser J, Boyd AE, Liston A, Spatafora JW, Dharmwardhana P, Raja R, Sullivan C, Romanel E, Alves-Ferreira M, Külheim C, Foley W, Carocha V, Paiva J, Kudrna D, Brommonschenkel SH, Pasquali G, Byrne M, Rigault P, Tibbits J, Spokevicius A, Jones RC, Steane DA, Vaillancourt RE, Potts BM, Joubert F, Barry K, Pappas GJ, Strauss SH, Jaiswal P, Grima-Pettenati J, Salse J, Van de Peer Y, Rokhsar DS, Schmutz J (2014) The genome of *Eucalyptus grandis*. Nature 510: 356-362. doi:10.1038/nature13308

Mysore KS, Ryu CM (2004) Nonhost resistance: how much do we know? Trends in Plant Science 9: 97-104. doi:10.1016/j.tplants.2003.12.005

Naidoo S, Külheim C, Zwart L, Mangwanda R, Oates CN, Visser EA, Wilken FE, Mamni TB, Myburg AA (2014) Uncovering the defence responses of *Eucalyptus* to pests and pathogens in the genomics age. Tree Physiology 34: 931-943. doi:10.1093/treephys/tpu075

Neale DB, Kremer A (2011) Forest tree genomics: growing resources and applications. Nature Reviews Genetics 12: 111-122. doi:10.1038/nrg2931

Nei M (2005) Selectionism and neutralism in molecular evolution. Molecular Biology and Evolution 22: 2318-2342. doi:10.1093/molbev/msi242

Neilson EH, Goodger JQD, Woodrow IE, Møller BL (2013) Plant chemical defense: at what cost? Trends in Plant Science 18: 250-258. doi:10.1016/j.tplants.2013.01.001

Nesbitt KA, Potts BM, Vaillancourt RE, West AK, Reid JB (1995) Partitioning and distribution of RAPD variation in a forest tree species, *Eucalyptus globulus* (Myrtaceae). Heredity 74: 628-637. doi:10.1038/hdy.1995.86

Neu C, Keller B, Feuillet C (2003) Cytological and molecular analysis of the *Hordeum vulgare-Puccinia triticina* nonhost interaction. Molecular Plant-Microbe Interactions 16: 626-633. doi:10.1094/MPMI.2003.16.7.626

Nevill PG, Bossinger G, Ades PK (2010) Phylogeography of the world's tallest angiosperm, *Eucalyptus regnans*: evidence for multiple isolated Quaternary refugia. Journal of Biogeography 37: 179-192. doi:10.1111/j.1365-2699.2009.02193.x

Nevill PG, Després T, Bayly MJ, Bossinger G, Ades PK (2014) Shared phylogeographic patterns and widespread chloroplast haplotype sharing in *Eucalyptus* species with different ecological tolerances. Tree Genetics & Genomes 10: 1079-1092. doi:10.1007/s11295-014-0744-y

Newcombe G, Chastagner GA (1993) First report of the Eurasian poplar leaf rust fungus, *Melampsora larici-populina*, in North America. Plant Disease 77: 532-535. doi:10.1094/PD-77-0532

Neyland M, Hickey J, Beadle C, Bauhus J, Davidson N, Edwards L (2009) An examination of stocking and early growth in the Warra silvicultural systems trial confirms the importance of a burnt seedbed for vigorous regeneration in *Eucalyptus obliqua* forest. Forest Ecology and Management 258: 481-494. doi:10.1016/j.foreco.2008.10.039

Nichol NS, Wingfield MJ, Swart WJ (1992) Differences in susceptibility of *Eucalyptus* species to *Phaeoseptoria eucalypti*. European Journal of Forest Pathology 22: 418-423. doi:10.1111/j.1439-0329.1992.tb00315.x

Niks RE (2014) How specific is non-hypersensitive host and nonhost resistance of barley to rust and mildew fungi? Journal of Integrative Agriculture 13: 244-254. doi:10.1016/S2095-3119(13)60648-6

Niks RE, Dekens RG (1991) Prehaustorial and posthaustorial resistance to wheat leaf rust in diploid wheat seedlings. Phytopathology 81: 847-851. doi:10.1094/Phyto-81-847

Niks RE, Marcel TC (2009) Nonhost and basal resistance: how to explain specificity? New Phytologist 182: 817-828. doi:10.1111/j.1469-8137.2009.02849.x

Noble IR (1989) Ecological traits of the *Eucalyptus* L'Herit. subgenera *Monocalyptus* and *Symphyomyrtus*. Australian Journal of Botany 37: 207-224. doi:10.1071/BT9890207

Nordborg M, Weigel D (2008) Next-generation genetics in plants. Nature 456: 720-723. doi:10.1038/nature07629

Novaes E, Drost DR, Farmerie WG, Pappas Jr. GJ, Grattapaglia D, Sederoff RR, Kirst M (2008) High-throughput gene and SNP discovery in *Eucalyptus grandis*, an uncharacterized genome. BMC Genomics 9, Article 312: 1-14. doi:10.1186/1471-2164-9-312

Nuismer SL, Thompson JN, Gomulkiewicz R (1999) Gene flow and geographically structured coevolution. Proceedings of the Royal Society of London B: Biological Sciences 266: 605-609. doi:10.1098/rspb.1999.0679

Nürnberger T, Brunner F, Kemmerling B, Piater L (2004) Innate immunity in plants and animals: striking similarities and obvious differences. Immunological Reviews 198: 249-266. doi:10.1111/j.0105-2896.2004.0119.x

Nürnberger T, Lipka V (2005) Non-host resistance in plants: new insights into an old phenomenon. Molecular Plant Pathology 6: 335-345. doi:10.1111/j.1364-3703.2005.00279.x

O'Brien EK, Mazanec RA, Krauss SL (2007) Provenance variation of ecologically important traits of forest trees: implications for restoration. Journal of Applied Ecology 44: 583-593. doi:10.1111/j.1365-2664.2007.01313.x

O'Reilly-Wapstra JM, Freeman JS, Davies NW, Vaillancourt RE, Fitzgerald H, Potts BM (2011) Quantitative trait loci for foliar terpenes in a global eucalypt species. Tree Genetics & Genomes 7: 485-498. doi:10.1007/s11295-010-0350-6

O'Reilly-Wapstra JM, Humphreys JR, Potts BM (2007) Stability of genetic-based defensive chemistry across life stages in a *Eucalyptus* species. Journal of Chemical Ecology 33: 1876-1884. doi:10.1007/s10886-007-9366-2

O'Reilly-Wapstra JM, McArthur C, Potts BM (2004) Linking plant genotype, plant defensive chemistry and mammal browsing in a *Eucalyptus* species. Functional Ecology 18: 677-684. doi:10.1111/j.0269-8463.2004.00887.x

O'Reilly-Wapstra JM, Miller A, Potts BM (2014) Variable patterns of inheritance of ecologically important plant secondary metabolites in an inter-specific eucalypt hybrid. Forest Ecology and Management 318: 71-77. doi:10.1016/j.foreco.2014.01.016

O'Reilly-Wapstra JM, Potts BM, McArthur C, Davies NW, Tilyard P (2005) Inheritance of resistance to mammalian herbivores and of plant defensive chemistry in a *Eucalyptus* species. Journal of Chemical Ecology 31: 519-537. doi:10.1007/s10886-005-2030-9

Old KM (1981) Western gall rust, a serious disease of *Pinus radiata* in California. Australian Forestry 44: 178-184. doi:10.1080/00049158.1981.10674311

Osbourn AE (1996) Preformed antimicrobial compounds and plant defense against fungal attack. Plant Cell 8: 1821-1831. doi:10.1105/tpc.8.10.1821

Ottewell KM, Donnellan SC, Moran GF, Paton DC (2005) Multiplexed microsatellite markers for the genetic analysis of *Eucalyptus leucoxylon* (Myrtaceae) and their utility for ecological and breeding studies in other *Eucalyptus* species. Journal of Heredity 96: 445-451. doi:10.1093/jhered/esi057

Padovan A, Keszei A, Foley WJ, Külheim C (2013) Differences in gene expression within a striking phenotypic mosaic *Eucalyptus* tree that varies in susceptibility to herbivory. BMC Plant Biology 13, Article 29: 1-12. doi:10.1186/1471-2229-13-29

Padovan A, Keszei A, Köllner TG, Degenhardt J, Foley WJ (2010) The molecular basis of host plant selection in *Melaleuca quinquenervia* by a successful biological control agent. Phytochemistry 71: 1237-1244. doi:10.1016/j.phytochem.2010.05.013

Padovan A, Keszei A, Külheim C, Foley WJ (2014) The evolution of foliar terpene diversity in Myrtaceae. Phytochemistry Reviews 13: 695-716. doi:10.1007/s11101-013-9331-3

Padovan A, Keszei A, Wallis IR, Foley WJ (2012) Mosaic eucalypt trees suggest genetic control at a point that influences several metabolic pathways. Journal of Chemical Ecology 38: 914-923. doi:10.1007/s10886-012-0149-z

Paine TD, Steinbauer MJ, Lawson SA (2011) Native and exotic pests of *Eucalyptus*: a worldwide perspective. Annual Review of Entomology 56: 181-201. doi:10.1146/annurev-ento-120709-144817

Park RF, Keane PJ (1982) Leaf diseases of *Eucalyptus* associated with *Mycosphaerella* species. Transactions of the British Mycological Society 79: 101-115. doi:10.1016/S0007-1536(82)80195-2

Park RF, Keane PJ, Wingfield MJ, Crous PW (2000) Fungal diseases of eucalypt foliage. In: Keane PJ, Kile GA, Podger FD, Brown BN (eds) Diseases and Pathogens of Eucalypts. CSIRO Publishing, Collingwood VIC, pp 153-239

Pearce RB (1996) Antimicrobial defences in the wood of living trees. New Phytologist 132: 203-233. doi:10.1111/j.1469-8137.1996.tb01842.x

Pederick LA (1979) Natural variation in shining gum (*Eucalyptus nitens*). Australian Forest Research 9: 41-63

Pegg G, Perry S, Carnegie A, Ireland K, Giblin F (2012) Understanding myrtle rust epidemiology and host specificity to determine disease impact in Australia. CRC70186 Final Rerpot. Cooperative Research Centre for National Plant Biosecurity, Canberra

Pegg GS, Brawner JT, Lee DJ (2014a) Screening *Corymbia* populations for resistance to *Puccinia psidii*. Plant Pathology 63: 425-436. doi:10.1111/ppa.12097

Pegg GS, Giblin FR, McTaggart AR, Guymer GP, Taylor H, Ireland KB, Shivas RG, Perry S (2014b) *Puccinia psidii* in Queensland, Australia: disease symptoms, distribution and impact. Plant Pathology 63: 1005-1021. doi:10.1111/ppa.12173

Penfold AR, Morrison FR (1936) The occurrence of a number of varieties of *Eucalyptus radiata* (*E. numerosa*) as determined by chemical analyses of the essential oils. Part II. Journal and Proceedings of the Royal Society of New South Wales 70: 375-377

Penfold AR, Willis JL (1953) Physiological forms of *Eucalyptus citriodora* Hooker. Nature 171: 883-884. doi:10.1038/171883b0

Petersen RH (1974) The rust fungus life cycle. The Botanical Review 40: 453-513. doi:10.1007/BF02860021

Peterson RS (1971) Wave years of infection by western gall rust on pine. Plant Disease Reporter 55: 163-167

Pink B (2012) Year Book Australia. Australian Bureau of Statistics, Canberra

Pinto CS, da Costa RML, de Moraes CB, de Pieri C, Tambarussi EV, Furtado EL, Mori ES (2014) Genetic variability in progenies of *Eucalyptus dunnii* Maiden for resistance to *Puccinia psidii*. Crop Breeding and Applied Biotechnology 14: 187-193. doi:10.1590/1984-70332014v14n3a28

Pirralho M, Flores D, Sousa VB, Quilhó T, Knapic S, Pereira H (2014) Evaluation on paper making potential of nine *Eucalyptus* species based on wood anatomical features. Industrial Crops and Products 54: 327-334. doi:10.1016/j.indcrop.2014.01.040

Piza SMT, Ribeiro IJA (1988) Influence of light and temperature on uredospores germination of *Puccinia psidii* Winter. Bragantia 47: 75-78. doi:10.1590/S0006-87051988000100009

Plant Health Australia (2011) Plan for trasition to management of myrtle rust. Plant Health Australia Ltd, Canberra. http://myrtlerust.net.au/wordpress/wp-content/uploads/2011/11/Transition-to-Management-Plan-v1-Nov-2011.pdf. Accessed 7 August 2017

Plomion C, Bastien C, Bogeat-Triboulot M-B, Bouffier L, Déjardin A, Duplessis S, Fady B, Heuertz M, Le Gac A-L, Le Provost G, Legué V, Lelu-Walter M-A, Leplé J-C, Maury S, Morel A, Oddou-Muratorio S, Pilate G, Sanchez L, Scotti I, Scotti-Saintagne C, Segura V, Trontin J-F, Vacher C (2016) Forest tree genomics: 10 achievements from the past 10 years and future prospects. Annals of Forest Science 73: 77-103. doi:10.1007/s13595-015-0488-3

Podger FD, Batini F (1971) Susceptibility to *Phytophthora cinnamomi* root-rot of thirty six species of *Eucalyptus*. Australian Forest Research 5: 9-20

Poelman EH, van Loon JJA, Dicke M (2008) Consequences of variation in plant defense for biodiversity at higher trophic levels. Trends in Plant Science 13: 534-541. doi:10.1016/j.tplants.2008.08.003

Poke FS, Vaillancourt RE, Elliott RC, Reid JB (2003) Sequence variation in two lignin biosynthesis genes, cinnamoyl CoA reductase (*CCR*) and cinnamyl alcohol dehydrogenase 2 (*CAD2*). Molecular Breeding 12: 107-118. doi:10.1023/A:1026006828721

Porth I, Klapšte J, Skyba O, Hannemann J, McKown AD, Guy RD, DiFazio SP, Muchero W, Ranjan P, Tuskan GA, Friedmann MC, Ehlting J, Cronk QCB, El-Kassaby YA, Douglas CJ, Mansfield SD (2013) Genome-wide association mapping for wood characteristics in *Populus* identifies an array of candidate single nucleotide polymorphisms. New Phytologist 200: 710-726. doi:10.1111/nph.12422

Potts BM, Jordan GJ (1994a) Genetic variation in the juvenile leaf morphology of *Eucalyptus globulus* Labill. ssp *globulus*. Forest Genetics 1: 81-95

Potts BM, Jordan GJ (1994b) The spatial pattern and scale of variation in *Eucalyptus globulus* ssp *globulus*: Variation in seedling abnormalities and early growth. Australian Journal of Botany 42: 471-492. doi:10.1071/BT9940471

Potts BM, Sandhu KS, Wardlaw T, Freeman J, Li H, Tilyard P, Park RF (2016) Evolutionary history shapes the susceptibility of an island tree flora to an exotic pathogen. Forest Ecology and Management 368: 183-193. doi:10.1016/j.foreco.2016.02.027

Pritchard L, Birch P (2011) A systems biology perspective on plant-microbe interactions: biochemical and structural targets of pathogen effectors. Plant Science 180: 584-603. doi:10.1016/j.plantsci.2010.12.008

Pryor LD, Johnson LAS (1971) A Classification of the Eucalypts. Australian National University Press, Canberra

Quilliam RS, Shattock RC (2003) Haustoria of microcyclic rust fungi *Uromyces ficariae* and *Puccinia tumida* and other gall-forming species, *U. dactylidis* (macrocyclic) and *P. smyrnii* (demicyclic). Plant Pathology 52: 104-113. doi:10.1046/j.1365-3059.2003.00800.x

R Core Team (2016) R: A language and environment for statistical computing. R Foundation for Statistical computing. Vienna, Austria. URL https://www.R-project.org/

Rae AM, Street NR, Rodríguez-Acosta M (2007) *Populus* Trees. In: Kole C (ed) Forest Trees. Springer-Verlag, Berlin, Heidelberg, pp 1-28. doi:10.1007/978-3-540-34541-1_1

Rafalski A (2002) Applications of single nucleotide polymorphisms in crop genetics. Current Opinion in Plant Biology 5: 94-100. doi:10.1016/S1369-5266(02)00240-6

Rapley LP, Allen GR, Potts BM, Davies NW (2007) Constitutive or induced defences - how does *Eucalyptus globulus* defend itself from larval feeding? Chemoecology 17: 235-243. doi:10.1007/s00049-007-0382-z

Rathbone DA, McKinnon GE, Potts BM, Steane DA, Vaillancourt RE (2007) Microsatellite and cpDNA variation in island and mainland populations of a regionally rare eucalypt, *Eucalyptus perriniana* (Myrtaceae). Australian Journal of Botany 55: 513-520. doi:10.1071/BT06203

Rayachhetry MB, Elliott ML, Van TK (1997) Natural epiphytotic of the rust *Puccinia psidii* on *Melaleuca quinquenervia* in Florida. Plant Disease 81: 831-831. doi:10.1094/PDIS.1997.81.7.831A

Rayachhetry MB, Van TK, Center TD, Elliott ML (2001) Host range of *Puccinia psidii*, a potential biological control agent of *Melaleuca quinquenervia* in Florida. Biological Control 22: 38-45. doi:10.1006/bcon.2001.0949

Rédei GP (2008) *Forma specialis*. In: Encyclopedia of Genetics, Genomics, Proteomics and Informatics. Springer Netherlands, Dordrecht, pp 706-706. doi:10.1007/978-1-4020-6754-9_6165

Resende MDV, Resende Jr. MFR, Sansaloni CP, Petroli CD, Missiaggia AA, Aguiar AM, Abad JM, Takahashi EK, Rosado AM, Faria DA, Pappas Jr. GJ, Kilian A, Grattapaglia D (2012a) Genomic selection for growth and wood quality in *Eucalyptus*: capturing the missing heritability and accelerating breeding for complex traits in forest trees. New Phytologist 194: 116-128. doi:10.1111/j.1469-8137.2011.04038.x

Resende MFR, Muñoz P, Resende MDV, Garrick DJ, Fernando RL, Davis JM, Jokela EJ, Martin TA, Peter GF, Kirst M (2012b) Accuracy of genomic selection methods in a standard data set of loblolly pine (*Pinus taeda* L.). Genetics 190: 1503-1510. doi:10.1534/genetics.111.137026

Resende RT, Resende MDV, Silva FF, Azevedo CF, Takahashi EK, Silva-Junior OB, Grattapaglia D (2017a) Assessing the expected response to genomic selection of individuals and families in *Eucalyptus* breeding with an additive-dominant model. Heredity 119: 245-255. doi:10.1038/hdy.2017.37

Resende RT, Resende MDV, Silva FF, Azevedo CF, Takahashi EK, Silva-Junior OB, Grattapaglia D (2017b) Regional heritability mapping and genome-wide association identify loci for complex growth, wood and disease resistance traits in *Eucalyptus*. New Phytologist 213: 1287-1300. doi:10.1111/nph.14266

Ribeiro IJA, Pommer CV (2004) Breeding guava (*Psidium guajava*) for resistance to rust caused by *Puccinia psidii*. Acta Horticulturae 632: 75-78. doi:10.17660/ActaHortic.2004.632.9

Rodrigues P, Garrood JM, Shen QH, Smith PH, Boyd LA (2004) The genetics of non-host disease resistance in wheat to barley yellow rust. Theoretical and Applied Genetics 109: 425-432. doi:10.1007/s00122-004-1648-2

Rosado CCG, Guimarães LMS, Faria DA, de Resende MDV, Cruz CD, Grattapaglia D, Alfenas AC (2016) QTL mapping for resistance to Ceratocystis wilt in *Eucalyptus*. Tree Genetics & Genomes 12, Article 72: 1-10. doi:10.1007/s11295-016-1029-4

Rosado TB, Tomaz RS, Ribeiro Junior MF, Rosado AM, Guimarães LMS, de Araújo EF, Alfenas AC, Cruz CD (2010) Detection of QTL associated with rust resistance using IBD-based methodologies in exogamic *Eucalyptus* spp. populations. Crop Breeding and Applied Biotechnology 10: 321-328. doi:10.1590/S1984-70332010000400006

Roux J, Germishuizen I, Nadel R, Lee DJ, Wingfield MJ, Pegg GS (2015) Risk assessment for *Puccinia psidii* becoming established in South Africa. Plant Pathology 64: 1326-1335. doi:10.1111/ppa.12380

Roux J, Granados GM, Shuey L, Barnes I, Wingfield MJ, McTaggart AR (2016) A unique genotype of the rust pathogen, *Puccinia psidii*, on Myrtaceae in South Africa. Australasian Plant Pathology 45: 645-652. doi:10.1007/s13313-016-0447-y

Roux J, Greyling I, Coutinho TA, Verleur M, Wingfield MJ (2013) The Myrtle rust pathogen, *Puccinia psidii*, discovered in Africa. IMA Fungus 4: 155-159. doi:10.5598/imafungus.2013.04.01.14

Rupe J, Sconyers L (2008) Soybean rust. The Plant Health Instructor. The American Phytopathological Society. doi: 10.1094/PHI-I-2008-0401-01. http://www.apsnet.org/edcenter/intropp/lessons/fungi/Basidiomycetes/Pages/SoybeanRust.aspx. Accessed 6 March 2017

Saini RG, Kaur M, Singh B, Sharma S, Nanda GS, Nayar SK, Gupta AK, Nagarajan S (2002) Genes *Lr48* and *Lr49* for hypersensitive adult plant leaf rust resistance in wheat (*Triticum aestivum* L.). Euphytica 124: 365-370. doi:10.1023/A:1015762812907

Sánchez Márquez S, Bills GF, Zabalgogeazcoa I (2011) Fungal species diversity in juvenile and adult leaves of *Eucalyptus globulus* from plantations affected by Mycosphaerella leaf disease. Annals of Applied Biology 158: 177-187. doi:10.1111/j.1744-7348.2010.00449.x

Sandhu KS, Karaoglu H, Zhang P, Park RF (2016) Simple sequence repeat markers support the presence of a single genotype of *Puccinia psidii* in Australia. Plant Pathology 65: 1084-1094. doi:10.1111/ppa.12501

Sandhu KS, Park RF (2013) Genetic basis of pathogenicity in *Uredo rangelii*. National Myrtle Rust Transition to Management (T2M) Program Final Report. The University of Sydney, Cobbitty NSW

Santos MR, Guimarães LMS, de Resende MDV, Rosse LN, Zamprogno KC, Alfenas AC (2014) Resistance of *Eucalyptus pellita* to rust (*Puccinia psidii*). Crop Breeding and Applied Biotechnology 14: 244-250. doi:10.1590/1984-70332014v14n4a38

SAS Institute (2014) JMP Genomics, Version 7.0. SAS Institute Inc., Cary, NC

Schmidt RA (2003) Fusiform rust of southern pines: a major success for forest disease management. Phytopathology 93: 1048-1051. doi:10.1094/PHYTO.2003.93.8.1048

Schroth G, Krauss U, Gasparotto L, Aguilar JAD, Vohland K (2000) Pests and diseases in agroforestry systems of the humid tropics. Agroforestry Systems 50: 199-241. doi:10.1023/A:1006468103914

Schulze-Lefert P, Panstruga R (2011) A molecular evolutionary concept connecting nonhost resistance, pathogen host range, and pathogen speciation. Trends in Plant Science 16: 117-125. doi:10.1016/j.tplants.2011.01.001

Schumann GL, Leonard KJ (2000) Stem rust of wheat. The Plant Health Instructor. The American Phytopathological Society. doi: 10.1094/PHI-I-2000-0721-01. http://www.apsnet.org/edcenter/intropp/lessons/fungi/Basidiomycetes/Pages/StemRust.aspx. Accessed 6 March 2017

Schwab W, Davidovich-Rikanati R, Lewinsohn E (2008) Biosynthesis of plant-derived flavor compounds. Plant Journal 54: 712-732. doi:10.1111/j.1365-313X.2008.03446.x

Schwab W, Wüst M (2015) Understanding the constitutive and induced biosynthesis of mono- and sesquiterpenes in grapes (*Vitis vinifera*): a key to unlocking the biochemical secrets of unique grape aroma profiles. Journal of Agricultural and Food Chemistry 63: 10591-10603. doi:10.1021/acs.jafc.5b04398

Sebei K, Sakouhi F, Herchi W, Khouja ML, Boukhchina S (2015) Chemical composition and antibacterial activities of seven *Eucalyptus* species essential oils leaves. Biological Research 48, Article 7: 1-5. doi:10.1186/0717-6287-48-7

Segonzac C, Zipfel C (2011) Activation of plant pattern-recognition receptors by bacteria. Current Opinion in Microbiology 14: 54-61. doi:10.1016/j.mib.2010.12.005

Seigler DS (2012) Plant Secondary Metabolism. Springer Science & Business Media, New York. doi:10.1007/978-1-4615-4913-0

Senior JK, Potts BM, Davies NW, Wooliver RC, Schweitzer JA, Bailey JK, O'Reilly-Wapstra JM (2016) Phylogeny explains variation in the root chemistry of *Eucalyptus* species. Journal of Chemical Ecology 42: 1086-1097. doi:10.1007/s10886-016-0750-7

Senthil-Kumar M, Mysore KS (2013) Nonhost resistance against bacterial pathogens: retrospectives and prospects. Annual Review of Phytopathology 51: 407-427. doi:10.1146/annurev-phyto-082712-102319

Serrano M, Coluccia F, Torres M, L'Haridon F, Métraux JP (2014) The cuticle and plant defense to pathogens. Frontiers in Plant Science 5, Article 274: 1-8. doi:10.3389/fpls.2014.00274

Shao ZQ, Xue JY, Wu P, Zhang YM, Wu Y, Hang YY, Wang B, Chen JQ (2016) Large-scale analyses of angiosperm nucleotide-binding site-leucine-rich repeat genes reveal three anciently diverged classes with distinct evolutionary patterns. Plant Physiology 170: 2095-2109. doi:10.1104/pp.15.01487

Shao ZQ, Zhang YM, Hang YY, Xue JY, Zhou GC, Wu P, Wu XY, Wu XZ, Wang Q, Wang B, Chen JQ (2014) Long-term evolution of nucleotide-binding site-leucine-rich repeat genes: understanding gained from and beyond the legume family. Plant Physiology 166: 217-234. doi:10.1104/pp.114.243626

Shepherd M, Sexton TR, Thomas D, Henson M, Henry RJ (2010) Geographical and historical determinants of microsatellite variation in *Eucalyptus pilularis*. Canadian Journal of Forest Research 40: 1051-1063. doi:10.1139/X10-049

Silva MC, Várzea V, Guerra-Guimarães L, Azinheira HG, Fernandez D, Petitot AS, Bertrand B, Lashermes P, Nicole M (2006) Coffee resistance to the main diseases: leaf rust and coffee berry disease. Brazilian Journal of Plant Physiology 18: 119-147. doi:10.1590/S1677-04202006000100010

Silva PHM, Miranda AC, Moraes MLT, Furtado EL, Stape JL, Alvares CA, Sentelhas PC, Mori ES, Sebbenn AM (2013) Selecting for rust (*Puccinia psidii*) resistance in *Eucalyptus grandis* in Sao Paulo State, Brazil. Forest Ecology and Management 303: 91-97. doi:10.1016/j.foreco.2013.04.002

Simpson JA, Thomas K, Grgurinovic CA (2006) Uredinales species pathogenic on species of Myrtaceae. Australasian Plant Pathology 35: 549-562. doi:10.1071/AP06057

Sinapidou E, Williams K, Nott L, Bahkt S, Tör M, Crute I, Bittner-Eddy P, Beynon J (2004) Two TIR:NB:LRR genes are required to specify resistance to *Peronospora parasitica* isolate Cala2 in *Arabidopsis*. Plant Journal 38: 898-909. doi:10.1111/j.1365-313X.2004.02099.x

Singh RP, Hodson DP, Huerta-Espino J, Jin Y, Bhavani S, Njau P, Herrera-Foessel S, Singh PK, Singh S, Govindan V (2011) The emergence of Ug99 races of the stem rust fungus is a

threat to world wheat production. Annual Review of Phytopathology 49: 465-481. doi:10.1146/annurev-phyto-072910-095423

Singh RP, Hodson DP, Huerta-Espino J, Jin Y, Njau P, Wanyera R, Herrera-Foessel SA, Ward RW (2008) Will stem rust destroy the world's wheat crop? Advances in Agronomy 98: 271-309. doi:10.1016/S0065-2113(08)00205-8

Soria F, Borralho NMG (1997) The genetics of resistance to *Phoracantha semipunctata* attack in *Eucalyptus globulus* in Spain. Silvae Genetica 46: 365-369

Spiers AG, Hopcroft DH (1994) Comparative studies of the poplar rusts *Melampsora medusae*, *M. larici-populina* and their interspecific hybrid *M. medusae-populina*. Mycological Research 98: 889-903. doi:10.1016/S0953-7562(09)80260-8

Stackpole DJ, Vaillancourt RE, Alves A, Rodrigues J, Potts BM (2011) Genetic variation in the chemical components of *Eucalyptus globulus* wood. G3: Genes | Genomes | Genetics 1: 151-159. doi:10.1534/g3.111.000372

Stahl EA, Dwyer G, Mauricio R, Kreitman M, Bergelson J (1999) Dynamics of disease resistance polymorphism at the Rpm1 locus of Arabidopsis. Nature 400: 667-671. doi:10.1038/23260

Stamp N (2003) Out of the quagmire of plant defense hypotheses. The Quarterly Review of Biology 78: 23-55. doi:10.1086/367580

Stanturf JA, Vance ED, Fox TR, Kirst M (2013) Eucalyptus beyond its native range: Environmental issues in exotic bioenergy plantations. International Journal of Forestry Research 2013, Article 463030: 1-5. doi:10.1155/2013/463030

Steane DA, Byrne M, Vaillancourt RE, Potts BM (1998) Chloroplast DNA polymorphism signals complex interspecific interactions in *Eucalyptus* (Myrtaceae). Australian systematic botany 11: 25-40. doi:10.1071/SB96028

Steane DA, Conod N, Jones RC, Vaillancourt RE, Potts BM (2006) A comparative analysis of population structure of a forest tree, *Eucalyptus globutus* (Myrtaceae), using microsatellite markers and quantitative traits. Tree Genetics & Genomes 2: 30-38. doi:10.1007/s11295-005-0028-7

Steane DA, Potts BM, McLean EH, Collins L, Holland BR, Prober SM, Stock WD, Vaillancourt RE, Byrne M (2017) Genomic scans across three eucalypts suggest that adaptation to aridity is a genome-wide phenomenon. Genome Biology and Evolution 9: 253-265. doi:10.1093/gbe/evw290

Steane DA, West AK, Potts BM, Ovenden JR, Reid JB (1991) Restriction fragment length polymorphisms in chloroplast DNA from six species of *Eucalyptus*. Australian Journal of Botany 39: 399-414. doi:10.1071/BT9910399

Stein M, Dittgen J, Sánchez-Rodríguez C, Hou BH, Molina A, Schulze-Lefert P, Lipka V, Somerville S (2006) *Arabidopsis* PEN3/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. Plant Cell 18: 731-746. doi:10.1105/tpc.105.038372

Steinbauer MJ (2010) Latitudinal trends in foliar oils of eucalypts: environmental correlates and diversity of chrysomelid leaf-beetles. Austral Ecology 35: 204-213. doi:10.1111/j.1442-9993.2009.02028.x

Steppuhn A, Gase K, Krock B, Halitschke R, Baldwin IT (2004) Nicotine's defensive function in nature. PLoS Biology 2, e217: 1074-1080. doi:10.1371/journal.pbio.0020217

Stich B, Möhring J, Piepho HP, Heckenberger M, Buckler ES, Melchinger AE (2008) Comparison of mixed-model approaches for association mapping. Genetics 178: 1745-1754. doi:10.1534/genetics.107.079707

Stone C, Bacon PE (1994) Relationships among moisture stress, insect herbivory, foliar cineole content and the growth of river red gum *Eucalyptus camaldulensis*. Journal of Applied Ecology 31: 604-612. doi:10.2307/2404151

Stone C, Simpson JA, Gittins R (1998) Differential impact of insect herbivores and fungal pathogens on the *Eucalyptus* subgenera *Symphyomyrtus* and *Monocalyptus* and genus *Corymbia*. Australian Journal of Botany 46: 723-734. doi:10.1071/BT97077

Strauss SY (2001) Benefits and risks of biotic exchange between *Eucalyptus* plantations and native Australian forests. Austral Ecology 26: 447-457. doi:10.1046/j.1442-9993.2001.01136.x

Sturrock RN, Frankel SJ, Brown AV, Hennon PE, Kliejunas JT, Lewis KJ, Worrall JJ, Woods AJ (2011) Climate change and forest diseases. Plant Pathology 60: 133-149. doi:10.1111/j.1365-3059.2010.02406.x

Sumathi M, Yasodha R (2014) Microsatellite resources of *Eucalyptus*: current status and future perspectives. Botanical Studies 55, Article 73: 1-16. doi:10.1186/s40529-014-0073-3

Szatmári Á, Zvara Á, Móricz ÁM, Besenyei E, Szabó E, Ott PG, Puskás LG, Bozsó Z (2014) Pattern triggered immunity (PTI) in tobacco: isolation of activated genes suggests role of the phenylpropanoid pathway in inhibition of bacterial pathogens. PloS One 9, e102869: 1-16. doi:10.1371/journal.pone.0102869

Tabor HK, Risch NJ, Myers RM (2002) Candidate-gene approaches for studying complex genetic traits: practical considerations. Nature Reviews Genetics 3: 391-397. doi:10.1038/nrg796

Tang Y, Liu X, Wang J, Li M, Wang Q, Tian F, Su Z, Pan Y, Liu D, Lipka AE, Buckler ES, Zhang Z (2016) GAPIT version 2: an enhanced integrated tool for genomic association and prediction. Plant Genome 9: 1-9. doi:10.3835/plantgenome2015.11.0120

Telfer EJ, Stovold GT, Li Y, Silva-Junior OB, Grattapaglia DG, Dungey HS (2015) Parentage reconstruction in *Eucalyptus nitens* using SNPs and microsatellite markers: a comparative analysis of marker data power and robustness. PloS One 10, e130601: 1-18. doi:10.1371/journal.pone.0130601

Thavamanikumar S, McManus LJ, Ades PK, Bossinger G, Stackpole DJ, Kerr R, Hadjigol S, Freeman JS, Vaillancourt RE, Zhu P, Tibbits JFG (2014) Association mapping for wood quality and growth traits in *Eucalyptus globulus* ssp. *globulus* Labill identifies nine stable marker-trait associations for seven traits. Tree Genetics & Genomes 10: 1661-1678. doi:10.1007/s11295-014-0787-0

Thavamanikumar S, McManus LJ, Tibbits JFG, Bossinger G (2011) The significance of single nucleotide polymorphisms (SNPs) in *Eucalyptus globulus* breeding programs. Australian Forestry 74: 23-29. doi:10.1080/00049158.2011.10676342

Thavamanikumar S, Southerton SG, Bossinger G, Thumma BR (2013) Dissection of complex traits in forest trees — opportunities for marker-assisted selection. Tree Genetics & Genomes 9: 627-639. doi:10.1007/s11295-013-0594-z

Thompson JN (1999) Specific hypotheses on the geographic mosaic of coevolution. The American Naturalist 153: S1-S14. doi:10.1086/303208

Thompson JN, Burdon JJ (1992) Gene-for-gene coevolution between plants and parasites. Nature 360: 121-125. doi:10.1038/360121a0

Thornhill AH, Ho SYW, Kulheim C, Crisp MD (2015) Interpreting the modern distribution of Myrtaceae using a dated molecular phylogeny. Molecular Phylogenetics and Evolution 93: 29-43. doi:10.1016/j.ympev.2015.07.007

Thumma B, Pegg G, Warburton P, Brawner J, Macdonell P, Yang X, Southerton S (2013) Molecular tagging of rust resistance genes in eucalypts. Final Report PHA_6.2. Plant Health Australia Limited, Canberra

Thumma BR, Matheson BA, Zhang D, Meeske C, Meder R, Downes GM, Southerton SG (2009) Identification of a *cis*-acting regulatory polymorphism in a eucalypt *COBRA*-like gene affecting cellulose content. Genetics 183: 1153-1164. doi:10.1534/genetics.109.106591

Thumma BR, Nolan MR, Evans R, Moran GF (2005) Polymorphisms in *cinnamoyl CoA reductase* (*CCR*) are associated with variation in microfibril angle in *Eucalyptus* spp. Genetics 171: 1257-1265. doi:10.1534/genetics.105.042028

Thumma BR, Sharma N, Southerton SG (2012) Transcriptome sequencing of *Eucalyptus camaldulensis* seedlings subjected to water stress reveals functional single nucleotide polymorphisms and genes under selection. BMC Genomics 13, Article 364: 1-21. doi:10.1186/1471-2164-13-364

Tibbits JFG, McManus LJ, Spokevicius AV, Bossinger G (2006) A rapid method for tissue collection and high-throughput isolation of genomic DNA from mature trees. Plant Molecular Biology Reporter 24: 81-91. doi:10.1007/BF02914048

Tippett JT, Hill TC, Shearer BL (1985) Resistance of *Eucalyptus* spp. to invasion by *Phytophthora cinnamomi*. Australian Journal of Botany 33: 409-418. doi:10.1071/BT9850409

Tobias PA (2012) *Eucalyptus grandis* resistance genes: an *in silico* search for potential targets in response to myrtle rust. Macquarie Matrix 2.2: 134-149

Tobias PA, Guest DI, Külheim C, Hsieh JF, Park RF (2016) A curious case of resistance to a new encounter pathogen: myrtle rust in Australia. Molecular Plant Pathology 17: 783-788. doi:10.1111/mpp.12331

Troncoso C, Becerra J, Bittner M, Perez C, Sáez K, Sánchez-Olate M, Ríos D (2011) Chemical defense responses in *Eucalyptus globulus* (Labill) plants. Journal of the Chilean Chemical Society 56: 768-770. doi:10.4067/S0717-97072011000300010

Tucker SL, Talbot NJ (2001) Surface attachment and pre-penetration stage development by plant pathogenic fungi. Annual Review of Phytopathology 39: 385-417. doi:10.1146/annurev.phyto.39.1.385

Turnbull CRA, McLeod DE, Beadle CL, Ratkowsky DA, Mummery DC, Bird T (1993) Comparative early growth of *Eucalyptus* species of the subgenera *Monocalyptus* and *Symphyomyrtus* in intensively-managed plantations in southern Tasmania. Australian Forestry 56: 276-286. doi:10.1080/00049158.1993.10674615

Turnbull JW (1995) Biodiversity in Australian forest ecosystems. Paper presented at the IUFRO XX World Congress, Tampere, Finland, 6-12 August 1995

Turnbull JW (1999) Eucalypt plantations. New Forests 17: 37-52. doi:10.1023/A:1006524911242

Tuskan GA, DiFazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U, Putnam N, Ralph S, Rombauts S, Salamov A, Schein J, Sterck L, Aerts A, Bhalerao RR, Bhalerao RP, Blaudez D, Boerjan W, Brun A, Brunner A, Busov V, Campbell M, Carlson J, Chalot M, Chapman J, Chen GL, Cooper D, Coutinho PM, Couturier J, Covert S, Cronk Q, Cunningham R, Davis J, Degroeve S, Déjardin A, dePamphilis C, Detter J, Dirks B, Dubchak I, Duplessis S, Ehlting J, Ellis B, Gendler K, Goodstein D, Gribskov M, Grimwood J, Groover A, Gunter L, Hamberger B, Heinze B, Helariutta Y, Henrissat B, Holligan D, Holt R, Huang W, Islam-Faridi N, Jones S, Jones-Rhoades M, Jorgensen R, Joshi C, Kangasjärvi J, Karlsson J, Kelleher C, Kirkpatrick R, Kirst M, Kohler A, Kalluri U, Larimer F, Leebens-Mack J, Leplé JC, Locascio P, Lou Y, Lucas S, Martin F, Montanini B, Napoli C, Nelson DR, Nelson C, Nieminen K, Nilsson O, Pereda V, Peter G, Philippe R, Pilate G, Poliakov A, Razumovskaya J, Richardson P, Rinaldi C, Ritland K, Rouzé P, Ryaboy D, Schmutz J, Schrader J, Segerman B, Shin H, Siddiqui A, Sterky F, Terry A, Tsai CJ, Uberbacher E, Unneberg P, Vahala J, Wall K, Wessler S, Yang G, Yin T, Douglas C, Marra M, Sandberg

G, Van de Peer Y, Rokhsar D (2006) The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). Science 313: 1596-1604. doi:10.1126/science.1128691

Uchida J, Zhong S, Killgore E (2006) First report of a rust disease on ohia caused by *Puccinia psidii* in Hawaii. Plant Disease 90: 524-524. doi:10.1094/PD-90-0524C

Underwood W, Somerville SC (2013) Perception of conserved pathogen elicitors at the plasma membrane leads to relocalization of the *Arabidopsis* PEN3 transporter. Proceedings of the National Academy of Sciences of the United States of America 110: 12492-12497. doi:10.1073/pnas.1218701110

Unterseer S, Bauer E, Haberer G, Seidel M, Knaak C, Ouzunova M, Meitinger T, Strom TM, Fries R, Pausch H, Bertani C, Davassi A, Mayer KFX, Schön CC (2014) A powerful tool for genome analysis in maize: development and evaluation of the high density 600 k SNP genotyping array. BMC Genomics 15, Article 823: 1-15. doi:10.1186/1471-2164-15-823

Uppalapati SR, Ishiga Y, Doraiswamy V, Bedair M, Mittal S, Chen J, Nakashima J, Tang Y, Tadege M, Ratet P, Chen R, Schultheiss H, Mysore KS (2012) Loss of abaxial leaf epicuticular wax in *Medicago truncatula irg1/palm1* mutants results in reduced spore differentiation of anthracnose and nonhost rust pathogens. Plant Cell 24: 353-370. doi:10.1105/tpc.111.093104

Van Alfen NK (2001) Fungal pathogens of plants. In: Encyclopedia of Life Sciences (eLS). John Wiley & Sons, Ltd, Chichester. doi:10.1038/npg.els.0000362

van der Biezen EA, Jones JDG (1998) Plant disease-resistance proteins and the gene-for-gene concept. Trends in Biochemical Sciences 23: 454-456. doi:10.1016/S0968-0004(98)01311-5

van der Hoorn RAL, Kamoun S (2008) From guard to decoy: a new model for perception of plant pathogen effectors. Plant Cell 20: 2009-2017. doi:10.1105/tpc.108.060194

van Ooijen G, van den Burg HA, Cornelissen BJC, Takken FLW (2007) Structure and function of resistance proteins in solanaceous plants. Annual Review of Phytopathology 45: 43-72. doi:10.1146/annurev.phyto.45.062806.094430

VanEtten HD, Mansfield JW, Bailey JA, Farmer EE (1994) Two classes of plant antibiotics: phytoalexins versus "phytoanticipins". Plant Cell 6: 1191-1192. doi:10.1105/tpc.6.9.1191

Vaz Patto MC, Niks RE (2001) Leaf wax layer may prevent appressorium differentiation but does not influence orientation of the leaf rust fungus *Puccinia hordei* on *Hordeum chilense* leaves. European Journal of Plant Pathology 107: 795-803. doi:10.1023/A:1012410330287

Venables WN, Ripley BD (2002) Modern Applied Statistics with S. 4 edn. Springer-Verlag, New York. doi:10.1007/978-0-387-21706-2

Vorwerk S, Somerville S, Somerville C (2004) The role of plant cell wall polysaccharide composition in disease resistance. Trends in Plant Science 9: 203-209. doi:10.1016/j.tplants.2004.02.005

Wallis IR, Keszei A, Henery ML, Moran GF, Forrester R, Maintz J, Marsh KJ, Andrew RL, Foley WJ (2011) A chemical perspective on the evolution of variation in *Eucalyptus globulus*. Perspectives in Plant Ecology, Evolution and Systematics 13: 305-318. doi:10.1016/j.ppees.2011.05.005

Wallis IR, Nicolle D, Foley WJ (2010a) Available and not total nitrogen in leaves explains key chemical differences between the eucalypt subgenera. Forest Ecology and Management 260: 814-821. doi:10.1016/j.foreco.2010.05.040

Wallis IR, Smith HJ, Henery ML, Henson M, Foley WJ (2010b) Foliar chemistry of juvenile *Eucalyptus grandis* clones does not predict chemical defence in maturing ramets. Forest Ecology and Management 260: 763-769. doi:10.1016/j.foreco.2010.05.034

Wang B, Wang Y, Wang Q, Luo G, Zhang Z, He C, He SJ, Zhang J, Gai J, Chen S (2004) Characterization of an NBS-LRR resistance gene homologue from soybean. Journal of Plant Physiology 161: 815-822. doi:10.1016/j.jplph.2004.01.007

Wang JF, Stall RE, Vallejos CE (1994) Genetic analysis of a complex hypersensitive reaction to bacterial spot in tomato. Phytopathology 84: 126-132. doi:10.1094/Phyto-84-126

Wang M, Jiang N, Jia T, Leach L, Cockram J, Waugh R, Ramsay L, Thomas B, Luo Z (2012) Genome-wide association mapping of agronomic and morphologic traits in highly structured populations of barley cultivars. Theoretical and Applied Genetics 124: 233-246. doi:10.1007/s00122-011-1697-2

Wang S, Wong D, Forrest K, Allen A, Chao S, Huang BE, Maccaferri M, Salvi S, Milner SG, Cattivelli L, Mastrangelo AM, Whan A, Stephen S, Barker G, Wieseke R, Plieske J, International Wheat Genome Sequencing Consortium, Lillemo M, Mather D, Appels R, Dolferus R, Brown-Guedira G, Korol A, Akhunova AR, Feuillet C, Salse J, Morgante M, Pozniak C, Luo MC, Dvorak J, Morell M, Dubcovsky J, Ganal M, Tuberosa R, Lawley C, Mikoulitch I, Cavanagh C, Edwards KJ, Hayden M, Akhunov E (2014) Characterization of polyploid wheat genomic diversity using a high-density 90 000 single nucleotide polymorphism array. Plant Biotechnology Journal 12: 787-796. doi:10.1111/pbi.12183

Wang Z, Taramino G, Yang D, Liu G, Tingey SV, Miao GH, Wang GL (2001) Rice ESTs with disease-resistance gene- or defense-response gene-like sequences mapped to regions containing major resistance genes or QTLs. Molecular Genetics and Genomics 265: 302-310. doi:10.1007/s004380000415

Wanyera R, Kinyua MG, Jin Y, Singh RP (2006) The spread of stem rust caused by *Puccinia graminis* f. sp. *tritici*, with virulence on *Sr31* in wheat in Eastern Africa. Plant Disease 90: 113-113. doi:10.1094/PD-90-0113A

Weihs C, Ligges U, Luebke K, Raabe N (2005) klaR analyzing German business cycles. In: Baier D, Decker R, Schmidt-Thieme L (eds) Data Analysis and Decision Support. Springer-Verlag, Berlin, pp 335-343. doi:10.1007/3-540-28397-8

Wen Z, Boyse JF, Song Q, Cregan PB, Wang D (2015) Genomic consequences of selection and genome-wide association mapping in soybean. BMC Genomics 16, Article 671: 1-14. doi:10.1186/s12864-015-1872-y

Wheeler GS (2006) Chemotype variation of the weed *Melaleuca quinquenervia* influences the biomass and fecundity of the biological control agent *Oxyops vitiosa*. Biological Control 36: 121-128. doi:10.1016/j.biocontrol.2005.10.005

Wheeler GS, Ordung KM (2005) Secondary metabolite variation affects the oviposition preference but has little effect on the performance of *Boreioglycaspis melaleucae*: a biological control agent of *Melaleuca quinquenervia*. Biological Control 35: 115-123. doi:10.1016/j.biocontrol.2005.07.006

Whiteley SB (1999) Calculating the sustainable yield of Tasmania's State forests. Tasforests 11: 23-34

Whitham TG, Slobodchikoff CN (1981) Evolution by individuals, plant-herbivore interactions, and mosaics of genetic variability: the adaptive significance of somatic mutations in plants. Oecologia 49: 287-292. doi:10.1007/BF00347587

Wiggins NL, Marsh KJ, Wallis IR, Foley WJ, McArthur C (2006) Sideroxylonal in *Eucalyptus* foliage influences foraging behaviour of an arboreal folivore. Oecologia 147: 272-279. doi:10.1007/s00442-005-0268-0

Wilkinson GR (2008) Population differentiation within *Eucalyptus obliqua*: implications for regeneration success and genetic conservation in production forests. Australian Forestry 71: 4-15. doi:10.1080/00049158.2008.10676266

Williams KJ, Potts BM (1996) The natural distribution of *Eucalyptus* species in Tasmania. Tasforests 8: 39-165

Wilson PG, O'Brien MM, Heslewood MM, Quinn CJ (2005) Relationships within Myrtaceae sensu lato based on a *mat*K phylogeny. Plant Systematics and Evolution 251: 3-19. doi:10.1007/s00606-004-0162-y

Wingen LU, Shaw MW, Brown JKM (2013) Long-distance dispersal and its influence on adaptation to host resistance in a heterogeneous landscape. Plant Pathology 62: 9-20. doi:10.1111/j.1365-3059.2012.02621.x

Wingfield MJ, Slippers B, Roux J, Wingfield BD (2001) Worldwide movement of exotic forest fungi, especially in the tropics and the Southern Hemisphere. Bioscience 51: 134-140. doi:10.1641/0006-3568(2001)051[0134:WMOEFF]2.0.CO;2

Winter G (1884) Repertorium. Rabenhorstii fungi europaei et extraeuropaei exsiccati cura Dr. G. Winter, Centuria XXXI et XXXII. Hedwigia 23: 164-172

Wise ML, Savage TJ, Katahira E, Croteau R (1998) Monoterpene synthases from common sage (*Salvia officinalis*) - cDNA isolation, characterization, and functional expression of (+)-sabinene synthase, 1,8-cineole synthase, and (+)-bornyl diphosphate synthase. Journal of Biological Chemistry 273: 14891-14899. doi:10.1074/jbc.273.24.14891

Wolff JLC, McDowell SW, Machado MA (1993) Analysis of the restriction fragment length polymorphism of *Eucalyptus urophylla*, *E. grandis* and *E. citriodora*. Brazilian Journal of Genetics 16: 431-439

Xavier AA, Alfenas AC, Matsuoka K, Hodges CS (2001) Infection of resistant and susceptible *Eucalyptus grandis* genotypes by urediniospores of *Puccinia psidii*. Australasian Plant Pathology 30: 277-281. doi:10.1071/AP01038

Xavier AA, da Silva AC, Guimarães LMD, Matsuoka K, Hodges CS, Alfenas AC (2015) Infection process of *Puccinia psidii* in *Eucalyptus grandis* leaves of different ages. Tropical Plant Pathology 40: 318-325. doi:10.1007/s40858-015-0043-7

Yamamoto H (1995) Pathogenesis and host-parasite specificity in rusts. In: Singh US, Singh RP, Kohmoto K (eds) Pathogenesis and host specificity in plant diseases: histopathological, biochemical, genetic, and molecular bases, vol 2: Eukaryotes. Oxford, Pergamon, pp 203-215

Yang S, Feng Z, Zhang X, Jiang K, Jin X, Hang Y, Chen JQ, Tian D (2006) Genome-wide investigation on the genetic variations of rice disease resistance genes. Plant Molecular Biology 62: 181-193. doi:10.1007/s11103-006-9012-3

Yang S, Zhang X, Yue JX, Tian D, Chen JQ (2008) Recent duplications dominate NBS-encoding gene expansion in two woody species. Molecular Genetics and Genomics 280: 187-198. doi:10.1007/s00438-008-0355-0

Yeoh SH, Bell JC, Foley WJ, Wallis IR, Moran GF (2012) Estimating population boundaries using regional and local-scale spatial genetic structure: an example in *Eucalyptus globulus*. Tree Genetics & Genomes 8: 695-708. doi:10.1007/s11295-011-0457-4

Yu J, Pressoir G, Briggs WH, Bi IV, Yamasaki M, Doebley JF, McMullen MD, Gaut BS, Nielsen DM, Holland JB, Kresovich S, Buckler ES (2006) A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. Nature Genetics 38: 203-208. doi:10.1038/ng1702

Zambino PJ, Nolan PA (2011) First report of rust caused by *Puccinia psidii* on paperbark, *Melaleuca quinquenervia*, in California. Plant Disease 95: 1314-1314. doi:10.1094/PDIS-05-11-0436

Zang D, Wang H, You Y (1995) Performance and selection of a 4-year *Eucalyptus globulus* seedling seed orchard in Yunnan, China. In: Potts BM, Borralho NMG, Reid JB, Cromer RN, Tibbits WN, Raymond CA (eds) Eucalypt Plantations: Improving Fibre Yield and

Quality. Proceedings of the CRCTHF-IUFRO Conference, CRC for Temperate Hardwood Forestry, Hobart, pp 226-229

Zauza EAV, Alfenas AC, Old K, Couto MMF, Graca RN, Maffia LA (2010) Myrtaceae species resistance to rust caused by *Puccinia psidii*. Australasian Plant Pathology 39: 406-411. doi:10.1071/AP10077

Zhang M, Wu YH, Lee MK, Liu YH, Rong Y, Santos TS, Wu C, Xie F, Nelson RL, Zhang HB (2010) Numbers of genes in the NBS and RLK families vary by more than four-fold within a plant species and are regulated by multiple factors. Nucleic Acids Research 38: 6513-6525. doi:10.1093/nar/gkq524

Zhang X, Feng Y, Cheng H, Tian D, Yang S, Chen JQ (2011) Relative evolutionary rates of NBS-encoding genes revealed by soybean segmental duplication. Molecular Genetics and Genomics 285: 79-90. doi:10.1007/s00438-010-0587-7

Zhao K, Tung CW, Eizenga GC, Wright MH, Ali ML, Price AH, Norton GJ, Islam MR, Reynolds A, Mezey J, McClung AM, Bustamante CD, McCouch SR (2011) Genome-wide association mapping reveals a rich genetic architecture of complex traits in *Oryza sativa*. Nature Communications 2, Article 467: 1-10. doi:10.1038/ncomms1467

Zhou C, He X, Li F, Weng Q, Yu X, Wang Y, Li M, Shi J, Gan S (2014) Development of 240 novel EST-SSRs in *Eucalyptus* L'Herit. Molecular Breeding 33: 221-225. doi:10.1007/s11032-013-9923-z

Zhou T, Wang Y, Chen JQ, Araki H, Jing Z, Jiang K, Shen J, Tian D (2004) Genome-wide identification of NBS genes in *japonica* rice reveals significant expansion of divergent non-TIR NBS-LRR genes. Molecular Genetics and Genomics 271: 402-415. doi:10.1007/s00438-004-0990-z

Zhu C, Gore M, Buckler ES, Yu J (2008) Status and prospects of association mapping in plants. Plant Genome 1: 5-20. doi:10.3835/plantgenome2008.02.0089

Zhuang JY, Wei SX (2011) Additional materials for the rust flora of Hainan Province, China. Mycosystema 30: 853-860

Ziegler J, Facchini PJ (2008) Alkaloid biosynthesis: metabolism and trafficking. Annual Review of Plant Biology 59: 735-769. doi:10.1146/annurev.arplant.59.032607.092730

Zobel B, Talbert J (1984) Applied Forest Tree Improvement. John Wiley & Sons, New York