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Investigating the cause of dieback in the invasive plant, *Parkinsonia aculeata*

BY

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“Watch with glittering eyes the whole world around you because the greatest secrets are always hidden in the most unlikely places. Those who don't believe in magic will never find it”

-- Roald Dahl

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Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full, or in part, for a degree at this or any other institution.

Signed: ___  ___

Tracey Vivien Steinrucken

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TABLE OF CONTENTS

List of Figures v
List of Tables vii
Abbreviations viii
Thesis Abstract ix

CHAPTER 1: Microbe-plant interactions, their influence on invasion ecology, and the use of dieback in the biocontrol of invasive plants: a review 1
1.1 Introduction 1
1.2 Plant-microbe interactions 4
1.3 The roles of endophyte and other microorganisms in plant invasion 6
1.4 Plant dieback 8
1.4.1 Pathogen-related dieback of trees 10
1.5 Dieback in Weeds of National Significance (WONS) 13
1.5.1 *Parkinsonia aculeata* 14
1.5.2 The potential for using dieback in the biological control of parkinsonia 22
1.6 Summary 23
1.7 Thesis outline 24
1.8 Publications 26

CHAPTER 2: Endophyte community composition is associated with dieback occurrence in an invasive tree 30
2.1 Abstract 30
2.2 Introduction 31
2.3 Materials and methods 36
2.3.1 Sampling and sample preparation 36
2.3.2 Nucleic acid analysis 40
2.3.3 Statistical data analysis 41
2.4 Results 42
2.4.1 Sampling effort 42
2.4.2 Plant part and disease status effects 42
2.4.3 Indicator species analysis 46
2.5 Discussion 46
2.6 Conclusion 51

CHAPTER 3: Triggering dieback in an invasive plant: endophyte diversity and pathogenicity 52
3.1 Abstract 52
3.2 Introduction 53
3.3 Materials and method 57
3.3.1 Sampling, identification and analysis of the fungal endophyte community 57

3.3.2 Glass house pathogenicity trial 59
3.4 Results 64
3.4.1 Fungal endophytes in healthy and dieback-affected *Parkinsonia aculeata* 64
3.4.2 Pathogenicity testing of isolates with a water stress interaction 69
3.5 Discussion 73

CHAPTER 4: First report of oomycetes associated with the invasive tree *Parkinsonia aculeata* (family: Fabaceae) 78
4.1 Abstract 78
4.2 Introduction 79
4.3 Materials and Methods 82
4.3.1 Field survey and sampling 82
4.3.2 Isolation of *Phytophthora* species 85
4.3.3 DNA isolation, PCR and sequencing of isolated species 86
4.4 Results 87
4.5 Discussion 93

CHAPTER 5: Host and range specificity of *Parkinsonia aculeata* endophyte communities 97
5.1 Abstract 97
5.2 Introduction 99
5.3 Materials and Methods 101
5.3.1 Sampling 101
5.3.2 DNA extraction 102
5.3.3 PCR amplification and sequencing 104
5.3.4 Amplicon sequence analysis 106
5.3.5 Data analysis 107
5.4 Results 108
5.4.1 Fungal and bacterial endophyte sequence diversity 108
5.4.2 Host species affected fungal endophyte community composition 109
5.4.3 Host species was not observed to affect bacterial endophyte community composition 111
5.4.4 Fungal endophyte community composition in *P. aculeata* differed by host location .. 111
5.4.5 Bacterial endophyte community composition in *P. aculeata* differed by host range .. 112
5.5 Discussion 115
5.5.1 *Parkinsonia* fungal endophyte communities are driven by host species 115
5.5.2 *Parkinsonia* endophyte community composition by host range 117
5.6 Conclusion 122

CHAPTER 6: Combining culture-independent and culture-based methods to investigate fungal and bacterial endophyte communities associated with dieback occurence in *Parkinsonia aculeata* 123
6.1 Abstract 123

6.2 Introduction 124
6.3 Materials and Methods 127
6.3.1 Sampling 127
6.3.2 DNA extraction 129
6.3.3 PCR amplification and sequencing 129
6.3.4 Amplicon sequence analysis 130
6.3.5 Data analysis 131
6.4 Results 132
6.4.1 Fungal and bacterial endophyte sequence diversity 132
6.4.2 Dieback occurrence affected fungal endophyte community composition 134
6.4.3 Dieback occurrence was not shown to affect bacterial community composition 139
6.4.4 Fungal community composition varies when using culture-independent vs. culture-based methods 143
6.5 Discussion 145
6.5.1 Dieback occurrence is associated with fungal community composition 145
6.5.2 Dieback occurrence was not shown to be associated with bacterial community composition 150
6.5.3 Comparison to previous work on parkinsonia dieback: T-RFLP 151
6.5.4 Comparison to previous work on parkinsonia dieback: isolations and pathogenicity screening 152
6.6 Conclusion 153

CHAPTER 7: Synthesis and conclusions 155
7.1 Summary of the results in the context of the key research questions 155
7.2 Fungal endophyte community composition and parkinsonia dieback 156
7.3 Pathogenicity screening of putative fungal pathogens for dieback symptoms in parkinsonia 157
7.3.1 Potential workflow for diagnosing plant disease associated with fungal endophyte communities 158
7.4 Other microbial communities associated with parkinsonia 160
7.4.1 Archaea 160
7.4.2 Bacteria 160
7.4.3 Oomycetes 161
7.5 Conclusion 162

APPENDIX A: Supplementary material by chapter 164
Chapter 2 Supplementary Material 164
Chapter 3 Supplementary Material 165
Chapter 4 Supplementary Material 166
Chapter 5 Supplementary Material 169
Chapter 6 Supplementary Material 175

APPENDIX B: Details of electronic supplementary material 179
APPENDIX C: Detailed protocol for multiplex dual-index Illumina sequencing of endophyte DNA 180
REFERENCES 186

List of Figures

Figure 1.1 Endophytes convey multiple benefits to their host to improve health, increase growth and protect against stress and disease occurrence 5

Figure 1.2 The Tree Decline Spiral illustrating how potential predisposing, inciting and contributing factors result in decline 9

Figure 1.3 *Parkinsonia aculeata* 15

Figure 1.4 Potential distribution of *Parkinsonia aculeata* in the event of no management biodiversity 17

Figure 1.5 Symptoms of dieback in *Parkinsonia aculeata* 19

Figure 1.6 Thesis outline flowchart showing progression from each chapter to the next, including summary of methods used, the purpose of each study and which research question is addressed. 25

Figure 2.1 *Parkinsonia aculeata* and native tree sampling sites for this study 37

Figure 2.2 Healthyand dieback-affected *Parkinsonia aculeata* trees, stem of a dieback-affected tree and stem vascular staining of a dieback-affected tree 38

Figure 2.3 OTU accumulation curves for archaea, bacteria and fungi by disease status of host 43

Figure 2.4 Unconstrained principle coordinates ordination (PCO) analyses showing separation along the axes by *Parkinsonia aculeata* disease status and/or plant part for archaeal, bacterial and fungal OTU community data 45

Figure 3.1 Neighbour-joining tree on a 458 bp length MUSCLE alignment of ITS1-ITS4 sequences from representative endophytic fungal taxa including the number of those isolates isolated from each plant tissue type. 60

Figure 3.2 Fungal community overview showing the proportion of taxonomic orders represented, the proportion of isolates isolated by tissue type and host disease status, and the percentage of isolates found in healthy, dieback-affected *Parkinsonia aculeata*, or both. 68

Figure 3.3 Average underbark lesion length on one-year-old *Parkinsonia aculeata* seedlings by isolate and water treatment, at the conclusion of this 10 week glasshouse pathogenicity trial 70

Figure 3.4 The effects of water treatment on the average change in *Parkinsonia aculeata* plant height and stem circumference from the start to the end of the 10 week pathogenicity trial, and average post-harvest dry mass of roots, stems and foliage at the conclusion of the trial 72

Figure 3.5 Mite damage to *Parkinsonia aculeata* plants by water treatment at the conclusion of this glasshouse pathogenicity trial 72

Figure 4.1 Maps of Kununurra, Western Australia and central-east Queensland showing locations of *Parkinsonia aculeata* sampling sites, and proportion of oomycete isolate genotypes identified in this study 83

Figure 4.2 Total monthly rainfall (2014) and average monthly rainfall (1992-2016) for Charters Towers QLD and Kununurra WA from the Bureau of Meteorology 84

Figure 4.3 Symptoms of *Parkinsonia aculeata* dieback in Kununurra WA in May 2014 and in Charters Towers QLD in October 2014 89

Figure 4.4 Jukes-Cantor UPGMA tree derived from an alignment (981 bp) of partial sequences of the internal transcribed spacer (ITS) region of isolates classified as oomycetes from *Parkinsonia aculeata* roots and soil in this study 90

Figure 5.1 Satellite images of *Parkinsonia aculeata* sampling sites used in this study, showing sites in Australia and the USA, and sites where samples from all three *Parkinsonia* spp. (*P. aculeata*, *P. florida* and *P. microphylla*) were collected 103

Figure 5.2 A schematic diagram illustrating the multiplex dual-indexed PCR approach used in this study, with the fungal ITS1 amplicon-specific primers in this example 105

Figure 5.3 Fungal and bacterial OTU accumulation curves comparing leaf, stem and stem tip endophyte communities hosted by healthy trees from three *Parkinsonia* species (*P. aculeata*, *P. florida* and *P. microphylla*) sampled in Arizona (AZ) USA; and stem and stem tip endophyte communities hosted by healthy trees sampled from native range (USA) *P. aculeata* in AZ and TX to healthy trees sampled from invasive range (Australia) *P. aculeata* in QLD, NT and WA 110

Figure 5.4. Fungal endophyte taxa that differ significantly ($q < 0.05$) in abundance between *Parkinsonia aculeata* in the invasive Australian range and native USA range. 114

Figure 5.5 Bacterial endophyte taxa that differ significantly ($q < 0.05$) in relative abundance between *Parkinsonia aculeata* in the native range and invasive range 114

Figure 6.1 Fungal and bacterial OTU accumulation curves comparing leaf, stem and stem tip endophyte communities hosted by dieback-affected and healthy *Parkinsonia aculeata* sampled near Charters Towers, QLD 133

Figure 6.2 Principle coordinate ordination plots of *Parkinsonia aculeata* fungal and bacterial endophyte community data showing separation of samples by disease status and plant part. 135

Figure 6.3 The number of Illumina sequencing reads by ordinal group recovered from fungal and bacterial endophyte communities from healthy and dieback-affected *Parkinsonia aculeata*. 136

Figure 6.4 Fungal endophyte genera that differ significantly ($p < 0.05$) in relative abundance between dieback-affected and healthy *Parkinsonia aculeata* 138

Figure 6.5 OTU accumulation curves for fungal ITS sequencing in this study, T-RFLP analysis in Chapter 2, and fungal culture isolations from Chapter 3 showing endophyte communities hosted by dieback-affected and healthy *Parkinsonia aculeata* sampled near Charters Towers, QLD (Australia). 144

Figure 7.1 Diversity of higher and lower fungal taxa derived from NGS sequencing using ITS6 and ITS7 primers 163

List of Tables

Table 2.1 PCR primers used in this study 40

Table 2.2 PERMANOVA of the *Parkinsonia aculeata* OTU community for the main effects of disease status and plant part and the interaction between these effects 45

Table 2.3 *t*-statistics from pairwise PERMANOVA comparisons testing for differences in *Parkinsonia aculeata* endophyte OTU composition in healthy compared to dieback-affected plant parts 45

Table 2.4 The number of indicator OTUs that were significantly associated with healthy or dieback-affected *Parkinsonia aculeata*, grouped by plant part. 46

Table 3.1 Fungal species information for identified isolates used in the pathogenicity trial 62

Table 3.2 Endophytes isolated in this study and identified by ITS sequencing to closest match in the NCBI nucleotide database 65

Table 3.3 ANOVA testing the effects of water treatment and inoculated isolate on lesion length and three measures of *Parkinsonia aculeata* plant health 70

Table 3.4 Tukey Q statistics for post-hoc pairwise analysis of the effect of inoculated isolate on underbark lesion length in *Parkinsonia aculeata* 70

Table 3.5 Tukey Q statistics (where $P < 0.05$) for post-hoc pairwise analysis of the effect of water treatment on three measurements of plant health at the end of the 10 week *Parkinsonia aculeata* inoculation trial 72

Table 4.1 Primers used in this study 87

Table 4.2 Isolates classified as oomycetes from soil and roots of *Parkinsonia aculeata*, identified to closest match on the NCBI database and by phylogenetic analysis using the ITS backbone from Robideau et al. (2011) 91

Table 4.3 The number of oomycetes recovered and identified in this study from dieback-affected or healthy *Parkinsonia aculeata* in Queensland and Western Australia. 92

Table 5.1 Primers used in the first round of PCR in this study for fungal ITS and bacterial 16s amplification ... 105

Table 5.2 Comparisons between fungal communities hosted by *Parkinsonia* spp. from Arizona USA, by plant part, host species and sampling site, as calculated by PERMANOVA 110

Table 5.3 Three pairwise PERMANOVA tests of significant variation effects in fungal endophyte communities hosted by *Parkinsonia* spp. from Arizona USA, by host species, plant part and sampling site 110

Table 5.4 Pairwise PERMANOVA showing variation in fungal endophyte community by geographic region (state) and the average similarity of these communities between each state/group 111

Table 6.1 PERMANOVAs of the *Parkinsonia aculeata* OTU community, testing the effects of disease status and plant part on fungal and bacterial endophyte community composition 135

Table 6.2 Summary of significant associations of fungal genera with healthy or dieback-affected *Parkinsonia aculeata* using results from SIMPER, extended error bars, isolations, and the ecological classification of each genus as determined by a search of the literature. 140

Table 6.3 Comparison of PERMANOVA results from data obtained via Illumina sequencing and T-RFLP 152

Abbreviations

ANOVA	Analysis of variance
AZ	Arizona, USA
CSIRO	Commonwealth Scientific and Industrial Research Institute
CTQ	Charters Towers, Queensland
DAFF	Department of Agriculture, Forestry and Fisheries
dNTPs	Deoxynucleotide triphosphates
ITS	Internal transcribed spacer (region)
KWA	Kununurra, Western Australia
NaClO	Sodium hypochlorite (bleach)
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
NT	Northern Territory, Australia
OTU	Operational taxonomic unit
PCO	Principle coordinates ordination
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
sPDA	Potato dextrose agar amended with streptomycin
PERMANOVA	Permutational analysis of variance
QLD	Queensland, Australia
SIMPER	Similarity percentages (analysis)
T-RFLP	Terminal restriction fragment length polymorphism
TX	Texas, USA
UV	Ultra violet (light)
WA	Western Australia, Australia
WONS	Weed of National Significance

THESIS ABSTRACT

Invasive plants cost Australia, directly and indirectly, around AU$4 billion pa; displacing native species, changing sensitive ecosystems and sometimes affecting human health and safety. Developing novel tools to control invasive species will benefit landholders and the environment, not just in Australia, but globally. Biocontrol of invasive plants via dieback causative agents is one such potential tool. Dieback causes a progressive reduction in plant population health, resulting in the death of plant parts and often complete plant death. It is prevalent in many invasive woody weeds in Australia and has been suggested as a potential mechanism for their biocontrol, particularly because local native plants appear unaffected.

Parkinsonia aculeata L. (Fabaceae; referred to hereafter as "parkinsonia") is an invasive tree in northern Australia, with native populations in South and Central America and southern USA. It is a perennial thorny shrub that forms dense thickets along waterways, floodplains and throughout paddocks, seriously impacting the pastoral industry, local biodiversity, and providing shelter to other invasive species such as feral pigs. Some Australian parkinsonia populations are affected by dieback, resulting in localised control. Despite previous and ongoing research, the cause of parkinsonia dieback remains elusive and dieback has not been observed in parkinsonia's native range. This thesis investigates the potential cause(s) of dieback in parkinsonia to contribute towards research on determining its suitability as a biological control tool. My goals were to describe the microbial endophytes of parkinsonia, identify correlations of microbial community composition and dieback occurrence, and identify patterns and pathogens that might be involved in dieback.

First, I analysed the community composition of archaeal, bacterial and fungal endophytes from the roots, stems and stem tips of healthy and dieback-affected parkinsonia. Samples were taken from Charters Towers in Queensland (QLD), Australia in May 2013. I used terminal restriction fragment length polymorphism (T-RFLP) analysis with taxon-specific primers for archaea, bacteria and fungi, followed by statistical analysis to determine how endophyte community composition relates to plant part and disease status. Archaeal and fungal community structures were significantly correlated with dieback occurrence and plant part. Bacterial community composition showed significant correlation to dieback occurrence but not plant part. The results showed that endophyte community composition in parkinsonia is associated with the occurrence of dieback and that endophyte communities vary across plants parts. I hypothesised that dieback occurrence may be due to the lack of potentially protective endophytes or the presence of putative pathogens.

As a complimentary study to the T-RFLP analysis, I used the same samples collected in QLD to characterise the culturable fungal endophyte communities in healthy and dieback-affected parkinsonia. I identified 219 isolates via amplicon sequencing of the internal transcribed spacer (ITS) to reveal a library of 54 unique species from 25 families. Eight isolates, identified as putative pathogens, were selected for a 10-week pathogenicity trial, including water stress treatments, on parkinsonia seedlings to determine whether inoculations of parkinsonia with these isolates would result in dieback-like symptoms, and whether stress due to drought or inundation enhanced these responses. Of the eight putative pathogenic isolates tested in the pathogenicity trial, inoculation with *Lasiodiplodia pseudotheobromae*, *Botryosphaeria dothidea* and *Pestalotiopsis mangiferae* resulted in the largest lesions, but systemic infection or dieback-like symptoms were not observed, despite

significant reductions in plant health due to water stress. As systemic infection or dieback symptoms were not observed, I determined that these pathogens are either not involved in parkinsonia dieback, that different or more extreme abiotic or biotic stress levels are required to trigger dieback-like symptoms, or that changes to the inoculation method are needed. Combining these factors will be essential in evaluating which factors are most important in initiating dieback in parkinsonia.

Next, I conducted the first survey of *Phytophthora* and other oomycetes associated with parkinsonia dieback. Despite years of parkinsonia dieback research and the involvement of *Phytophthora* in dieback symptoms for other tree species, these soil-borne pathogens had not been previously studied in the parkinsonia dieback system. Using zoospore baiting, I recovered multiple isolates from roots and soil of healthy and dieback-affected parkinsonia sampled from Kununurra, WA in May 2014 and Charters Towers, QLD in October 2014. Using molecular taxonomy of the ITS region, I identified 37 unique oomycetes, predominantly composed of *Phytophthora palmivora*, *P. nicotianae* and *Phytopythium vexans*. There were fewer oomycete isolates recovered from soil and roots in drought-affected Charters Towers than Kununurra, which had experienced recent rainfall. Oomycetes require soil moisture for the dispersal of zoospores, and parkinsonia dieback occurs across multiple climatic zones, including those experiencing severe drought. None of the species identified were consistently isolated from dieback-affected trees suggesting that any association with parkinsonia dieback may be coincidental or localised, or that they were being detected before dieback symptoms had become apparent. More extensive surveys and pathogenicity screenings of isolated oomycetes are required to evaluate their suitability as potential biocontrol agents.

Endophytes accompanying invasive plants upon introduction to a new range are often important for their successful establishment, their ability to outcompete native plants, and for resistance against newly encountered pathogens or stress factors. To investigate why parkinsonia dieback has not been observed in the native range, I described and compared the endophytic microbial communities of parkinsonia from the native and introduced ranges. Samples from seven sites in QLD, WA and Northern Territory (NT), Australia were collected in May and June 2015, and DNA was extracted from them in Brisbane. While based at the University of California Berkeley, I sampled and extracted endophyte DNA from native-range parkinsonia in southern USA (Arizona and Texas) in October and November 2015. I also sampled from two closely-related *Parkinsonia* species (*P. florida* and *P. microphylla*), which co-occurred with *P. aculeata* at three sites in Arizona. Using multiplex Illumina sequencing, I characterised fungal and bacterial endophyte community compositions and found significant variation in fungal community composition by host species, by plant part and when comparing *P. aculeata* from the invasive vs. native ranges. There were no site, plant part or host species effects for bacterial endophytes, but there was a significant range effect when comparing invasive vs. native *P. aculeata*. The results suggested that *Parkinsonia* spp. fungal endophyte communities are more strongly affected by host species than geographic location within the native range, and that endophyte communities in invasive and native range *P. aculeata* populations are significantly different. Identification of the dominant endophytes in invasive *P. aculeata* did not support the hypothesis that a unique community accompanying invasive *P. aculeata* to Australia might have aided in its establishment and spread, it is perhaps more likely that there is simply a different pool of species in the metacommunity associated with each range. The methodology from this study could

be used for developing mechanisms to screen endophytic pathogens as potential biological control agents, if followed up by isolations and pathogenicity testing.

Finally, for Chapter 6, I used the same culture-independent (Illumina sequencing) technique as in Chapter 5 to characterise the fungal and bacterial endophyte communities of healthy and dieback-affected parkinsonia from the invasive Australian population in Charters Towers, QLD. The composition of fungal endophyte communities had been correlated with parkinsonia dieback occurrence in Chapter 2, however putative dieback-causing pathogens were not identified. As in the T-RFLP study (Chapter 2), fungal and bacterial communities from roots, stems and stem tips were analysed for the effects of health status. Health status was shown to significantly correspond with fungal community composition, as in the T-RFLP study, but not with bacterial community composition. The sequencing enabled the identification of taxa most likely to be associated with healthy parkinsonia as potentially protective endophytes, or as most likely to be associated with dieback occurrence as pathogens. No fungal or bacterial taxa were identified as likely to confer pathogen resistance to parkinsonia as determined by their assigned ecological guild or relevant publications. *Fusarium*, *Neurospora*, *Cladosporium*, *Phoma*, *Curvularia* and *Acremonium* were identified as potentially pathogenic fungal genera typical of dieback-affected parkinsonia. When comparing the results to the culture-based study in Chapter 3, I found that *Fusarium*, *Phoma* and *Neurospora* were the most likely to be involved in dieback occurrence, and are good candidates for future pathogenicity screening following targeted isolations. This study showed the usefulness of combining culture-based and culture-independent methodology in the diagnosis of endophyte-associated plant disease, but emphasises the need for ecological testing, regardless of guild classifications due to assigned taxonomy.

In conclusion, my PhD research was not able to identify the cause(s) of parkinsonia dieback. The results suggest that a lot of work remains in order to get to the point where it is viable to test a dieback-causing pathogen(s) against parkinsonia (and ensure that it is host specific), if indeed one exists. The use of traditional culture-based techniques are essential for testing putative pathogens, but high-throughput next-generation sequencing techniques such as Illumina, and the resulting analyses are potentially a better starting point when not much is known about a dieback system. This could be followed by isolating endophytes using a variety of media and isolation techniques in order to maximise isolate species diversity. Next it would be prudent to test putative pathogens shown to be significantly associated with the dieback-affected host, potentially alongside stress-factors such as drought, low soil quality or heat. A potential workflow for such a study is detailed in Chapter 7.

Determining the cause of dieback in affected weeds may present land managers with a ‘silver bullet’ of biological control that could become a self-managed, perpetual instrument, reducing weed management costs and increasing biodiversity and land productivity. As such, future work in the use of dieback and host-specific phytopathogens for biological control of invasive plants should continue.

1

MICROBE-PLANT INTERACTIONS, THEIR INFLUENCE ON INVASION ECOLOGY, AND THE USE OF DIEBACK IN THE BIOCONTROL OF INVASIVE PLANTS: A REVIEW

1.1 Introduction

Throughout the primary literature, there are multiple examples of research on economically or environmentally important invasive plants. Darwin himself (1859) explored theories on invasive plants and animals in "The Origin of Species". This was followed by Elton (1958) with the book "The ecology of invasions by animals and plants", and since then multiple theories have attempted to explain weed invasion success, some of which are summarised and grouped in reviews such as those by Catford et al. (2009) or Barney and Whitlow (2007), among others, which discuss theories that may promote or hinder the success of an invasive plant in a new range. For example, 'novel weapons' theory highlights incidences where invasive plants or co-introduced microorganisms release allelopathic compounds that adversely affect indigenous plants (Callaway & Ridenour 2004). Case studies and particular invasion events, such as those involving novel weapons, are often recorded, and resources are committed into preventing exotic plant introduction opportunities, but it is difficult and perhaps impossible to discover why certain plants do not become invasive, how to prevent invasion by weeds, or why certain habitats remain free of invasive plants.

This is primarily because, by nature, these studies usually only begin after a successful invasion event, once stakeholders become affected.

Other theories relating to the inability of some invasive species to establish in the invaded range include 'new associations', which refers to incidences where an invasive species encounters a pathogen or pest in the new range, which it had not previously faced, and therefore is more likely to be negatively impacted (Colautti et al. 2004). Alternatively, 'missed mutualisms' occur when an invasive species leaves behind co-evolved beneficial or protective microorganisms in the native range and therefore is not as competitive or adaptable in the invasive range, which can also lead to increased susceptibility to disease (Mitchell et al. 2006). The latter two invasion ecology theories (new associations and missed mutualisms) are developed further in the extensive work by Evans (2008) on the endophyte-enemy release hypothesis. This occurs when invasive plants leave behind their natural co-evolved enemies (enemy release), but bring along their co-evolved endophytes, so they have a double advantage over local plants in the invaded range (Evans 2008). They may then form associations with local endophytes (positive new associations), further increasing their competitiveness and preventing successful control. However, if an invasive plant species leaves its endophytes behind (missed mutualisms) and has no obvious enemies in the new environment, it may also have an advantage because it no longer needs to allocate resources to its mutualists. In the latter case, this invasive species may become extremely vulnerable when a co-evolved enemy is subsequently introduced (the 'silver bullet' of ideal biological control; Evans 2008).

My research is a novel approach to invasion ecology since it investigates the natural and unexplained decline of a successful weed, *Parkinsonia aculeata* L. (Fabaceae;

hereafter referred to as parkinsonia), due to dieback. Dieback is the death of groups of adjacent trees in a population, most commonly attributed to a biotic agent (insects, fungi or bacteria), and initially diagnosed by loss of canopy cover, leading to reduced fitness and finally death of the trees (Mueller-Dombois 1987). The cause of dieback in plants has been most commonly attributed to pathogenic fungi (Agrios 2005); however, the mechanisms and epidemiology of infection are often unclear.

The benefit of dieback in weeds such as parkinsonia, is that the population is controlled to some extent, naturally. The motivation for researching parkinsonia dieback is that, should a putative, dieback-causing pathogen be identified, the agent responsible could be tested as a potential biological control agent for parkinsonia (Toh 2009; Diplock 2016). Similar studies on other dieback-affected weeds of national significance (WONS) have also explored this possibility, with focus on putative fungal pathogens as potential biological control agents (Aghighi et al. 2014; Haque 2015; Sacdalan 2015; Raghavendra et al. 2017).

Parkinsonia dieback is most likely to be the result of pathogenic microorganisms associated with the weed (Toh 2009; Diplock 2016), but may also involve a lack of protective/beneficial microorganisms (i.e. missed mutualisms). Hence, this review addresses the roles that endophytes and other plant-associated microorganisms play in general plant health, and how they might transform an introduced plant into a successful invader, or render them more susceptible to disease. I review both pathogenic and mutualistic (e.g. endophytes) plant-microbial interactions, and thereby give due consideration to factors that may have not been taken into account in prior studies on parkinsonia dieback (Toh 2009; Diplock 2016; Raghavendra et al. 2017). The aim of this thesis is to determine the cause of parkinsonia dieback, and how

dieback might be linked to parkinsonia invasiveness. Should a cause be identified, this work would subsequently contribute to investigations into whether dieback can be used as a biological control agent of parkinsonia. In this review I consider the roles endophytes play in plant health, previous research on parkinsonia dieback, and I present established methods of biological control using pathogens. Finally, I present the structure of this thesis and how each research chapter flows on to the next with the aim of addressing my research questions.

1.2 Plant-microbe interactions

Plant-associated microbial communities comprise multiple taxa including protists, fungi, bacteria and archaea (Lavelle & Spain 2001; Willey et al. 2008; Ma et al. 2013). Those that live within the host plant's tissue for all or part of their life cycle are known as endophytes (Ghimire & Hyde 2004) and are usually transmitted horizontally (Wilson 1996). Endophytes exhibit a continuum of ecological roles from parasitism to commensalism and mutualism (Saikkonen et al. 1998). Their specific roles (Fig 1.1) may include enhanced photosynthetic ability of infected tissue, thereby extension of tissue and host life; sharing and processing of nutrients and chemicals between host and microorganism; and defence of the host by the production of compounds that are unpalatable or poisonous to herbivores (Ghimire & Hyde 2004; Zhang et al. 2012). Many endophytes also cause disease in their host (Agrios 2005), and some persist as asymptomatic infections prior to being triggered into pathogenicity. These are called latent pathogens (Sinclair & Cerkauskas 1996).

The role of fungal and bacterial endophytes as mutualists or latent pathogens have previously been exploited in a number of ways: for biological control of plant diseases,

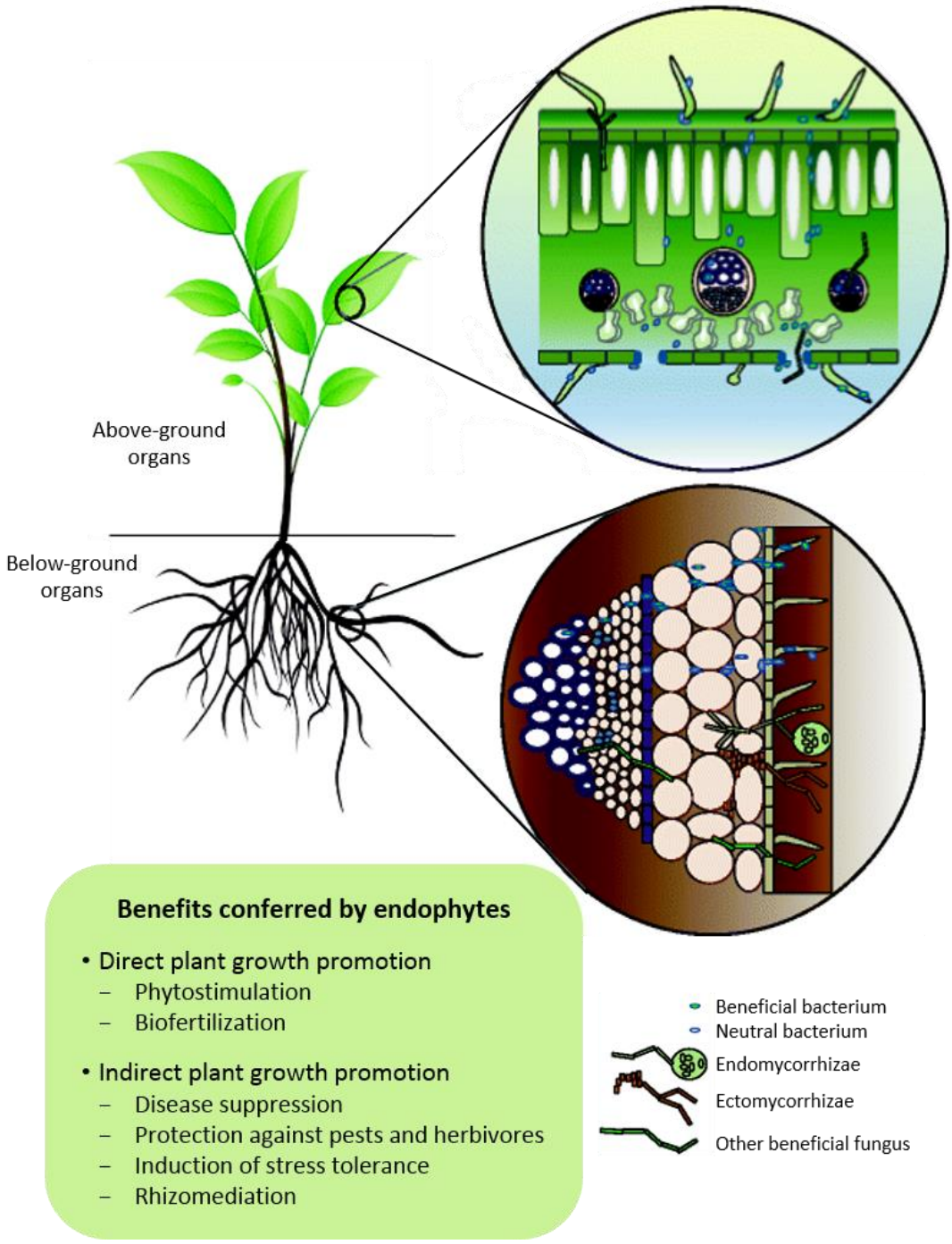


Figure 1.1 Endophytes convey multiple benefits to their host to improve health, increase growth and protect against stress and disease occurrence (Image from Compant et al. 2016)

to enhance plant physiology for agricultural benefit, as delivery methods for herbicides, and for environmental decontamination (Bacon & Hinton 2007). Both fungal and bacterial endophytes, therefore, have important roles to play in the defence of their host against pathogens or herbivores (Sinclair & Cerkauskas 1996), and since the absence of a mutualistic endophyte might mean the equivalent of an inferior immune system, endophytic communities should be considered when researching plant diseases including dieback.

1.3 The roles of endophyte and other microorganisms in plant invasion

Although abiotic conditions should not be discounted in the search for dieback causes, the focus of this review is on biotic conditions and microbial community interactions with invasive species that may give rise to disease and plant death. In order to establish the mechanisms by which endophytes influence invasive plants, it is important to address the potential ecological relationships between invasive plant hosts and their endophytes within the invaded and native range. Certain weed species have been shown to have superior invasion ability when colonised by co-evolved or newly-acquired plant-associated microorganisms (Evans 2008; Newcombe et al. 2009; Rudgers et al. 2010; Uchitel et al. 2011; Aschehoug et al. 2012; Kurose et al. 2012). For example, plant-soil feedback that enhances invasion may occur when an invasive plant is able to change the soil community (via exudates or co-introduction of microbes) to increase invasion pressure and decrease competition from native plants (Wolfe & Klironomos 2005; de la Peña et al. 2010). Facilitative or exclusionary interactions like these may be due to feedback with microbes already present in the

invaded habitat via host-jumping (Shipunov et al. 2008) or new associations (Colautti et al. 2004). These interactions may also arise from microbes that were introduced alongside the exotic plant as coevolved endophytes (Shipunov et al. 2008; Newcombe et al. 2009; Aschehoug et al. 2012; Xiao et al. 2012) or novel weapons, some of which are able to use allelopathic compounds to alter the microbial community and limit microbial interactions in neighbouring plants (Bennett 2013). The influence of these mechanisms on the endophytic or soil microbial community may vary during the stages of invasion and it is possible that not all invasive plants are subject to them (Wolfe & Klironomos 2005).

Hypotheses relating to biotic factors that inhibit successful invasion include increased susceptibility to generalist pathogens, possibly due to lack of genetic variability in the invasive plant (Colautti et al. 2004); new associations with previously encountered pathogens for which the invasive plant has no co-evolved defence mechanism (Callaway & Ridenour 2004; Colautti et al. 2004; Mitchell et al. 2006); or missed mutualism due to the loss of potentially 'protective' endophytes from the native range (Mitchell et al. 2006). The application of these hypotheses concerns the failure of an exotic plant to establish in a new habitat and form an invasive population, however, as Levine et al. (2004) argue, it is possible for an already established weed to experience an ecological response, like biotic resistance, which will limit further dispersal or devastate existing populations. Biotic resistance occurs when the local native community is able to kill-off or outcompete an invading plant and may be caused by other plants, microorganisms or herbivores at any time after the invasion event (Levine et al. 2004).

1.4 Plant dieback

Disease is common in plants in both natural and managed ecosystems, and many diseases are attributed to pathogenic fungi (McRae & Auld 2000). One such disease is plant dieback. I define dieback according to the definition of Mueller-Dombois (1987): the death of groups of adjacent trees in a population, attributed to a biotic agent (insects, fungi or bacteria), and initially diagnosed by loss of canopy cover, leading to reduced fitness and finally tree death.

A number of models have been designed to explain the high level of complexity involved in dieback. The first is the Tree Decline Spiral model by Manion (1991) which details several pre-disposing (e.g. genetic potential, fertility, climate change), inciting (e.g. defoliating insects, drought, frost) and contributing (e.g. pathogens, nematodes, wood-boring insects) factors which lead to eventual tree death (Fig 1.2). The Blackberry Decline Spiral (Aghighi et al. 2014) further developed the original framework by customising it to the context of blackberry decline in Western Australia. Wang et al. (2012) subsequently developed the Drought-Induced Tree Mortality model, which focused on abiotic factors starting with hydraulic failure, leading to carbon starvation and carbon mobilization failure. Most recently, the Tree Decline Recovery Seesaw model (Whyte et al. 2016) details how trees may be impacted by pathogens and/or insect pests, thereby starting a chain reaction involving attraction of opportunistic borers and the ‘activation’ of latent pathogens in the stressed host. From there, the authors (Whyte et al. 2016) propose it is possible for the host to recover if pathogens are disadvantaged by dry conditions, alternatively, the severely stressed tree dies and saprophytes develop on necrotic tissue. These models are helpful in detailing the complexity in tree dieback events, however without specific symptoms such as

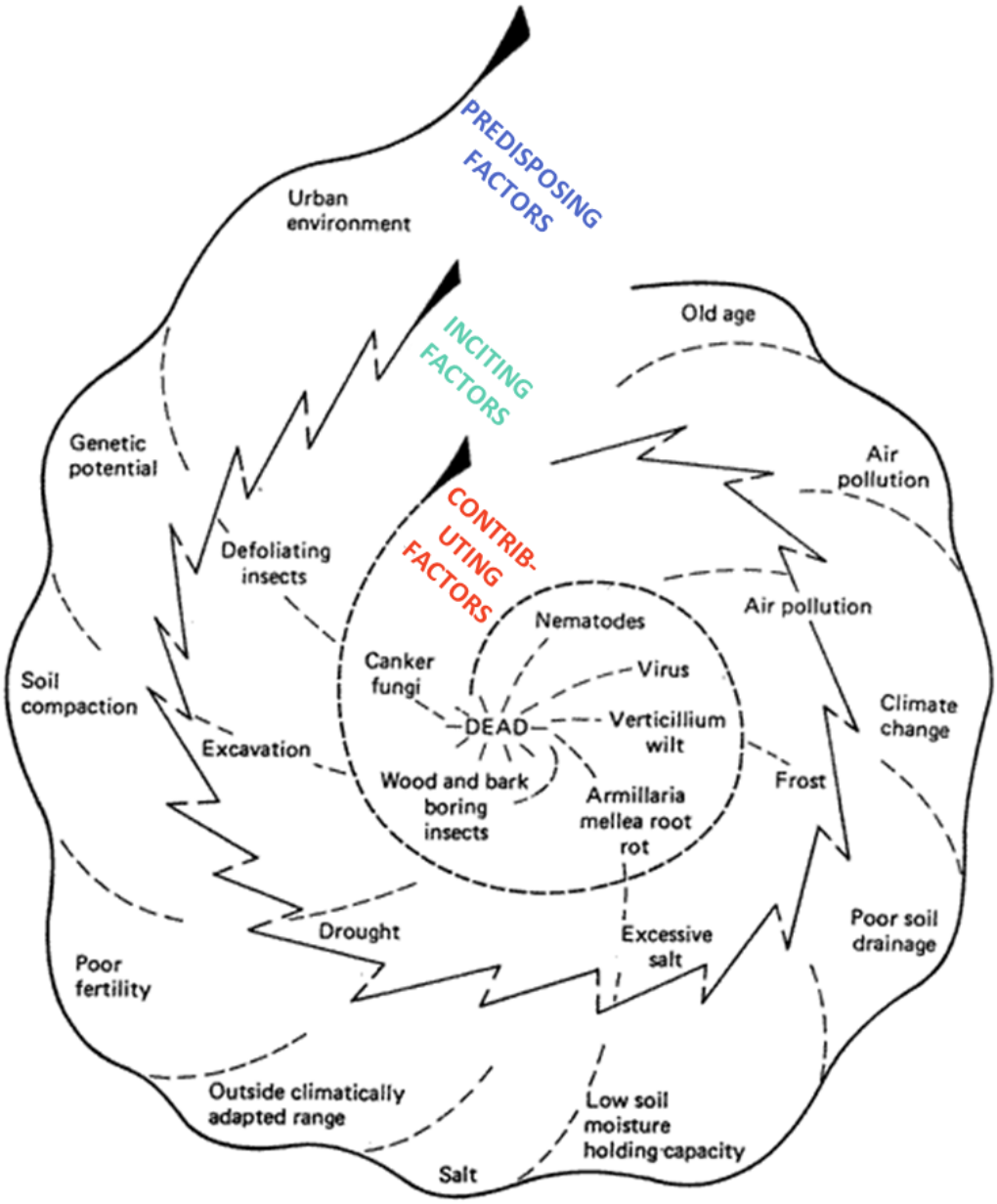


Figure 1.2 The Tree Decline Spiral by Manion (1991) illustrating how potential predisposing, inciting and contributing factors result in decline

lesions, fruiting bodies or pests, host stress due to environmental factors can appear very similar to stress due to pathogen attack. Additionally, by the time stress is observed, it may be too late to determine which factors are pre-disposing, inciting or contributing.

1.4.1 Pathogen-related dieback of trees

Some of the most commonly observed dieback-causing fungi are members of the ascomycete family, Botryosphaeriaceae. Slippers and Wingfield (2007) reviewed the ecology, diversity, occurrence and pathology of this fungal family as important environmental and agricultural pathogens of angiosperms and gymnosperms. Members of the Botryosphaeriaceae include *Fusicoccum*, *Diplodia*, *Lasiodiplodia* and *Neoscytalidium.* These species have been shown to cause dieback of tropical and temperate trees including oaks, citrus, willow and coconut (Agrios 2005), eucalyptus (Slippers et al. 2009; Taylor et al. 2009) and invasive weeds (Toh 2009; Haque 2015; Sacdalan 2015; Diplock 2016). Slippers and Wingfield (2007) showed that as endophytes or latent pathogens, some Botryosphaeriaceae species are opportunistic, and take advantage of a host when it is under stress from abiotic or biotic factors, causing symptoms such as cankers, brown fruit rot, loss of canopy, damping off and blight. Due to the opportunistic nature of these pathogens, increases in environmental stress of potential hosts due to climate change are likely to increase the prevalence of Botryosphaeriaceae diseases, such as dieback (Pitt et al. 2010; Urbez-Torres et al. 2010; Wunderlich et al. 2010).

In a study on dieback of *Prunus* species in South Africa, several Botyrosphaeriaceae species were morphologically and genetically identified, and

some were shown to be pathogenic to *Prunus* species (Damm et al. 2007). The study sampled symptomatic wood and pruning debris from plum, peach and apricot orchards in a range of locations with differing rainfall. The isolated endophytes formed four clades: *Lasiodiplodia*, *Diplodia*, *Dothiorella* and *Neofusicoccum* and also included two new species (Damm et al. 2007). Slippers et al. (2009) then reviewed the occurrence and global distribution of Botryosphaeriaceae in Eucalypts. *Lasiodiplodia theobromae* was most frequently isolated in Australia, South America and Africa, however other common species included *N. austral*, *Botryosphaeria mamane* and *L. pseudotheobromae* (Slippers et al. 2009).

There have been a number of recent studies that compare Botryosphaeriaceae diversity between healthy and dieback-affected plants (Jami et al. 2013; Sacdalan 2015) and interestingly, there seems to be greater or equal diversity in healthy tree tissues compared to diseased tree tissues. This was shown to be the case in *Acacia karroo* trees in Pretoria, South Africa (Jami et al. 2013), and the authors hypothesised that this opportunistic, latent endophyte may take advantage of stressed tissues (possibly caused by biological agents such as burrowing larvae) to become pathogenic. The findings are similar to a subsequent study by (Sacdalan 2015) on mimosa, which showed similar Botryosphaeriaceae diversity on healthy mimosa compared to dieback mimosa (*Mimosa pigra*).

There are multiple other dieback-associated fungal pathogens apart from the Botryosphaeriaceae. Dieback in ash caused by the ascomycete *Hymenoscyphus fraxineus* (previously *Chalara fraxinea*, family: Helotiaceae), results in symptoms including canopy loss, failure to flush, diamond-shaped lesions and widespread tree death (Forestry Commission 2013). Another ascomycete causing dieback is *Eutypa*

lata, a member of Diatrypaceae that has been shown to infect grapevines in California and throughout Australia (Pitt et al. 2013). Infection is via fresh pruning wounds and the associated symptoms are cankers and lesions around the wound, chlorosis, necrosis, destruction of vascular tissue, and eventually death (Pitt et al. 2013). Strict import and transport restrictions on grapevines and associated material are already in place throughout Australia, yet *E. lata* is present across SE Australia (Pitt et al. 2013), suggesting that controls are not sufficiently stringent. The authors hypothesised that *E. lata* spores are probably able to travel long distances unaccompanied by host material, and therefore even strict control measures were unable to halt the spread of this disease (Pitt et al. 2013).

Fusarium (family: Nectriaceae) are primarily soil-dwelling ascomycetes that infect root tissue of host plants, interfering with the water supply and causing vascular wilt (Agrios 2005). They are able to both inactivate toxic substances produced by the host, and produce their own, enhancing their virulence (Agrios 2005). A number of species have been shown to be responsible for dieback across a range of plant hosts, for example, dieback of *Euphorbia larica* in Oman has recently been shown to be caused by *F. brachygibbosum* (Al-Mahmooli et al. 2013); and avocado (*Persea americana*), castor bean (*Ricinus communis*) and other woody plants have experienced dieback in California caused by an unknown *Fusarium* fungus that is spread by a beetle vector (Eskalen et al. 2013).

Phytophthora are oomycetes (lower fungi) that are responsible for a large proportion of plant diseases around the world, including dieback (Erwin & Ribeiro 1996). *Phytophthora* cause root and fruit rot, cankers, root lesions and damping off of seedlings and are the cause of multiple economically important diseases including late

blight of potatoes and tomatoes (Agrios 2005), Sudden Oak Death (McPherson et al. 2001), and the environmentally and culturally significant kauri dieback in New Zealand (Beever et al. 2008). Recent observations by Akilli et al. (2013) showed extensive dieback in the old ash (*Fraxinus augustifolia*) forests of Turkey caused by *P. lacustris*; while *Phytophthora* dieback was also observed in Lawson cypress (*Chamaecyparis lawsoniana*) in Great Britain, with the responsible species being *P. lateralis*, thought to be spread in infested nursery stock (Green et al. 2013).

1.5 Dieback in Weeds of National Significance (WONS)

Mimosa, prickly acacia (*Vachellia nilotica subsp. indica*), blackberry (*Rubus anglocandicans*), athel pine (*Tamarix aphylla*) and bellyache bush (*Jatropha gossypiifolia*) are WONS in Australia (Thorp & Lynch 2000), and dieback has been observed in a number of populations of each species. Sacdalan (2015) isolated 284 fungi from mimosa trees and showed that five *Lasiodiplodia theobromae* (Botryosphaeriaceae) isolates resulted in lesions in mimosa seedlings during pathogenicity screening. Haque (2015) cultured stem segments from prickly acacia and inoculated plants with fungal pathogens chosen from the isolations. He showed that *Botryosphaeria mamane* may be a causal agent of dieback in prickly acacia, along with another *Botryosphaeria* sp. (unidentified) and *L. pseudotheobromae*. Haque (2015) suggested that both could be used in a mycoherbicide targeting prickly acacia if determined to be effective in subsequent testing. A new species of *Phytophthora*, *P. bilorbang*, and the pathogen *P. cryptogea* were found associated with blackberry dieback in Western Australia (Aghighi et al. 2015). Blackberry decline is also the only system where good experimental support for causation was found in association with

flood stress. In all cases, the focus of WONS dieback research has been to investigate the potential for the use of dieback-causing pathogens as biological control agents for these invasive plants (Aghighi et al. 2015; Haque 2015; Sacdalan 2015; Raghavendra et al. 2017).

These studies have two things in common. Firstly, dieback has not been observed in the native range of any of the studied weeds (Aghighi et al. 2015; Haque 2015; Sacdalan 2015; Raghavendra et al. 2017). Secondly, Aghighi et al. (2015), Haque (2015), Sacdalan (2015) and Diplock (2016) all used traditional culture-based techniques to identify endophytes associated with each plant. While these techniques have merit, they are likely to miss scores of other species that for various reasons (Peršoh 2015), cannot be isolated. Thus, the use of new technology such as Next-Gen sequencing, may give a more accurate picture of the endophyte community.

1.5.1 Parkinsonia aculeata

My thesis focuses on another dieback-affected WONS, parkinsonia, a pan-tropical shrub that is a serious invader of diverse habitats and climatic zones across northern Australia (Deveze et al. 2004). The Australian population has been around since the 1800s, originating from Venezuelan/Meso-American populations (Hawkins et al. 2007; Van Klinken & Heard 2012). It was spread in Australia as an ornamental tree due to its bright yellow flowers and luscious foliage (Fig 1.3) and was also used for hedging, feed and land rehabilitation (Van Klinken & Heard 2012). It is estimated to be present on over 3.3 million ha of Australia (van Klinken et al. 2009), with over 590 records in the Atlas of Living Australia (Fig 1.4; Global Biodiversity Information Facility 2016). It is a perennial thorny shrub that forms dense thickets along

Figure 1.3 *Parkinsonia aculeata*: (a) a young healthy tree; (b) flowers; (c) seed pods and spines; and (d) a dense stand (TV Steinrucken March 2013, Charters Towers).

waterways, floodplains and throughout paddocks, and seriously impacts the pastoral industry and local biodiversity (Van Klinken & Heard 2012). The thickets provide shelter for pest animals such as feral pigs, exclude native vegetation, and present extreme difficulties in cattle mustering and management (Deveze et al. 2004). Parkinsonia was ranked as the number one WONS, mostly due to its potential distribution should it go unmanaged (Fig 1.4; Thorp & Lynch 2000).

Since escaping cultivation, parkinsonia has been the target of a range of manual, herbicidal and biological control programs (Deveze et al. 2004). Manual control is mostly impractical for established populations due to their size and density, and the use of herbicides in general are socially and environmentally controversial due to potential off-target effects on other plants and animals (Deveze et al. 2004; Lawes & Grice 2007). Native range surveys for insect biocontrol agents began in 1983 and by 1993 a seed-feeding bruchid *Mimosestes ulkei* and a sap-sucking myrid *Rhinacloa callicrates* were released. Then in 1995 another seed-feeding bruchid *Penthobruches germaini* (found in Argentina) was also released and this was the only agent that spread; although none of the three were effective at limiting parkinsonia (Heard 2006). The most successful introduction of insect biological control for parkinsonia is currently making headway. The Queensland Department of Agriculture and Fisheries' Tropical Weed Research Centre and CSIRO Health and Biosecurity have managed to successfully establish *Eueupithecia cisplatensis*, a seed-feeding caterpillar, which is slowly reducing viable parkinsonia seed in the areas of release (Minister for Agriculture and Fisheries 2016), however progress is slow and complimentary controls are being sought.

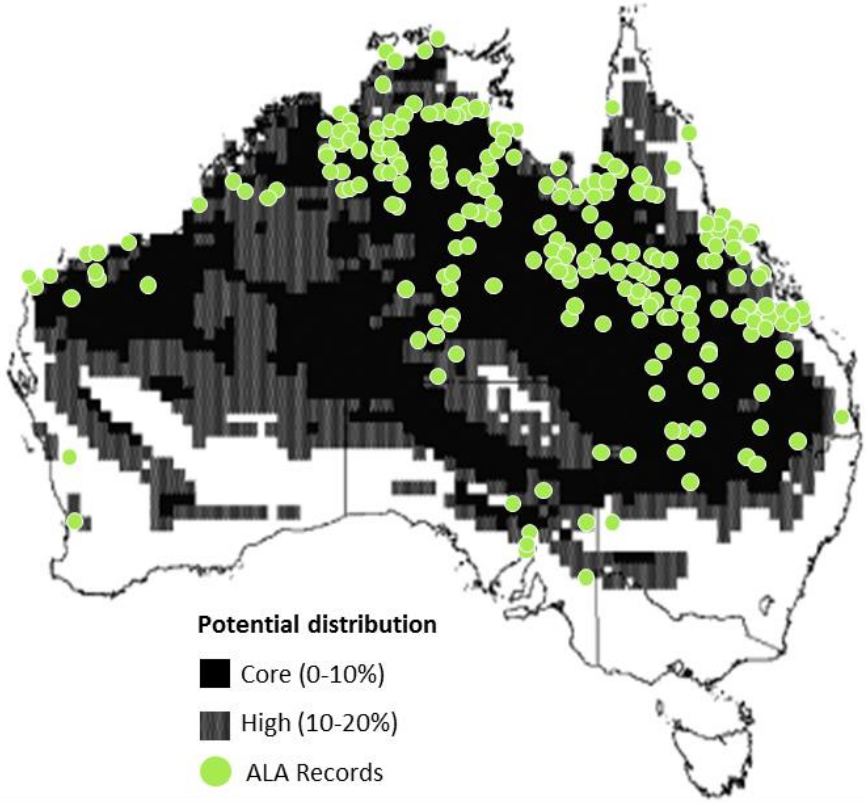


Figure 1.4 Potential distribution of *Parkinsonia aculeata* in the event of no management biodiversity (Van Klinken & Heard 2012). Green circles indicate approximate location of herbarium records submitted to the Atlas of Living Australia (Global Biodiversity Information Facility 2016)

Dieback has been observed in parkinsonia populations for decades (Diplock 2016) and may be one of the reasons that parkinsonia has not spread more widely (van Klinken et al. 2009). Parkinsonia dieback symptoms include leaf loss, death of stems starting at the stem tips and gradually moving towards the base, usually brown staining of inner stem tissue and eventually outright tree mortality (Fig 1.5). The cause of dieback in parkinsonia has been previously researched but no agent has been definitively linked to dieback and many questions remain unanswered. One study (Toh 2009) found that five fungal species isolated from parkinsonia were pathogenic and all were members of the Botryosphaeriaceae family. *Lasiodiplodia pseudotheobromae* was the most virulent of these species, which killed seedlings prior to emergence, caused greatest glasshouse mortality rates and resulted in rapid seedling death. The other fungal species tested by Toh (2009) included *L. theobromae* (also caused rapid seedling death), *Neoscytalidium dimidiatum* and *Botryosphaeriaceae* sp. The study was conducted on two-day old parkinsonia seedlings, and the results were not replicated in larger-stemmed plants (Toh 2009), so a similar approach may not produce the same results in more mature trees.

Another previous study (Diplock 2016) showed that inoculations with *Lasidioplodia pseudotheobromae, Fusarium* sp. and *Fusicoccum dimidiatum* collected from dieback parkinsonia caused some disease symptoms in adult trees. The invasive inoculation method was used on adult trees in the field and involved insertion of colonised millet seed rolled in thin paper into a wound in the stem. This resulted in small lesions associated with the inoculation wounds and may have caused initial stress resulting in enhanced dieback symptoms as a secondary response. Some trees

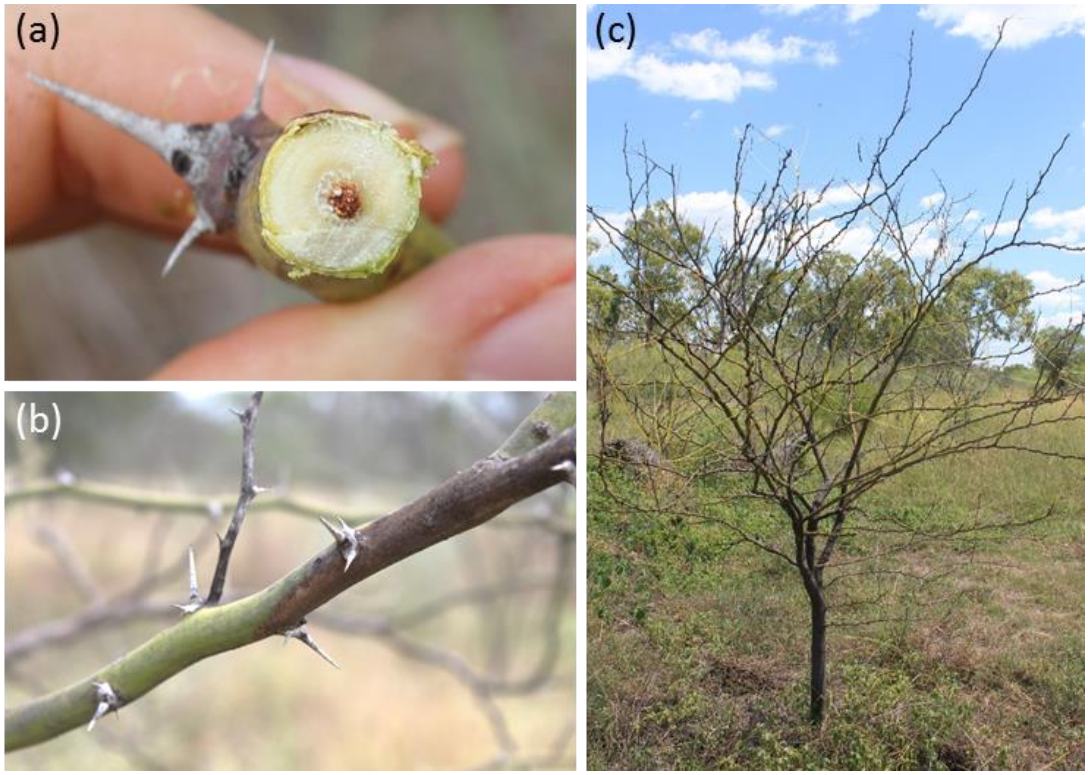


Figure 1.5 Symptoms of dieback in *Parkinsonia aculeata*: staining of vascular tissue (a), browning of stems (b), defoliation and tree death (c). Charters Towers, Queensland March 2013

also had existing dieback-like symptoms or poor health (Diplock 2016). Dieback symptoms observed in the field were not replicated in either study (Toh 2009; Diplock 2016), suggesting that any growth effects may have been a physical consequence of the inoculation on translocation. Even if it had been successful, Diplock's inoculation technique would not be economically viable for large-scale inoculations or biological control as it is time consuming and labour intensive, although efforts are being made to reduce the labour involved (School of Agriculture and Food Sciences 2016), with mixed results (Galea 2012). Both studies (Toh 2009; Diplock 2016) showed some evidence to support the involvement of Botryosphaeriaceae species (*L. pseudotheobromae, L. theobromae, N. dimidiatum)* in dieback. However, due to the mixed results, Toh (2009) hypothesised that putative pathogenic isolates may be a part of a disease complex that relies on the presence of certain other parameters or microbes, and are therefore not as pathogenic in the field where antagonists exist, or the required microorganisms are absent. Other microbial groups such as bacteria have not yet been considered in the parkinsonia dieback system, and neither have soil-borne pathogens such as *Phytophthora*.

The results from these studies highlight the need for an open-minded approach to discovering the cause of parkinsonia dieback. Instead of continuing on from where Diplock (2016) and Toh (2009) left off, it is important to first investigate the impact of microbial (fungal, oomycete and bacterial) communities associated with parkinsonia, and investigate how this contributes to the promotion or prevention of dieback. Dieback research has usually focused on fungi as the probable cause, but the possibility of dieback-inducing bacteria has never been considered and should not be overlooked. Also, by considering the factors that result in successful plant invasions

and their association with microbial activity, certain aspects of dieback may be clarified. These points should all be taken into account when assessing the pathology of parkinsonia dieback. Once a suspect is identified (Botryosphaeriaceae or not), there is potential for its use as a biological control agent which could work alongside the moth *E. cisplatensis* to control parkinsonia populations in northern Australia.

Although the studies discussed above are able to explain some aspects of parkinsonia dieback, many unanswered questions remain. For example, is parkinsonia susceptibility in the field related to external stress factors? Is dieback caused by a disease complex – rather than one or two species – and is it possible that the initiators are not fungi but actually another agent (e.g., bacteria) or abiotic factor? Why is dieback not observed in the native range of parkinsonia (South America, or in North America)? Why is Australian parkinsonia susceptible to dieback in the field, while native, co-occurring or closely related species are not? We also don't yet know if dieback is partly due to a loss of potentially protective endophytes, left behind in the native range as missed mutualisms; if potential dieback-causing pathogens were encountered by parkinsonia in Australia as new associations; or if these pathogens were brought with the plant during introduction. Furthermore, bacterial and oomycete species have not yet been considered in this system and the effects of abiotic factors on parkinsonia disease susceptibility have not been tested. Answers to these questions are likely to provide insight into how and why dieback occurs and whether the agents of dieback may be useful in biological control of parkinsonia.

1.5.2 The potential for using dieback in the biological control of parkinsonia

Biological control (or 'biocontrol') of invasive plants is the exploitation of natural methods used by pathogens or herbivores to suppress weed populations (Wapshere et al. 1989). There are many examples of successful and failed biocontrol programs, and these can be found in publications such as "Biological control of weeds: a world catalogue of agents and their target weeds" (Julien & Griffiths 1998) and "Failure in Biological Control of Weeds" (Myers 2000). The relevance of biocontrol in my research is the potential for it to be applied to parkinsonia using the agent or agents responsible for dieback.

There are a number of methods of ensuring a biocontrol agent is safe to use and is specific to the target plant (Wapshere 1974; Evans 2000). In the case of dieback in parkinsonia, the agent responsible is already in Australian populations, so if it is able to be augmented and used as a biocontrol agent, none of the more rigorous import regulations need to be dealt with. It will be necessary, however, to ensure that Australian parkinsonia populations are susceptible to the pathogen, and that local native or desired plants are not. Also, much of the research effort would need to be spent on developing effective methodology for inoculation and maintenance of the selected pathogen(s) in the field. Although direct application of a putative agent is ideal, it would be highly impractical and expensive if the target weed is inaccessible or distributed across wide-spaced regions (like parkinsonia is), and may also be unnecessary if the agent has a mechanism for dispersal. Toh (2009) was able to show in seedling inoculation trials, that there is potential for development of a mycoherbicide against parkinsonia, possibly using *Lasiodiplodia* or *Fusicoccum*

isolates. As mentioned, the inoculation achieved significant mortality of seedlings in lab conditions (Toh 2009), however for biocontrol to be effective, this needs to occur in adult plants in the field, and the agent should ideally be able to persist and spread without artificial augmentation.

1.6 Summary

Parkinsonia is an example of an extremely successful invasive species that has mostly managed to avoid barriers to invasion such as biotic resistance, negative new associations and missed mutualisms. Since naturalisation however, its spread has been limited by dieback occurrence in some regions (van Klinken et al. 2009). As a wide-spread weed, parkinsonia may have been introduced to Australia alongside its co-evolved mutualist endophytes and/or without any co-evolved pathogens, in line with Evans' (2008) endophyte-enemy release hypothesis. There is a possibility that co-evolved pathogens in the native range are partly responsible for keeping parkinsonia populations under control there, and that this pathogen has been introduced to Australia recently, causing parkinsonia dieback, however dieback has not been observed in the native range. These explanations would be justified if a protective endophyte that had co-evolved with parkinsonia was no longer associated with the host, leaving parkinsonia more vulnerable to pathogens ("silver bullet" theory; Evans, 2008).

Biotic agents responsible for causing dieback have most commonly been shown to be fungi, some of which may be latent endophytes, including those from the Botryosphaeriaceae. However, since there are multiple beneficial, mutualistic or detrimental interactions between a plant and its associated microbial community,

dieback may not be simply due to the presence of a pathogen, but could be as a result of a pathogen complex or due to the lack of an endophyte that normally aids in defence.

The issue of abiotic stress is also important by reason of the location of parkinsonia populations – often in zones with high temperatures, frequent flooding or desiccation and increased soil disturbance – and thereby potential opportunistic action by certain pathogens when the plant is stressed (Slippers & Wingfield 2007).

There are many variables involved in the promotion or prevention of invasion by an exotic plant, but more insight into the reasons for decline of a successful invader is required to determine what causes parkinsonia dieback, and if it can be exploited for biological control.

1.7 Thesis outline

There are two primary objectives for this thesis:

1. To find the cause of parkinsonia dieback in Australia, and;
2. To determine whether the cause of parkinsonia dieback can be used in biological control of this weed.

In order to realise the above objectives, there are a number of research questions that are addressed in this thesis. These have been compiled along with the summarised methodology and experimental purposes in a flowchart (Fig 1.7).

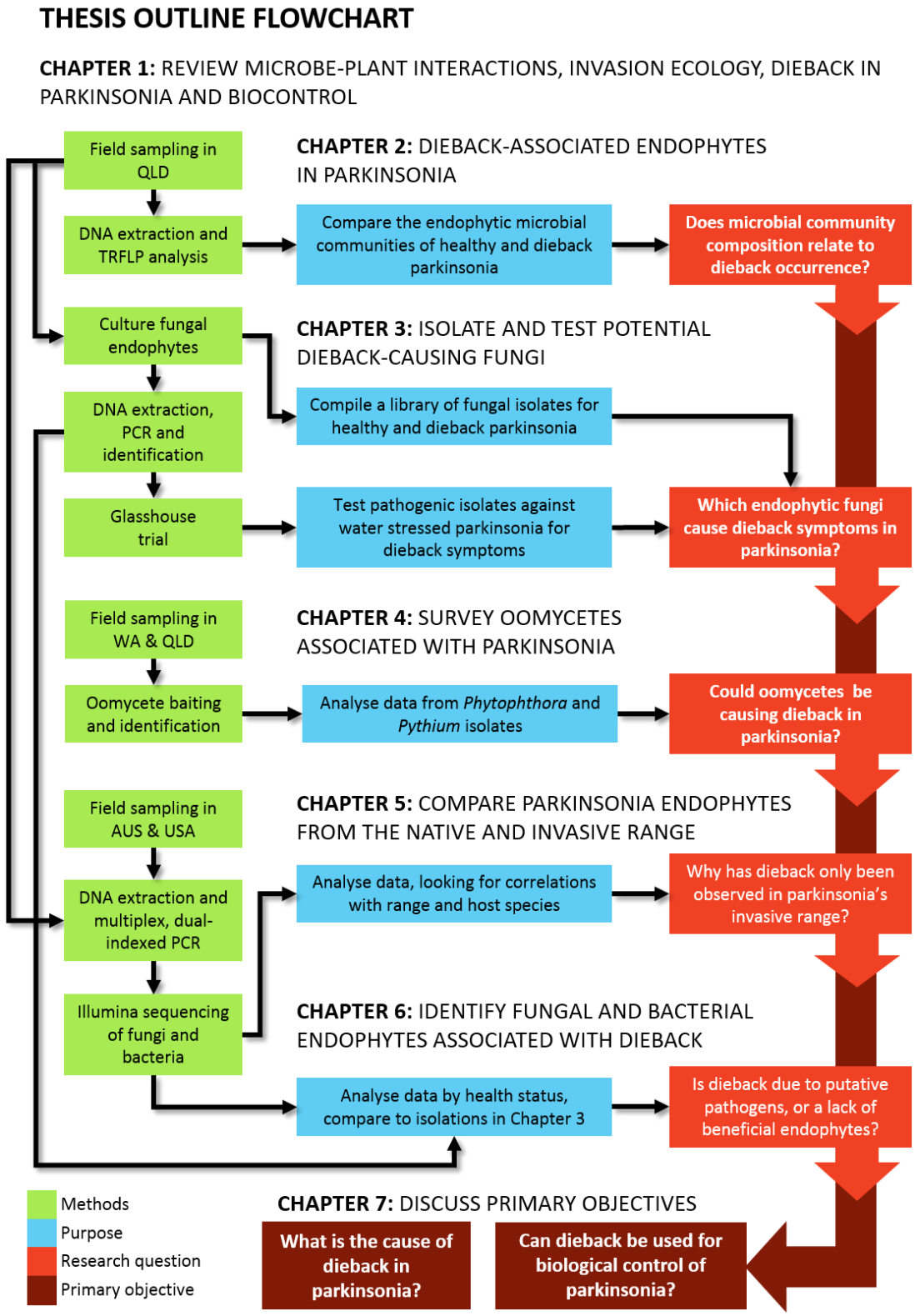


Figure 1.6 Thesis outline flowchart showing progression from each chapter to the next, including summary of methods used, the purpose of each study and which research question is addressed.

1.8 Publications

Apart from Chapters 1 and 7, this thesis was compiled as a collection of publications or potential publications. There is therefore some repetition throughout the thesis, particularly when describing the background of each study. The publications associated with each chapter in this thesis are listed below. Chapters 2, 3 and 4 were published in their entirety in the specified journals. Chapters 5 and 6 are in preparation. Except where explicitly stated, I led all the work, generated all the data, did all the analyses and wrote the entire manuscript for each publication.

Chapter 2

- Steinrucken TV, Bissett A, Powell JR, Raghavendra AKH & van Klinken RD (2016) Endophyte community composition is associated with dieback occurrence in an invasive tree. Plant Soil, 405, 311-323. DOI: 10.1007/s11104-015-2529-y

I led all the work, generated all the data, did all the analyses and wrote the entire manuscript expect that the T-RFLP digestion and sequencing was carried out by Shamsul Hoque (CSIRO Agriculture & Food, Canberra). My co-authors contributed ideas on the types of analyses I should conduct, assisted me with the interpretation of results and helped with editing the manuscript for publication.

Chapter 3

- Steinrucken TV, Raghavendra AKH, Powell JR, Bissett A & van Klinken RD (2017) Triggering dieback in an invasive plant: endophyte diversity and pathogenicity. Australasian Plant Pathology, 46: 157-170. DOI: 10.1007/s13313-017-0472-5.

I led all the work, generated all the data, did all the analyses and wrote the entire manuscript expect that Anil Raghavendra (CSIRO Health & Biosecurity) advised on isolation and morphological identification of some fungal endophytes, and sequencing was carried out by Marcus Klein (Hawkesbury Institute for the Environment, Western Sydney University). My co-authors contributed ideas on the types of analyses I should conduct, assisted me with the interpretation of results and helped with editing the manuscript for publication.

Chapter 4

- Steinrucken TV, Aghighi S, Hardy GESJ, Bissett A, Powell JR & van Klinken RD (2017) First report of Oomycetes associated with the invasive plant, *Parkinsonia aculeata*. Australasian Plant Pathology, 46: 313-321. DOI: 10.1007/s13313-017-0494-z

I led all the work, generated all the data, did all the analyses and wrote the entire manuscript except that Sonia Aghighi (CPSM, Murdoch University) helped with the isolation and morphological identification of oomycete isolates, Giles Hardy (CPSM, Murdoch University) led the fieldwork in Kununurra WA, and Diane White (CPSM, Murdoch University) did the sequencing of oomycete DNA. My co-authors contributed ideas on the types of analyses I should conduct, assisted me with the interpretation of results and helped with editing the manuscript for publication.

Chapter 5

- Steinrucken TV, Bissett A, Powell JR, Garbelotto M & van Klinken RD *In preparation.* Fungal endophyte communities differ across the native and invasive range of *Parkinsonia aculeata*. Biological Invasions

I led all the work, generated all the data, did all the analyses and wrote the entire manuscript expect that Dylan Smith (UC Berkeley) ran the Illumina sequencing protocol for this study, and Andrew Bissett (CSIRO Oceans and Atmosphere) generated the target sequence data from the Illumina sequencing reads. My co-authors helped plan fieldwork, contributed ideas on the types of analyses I should conduct, assisted me with the interpretation of results and helped with editing the manuscript for publication.

Chapter 6

- Steinrucken TV, Bissett A, Powell JR, Garbelotto M & van Klinken RD *In preparation.* Narrowing down the suspects: molecular and culture-based methods combine in the diagnosis of endophyte-associated disease in invasive *Parkinsonia aculeata*. Phytopathology

I led all the work, generated all the data, did all the analyses and wrote the entire manuscript except that Dylan Smith and other technical staff (UC Berkeley) ran the Illumina sequencing protocol for this study, and Andrew Bissett (CSIRO Oceans and Atmosphere) generated the target sequence data from the Illumina sequencing reads. My co-authors contributed ideas on the types of analyses I should conduct, assisted me with the interpretation of results and helped with editing the manuscript for publication.

A note on experimental work for Chapters 5 and 6:

The Illumina data for these chapters were fortuitous, generated after the T-RFLP data were published when I travelled to the USA on a Fulbright Fellowship. Thus, it was not possible to publish the data together with Chapter 2, which uses the same samples as in Chapter 6. Additionally, I had planned on conducting fieldwork in Venezuela (the genetic origin of Australian *P. aculeata*) while on the Fellowship, however my travel there was not approved by Fulbright due to safety concerns. I also considered having locals in Venezuela sample on my behalf, however transporting vegetative or soil material into the USA would not have been possible due to biosecurity concerns, and we did not have contacts there that would be able to conduct DNA extractions on my behalf. As a result, *Parkinsonia* sampling sites in the USA were chosen instead.

2

ENDOPHYTE COMMUNITY COMPOSITION IS ASSOCIATED WITH DIEBACK OCCURRENCE IN AN INVASIVE TREE

2.1 Abstract

Dieback is the general nature of observed disease patterns in a plant population leading to reduced fitness and finally death. Most dieback research is limited to one or two taxa. I aim to determine if microbial community composition of archaeal, bacterial and fungal endophytes are associated with the presence of dieback in multiple plant parts of the dieback-affected invasive tree, *Parkinsonia aculeata L*. Roots, stems and stem tips were sampled from healthy and dieback-affected parkinsonia populations. I conducted terminal restriction fragment length polymorphism (T-RFLP) analysis on the extracted DNA using taxon-specific primers for archaea, bacteria and fungi, followed by statistical analysis to determine how endophyte community composition relates to plant part and disease status. Archaeal and fungal community structures were significantly correlated to dieback presence and plant part, particularly in parkinsonia stem tips and roots. Bacterial community composition showed significant correlation to dieback presence but not plant part. This study shows that endophyte community composition in parkinsonia tips and roots is associated with the presence of dieback and that both protective and pathogenic endophytes may be involved in the onset of dieback in parkinsonia.

2.2 Introduction

Dieback is described as the general nature of observed disease patterns in a plant population which are the loss of canopy cover, leading to reduced fitness and finally death of the trees (Mueller-Dombois 1987). Dieback seems to be increasingly common in native and invasive plant species globally (La Porta et al. 2008) and microorganisms are often implicated (e.g. Mueller-Dombois 1987; Kowalski & Holdenrieder 2009; Herrero et al. 2011; Ismail et al. 2012; Mehl et al. 2013). Dieback in some tree and shrub species has been found to result from single pathogens, sometimes in the presence of stressors (Rice et al. 2004; La Porta et al. 2008; Pautasso et al. 2013; Scarlett et al. 2013). More often, causal explanations remain elusive (Houston 1992; Slippers & Wingfield 2007; Sakalidis et al. 2011; Jami et al. 2013). This is due in part to complex interactions between pathogens, the host and the environment, as described in dieback models by Houston (1992) and Manion (1991). Isolating potential pathogens is also made more difficult if using techniques that do not necessarily obtain a true representation of all microorganisms within the host. Many studies that aim to identify the pathogen(s) responsible are limited by traditional culturing techniques, or the use of targeted studies that only focus on one group of microorganisms. Additionally, other than pathogens, the presence of beneficial endophytes may prevent disease in some hosts and therefore should also be considered as part of the system. In order to identify possible causal-pathogens or protective endophytes, it is important to determine if dieback correlates with microbial community structure and if it does, with which component(s) of the microbial community.

Manion's (1991) Tree Decline Spiral is a classical dieback model which aims to capture the many potential and diverse drivers of dieback. These include abiotic pre-

disposing factors such as climate change and local flooding; inciting factors such as genetic diversity, competition and loss of protective endophytes; and contributing factors like pathogens and arthropod damage. The process of narrowing down the contributing factors is complex due to the difficulty in proving Koch's postulates when multiple pathogens or protective endophytes could potentially be involved. As such, there are many examples of studies on dieback with both diagnosed and idiopathic causes. Some of the most commonly observed dieback-causing fungi are members of Botryosphaeriaceae. Botryosphaeriaceae fungi are ascomycetes that have been shown to cause dieback of tropical and temperate trees including oaks, citrus, willow and coconut (Agrios 2005), *Eucalyptus* (Slippers et al. 2009; Taylor et al. 2009), *Prunus* (Damm et al. 2007) and grapevines (Pitt et al. 2010; Urbez-Torres et al. 2010; Wunderlich et al. 2010). The focus on this family of opportunistic pathogens is partly due to their ability to take advantage of a host when it is under stress from environmental or biotic factors, and cause symptoms such as cankers, brown fruit rot, loss of canopy, damping off and blight (Slippers & Wingfield 2007). Despite this, some of the most common dieback-causing species in this family are more frequently found in healthy hosts than their dieback-affected counterparts (Jami et al. 2013), suggesting that their presence in the host does not naturally infer dieback occurrence.

There are an increasing number of reports of dieback in invasive shrubs and tree species in Australia over the past two decades, including many of the 20 taxa ranked as weeds of national significance (WONS). Symptoms include loss of leaves, browning of the stem tips that gradually moves down the plant, loss of vigour and eventually death. Population decline can be local or extensive and in some cases results in populations dying out, moreover, there is no evidence that dieback occurs in these

weeds' native populations. *Parkinsonia aculeata* L. is a tree invasive to northern Australian rangelands, originally introduced from central and South America in 1860 (van Klinken et al. 2009). Parkinsonia now covers over 1 million hectares of northern Australia, with its dense, thorny thickets impacting heavily on the pastoral industry and the environment (Van Klinken & Heard 2012). Dieback has been observed in invasive parkinsonia populations in northern Queensland, the Northern Territory and northern Western Australia (Diplock 2016) and may be one of the reasons that parkinsonia has not spread more than it already has (van Klinken et al. 2009). Other WONS affected by dieback include mimosa (*Mimosa pigra*; Sacdalan 2015), prickly acacia (*Acacia nilotica* ssp. *indica*; Haque 2015) and bitou bush (*Chrysanthemoides monilifera* ssp. *rotundata*; Morin et al. 2012). Since there is potential for dieback to be an effective method of natural weed control, identification of the causal agent(s) is essential. For example, using classical pathology techniques Haque (2015) showed that *Botryosphaeria mamane* was most likely to be causal agent of dieback in prickly acacia and concluded that these potential pathogens could be developed as a mycoherbicide targeting prickly acacia.

The cause of dieback in parkinsonia has been previously researched (Toh 2009; Diplock 2016) and although there is some evidence that inoculations with *Lasiodiplodia theobromae, L. pseudotheobromae, Neoscytalidium dimidiatum, Macrophomina phaseolina* and *Botryosphaeria sp.* (all members of Botryosphaeriaceae) isolated from dieback-affected parkinsonia may have caused dieback-like symptoms in inoculated seedlings (Toh 2009), there is little evidence of their pathogenicity in the field. Although classical pathology techniques have been used extensively in dieback research, their use has not shown a correlation between

pathogen presence and absence in healthy parkinsonia compared to dieback parkinsonia. Therefore, even though a number of putative pathogens have been found, it is necessary to do a more comprehensive analysis of endophytic microbial communities that occur within healthy and dieback-affected parkinsonia populations, without being limited by taxonomic group or culturability in the lab.

The examples above illustrate that the focus for diagnosing dieback has been on the identification of pathogenic fungal species alone, and does not consider the role of other taxa in the system, such as the prokaryotes bacteria and archaea. Ruppel et al. (2013) show that all three taxa (archaea, bacteria and fungi) contribute to plant performance. It is essential to consider that all three groups may be involved as pathogens, beneficials or commensals and that communities, not individual species, might contribute to the occurrence of dieback. Mendes et al. (2011) detected over 33,000 archaeal and bacterial species in disease-suppressive soils and showed that certain soil-borne bacterial groups were consistently associated with disease suppression of a specific fungal root pathogen. Metabolites produced by endophytic bacteria (Brader et al. 2014) or fungi (Aly et al. 2011) may also play a role in disease suppression in their host, and plants are constantly challenged with infection by potential pathogens from multiple microbial consortia (Mendes et al. 2013). As such, a true indication of microbial community composition is needed in order to draw inference about the cause of dieback, and this cannot be obtained from culture studies alone, nor should it focus on only one group of microorganisms. Additionally, classical pathology research on dieback has usually focused separately on either root-associated pathogens, or on endophytic pathogens from stems of affected plants. Research has shown that microbial community structure varies across different parts of plants

(Rudgers & Orr 2009; Knief et al. 2012; Thongsandee et al. 2012; Ruppel et al. 2013), and since microbial associations with their hosts differ depending on their location within the plant (e.g. mychorrizal fungi vs. other endophytes), it is important to also consider above- and below-ground communities.

I hypothesise that the occurrence of dieback in parkinsonia populations is associated with a change in endophytic microbial community structure, and that this change varies between different parts of the plant. To encapsulate a broader picture of the endophyte community response to a variable such as dieback, taxa from the three microbial domains of life: archaea, bacteria and fungi, should be considered across the whole plant. It is possible that although the symptoms are most noticeable in the stem tips and stems of the trees, pathogens might be infecting the roots and are preventing the upper limbs from receiving water and nutrients. It is as equally possible that infection is initially in the upper limbs and then moves down the plant as the disease progresses. I suggest that more than one pathogen or latent endophyte is responsible for dieback, possibly as a disease complex (multiple taxa causing an infection), and that these are opportunistic pathogens, possibly triggered by host stress. I also expect that protective endophytes, or a lack thereof, may play a role in the level of population susceptibility to dieback or disease, as has been shown previously in tropical trees (Arnold et al. 2003) and invasive plants (Newcombe et al. 2009) among others. If multiple pathogens and/or protective endophytes are involved, this would explain why proving Koch's postulates in dieback-affected populations is difficult. Both hypotheses require the use of techniques that can provide a more complete overview of microbial communities. Using terminal restriction fragment length polymorphism (T-RFLP) analysis, I assessed how the composition of the entire microbial endophyte

community changes in the presence of dieback. To the best of my knowledge, no analysis has been conducted on an endophytic ecosystem to include all three taxa across a range of plant parts.

2.3 Materials and methods

2.3.1 Sampling and sample preparation

I sampled five trees from each of three replicate dieback-affected and three healthy populations of parkinsonia on pastoral properties between 20 and 40 km north of Charters Towers, Queensland (Fig 2.1; Table S2.1, Appendix A). This area has large populations of healthy and dieback-affected parkinsonia and the land managers have an interest in controlling the weed on their properties. For each tree I sampled three roots, stems and stem tips and pooled the samples by plant part. Sites were grouped into one healthy and one dieback-affected parkinsonia population such that, where possible, groups were closer to each other than to other replicates and I did not observe variation in abiotic factors within the groups. (Fig 2.1). Parkinsonia was categorised as healthy if plants were unambiguously healthy with 70-100% foliage cover, all major and minor stems healthy, and no consistent vascular staining or browning of branches (Fig 2.2a). Dieback-affected parkinsonia trees (Fig 2.2b) were classified as live specimens that showed evidence of dieback including defoliation (1-40% foliage cover), dead or dying stems (Fig 2.2c), vascular staining when sampled (Fig 2.2d) and evidence of tissue death in the stem tips. All trees sampled were mature: >25 cm trunk circumference at 30 cm above ground level and >2 m tall.

Tools for collecting samples were sterilized between sites by immersing in 50% NaClO for 5 min, and dried with sterile paper towel before next use. Where possible,

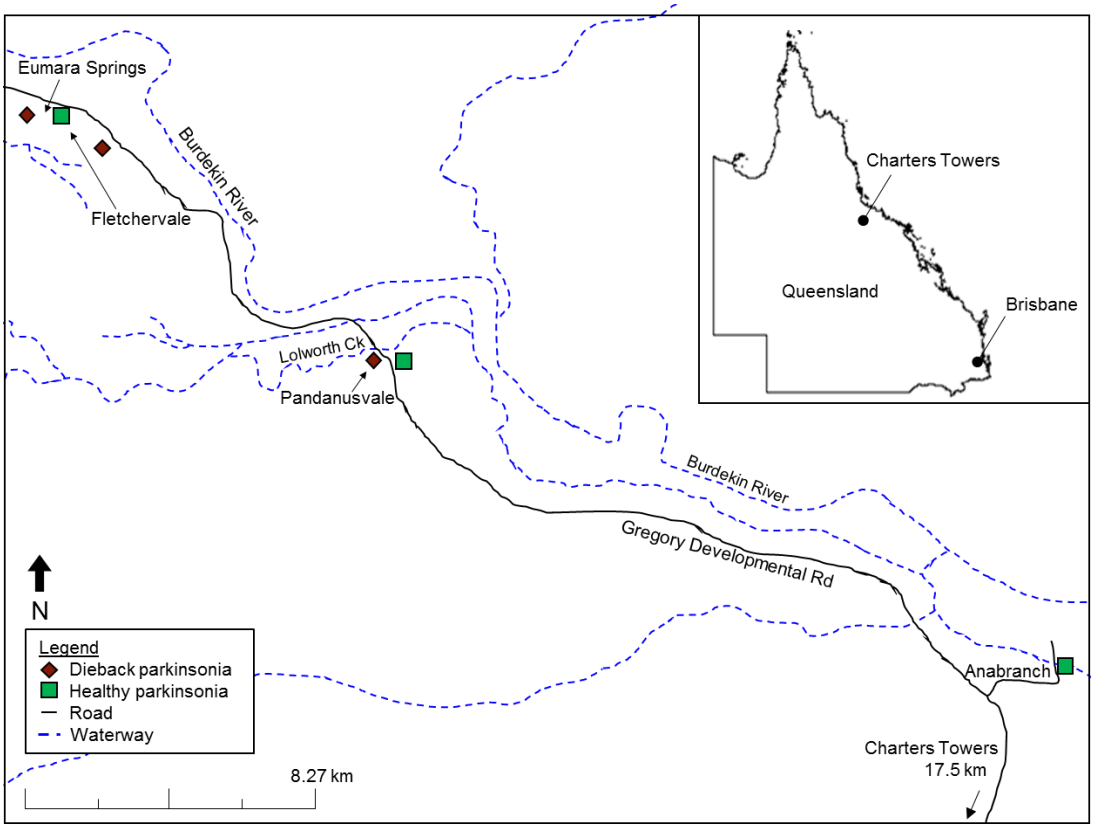


Figure 2.1 *Parkinsonia aculeata* and native tree sampling sites for this study, March 2013. Property names are indicated by arrows and the insert shows the location of Charters Towers in Queensland

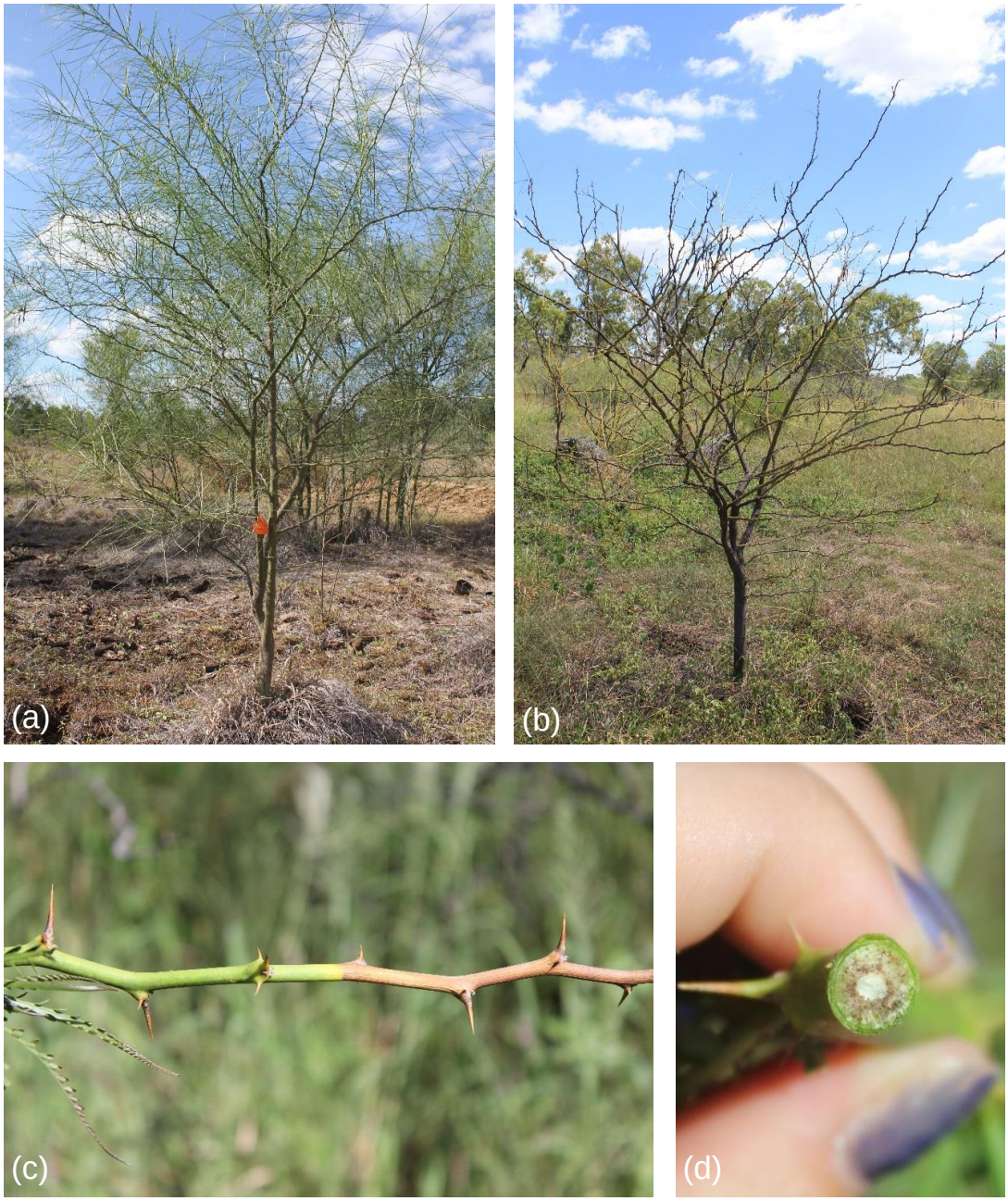


Figure 2.2 Healthy (a) and dieback-affected (b) *Parkinsonia aculeata* trees, stem of a dieback-affected tree (c) and stem vascular staining of a dieback-affected tree (d). Charters Towers, Queensland March 2013

samples were collected first from healthy parkinsonia, followed by dieback-affected trees. Three roots, stems and stem tips (each 5 – 8 cm long) were sampled from each individual tree. Stems were sourced from secondary branches of at least 1 cm diameter. Stem tips were defined as the terminal points of the tertiary stems from where only rachi grew. Roots were primary or secondary roots which I could see as connected to the sampled tree and were at least 1 cm diameter. These were rinsed free of excess soil immediately using tap water. Samples from the same plant part of the same plant were stored together in sealed plastic bags with silicon packets. All samples were maintained at 4°C until processed in the lab within 48-72 h.

Sample processing was conducted in a UV-sterilized laminar flow cabinet and all instruments were surface sterilised between samples using 95% ethanol and a flame. Plant parts were vigorously pre-washed in sterile, distilled H_2O for 20 s. For stems and stem tips a three stage ethanol-bleach-ethanol surface sterilization method was then used as recommended by Bills (1996). Roots were washed for 30 s in sterile, distilled H_2O containing 0.1% Tween-20™ since harsher sterilization techniques are not recommended for roots (Thorn et al. 2007). All samples were blotted dry with sterile filter paper and surface sterilization was checked by sliding sterilized tissue over the surface of media as an imprint, and incubating at 30°C for a week or longer (Bacon and Hinton 2007). The epidermis of stem tips and stems, and a small portion of root cortex was then removed using a sterile scalpel. Samples were stored in sealed, individual sterile plastic containers at -20°C until surface sterilisation was established. Once surface sterilized, the inner tissue was shaved and placed in sterile paper envelopes for DNA extraction.

2.3.2 Nucleic acid analysis

Sample shavings were freeze-dried for 48 h and then ground to a fine powder using a beadbeater for 2-3 min in 2 mL Eppendorf tubes containing two sterile 2 mm steel ball bearings. The MO BIO PowerSoil® DNA Isolation Kit was then used to extract total DNA from approximately 20 mg of each powdered sample, according to manufacturer's instructions, and DNA presence was confirmed on an agarose gel.

For T-RFLP, extracted DNA was amplified using taxon-specific primers (Table 2.1) as per Singh et al. (2006), except that amplification for each taxon was performed individually, rather than in a multiplex reaction. PCR reactions contained: MyTaq DNA polymerase (Bioline), 4µl 5x MyTaq Reaction Buffer, 0.5 µL MyTaq polymerase, 5 µL extracted DNA, and varying concentrations of primer depending on target DNA (0.4 µM for fungi, 0.2 µM for bacteria and 1.0 µM for fungi). PCR conditions for archaea and bacteria comprised 1 cycle of 95°C for 2 min; 30 cycles of 95°C for 15 s, 55°C for 15s and 72°C for 50 s, and a final extension step of 72°C for 10 min. PCR conditions for fungi were optimised as 1 cycle of 94°C for 3 min; 30 cycles of 95°C for 15 s, annealing at 56-50°C, dropping 1°C each cycle then maintained at 50°C for subsequent cycles, 72°C for 1 min, and a final extension step of 72°C for 10 min.

Table 2.1 PCR primers used in this study

Primer	Target region	Sequence (5' to 3')	Reference	Fluorescent label
Ar3f	Archaea 16S rRNA	TTCCGGTTGATCCTGCCGGA	(Giovannoni et al. 1988)	none
AR927r	Archaea 16S rRNA	CCCGCCAATTCCTTTAAGTTTC	(Jurgens et al. 1997)	NED
63f	Eubacteria 16S rRNA	AGGCCTAACACATGCAAGTC	(Marchesi et al. 1998)	none
1087r	Eubacteria 16S rRNA	CTCGTTGCGGGACTTACCCC	(Hauben et al. 1997)	VIC
ITS1f	Fungi ITS	CTTGGTCATTTAGAGGAAGTAA	(Gardes and Bruns 1993)	FAM
ITS4r	Fungi ITS	TCCTCCGCTTATTGATATGC	(White et al. 1990)	none

PCRs were purified using the Wizard®SV Gel and PCR Clean-Up System (Promega), quantified spectrophotometrically (Nanodrop ND-1000, Thermoscientific) and 30 ng PCR product digested with the restriction endonuclease *HindII*. Digests were isopropanol precipitated, resuspended in 10 µl formamide containing LIZ600 size standard (Applied Biosystems), denatured at 95 °C (3 min) and separated by size using capillary electrophoresis (ABI PRISM 3130xl Genetic 110 Analyzer, Applied Biosystems).

2.3.3 Statistical data analysis

True peaks were selected in R (R Core Team 2014) using the method by Abdo et al. (2006) with minimum peak size 40, maximum at 600 by "Area" with 1 standard deviation and minimum threshold 20. Peaks were then 'binned' using Interactive Binner v1.4 (Ramette 2009) with the following constraints: min = 40, max = 600, MinRFI = 0.09, window size = 1, shift size = 0.9. The resulting file contained the raw relative abundance data, which were then analysed in Primer v6 (Clarke and Gorley 2006). Both presence/absence data and relative abundance data were analysed, but since the analyses showed similar results, only relative abundance data are reported here. OTU accumulation plots for dieback-affected and healthy samples were then constructed for each of the three taxa using the Chao1 index and 9999 permutations.

To estimate the influence of dieback occurrence and plant part on microbial community composition, I constructed Bray-Curtis dissimilarity matrices (Legendre and Legendre 1998) after square-root transformation of the archaeal, bacterial and fungal OTU abundance data. The transformed data were subjected to permutational multivariate analyses of variance (PERMANOVA) using type III sum of squares, permutation of residuals under a reduced model with 9999 permutations. This was

conducted for interactions between plant part and disease status. Since the samples were gathered from multiple sites (Fig 2.1), I also tested for geographic location effects on OTU community. I constructed principle coordinates ordination (PCO) plotskm on the Bray-Curtis resemblance matrices showing samples by plant part and disease status. Finally, I conducted indicator species analysis by plant part to test whether there were OTUs significantly associated with either dieback parkinsonia (potentially pathogenic endophytes) or healthy parkinsonia (potentially protective endophytes). This was done using the statistical package IndicSpecies v1.7.4 (De Cáceres and Legendre 2009) available through R (R Core Team 2014), using 999 permutations and a 0.01 alpha significance level.

2.4 Results

2.4.1 Sampling effort

The number of bacterial OTUs recovered from the T-RFLP analysis was far fewer than the number recovered from the archaeal and fungal analyses (Fig 2.3) and for some samples there was no bacterial amplification (data not shown). With the exception of archaeal diversity in dieback plants, there were indications of OTU richness plateauing as the number of samples increased (Fig 2.3). OTU richness in dieback samples was double that observed in healthy samples for archaea and fungi, but was unaffected by plant disease status for bacteria.

2.4.2 Plant part and disease status effects

The effects of plant part, disease status and the interaction between the two, on endophyte community composition were significant for each microbial group (Table

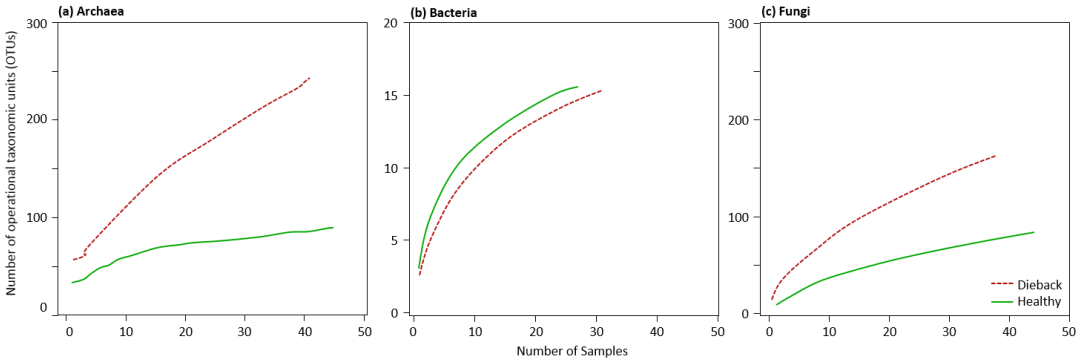


Figure 2.3 OTU accumulation curves for (a) archaea, (b) bacteria and (c) fungi by disease status of host. Broken line indicates OTUs and samples from dieback-affected *Parkinsonia aculeata*, solid line indicates healthy *P. aculeata*

2.2). There were no effects on community composition due to the geographic location of sites (data not shown). Archaeal community composition was highly structured within healthy plants, being distinctive in each plant part (Fig 2.4a). Archaeal communities in stem tips and roots was significantly different in dieback-affected plants compared to healthy plants (Table 2.3), with stem tip communities showing similarities with stem communities (Fig 2.4a). Archaeal community composition in stems was similar for healthy and dieback plants (Table 2.3). Bacterial community composition differed significantly with health status for stems and tips but not roots (Table 2.3). However, it was not as structured as archaeal communities (Fig 2.4a), with considerable unexplained variation apparent between samples (Fig 2.4b). Explanations for this pattern are limited since figure 2.4 only describes the first two axes of ordination in the PCO analysis. The archaeal data was also plotted along the other axes (data not shown) but I did not find any evidence that archaeal community structure in dieback-affected stem tips differed to that in healthy or dieback stems. Bacterial community composition showed diversity within both stem and stem tip samples for healthy plants (Table 2.3), but this is greatly reduced in dieback plants with communities becoming more homogenous throughout the plant (and possibly more like that in healthy/dieback roots; Fig 2.4b). Although fungal community composition was affected by disease status in all three plant parts (Table 2.3), it was not strongly structured (Fig 2.4c). The effect of disease status on fungal communities was most apparent in roots (along PCO axis 1 in Fig 2.4c) and stem tips (along PCO axes 2 in Fig 2.4c).

Table 2.2 PERMANOVA of the *Parkinsonia aculeata* OTU community for the main effects of disease status and plant part and the interaction between these effects

	df[a]	Pseudo-F	P-value
Archaea			
Plant part	2	6.9187	0.0001
Disease status	1	11.917	0.0001
Plant part x Disease status	2	7.1118	0.0001
Residuals	80		
Bacteria			
Plant part	2	3.0066	0.0031
Disease status	1	7.1639	0.0002
Plant part x Disease status	2	4.442	0.0001
Residuals	52		
Fungi			
Plant part	2	3.5693	0.0001
Disease status	1	3.1459	0.0004
Plant part x Disease status	2	3.2821	0.0001
Residuals	76		

[a] differences in df due to lack of amplification in some samples which were not included in the analysis

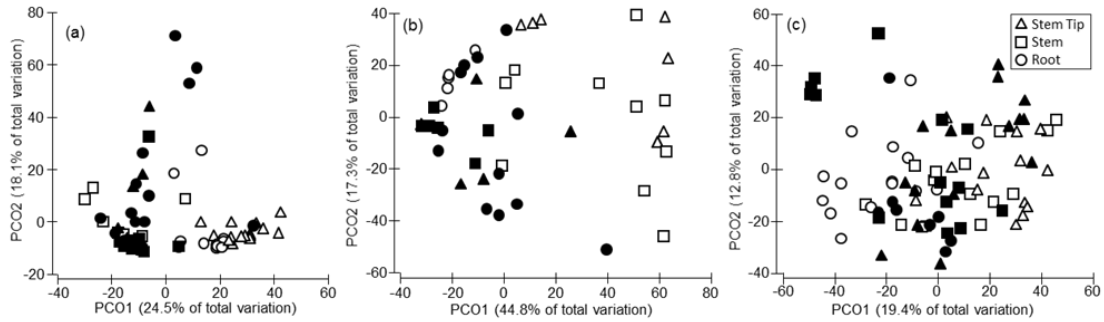


Figure 2.4 Unconstrained principle coordinates ordination (PCO) analyses showing separation along the axes by *Parkinsonia aculeata* disease status and/or plant part for (a) archaeal, (b) bacterial and (c) fungal OTU community data. Shaded symbols indicate samples from dieback-affected trees and open symbols from healthy trees.

Table 2.3 *t*-statistics from pairwise PERMANOVA comparisons testing for differences in *Parkinsonia aculeata* endophyte OTU composition in healthy compared to dieback-affected plant parts

	Archaea	Bacteria	Fungi
Stem Tip	4.18*	2.22*	1.85*
Stem	1.26	2.44*	1.70*
Root	2.64*	1.75	1.90*

* significant ($p < 0.01$)

2.4.3 Indicator species analysis

The indicator species analysis (Table 2.4) supports the patterns observed in the canonical analysis (Fig 2.4). There were 12 archaeal OTUs significantly associated with specific plant parts within healthy plants, and a further two associated with stem tips and roots (Table 2.4). Most notably there were 18 archaeal OTUs associated with roots of dieback plants, representing 8% of the archaeal community in dieback plants (Table 2.4). There were only four bacterial OTUs associated with healthy plants and one with dieback plants (Table 2.4) although this also reflected low OTU diversity (Fig 2.2). OTUs associated with health status were proportionally lowest for fungi, of which most were in stem tips (Table 2.4).

Table 2.4 The number of indicator OTUs that were significantly associated with healthy or dieback-affected *Parkinsonia aculeata*, grouped by plant part. All OTUs are only represented once, and some are indicators for multiple plant parts (e.g. Stems and Roots: S+R). Brackets indicate the percentage of OTUs which are significant indicators ($p < 0.01$).

Plant Part(s)	Archaea		Bacteria		Fungi	
	Healthy	Dieback	Healthy	Dieback	Healthy	Dieback
Stem Tip (T)	7 (3.14%)	-	2 (10.53%)	-	2 (1.12%)	3 (1.69%)
Stem (S)	3 (1.35%)	-	-	-	-	1 (0.56%)
Root (R)	2 (0.90%)	18 (8.07%)	-	-	1 (0.56%)	-
T+S	-	1 (0.45%)	2 (10.53%)	-	-	-
T+R	2 (0.90%)	1 (0.45%)	-	-	-	2 (1.12%)
S+R	-	1 (0.45%)	-	1 (5.27%)	1 (0.56%)	-
T+S+R	-	-	-	-	-	-
Total	14 (6.29%)	21 (9.42%)	4 (21.06%)	1 (5.27%)	3 (2.24%)	6 (3.37%)

2.5 Discussion

Dieback in plant populations is widely observed globally but in most cases remains difficult to explain, and its presence in invasive species is counter to general expectations (Callaway and Ridenour 2004). This study is the first to show significant difference in microbial community composition between healthy and dieback-affected plants of an invasive species. Differences were evident in all studied microbial groups (archaea, bacteria and fungi), but were strongest for archaea, which is also the least

understood group of microorganisms. Community composition was structured across plant parts, most strongly in archaea, which also showed interaction with health status. Differences in community structure was partially explained by OTUs that were affiliated either with healthy or dieback-affected plants. This provides some support for my hypothesis that both pathogenic and potentially protective endophytes are involved in parkinsonia dieback. These results extend current thinking, that multiple organisms within multiple taxonomic domains may be involved in the onset of disease or disease suppression in plants (Coats and Rumpho 2014).

Most studies on microbial endophyte communities and dieback investigate one specific microbial taxon, typically fungi (Wilson and Pitkethley 1992; Diplock et al. 2006; Kowalski and Holdenrieder 2009; Haque 2015; Ismail et al. 2012; Sacdalan et al. 2012), although there are exceptions (Mendes et al. 2011; Knief et al. 2012; Ma et al. 2013; Oliveira et al. 2013; Ruppel et al. 2013). My study found a very diverse microbial community (as assessed using OTUs) whose structure differed with plant health status. OTU diversity was high for archaea and fungi, and low for bacteria. However, the lack of resolution in the bacterial OTU data might be due to the use of only one restriction enzyme, since one bacterial OTU may represent more than one bacterial species (Avaniss-Aghajani et al. 1996). At least for fungi, high endophytic fungal biodiversity has previously been observed in plants, which can host hundreds of fungal species or genotypes (Hawksworth 2001). High diversity of bacterial and archaeal endophytes was also expected (Ma et al. 2013). Archaeal and fungal OTUs were twice as numerous per sample in dieback-affected plants compared to those in healthy plants. This may be due to the subsequent colonisation of dieback-affected tissue by opportunists or saprotrophs (Arnold 2007), although the indicator species

analysis did not show a consistent pattern to support this. Alternatively, I suggest that the observed OTUs may have to co-occur to trigger dieback symptoms.

Microbial communities can differ by plant part due to their role as endophytes (e.g. production of alkaloids as herbivory inhibitors in above ground parts; Alexopoulos et al. 1996), the way in which these microorganisms are transmitted between hosts (via wind dispersal to aerial tissues or animal dispersal to roots or flowers), or whether infection by these microorganisms is systemic or local (Rudgers and Orr 2009; Knief et al. 2012; Thongsandee et al. 2012). Expectations of within-plant structuring were supported in my study irrespective of health status. Changes in community composition with plant health status differed according to plant part and endophyte taxonomic group. Structuring was strongest for archaea and not as obvious for fungi, a comparison which has not been previously tested in the literature. Structuring of fungal community composition across plant parts may be the result of the function of fungal species differing with plant part (Rudgers and Orr 2009). However, since the majority of OTUs were not restricted to specific plant parts, I suggest that community structure across plant parts was driven by the presence or absence of the indicator OTUs. Most previous studies on dieback in parkinsonia have taken fungal isolations from stems (Diplock et al. 2006; Toh 2009). These and many other dieback studies could therefore be biased by limiting sampling to a particular plant part. Archaeal communities in stems were similar, regardless of plant disease status, but changed substantially in roots and stem tips of dieback-affected trees, with community structure in stem tips converging with that in stems. The functional significance of this, if any, is yet to be determined.

Endophytes in invasive plants can enhance their competitiveness and invasiveness in non-native ranges (Callaway et al. 2008; Mangla and Callaway 2008; Rudgers and Orr 2009; Rout et al. 2013) and the protective role of fungi and bacteria have been observed in other systems (Newcombe et al. 2009; Rout et al. 2013). The apparent absence of dieback in parkinsonia from the native range suggests that any potential dieback-causing pathogen(s) would have been acquired in Australia, contrary to the enemy-release hypothesis (Keane and Crawley 2002), or that endophytes protecting parkinsonia from pathogens in the native range are absent in Australia, which aligns more with the endophyte-enemy release hypothesis (Evans 2008). The association in my study of archaea, bacteria and fungi with both healthy and dieback-affected parkinsonia provides support that the relevant indicator OTUs may have either pathogenic or protective roles that contribute to, or prevent, dieback in Australia, potentially as new associations. Most notable was a diverse archaeal community associated with dieback-affected roots. The OTUs associated with dieback-affected plants may be opportunists that take over the niche left by those endophytes which have been displaced as a result of dieback-causing organisms. Conversely, these OTUs may be pathogens in their own right and infected parkinsonia upon introduction to Australia, causing dieback either before or after the loss of OTUs unique to healthy parkinsonia.

I found preliminary support for the presence of potentially protective archaeal endophytes in healthy stem tips. I also found evidence for potential protective endophytes from the fungi and bacteria, as expected (although it should be noted that these OTUs might also represent endophytes which are neutral and are displaced by opportunists with the onset of dieback). Previous experimental studies have shown that

some fungal endophyte species do limit damage to their hosts by pathogens (Arnold et al. 2003) and some bacterial endophytes have a positive effect on disease-affected hosts (Bacon and Hinton 2007). However, the potential pathogenic and protective organisms identified in my study need to be identified, and whether they are causally related to dieback (or confer protection) is yet to be ascertained. To determine this, a sequencing-based approach is required, followed by isolation and pathogenicity trials. Since multiple taxa were identified as potential causal agents of dieback or to confer protection, a next step might be to utilise innovative inoculation strategies to test entire communities. This could be done in a minimally-invasive inoculation trial by inoculating healthy plants using ground-up material from different healthy or dieback-affected plant parts. Furthermore, it is important to compare endophyte community composition of invasive Australian parkinsonia in this study, to that of parkinsonia from native populations in meso-America. I also note that due to the specificity of the primers selected for this study, communities other than those characterised might also contribute to dieback occurrence.

Historically, the focus in dieback studies has been on potential fungal pathogens, but my results have shown that a diverse community of archaea are present in both healthy and dieback-affected parkinsonia. Despite archaeal communities showing the most significant difference between healthy and dieback-affected parkinsonia, very few archaeal species have been identified in the literature – e.g. methanogens associated with human or animal health – and even fewer have been cultured (Schleper et al. 2005). Without representative cultures of these species, it is difficult to observe or analyse their ecosystem function, physiology or pathogenic potential (Killham and Prosser 2007). Additionally, no known archaeal pathogen has been identified or shown

to be the primary cause of disease in any plant or animal (Cavicchioli 2011), so I can only make assumptions about the significance of the results for archaea presented in this study. At most I can conclude that archaea are more involved in dieback than previously thought, and should not be ignored in future studies. From this perspective, the next step might be to evaluate gene expression associated with archaea in healthy and dieback-affected tissue in order to infer ecological functions.

2.6 Conclusion

My study demonstrated the value of taking a community-level analysis approach to investigating the complex phenomenon that is dieback, which has allowed me to analyse the composition and structure of these communities. Using this approach, I have shown that there are strong correlations between changes in endophytic archaeal and fungal community structure, and the occurrence of dieback in parkinsonia, and that the degree of structuring also varies by plant part. Nevertheless, one difficulty is that very little is known about one of these domains: archaea, but I know now from these results that archaea should be considered an important part of the dieback phenomenon. Despite this, a number of OTUs have been implicated as potentially pathogenic or protective endophytes. These results open new avenues for research into understanding the dieback phenomenon. For invasive species this may also lead to novel management solutions.

3

TRIGGERING DIEBACK IN AN INVASIVE PLANT: ENDOPHYTE DIVERSITY AND PATHOGENICITY

3.1 Abstract

Dieback causes a progressive reduction in plant population health, resulting in the death of plant parts and often plant death. It is prevalent in many invasive woody weeds in Australia and has been suggested as a potential mechanism for biocontrol of these species. *Parkinsonia aculeata* is one such invasive tree in northern Australia. It has naturalised across a wide range of climatic zones and some populations have been heavily reduced by dieback occurrence. The cause(s) of dieback in parkinsonia remain elusive, although fungal endophytes have been previously implicated.

In this study, I characterised the culturable fungal endophyte community of healthy and dieback-affected parkinsonia using culture-based techniques, and identified cultured isolates via amplicon sequencing of the internal transcribed spacer (ITS) of the rDNA operon. Eight isolates, identified as pathogens, were selected for a 10-week pathogenicity trial, including water stress treatments, on parkinsonia seedlings.

I isolated a taxonomically diverse fungal community from parkinsonia, representing 54 unique species from 25 families. Communities were similar across healthy and dieback-affected plants, but differed by plant tissue. Of the eight putative pathogenic isolates tested in the pathogenicity trial, inoculation with *Lasiodiplodia*

pseudotheobromae, *Botryosphaeria dothidea* and *Pestalotiopsis mangiferae* resulted in the largest lesions, but systemic infection or dieback-like symptoms were not observed in any treatment despite plant stress being induced by drought or inundation. I concluded that inoculation of parkinsonia with the tested putative fungal pathogens is unlikely to result in dieback, which has implications for future work in biocontrol of parkinsonia.

3.2 Introduction

Parkinsonia aculeata (parkinsonia, family: Fabaceae) is a spiny, leguminous, thicket-forming tree, native to the Americas, but a serious invader in northern Australia (Thorp & Lynch 2000). The management of parkinsonia is expensive and labour-intensive and usually involves the use of herbicides followed by manual removal of dead trees (Deveze et al. 2004). Since the most recent estimates of population extent exceeds 3 million ha (van Klinken et al. 2009), more efficient and autonomous control methods are sought. The most promising mechanism for parkinsonia control has been the occurrence of dieback in some populations (van Klinken et al. 2009). I define dieback as a progressive reduction in plant health, resulting in the death of plant parts, often followed by outright tree death that may result in local population decline, either as a gradual or sudden occurrence (Mueller-Dombois 1987). Parkinsonia dieback begins with defoliation, followed by browning of the stems starting at the stem tips, and eventually usually causes whole tree mortality (Diplock 2016). Dieback has been observed in a number of Australian Weeds of National Significance (WONS), but has not been observed in locally-occurring native species (Wilson & Pitkethley 1992; van Klinken et al. 2009; Raghavendra et al. 2017) and there is no evidence that dieback

occurs in these WONS' native ranges. If the cause of parkinsonia dieback is identified there is potential for its use as a self-sustaining biological control agent to be used alongside other control methods.

Plants host a diverse community of fungal species, the vast majority of which are mutualistic or benign endophytes but some may be pathogenic or saprophytic (Hawksworth 2001). In previous work, endophyte communities (archaea, bacteria and fungi) were analysed for correlation with dieback occurrence in parkinsonia using terminal restriction fragment length polymorphism (T-RFLP) analysis (Chapter 2; Steinrucken et al. 2016). Bacterial community composition was not significantly correlated to parkinsonia dieback, and although significant correlations with archaeal OTUs and dieback were observed, little is known about archaeal endophytes and few archaea have ever been cultured (Schleper et al. 2005). With regard to endophytic fungal communities, in their previous work Steinrucken et al. (2016; Chapter 2) also found a significant correlation between fungal community composition and dieback occurrence, suggesting the involvement of multiple fungal endophytic species which differ in composition across plant parts. Although this work demonstrated the potential involvement of fungal endophytes in parkinsonia dieback, the method of community fingerprinting with T-RFLP did not allow assignment of taxonomy or ecological roles.

Diplock (2016) and Toh (2009) isolated, identified and tested a number of endophytic fungal pathogens reported to be involved in parkinsonia dieback. In their studies one species stood out as a potential causal agent: *Lasiodiplodia pseudotheobromae*. This species has been implicated in dieback of other non-native tree species globally including multiple Australian leguminous and woody WONS (Toh 2009; Haque 2015; Sacdalan 2015; Diplock 2016), *Prunus* spp. in South Africa

(Damm et al. 2007), mango in Egypt (Ismail et al. 2012) and *Acacia* spp. in Australia (Adair et al. 2009). In testing the pathogenicity of *L. pseudotheobromae* on parkinsonia, Toh (2009) inoculated sterile vermiculite substrate with colonised millet seed before transplanting parkinsonia seedlings into the mixture one week post-emergence. This study showed that *L. pseudotheobromae* (isolate NT039; Genbank Accession no. KX893409) was the most virulent of 83 tested, including other Botryosphaeriaceae. A concurrent four-year field trial on adult parkinsonia trees involved inserting colonised millet seed into holes drilled into the base of the trees (Diplock 2016). On some sites, the treatment resulted in lesion formation by *L. pseudotheobromae*, but was unable to recreate dieback symptoms or tree mortality. The study was further complicated by wounding reactions, bacterial contamination and adverse environmental conditions including a flood and fire (Diplock 2016).

Although it has been implicated in disease and dieback of woody hosts, *L. pseudotheobromae* has also been associated with healthy hosts as a non-pathogenic endophyte (Slippers & Wingfield 2007; Jami et al. 2013). A number of other Botryosphaeriaceae species have both pathogenic and endophytic associations with their host and many can be triggered to become pathogenic in the presence of abiotic factors such as water stress (Schulz et al. 1998; Mehl et al. 2013). These species are termed ‘latent pathogens’: microorganisms that remain benign or mutualistic until triggered to be pathogenic by an external factor such as environmental stress to the host, or co-infection by a more virulent pathogen (Slippers & Wingfield 2007). It is therefore difficult to predict whether endophytic fungi could be pathogenic under certain circumstances or if they are simply opportunistic, becoming pathogenic or saprophytic when the plant is stressed.

The interaction between the host, its environment and pathogens plays an integral part in the occurrence of disease (Agrios 2005). Conceptual models described by Houston (1992), Manion (1991) and Whyte et al. (2016) attempt to characterise the interactions between these inciting and contributing factors and how they relate to dieback occurrence. This complexity means it is unclear whether symptoms of dieback in parkinsonia are the primary cause of dieback or are the results of secondary infections by opportunistic pathogens, triggered by other biotic or abiotic factors. Parkinsonia and many other dieback-affected WONS are spread across regions of northern Australia that are subject to long-term drought and intermittent flooding, so it is possible that dieback is partly triggered by water availability. This has been observed in the decline of black alder (*Alnus glutinosa*) by the pathogen *Phytophthora alni*, whose virulence is associated with flooding episodes (Webber et al. 2004). Similarly, in drought-stressed oak trees, a number of ascomycete pathogens such as *Biscogniauxia mediterranea* take advantage of weakened host tissues and become more virulent, causing decline in several species (La Porta et al. 2008).

In this study I describe the culturable fungal endophyte community in healthy and dieback-affected parkinsonia from regions previously shown to have dieback/endophyte community correlations. I also identify putative pathogens to test against parkinsonia seedlings exposed to excessive, limiting, or optimal water treatments in a glasshouse inoculation study. I consequently address the following question: Can I induce systemic infection and dieback-like symptoms in parkinsonia, by inoculating plants with the selected putative fungal pathogens, and will water stress enhance this effect?

3.3 Materials and method

3.3.1 Sampling, identification and analysis of the fungal endophyte community

I sampled sub-dermal tissue from three roots, three secondary stems and three stem tips and seeds (when available) of five trees in each of three healthy and three dieback-affected parkinsonia populations near Charters Towers, Queensland (Table S2.1, Appendix A). Endophyte communities have been previously shown to be structured by plant part (Rudgers & Orr 2009; Chapter 2, Steinrucken et al. 2016). These plant parts were chosen to ensure any stratification of endophyte communities across an individual plant was accounted for, and since leaves and seeds were not always available, stem tips were collected. Sampling was conducted in March 2013 and repeated on the same trees in May 2013 in order to ensure sampled healthy trees did not develop dieback-like symptoms between sampling periods (they did not) and to avoid isolating a community of endophytes representative of only one point in time. Between sampling of different trees and plant parts, all tools were sterilized using 50% NaClO and then rinsed with sterile water. Samples from different plant parts and trees were stored in separate paper bags at 5°C for up to 48 h until processing. Plant parts were vigorously pre-washed in distilled H_2O for 20 s. An ethanol (70%) and UV-sterilized laminar flow cabinet was used for subsequent steps. For stems, stem tips and seeds a three-stage ethanol-bleach-ethanol surface sterilization method was used as recommended by Bills (1996). Seeds were then imbibed in 95°C sterile, distilled H_2O for 12 h. Roots were washed for 30 s in sterile, distilled H_2O containing 0.1% Tween-20™ (Sigma-Aldrich, St Louis, MO, USA) since harsher sterilization techniques are not recommended for roots (Thorn et al. 2007). All samples were blotted dry with

sterile filter paper and surface sterilization was checked by sliding tissue over the surface of 50% Potato Dextrose Agar amended with streptomycin (sPDA; 35 mg L^{-1}) and incubating at 30°C for seven days (Bacon & Hinton 2007). The bark of stem tips and stems, the seed coat of imbibed seeds and a small portion of root cortex was then removed using a sterile scalpel. Three tissue plugs (3-5 mm^2) from each sample were placed on sPDA media and were maintained at room temperature in the dark for seven days. Isolates were subcultured daily, or when mycelial growth was observed.

Once pure fungal isolates were obtained, genomic DNA was isolated using a MO BIO Powersoil® DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA), and identified via sequencing of amplified ITS rDNA amplicons. PCR reactions were undertaken in a total volume of 20 µl and consisted of 0.2 U BIOTAQ™ DNA polymerase (Bioline, London, UK), 10x NH_4 Buffer (2 uL per reaction), $MgCl_2$ (60 mM per reaction), dNTPs (50 mM each per reaction), ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') primers (4 mM each per reaction; Gardes & Bruns 1993), and 2 uL extracted DNA per reaction. PCR reactions were run at 94°C for 3 min; 34 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min; and a final extension step of 72°C for 10 min. PCR products were purified using the Wizard®SV Gel and PCR Clean-Up System (Promega Madison, WI, USA), and sequenced by Sanger sequencing using the same forward primer (ITS1), in one direction, at the Hawkesbury Institute for the Environment using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) as per the manufacturer's instructions. Sequence chromatograms were analysed in Geneious® V6.1.6 (Biomatters Ltd., Auckland, New Zealand) and underwent BLASTn searches on the National Center for Biotechnology Information (NCBI) nucleotide

database on 6th May 2016. Closest match was determined by comparing maximum sequence length and lowest e-values. The cut-off point for assigning species names to closest match on the database was 97–100% identity; genus names were 94–97% identity; family name 90–94% identity; and sequences with lower identity with members of several families were identified only at the ordinal level (Vega et al. 2010). Sequences sharing less than 85% identity with closest match sequences or sharing higher identity to unidentified sequences in GenBank, were identified only to class or phylum (Vega et al. 2010). All taxonomic classifications required >95% query coverage and sequences with the same % ID for different organisms were identified to the closest common taxonomic level. I aligned unique sequences using MUSCLE Alignment (Edgar 2004) with eight iterations over 456 bases as implemented in Geneious® v8.1 and constructed a neighbour-joining tree based on the UPGMA model with a *Phytophthora ramorum* voucher sequence as the outgroup (Fig 3.1).

3.3.2 Glass house pathogenicity trial

One month old parkinsonia seedlings grown from seed and collected from healthy populations in Charters Towers (QLD) were re-potted in 0.8 L free-draining square plastic pots in media consisting of 8 parts fine/medium pit sand, 1 part Mikskaar White Peat and 1 part Mikskaar Professional® substrate 250 (pH 5.2–6; Mikskaar AS, Tallinn, Estonia) and amended with 2.8 g/L Basacote® Plus Prilled slow release fertilizer, 1.5 g/L Osmoform® slow release fertiliser (Everris International B.V., Geldermalsen, The Netherlands) and 0.2 g/L SierraForm GT® Anti Stress (Everris International B.V). Plants were grown in an evaporatively-cooled glasshouse (21–27°C) watered every second day, fertilised monthly with All Purpose Soluble

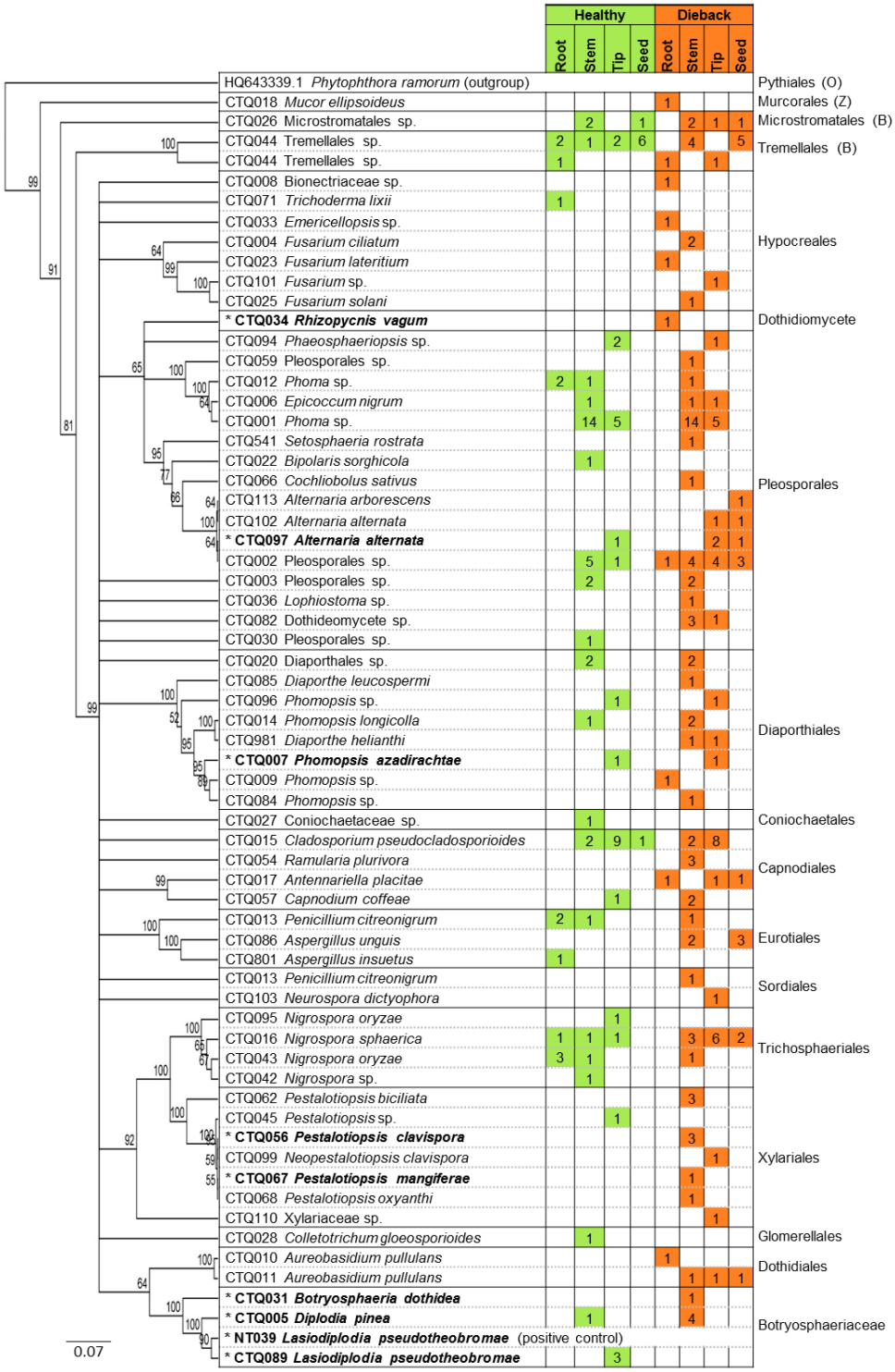


Figure 3.1 Neighbour-joining tree (TreeBASE submission no. 20057) based on the UPGMA Model constructed using Geneious® v8.1 on a 458 bp length MUSCLE alignment of ITS1-ITS4 sequences from representative endophytic fungal taxa (Table 3.2) including the number of those isolates isolated from each plant tissue type. Bootstrap values (n=1000 replicates) are shown on the intercepts. Outgroup is a *Phytophthora ramorum* (HQ643339.1). Isolates used in the pathogenicity trial are in bold and indicated with *. Ordinal groups indicated on right. All isolates are ascomycetes apart from those in orders marked with (B) basidiomycete, (Z) zygomycete and (O) Oomycete.

Fertilizer (Hortico®, Padstow NSW, Australia), and treated for mites, thrips, scale and powdery mildew with Crown® SureGrow (Everris International B.V) at 2.5 mL/L at 3 and 6 months, and weekly with predatory mites. After ten months, 258 healthy plants were selected for this trial, and randomly arranged in a temperature-controlled glasshouse. Plants were not fertilized after this point and, during the trial, glasshouse pests were controlled only using predatory mites (*Neoseiulus californicus*; Bugs for Bugs, Mundubbera, QLD Australia*)*.

Eighty-six plants (the control group) were watered as before with 100 mL water every second day; 86 were placed in white plastic trays with the water level maintained at 5 cm depth, inundating the roots and the third group of 86 plants were drip-fed 8–10 mL water twice a week to simulate drought conditions. Glasshouse conditions were set at 28°C during the day, 21°C at night and 60% constant humidity for one week before inoculation and then a further ten weeks from January to March 2015 at the Ecosciences Precinct, Brisbane, Australia.

Eight fungal isolates were chosen from the identified endophytic species, identified via sequencing as species previously reported to be pathogenic and cause dieback in their host (Table 3.1; see Fig S3.1 in Appendix A for decision flowchart on how the eight isolates were chosen). Representing five families (Table 3.1), all but one (CTQ089 *L. pseudotheobromae*) were isolated from dieback-affected plants. For a positive control I also included a *L. pseudotheobromae* isolate (NT039), obtained from the University of Queensland culture collection, which had been isolated and tested in previous dieback studies, and shown to be pathogenic on parkinsonia (Toh 2009; Diplock 2016). All nine isolates tested were ascomycetes. I tested the effect of three

Table 3.1 Fungal species information for identified isolates used in the pathogenicity trial

Isolate	GenBank Accession	Host[a]	Species	Family	Previous implications in host dieback
CTQE056	KT699873	D: S	*Pestalotiopsis clavispora*	Amphisaeriaceae	Mango (Ismail et al. 2013), blueberry (González et al. 2012)
CTQE067	KT699874	D: S	*Pestalotiopsis mangiferae*	Amphisaeriaceae	Mango (Johnson et al. 1992), Chinese Bayberry (Chen et al. 2013)
CTQE005	KT699869	D: S, H: S	*Diplodia pinea*	Botryosphaeriaceae	*Pinus* spp. (de Wet et al. 2000)
CTQE089	KT699875	H: T	*Lasiodiplodia pseudotheobromae*	Botryosphaeriaceae	Invasive trees in Australia (Haque 2015; Sacdalan 2015; Toh 2009)
CTQE031	KT699871	D: S	*Botryosphaeria dothidea*	Botryosphaeriaceae	Fruit and nut trees (Slippers and Wingfield 2007)
NT039 [b]	KX893409	D:S	*Lasiodiplodia pseudotheobromae*	Botryosphaeriaceae	Isolated from dieback-affected *Parkinsonia aculeata* (Diplock 2016)
CTQE034	KT699872	D: R	*Rhizopycnis vagum*	Morosphaeriaceae	Musk-melon and *Medicago sativa* (Armengol et al. 2003)
CTQE097	KT699876	D: TE, H: T	*Alternaria alternata*	Pleosporaceae	Kiwi (Tsahouridou and Thanassoulopoulos 2000), *Fraxinus excelsior* (Bakys et al. 2009), grape (Ferreira et al. 1989).
CTQE007	KT699870	D: T, H: T	*Phomopsis azadirachtae*	Valsaceae	*Azadirachta indica* (Zwolinski et al. 1990)

[a] Disease status (D = dieback, H = healthy): plant part (E = seed, T = stem tip, S = stem, R = root) of *Parkinsonia aculeata* tree from which this species was isolated; [b] Obtained from the University of Queensland culture collection

water stress treatments (drought, inundation and 'normal') on the pathogenicity of the selected fungal isolates and the growth of twelve month-old parkinsonia seedlings.

The nine isolates were passaged through Granny Smith apples to ensure they had not lost their pathogenicity due to prolonged subculturing (Erwin & Ribeiro 1996), and then re-isolated on PDA without streptomycin for use in subsequent inoculations. After seven days, underbark inoculation was carried out on surface-sterilised stems at approximately 7 cm above soil surface. Incisions of 8–10 mm long were made with a sterile scalpel blade. A 5 mm^2 mycelial plug was fully inserted into the wound and the stem was bound with Parafilm® (Bemis, Oshkosh, WI, USA). The negative control (five of the 86 plants in each water treatment) consisted of a sterile PDA plug. Plants were arranged randomly within a split-plot design, with each fungal inoculant (subplot) occurring once nested within each water treatment (main plot), each with

nine replicates (therefore, 9 isolates x 9 replicates = 81, + 5 negative controls = 86 plants x 3 water treatments = 258 plants in total), using isolate and water treatment as fixed effects and the nesting of 'subplot' in 'plot' as a random effect.

Immediately prior to inoculation and at the end of the trial plant growth by height (cm) from the soil surface and stem girth (mm) at the site of inoculation was measured. I also monitored any damage by mites (% foliage damage). At the conclusion of the trial (10 weeks following inoculation) plants were harvested at the root collar. After harvest, lesions were bisected with a sterile blade. Underbark lesion size, identified by discolouration from the site of inoculation, and any scarring was measured. To confirm that lesions were associated with the inoculated pathogen and to look for any systemic infection by the inoculated pathogen, a small amount of tissue was sampled from the lesion or cut site of three plants in each replicate group; 1 cm above and below the lesion; and 10 cm above the lesion. Tissue samples were plated on sPDA and incubated for 1 week at room temperature in the dark, and isolates were identified via ITS sequencing as above. Roots were freed from soil by carefully running them under water, being careful not to wash away fine roots and as with the above-ground parts, were placed in paper bags and dried in an oven at 60°C for 14 days. I recorded the dry weight of above and below-ground parts.

I tested the effects of water treatment and inoculated isolate on lesion length and three measures of plant health: the change in height and stem circumference over the ten-week inoculation trial and post-harvest dry mass at the conclusion of the trial. Data were treated as a split-plot design (Schwarz 2015) in R (R Core Team 2016) using the 'lme4' (Bates et al. 2015) and 'lmerTest' (Kuznetsova et al. 2016) packages for analysis of variance (ANOVA) with Kenward-Roger approximation for degrees of

freedom. ANOVAs were followed by post-hoc testing using Tukey HSD (Tukey 1949) using the ‘multcomp’ (Hothorn et al. 2008) package.

3.4 Results

3.4.1 Fungal endophytes in healthy and dieback-affected *Parkinsonia aculeata*

I cultured a total of 213 fungal isolates from multiple plant parts in healthy and dieback-affected parkinsonia and identified 54 unique operational taxonomic units (OTUs) through DNA sequencing of the ITS rDNA region (Table 3.2). The identified isolates (GenBank Accessions KT699870–KT699873; KX893353–KX893409) represented 16 fungal orders and 25 families. The majority (90%) were ascomycetes while seven basiodiomycetes and one zygomycete were also isolated. Fungi from the order Pleosporales had the greatest number of representative isolates (Fig 3.2) with a total of 85 isolates, but only 5 unique OTUs. The Xylariales were well represented (12 isolates, 9 unique species), as were the Hypocreales (10 isolates, 8 unique species) and Eurotiales (9 isolates, 5 unique species). I recovered 31 isolates from samples collected in March 2013 and 58 isolates from samples collected in May 2013. I isolated the greatest number of endophytes from parkinsonia stems with 28% of isolates from dieback trees and 11% from healthy trees (Fig 3.2). This was followed by the branch tips (17% from dieback, 12% from healthy), the seeds (9% dieback, 4% healthy) and roots (4% dieback, 5% healthy). I isolated four species of Botryosphaeriaceae (8 isolates), and although four of these isolates were *L. pseudotheobromae*, this species was only isolated from healthy parkinsonia (Table 3.2). Overall, I isolated more endophytes from dieback-affected parkinsonia (61%) compared to healthy parkinsonia

Table 3.2 Endophytes isolated in this study and identified by ITS sequencing to closest match (CM) in the NCBI nucleotide database. When the sequences of two or more isolates were identical, a representative isolate was chosen and taxonomic identities were assigned[†] (GenBank Accessions KX893353-KX893409; KT699869-KT699876)

Isolate[a]	CM Order	CM Family	CM Species	CM Accession	% Similarity	% Coverage	Rep. isolate	Rep. isolate identification[†]	Assigned Accession
CTQE031[b]	Botryosphaeriales	Botryosphaeriaceae	*Botryosphaeria dothidea*	LC120711	100	100	CTQE031	*Botryosphaeria dothidea*	KT699871
CTQE005[b]			*Diplodia pinea*	KU319042	98	100	CTQE005	*Diplodia pinea*	KT699869
CTQE064			*Diplodia pinea*	KU319042	97.8	100	CTQE005	*Diplodia pinea*	KT699869
CTQE065			*Diplodia pinea*	KU319042	97.7	100	CTQE005	*Diplodia pinea*	KT699869
CTQE089[b]			*Lasiodiplodia pseudotheobromae*	KT075144	99.8	100	CTQE089	*Lasiodiplodia pseudotheobromae*	KT699875
CTQE090			*Lasiodiplodia pseudotheobromae*	KT075144	100	100	CTQE089	*Lasiodiplodia pseudotheobromae*	KT699875
CTQE092			*Lasiodiplodia pseudotheobromae*	KT075144	100	100	CTQE089	*Lasiodiplodia pseudotheobromae*	KT699875
CTQE017	Capnodiales	Antennulariellaceae	*Antennariella placitae*	JN116688	97.6	100	CTQE017	*Antennariella placitae*	KX893367
CTQE024			*Antennariella placitae*	JN116688	97.6	100	CTQE017	*Antennariella placitae*	KX893367
CTQE057	Capnodiales	Capnodiaceae	*Capnodium coffeae*	DQ491515	99.2	96.08	CTQE057	*Capnodium coffeae*	KX893384
CTQE091			*Capnodium coffeae*	DQ491515	99.2	96.06	CTQE057	*Capnodium coffeae*	KX893384
CTQE015	Capnodiales	Mycosphaerellaceae	*Cladosporium pseudocladosporioides*	KT877407	100	100	CTQE015	*Cladosporium pseudocladosporioides*	KX893365
CTQE054			*Ramularia plurivora*	KJ504782	100	100	CTQE054	*Ramularia plurivora*	KX893383
CTQE027	Coniochaetales	Coniochaetaceae	*Lecythophora hoffmannii*	JN942898	92.7	98.68	CTQE027	Coniochaetaceae	KX893374
CTQE981	Diaporthales	Valsaceae	*Diaporthe helianthi*	KM979834	99.2	100	CTQE981	*Diaporthe helianthi*	KX893408
CTQE085			*Diaporthe leucospermi*	KT323120	100	100	CTQE085	*Diaporthe leucospermi*	KX893394
CTQE020			*Diatractium cordianum*	EU541488	87.6	98.21	CTQE020	Diaporthales	KX893369
CTQE007[b]			*Phomopsis azadirachtae*	KJ427811	100	99.07	CTQE007	*Phomopsis azadirachtae*	KT699870
CTQE014			*Phomopsis longicolla*	FJ462759	99.3	100	CTQE014	*Phomopsis longicolla*	KX893364
CTQE009			*Phomopsis* sp.	JQ341094	95.8	100	CTQE009	*Phomopsis* sp.	KX893359
CTQE084			*Phomopsis* sp.	KP006360	99.8	100	CTQE084	*Phomopsis* sp.	KX893393
CTQE096			*Phomopsis* sp.	DQ780461	98	100	CTQE096	*Phomopsis* sp.	KX893398
CTQE010	Dothideales	Dothioraceae	*Aureobasidium pullulans*	FJ744598	99.8	100	CTQE010	*Aureobasidium pullulans*	KX893360
CTQE011			*Aureobasidium pullulans*	JQ235065	100	100	CTQE011	*Aureobasidium pullulans*	KX893361
CTQE034[b]	Dothidiomycete	Dothidiomycete	*Rhizopycnis vagum*	KF494167	99.9	100	CTQE034	*Rhizopycnis vagum*	KT699872
CTQE801	Eurotiales	Trichocomaceae	*Aspergillus insuetus*	NR_131292	100	100	CTQE801	*Aspergillus insuetus*	KX893407
CTQE086			*Aspergillus unguis*	KC478524	99.2	100	CTQE086	*Aspergillus unguis*	KX893395
CTQE013			*Penicillium citreonigrum*	KT316706	99.6	100	CTQE013	*Penicillium citreonigrum*	KX893363
CTQE076			*Penicillium citreonigrum*	KT316706	100	100	CTQE013	*Penicillium citreonigrum*	KX893363
CTQE079			*Penicillium citreonigrum*	KT316706	100	100	CTQE013	*Penicillium citreonigrum*	KX893363
CTQE111			*Penicillium citreonigrum*	KT316706	98.2	100	CTQE013	*Penicillium citreonigrum*	KX893363
CTQE028	Glomerellales	Glomerellaceae	*Colletotrichum gloeosporioides*	KU820630	100	100	CTQE028	*Colletotrichum gloeosporioides*	KX893375

Isolate[a]	CM Order	CM Family	CM Species	CM Accession	% Similarity	% Coverage	Rep. isolate	Rep. isolate identification[†]	Assigned Accession
CTQE008	Hypocreales	Bionectriaceae	*Clonostachys rosea*	KR093840	91.8	99.81	CTQE008	Bionectriaceae	KX893358
CTQE071		Hypocreaceae	*Trichoderma lixii*	KU934235	100	100	CTQE071	*Trichoderma lixii*	KX893390
CTQE033		Hypocreales	*Emericellopsis* sp.	KF1915990	100	100	CTQE033	*Emericellopsis* sp.	KX893377
CTQE004		Nectriaceae	*Fusarium ciliatum*	HQ897818	100	100	CTQE004	*Fusarium ciliatum*	KX893356
CTQE060			*Fusarium ciliatum*	HQ897818	100	100	CTQE004	*Fusarium ciliatum*	KX893356
CTQE023			*Fusarium lateritium*	JN198452	100	10	CTQE023	*Fusarium lateritium*	KX893371
CTQE025			*Fusarium solani*	KF918565	100	100	CTQE025	*Fusarium solani*	KX893372
CTQE101			*Fusarium* sp.	KU881904	95.5	100	CTQE101	*Fusarium* sp.	KX893401
CTQE026	Microstromatales	Microstromataceae	*Microstromatales*	EF060728	96	99.67	CTQE026	Microstromatales	KX893373
CTQE018	Mucorales	Mucoraceae	*Mucor ellipsoideus*	NR_111683	99.6	100	CTQE018	*Mucor ellipsoideus*	KX893368
CTQE082	Pleosporales	Didymellaceae	*Leptosphaeria senegalensis*	KJ439197	83.8	89.81	CTQE082	Dothideomycete	KX893392
CTQE083			*Leptosphaeria senegalensis*	KJ439197	85	87.29	CTQE082	Dothideomycete	KX893392
CTQE036	Pleosporales	Lophiostomataceae	*Lophiostoma* sp.	GQ254683	99.4	100	CTQE036	*Lophiostoma* sp.	KX893378
CTQE030			*Massarina armatispora*	AF383955	88.8	98.61	CTQE030	Pleosporales	KX893376
CTQE002		Pleosporaceae	*Alternaria alternata*	KU041713	100	100	CTQE002	*Alternaria alternata*	KX893354
CTQE097[b]			*Alternaria alternata*	KU041713	99	100	CTQE097	*Alternaria alternata*	KT699876
CTQE102			*Alternaria alternata*	KT192219	99.9	100	CTQE102	*Alternaria alternata*	KX893402
CTQE113			*Alternaria arborescens*	KP942903	98.3	100	CTQE113	*Alternaria arborescens*	KX893405
CTQE022			*Bipolaris sorghicola*	KU232899	100	100	CTQE022	*Bipolaris sorghicola*	KX893370
CTQE066			*Cochliobolus sativus*	JQ753975	100	100	CTQE066	*Cochliobolus sativus*	KX893387
CTQE541			*Setosphaeria rostrata*	KT933715	100	100	CTQE541	*Setosphaeria rostrata*	KX893406
CTQE006		Pleosporales	*Epicoccum nigrum*	KR095197	100	100	CTQE006	*Epicoccum nigrum*	KX893357
CTQE059		*incertae sedis*	*Peyronellaea glomerata*	JN850981	90	100	CTQE059	Pleosporales sp.	KX893385
CTQE094			*Phaeosphaeriopsis* sp.	KP230840	99.2	100	CTQE094	*Phaeosphaeriopsis* sp.	KX893396
CTQE001			*Phoma* sp.	FJ985695	99.8	100	CTQE001	*Phoma* sp.	KX893353
CTQE039			*Phoma* sp.	FJ985695	99.8	100	CTQE001	*Phoma* sp.	KX893353
CTQE088			*Phoma* sp.	FJ985695	99.8	100	CTQE001	*Phoma* sp.	KX893353
CTQE961			*Phoma* sp.	FJ985695	99.8	100	CTQE001	*Phoma* sp.	KX893353
CTQE012			*Phoma* sp.	KM259932	100	100	CTQE012	*Phoma* sp.	KX893362
CTQE080			*Phoma* sp.	KM259932	100	100	CTQE012	*Phoma* sp.	KX893362
CTQE003			*Pleosporales*	KR909157	99.4	100	CTQE003	Pleosporales	KX893355
CTQE098	Sordariales	Chaetomiaceae	*Chaetomium* sp.	KM520348	100	100	CTQE098	*Chaetomium* sp.	KX893399
CTQE103		Sordariaceae	*Neurospora dictyophora*	AY681181	98.9	100	CTQE103	*Neurospora dictyophora*	KX893403
CTQE044	Tremellales	Tremellaceae	*Cryptococcus* sp.	HQ623585	88.9	90.41	CTQE044	Tremellales	KX893381

Isolate[a]	CM Order	CM Family	CM Species	CM Accession	% Similarity	% Coverage		Rep. isolate	Rep. isolate identification[†]	Assigned Accession
CTQE073	…Tremellales		…Tremellaceae	*Cryptococcus* sp.	HQ623585	88.9	88.49	CTQE044	Tremellales	KX893381
CTQE075				*Cryptococcus* sp.	HQ623585	88.7	90.41	CTQE044	Tremellales	KX893381
CTQE077				*Cryptococcus* sp.	HQ623585	88.6	88.49	CTQE044	Tremellales	KX893381
CTQE078				*Cryptococcus* sp.	HQ623585	88.7	88.68	CTQE044	Tremellales	KX893381
CTQE087				*Cryptococcus* sp.	HQ623584	88.7	88.49	CTQE077	Tremellales	KX893391
CTQE043	Trichosphaeriales		Trichosphaeriaceae	*Nigrospora oryzae*	KC771471	100	100	CTQE043	*Nigrospora oryzae*	KX893380
CTQE072				*Nigrospora oryzae*	KC771471	99	100	CTQE043	*Nigrospora oryzae*	KX893380
CTQE095				*Nigrospora oryzae*	KF23404	98.4	100	CTQE095	*Nigrospora oryzae*	KX893397
CTQE042				*Nigrospora* sp.	KF128783	99.8	99.61	CTQE042	*Nigrospora* sp.	KX893379
CTQE016				*Nigrospora sphaerica*	KU878079	100	100	CTQE016	*Nigrospora sphaerica*	KX893366
CTQE041				*Nigrospora sphaerica*	KU878079	100	100	CTQE016	*Nigrospora sphaerica*	KX893366
CTQE055				*Nigrospora sphaerica*	KU878079	95.9	100	CTQE016	*Nigrospora sphaerica*	KX893366
CTQE070				*Nigrospora sphaerica*	KU878079	100	100	CTQE016	*Nigrospora sphaerica*	KX893366
CTQE100				*Nigrospora sphaerica*	KU878079	99	100	CTQE016	*Nigrospora sphaerica*	KX893366
CTQE093				*Nigrospora sphaerica*	KU878079	99.8	100	CTQE016	*Nigrospora sphaerica*	KX893366
CTQE099	Xylariales		Amphisphaeriaceae	*Neopestalotiopsis clavispora*	KP075005	100	100	CTQE099	*Neopestalotiopsis clavispora*	KX893400
CTQE062				*Pestalotiopsis biciliata*	KM199305	99.6	100	CTQE062	*Pestalotiopsis biciliata*	KX893386
CTQE063				*Pestalotiopsis biciliata*	KM199305	99.6	100	CTQE062	*Pestalotiopsis biciliata*	KX893386
CTQE056[b]				*Pestalotiopsis clavispora*	JX045815	100	100	CTQE056	*Pestalotiopsis clavispora*	KT699873
CTQE067[b]				*Pestalotiopsis mangiferae*	KM510410	100	100	CTQE067	*Pestalotiopsis mangiferae*	KT699874
CTQE068				*Pestalotiopsis oxyanthi*	KT716303	100	100	CTQE068	*Pestalotiopsis oxyanthi*	KX893388
CTQE045				*Pestalotiopsis* sp.	GU592002	100	100	CTQE045	*Pestalotiopsis* sp.	KX893382
CTQE110			Xylariaceae	Xylariaceae	AB741586	99.2	100	CTQE110	Xylariaceae	KX893404

[a] Isolates CTQ001-CTQ036 (n = 31) were recovered from samples collected in March 2013, all others were isolated from samples collected in May 2013 (n = 58); [b] Isolates tested in the pathogenicity trial (Table 3.1); [†] Assigned identities: Species name approximately 97-100% sequence identity with closest match; genus ~94–97%; family ~90–94%; order 85-90%; class or phylum < 85 % (Vega et al. 2010)

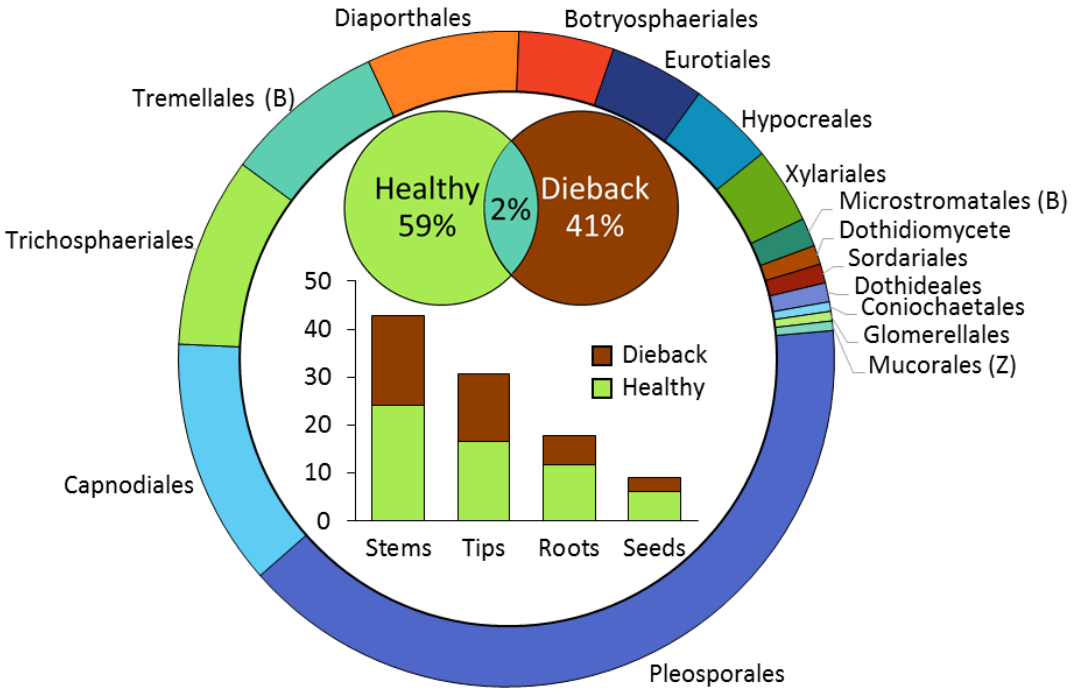


Figure 3.2 Fungal community overview showing the proportion of taxonomic orders represented where (B) are basidiomycetes, (Z) is a zygomycete and all others are Ascomycetes; the proportion of isolates isolated by tissue type and host disease status; and the percentage of isolates found in healthy, dieback-affected *Parkinsonia aculeata*, or both.

(39%) trees. Of the isolated taxa, 32 were previously shown to be pathogenic according to the literature, 27 had history as dieback pathogens, but only nine were involved in dieback of trees. Three of these were *Pestalotiopsis* (*P. clavispora*, *P. mangiferae* and *P. visimae*). Due to the number of available plants and my desire to maximise statistical power and therefore the number of replicates, I decided to exclude *P. visimae* from this trial.

3.4.2 Pathogenicity testing of isolates with a water stress interaction

There was a statistically significant difference in lesion length, explained by inoculated isolate (F = 2.347, P = 0.01), but no effect by water treatment or the interaction between these factors ($P > 0.05$; Table 3.3). Universally, lesion size was greater underbark than on the surface. The length of incision in the negative controls (sterile ½ PDA plug) was consistent with the length of the underbark "lesion", which I assume was a result of scarring, so I concluded that no lesion was formed for the negative control treatment. The positive control (*L. pseudotheobromae*, NT039) consistently formed larger lesions than any other pathogen tested (40.57 ± 0.51 mm; Fig 3.3) and contributed significantly to variation in lesion length in post-hoc testing (Table 3.4). *Pestalotiopsis mangiferae* (CTQE067), *L. pseudotheobromae* (CTQE089), *Botryosphaeria dothidea* (CTQE031) and *Pestalotiopsis clavispora* (CTQE056) caused similar sized underbark lesions (23.94 ± 0.47 mm; Fig 3.3). *Diplodia pinea* (CTQE005) and *Phomopsis azadirachtae* (CTQE007) resulted in the smallest lesions, with *P. azadirachtae* (12.83 ± 0.15 mm) only just exceeding the inoculation site scar length (10.67 ± 0.02 mm) but was greater than the negative control (11.77 ± 0.33 mm).

Table 3.3 ANOVA testing the effects of water treatment and inoculated isolate on lesion length and three measures of *Parkinsonia aculeata* plant health, where 'growth' is the change in height or stem circumference over the ten-week inoculation trial

		Lesion length		Growth (Height)		Growth (Circumference)		Post-harvest biomass	
Source of Variation	***df***	***F***	***P*-value**	***F***	***P*-value**	***F***	***P*-value**	***F***	***P*-value**
Isolate	8	2.35	0.02	0.20	0.99	0.07	1.00	0.18	0.99
Water Treatment	2	0.36	0.70	14.08	<0.001	10.82	<0.001	8.38	<0.001
Interaction	16	0.12	0.99	0.22	1.00	0.05	1.00	0.18	1.0

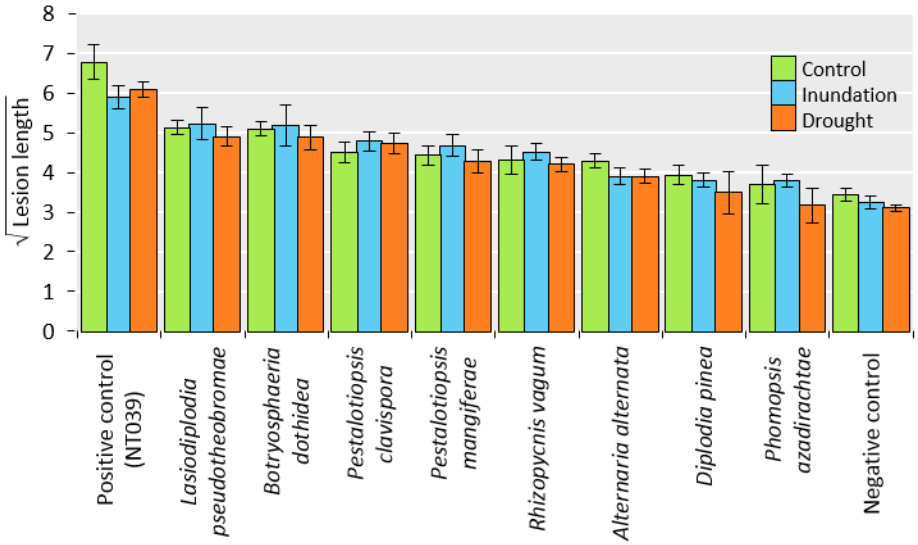


Figure 3.3 Average underbark lesion length on one-year-old *Parkinsonia aculeata* seedlings by isolate and water treatment, at the conclusion of this 10 week glasshouse pathogenicity trial

Table 3.4 Tukey Q statistics (where $P < 0.05$) for post-hoc pairwise analysis of the effect of inoculated isolate[a] on underbark lesion length in *Parkinsonia aculeata*

	CTQE005	**CTQE007**	**CTQE031**	**CTQE034**	**CTQE056**	**CTQE067**	**CTQE089**	**CTQE097**
CTQE007	-							
CTQE031	5.11	6.80						
CTQE034	-	-	-					
CTQE056	-	5.65	-	-				
CTQE067	5.44	7.14	-	-	-			
CTQE089	5.58	7.28	-	-	-	-		
CTQE097	-	-	-	-	-	4.62	4.76	
NT039	14.48	16.33	9.59	12.25	10.73	9.26	9.12	13.66

[a]CTQE005 *Diplodia pinea;* CTQE007 *Phomopsis azadirachtae;* CTQE031 *Botryosphaeria dothidea;* CTQE034 *Rhizopycnis vagum;* CTQE056 *Pestalotiopsis clavispora;* CTQE067 *Pestalotiopsis mangiferae;* CTQE089 *Lasiodiplodia pseudotheobromae;* CTQE097 *Alternaria alternata;* NT039 Positive control *L. pseudotheobromae*

- Not significant where $P > 0.05$

There was a statistically significant difference in plant health measurements by water treatment. Water treatment affected plant growth by height (F = 14.08, $P < 0.001$) stem circumference (F = 10.82, $P < 0.001$), and post-harvest biomass (F = 8.38, $P < 0.001$) but I found no significant effect of inoculated isolate or the interaction between isolate and water treatment on any recorded plant heath measurements ($P > 0.05$). Drought-affected plants showed the greatest levels of water stress (Table 3.5), which was reflected in their relatively slower growth rate over 10 weeks during the trial with smaller changes in stem circumference and height (Fig 3.4a,b); lower post-harvest dry-mass (Fig 3.4c); and their increased susceptibility to pests (Fig 3.5). They also had a lower above-ground:below-ground plant dry mass ratio (1.43 ± 0.09) than inundated plants (2.08 ± 0.08) or control plants (2.13 ± 0.10). Plants that were inundated had reduced height compared to the control treatment (Fig 3.4a); however, stem girth and post-harvest dry mass were similar between inundated plants and those in the control water regime (Table 3.5).

Despite confirming local infection by the inoculated pathogen via re-isolation from lesions, I was unable to re-isolate the pathogen more than 2 cm away from the lesion in any of the plants post-harvest. Isolates from post-harvest, healthy, plant tissue were identified as *Myrothecium verrucaria*, a ubiquitous contaminant and plant pathogen; *Phoma sp*. and *Chaetomium globosum* – both endophytes; and *Fusarium oxysporum* and *Penicillium verruculosum*, which are common saprotrophs and endophytes (Nguyen et al. 2016). I did not observe any dieback-like symptoms in the plants such as loss of foliage, internal staining or death of plant parts, other than lesions at the inoculation site. Any loss of foliage in drought-affected plants was consistent across

Table 3.5 Tukey Q statistics (where $P < 0.05$) for post-hoc pairwise analysis of the effect of water treatment on three measurements of plant health at the end of the 10 week *Parkinsonia aculeata* inoculation trial

Water Treatments	Growth (Height)	Growth (Circumference)	Post-harvest Biomass
Control vs Inundate	2.73	-	-
Control vs Drought	5.31	4.00	3.85
Inundate vs Drought	2.58	4.01	3.14

- Not significant where $P > 0.05$

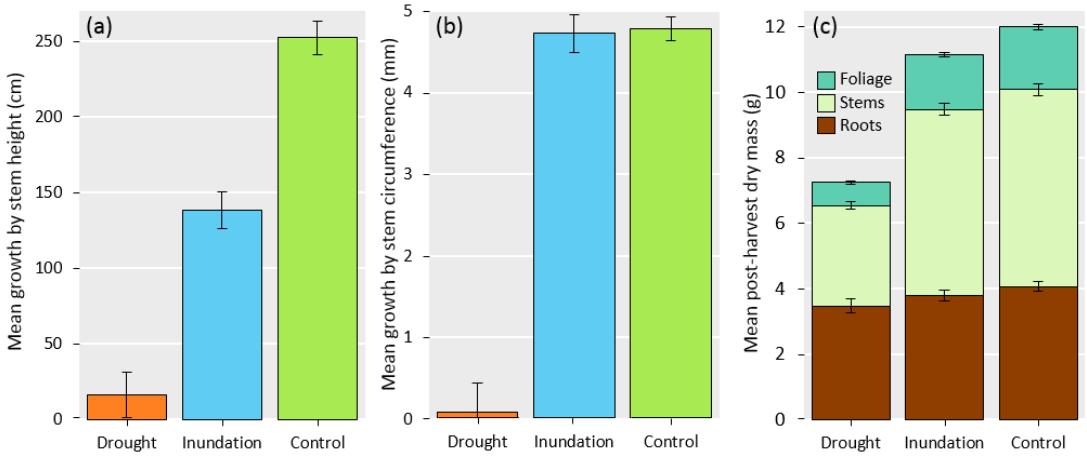


Figure 3.4 The effects of water treatment on the average change in *Parkinsonia aculeata* plant height (a) and stem circumference (b) from the start to the end of the 10 week pathogenicity trial, and average post-harvest dry mass of roots, stems and foliage (c) at the conclusion of the trial

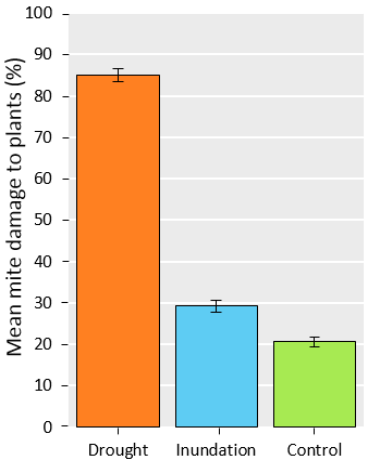


Figure 3.5 Mite damage to *Parkinsonia aculeata* plants by water treatment at the conclusion of this glasshouse pathogenicity trial

inoculated pathogens, so was presumed to be due to water availability, not infection by a pathogen. I therefore found no evidence of systemic infection by any inoculated pathogen, regardless of lesion size or level of stress.

3.5 Discussion

Despite significant levels of water stress, and a decrease in the health of stressed plants, underbark inoculation by any of the chosen fungal isolates did not cause systemic infection or dieback-like symptoms in parkinsonia. Four of the isolates tested in this trial were members of the Botryosphaeriaceae, many of which are known to persist as latent pathogens within their host (Slippers & Wingfield 2007; Jami et al. 2013; Mehl et al. 2013). This family of pathogens grow both intracellularly and intercellularly and after infection, are known to move via the mesophyll and vascular bundle (Mehl et al. 2013). Host response involving the formation of a new periderm can also lead to infection of the xylem and phloem tissue (Rayachhetry et al. 1996) leading to systemic infection within eight weeks. *Lasiodiplodia pseudotheobromae* (CTQ089) was only isolated from healthy, symptomless parkinsonia during field sampling, yet formed a significantly larger lesion in the pathogenicity trial than some of the other isolates that were isolated from dieback-affected parkinsonia. This supports the idea that at least this strain of *L. pseudotheobromae* is a latent pathogen (Jami et al. 2013). Lesions formed by *Diplodia pinea* were relatively small, and this may be because it is potentially more pathogenic to other tree species such as *Pinus* sp. (de Wet et al. 2000). *Botryosphaeria dothidea* has been observed to cause girdling and death in defoliated downy birch (*Betula pubescens*) stems after just four weeks,

suggesting that defoliation stress might be essential for increased *B. dothidea* virulence (Crist & Schoeneweiss 1974).

Pestalotiopsis spp. are responsible for a number of plant diseases, mostly in the tropics (Keith et al. 2006; Espinoza et al. 2008; Chen et al. 2013; Ismail et al. 2013) and are commonly isolated as saprobes, although some are likely to have both endophytic and pathogenic stages in their lifecycles (Maharachchikumbura et al. 2011). Endophytes from this group are ubiquitous and not associated with geographic limits, but their host colonisation rates are lower in monsoon seasons than in the dryer winter season (Tejesvi et al. 2005). This indicates that they may be limited by drought-like conditions, and take advantage of their host in sustained wet weather. In my study, I did not observe significant variation in lesion length between inundated and drought-affected plants by either *P. clavispora* or *P. mangiferae*. This might be because parkinsonia is relatively healthy in inundated conditions compared to drought-affected conditions (Fig 3.4), thereby not presenting with the stress required by the two *Pestalotiopsis* spp. for increased colonisation or pathogenicity. There are no records of dieback occurrence in parkinsonia in relation to rainfall conditions in the field.

Out of the other three isolates used in this study, only *Alternaria alternata* and *Rhizopycnis vagum* caused underbark lesions that were significantly greater than the negative control. *Alternaria alternata* is known to produce host-specific phytotoxins which may cause defoliation (Babu et al. 2003). This species may therefore require a susceptible host for it to be more virulent. *Rhizopycnis vagum* is most frequently a root-colonizing endophyte (Knapp et al. 2012), although some studies have shown it to be pathogenic to musk-melon roots (Armengol et al. 2003) and involved in mature watermelon vine decline (Westphal et al. 2011). I isolated it from the roots of dieback-

affected parkinsonia, but it too, only resulted in small localised lesions when inoculated. Westphal et al. (2011) suggests *Rhizopycnis vagum* may require other factors to increase disease severity, such as soil inoculation.

I attempted to ensure that each inoculated isolate was triggered into pathogenicity by first passaging the isolate through an apple. Although I achieved local infection in the plant, I did not observe systemic infection, despite first ensuring that the host was under water stress. It is possible that extending the length of the trial past 10 weeks may have resulted in eventual mortality. Incubation times vary between studies (Ismail et al. 2013; Pitt et al. 2013) but many report significant results within 10 days of inoculation (e.g., Stukely & Crane 1994; Armengol et al. 2003). Additionally, any response observed in an inoculation trial may be different to that observed in the field, even under similar conditions. The age of the plant tissue may affect the plant's response to inoculation, and the endophyte community hosted by plants in the field may be different to those hosted by glasshouse plants grown from seed. A latent pathogen may only be triggered into pathogenicity by a combination of these factors which may also explain the lack of dieback symptoms observed in this glasshouse trial. Plants are complex organisms, playing host to multiple taxonomic and trophic groups, with environmental responses ranging from inherent to symbiotically-assisted. It is therefore difficult to predict or monitor infection from inoculation with one organism, without distinctive symptoms. Future pathogenicity work in this system, as demonstrated in Toh (2009) using seedlings, should be assessed histologically during and after the trial.

I isolated a taxonomically diverse range of fungal endophytes from multiple plant parts of healthy and dieback parkinsonia, including some reportedly pathogenic

species, with a total of 54 unique taxa from 204 isolates as identified by ITS amplicon sequencing. These species came mostly from dieback-affected plants, and the greatest number were isolated from stems. The fungal endophyte community of other invasive plants is similar in regards to culturable endophyte species found in this study. Diplock (2016) only isolated fungi from stems and identified 20 unique fungal endophyte species of 48 isolates associated with dieback-affected parkinsonia. I identified 31 taxa from dieback stems collected in the same region. Twenty-four fungal endophyte taxa (out of 1,352 isolates) were recovered from healthy and dieback-affected *Mimosa pigra* stems by Sacdalan (2015), and 23 taxa from 166 isolates from healthy and dieback-affected *Vachellia nilotica* subsp. *indica* stems and roots (Haque 2015). Overall, the number of taxa recovered from dieback-affected plants in this study was greater than from healthy plants, which is expected if additional dieback-causing pathogens are present, or as the host is colonised by incoming saprophytes during cell death brought on by dieback. The composition of endophyte communities between individual hosts and host species may also differ due to local environmental conditions, distance decay (i.e. increasing dissimilarity between communities with increasing spatial distance; Peršoh 2015) and mode of endophyte transmission (i.e. vertical vs. horizontal). However, there is a high chance that the isolates recovered in this study are dominant and/or fast growing members of the parkinsonia endophyte community, since these species are more likely to be isolated. Conversely, this also implies that slower-growing or more benign species may not have been recovered and that unculturable taxa were missed. Steinrucken et al. (2016; Chapter 2) recovered over 150 unique OTUs from dieback-affected plant parts and over 70 from healthy plant parts, which is more than double those isolated in this study. The availability of

molecular techniques and the decreasing price of high-throughput sequencing technology has consistently demonstrated that the diversity of fungi is grossly underestimated by culture-based studies (Peay et al. 2016).

Our observations support the idea that some of the fungal endophytes isolated from parkinsonia, particularly the Botryosphaeriaceae, exist commonly as endophytes and may act as latent pathogens but in order to cause disease in their host some external environmental trigger is required. Despite the formation of localised lesions, no dieback-like symptoms were observed via underbark inoculation of parkinsonia with the eight chosen isolates in this study. Under the right conditions (e.g., a specific environmental stress or the presence of other microorganisms) however, underbark inoculation may still be an appropriate method for testing other potential putative pathogens. In the future, other factors such as salinity, heat, and defoliation stress could be used during pathogenicity screening, and may provide insight into host susceptibility to dieback-associated pathogens. More thorough reporting of dieback occurrence in the field, and any associated environmental conditions, would also aid greatly in determining which stress factors are important for disease expression. Any potential dieback-causing agent(s) identified should be systematically tested for host-specificity (see Wapshere 1974; Evans 2000) – particularly against locally occurring native plant species – prior to release and widespread use as a biocontrol agent(s). Dieback syndromes adversely affect many desired tree species globally, but with the right combination of effective and specific dieback-causing pathogens, efficient inoculation techniques and conducive conditions, dieback may become an alternative tool for use in large scale weed management.

4

FIRST REPORT OF OOMYCETES ASSOCIATED WITH THE INVASIVE TREE *PARKINSONIA ACULEATA* (FAMILY: FABACEAE)

4.1 Abstract

Phytophthora species have caused the decline and dieback of multiple tree species in Australia and around the world. Dieback in invasive trees in Australia has been observed for decades, motivating research into the potential causes of dieback to be used for biological control of these invasive species. Despite wide-ranging and ongoing research into invasive plant dieback, *Phytophthora* species have been largely ignored as potential causal agents of dieback, with the focus more on latent fungal pathogens living as endophytes.

I conducted the first survey of *Phytophthora* and other oomycetes to determine their association with dieback of the invasive tree, *Parkinsonia aculeata* L. (Fabaceae). Using zoospore baiting, I recovered 37 oomycete isolates from roots and soil of healthy and dieback-affected Parkinsonia in Kununurra, Western Australia and Charters Towers, Queensland. Using molecular taxonomy, I identified ten unique oomycete taxa, predominantly composed of *Phytophthora palmivora*, *P. nicotianae* and *Phytopythium vexans*.

Parkinsonia dieback occurs across multiple climatic zones including those experiencing severe drought. I recovered fewer oomycete isolates from soil and roots in drought-affected Charters Towers than Kununurra, which had experienced recent rainfall. This may be because oomycetes require soil moisture for the dispersal of zoospores. None of the genotypes identified were consistently isolated from dieback-affected trees suggesting that any association with parkinsonia dieback may be localised. More extensive surveys and pathogenicity screenings of isolated oomycetes are required to evaluate their role in the parkinsonia dieback phenomenon.

4.2 Introduction

Dieback is defined in perennial plants as a progressive reduction in plant health resulting in the death of plant parts and often outright death of the whole plant, potentially resulting in local population extinctions, either as a gradual process over many years or as a more sudden occurrence (Manion & Lachance 1992; Ciesla & Donaubauer 1994; Pautasso et al. 2013; Steinrucken et al. 2016, Chapter 2). Dieback is not age-related or explained by a specific stress such as fire or prolonged flooding (Mueller-Dombois 1987). Symptoms may include defoliation, browning of stems, and staining of stem tissue.

Phytophthora species are implicated in dieback of multiple tree species in Australia and around the world. *Phytophthora ramorum*, for example, is the cause of Sudden Oak Death (Rizzo & Garbelotto 2003; Garbelotto & Hayden 2012) and is also responsible for dieback of many other tree species including larch (*Larix* spp.; Brasier & Webber 2010), beech and chestnut (*Fagus* spp. and *Castanea* spp.; Brasier et al. 2004). *Phytophthora cinnamomi* causes oak decline in Iberia (Brasier et al. 1993) and

little leaf disease in pine (*Pinus* spp.; Otrosina & Marx 1975). In Australia, *Phytophthora cinnamomi* has resulted in extensive dieback of native forests and bushland (Pratt & Heather 1973), with extensive damage to jarrah (*Eucalyptus marginata*) forest, as well as *Prunus*, *Camellia*, *Erica*, *Rhododendron* and *Jacaranda* species (Newhook & Podger 1972; Dell & Malajczuk 1989). *Phytophthora*, like other oomycetes, disperse via flagellated zoospores that swim towards potential hosts, often via soil moisture or water flow (Scott et al. 2013). Many species might also remain viable in the soil for years due to environmentally-resistant structures such as oospores and chlamydospores (Judelson & Blanco 2005). *Phytophthora* often have a wide host range making them one of the most important groups of plant pathogens globally (Scott et al. 2013).

Dieback in several important woody weedy trees and shrubs in arid, semi-arid and wet-dry tropical habitats is a widely researched phenomenon due to the potential for the biotic causal agent(s) of dieback to be used in biological control of these invasive trees (Raghavendra et al. 2017). However, most invasive plant dieback research has focused on putative pathogens derived from stem tissue, in the form of endophytic higher fungi (Haque 2015; Sacdalan 2015; Diplock 2016), and few have considered the involvement of soil-borne *Phytophthora* species. An exception is the research on European blackberry (*Rubus anglocandicans*) decline in Western Australia (WA; Aghighi et al. 2015), associated with *Phytophthora cryptogea* and *P. bilorbang*. Above- and below-ground biomass of blackberry decreased during regular flooding events, when infected with these species, which is consistent with the common method of *Phytophthora* dispersal (Aghighi et al. 2015). In other research on woody weed dieback in arid zones, *Phytophthora* species were likely assumed to be unimportant

since many dieback-affected invasive tree species are found in very dry climatic conditions (Raghavendra et al. 2017) with little evidence of root disease, and few studies focused on sampling roots (Haque 2015; Sacdalan 2015; Diplock 2016). If dieback is observed in regions with intermittent flooding or extended wet seasons, it is more likely that *Phytophthora* species are involved. I believe it is therefore important to consider the role of *Phytophthora* in dieback of other weed species, particularly in more mesic habitats.

Parkinsonia aculeata L. (hereafter referred to as parkinsonia) is a weed of national significance in Australia (Thorp & Lynch 2000) adversely affecting livestock management, water access and biodiversity, with populations found across northern Queensland (QLD), northern Western Australia (WA) the Northern Territory (van Klinken et al. 2009). Introduced from meso-America in the 19th century, this spiny, leguminous tree has become a target species for biological control due to its vast range, presence in remote locations and large populations (van Klinken et al. 2006). Dieback has been observed across its invasive range for two decades with some anecdotal reporting of dieback from the 1950s (van Klinken et al. 2009). Despite extensive research (Toh 2009; Diplock 2016; Steinrucken et al. 2016, Chapter 2; Raghavendra et al. 2017; Steinrucken et al. 2017, Chapter 3), the cause of parkinsonia dieback remains unknown.

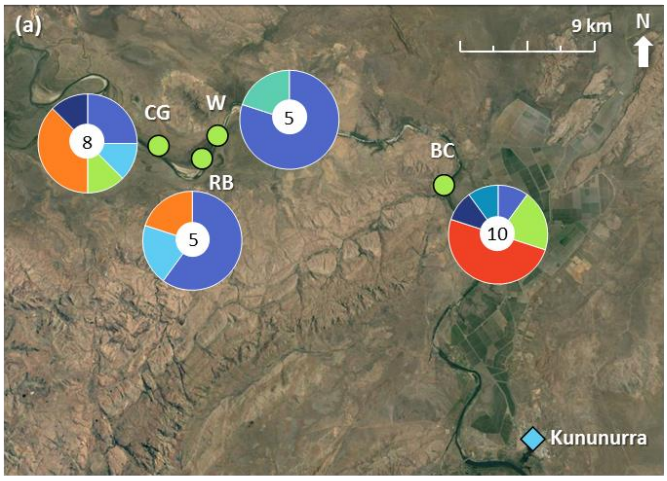
In a molecular study using terminal-fragment length polymorphism (T-RFLP) analysis, endophytic higher fungal community composition was shown to be related to dieback occurrence in parkinsonia (Steinrucken et al. 2016, Chapter 2). However, due to the methodology, taxonomic classifications could not be attached to the operational taxonomic units (OTUs). Subsequently, pathogenicity screening of

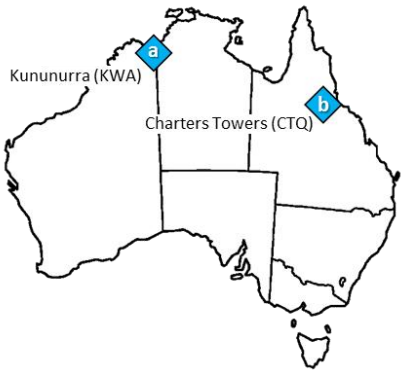
selected endophytic fungal pathogens isolated from parkinsonia tissue did not result in dieback-like symptoms or systemic infection in one-year old parkinsonia plants, despite the addition of water-stress treatments which reduced plant health (Steinrucken et al. 2017, Chapter 3). It is possible that parkinsonia dieback may be due to a combination of environmental stress factors and/or a disease complex involving multiple pathogenic species. Oomycetes have not yet been explored as potential dieback-causing pathogens in this system, but are putative pathogens in other dieback systems in Australian weeds (Aghighi et al. 2015). No environmental surveys have been conducted to determine whether flooding is important for the onset or development of dieback in parkinsonia. However, since parkinsonia can survive for up to 9 months with the lower portion of their trunks under water, conditions which favour oomycete dispersal and infection, I have conducted the following survey of oomycetes associated with parkinsonia to determine their involvement, or lack thereof, in dieback of this invasive tree.

4.3 Materials and Methods

4.3.1 Field survey and sampling

Four sites near Kununurra, WA and three sites near Charters Towers, QLD (Fig 4.1) were sampled in May and October 2014, respectively (Fig 4.2). The sites were chosen because expert knowledge (Kelli Pukallus, Tropical Weeds Research Centre DAFF, and Rieks van Klinken and Andrew White from CSIRO), indicated that I could obtain healthy and dieback samples within a short distance of each other, lessening climate variation between sites. These sites are also likely to be representative because the symptoms described in the literature (Diplock 2017, Toh 2009, van Klinken 2009)





Site		Name	South	East
KWA	BC	Button's Crossing	15°37.255′	128°41.500′
(a)	W	Wetland	15°34.822′	128°31.126′
	RB	Riverbank	15°35.563′	128°30.064′
	CG	Manbi Campground	15°34.613′	128°28.087′
CTQ	BS	Burdekin South	20°03.436′	146°15.368′
(b)	BN	Burdekin North	19°48.694′	146°02.963′
	GC	Groper Creek	19°42.995′	147°31.856′

Oomycete isolate taxa

- UC Pythiaceae
- UC Pythiales
- *Phytophthora* sp.
- *Phytophthora insolita*
- *Phytophthora nicotianae*
- *Phytophthora palmivora*
- *Phytopythium* sp.
- *Phytopythium vexans*
- *Pythium aphanidermatum*
- *Pythium graminicola*

UC = unclassified

Legend

- Sampling site
- City/Town

Figure 4.1 Maps of Kununurra, Western Australia (KWA; a) and central-east Queensland (CTQ; b) showing locations of *Parkinsonia aculeata* sampling sites, and proportion of oomycete isolate genotypes identified in this study. The number inside each pie chart is the number of oomycete isolates in total from that site.

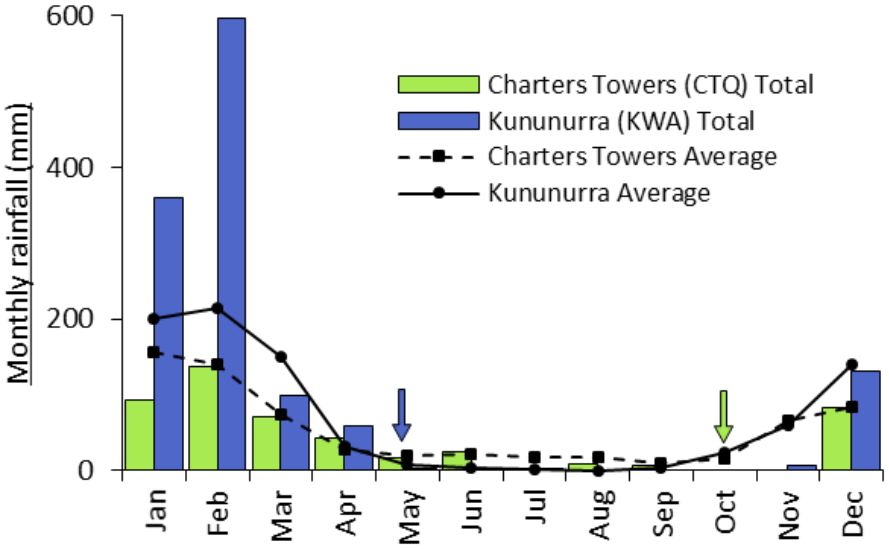


Figure 4.2 Total monthly rainfall (2014) and average monthly rainfall (1992-2016) for Charters Towers QLD (dark) and Kununurra WA (light) from the Bureau of Meteorology (2016). Sampling times for this study indicated by the arrows

and observed on the ground were similar to that seen at other sites, including those that are more remote. Sampling sites from both regions were alongside water bodies or in areas where flooding occurs. Only parkinsonia trees that were unambiguously healthy were classified as healthy, and trees with symptoms consistent with parkinsonia dieback, as described in the introduction, were sampled as such. I collected between five and seven roots (5-20 cm by 0.5-1.5 cm) and approximately 500 g rhizosphere soil material from five healthy and dieback-affected parkinsonia trees at each site. The health of trees were assessed by scoring percentage canopy and foliage cover (Steinrucken et al. 2016, Chapter 2), and by inspecting the tree for signs of dieback or disease such as defoliation, lesions, browning of the stems and signs of root infection. Tools were surface-sterilized between trees with 50% NaHCl for 3 min, and samples were placed in individual zip lock bags, sealed and stored at 5°C for up to 48 h before further processing.

4.3.2 Isolation of *Phytophthora* species

Zoospores from parkinsonia soil and root samples (pooled by plant) were baited in a cool, naturally-lit room, using a modified version of the method of Rea et al. (2010). Soil and root samples from individual trees were placed in 1 L plastic take-away containers and covered with distilled water (1:2 volume to volume sample/water). Containers were placed on a heating mat set at 28°C, and left undisturbed for 2 hours to allow sediment to settle. Approximately 15 "baits" in the form of youngest, fully emerged leaves, picked fresh from multiple plants (*Leucadendron* sp., *Pittosporum tobira*, *Scholtzia* sp., *Lantana camara*, *Melaleuca scabia*, parkinsonia and *Origanum vulgare*) were floated on the water, lower surface down, and left undisturbed for 24 h

(Fig S4.1, Appendix A). Baits were then checked twice a day for 5 days and any baits with brownish or water-soaked lesions were plated onto NARPH, a *Phytophthora* selective medium containing Nilstat, Ampicillin, Rifadin, Hymexazol and cornmeal agar (Hüberli et al. 2000) and incubated in the dark at room temperature. Once aseptate hyphae were observed growing from the plated lesion section, a random selection of isolates were examined with a light microscope to confirm isolation of oomycete species (Fig S4.2, Appendix A). Isolates were then subcultured onto fresh NARPH medium and repeatedly subcultured until a pure culture was obtained, before being transferred onto half-strength PDA (potato dextrose agar; 19.25 gL^{-1}) amended with 35 mg L^{-1} streptomycin and incubated in the dark at room temperature. Water was carefully poured off the samples, which were then left to air-dry for 6 weeks in the bait containers. The baiting procedure was repeated, followed by isolation and culturing of recovered isolates using the methods above.

4.3.3 DNA isolation, PCR and sequencing of isolated species

Mycelia were scraped from pure 7-day old cultures using a sterile blade and added to 400 µL sterile H_2O. DNA was extracted using the MO BIO Powersoil® Isolation kit (Qiagen, Carlsbad, California), PCR-amplified with MyTaq® (Bioline, London UK) according to the manufacturer with 0.2 µM each of forward primer DC6 and reverse primer ITS4 (Table 4.1). PCR conditions consisted of 96°C 2 min; 10 cycles of 95°C for 30 s, 54°C for 30 s and 72°C for 1 min; 25 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 1 min; and a final round of 72°C for 7 min. Amplicons were then purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin), and were Sanger sequenced with the BigDye Terminator Kit v3.1

(Applied Biosystems, Foster City, California) at the Centre for *Phytophthora* Science & Management at Murdoch University, Perth.

The initial identification of all sequences was obtained via BLAST search (http://www.ncbi.nlm.nih.gov/BLAST). Further identification of isolates was derived by interpreting a combination of the first 100 BLAST matches. Using an approach similar to Vega et al. (2010), I was cautious in identifying isolates at the genus level

Table 4.1 Primers used in this study

Primer	Location	Direction	Sequence (5`-3`)	Reference
DC6	18S	Forward	GAG GGA CTT TTG GGT AAT CAG AGG GAC TTT TGG GTA ATC A	Cooke et al. (2000)
ITS4	28S	Reverse	TCC TCC GCT TAT TGA TAT GCT CCT CCG CTT ATT GAT ATG C	Gardes and Bruns (1993)

or above, given the occurrence of misidentified sequences in GenBank (Vilgalys 2003). The cut-off point for assigning species names was 99% identity; genus names was 95–99% identity, family name 90–95% identity; sequences with lower identity with members of several families were identified only at the ordinal level. Once taxa were assigned, those classified as oomycetes were MUSCLE aligned to 796 bp, and a Jukes-Cantor UPGMA phylogenetic tree (TreeBASE Submission # 20917) was built using bootstrap resampling and 1000 replicates using Geneious® v8.1.6 (Biomatters, Auckland, New Zealand). I used an *Eurychasma dicksonii* voucher sequence (HQ643131) as the outgroup and voucher specimen sequences downloaded from GenBank were included (when available) for each identified taxon.

4.4 Results

The symptoms of parkinsonia dieback in WA (leaf death, minimal defoliation, red streaking of the trunk and roots under the bark; Fig 4.3 a-c) were different to those

observed in QLD (leaf loss, no red streaking of the trunk or roots under bark; Fig 4.3 d-f). In both cases, these symptoms were consistent across dieback-affected parkinsonia trees in the area. Locally-occurring species in QLD included bellyache bush (*Jatropha gossypiifolia*), *Melaleuca* spp., Noogoora burr (*Xanthium occidentale*) and *Lysiphyllum cunninghamii*. In WA, bellyache bush, *Melaleuca* spp., buffel grass (*Cenchrus ciliaris*), caltrop (*Tribulus terrestris*) and Noogoora burr were most common. At all sites, the ground beneath parkinsonia was usually bare soil and the trees were generally 1 - 3 m apart.

The most effective baits were leaves from *Scholtzia* sp., *Leucadendron* sp., *Pittosporum tobira*, and leaflets from parkinsonia, and no additional taxa were isolated in the second round of baiting. From a total of 141 isolates recovered from parkinsonia, sequence data resulted in their taxonomic classification to two zygomycetes (*Rhizopus* spp.), one basidiomycete (*Rhizoctonia* sp.) and 22 ascomycetes (mostly *Epicoccum* sp. and *Fusarium* spp.). The 37 isolates classified as oomycetes (Table 4.2) included fifteen which were classified as *Phytophthora* spp. (including *Phytophthora nicotianae*, *P. insolita* and *P. palmivora*), two classified as *Pythium* spp. (*Pythium graminicola* and *P. aphanidermatum*), twelve classified as *Phytopythium* spp. (including one *Phytopythium vexans*) and two *Pilasporangium* spp. Three were classified only to Pythiaceae, and another three only classified to Pythiales (Table 4.3). The taxonomic classifications of each isolate was supported by the phylogenetic tree (Fig 4.4).

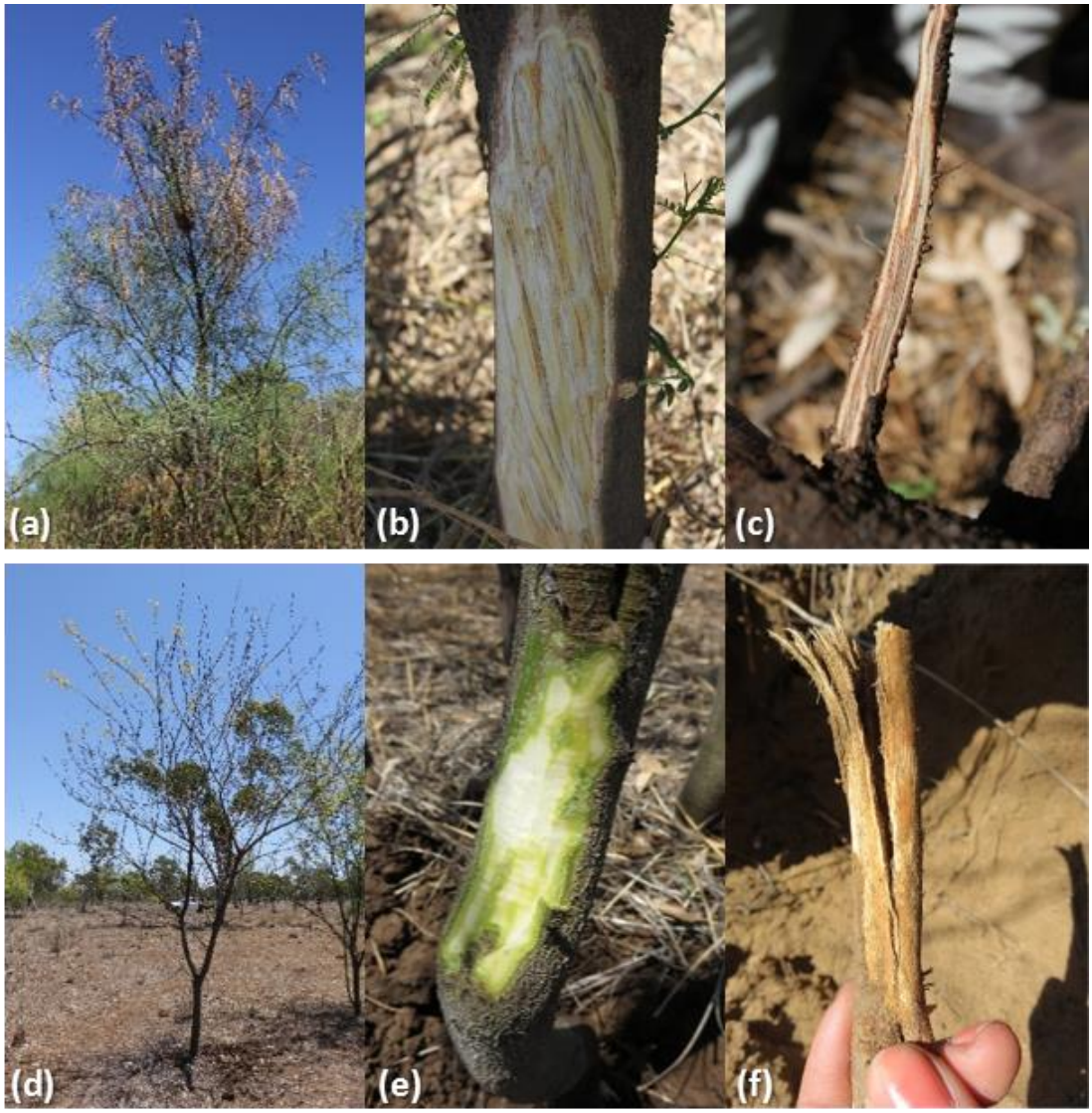


Figure 4.3 Symptoms of *Parkinsonia aculeata* dieback in Kununurra WA in May 2014: (a) brown leaves and crown dieback, (b) underbark trunk streaking not associated with a lesion, (c) root streaking; and in Charters Towers QLD in October 2014 (d) defoliation from crown, (e) no trunk streaking (f) no root streaking

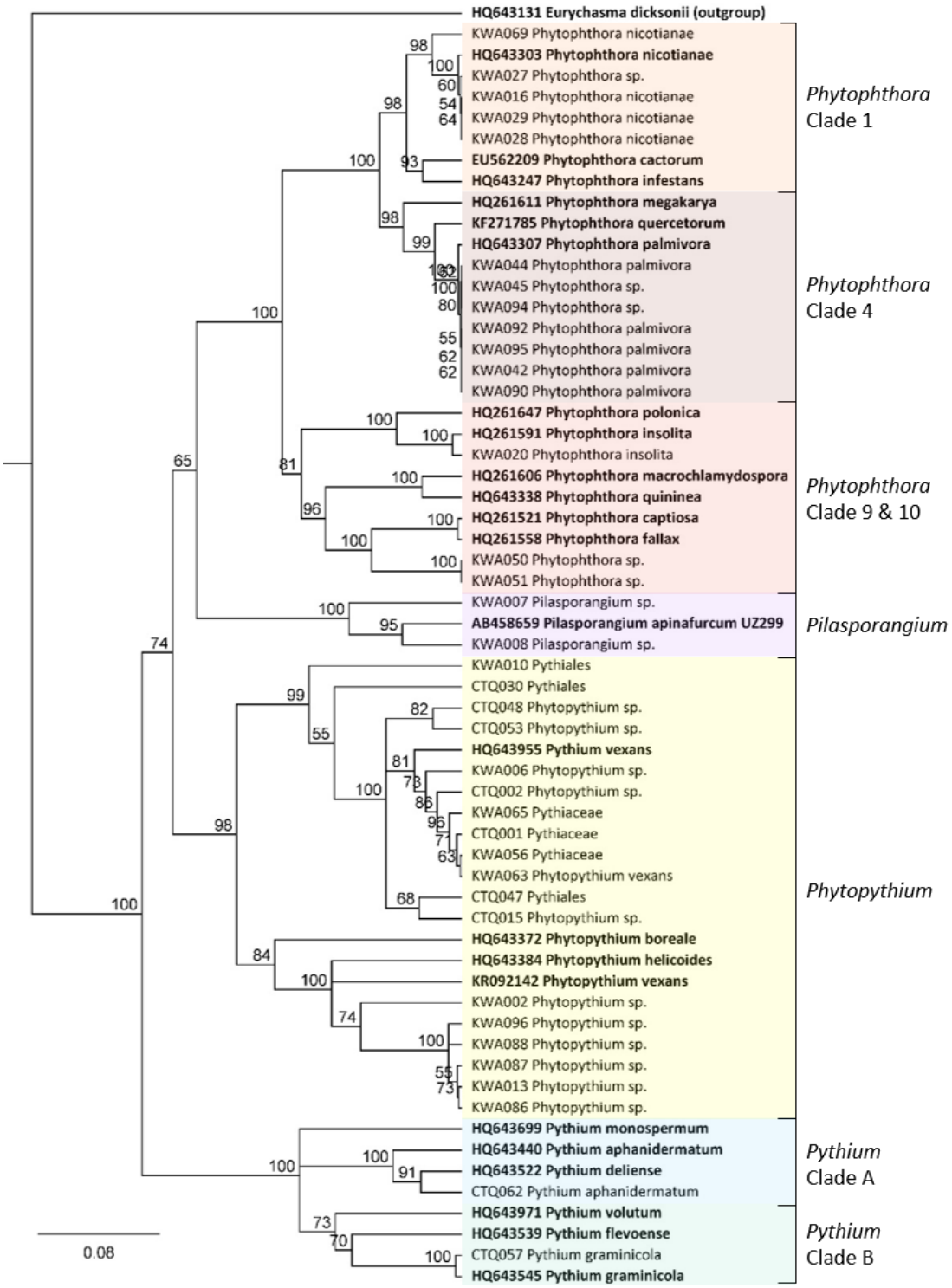


Figure 4.4 Jukes-Cantor UPGMA tree derived from an alignment (981 bp) of partial sequences of the internal transcribed spacer (ITS) region of isolates classified as oomycetes from *Parkinsonia aculeata* roots and soil in this study (TreeBASE Submission # 20917). Isolate codes indicate geographic origin, i.e. KWA: Kununurra Western Australia, CTQ: central east Queensland. Sequences in **bold** are reference sequences from GenBank and are from voucher specimens where possible. Apart from *Pilasporangium* spp., taxonomic assignation to clades was as per the ITS backbone in Robideau et al. (2011).. Sequences from isolates identified in this study have been submitted to Genbank (Accessions: KY938843 to KY938875)

Table 4.2 Isolates classified as oomycetes from soil and roots of *Parkinsonia aculeata*, identified to closest match (CM) on the NCBI database and by phylogenetic analysis using the ITS backbone from Robideau et al. (2011). Site of sampling in Kununurra (KWA) and Charters Towers (CTQ) from Fig 4.1, and disease status of host plant are also provided

Isolate	CM Taxon	CM Accession	%PWI[a]	%QC[b]	Assigned Taxonomic ID[c]	Site[d]	H/D[e]
CTQ001	*Pythium vexans*[†]	HQ643954	93.9	100.0	Pythiaceae	BN	H
CTQ002	*Pythium vexans*[†]	GU133601	97.1	99.6	*Phytopythium* sp.	BS	D
CTQ015	*Pythium vexans*[†]	GU133601	97.1	99.6	*Phytopythium* sp.	BS	D
CTQ030	*Pythium vexans*[†]	GU133578	88.8	71.5	Pythiales	BS	D
CTQ047	*Pythium vexans*[†]	GU133601	87.2	100.0	Pythiales	BS	D
CTQ048	*Phytopythium aff. vexans*	HQ643371	95.2	95.1	*Phytopythium* sp.	BS	D
CTQ053	*Pythium vexans*[†]	GU133593	98.9	94.5	*Phytopythium* sp.	BS	D
CTQ057	*Pythium graminicola*	HQ643545	100.0	100.0	*Pythium graminicola*	BN	H
CTQ062	*Pythium aphanidermatum*	KF667387	100.0	100.0	*Pythium aphanidermatum*	BS	D
KWA002	Pythium sp.	KP183943	96.6	100.0	*Phytopythium* sp.	BC	D
KWA006	*Pythium vexans*[†]	GU133593	96.3	100.0	*Phytopythium* sp.	CG	D
KWA007	*Pilasporangium apinafurcum*	AB458659	84.9	97.2	*Pilasporangium* sp.	CG	D
KWA008	*Pilasporangium apinafurcum*	AB458659	91.9	97.2	*Pilasporangium* sp.	RB	H
KWA010	Phytopythium aff. vexans	HQ643371	87.1	100.0	Pythiales	RB	H
KWA013	*Phytopythium vexans*	KR092142	91.4	89.9	*Phytopythium* sp.	RB	H
KWA016	*Phytophthora nicotianae*	LT628539	100.0	100.0	*Phytophthora nicotianae*	CG	D
KWA020	*Phytophthora insolita*	GU111612	99.0	100.0	*Phytophthora insolita*	W	D
KWA027	*Phytophthora nicotianae*	LT628539	97.6	100.0	*Phytophthora* sp.	CG	D
KWA028	*Phytophthora nicotianae*	KU248811	99.4	97.5	*Phytophthora nicotianae*	CG	D
KWA029	*Phytophthora nicotianae*	LT628539	99.4	100.0	*Phytophthora nicotianae*	CG	D
KWA042	*Phytophthora palmivora*	KY357521	99.5	100.0	*Phytophthora palmivora*	BC	D
KWA044	*Phytophthora palmivora*	KY357521	99.3	100.0	*Phytophthora palmivora*	BC	D
KWA045	*Phytophthora palmivora*	KY357521	98.3	100.0	*Phytophthora* sp.	BC	D
KWA050	*Phytophthora fallax*	HQ261559	91.3	98.9	*Phytophthora* sp.	CG	H
KWA051	*Phytophthora fallax*	HQ261559	91.0	98.9	*Phytophthora* sp.	W	D
KWA056	Phytopythium aff. vexans	HQ643371	93.5	100.0	Pythiaceae	BC	D
KWA063	Phytopythium aff. vexans	HQ643371	99.1	100.0	*Phytopythium vexans*	BC	D
KWA065	Phytopythium aff. vexans	HQ643371	92.6	93.5	Pythiaceae	CG	D
KWA069	*Phytophthora nicotianae*	KJ506196	99.0	89.7	*Phytophthora nicotianae*	RB	H
KWA086	*Phytopythium vexans*	KR092142	92.5	85.7	*Phytopythium* sp.	W	D
KWA087	*Phytopythium vexans*	KR092142	92.3	85.0	*Phytopythium* sp.	W	D
KWA088	*Phytopythium vexans*	KR092142	92.5	86.0	*Phytopythium* sp.	W	D
KWA090	*Phytophthora palmivora*	KY357521	99.8	100.0	*Phytophthora palmivora*	BC	D
KWA092	*Phytophthora palmivora*	KY357521	99.9	100.0	*Phytophthora palmivora*	BC	D
KWA094	*Phytophthora palmivora*	KY357521	98.8	100.0	*Phytophthora* sp.	BC	D
KWA095	*Phytophthora palmivora*	KY357521	99.8	100.0	*Phytophthora palmivora*	BC	D
KWA096	*Phytopythium vexans*	KR092142	90.7	84.0	*Phytopythium* sp.	RB	H

[a] % PWI: percentage pairwise identity

[b] % QC: percentage query coverage

[c] Sequences deposited in GenBank under accession numbers KY938843 to KY938875

[d] Site key: BC – Button's Crossing KWA, CG – Manbi camp ground KWA, RB – river bank KWA, W – Wetland KWA, BN – Burdekin North CTQ, BS – Burdekin South CTQ, GC – Groper Creek CTQ

[e] Disease status of host plant: H – healthy, D – dieback

[†] Also known as *Phytopythium vexans* (de Cock et al. 2015)

Table 4.3 The number of oomycetes recovered and identified in this study from dieback-affected (D) or healthy (H) *Parkinsonia aculeata* in Queensland (QLD) and Western Australia (WA). For those isolates identified to species or genus, reference to their association with plant disease in the literature is provided.

	QLD		WA			
Identified Species	**D**	**H**	**D**	**H**	**Total**	**Susceptible host species**
Phytophthora sp.			4	1	5	Multiple (Erwin & Ribeiro, 1996)
Phytophthora insolita			1		1	*Rhododendron* sp., apple, cucumber (Erwin & Ribeiro, 1996)
Phytophthora nicotianae			3	1	4	Multiple (Widmer et al. 1998; Cline et al. 2008; Valencia et al. 2011)Multiple (Widmer et al. 1998; Cline et al. 2008; Valencia et al. 2011)
Phytophthora palmivora			5		5	Multiple (Kaosiri et al. 1980; Erwin & Ribeiro 1996; Vawdrey et al. 2005)Multiple (Kaosiri et al. 1980; Erwin & Ribeiro 1996; Vawdrey et al. 2005)
Pythium aphanidermatum	1				1	Bean, sugarbeet, cucumber, tobacco (Middleton 1943)Bean, sugar beet, cucumber, tobacco (Middleton 1943)
Pythium graminicola		1			1	Grasses, grains (Middleton 1943; Kageyama et al. 2005)Grasses, grains (Middleton 1943; Kageyama et al. 2005)
Phytopythium sp.	4		5	2	11	Multiple (Farr et al. 1989)Multiple (Farr et al. 1989)
Phytopythium vexans			1		1	Multiple (Farr et al. 1989; Vawdrey et al. 2005; Spies et al. 2011; Polat et al. 2017)Multiple (Farr et al. 1989; Vawdrey et al. 2005; Spies et al. 2011; Polat et al. 2017)
Pilasporangium sp.			1	1	2	NA – soil saprophyte (Uzuhashi et al. 2010)
Pythiales	2			1	3	NA
Pythiaceae		1	2		3	NA
Total number of isolates	7	2	22	6	**37**	

Diversity of recovered isolates was lower in QLD compared to WA, with no isolates identified as *Phytophthora* spp. recovered from QLD (Fig 4.1). *Phytophthora nicotianae* was isolated four times from two sites in WA, and *Phytopythium vexans* was isolated once from one site in QLD. Five isolates classified as *Phytophthora palmivora* were isolated from dieback-affected parkinsonia in WA (Table 4.3). *Pythium aphanidermatum* was isolated once from a dieback-affected tree in WA, *Pythium graminicola* was isolated once from dieback-affected parkinsonia in QLD, and dieback-affected parkinsonia in WA.

4.5 Discussion

I isolated multiple species of *Phytophthora* and *Pythium* from the roots and soil of healthy and dieback affected parkinsonia in WA, but no isolates identified as *Phytophthora* spp. in QLD. Diversity of oomycetes in QLD was therefore lower compared to WA from where *Phytophthora palmivora*, *Phytophthora nicotianae*, and *Phytopythium vexans* were isolated. This is also the first time a difference in dieback symptoms for this species has been observed, which suggests that parkinsonia dieback in Kununurra WA may be caused by a different agent(s) or stress factors than in QLD. Future investigations should involve surveying more dieback sites and further comparisons of symptoms in the populations as well as testing of putative pathogens. Although *Phytophthora palmivora* was isolated only from samples collected in association with dieback-affected trees, this was only at one site in WA. Therefore the results from this preliminary survey did not allow me to determine if there was a significant association between *P. palmivora* or other oomycetes and parkinsonia, or parkinsonia dieback.

The most commonly isolated oomycetes were *Phytophthora palmivora*, *Phytophthora nicotianae* and *Phytopythium vexans*. In order to determine the likelihood of these taxa being associated with parkinsonia dieback, and their potential for use as biological control agents, I describe them in more detail below.

Phytophthora palmivora has a wide host range. Most notably it has resulted in 20-30% reduction of the global cocoa crop (Erwin & Ribeiro 1996). Pathogenicity trials implicated *P. palmivora* in dieback of durian (*Durio zibethinus*) in multiple orchards in far north QLD, causing significant root rot (Vawdrey et al. 2005). *Phytophthora palmivora* has been used as a mycoherbicide for the biological control of the invasive

plant, Milkweed vine (*Morrenia ororata*), a pest in citrus orchards in Florida, USA (Templeton & Greaves 1984). However, since *P. palmivora* is not host-specific and can persist in the soil for several seasons, the use of this mycoherbicide was restricted (Templeton & Greaves 1984). There is evidence that both *P. palmivora* and *P. nicotianae* infect and cause rot in citrus (Widmer et al. 1998). *Phytophthora nicotianae* also has a very wide host range with a global cosmopolitan distribution and is a major pathogen of tobacco, ornamentals, tomato and some *Banksia* and *Eucalyptus* species (Erwin & Ribeiro 1996; Cline et al. 2008).

Phytopythium vexans (formally *Pythium vexans,* de Cock et al. 2015) has been reported to cause root and collar rot of kiwifruit in Turkey (Polat et al. 2017), is pathogenic to apple trees (Tewoldemedhin et al. 2011) and grapevines, and has been isolated from several other woody hosts (Spies et al. 2011). It has also been implicated alongside *Phytophthora palmivora* in dieback of durian in northern QLD (Vawdrey et al. 2005).

Differences in the number of isolates recovered from QLD compared to WA may be accounted for by average rainfall in the time leading up to sampling (Bureau of Meteorology, 2016). Kununurra WA experienced 1113.6 mm rain in the 5 months leading up to the sampling date in May, whereas Charters Towers QLD was at the end of the dry season and had received only 41.8 mm rain for the same period of time before sampling in October (Fig 4.2) and the dry spell was preceded by a long drought. Zoospores produced by oomycetes swim in water to infect new plant tissue (Scott et al. 2013), a feature which zoospore baiting takes advantage of (Rea et al. 2010). Diseases caused by *Phytophthora* species are therefore often correlated with increased soil moisture or rainfall, as in the case of blackberry decline (Aghighi et al. 2015).

Plants are known to become more susceptible to pathogen infection when stressed (Agrios 2005) and in the case of parkinsonia, plant vigour deteriorates significantly more under drought stress compared to inundation stress (Steinrucken et al. 2017, Chapter 3). This is especially the case with *Phytophthora* diseases, which frequently knock off the lateral and finer roots during infection, and then by the drought periods (e.g. the long, hot, dry summers in the Mediterranean climate of WA), infected plants do not have sufficient roots to extract water from soil, so they succumb (Erwin & Ribeiro 1996; Judelson & Blanco 2005). While I did observe red streaking of the roots (Fig 4.3c), there was no evidence of dead or dying lateral or fine roots in dieback-affected parkinsonia. Despite relatively low abundance, oomycetes were isolated even in the drought-affected sites in QLD, so it is likely that these species are able to remain dormant but viable during drought.

Oospores from species such as *P. palmivora*, and *P. nicotianaea* are particularly adapted to remaining dormant through drought, avoiding desiccation and microbial degradation due to their thick walls, chemical composition, and ability to reproduce via selfing (Ashby 1928; Kaosiri et al. 1980). In the case of *P. palmivora*, and likely other *Phytophthora* species, they may also persist in perennial water bodies (Ko 2003), to then be moved across the landscape during flooding events. Many Australian parkinsonia populations are located in drought-affected areas that also experience seasonal flooding (van Klinken et al. 2009). These conditions are likely to be suited to a number of oomycetes due to their life history, since zoospores require soil moisture to move and infect new hosts (Scott et al. 2013). Consequently, they may take advantage of drought-stressed parkinsonia at the first sign of soil moisture sufficient for infection. Alternatively, when parkinsonia puts out new roots in more favourable,

wetter conditions, the new roots are attacked by pathogens in the rhizosphere. Lower rates of infection in drier conditions may be because desiccated plants have fewer young roots to infect, however this does not necessarily make them less susceptible to infection (G.E.St.J. Hardy, unpublished data). Pathogenicity screening of oomycetes isolated from dieback-affected parkinsonia would be essential as a next step in testing this conjecture.

Although this survey did not allow me to determine whether oomycetes are associated with parkinsonia dieback, I believe it is likely that some *Phytophthora* species (e.g. *P. palmivora*) might be associated with parkinsonia, at least in WA. It would be beneficial for future work to consider sampling at multiple times throughout the year and also to include more sampling sites. This may open up a new perspective on parkinsonia dieback in Kununurra compared to Charters Towers. Hence, the cause of dieback in parkinsonia is, as yet, unknown, but the diversity of species isolated in this study suggest that oomycetes should be considered in future community and pathogenicity work when investigating dieback in parkinsonia. All of the oomycetes isolated in this study have relatively broad host ranges, meaning their suitability for use as biological control agents for parkinsonia is unlikely. If, after further studies, putative dieback-causing agents are identified via more extensive surveys, pathogenicity testing may determine if dieback can be used for biological control of parkinsonia and could open the door to the development of novel technology for improving management and control of widespread weed populations.

5

HOST AND RANGE SPECIFICITY OF *PARKINSONIA ACULEATA* ENDOPHYTE COMMUNITIES

5.1 Abstract

Parkinsonia aculeata is a weed of national significance in Australia, with native populations in South and Central America and southern USA. Invasive dieback populations have been reported to experience dieback events, the causes of which in *P. aculeata* have not yet been determined. Dieback has not been reported in the native range of *P. aculeata*, nor has it been observed in co-occurring or closely-related species. Thus, indications suggest that dieback occurrence is host and/or range-specific. Endophyte community composition has been previously shown to be associated with *P. aculeata* dieback, but the identities of potential causal agents remain unknown. Identification of taxa from dieback associated endophyte communities may give insight as to the causative agent/s of dieback and their potential for use in biocontrol.

To investigate relationships between host species, host range (native/introduced) and endophyte community composition, I sampled above-ground tissue from *P. aculeata* at seven sites in southern USA (Arizona and Texas) and seven sites in northern Australia (Queensland, Northern Territory and Western Australia) using high-throughput ITS region (fungi) and 16S rRNA genes (bacteria) sequencing of

surface-sterilized plant tissue. I also sampled *P. florida* and *P. microphylla*, which co-occurred with *P. aculeata,* at three sites in Arizona.

I found significant variation in endophyte community composition by host species and range for fungi. Operational taxonomic units (OTUs) classified as *Malassezia restricta, Penicillium spinulosum* and *Fusarium graminearum* were dominant in Australian *P. aculeata* populations, and an OTU classified as *Aureobasidium* sp. was more dominant in the North American *P. aculeata* populations. There were no observed host species effects on bacterial endophyte communities, but there was a significant range effect when comparing North American vs. invasive Australian *P. aculeata*. Bacterial OTUs classified as *Escherichia coli, Sphingomonas yabuuchiae* and *Staphylococcus equorum* were more typical of invasive *P. aculeata* populations than those from the native range. Of all the taxa identified (fungal or bacterial) as being significantly associated with Australian *P. aculeata*, only *Fusarium graminearum* has been shown to be a plant pathogen, but not in woody plants.

Since *Parkinsonia* fungal and bacterial endophyte communities differed significantly by host species and *P. aculeata* endophyte communities were affected by range, my results suggest that the communities in the native range are different to those (if any) that were introduced alongside *P. aculeata* at the time of introduction. Invasion success of *P. aculeata* in Australia could not be explained by the presence of potential beneficial endophytes. To determine the subsequent occurrence of dieback in these invasive *P. aculeata* populations, a similar analysis on the endophyte communities in dieback-affected *P. aculeata* is required.

5.2 Introduction

All plants host multiple endophyte species from taxonomic groups that include bacteria (Rosenblueth & Martínez-Romero 2006), fungi (Peay et al. 2016) and archaea (Cavicchioli 2011; Steinrucken et al. 2016, Chapter 2). The term 'endophyte' typically applies to members of the plant microbial community that spend part of their lifecycle completely within plant tissue as a symptomless-infection, sometimes emerging to sporulate (Carroll 1988; Rodriguez et al. 2009). In this study I include mutualistic, symbiotic, saprophytic and commensal microorganisms in this definition, but also latent pathogenic taxa, since there are many cases of symptomless endophytic infections becoming pathogenic when the host is under stress (Carroll 1988; Sinclair & Cerkauskas 1996; Slippers & Wingfield 2007). The composition of endophyte communities depends on multiple factors, including plant tissue type (Ma et al. 2013; Steinrucken et al. 2016, Chapter 2), host geographic location (Faeth et al. 2006; Dickie et al. 2010) and evolutionary history of the host and symbiont (Krings et al. 2007).

Parkinsonia aculeata L. (Fabaceae) is a weed of national significance (WONS) in Australia, with populations spread across Queensland (QLD), the Northern Territory (NT) and Western Australia (WA). Originally from meso-America (Hawkins et al. 2007), there are naturalised populations across South and Central America and southern USA (Hawkins et al. 2007). Due to its size (3-8 m tall), form (a thicket-forming spiny legume) and habitat preference (primarily riparian, but successful in drought- and inundation-affected areas), it is an environmental weed that has major impacts on the beef cattle and pastoral industries in northern Australia (van Klinken et al. 2009). *Parkinsonia aculeata* was a highly successful invader, escaping cultivation and spreading across northern Australia where it tolerates and persists in a

range of climatic and environmental conditions (Van Klinken & Heard 2012). Some invasive Australian *P. aculeata* populations are affected by dieback, which has been shown to reduce population size, leading to suggestions that dieback may have potential as a biocontrol agent for *P. aculeata* (van Klinken et al. 2009). *Parkinsonia aculeata* dieback begins with leaf loss, leading to death of outer stem tips, then stems, and usually results in whole tree mortality (Diplock 2016; Steinrucken et al. 2016, Chapter 2; Steinrucken et al. 2017, Chapter 3). Locally occurring plants of other species are unaffected (Diplock 2016) and *P. aculeata* dieback has not been observed in its native range.

Although *P. aculeata* and other dieback-affected weeds are generally successful invasive species, their subsequent collapse has so far been unexplained. Several ecological hypotheses have been postulated to explain the success, and sometimes subsequent failure, of plant invasion events as a result of interactions with the plant's endophytic communities (Elton 1958; Catford et al. 2009; Blackburn et al. 2011; Foxcroft et al. 2011; Gurevitch et al. 2011; Luke Flory & Clay 2013; Vestergård et al. 2015). "Invasional meltdown" hypothesis (Simberloff & Holle 1999; Mack 2003) describes how co-evolved beneficial endophyte communities may increase the competitiveness and invasion success of their host species upon introduction to a new range (Evans 2008; Catford et al. 2009; Aschehoug et al. 2014; Day et al. 2016). This is particularly effective if co-evolved natural enemies (including pathogens and pests) do not accompany the invasive plant upon introduction ("enemy release theory"; Keane & Crawley 2002). Conversely, theories such as "missed mutualism" (beneficial mutualist relationships with organisms in the home range are lost; Mitchell et al. 2006) and "new associations" (microorganisms in the new habitat form relationships with

the invader that may hinder further invasion; Colautti et al. 2004) may prevent establishment and spread of an introduced plant.

Since dieback has not been observed in co-occurring native plants, I aimed to determine firstly whether *Parkinsonia* endophyte communities are host-specific within *Parkinsonia* species by comparing them across congeneric species. Dieback might be caused by generalist pathogens in the invasive range which Australian *P. aculeata* became susceptible to due to a loss of potentially protective endophytes when introduced. This can be tested by comparing endophytes that are host-specific to *P. aculeata* in the native range with those from *P. aculeata* in the invasive range. There are no congeneric *Parkinsonia* species in Australia, so I surveyed endophyte communities hosted by *P. aculeata* in native-range sites in Arizona, and two co-occurring *Parkinsonia* species: *P. florida* and *P. microphylla*. My second aim was to determine whether *P. aculeata* endophyte communities from the native range (Texas and Arizona, USA) vary significantly compared to the trans-continental populations in the invasive range (QLD, NT and WA, Australia). Given that *P. aculeata* in Australia is exposed to different microbial communities than populations in the native range, and given that dieback is only observed in the Australian range, addressing this latter aim may help to identify endophytic agents that facilitated its invasion into Australia and/or are associated with dieback.

5.3 Materials and Methods

5.3.1 Sampling

I sampled stems and stem tips from five healthy *Parkinsonia aculeata* trees at each of seven sites in Australia from April to June 2015 (invasive populations; Fig 5.1

a,b,c); stems, stem tips and leaves from five healthy *P. aculeata* trees at seven sites in the USA, and between two and three healthy *P. florida* and *P. microphylla* trees in November 2015 (native populations; Fig 5.1d,e; See Appendix A Fig S5.1 for geographic coordinates of all sites and Table S5.1 for site and sample information).. Blades and secateurs for cutting plant parts were surface sterilised with 50% NaClO for 2 min between samples. Three samples from the stems (>5 cm long, secondary stems) and stem tips (>8 cm long, tips of stems) were collected from each tree. In addition in the USA, rachi attached to the stem tips (with leaflets) were collected. Samples were pooled by plant part and tree, stored in Ziplock plastic bags containing silica gel, and maintained at 5°C for up to 72 h before processing. Sites in Australia were chosen based on the presence of healthy and/or dieback parkinsonia populations in areas accessible for fieldwork. Some sampling was outsourced so only five trees were sampled at each site to maintain consistency across sampling and reduce the complexity in sampling strategies for the outsourced fieldwork. Samples used in this study only included above-ground plant parts due to restrictions on transporting soil and root material interstate within the USA.

5.3.2 DNA extraction

Samples were surface sterilised using a three-stage ethanol-bleach-ethanol method as described by Bills (1996) using an initial wash of 1 min in 70% ETOH, followed by 3 min in 50% NaClO and a further 1 min in 75% ETOH, before being dried with sterile paper towel. The epidermis of stem tips and stems was then removed using a sterile scalpel. Surface-sterilised leaflets and rachi were placed into sterile paper envelopes without epidermis removal. The inner tissue of stems and stem tips were also placed

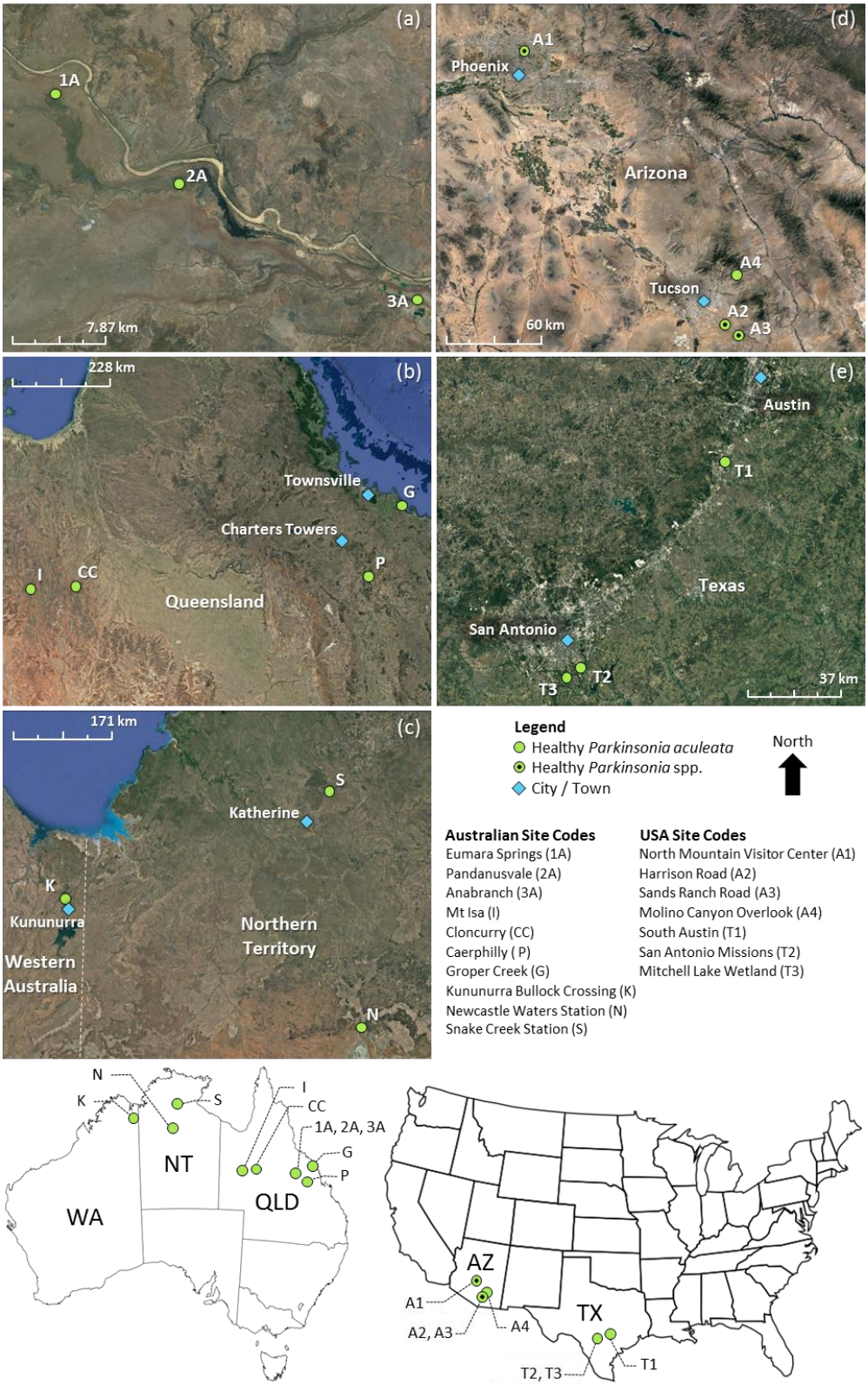


Figure 5.1 Satellite images of *Parkinsonia aculeata* sampling sites used in this study, showing sites in Australia (a,b,c) and the USA (d,e); and sites where samples from all three *Parkinsonia* spp. (*P. aculeata*, *P. florida* and *P. microphylla*) were collected (images: ©2016 Google). Schematic maps below satellite images also indicate approximate locations of each site by that country's states. Country maps are not to scale.

into sterile paper envelopes. Samples were freeze-dried for 72 h and ground to a fine powder in a MP Biomedical FastPrep® homogenizer for 2–3 min in 2 mL mL screw-cap Eppendorf® tubes containing two sterile 6 mm glass beads. The MO BIO PowerSoil® DNA Isolation Kit was then used to extract total DNA from approximately 20 mg of each powdered sample, according to manufacturer's instructions. DNA was quantified on a QuBit® Fluorometer (Thermo Fisher Scientific), normalised to 5 ng/µL with sterile dH_2O, and stored at -20°C.

5.3.3 PCR amplification and sequencing

DNA was amplified using PCR in two rounds (Fig 5.2). For the first round I used taxon-specific primers (Table 5.1) for the bacterial 16S rRNA gene or the fungal internal-transcribed spacer (ITS) region. The PCR reactions (16S, ITS) consisted of 5× MyTaq Reaction Buffer, 0.5 µL MyTaq polymerase, 10 ng extracted DNA, and 10 µM of each primer, and dsH_2O replaced DNA for the negative control. Conditions for fungal reactions consisted of 95°C for 2 min; 35 cycles of 95°C for 20 s, 50°C for 30 s, 72°C for 50s; then 72°C for 10 min. For bacterial 16S amplification, PCRs were run at 95°C for 2 min; 35 cycles of 95°C for 20 s, 50°C for 30 s, 72°C for 50s; then 72°C for 10 min. I then ran a 1.5% agarose gel testing the first column of each plate (including negative control and DNA samples) to confirm amplification of samples at the expected size. Samples were then purified using the AMPure XP kit according to the manufacturer's instructions, and normalised to 10 ng/µL with sterile dH_2O. Normalised amplicons were pooled by sample (i.e., pool ITS and 16s amplicons from sample X in one well) by adding an equal volume (5 µL) of each, and normalised to 5 ng/µL with sterile dH_2O.

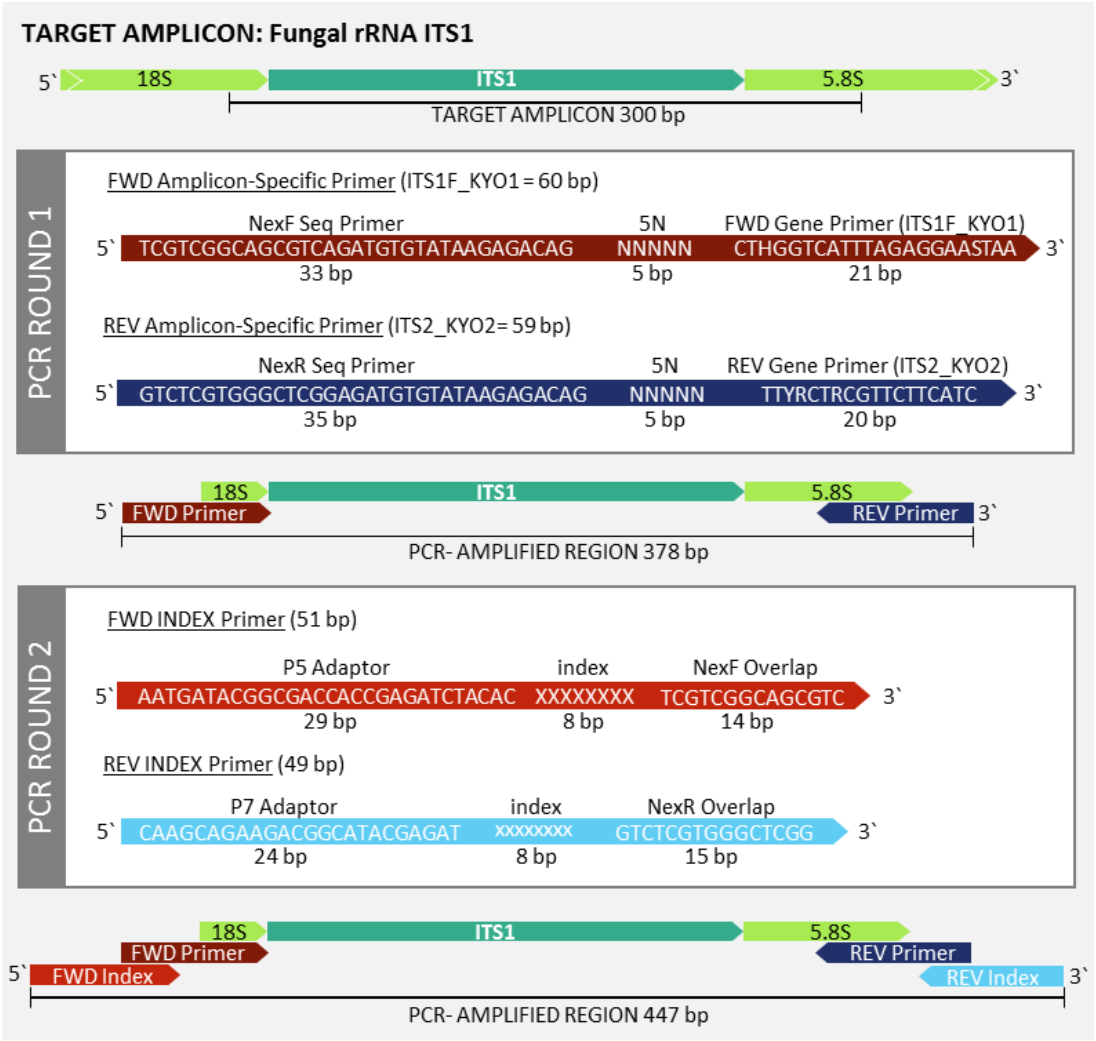


Figure 5.2 A schematic diagram illustrating the multiplex dual-indexed PCR approach used in this study, with the fungal ITS1 amplicon-specific primers in this example. See Table 5.1, the electronic supplementary material in Appendix B, and Appendix C (Table C5) for full list of primers used in this study.

Table 5.1 Primers used in the first round of PCR in this study for fungal ITS and bacterial 16s amplification

Primer	Sequence (5'- sequencing primer - 5mer Ns - amplicon specific primer - 3')	Product size	Reference
Fungal ITS1:			
nexF-N5-ITS1F_KYO1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-NNNNN-CTHGGTCATTTAGAGGAASTAA	350 bp	Toju et al. (2012)
nexR-N5-ITS2_KYO2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-NNNNN-TTYRCTRCGTTCTTCATC		
Bacterial 16s:			
nexF-N5-515f	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-NNNNN-GTGYCAGCMGCCGCGGTAA	300 bp	Apprill et al. (2015)
nexR-N5-806rB	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-NNNNN-GGACTACNVGGGTWTCTAAT		

For the second PCR round, primers were designed according to the dual-indexing approach from Toju et al. (2016) to contain the Illumina P5 or P7 adaptor, an 8 bp index sequence and a nexF or nexR overlap to anneal to the sequencing primer from the first PCR round amplicons (Forward primer: 5'- AAT GAT ACG GCG ACC ACC GAG ATC TAC AC - XXX XXX XX - TCG TCG GCA GCG TC -3'; Reverse primer: 5'- CAA GCA GAA GAC GGC ATA CGA GAT - XXX XXX XX - GTC TCG TGG GCT CGG -3'; see Table C5, Appendix C for a full list of index primers used in this study). PCRs consisted of 16 µL Accumprime Pfx Supermix, 10 ng pooled first-round PCR amplicons, and 10 µM each primer in a 20 µL reaction. They were run at 95°C for 2 min; 8 cycles of 95°C for 20 s, 52°C for 30 s, 72°C for 50s; then 72°C for 10 min. I purified PCR products by size selection to >200 bp using the AMPure XP kit. Samples were run on a Bioanalyzer and QuBit for quantification, and then sequenced on the MiSeq 3000 Illumina sequencing platform with 20% PhiX at the Vincent J. Coates Genomics Sequencing Laboratory (UC Berkeley). For the detailed protocol used here, see Appendix C.

5.3.4 Amplicon sequence analysis

Illumina sequencing reads were cleaned up and curated after Bissett et al. (2016). The quality of all Illumina R1 and R2 reads was assessed visually using FastQC (Andrews 2016). Sequences were merged using FLASH (Magoč & Salzberg 2011). Merged sequences were converted to FASTA format. For 16S rRNA sequences, sequences < 270 bp, or containing N or homopolymer runs of > 8 bp, were removed using MOTHUR v1.34.1 (Schloss et al. 2009). For ITS region sequences, full ITS1 region sequences were extracted using ITSx (Bengtsson-Palme et al. 2013).

For both amplicons, sequences were clustered and assigned to OTUs using USEARCH 64 bit v8.0.1517 (usearch –cluster_otus -strand plus –id 0.97 and usearch –usearch_global –strand plus –maxaccepts 10 –maxrejects 256). Finally, sequences were classified against the green genes database (May, 2013) or the UNITE fungal database (v7.1) using the rdp classifier as implemented in MOTHUR, using 60% probability cut-off. Any OTUs not belonging to bacteria or fungi were removed from the dataset (including mitochondria and chloroplasts). Remaining target OTUs were blasted (default blast+) against NCBI-nt for more thorough identification in November 2016. Raw FASTQ files of all generated sequences are available from Genbank (Bioproject SUB2552391). Target sequence data can be found in the electronic supplementary material (Appendix B)

5.3.5 Data analysis

OTU accumulation curves were calculated with the rarecurve function of the VEGAN v2.4-0 package (Oksanen et al. 2016) in the R statistical environment (R Core Team 2016). Bray-Curtis resemblance matrices were constructed in PRIMER v6 (Clarke & Gorley 2013) prior to analysis of variance for each effect (host species, host range and plant part) using permutational ANOVA (PERMANOVA; Anderson 2001). This determines which effects are significantly correlated with community composition. Significant effects were further explored in PRIMER v6 using unconstrained Principle Coordinate Ordinations (PCO; Gower 1966) to view community patterns, SIMPER (similarity percentages) to determine which OTUs contributed to the observed effects, and RELATE (Clarke & Gorley 2013) to look for correlations between community composition and the geographic distance between

sites. I then compared the contributions of taxonomic groups to the effect of each significant variable by mapping extended error bar plots (Welch's t-test with 95% confidence intervals) in STAMP v2.1.3 (Parks et al. 2014). These plots show the difference in mean proportion between two groups, accounting for multiple comparisons using q-value correction (Benjamini-Hochberg's false discovery rate; Benjamini & Hochberg 1995).

5.4 Results

5.4.1 Fungal and bacterial endophyte sequence diversity

The average number of target sequences recovered per sample varied for fungi and bacteria. The fungal primers amplified over 600,000 sequences, of which 79% were classified as fungal OTUs, with an average of 1,282 ± 28.7 target sequences per North American sample and 1,550 ± 76.8 per Australian sample. The 16S rRNA primers amplified 7,967,376 sequences, of which only 0.019% were classified as bacterial OTUs (the majority of the non-target OTUs were closest matches to plant plasmid DNA), with an average of 1.1 ± 0.2 target sequences per North American sample and 15.5 ± 6.1 target sequences per Australian sample.

The OTU accumulation curves for samples pooled by location and tree species indicate that the numbers of OTUs were nearly saturated for the fungal ITS region in *P. aculeata*, and in particular for *P. aculeata* sampled in Arizona (Fig 5.3a). For bacteria, only sequences from Australian *P. aculeata* neared saturation (Fig 5.3b). For fungal and bacterial OTUs, *P. microphylla* and *P. florida* were under sampled.

5.4.2 Host species affected fungal endophyte community composition

There were significant host species (Pseudo-F = 2.607, $P < 0.001$), plant part (Pseudo F = 2.4174, $P < 0.001$) and sampling site (Pseudo-F = 1.43, P = 0.011) effects on fungal endophyte community composition (Table 5.2). Significant differences in community composition were observed among all three host species and between leaves and either stems or tips. It also showed that communities in tips and stems were most similar to each other (Table 5.3).

The most abundant fungal OTUs in *P. aculeata* were classified as most closely related to Ascomycota sp., Dothioraceae sp., *Alternaria* sp., Dothioraceae sp., *Cladosporium ramotenellum, Phoma* sp. and *Alternaria penicillatum* (Table S5.2a, Appendix A). These fungal OTUs made up 51.67% of fungal sequences recovered from *P. aculeata*. OTUs classified as closest matches to *Alternaria* sp., *Aureobasidium* sp. and *Alternaria penicillatum* made up 55.32% of sequences recovered from *P. florida* (Table S5.2b, Appendix A).

For *P. microphylla*, OTUs classified as *Capnodium* sp., *Gymnopus brassicolens*, *Capnobotryella* sp. and *Alternaria penicillatum* made up 54.30% of recovered sequences (Table S5.2c, Appendix A). Despite the differences in abundance of taxa within each host species, further analyses of target OTUs using STAMP were unable to show that particular OTUs contributed significantly to the variation in community composition among hosts.

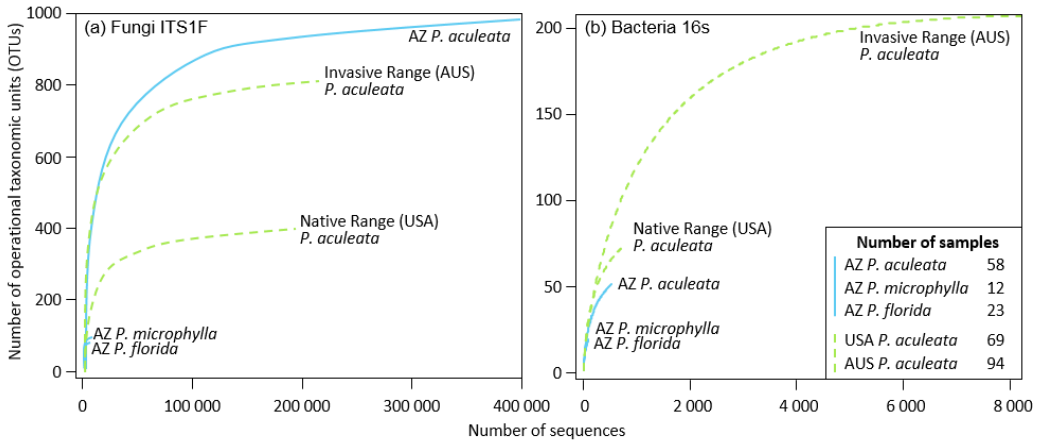


Figure 5.3 Fungal (a) and bacterial (b) OTU accumulation curves comparing leaf, stem and stem tip endophyte communities hosted by healthy trees from three *Parkinsonia* species (*P. aculeata*, *P. florida* and *P. microphylla*) sampled in Arizona (AZ) USA (solid blue lines); and stem and stem tip endophyte communities hosted by healthy trees sampled from native range (USA) *P. aculeata* in AZ and TX to healthy trees sampled from invasive range (Australia) *P. aculeata* in QLD, NT and WA (dotted green lines). The number of samples analysed for both fungi and bacteria are indicated in the box on the bottom right.

Table 5.2 Comparisons between fungal communities hosted by *Parkinsonia* spp. from Arizona USA, by plant part, host species and sampling site, as calculated by PERMANOVA (pseudo-*F*) where $P < 0.05$

	Fungi		**Bacteria**	
Effect	**Pseudo-*F***	***P***	**Pseudo-*F***	***P***
Site[a] (A1, A2, A3)	1.4300*	0.011	0.7996	0.711
Species[b] (PA, PM, PF)	2.6070*	0.001	1.2783	0.185
Plant part (stems, tip, leaves)	2.4174*	0.001	2.2156	0.007

[a]Three sites in Arizona – see Figure 5.1d

[b]*Parkinsonia aculeata*: PA, *P. florida*: PF, and *P. microphylla*: PM

Table 5.3 Three pairwise PERMANOVA tests of significant variation effects from Table 5.2 in fungal endophyte communities hosted by *Parkinsonia* spp. from Arizona USA, by host species, plant part and sampling site where $P < 0.05$

	Comparison	**Pseudo-*t***	***P***	**Average similarity %**
Site[a]	A1, A2	1.0271	0.383	8.35
	A1, A3	1.1732	0.053	8.36
	A2, A3	1.2442*	0.035	9.49
Host species[b]	PA, PF	1.8758*	0.001	7.90
	PA, PM	1.5668*	0.001	6.05
	PF, PM	1.3932*	0.004	4.99
Plant part	Leaf, Tip	1.5796*	0.001	9.30
	Leaf, Stem	1.4275*	0.001	7.13
	Tip, Stem	0.96839	0.571	7.27

[a]Three sites in Arizona – see Figure 5.1d

[b]*Parkinsonia aculeata*: PA, *P. florida*: PF, and *P. microphylla*: PM

5.4.3 Host species was not observed to affect bacterial endophyte community composition

I did not find any significant effects of host species or sampling sites on the composition of bacterial endophyte communities, but there was a significant plant part effect (Table 5.2). Some of the most abundant bacterial OTUs in *Parkinsonia* were classified as *Sphingomonas yabuuchiae*, *Escherichia coli* and *Corynebacterium variabile* (Table S5.3a,b,c; Appendix A).

5.4.4 Fungal endophyte community composition in *P. aculeata* differed by host location

The composition of fungal endophyte communities in *P. aculeata* differed significantly between the Australian and North American range (Pseudo F = 4.9437, P = 0.001). Fungal community composition also differed among sites within each range (Pseudo F = 3.5015, P = 0.001), in all plant part and sampling site combinations, except for sites in NT and WA, which shared 43% average similarity (Table 5.4). A comparison of fungal endophyte community composition against geographic distance between sites was also significant (RELATE ρ = 0.273, P = 0.005).

Table 5.4 Pairwise PERMANOVA showing variation in fungal endophyte community by geographic region (state) and the average similarity of these communities between each state/group

Group	Pseudo-*t*	*P*	Av. similarity (%)
AZ, TX	2.1769*	0.001	21.58
AZ, QLD	2.4498*	0.001	13.03
AZ, NT	2.7411*	0.001	23.80
AZ, WA	2.1522*	0.001	19.47
TX, QLD	2.4499*	0.001	19.64
TX, NT	1.8381*	0.005	39.33
TX, WA	1.5735*	0.010	32.78
QLD, NT	2.5032*	0.001	24.28
QLD, WA	1.7693*	0.001	21.13
NT, WA	1.2925	0.053	43.26

In North American *P. aculeata*, the most abundant OTUs were classified as most similar to *Cladosporium ramotenellum*, *Alternaria* sp., and *Phoma* sp. (51.87% cumulative relative abundance; Table S5.4a, Appendix A). The most abundant OTUs in Australian *P. aculeata* were classified as most similar to *Cladosporium ramotenellum*, *Alternaria* sp., *Phoma* sp. and Trichosphaeriales sp. (50.61% cumulative relative abundance; Table S5.4b, Appendix A). Although there is similarity between the most abundant OTUs in either group, SIMPER (similarity percentages) analysis showed that the abundance of these taxa were highly dissimilar, with *Phoma* spp. contributing 30.32% to the total dissimilarity between communities by range (72.45%), followed by *Cladosporium* (7.26%), unclassified Pleosporales (6.77%), *Alternaria* (5.52%) and *Penicillium* (4.86%). Further analysis of the most abundant OTUs that make up 90% of the reads recovered from each range using STAMP, indicated that OTUs classified as most similar to *Malassezia restricta*, *Penicillium spinulosum* and *Fusarium graminearum* were significantly associated with the Australian range, and *Aureobasidium* sp. was significantly associated with the North American range (Fig 5.4).

5.4.5 Bacterial endophyte community composition in *P. aculeata* differed by host range

Bacterial endophyte composition differed significantly between host ranges (Pseudo $F = 8.708$, $P = 0.0001$). Bacterial community composition also differed within each range (Pseudo $F = 2.8862$, $P = 0.001$), but within-range variation was only significant (where $P < 0.01$) when comparing QLD sites to NT sites (Pseudo-$t = 1.809$, $P = 0.003$). I found no significant correlation between variation in bacterial endophyte

communities by geographic region with the matched geographic distances between sites (RELATE ρ = 0.053, P = 0.253).

The OTU classified as most similar to *Sphingomonas yabuuchiae* made up 52.73% relative abundance in Australian range samples (Table S5.5a, Appendix A), followed by *Phytoplasma australiense* (10.19%) and *Escherichia coli* (9.95%). In comparison, seven OTUs made up 54.43% cumulative relative abundance in North American samples (Table S5.5b, Appendix A). These OTUs were classified as most similar to *Massilia niastensis*, *Sphingomonas yabuuchiae*, *Curtobacterium* sp., *Sphingomonas* sp., *Corynebacterium* sp. and *Kineococcus* sp. Using SIMPER analysis, the average abundance of *Sphingomonas* in the native range contributed 19.51% to the average dissimilarity between ranges, while *Escherichia* in the Australian range contributed 9.62% where total dissimilarity between ranges was 89.46%. The dissimilarity of *Escherichia* and *Sphingomonas* abundance between the two ranges was confirmed by STAMP analysis (Fig 5.5), which also showed that OTUs identified as most similar to *Staphylococcus equorum* were also significantly associated with Australian range *P. aculeata*.

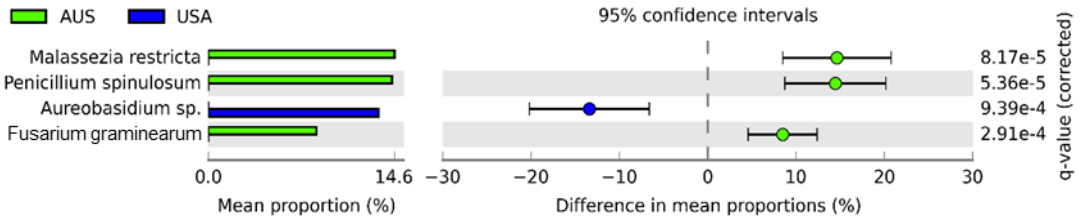


Figure 5.4. Fungal endophyte taxa that differ significantly ($q < 0.05$) in abundance between *Parkinsonia aculeata* in the invasive Australian range (green) and native USA range (blue). Only OTUs with a cumulative relative abundance of >90% were considered in this analysis. Differences between proportions of each genus are shown with 95% confidence intervals. The bar plot indicates the mean proportion of fungal sequences assigned to the range for each genus.

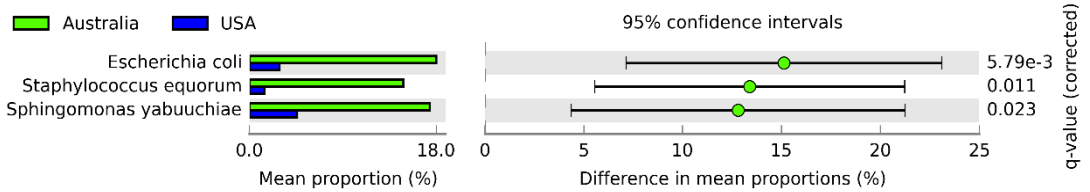


Figure 5.5 Bacterial endophyte taxa that differ significantly ($q < 0.05$) in relative abundance between *Parkinsonia aculeata* in the native range (blue) and invasive range (green). Only OTUs with a cumulative relative abundance of >90% were considered in this analysis. Differences between proportions of each taxon are shown with 95% confidence intervals.

5.1 Discussion

5.1.1 *Parkinsonia* fungal endophyte communities are driven by host species

I found that the composition of fungal endophyte communities in co-occurring congeneric *Parkinsonia* species (*P. aculeata*, *P. florida* and *P. microphylla*) differ significantly. Although I cannot determine how their ecological roles affect their hosts, my results indicate that fungal endophyte community composition is affected by host species within the *Parkinsonia* genus.

Variation in the composition of fungal endophyte communities is often shaped strongly by host plant identity, as shown in studies comparing endophytes in mistletoes with their pine hosts (Peršoh et al. 2013), coastal dune grass species (David et al. 2016), and angiosperm with gymnosperm host trees (Weig et al. 2013). Host effects on endophyte communities are, however, not always apparent and often endophytes are indistinguishable between closely related hosts (Peršoh 2015). This was illustrated in the case of eleven tropical grass species, where endophyte community composition was similar, regardless of host (Higgins et al. 2014). Species-level host-specificity in *Parkinsonia* spp. fungal communities might have suggested that if *P. aculeata* was introduced to Australia alongside co-evolved beneficial fungal endophytes, these endophytes may have given *P. aculeata* a competitive advantage in the new range via "Invasional meltdown" (Simberloff & Holle 1999; Mack 2003). However, the significant effect of range on fungal community composition between native and invasive *P. aculeata* further implies that these communities were not introduced alongside *P. aculeata* and therefore this hypothesis was not supported by the results. The PERMANOVA analysis showed that fungal endophyte community

composition was affected by sampling site location (within the 240 km sampling distance in Arizona). Based on the PERMANOVA results it is likely, therefore, that at a more local scale, the effect of host species is marginally stronger (Pseudo-$F_{\text{host species}} = 2.607$, $P < 0.001$) than geographic location (Pseudo-$F_{\text{sampling site}} = 1.43$, $P = 0.011$) on fungal endophyte communities. This suggests that at this geographic scale at least, endophyte communities are more likely to preference a host based on its identity more so than solely on local geographic convenience for colonization. Since a host species effect was observed, the next step was to determine which, if any, endophytes were likely to have accompanied *P. aculeata* to Australia, and whether these taxa can be classified as beneficial to invasion success.

I expected that bacterial endophytes might face similar barriers to colonization as fungal endophytes. Unlike fungal endophytes however, there were no significant or consistent effects due to sampling site or host species on the composition of bacterial endophyte communities. This is the first time bacteria have been sequenced from *Parkinsonia* species, but the lack of a significant effect on bacterial communities by host species was unexpected since host specificity of some root-associated bacterial symbionts has been previously established (Bokulich & Mills 2013). Bacterial communities are also host specific in the seaweed species A*sparagopsis taxiformis* and *A. armata* (Aires et al. 2016), and variation in bacterial endophyte communities by plant part (Ma et al. 2013) and host habitat has also been shown in *Stellera chamaejasme* (Jin et al. 2014). Bacterial coverage curves for *P. microphylla* and *P. florida* indicated significant under sampling of the diversity within bacterial endophyte communities. Lack of significance may therefore be due to sampling depth or sequencing strategy. As in this study, Steinrucken et al. (2016; Chapter 2) also found

significant variation in bacterial community composition by *P. aculeata* plant part using different PCR primers (1087r; Hauben et al. 1997; and 63f; Marchesi et al. 1998). Additionally, the DNA extraction kit (Zielińska et al. 2017) or amplification approach may not have been optimal for bacterial endophytes due to the high percentage of plastid DNA recovered, so alternative primers such as those used by Redford et al. (2010) could be considered in future work. Without saturated rarefaction curves, it is difficult to draw conclusions about the influence of host specificity on bacterial communities. Regardless, the results suggest that the role of host specificity and aboveground host-endophyte associations for fungal communities is greater than for bacterial communities.

5.1.2 *Parkinsonia* endophyte community composition by host range

I expected to find variation in endophyte community composition between host *P. aculeata* populations from the North American range compared to the invasive Australian range. The purpose of doing so was to gain insight into the invasion success and the occurrence of *P. aculeata* dieback in the invasive range. Despite extensive native range surveys (Heard 2006), dieback has not previously been observed in the native range (North, South and Central America), and therefore may be caused by a pathogen(s) that only affects invasive range (Australian) *P. aculeata* populations. While I did find a significant difference in fungal and bacterial endophyte community composition between the native and invasive ranges of *P. aculeata*, I could not attribute it to a difference in range alone.

It is possible that at the time of introduction, co-evolved natural enemies (diseases or pests) were not introduced with *P. aculeata*, allowing the plant to spread and

colonise northern Australia effectively, as per "enemy-release" (Keane & Crawley 2002; Colautti et al. 2004). Two hypotheses might help to explain the subsequent occurrence of dieback in the invasive range and not the native range. First, dieback may be due to new pathogens that have been encountered by the invasive range population since arrival in Australia. To test this, sequencing of endophyte communities was essential as, although other methods such as T-RFLP are informative (Steinrucken et al. 2016; Chapter 2), I needed to identify taxa that might be pathogenic to *P. aculeata.* In particular I was looking for patterns that are consistent with the presence of latent pathogens, which are symptomless infections prior to being triggered into pathogenicity by the host, environment or other cues (Sinclair & Cerkauskas 1996). Such a pattern would consist of the presence of OTUs classified as pathogens in the healthy samples analysed in this study. Ecological studies and/or pathogenicity testing would be required to confirm such a pathogen is latent.

Three fungal OTUs were more highly associated with *P. aculeata* in the invasive range compared to the native range. These OTUs were classified as *Fusarium graminearum* (Order: Hypocreales), *Penicillium spinulosum* (Order: Eurotiales), and *Malassezia restrica* (Order: Malasseziales). Of these three, only *Fusarium graminearum* is a plant pathogen, but it is most commonly associated with headblight in grains, and dieback of carnations (Wright et al. 1997). I could not, however, find reference to *F. graminearum* causing dieback or plant disease in woody plants. Isolation and pathogenicity screening of this species would be essential next steps in determining whether it is pathogenic in *P. aculeata*. The OTU classified as *Penicillium spinulosum* was also abundant in healthy invasive *Parkinsonia aculeata.* Antimicrobial metabolites produced by *Penicillium* are frequently investigated for

application in human health, with the flagship case being penicillin from *P. notatum*, which has been used for decades against gram-positive bacteria (Fleming 1980). It is possible that *P. spinulosum* plays a role in protecting the host from newly encountered pathogens in the invasive range, although extensive culture-based surveys, extractions of *Penicillium* metabolites, comparison with dieback-affected *Parkinsonia aculeata* and subsequent ecological studies would be needed to support this hypothesis. Another OTU, classified as *Malassezia restrica*, was also shown to be dominant in invasive *P. aculeata*. Although *M. restrica* is known as a human skin pathogen with no known plant pathogenic traits, the OTU may have been misclassified under this taxa since there are other *Malassezia* spp. which are plant pathogens.

Bacterial OTUs classified as *Sphingomonas yabuuchiae*, *Escherichia coli* (Phylum: Proteobacteria) and *Staphylococcus equorum* (Phylum: Firmicutes) were characteristic of invasive range *P. aculeata* bacterial communities. *Sphingomonas* species are common endophytes, often abundant in their host (Ulrich et al. 2008), and some species have been shown to benefit their hosts by improving growth, stress tolerance (Khan et al. 2014) and nutrient processing (Chen et al. 2014). In one study, "sphingomonads" were found in abundance on below-ground and aerial surfaces of a variety of plant species, with some indication they may promote plant growth (Kim et al. 1998). The dominance of the OTU classified as *Sphingomonas yabuuchiae* in healthy invasive *P. aculeata* may have positively influenced the success of these trees as invasive species, however I did not find evidence that this species in particular has these traits. *Escherichia coli* is most commonly considered a foodborne pathogen often associated with fresh produce as an endophyte within plant tissue (Wright et al. 2013), however I found no evidence of this OTU being phytopathogenic or beneficial to plant

hosts, and therefore cannot speculate as to its ecological role within *P. aculeata* or how it might affect invasion success. *Staphylococcus equorum* is an endophyte (Chaudhry et al. 2017) with no documented plant-pathogenic or beneficial traits, so despite the OTU classified as *S. equorum* having a significant association with invasive *P. aculeata*, I could not infer any ecological or biological role from its presence alone.

The second hypothesis which may explain the occurrence of dieback in the invasive and not the native range, is that dieback might be caused by generalist pathogens in the invasive range which *P. aculeata* is now susceptible to due to a loss of potentially protective endophytes upon introduction. I intended to test this by looking for endophytes that were host-specific to *P. aculeata* in the native range and in *P. aculeata* from the invasive range. This might indicate they were "lost" upon introduction (there are also other explanations for such a difference, and these are discussed further below). For the fungal endophyte communities, OTUs classified as *Aureobasidium* were more dominant in *P. aculeata* from the native range. When associated with plants, *Aureobasidium* species are most commonly saprotrophs (Tedersoo et al. 2014), and are also common airborne fungal species (CDC 2015) which may contaminate experiments. Once again, no bacterial OTUs were shown to be more significantly associated with native-range *P. aculeata*. These results therefore don't support the hypothesis that potentially protective, host-specific endophytes were lost upon introduction to the invasive range, at least with respect to those that I could detect in abundance in aboveground tissues.

I found that *P. aculeata* fungal communities from AZ were significantly different from all other sites, but that fungal communities in TX were more similar to Australian

P. aculeata than AZ *P. aculeata*. Australia's population of *P. aculeata* was more likely introduced from Venezuela and meso-America (Hawkins et al. 2007) and not the USA, so it is highly likely that *P. aculeata* in the source population hosts endophyte communities that differ from those analysed here. Importantly, any change in endophyte community composition between plants, particularly over large geographic distances, may be explained by other mechanisms. Endophyte communities are dynamic over space and time (Peršoh 2015) and, in the case of invasive plant introductions, endophyte composition at the time of establishment would depend upon mode of introduction (ranging from multiple whole plants to a single seed) to the invasive range. Whole plants may host a range of endophytes that are not present in the new range, whereas only vertically transmitted endophytes, and possibly some on the seed surface, are likely to accompany seed. These initial 'baseline' communities would then be expected to change over time (Peršoh 2015) due to factors such as soil conditions, the presence of locally-occurring plant species, climatic tolerance of existing endophytes, microbe–microbe interactions and plant-microbe interactions (Gaiero et al. 2013). Endophyte community composition has also been shown to differ with local environmental conditions (Peršoh 2015), distance decay (Toju et al. 2014) and mode of endophyte transmission (i.e., vertical vs. horizontal). Communities are also highly likely to have changed over time since the populations diverged.

Although I found significant host species and range effects on the composition of *P. aculeata* endophyte communities and a potential plant pathogen (OTU classified as *F. graminearum*) associated with Australian invasive *P. aculeata*, these data indicate that conclusions about the nature of host + endophyte community co-introduction may be premature. A reciprocal transplant would be necessary to determine whether this

pathogen can infect and trigger dieback in *P. aculeata* from the native range. This would also need to be accompanied by isolations from dieback-affected invasive *P. aculeata* and pathogenicity screening.

5.2 Conclusion

The primary motivation for this study was to determine whether *P. aculeata* in the North American native range are host to a different community of endophyte species compared to those in the invasive Australian range. I found a significant host species effect on fungal endophyte communities between co-occurring *Parkinsonia* spp. populations in the native range. I also found a significant difference between *P. aculeata* fungal and bacterial endophyte communities in the native range compared to the invasive range, however the results did not support the hypothesis that dieback occurrence in the invasive range is associated with the loss of potentially beneficial endophytes upon introduction of *P. aculeata* to Australia. The next step will be to perform similar analyses on healthy and dieback-affected *P. aculeata* in the invasive range, to identify potential pathogenic taxa which may be causing dieback. With the addition of pathogenicity testing, endophyte community composition results may lead to methods for use in biological control of invasive plants, by determining which endophytes are likely to have host-protective roles, and which are potential pathogens.

6

COMBINING CULTURE-INDEPENDENT AND CULTURE-BASED METHODS TO INVESTIGATE FUNGAL AND BACTERIAL ENDOPHYTE COMMUNITIES ASSOCIATED WITH *PARKINSONIA ACULEATA* DIEBACK[1]

6.1 Abstract

Dieback has been considered as a potential biological control tool for invasive *Parkinsonia aculeata* L. (parkinsonia) in Australia. Dieback results in high levels of parkinsonia mortality, does not affect local native species, and does not occur in parkinsonia's native range in the Americas. Parkinsonia fungal and bacterial endophyte community composition has been correlated with parkinsonia dieback occurrence previously, however putative dieback-causing pathogens have not yet been identified.

In this study I used culture-independent techniques to characterise the fungal and bacterial endophyte communities of healthy and dieback-affected parkinsonia from an invasive Australian population in Charters Towers, Queensland. I analysed endophyte communities from roots, stems and stem tips for significant effects on health status and plant part, and classified these taxa as most likely to be involved in dieback

[1] Please note that, when a reference to 'parkinsonia' is made in this chapter, it refers to *Parkinsonia aculeata*, unlike in the previous chapter wherein three congeneric species were analysed.

occurrence by assigning ecological guild classifications to OTUs by taxonomic groups. These results were then compared to a previous culture-based mycological study (Chapter 3) to determine which fungal taxa could be targeted in future pathogenicity screening.

Composition of the fungal community, but not the bacterial community, was shown to vary significantly with health status. *Fusarium*, *Neurospora*, *Cladosporium*, *Phoma*, *Curvularia* and *Acremonium* were identified as potentially pathogenic fungal genera typical of dieback-affected parkinsonia, with *Fusarium*, *Phoma* and *Neurospora* the most likely to be involved in dieback occurrence, in the absence of pathogenicity testing. OTUs classified as the common bacterial genera *Sphingomonas* and *Escherichia* were the only genera typical of parkinsonia regardless of health status. No fungal or bacterial taxa were identified as likely to confer pathogen resistance to parkinsonia.

The novel aspect of this work was that the approach allowed me to address questions that arose, but were not answerable in the previous T-RFLP study from Chapter 2 (which did not identify endophyte taxa) and the culture-based study from Chapter 3 (which likely only isolated a subset of the total fungal community). This study instead showed the usefulness of combining culture-based and culture-independent methodology in the diagnosis of plant disease, but emphasised the need for ecological testing, regardless of guild classifications due to assigned taxonomy.

6.2 Introduction

Dieback is characterised by a progressive reduction in plant health, beginning with defoliation, browning of the stems from the tips to the trunk, and often leading to

outright tree mortality (Chapter 2; Steinrucken et al. 2016). As with the majority of plant health phenomena, dieback occurrence is likely to be influenced by abiotic, biotic and host-centric factors (Manion 1991). Dieback affects both native and invasive plant species. Examples of native species dieback include that of the native Australian eucalypt Marri (*Corymbia calophylla*) in Western Australia by fungal pathogen *Quambalaria coyrecup* (Sapsford et al. 2016), and dieback in Kauri (*Agathis australis*) by *Phytophthora agathidicida* in New Zealand (Beauchamp & Waipara 2014). Populations of multiple invasive plant species in Australia have also been affected by dieback, which often reduces local density more effectively than other anthropogenic weed control methods (Aghighi et al. 2014; Haque 2015; Sacdalan 2015; Diplock 2016; Chapter 2, Steinrucken et al. 2016; Raghavendra et al. 2017). Interest in invasive plant dieback is spurred on by the potential for causative agents to be used in biological control (Templeton & Greaves 1984; Charudattan & Dinoor 2000; Boyetchko et al. 2002; Toh 2009). Dieback has not been observed in the native range of any of these Australian invasive species, and locally co-occurring species appear to remain unaffected.

Dieback may occur in invasive plants upon introduction or during spread due to new encounters with novel pathogens (Callaway & Ridenour 2004), the loss of co-evolved protective endophytes, which increase susceptibility to generalist pathogens (Evans 2008; Chapter 2, Steinrucken et al. 2016), or re-association with a co-evolved pathogen(s) from the native range when this pathogen(s) is subsequently introduced to the invasive range (Evans 2008). Additionally, invasive organisms usually have greater susceptibility to generalist pathogens due to lack of genetic variability within

the invasive population when introduced as only a few individuals (Colautti et al. 2004).

Parkinsonia aculeata L. (hereafter referred to as 'parkinsonia') is one such dieback-affected tree, invasive in Australia. Dieback has dramatically reduced some parkinsonia populations, but its cause remains unexplained. One specific complication in diagnosing the cause of dieback, is the lack of consistent symptoms within dieback-affected weed populations (e.g. fruiting bodies, lesions or consistently isolating the same pathogens). Climate also complicates diagnosis as climate extremes may cause initial host stress (Chapter 3; Steinrucken et al. 2017), resulting in increased susceptibility to secondary infections and possibly dieback-like symptoms such as leaf loss. Relationships between endophyte community structures and dieback-affected parkinsonia populations have been previously characterised by T-RFLP analysis, which showed a correlation between dieback occurrence and endophyte community composition (Chapter 2; Steinrucken et al. 2016). Culture-based studies have further characterised parkinsonia's culturable fungal (Chapter 3; Steinrucken et al. 2017) and oomycete taxa (Chapter 4; Steinrucken et al. 2017). Additionally, Illumina sequencing of endophyte communities in native and invasive parkinsonia populations have indicated that fungal endophyte communities are host specific within the *Parkinsonia* genus, and that fungal and bacterial communities differ between the native and invasive ranges (Chapter 5). Although the distinction between parkinsonia endophyte communities from each range was significant, none of the taxa identified in that study (Chapter 5) suggested that invasive populations were accompanied by potentially protective endophytes upon introduction to Australia. Despite evidence that the composition of fungal, bacterial and archaeal endophyte communities are associated

with dieback occurrence (Chapter 2; Steinrucken et al. 2016), the specific causative agent(s) of parkinsonia dieback remain unknown.

To determine which endophytes are likely to be involved in dieback of invasive parkinsonia, I investigated endophyte communities in healthy and dieback parkinsonia using high throughout sequencing. This method extended previous findings, via endophyte community structure analysis, that endophyte community shifts are associated with plant health (Chapter 2; Steinrucken et al. 2016) and identified taxa closely associated with dieback-affected parkinsonia. Taxonomic information allowed the derivation of likely ecological traits for identified endophytes (using, for example, Nguyen et al. 2016), which were then combined with results from the culture-based study and results from the pathogenicity trial (Chapter 3; Steinrucken et al. 2017) to enable predictions of taxa likely to be involved in dieback.

6.3 Materials and Methods

I used the same samples collected in March 2013, as described in Chapter 2 (Steinrucken et al. 2016), along with the same sterilization and DNA extraction protocols, as described below.

6.3.1 Sampling

Five trees were sampled from each of three dieback-affected and three healthy populations of parkinsonia, located between 20 and 40 km north of Charters Towers, Queensland (Fig 2.1; Table S2.1, Appendix A). Where possible, sites were arranged into one healthy and one dieback-affected population such that pairs were closer to each other than to other replicates. I categorised parkinsonia as healthy if the plants had 70-100% foliage cover and all major and minor stems were healthy with no

consistent vascular staining or browning of branches (Fig 2.2a). Dieback-affected parkinsonia trees (Fig 2.2b) were classified as showing evidence of dieback, including defoliation (1-40% foliage cover), dead or dying stems (Fig 2.2c), vascular staining when sampled (Fig 2.2d) and evidence of tissue death in the stem tips, but were also confirmed as living specimens. Secateurs, shovels and blades used for sample collection were sterilized between sites by immersing in 50% NaClO for 5 min, and dried with sterile paper towel before next use. I sampled three roots, stems and stem tips (each 5 – 8 cm long) from each individual tree. Roots were rinsed free of excess soil after sampling using tap water. Samples from the same plant part of the same tree were stored together in sealed plastic bags containing silica gel packets. All samples were maintained for 48 - 72 h at 4°C until processed in the lab.

Surface sterilization was conducted in a UV-sterilized laminar flow cabinet and all instruments were sterilised between samples using 95% ETOH and a flame. All plant parts were firstly vigorously washed in sterile, distilled H_2O for 20 s. I used a three-stage ethanol-bleach-ethanol surface sterilization method for stems and stem tips as recommended by Bills (1996). Harsh sterilization techniques are not recommended for roots (Thorn et al. 2007), so they were washed for 30 s in sterile, distilled H_2O containing 0.1% Tween-20 ™. All samples were blotted dry with sterile filter paper and I checked surface sterilization by sliding the sterilized tissue over the surface of 50% potato dextrose agar (PDA) media as an imprint, incubating the plate at 30°C for a week or longer (Bacon and Hinton 2007). I removed the epidermis of stem tips and stems, and a small portion of root cortex using a scalpel. Until surface sterilisation was established by checking for growth on the PDA imprint, samples were stored in sealed, individual sterile plastic containers at -20°C. After confirmation of no growth on PDA

the inner tissue was then shaved and placed in sterile paper envelopes for DNA extraction.

6.3.2 DNA extraction

Sample shavings were freeze-dried for 48 h and placed in a MP Biomedical FastPrep® homogenizer for 2-3 min in 2 mL microcentrifuge tubes containing two sterile 2 mm steel ball bearings until a fine powder formed. I used a MO BIO PowerSoil® DNA Isolation Kit to extract total DNA from 20 mg of each powdered sample, according to manufacturer's instructions.

6.3.3 PCR amplification and sequencing

For a more detailed version of the sequencing protocol used in this study, refer to Appendix C.

The two rounds of PCR and the Illumina sequencing protocol used in this study is the same as described in Chapter 5. For the first round of PCR amplification, I used taxon-specific primers (Table 5.1) for the bacterial 16S rRNA gene and the fungal internal-transcribed spacer (ITS) region. These reactions consisted of 5× MyTaq Reaction Buffer, 0.5 µL MyTaq polymerase, 10 ng extracted DNA, and 10 µM of each primer. Conditions for the ITS-amplicon reactions consisted of 95°C for 2 min; 35 cycles of 95°C for 20 s, 50°C for 30 s, 72°C for 50s; then 72°C for 10 min. For the bacterial 16S amplicon, PCRs were run at 95°C for 2 min; 35 cycles of 95°C for 20 s, 50°C for 30 s, 72°C for 50s; then 72°C for 10 min. Samples were then purified with the AMPure XP kit according to the manufacturer's instructions, and normalised to 10 ng/µL with sterile, distilled H_2O. Normalised PCRs were pooled by sample by

adding an equal volume (5 µL) of each, and were then normalised to 5 ng/µL with sterile, distilled H_2O.

For the second PCR round, primers were designed according to the dual-indexing approach from Toju et al. (2016) to include the Illumina P5 or P7 adaptor, an 8 bp index sequence and a nexF or nexR overlap to anneal to the sequencing primer from the first PCR round (see Table C5 in Appendix C for a full list of index primers used in this study). PCRs (16 µL Accumprime Pfx Supermix, 10 ng pooled first-round PCR amplicons, and 10 µM each primer in a 20 µL reaction) were run at 95°C for 2 min; 8 cycles of 95°C for 20 s, 52°C for 30 s, 72°C for 50s; then 72°C for 10 min. PCR products were purified by size selection to >200 bp using the AMPure XP kit. Samples were quantified on a Bioanalyzer and QuBit, and then sequenced on the MiSeq 3000 Illumina sequencing platform with 20% PhiX at the Vincent J. Coates Genomics Sequencing Laboratory (UC Berkeley).

6.3.4 Amplicon sequence analysis

Illumina sequencing reads were cleaned up and curated using the same methodology as in Bissett et al. (2016) and analysed as in Chapter 5. The quality of all Illumina R1 and R2 reads was assessed visually using FastQC (Andrews 2016). Sequences were merged using FLASH (Magoč & Salzberg 2011). Merged sequences were converted to FASTA format. For 16S rRNA sequences, sequences < 270 bp, or containing N or homopolymer runs of > 8 bp, were removed using MOTHUR v1.34.1 (Schloss et al. 2009). For ITS region sequences, full ITS1 region sequences were extracted using ITSx (Bengtsson-Palme et al. 2013).

For both genes, sequences were clustered and assigned to OTUs using USEARCH 64 bit v8.0.1517 (usearch –cluster_otus -strand plus –id 0.97 and usearch –usearch_global –strand plus –maxaccepts 10 –maxrejects 256). Finally, sequences were classified against the green genes database (May, 2013) or the UNITE fungal database (v7.1) using the rdp classifier as implemented in MOTHUR, using 60% probability cut-off. Any OTUs not belonging to bacteria or fungi were removed from the dataset (including mitochondria and chloroplasts). Remaining OTUs were blasted (default blast+) against NCBI-nt for more thorough identification. Raw OTU and taxonomic data can be found in the electronic supplementary material (refer to Appendix B).

6.3.5 Data analysis

OTU tables were then subsampled where only those OTUs identified as fungi (for ITS) or bacteria (for 16S rRNA) were kept for further analysis. The rarecurve function of the VEGAN v2.4-0 package (Oksanen et al. 2016) was used to construct OTU accumulation curves in the R statistical environment (R Core Team 2016). PRIMER v6 (Clarke & Gorley 2013) was used to calculate Bray-Curtis resemblance matrices prior to using permutational ANOVAs (PERMANOVA; Anderson 2001) to analyse each effect (plant part and health status). Multiple plant parts were included to better partition variation and potentially use any interaction effect with health status to better locate causative agents. Significant effects were further explored using unconstrained Principle Coordinate Ordinations (PCO; Gower 1966), SIMPER (similarity percentages) and RELATE (Clarke & Gorley 2013). I subsequently compared the contributions of taxonomic groups to the effect of each significant variable by

mapping extended error bar plots (Welch's t-test with 95% confidence intervals) in STAMP v2.1.3 (Parks et al. 2014) with q-value correction using Benjamini-Hochberg's false discovery rate (Benjamini & Hochberg 1995).

In addition to the above analysis, I also compared my fungal sequencing results to data from Chapter 3 (Steinrucken et al. 2017) in which I isolated fungi from the same samples. Combining the results from the extended error bar plots generated in STAMP and the dominant fungal genera typical of either healthy or dieback samples, I then used the FUNGuild database (Nguyen et al. 2016) and literature searches to classify each genus by ecological guild and predict the likelihood of the organisms' involvement in dieback causation. Genera were classified as more likely to be involved in dieback occurrence due to a combination of SIMPER and STAMP analysis, taxon-associated ecological classification(s) and subsequent analysis of the literature.

6.4 Results

6.4.1 Fungal and bacterial endophyte sequence diversity

The average number of target sequences recovered per sample varied between fungi and bacteria. The fungal primers amplified over 177,000 sequences in total, of which 40% were classified as fungal OTUs, with an average of 299 ± 73.72 target sequences per healthy sample and 1,569 ± 410.32 per dieback sample. The OTU accumulation curves for samples pooled by plant part and health status indicated that the numbers of fungal OTUs were nearly saturated for the fungal ITS region in healthy parkinsonia and dieback-affected parkinsonia tips (Fig 6.1a).

For bacteria, the 16S rRNA primers amplified over 1,849,702 sequences in total, but only 0.25% were classified as bacterial. Non-target 16S sequences were classified

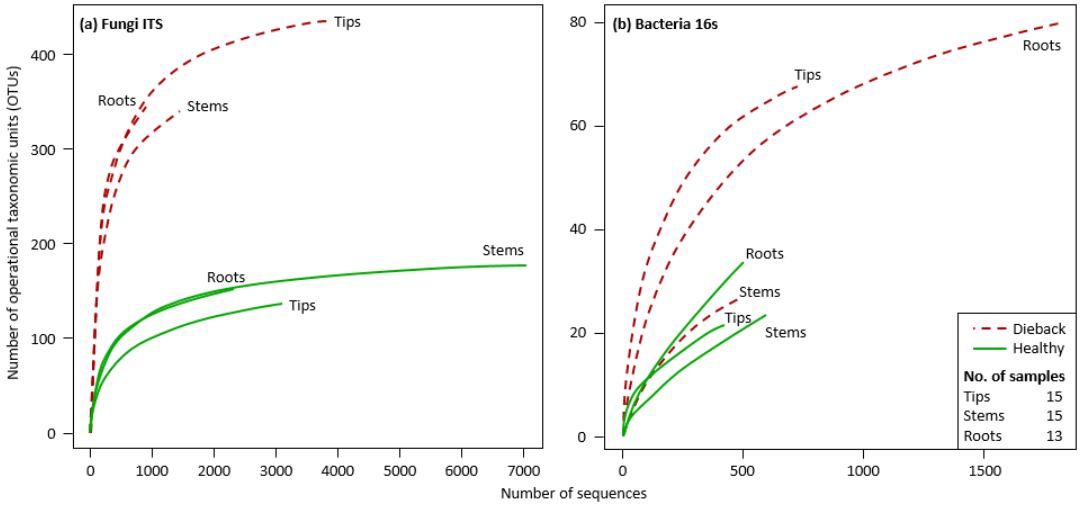


Figure 6.1 Fungal (a) and bacterial (b) OTU accumulation curves comparing leaf, stem and stem tip endophyte communities hosted by dieback-affected (red broken line) and healthy (green solid line) *Parkinsonia aculeata* sampled near Charters Towers, QLD (Australia). The number of samples analysed are indicated in the box on the bottom right.

mostly as plant plasmid DNA. There was an average of 36 ± 14.08 target bacterial sequences per healthy sample and 81 ± 28.20 bacterial sequences per dieback sample. OTU accumulation curves indicated that the bacterial endophyte community was under sampled, particularly in healthy parkinsonia and dieback-affected stems (Fig 6.1b).

6.4.2 Dieback occurrence affected fungal endophyte community composition

When testing the effects of health status and plant part on fungal endophyte communities (Table 6.1), by far the strongest significant effect was health status (Pseudo $F = 8.1117$, $P < 0.001$), followed by plant part (Pseudo $F = 1.7915$, $P = 0.002$). This was reflected in the PCO plot (Fig 6.2a) where dieback samples were mostly separated from healthy samples along axis 1 and most root samples were scattered near the bottom of axis 2. I did not find evidence of an interaction effect between plant part and disease status.

The most commonly sequenced fungal ordinal groups were Eurotiales (10,391 reads), Hypocreales (9,302 reads) and Pleosporales (8,745 reads; Fig 6.3a). The six most abundant OTUs, of the 233 recovered from healthy parkinsonia were classified as *Toxicocladosporium rubrigenum*, an unknown Ascomycota, *Penicillium spinulosum*, *Malassezia restricta* and *Fusarium graminearum* (49.11% cumulative relative abundance (RA); Table S6.1a, Appendix A). In dieback-affected parkinsonia, 14 unique OTUs out of a total of 568, made up 50% cumulative RA (Table S6.1b, Appendix A) including *Penicillium spinulosum* (10.20% RA), *Fusarium sublunatum* (6.69% RA), *Trichomerium foliicola* (2.51% RA) and *Antennariella placitae* (2.28% RA).

Table 6.1 PERMANOVAs of the *Parkinsonia aculeata* OTU community, testing the effects of disease status and plant part on fungal and bacterial endophyte community composition

	Fungi			**Bacteria**		
	df[a]	Pseudo-*F*	*P*-value	*df*[a]	Pseudo-*F*	*P*-value
Plant part	2	1.7915*	0.002	2	2.405*	0.001
Disease status	1	8.1117*	0.001	1	1.5508	0.066
Plant part x Disease status	2	1.1734	0.167	2	1.1868	0.2
Residuals	71			64		

[a] differences in df due to lack of amplification in some samples which were not included in the analysis
* Significant (where $P < 0.05$)

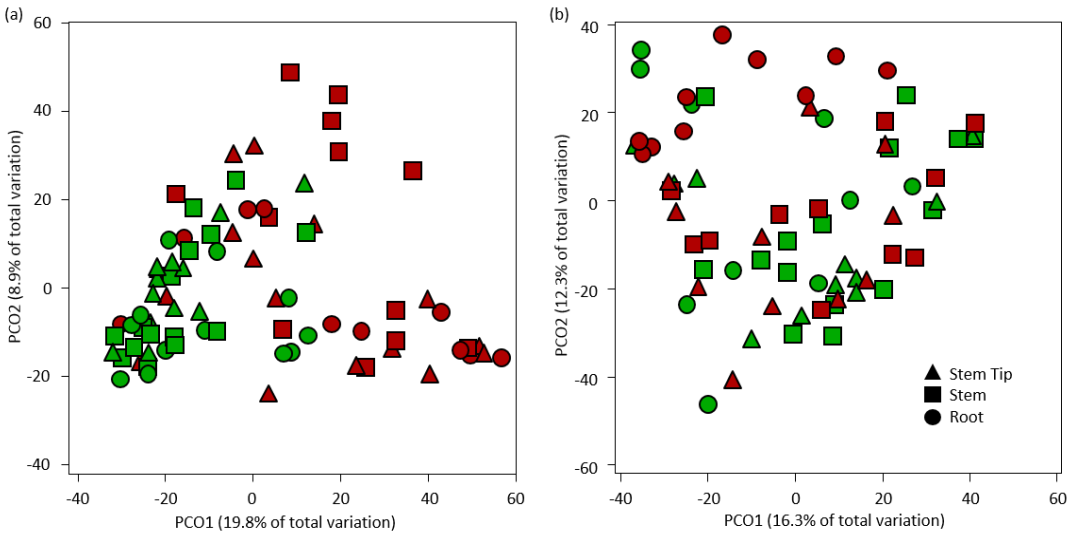


Figure 6.2 Principle coordinate ordination plots of *Parkinsonia aculeata* fungal (a) and bacterial (b) endophyte community data showing separation of samples by disease status (healthy = green, dieback = red) and plant part.

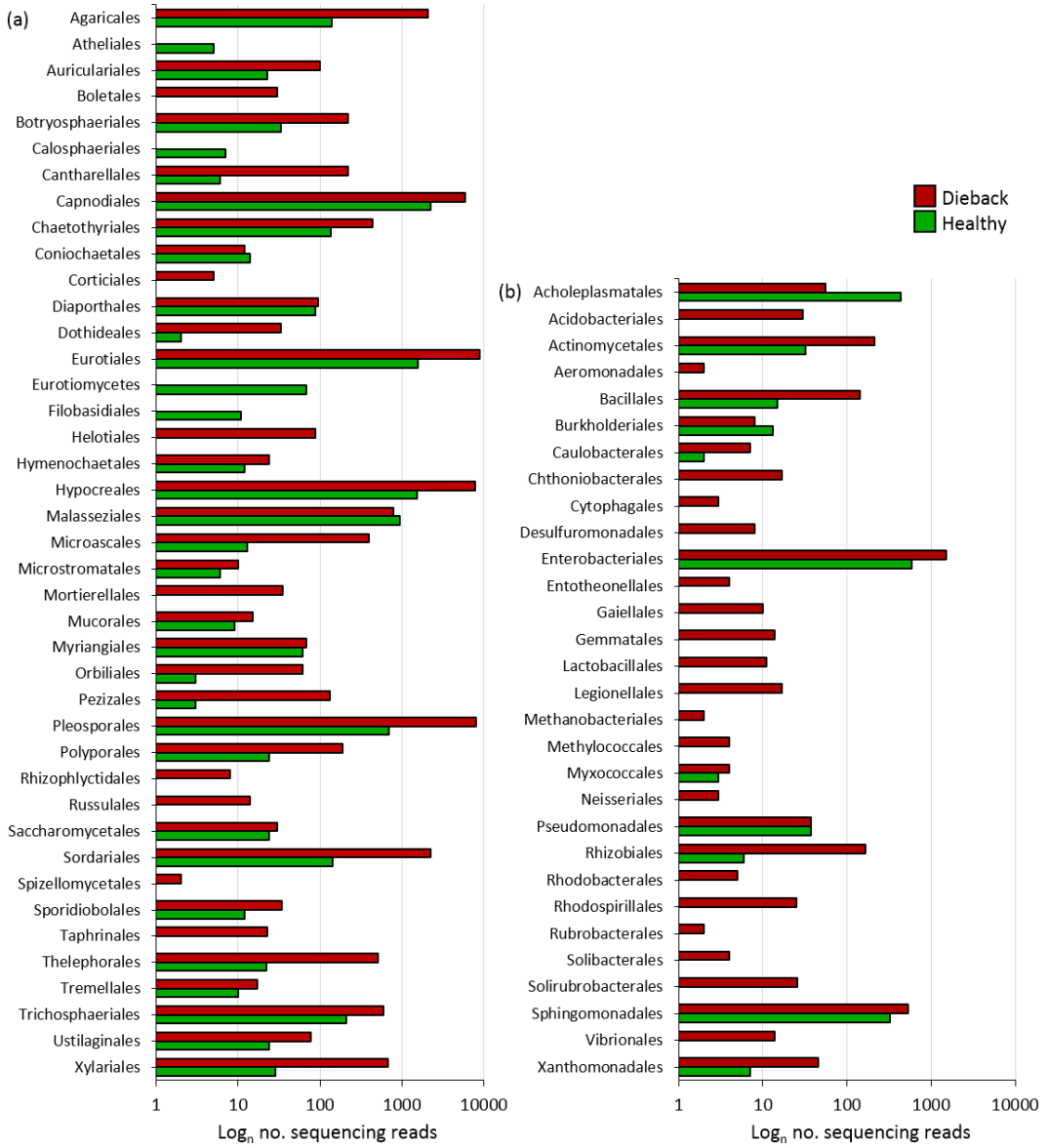


Figure 6.3 The number of Illumina sequencing reads by ordinal group recovered from fungal (a) and bacterial (b) endophyte communities from healthy (green) and dieback-affected (red) *Parkinsonia aculeata*.

All of the 26 genera classified by a genus that contributed significantly to the variation in fungal community by health status (STAMP analysis; Fig 6.4), none were more abundant in healthy plants. At the 0.05 q-value cut off, all had higher RA in dieback-affected plants. This included OTUs classified as *Neurospora, Acremonium, Hypocrea, Tomentella, Inocybe and Curvularia*.

From the SIMPER analysis (for summary see Table 6.2 or full results Table S6.3, Appendix A), the average community similarity among all healthy samples was 27.25% made of contributions from seven genera, including *Apergillus* (32.98%), *Fusarium* (21.77%) and *Penicillium* (15.8%), that could be said to be typical of healthy samples (Table 6.2). There were 17 genera typical of dieback-affected samples (average similarity of 29.06%) with contributions from *Penicillium* (16.29%), *Fusarium* (13.72%) and *Malassezia* (12.38%). The average of the Bray-Curtis dissimilarities among all pairs of healthy and dieback samples was 76.32%, made up of 9.43% contribution from *Penicillium*, 8.17% from *Malassezia* and 27.54% from *Giberella* (Table 6.2).

Of the fungal genera dominant in dieback-affected parkinsonia, as observed via SIMPER and STAMP analyses, many were classified as saprotrophs and some (e.g. *Fusarium, Acremonium, Curvularia, Hypocrea, Neurospora* and *Phoma*) were cited as plant pathogens (see citations in Table 6.2). There were fewer genera classified under ecological guilds known to confer benefits. They were *Cladosporium*, *Tomentella* and *Inocybe.*

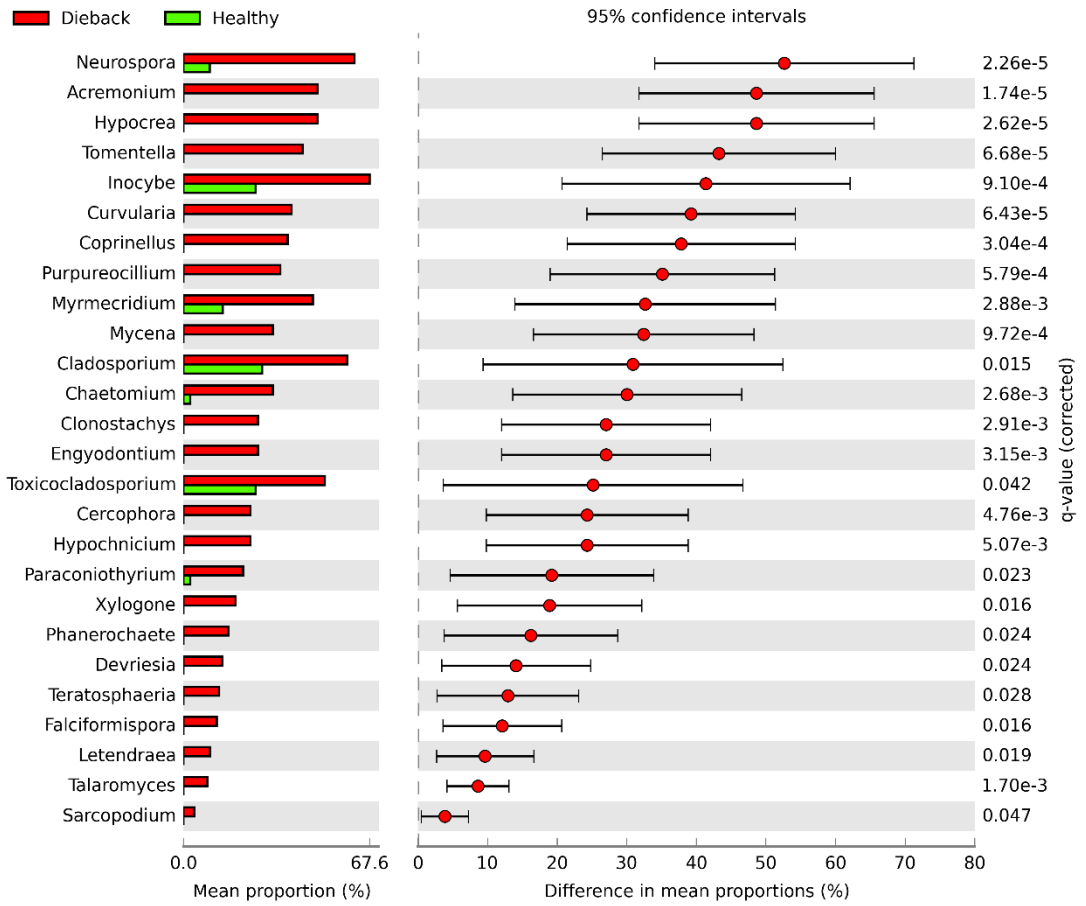


Figure 6.4 Fungal endophyte genera that differ significantly ($p < 0.05$) in relative abundance between dieback-affected (red) and healthy (green) *Parkinsonia aculeata*. Taxa are ordered by increasing q-value. Differences between proportions of each genus are shown with 95% confidence intervals.

6.4.3 Dieback occurrence was not shown to affect bacterial community composition

For the bacterial endophyte data, there was no health status effect (Table 6.1), but communities were structured by plant part (Pseudo $F = 2.405$, $P = 0.001$), as observed in the PCO where most root samples are near the top of axis 2 (Fig 6.2b).

Bacterial OTUs classified as *Escherichia coli*, *Phytoplasma australiense* and *Sphingomonas yabuuchiaewere* contributed 89.58% cumulative RA to the 49 total recovered for healthy parkinsonia samples (Table S6.2a, Appendix A). There were 135 bacterial OTUs recovered from dieback-affected parkinsonia samples, and unlike the healthy samples, 41 OTUs made up the top 90% cumulative RA, with *Escherichia coli* (49.21% RA), *Sphingomonas yabuuchiae* (9.72% RA) and *Sphingomonas wittichii* (3.27% RA) as the top three most abundant OTUs (Table S6.2b, Appendix A). The most commonly sequenced bacterial orders were OTUs classified as Enterobacteriales, Sphingomondales and Acholeplasmatales (Fig 6.3b). Since no significant effect of health status was found for bacterial communities, I did not build extended error bar plots in STAMP or conduct SIMPER analysis for these data.

Table 6.2 Summary of significant associations of fungal genera with healthy (H) or dieback-affected (D) *Parkinsonia aculeata* using results from SIMPER (similarity percentages, Primer-E), extended error bars (STAMP), isolations, and the ecological classification of each genus as determined by a search of the literature, with citations[e] listed below.

			SIMPER[a]			STAMP[b]			Ecological classification[d]									
Division	**Order**	**Genus**	**H Similarity (27.25%)**	**D Similarity (29.06 %)**	**Dissimilarity (76.32%)**	**H STAMP p-value**	**D STAMP p-value**	**Isolated in Chapter 3[c]**	**Plant pathogen**	**Mycoparasite**	**Plant saprotroph**	**Undefined saprotroph**	**Anti-microbial**	**Confers benefits**	**Symbiont/ Endophyte**	**Animal pathogen**	**Ubiquitous**	**Citation[e]**
Ascomycota	Botryosphaeriales	*Diplodia*	-	-	0.67	-	-	HD										1
	Capnodiales	*Capnodium*	-	-	2.31	-	-	HD										2
		*Cladosporium**	-	4.60	3.93	-	0.015	HD										3
		Devriesia	-	-	0.55	-	0.024	-										2
		Teratosphaeria	-	-	0.77	-	0.028	-										2
		Toxicocladosporium	2.14	3.13	4.58	-	0.042	-										4
	Chaetothyriales	*Cladophialophora*	-	-	0.59	-	-	-										5
		Cyphellophora	-	-	0.58	-	-	-										2
		Strelitziana	-	-	2.41	-	-	-										2
	Diaporthales	*Phomopsis*	-	-	0.97	-	-	HD										6
	Eurotiales	*Aspergillus*	2.64	6.80	4.67	-	-	HD										2
		Penicillium	15.80	16.29	9.43	-	-	D										2,7
		Talaromyces	-	4.32	3.12	-	0.002	-										2
	Helotiales	*Xylogone*	-	-	-	-	0.170	-										2
	Hypocreales	*Acremonium**	-	1.53	1.37	-	<0.001	-										1
		Clonostachys	-	-	0.51	-	0.003	-										2
		Engyodontium	-	-	0.43	-	0.003	-										2
		*Fusarium**	8.87	13.72	6.25	-	-	D										2,8
		Hypocrea	-	1.48	1.61	-	<0.001	-										8
		Purpureocillium	-	-	0.60	-	<0.001	-										2
	Jahnulales	*Xylomyces*	-	-	0.32	-	-	-										2
	Pleosporales	*Cochliobolus*	-	-	0.33	-	-	D										3

			SIMPER[a]			STAMP[b]			Ecological classification[d]									
Division	Order	Genus	H Similarity (27.25%)	D Similarity (29.06 %)	Dissimilarity (76.32%)	H STAMP p-value	D STAMP p-value	Isolated in Chapter 3[c]	Plant pathogen	Mycoparasite	Plant saprotroph	Undefined saprotroph	Anti-microbial	Confers benefits	Symbiont/ Endophyte	Animal pathogen	Ubiquitous	Citation[e]
…Ascomycota	…Pleosporales	*Curvularia**	-	1.72	1.77	-	<0.001	-										2,8
		Falciformispora	-	-	0.83	-	0.016	-										2
		Letendraea	-	-	0.35	-	0.019	-										9
		Ochrocladosporium	-	-	1.03	-	-	-										2,10
		Paraconiothyrium	-	-	0.44	-	0.023	-										2
		Periconia	-	-	0.95	-	-	-										1,10
		Phaeosphaeria	-	-	0.35	-	-	-										2,3
		*Phoma**	6.72	2.18	4.74	-	-	HD										6
		Alternaria	-	-	0.71	-	-	HD										1
	Saccharomycetales	*Saccharomyces*	-	-	0.32	-	-	-										11
	Savoryellales	*Savoryella*	-	-	0.33	-	-	-										2
	Sordariales	*Cercophora*	-	-	0.91	-	0.005	-										12
		Chaetomium	-	-	1.12	-	0.003	D										6
		*Neurospora**	-	3.01	1.96	-	<0.001	D										2,13
	Sordariomycetes[i]	*Myrmecridium*	-	2.60	2.30	-	<0.001	-										2
	Xylariales	*Monosporascus*	-	-	-	-	-	-										15
Basidiomycota	Agaricales	*Clitopilus*	-	-	-	-	-	-										2
		Coprinellus	-	2.21	2.20	-	<0.001	-										2
		Inocybe	-	4.82	3.45	-	<0.001	-										2,16
		Marasmius	-	-	0.63	-	-	-										2,3
		Mycena	-	-	0.58	-	<0.001	-										2
	Cantharellales	*Rhizoctonia*	-	-	0.55	-	-	-										2,6
	Cantharellales	*Thanatephorus*	-	-	0.32	-	-	-										2
	Malasseziales	*Malassezia*	32.98	12.38	8.17	-	-	-										17
	Polyporales	*Hypochnicium*	-	-	0.33	-	0.005	-										2,3

			SIMPER[a]			STAMP[b]			Ecological classification[d]										
Division	**Order**	**Genus**	**H Similarity (27.25%)**	**D Similarity (29.06 %)**	**Dissimilarity (76.32%)**	**H STAMP p-value**	**D STAMP p-value**	**Isolated in Chapter 3[c]**	**Plant pathogen**	**Mycoparasite**	**Plant saprotroph**	**Undefined saprotroph**	**Anti-microbial**	**Confers benefits**	**Symbiont/ Endophyte**	**Animal pathogen**	**Ubiquitous**	**Citation[e]**
…Basidiomycota	Thelephorales	*Tomentella*	-	1.08	1.65	-	<0.001	-										2,16
	Tremellales	*Cryptococcus*	-	-	0.39	-	-	-										18
Zygomycota	Mortierellales	*Mortierella*	-	-	0.29	-	-	-										2

[a]Results from SIMPER analysis where % in parenthesis is average similarity or dissimilarity for that group, and listed values are % contributions of each genera to the Bray-Curtis similarity within each group (either H or D) or the dissimilarity between groups, with a cut-off of 90% (Table S6.3, Appendix A)

[b]*P*-values associated with genera that differ significantly ($P < 0.05$) in relative abundance between H and D groups (Fig 6.3)

[c]Genera identified from isolations from Chapter 3 (Steinrucken et al., 2017) from healthy (H) and/or dieback-affected (D) plants

[d]Ecological classification definitions

Plant pathogen: cause disease in the plant host
Mycoparasite: a parasitic fungus whose host is another fungus
Plant saprotroph: obtain their nutrition from non-living plant cells (also called "saprophyte")
Undefined saprotroph: derive their nutrients from non-living organic matter
Anti-microbial: may be active against certain microbes
Confers benefits: benefit their host in some way such as providing nutrients or protecting against disease
Symbiont/ Endophyte: live in association with their plant host in a symptomless infection
Animal pathogen: cause disease in an animal host
Ubiquitous: present, appearing or found everywhere. Includes generalists.

[e]1. Duncan and Eslyn (1966); 2. Tedersoo et al. (2014); 3. Nguyen et al. (2016); 3. Zhang et al. (2011); 4. James et al. (2006); 5. Seehann et al. (1975); 6. Strobel et al. (2004); 7. Rodriguez et al. (2009); 9. Samuels (1973); 10. Costa (2012); 11. Agrios (2005); 12. Fernández et al. (1999); 13. Kuo et al. (2014); 14. Perdomo et al. (2013); 15. Martyn (2002); 16. Rinaldi et al. (2008); 17. Findley et al. (2013); 18. Kurtzman et al. (2011)

*Genera classified as more likely to be involved in dieback occurrence due to a combination of SIMPER and STAMP analysis, ecological classification(s) and subsequent analysis of the literature. These genera are discussed in more detail in the discussion.

6.4.4 Fungal community composition varies when using culture-independent vs. culture-based methods

The number of fungal sequencing reads recovered in this study (64,111 sequencing reads represented by 801 OTUs classified to 51 unique orders) was far greater than the number of isolates recovered in the culture based study (213 isolates classified to 19 orders) from the same samples (Chapter 3; Steinrucken et al. 2017). In Chapter 2's T-RFLP work, Steinrucken et al. (2016) binned T-RFLP peaks into 179 OTUs, but these could not be classified by taxa. The differences are illustrated in the OTU accumulation curve plots (Fig 6.5), which indicate that for ITS sequencing and T-RFLP, fungal OTUs are at or near saturation, whereas for isolations, isolates were under-sampled. In all cases however, there were more OTUs recovered for dieback parkinsonia samples as opposed to healthy samples.

From the Illumina sequencing in this study, OTUs identified as belonging to the ordinal group Capnodiales were the most common in healthy samples and OTUs identified as belonging to Eurotiales were the most common in dieback-affected samples (Fig 6.3), whereas Pleosporales was the most commonly isolated fungal order from both healthy and dieback samples (Chapter 3; Steinrucken et al. 2017). Of the 193 genera classified from OTUs sequenced from either healthy or dieback-affected parkinsonia, 20 were also isolated from these samples in Chapter 3 (Steinrucken et al. 2017). This included *Penicillium*, *Fusarium*, *Cochliobolus*, *Chaetomium* and *Neurospora* which were only isolated from dieback-affected parkinsonia. There were eight fungal genera unique to the culture-based work (e.g. *Epicoccum*, *Lasiodiplodia* and *Neopestalotiopsis*) and 139 which were unique to the sequencing work.

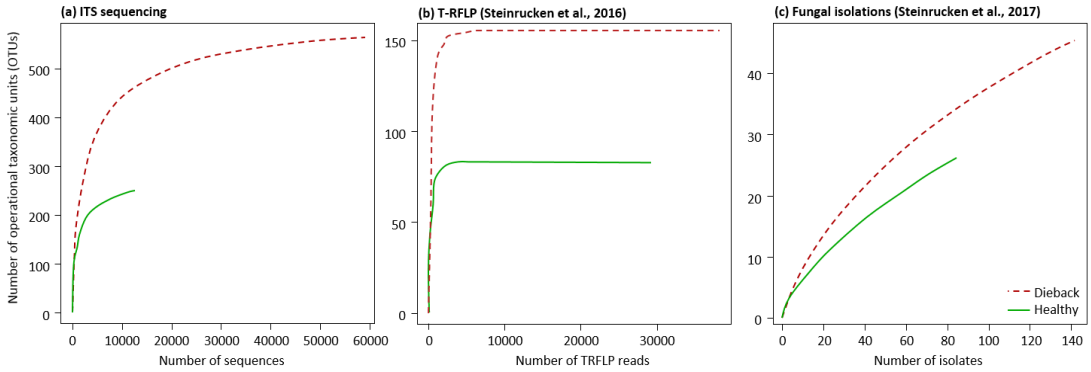


Figure 6.5 OTU accumulation curves for fungal ITS sequencing in this study (a), T-RFLP analysis in Chapter 2 (b), and fungal culture isolations from Chapter 3 (c) showing endophyte communities hosted by dieback-affected (red broken line) and healthy (green solid line) *Parkinsonia aculeata* sampled near Charters Towers, QLD (Australia).

6.5 Discussion

This study confirmed a significant correlation exists between fungal endophyte community composition and dieback occurrence in parkinsonia, as per Chapter 2 (Steinrucken et al. 2016), but also identified fungal taxa that were more closely associated with dieback-affected parkinsonia. By linking the taxonomic classification of fungi most closely associated with dieback-affected parkinsonia, with information about each taxon's ecological guild, I was able to hypothesise as to which fungal taxa are more likely to be involved in parkinsonia dieback.

6.5.1 Dieback occurrence is associated with fungal community composition

Although dieback has not been observed in the native range of parkinsonia (North, Central and South America), in Chapter 5 I did not find evidence to suggest that parkinsonia had left behind potentially protective endophytes in the native range upon introduction to Australia. Additionally, no potential dieback-causing pathogens were identified (using literature-derived data) in either native or invasive range (healthy) parkinsonia. Those results suggested that dieback-causing pathogens were encountered by parkinsonia once established in the invasive range. Therefore endophytic taxa from healthy and dieback-affected parkinsonia were analysed in this study to determine which taxa are more closely associated with dieback-affected parkinsonia, and which could be classified as potential dieback-causing plant pathogens. Assigning ecological roles to OTUs based on their taxonomic identity has some risks (Kõljalg et al. 2013). Taxa classified as belonging to one or another ecological guild might only express that phenotype when certain conditions (such as host species, environmental factors) align. Such assignations assume that taxonomic

classification of OTUs is correct. However, in the absence of pathogenicity testing post-isolation and despite potential inaccuracies, these ecological classifications allow for more meaningful analysis of the data than simply showing variation in diversity and composition, and can provide insight into targeted isolations for use in further pathogenicity testing.

None of the genera more closely associated with healthy parkinsonia are suspected to confer benefits to their host. Most of the fungal genera classified by taxa in this study are known saprophytes, the majority of which were more typical of dieback than healthy parkinsonia. This was expected since dieback-affected parkinsonia are likely to host opportunistic saprophytes which take advantage of the dead and dying tissue (Agrios 2005) typical of dieback.

In the absence of distinctive 'beneficial' fungal endophytes, I instead focused on putative fungal pathogens typical of, and abundant in, dieback-affected parkinsonia, that contributed to the Bray-Curtis dissimilarity between healthy and dieback samples. Of these, *Fusarium*, *Neurospora*, *Cladosporium*, *Phoma*, *Curvularia* and *Acremonium* are known phytopathogens:

- *Fusarium* (Order: Hypocreales) species are well-known plant pathogens with a global and diverse host range (Woolhouse et al. 2005; Goswami et al. 2008; Maciá-Vicente et al. 2008a; O'Donnell et al. 2012). It is the cause of dieback in a number of host plants in southern California due to its symbiosis with the invasive polyphagous shot-hole borer ambrosia beetle (Eskalen et al. 2013). Another *Fusarium* species, *F. brachygibbosum* is implicated in dieback of the spurge *Euphorbia larica* in Oman. Four unique *Fusarium* species were isolated

in Chapter 3 (Steinrucken et al. 2017): *F. ciliatum* (endophyte; Gerlach & Nirenberg 1982), *F. lateritium* (dieback in a number of species; Gerlach & Nirenberg 1982), *F. solani* (ubiquitous plant pathogen; Maciá-Vicente et al. 2008a) and an unidentified *Fusarium* sp..

- *Neurospora* (Order: Sordariales) species are most commonly known as endophytes (Qi et al. 2012) however, *N. crassa* is a latent pathogen in Scots pine, which may become pathogenic under adverse environmental conditions (Kuo et al. 2014). No *Neurospora* species were isolated in Chapter 3 (Steinrucken et al. 2017).
- *Cladosporium* species (Order: Capnodiales) are ubiquitous species, commonly found in air and on plant surfaces, and some are plant pathogens (Bensch et al. 2010; College of Agricultural and Environmental Sciences 2016). *Cladosporium pseudocladosporioides* was isolated frequently from healthy and dieback parkinsonia samples in Chapter 3 (Steinrucken et al. 2017).
- *Phoma* (Order Pleosporales) species are frequently isolated as endophytes (e.g. Kurose et al. 2012) and soil fungi (Rodriguez et al. 2009) and some have been shown to confer fitness benefits to their host by inhibiting root fungal pathogens (Maciá-Vicente et al. 2008b). Although many species are ubiquitous, more than 110 *Phoma* species are also important plant pathogens, including *P. medicaginis* and *P. lingam*. Many of these are opportunistic, infecting weakened plant tissue (Aveskamp et al. 2008). Some species (*P. herbarum*, *P. macrostoma* and *P. exigua*) have also been used as bioherbicides against broadleaf weeds (Aveskamp et al. 2008). Two unique *Phoma* spp. isolates were

recovered from healthy and dieback parkinsonia tissue in Chapter 3 (Steinrucken et al. 2017).

- *Curvularia* (Order: Pleosporales) species are most commonly saprophytes, but are also a weak plant pathogen of turf, hibiscus, wheat and corn (College of Agricultural and Environmental Sciences 2016). *Curvularia protuberata* has been shown to confer benefits to their host, while asymptomatically colonising host root tissue (Rodriguez et al. 2009). *Curvularia* was not isolated in Chapter 3 (Steinrucken et al. 2017).
- *Acremonium* (Order: Hypocreales) species are frequently isolated as endophytes (Evans et al. 2003; Maciá-Vicente et al. 2008a) but some species are also phytopathogenic and one is responsible for muskmelon collapse (Garcia-Jimenez et al. 1994). *Acremonium* was not isolated in Chapter 3 (Steinrucken et al. 2017).

A species from one of the fungal genera associated with healthy parkinsonia (*Fusarium*, Order: Hypocreales) is a known plant pathogen associated with headblight in grains and dieback of carnations (Wright et al. 1997). It was also found to be typical of healthy invasive parkinsonia (*P. aculea*ta) in Chapter 5. Its presence in healthy parkinsonia in this study and Chapter 5, and the fact that it was also present and abundant in dieback-affected parkinsonia may indicate that it is a latent pathogen. However, I have been unable to find reference to it being associated with disease or dieback in woody plants and as such, targeted isolation and pathogenicity screening would be required to further elucidate its ecological role in this system. *Fusarium* was isolated in Chapter 3 (Steinrucken et al. 2017).

From the information above, I can hypothesise which genera may be involved in parkinsonia dieback. While it is not possible to state whether organisms identified herein are responsible for dieback in parkinsonia, without more detailed ecological studies, identification of likely candidates by these methods is useful to reduce the targets of further testing. Most of the genera listed above are phylogenetically diverse, containing multiple species that are saprophytic, endophytic and pathogenic. Those most likely to be the cause(s) of parkinsonia dieback therefore, are those genera that can take advantage of weak plant tissue when environmental conditions are unfavourable for the host (such as drought; Chapter 3; Steinrucken et al. 2017), or those which have known histories as phytopathogens of trees, and in particular dieback. This leaves *Neurospora*, *Phoma* and *Fusarium*. As discussed in Chapter 3 (Steinrucken et al. 2017), many parkinsonia populations are in areas with persistent drought followed by intermittent flooding, poor soil quality and high temperatures. These abiotic conditions may result in increased susceptibility to opportunistic pathogens such as *Phoma* and *Neurospora*. The prevalence and dominance of *Fusarium* in dieback-affected parkinsonia, suggest that some species may be involved in dieback.

Previous studies implicated members of the Botryosphaeriaceae in dieback of parkinsonia (Toh 2009; Diplock 2016) and other invasive plants (Haque 2015; Sacdalan 2015). In this study *Diplodia* was the only Botryosphaeriaceae genus identified as significantly contributing to compositional variation between healthy and dieback samples (Table 6.2). Interestingly, although *Diplodia* has been previously shown to be associated with dieback in the literature (de Wet et al. 2000), and *D. pinea*

was isolated from dieback-affected parkinsonia in Chapter 3 (Steinrucken et al. 2017), the statistical tests conducted in this study showed it was neither abundant in, nor associated with, dieback-affected parkinsonia. Additionally, although *Lasiodiplodia* species have been implicated in multiple dieback studies (Haque 2015; Sacdalan 2015; Diplock 2016; Chapter 3, Steinrucken et al. 2017), that genus was not identified in the analysis of the sequencing data from this study. The previous focus of research on Botryosphaeriaceae species has meant that other potentially dieback-causing pathogens have been ignored. Although *Fusarium*, *Neurospora* and *Phoma* species were not tested in the pathogenicity trial, they should be considered in future work on parkinsonia dieback.

6.5.2 Dieback occurrence was not shown to be associated with bacterial community composition

There are known beneficial plant-associated bacteria, such as *Burkholderia gladioli*, which may play a role in protection of their host against phytopathogens (Shehata et al. 2016). In fact, bacteria have been used previously in the biological control of fungal pathogens (Bacon & Hinton 2007). However I found no health status effect on bacterial community composition in parkinsonia, and the most abundant bacterial genera did not include *Burkholderia,* but instead consisted mostly of common bacterial endophyte genera *Escherichia, Phytoplasma* and *Sphingomonas* in both healthy and dieback-affected parkinsonia. It is therefore unlikely that any bacterial endophytes play a direct role in dieback in parkinsonia.

6.5.3 Comparison to previous work on parkinsonia dieback: T-RFLP

I expected to find significant effects on fungal and bacterial endophyte communities due to parkinsonia disease status and plant part since that is what I found in the T-RFLP study (Table 6.3). While this was the case for fungal communities, bacterial endophyte communities were only affected by plant part. Since the DNA used for both studies was from the same samples and DNA extractions (Table S5.1, Appendix A), it is likely that the difference in results for bacteria was due to primer choice or sequencing technique, or may be due to undersampling of the bacterial community using T-RFLP leading to Type I error and a marginally non-significant effect on health status (Pseudo-F = 1.5508, P = 0.066). Primer choice is the most important aspect of environmental sequencing, particularly when doing multiplex sequencing due to an increased chance of forming primer dimers when multiple primers are used. Non-specific binding of primers may also result in amplification of unwanted sequences, a common occurrence for above-ground plant tissues due to lower microbial biomass relative to the plant host than roots or soil, and a tendency for the bacterial 16S rRNA gene to be outcompeted by chloroplast DNA (Ikeda et al. 2010). Repetition of the protocol and comparisons by Illumina sequencing with the bacterial primers used in the T-RFLP study would help to clarify this. Importantly, during sampling it is essential to ensure that as many endophytes as possible are collected. Since both bacterial and fungal endophyte communities were significantly affected by plant part, continuing to sample from multiple plant parts when conducting either culture based or culture-independent studies in parkinsonia is important (although in this study the

lack of an interaction effect suggests that any difference in community composition is likely to be systemic).

Table 6.3 Comparison of PERMANOVA results from data obtained via Illumina sequencing and T-RFLP

	Illumina sequencing [a]			**T-RFLP** [b]		
	df	Pseudo-*F*	*P*-value	*df*	Pseudo-*F*	*P*-value
Fungi[c]						
Plant part	2	1.7915*	0.002	2	3.5693*	0.0001
Disease status	1	8.1117*	0.001	1	3.1459*	0.0004
Bacteria[d]						
Plant part	2	2.405*	0.001	2	3.0066*	0.0031
Disease status	1	1.5508	0.066	1	7.1639*	0.0002

[a] results from Table 6.1; [b] results from Table 2.2 (Chapter 2; Steinrucken et al., 2016);
[c] Fungal primers ITS1F_KYO1-ITS2_KYO2 in this study, ITS1F-ITS4 in Chapter 2 (Steinrucken et al. 2016);
[d] Bacterial 16s primers 515f-806rB in this study, 63f-1087r in Chapter 2 (Steinrucken et al. 2016)
* significant (where *P*<0.05)

6.5.4 Comparison to previous work on parkinsonia dieback: isolations and pathogenicity screening

Parkinsonia dieback, as with dieback in other trees, is likely as a result of multiple abiotic and/or biotic factors (Manion 1991). These include environmental conditions such as water availability, soil salinity and temperature, as well as interactions with locally-occurring plants, grazing by herbivores and interactions with pathogens and pests. The effect of water availability was tested in previous pathogenicity screening for parkinsonia dieback, but failed to trigger dieback-like symptoms when parkinsonia was inoculated with selected pathogenic fungi (Chapter 3; Steinrucken et al. 2017). The limitations of culture-based techniques may have resulted in under-sampling of the fungal community, because isolates recovered from plant material are often the fastest growing or are simply more amenable to the chosen medium (Peršoh 2015). While culture-independent techniques increase the diversity of taxa recovered, this can sometimes also be misleading due to amplification of taxa that are not currently active

and only persist as spores (Peay 2014). It should be noted however that this might also be true for culture-based studies to some extent where the media used is preferable to *in planta* conditions. The diversity and abundance of fungal taxa sequenced in this study are far greater than those isolated in Chapter 3 (Steinrucken et al. 2017). Therefore, one advantage of this culture-independent technique is that it has widened the breadth of possible targets in the search for the causative agents of dieback (refer to the rarefaction curve, Fig 6.5), and I was able to test the significance of these extended community assemblages by factors such as disease (dieback) occurrence.

6.6 Conclusion

Next-generation sequencing produces vast quantities of data which can be tested in statistical analyses, and taxonomic delineation provides further information to connect raw sequences with ecological traits (Peay 2014). The resulting assumptions should be used carefully due to potential bias and inaccuracies in taxonomic classifications, but they are nevertheless useful (Peršoh 2015). Combining culture-based and culture-independent techniques in the diagnosis of plant disease has many more potential benefits, and is a more intensive approach than simply using one or the other. Isolates could be sequenced first, followed by sequencing and classification of OTUs from plant/soil tissue using a next-gen platform such as Illumina. Then the isolates could be matched with those in the sequencing data. The remainder of the data could be used to design isolation methods to target taxa that weren't previously isolated.

Analysis of the native and invasive parkinsonia endophyte communities (Chapter 5), and those in healthy and dieback-affected invasive Australian parkinsonia has shown that parkinsonia dieback is unlikely to have been caused by a lack of potentially

protective endophytes in healthy plants, as hypothesised in Chapter 2 (Steinrucken et al. 2016). Instead, I found a number of OTUs classified as fungal pathogens (*Fusarium*, *Neurospora* and *Phoma*), that were more closely associated with dieback-affected parkinsonia than healthy parkinsonia as well as a significant health status effect on fungal endophyte communities. This suggests that dieback is more likely due to infection by pathogens encountered in the invasive range. Future studies should consider these dominant pathogenic taxa in pathogenicity screening for parkinsonia dieback occurrence and as potential biocontrol agents.

7

SYNTHESIS AND CONCLUSIONS

7.1 Summary of the results in the context of the key research questions

The primary objectives of this work were to investigate the causes of dieback in *Parkinsonia aculeata* (parkinsonia), and subsequently if causes of dieback were found, to determine their potential suitability as biocontrol agents for the management of parkinsonia in Australia.

The following research questions were addressed:

i. Does microbial community composition in parkinsonia relate to dieback occurrence?
ii. Which endophytic fungi live in above- and below-ground parts of healthy and dieback parkinsonia and which of these cause dieback symptoms?
iii. Is parkinsonia dieback likely to be associated with oomycetes such as *Phytophthora*?
iv. Are endophyte communities in parkinsonia different between the native and introduced (Australian) ranges?
v. Is parkinsonia dieback caused by putative fungal or bacterial pathogens, or a lack of beneficial endophytes?

Dieback occurrence in parkinsonia is associated with a combination of factors including the composition of the fungal endophyte community, and the presence of putative pathogens. Pathogenicity screening of putative fungal pathogens isolated

from parkinsonia and considered to be involved in dieback in other studies (Toh 2009; Diplock 2016), failed to result in dieback-like symptoms in parkinsonia. However a drought stress treatment during the trial indicated that parkinsonia may be more susceptible to disease when under stress. A limited survey found oomycetes were associated with both healthy and dieback-affected parkinsonia. Oomycetes have been implicated in dieback in other plant species, but more work is needed to see if they play a role in parkinsonia dieback. Fungal endophyte communities were shown to be species-specific within the *Parkinsonia* genus, and the invasive parkinsonia population hosts a different fungal and bacterial endophyte community to that hosted by *P. aculeata* in the native range. This might explain why dieback has only been observed in the invasive range, and not in co-occurring local species. Despite these findings, it remains unclear whether dieback is caused by a single agent and whether that agent is the constant cause across its introduced range. What is clear from the outcomes of these studies, is that parkinsonia dieback is a complex phenomenon. What follows is some general discussion of the conclusions drawn across multiple chapters.

7.2 Fungal endophyte community composition and parkinsonia dieback

Using culture-independent techniques, I showed that fungal endophyte community assemblages vary significantly when comparing healthy parkinsonia to dieback-affected parkinsonia (Chapter 2 and Chapter 6). Moreover, these communities were different to those hosted by parkinsonia in the native range and were host-specific within the *Parkinsonia* genus (Chapter 5). It had been previously hypothesised that certain endophytic fungal pathogens are involved in parkinsonia dieback (Toh 2009;

Diplock 2016), however these putative pathogens (in particular *Lasiodiplodia pseudotheobromae*, Family: Botryosphaeriaceae) have not been consistently isolated from dieback-affected trees, nor have they been shown to consistently cause dieback-like symptoms when inoculated into parkinsonia. Had dieback been associated with one or more specific fungal species, I would have expected to have isolated or sequenced that (those) species consistently from dieback-affected parkinsonia. Instead, *L. pseudotheobromae* was only isolated from healthy parkinsonia in my culture-based study (Chapter 3) and was not recovered from dieback-affected parkinsonia using next-gen sequencing (NGS; Chapter 6). Only one Botryosphaeriaceae genus (*Diplodia*) contributed to variance between healthy and dieback parkinsonia, but only explained a very small amount of this variation (0.67%, Chapter 6), contradicting the tight assumed association of these taxa with parkinsonia dieback. OTUs classified as *Fusarium*, *Phoma* and *Neurospora*, stood out as genera which were significantly associated with parkinsonia dieback (Chapter 6). In the future, these taxa could be isolated and screened for pathogenicity in parkinsonia and hence their potential as biocontrol agents for parkinsonia might be tested.

7.3 Pathogenicity screening of putative fungal pathogens for dieback symptoms in parkinsonia

During pathogenicity screening (Chapter 3), drought and inundation significantly decreased plant health in comparison to the control, but none of the tested pathogens caused dieback-like symptoms or systemic infection. The largest lesions on inoculated plants were caused by *L. pseudotheobromae*, *Pestalotiopsis mangiferae* and *Botryosphaeria dothidea.* The findings don't eliminate the tested isolates from the list

of potential dieback-causing pathogens, but suggest that other factors may be involved in the induction of dieback-like symptoms. This may include variation in environmental stress factors imposed on the host or its associated microorganisms, a different pre-existing endophyte community to that found in parkinsonia from the field (Peršoh 2015), or the existence of a dieback-causing pathogen complex (as suggested in Chapter 2; Steinrucken et al. 2016). Testing of more potential putative fungal pathogens, isolated from dieback-affected parkinsonia, is recommended. Additionally, it is important to show that inoculation can initiate systemic infection in any trials to better replicate what is observed in the field.

7.3.1 Potential workflow for diagnosing plant disease associated with fungal endophyte communities

One limitation of testing putative fungal pathogens is the potentially limited pool of fungal isolates from which they are chosen. Many fungal species grow preferentially in different media, they require specific conditions for sporulation, and those which are slow-growing are outcompeted by faster-growing taxa. As a result, the community of isolates recovered from plant samples is likely not exhaustive. The findings of this thesis have led to the development of the following workflow for use when diagnosing plant disease thought to be associated with fungal endophyte communities. This workflow combines culture-based and culture-independent techniques and reduces the risk of assigning inaccurate ecological roles to taxa based solely on their taxonomic classification.

1. Isolate fungi from multiple plant parts of healthy and disease-affected or symptomatic endophytic tissue, using standard or available media such as potato dextrose agar (PDA) amended with streptomycin
2. Identify recovered isolates via sequencing with fungal-specific primers for the ITS region (e.g., ITS1F and ITS4; Gardes & Bruns 1993)
3. Sequence the greater fungal endophyte community (derived from the same tissues) using NGS platforms such as Illumina, with the same fungal specific primers as in the previous step
4. Analyse the NGS data and search for the isolate sequences obtained in step 2
5. Conduct further analyses to determine whether communities in healthy plant tissue differ significantly from those in diseased tissue. If the communities differ significantly, determine which OTUs (classified to taxonomic group) contribute to that variation, particularly in the diseased tissue
6. Using taxonomic data, consult the literature to determine which disease-associated fungal taxa are more likely to be pathogenic in your study system
7. Design and implement isolation strategies appropriate for use with any potential pathogen that has not yet been isolated but that contributes significantly to the variation between healthy and diseased plant tissue
8. Conduct pathogenicity screening of the amended pool of isolates and consider inoculations with more than one isolate at a time

Although not always practical or possible, it may be prudent to inoculate plants in or from the field, as they will likely host a specific community of endophytes that might affect the results of a pathogenicity screening. Alternatively, an assay could be conducted on seedlings initially, followed by further screening on plants from the field.

7.4 Other microbial communities associated with parkinsonia

7.4.1 Archaea

I also investigated whether parkinsonia dieback is associated with other microbial communities. Using T-RFLP (terminal restriction fragment length polymorphism), archaeal communities were shown to vary significantly with dieback occurrence in parkinsonia (Chapter 2). While some previous work has shown that archaea are structured by plant part in the reed *Phragmites australis* (Ma et al. 2013), no plant or animal archaeal pathogens have been discovered, and very few have been cultured (Cavicchioli 2011). This result was therefore unexpected and I hypothesise that the primers used to amplify archaea may have amplified another plant or microbial gene; I am exploring this possibility further. The purpose of including archaea was to explore the possibility of archaeal endophytes being involved in plant disease, but the difficulties involved in isolating and growing archaeal taxa (Schleper et al. 2005) prevented further study on this cryptic group.

7.4.2 Bacteria

In contrast to the T-RFLP study where bacterial communities varied significantly in healthy compared to dieback-affected parkinsonia (Chapter 2), bacterial communities in the same plant samples were not shown to be significantly affected by dieback when using NGS (Chapter 6), possibly due to primer choice or amplification of host DNA in the T-RFLP analysis which cannot be discounted. Additionally, no known bacterial plant pathogens were identified that contributed significantly to variation between healthy and dieback bacterial communities and the most abundant

taxa were classified as generalists and/or known endophyte taxa. So, I can provisionally conclude that bacterial endophytes are unlikely to be directly involved in parkinsonia dieback occurrence.

7.4.3 Oomycetes

Oomycetes, in particular *Phytophthora* species, cause widespread dieback in trees in Australia and globally (Erwin & Ribeiro 1996), but their potential role in parkinsonia dieback had not previously been examined. The most commonly recovered oomycete isolates from parkinsonia soil and root samples in Chapter 4 were *Phytophthora palmivora*, *P. nicotianae* and *Phytopythium* spp. Although some of the recovered isolates have been identified as plant pathogens (Erwin & Ribeiro, 1996), their pathogenicity in parkinsonia cannot be established without screening, and the results did not allow me to determine if some were more closely associated with dieback-affected parkinsonia than with healthy parkinsonia.

To follow on from the work in Chapter 4, oomycetes were also sequenced in the experiments for Chapter 5 and 6, but the sequences suggested a number of *Phytophthora* species were recovered from Australian parkinsonia that had not yet been recorded in Australia (such as *P. ramorum*; Matteo Garbelotto, pers. comm). Since the experimental portion of this work was carried out in a laboratory specialising in *Phytophthora* diseases (Matteo Garbelotto's Forest Mycology Laboratory, University of California Berkeley), the recovered sequences in question were possibly due to contamination during processing. The oomycete-specific primers, which I had designed specifically for this purpose, worked, but the results were unreliable due to the contamination risk. Although the negative control showed zero reads, a sequencing

read threshold could not be established. These primers also amplified multiple non-oomycete species (Fig 7.1). A primer set such as this, which amplifies oomycete species in environmental samples, would be very useful in areas where *Phytophthora*-disease is suspected, so further development of this protocol – with adequate anti-contamination measures – should be continued.

7.5 Conclusion

Developing novel methods to control invasive plant species will produce invaluable benefits to stakeholders and the environment, not just in Australia but on a global scale. Dieback is one such potential tool, and it has already been observed in multiple WONS in Australia including parkinsonia (this thesis), prickly acacia (Haque 2015), blackberry (Aghighi et al. 2014) and mimosa (Sacdalan 2015). Determining the cause of dieback in each of these species may present land managers with a 'silver bullet' of biological control (Evans 2008) which, as in the case of the *Cactoblastis* moth and prickly pear control in Australia (Biosecurity Queensland 2015), may become a self-managed, perpetual instrument, reducing weed management costs and increasing biodiversity and land productivity. My research has shown that dieback is a highly complex phenomenon, however the possibility of diagnosing parkinsonia dieback is not out of reach. Future work in this area that builds on my findings, hypotheses and recommended methodologies may lead to novel biocontrol solutions, and as such would be valuable to the biosecurity of important ecosystems.

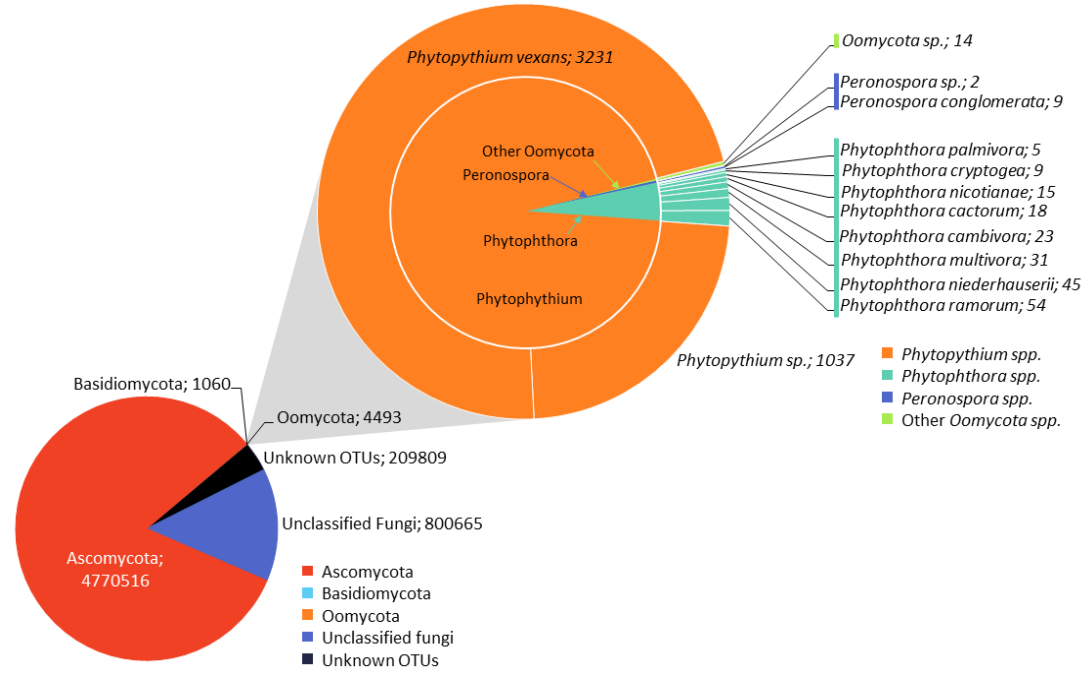


Figure 7.1 Diversity of higher and lower fungal taxa. Oomycete OTUs represent 0.4% of the total sequences recovered using these primers, and are represented by species name. *Phytopythium vexans* and *Phytopythium* sp. were the most frequently identified oomycete OTUs. The protocol for this experiment matches that in Appendix C, and the oomycete-specific primers were based on ITS6 and ITS7 (Cooke et al. 2000) with the following sequences: nexF-N5-ITS6 (TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG–NNN NN– GAA GGT GAA GTC GTA ACA AGG); and nexR-N5-ITS7 (GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G-NNN NN-AGC GTT CTT CAT CGA TGT GC).

APPENDIX A: SUPPLEMENTARY MATERIAL BY CHAPTER

Chapter 2 Supplementary Material

Table S 2.1 Details of *Parkinsonia aculeata* sampling sites from March and May 2013

Site	Property	Sample	Elevation	GPS coordinate	Description of habitat
1A	Eumara Springs	Healthy	937	19°45.257'S 145°57.782'E	90% grass cover, grazed area next to dam wall (no water). Parkinsonia not very dense
1C	Fletchervale	Dieback	951	19°45.269'S 145°57.690'E	Stand of diseased trees next to the fence. 95% grass cover.
2A	Pandanusvale	Healthy	913	19°49.419'S 146°03.0734'E	Adjacent to Lolworth creek, major highway and thick vegetation (rubber vine etc). Next to grazed pasture with cattle.
2C	Pandanusvale	Dieback	905	19°49.413'S 146°03.676'E	Highly scattered trees used for sampling (GPS recorded for each). Pasture with tall trees, mainly Eucalypts. Scattered parkinsonia, mainly dead. Very disturbed soil, prone to flooding. Cattle grazing. Near major road and Lolworth ck.
3A	Anabranch	Healthy	732	19°54.539'S 146°15.227'E	Along a river and adjacent to a campground. Many healthy parkinsonia around. 95% grass cover, horses grazing, many invasives, flooding evidence.
3C	Fletchervale	Dieback	917	19°45.854'S 145°58.516'E	Large infestation of parkinsonia, impenetrable in some areas. Area prone to flooding. 95% grass cover

Chapter 3 Supplementary Material

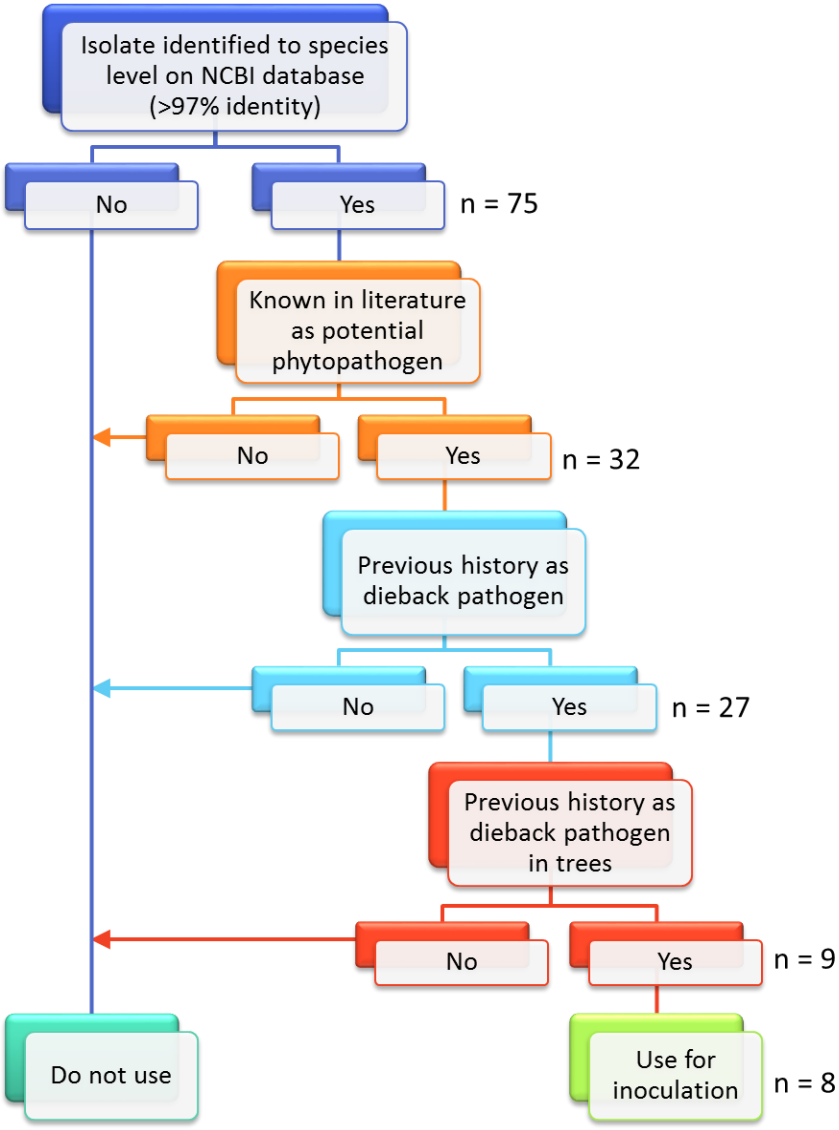


Figure S 3.1 Decision flow chart used to determine which isolates should be tested in the glasshouse pathogenicity trial

Chapter 4 Supplementary Material

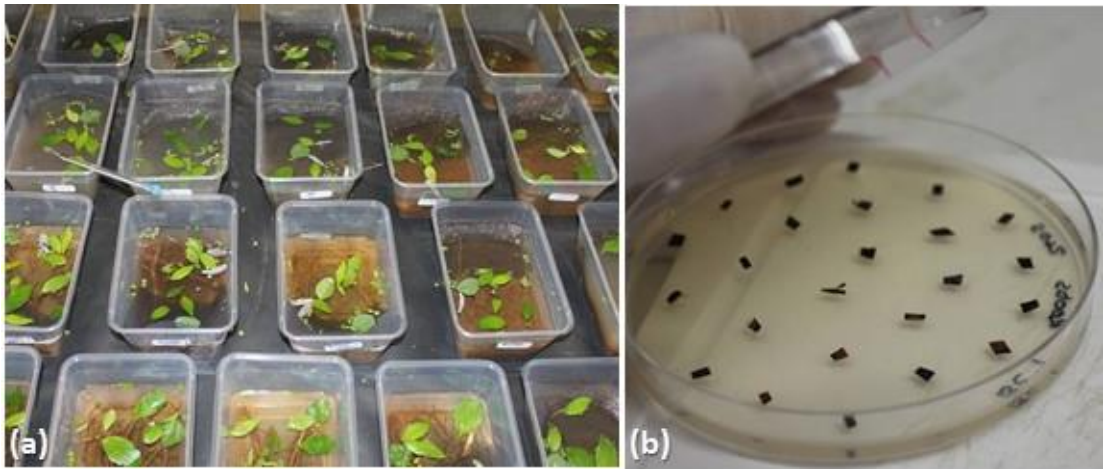


Figure S 4.1 'Baiting' for zoospores and isolation of oomycetes in *Parkinsonia aculeata;* processed soil and root materials (a) and plating out water-soaked infected leaves on *Phytophthora*-selective medium (b).

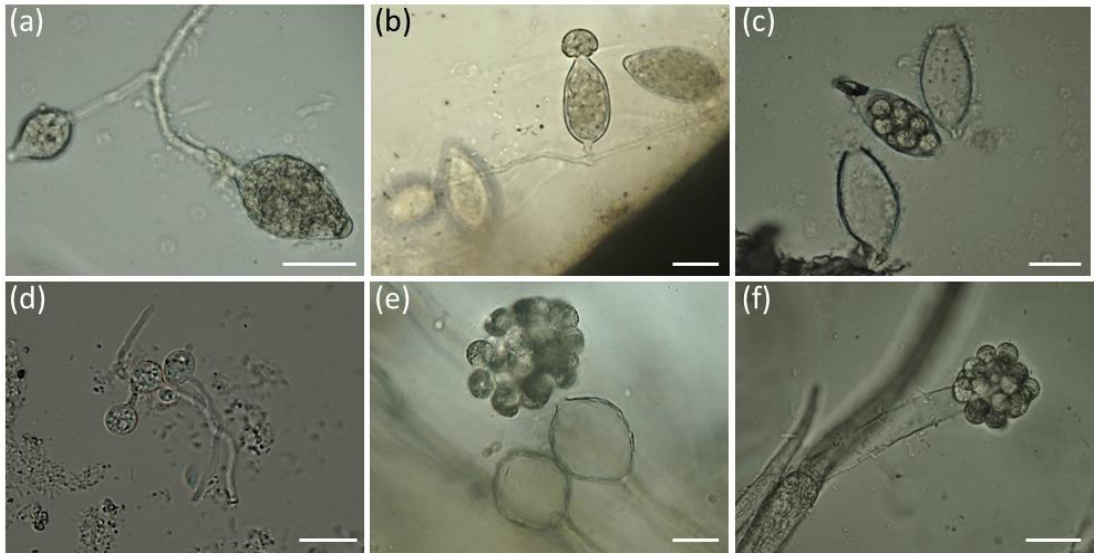


Figure S 4.2 Light microscope images of several isolates on potato dextrose agar amended with 35 mg L^{-1} streptomycin: (a) papillate sporangia of *Phytophthora nicotianae*, (b) *Phytophthora palmivora* sporangium releasing zoospores, (c) caduceus sporangia of *P. palmivora* with and without zoospores (d) *Phytophthora* sp. zoospores germinating, (e) *Pythium* sp. sporangium after release of zoospores, (f) encysted zoospores of *Aphanomyces* sp. Scale bars represent 20 µM. Images produced by Sonia Aghighi, Murdoch University.

Table S 4.1 All isolates from soil and roots of *Parkinsonia aculeata* recovered in this study, identified to closest match (CM) on the NCBI database and via phylogenetic analysis (for oomycetes; Fig 4.3). Site of sampling in Kununurra (KWA) and Charters Towers (CTQ) from Fig 4.1, and disease status of host plant are also provided

Isolate	CM Species	CM Accession	% PWI[a]	% QC[b]	Isolate Identification[c]	Site[d]	H/D[e]
CTQ001	*Pythium vexans*	HQ643954	93.9	100	Pythiaceae	BN	H
CTQ002	*Pythium vexans*	GU133601	97.1	99.56	*Phytopythium* sp.	BS	D
CTQ015	*Pythium vexans*	GU133601	97.1	99.56	*Phytopythium* sp.	BS	D
CTQ020	*Rhizoctonia solani*	JQ343829	99.2	99.62	*Rhizoctonia solani*	BN	D
CTQ022	*Fusarium* sp.	KX953666	100	100	*Fusarium* sp.	BN	D
CTQ025	*Fusarium* sp.	KX953666	100	100	*Fusarium* sp.	BS	D
CTQ030	*Pythium vexans*	GU133578	88.8	71.45	Pythiales	BS	D
CTQ043	*Fusarium* sp.	KX953666	99.3	100	*Fusarium* sp.	BN	H
CTQ047	*Pythium vexans*	GU133601	87.2	100	Pythiales	BS	D
CTQ048	*Phytopythium* aff. *vexans*	HQ643371	95.2	95.1	*Phytopythium* sp.	BS	D
CTQ051	*Fusarium chlamydosporum*	KY046256	100	100	*Fusarium chlamydosporum*	BN	H
CTQ053	*Pythium vexans*	GU133593	98.9	94.53	*Phytopythium* sp.	BS	D
CTQ057	*Pythium graminicola*	HQ643545	100	100	*Pythium graminicola*	BN	H
CTQ062	*Pythium aphanidermatum*	KF667387	100	100	*Pythium aphanidermatum*	BS	D
CTQ064	*Trichoderma harzianum*	KT278883	99.4	100	*Trichoderma harzianum*	BN	H
CTQ066	*Fusarium* sp.	KX953666	100	100	*Fusarium* sp.	GC	H
CTQ069	*Fusarium equiseti*	KX281176	100	100	*Fusarium equiseti*	BN	D
KWA001	*Epicoccum sorghinum*	KY454467	100	100	*Epicoccum sorghinum*	BC	D
KWA002	*Pythium* sp.	KP183943	96.6	100	*Phytopythium* sp.	BC	D
KWA006	*Pythium vexans*	GU133593	96.3	100	*Phytopythium* sp.	CG	D
KWA007	*Pilasporangium apinafurcum*	AB458659	84.9	97.18	*Pilasporangium* sp.	CG	D
KWA008	*Pilasporangium apinafurcum*	AB458659	91.9	97.18	*Pilasporangium* sp.	RB	H
KWA010	*Phytopythium* aff. *vexans*	HQ643371	87.1	100	Pythiales	RB	H
KWA012	*Epicoccum sorghinum*	KY454467	99.6	100	*Epicoccum sorghinum*	RB	H
KWA013	*Phytopythium vexans*	KR092142	91.4	89.89	*Phytopythium* sp.	RB	H
KWA014	*Epicoccum sorghinum*	KY454467	100	100	*Epicoccum sorghinum*	RB	H
KWA016	*Phytophthora nicotianae*	LT628539	100	100	*Phytophthora nicotianae*	CG	D
KWA017	*Fusarium solani*	JN882257	99.8	100	*Fusarium solani*	W	D
KWA018	*Fusarium solani*	JN882257	100	100	*Fusarium solani*	W	D
KWA020	*Phytophthora insolita*	GU111612	99	100	*Phytophthora insolita*	W	D
KWA021	Uncultured *Cenococcum*	LN829195	86.9	31.05	Mytilinidiales sp.	CG	D
KWA022	*Fusarium solani*	JN882257	100	100	*Fusarium solani*	W	D
KWA023	*Trichoderma harzianum*	KJ588244	99.5	100	*Trichoderma harzianum*	W	D
KWA024	*Epicoccum sorghinum*	KY454467	98.6	100	*Epicoccum* sp.	CG	D
KWA025	*Epicoccum sorghinum*	KY454467	99.7	100	*Epicoccum sorghinum*	RB	H
KWA027	*Phytophthora nicotianae*	LT628539	97.6	100	*Phytophthora* sp.	CG	D
KWA028	*Phytophthora nicotianae*	KU248811	99.4	97.49	*Phytophthora nicotianae*	CG	D
KWA029	*Phytophthora nicotianae*	LT628539	99.4	100	*Phytophthora nicotianae*	CG	D
KWA030	*Epicoccum sorghinum*	KY454467	97.4	100	*Epicoccum* sp.	CG	D
KWA031	*Epicoccum sorghinum*	KY454467	99.6	100	*Epicoccum sorghinum*	CG	D
KWA032	*Epicoccum sorghinum*	KY454467	98.8	100	*Epicoccum* sp.	RB	H
KWA035	*Epicoccum sorghinum*	KY454467	99.2	100	*Epicoccum sorghinum*	W	D
KWA042	*Phytophthora palmivora*	KY357521	99.5	100	*Phytophthora palmivora*	BC	D
KWA044	*Phytophthora palmivora*	KY357521	99.3	100	*Phytophthora palmivora*	BC	D
KWA045	*Phytophthora palmivora*	KY357521	98.3	100	*Phytophthora* sp.	BC	D
KWA048	*Rhizopus oryzae*	KU948382	96	100	*Rhizopus* sp.	CG	H
KWA050	*Phytophthora fallax*	HQ261559	91.3	98.9	*Phytophthora* sp.	CG	H
KWA051	*Phytophthora fallax*	HQ261559	91	98.91	*Phytophthora* sp.	W	D
KWA054	*Curvularia lunata*	KU715116	99.3	100	*Curvularia lunata*	W	D
KWA056	*Phytopythium* aff. *vexans*	HQ643371	93.5	100	Pythiaceae	BC	D
KWA058	*Rhizopus oryzae*	KU948382	99.9	100	*Rhizopus oryzae*	CG	D

Isolate	CM Species	CM Accession	% PWI[a]	% QC[b]	Isolate Identification[c]	Site[d]	H/D[e]
KWA063	*Phytopythium* aff. *vexans*	HQ643371	99.1	100	*Phytopythium vexans*	BC	D
KWA065	*Phytopythium* aff. *vexans*	HQ643371	92.6	93.47	Pythiaceae	CG	D
KWA069	*Phytophthora nicotianae*	KJ506196	99	89.71	*Phytophthora nicotianae*	RB	H
KWA086	*Phytopythium vexans*	KR092142	92.5	85.69	*Phytopythium* sp.	W	D
KWA087	*Phytopythium vexans*	KR092142	92.3	85.01	*Phytopythium* sp.	W	D
KWA088	*Phytopythium vexans*	KR092142	92.5	85.96	*Phytopythium* sp.	W	D
KWA090	*Phytophthora palmivora*	KY357521	99.8	100	*Phytophthora palmivora*	BC	D
KWA092	*Phytophthora palmivora*	KY357521	99.9	100	*Phytophthora palmivora*	BC	D
KWA094	*Phytophthora palmivora*	KY357521	98.8	100	*Phytophthora* sp.	BC	D
KWA095	*Phytophthora palmivora*	KY357521	99.8	100	*Phytophthora palmivora*	BC	D
KWA096	*Phytopythium vexans*	KR092142	90.7	83.97	*Phytopythium* sp.	RB	H

[a] % PWI: percentage pairwise identity

[b] % QC: percentage query coverage

[c] Oomycete sequences submitted to GenBank under Accession no's KY938843 to KY938879

[d] Site key: BC – Button's Crossing KWA, CG – Manbi camp ground KWA, RB – river bank KWA, W – Wetland KWA, BN – Burdekin North CTQ, BS – Burdekin South CTQ, GC – Groper Creek CTQ

[e] Disease status of sampled tree: H – healthy, D – dieback

Chapter 5 Supplementary Material

Table S 5.1 GPS coordinates and sampling details for each of the sites used in these studies

Site name (site code)	Country	State	Longitude	Latitude	Date collected	Collected by[a]	*P. aculeata*	*P. florida*	*P. microphylla*	Leaves	Stems Tips	Stems	Roots
Eumara Springs (1A) [b]	Australia	QLD	19.754335° S	145.962981° E	20/05/2013	TS, KP, RvK	5				✓	✓	✓
Fletchervale (1C) [b,c]	Australia	QLD	19.754483° S	145.961500° E	20/05/2013	TS, KP, RvK	5				✓	✓	✓
Pandanusvale (2A) [b]	Australia	QLD	19.823650° S	146.061717° E	20/05/2013	TS, KP, RvK	5				✓	✓	✓
Pandanusvale (2C) [b,c]	Australia	QLD	19.824728° S	146.060531° E	20/05/2013	TS, KP, RvK	5				✓	✓	✓
Anabranch (3A) [b]	Australia	QLD	19.909100° S	146.253883° E	21/05/2013	TS, KP, RvK	5				✓	✓	✓
Fletchervale (3C) [b,c]	Australia	QLD	19.764233° S	145.975267° E	21/05/2013	TS, KP, RvK	5				✓	✓	✓
Mt Isa (M)	Australia	QLD	20.645755° S	139.510168° E	15/04/2015	AW	7				✓	✓	
Cloncurry (C)	Australia	QLD	20.696415° S	140.492602° E	15/04/2015	AW	7				✓	✓	
Caerphilly (P)	Australia	QLD	20.976790° S	146.682764° E	22/04/2015	TS, KP, RvK	5				✓	✓	
Groper Creek (G)	Australia	QLD	19.690967° S	147.500705° E	23/04/2015	TS, KP, RvK	5				✓	✓	
Kununurra Bullock Crossing (K)	Australia	WA	15.781656° S	128.680350° E	21/05/2015	KB	5				✓	✓	
Newcastle Waters Station (N)	Australia	NT	17.415316° S	133.412480° E	27/05/2015	LE, BL	5				✓	✓	
Snake Creek Station (S)	Australia	NT	13.958131° S	132.652731° E	27/05/2015	LE, BL	5				✓	✓	
North Mountain Visitor Center (A1)	USA	AZ	33.598350° N	112.068300° W	28/10/2015	TS, BS	5	3	2	✓	✓	✓	
Harrison Road (A2)	USA	AZ	32.070690° N	110.791020° W	29/10/2015	TS, BS	5	4	1	✓	✓	✓	
Sands Ranch Road (A3)	USA	AZ	32.008960° N	110.690330° W	29/10/2015	TS, BS	5	2	1	✓	✓	✓	
Molino Canyon Overlook (A4)	USA	AZ	32.326930° N	110.700440° W	29/10/2015	TS, BS	2			✓	✓	✓	
South Austin (T1)	USA	TX	30.020570° N	97.856060° W	02/11/2015	TS, BS	5			✓	✓	✓	
San Antonio Missions (T2)	USA	TX	29.316520° N	98.436970° W	03/11/2015	TS, BS	5			✓	✓	✓	
Mitchell Lake Wetland (T3)	USA	TX	29.289412° N	98.501445° W	03/11/2015	TS, BS	5			✓	✓	✓	

[a] TS: T Steinrucken (WSU); KP: K Pukallus (Biosecurity Queensland); RvK: RD van Klinken; AW: A White (CSIRO); KB: K Bailey (Agriculture Western Australia); LE: L Elliot, BL: B Lukitsch (Northern Territory Dept. of Land Resource Management); BS: B Steinrucken (Big Red Ecology)

[b] Samples were also used in Chapter 2 (Steinrucken et al. 2016) and Chapter 3 (Steinrucken et al. 2017)

[c] Dieback-affected *P. aculeata*: samples used in Chapter 6.

Table S 5.2 Relative abundance (RA) of fungal ITS OTUs[a] recovered from stems, leaves and stem tips of each *Parkinsonia* host species sampled at sites A1, A2 and A3 in Arizona USA.

(a) *Parkinsonia aculeata*

OTU	Taxon	No. seqs	Seqs/sample	RA (%)	Cumulative RA (%)
ITS1F_0006	Ascomycota sp.	11315	707.2	12.61	
ITS1F_0007	Dothioraceae sp.	9709	485.5	10.82	23.44
ITS1F_0001	*Alternaria* sp.	9633	1204.1	10.74	34.17
ITS1F_1445	Dothioraceae sp.	5675	436.5	6.33	40.50
ITS1F_0002	*Cladosporium ramotenellum*	4456	891.2	4.97	45.47
ITS1F_1423	*Phoma* sp.	2868	1434	3.20	48.66
ITS1F_0222	*Alternaria penicillatum*	2669	190.6	2.98	51.64
ITS1F_0016	*Alternaria penicillatum*	2476	165.1	2.76	54.40
ITS1F_0015	Pleosporales sp.	2143	178.6	2.39	56.79
ITS1F_1429	*Phoma* sp.	2138	1069	2.38	59.17
ITS1F_1444	*Phoma* sp.	2083	1041.5	2.32	61.49
ITS1F_0405	Pleosporales sp.	1969	179	2.19	63.69
ITS1F_1581	*Phoma* sp.	1749	102.9	1.95	65.64
ITS1F_1461	*Phoma* sp.	1729	864.5	1.93	67.56
ITS1F_1475	*Phoma* sp.	1643	547.7	1.83	69.40
ITS1F_1699	*Phoma* sp.	1391	695.5	1.55	70.95
ITS1F_1708	*Phoma* sp.	1229	614.5	1.37	72.32
ITS1F_1137	*Aureobasidium* sp.	1218	76.1	1.36	73.67
ITS1F_1494	*Phoma* sp.	1026	513	1.14	74.82
ITS1F_1663	*Phoma* sp.	962	481	1.07	75.89
ITS1F_0062	Trichosphaeriales sp.	961	240.3	1.07	76.96
ITS1F_1720	*Phoma* sp.	930	465	1.04	78.00
ITS1F_1526	*Phoma* sp.	918	65.6	1.02	79.02

(b) *Parkinsonia florida*

OTU	Taxon	No. seqs	Seqs/sample	RA (%)	Cumulative RA (%)
ITS1F_0001	*Alternaria* sp.	1033	344.3	27.57	
ITS1F_1137	*Aureobasidium* sp.	605	151.3	16.15	43.71
ITS1F_0016	*Alternaria penicillatum*	435	62.1	11.61	55.32
ITS1F_0002	*Cladosporium ramotenellum*	387	193.5	10.33	65.65
ITS1F_0247	Lecanoromycetidae sp.	240	240	6.41	72.06
ITS1F_1420	*Phoma* sp.	151	75.5	4.03	76.09
ITS1F_0007	Dothioraceae sp.	135	16.9	3.60	79.69
ITS1F_0222	*Alternaria penicillatum*	93	13.3	2.48	82.17
ITS1F_0015	Pleosporales sp.	70	17.5	1.87	84.04

(c) *Parkinsonia microphylla*

OTU	Taxon	No. seqs	Seqs/sample	RA (%)	Cumulative RA (%)
ITS1F_0252	*Capnodium* sp.	855	855	25.81	
ITS1F_0051	*Gymnopus brassicolens*	495	495	14.94	40.75
ITS1F_0102	*Capnobotryella* sp.	175	175	5.28	46.03
ITS1F_0016	*Alternaria penicillatum*	153	51	4.62	50.65
ITS1F_0117	*Phoma tracheiphila*	151	151	4.56	55.21
ITS1F_1021	Ascomycota sp.	149	149	4.50	59.70
ITS1F_0405	Pleosporales sp.	143	71.5	4.32	64.02
ITS1F_0045	Ascomycota sp.	140	140	4.23	68.25
ITS1F_0188	*Chlorencoelia* sp.	99	99	2.99	71.23
ITS1F_0015	Pleosporales sp.	86	43	2.60	73.83
ITS1F_0002	*Cladosporium ramotenellum*	68	34	2.05	75.88
ITS1F_1796	Dothioraceae sp.	62	31	1.87	77.75
ITS1F_0287	*Candida quercitrusa*	56	56	1.69	79.44
ITS1F_0001	*Alternaria* sp.	55	55	1.66	81.10
ITS1F_1917	Pleosporales sp.	49	24.5	1.48	82.58
ITS1F_1445	Dothioraceae sp.	37	12.3	1.12	83.70
ITS1F_0222	*Alternaria penicillatum*	36	12	1.09	84.79

[a]Only OTUs with >1.0% relative abundance are presented. For the full list of recovered OTUs and the associated plant part/sampling site data, see Appendix B

Table S 5.3 Relative abundance (RA) of bacterial 16s OTUs[a] recovered from stems, leaves and stem tips of each *Parkinsonia* host species sampled at sites A1, A2 and A3 in Arizona USA.

(a) *Parkinsonia aculeata*

OTU	Taxon	No. seqs	Seqs/sample	RA (%)	Cumulative RA (%)
515F_004	*Sphingomonas yabuuchiae*	38	4.2	23.03	
515F_005	*Escherichia coli*	24	8	14.55	37.58
515F_033	*Ralstonia* sp.	19	19	11.52	49.09
515F_010	*Massilia niastensis*	14	2	8.48	57.58
515F_022	Bacteria sp.	10	1.1	6.06	63.64
515F_054	*Deinococcus* sp.	9	9	5.45	69.09
515F_057	*Curtobacterium* sp.	5	1.7	3.03	72.12
515F_104	*Hymenobacter* sp.	5	2.5	3.03	75.15
515F_144	*Sphingomonas* sp.	5	1.7	3.03	78.18
515F_081	*Kineococcus* sp.	4	2	2.42	80.61
515F_016	*Staphylococcus equorum*	3	1.5	1.82	82.42
515F_023	*Methylobacterium hispanicum*	3	1.5	1.82	84.24
515F_093	*Methylobacterium organophilum*	3	1.5	1.82	86.06
515F_167	Gammaproteobacteria sp.	3	3	1.82	87.88
515F_008	*Rhodococcus* sp.	2	1	1.21	89.09
515F_043	*Acinetobacter schindleri*	2	1	1.21	90.30
515F_048	*Blastomonas natatoria*	2	2	1.21	91.52
515F_092	Xanthobacteraceae sp.	2	2	1.21	92.73
515F_166	Pseudomonadaceae sp.	2	1	1.21	93.94
515F_186	*Snodgrassella alvi*	2	2	1.21	95.15

(b) *Parkinsonia florida*

OTU	Taxon	No. seqs	Seqs/sample	RA (%)	Cumulative RA (%)
515F_037	*Portiera aleyrodidarum*	28	28	29.17	
515F_004	*Sphingomonas yabuuchiae*	13	2.6	13.54	42.71
515F_005	*Escherichia coli*	8	8	8.33	51.04
515F_099	*Pseudomonas veronii*	6	6	6.25	57.29
515F_028	*Xanthomonas campestris*	4	4	4.17	61.46
515F_104	*Hymenobacter* sp.	3	1.5	3.13	64.58
515F_144	*Sphingomonas* sp.	3	3	3.13	67.71
515F_016	*Staphylococcus equorum*	2	1	2.08	69.79
515F_063	*Lactococcus* sp.	2	2	2.08	71.88
515F_090	Solirubrobacterales sp.	2	2	2.08	73.96
515F_092	Xanthobacteraceae sp.	2	2	2.08	76.04
515F_170	*Propionibacterium acnes*	2	1	2.08	78.13
515F_234	*Rubrobacter* sp.	2	2	2.08	80.21
515F_013	*Streptococcus* sp.	1	1	1.04	81.25
515F_022	Bacteria sp.	1	1	1.04	82.29
515F_023	*Methylobacterium hispanicum*	1	1	1.04	83.33
515F_026	*Bacillus longiquaesitum*	1	1	1.04	84.38
515F_027	*Pseudomonas* sp.	1	1	1.04	85.42
515F_029	*Salmonella enterica*	1	1	1.04	86.46
515F_033	*Ralstonia* sp.	1	1	1.04	87.50
515F_036	*Sphingobium yanoikuyae*	1	1	1.04	88.54
515F_042	*Corynebacterium* sp.	1	1	1.04	89.58
515F_045	*Bacillus fumarioli*	1	1	1.04	90.63
515F_055	Actinosynnemataceae sp.	1	1	1.04	91.67
515F_057	*Curtobacterium* sp.	1	1	1.04	92.71
515F_068	*Corynebacterium* sp.	1	1	1.04	93.75
515F_081	*Kineococcus* sp.	1	1	1.04	94.79
515F_085	*Bacillus cereus*	1	1	1.04	95.83
515F_091	*Peptoniphilus* sp.	1	1	1.04	96.88
515F_141	*Novosphingobium* sp.	1	1	1.04	97.92
515F_232	*Kineosporia* sp.	1	1	1.04	98.96
515F_233	*Hymenobacter* sp.	1	1	1.04	100.00

(c) *Parkinsonia microphylla*

OTU	Taxon	No. seqs	Seqs/sample	RA (%)	Cumulative RA (%)
515F_004	*Sphingomonas yabuuchiae*	38	19	41.30	
515F_017	*Corynebacterium variabile*	11	11	11.96	53.26
515F_088	*Pimelobacter* sp.	6	6	6.52	59.78
515F_131	Intrasporangiaceae sp.	5	5	5.43	65.22
515F_015	*Micrococcus luteus*	4	4	4.35	69.57
515F_029	*Salmonella enterica*	4	4	4.35	73.91
515F_079	*Finegoldia* sp.	4	2	4.35	78.26
515F_130	*Halomonas* sp.	4	4	4.35	82.61
515F_008	*Rhodococcus* sp.	3	3	3.26	85.87
515F_027	*Pseudomonas* sp.	2	2	2.17	88.04
515F_122	*Pseudomonas stutzeri*	2	2	2.17	90.22
515F_005	*Escherichia coli*	1	1	1.09	91.30
515F_016	*Staphylococcus equorum*	1	1	1.09	92.39
515F_022	Bacteria sp.	1	1	1.09	93.48
515F_033	*Ralstonia* sp.	1	1	1.09	94.57
515F_042	*Corynebacterium* sp.	1	1	1.09	95.65
515F_092	Xanthobacteraceae sp.	1	1	1.09	96.74
515F_172	*Snodgrassella alvi*	1	1	1.09	97.83
515F_193	*Acinetobacter lwoffii*	1	1	1.09	98.91
515F_211	*Comamonas* sp.	1	1	1.09	100.00

[a]Only OTUs with >1.0% relative abundance are presented. For the full list of recovered OTUs and the associated plant part/sampling site data, see the electronic supplementary material

Table S 5.4 Relative abundance (RA) of fungal ITS OTUs[a] recovered from stems and stem tips of *Parkinsonia aculeata* in the native range (Arizona and Texas USA), and the invasive range (Northern Territory, Queensland and Western Australia, Australia).

(a) Native range (USA)

OTU	Taxon	No. seqs	Seqs/sample	RA (%)	Cumulative RA (%)
ITS1F_0002	*Cladosporium ramotenellum*	10840	1355	27.67	
ITS1F_0001	*Alternaria* sp.	3367	673.4	8.59	36.26
ITS1F_1423	*Phoma* sp.	2426	808.7	6.19	42.46
ITS1F_1429	*Phoma* sp.	1973	493.3	5.04	47.49
ITS1F_1444	*Phoma* sp.	1715	571.7	4.38	51.87
ITS1F_1475	*Phoma* sp.	1488	297.6	3.80	55.67
ITS1F_1461	*Phoma* sp.	1351	450.3	3.45	59.12
ITS1F_1699	*Phoma* sp.	1211	403.7	3.09	62.21
ITS1F_1137	*Aureobasidium* sp.	1183	69.6	3.02	65.23
ITS1F_0062	Trichosphaeriales sp.	1182	394	3.02	68.24
ITS1F_1708	*Phoma* sp.	991	330.3	2.53	70.77
ITS1F_1494	*Phoma* sp.	873	291	2.23	73.00
ITS1F_1663	*Phoma* sp.	745	248.3	1.90	74.90
ITS1F_1720	*Phoma* sp.	717	239	1.83	76.73
ITS1F_0051	*Gymnopus brassicolens*	531	265.5	1.36	78.09
ITS1F_0016	*Alternaria penicillatum*	475	47.5	1.21	79.30
ITS1F_1822	*Phoma* sp.	464	154.7	1.18	80.49
ITS1F_0006	Ascomycota sp.	459	32.8	1.17	81.66
ITS1F_1422	*Phoma* sp.	440	146.7	1.12	82.78

(b) Invasive range (Australia*)*

OTU	Taxon	No. seqs	Seqs/sample	RA (%)	Cumulative RA (%)
ITS1F_0002	*Cladosporium ramotenellum*	14831	1483.1	25.71	
ITS1F_0001	*Alternaria* sp.	5142	1028.4	8.91	34.63
ITS1F_1423	*Phoma* sp.	2849	949.7	4.94	39.57
ITS1F_1429	*Phoma* sp.	2366	788.7	4.10	43.67
ITS1F_1444	*Phoma* sp.	2149	1074.5	3.73	47.39
ITS1F_0062	Trichosphaeriales sp.	1854	185.4	3.21	50.61
ITS1F_0025	*Cladosporium dominicanum*	1691	845.5	2.93	53.54
ITS1F_1461	*Phoma* sp.	1678	839	2.91	56.45
ITS1F_1475	*Phoma* sp.	1563	312.6	2.71	59.16
ITS1F_0017	*Toxicocladosporium rubrigenum*	1490	212.9	2.58	61.74
ITS1F_0005	*Penicillium spinulosum*	1414	35.4	2.45	64.19
ITS1F_1699	*Phoma* sp.	1413	471	2.45	66.64
ITS1F_0961	Ascomycota sp.	1206	603	2.09	68.73
ITS1F_1822	*Phoma* sp.	1035	129.4	1.79	70.53
ITS1F_1708	*Phoma* sp.	1031	515.5	1.79	72.32
ITS1F_1494	*Phoma* sp.	922	461	1.60	73.91
ITS1F_1720	*Phoma* sp.	832	208	1.44	75.36
ITS1F_1663	*Phoma* sp.	786	393	1.36	76.72
ITS1F_1422	*Phoma* sp.	758	126.3	1.31	78.03
ITS1F_0010	*Malassezia restricta*	618	17.7	1.07	79.11
ITS1F_1960	*Phoma* sp.	585	53.2	1.01	80.12

[a]Only OTUs with >1.0 relative abundance are presented. For the full list of recovered OTUs and the associated plant part/sampling site data, see the electronic supplementary material

Table S 5.5 Relative abundance (RA) of bacterial 16s OTUs[a] recovered from stems and stem tips of *Parkinsonia aculeata* in the native range (Arizona and Texas USA), and the invasive range (Northern Territory, Queensland and Western Australia, Australia).

(a) Native range (USA)

OTU	Taxon	No. seqs	Seqs/sample	RA (%)	Cumulative RA (%)
515F_022	Bacteria sp.	10	1.3	12.66	
515F_010	*Massilia niastensis*	9	1.8	11.39	24.05
515F_004	*Sphingomonas yabuuchiae*	6	1.5	7.59	31.65
515F_057	*Curtobacterium* sp.	5	1.7	6.33	37.97
515F_144	*Sphingomonas* sp.	5	1.3	6.33	44.30
515F_068	*Corynebacterium* sp.	4	4	5.06	49.37
515F_081	*Kineococcus* sp.	4	2	5.06	54.43
515F_187	*Sphingomonas wittichii*	4	4	5.06	59.49
515F_093	*Methylobacterium organophilum*	3	1	3.80	63.29
515F_104	*Hymenobacter* sp.	3	1	3.80	67.09
515F_005	*Escherichia coli*	2	1	2.53	69.62
515F_042	*Corynebacterium* sp.	2	2	2.53	72.15
515F_043	*Acinetobacter schindleri*	2	1	2.53	74.68
515F_079	*Finegoldia* sp.	2	2	2.53	77.22
515F_129	Aurantimonadaceae sp.	2	1	2.53	79.75
515F_210	*Blastococcus aggregatus*	2	2	2.53	82.28
515F_008	*Rhodococcus* sp.	1	1	1.27	83.54
515F_011	*Enterococcus* sp.	1	1	1.27	84.81
515F_016	*Staphylococcus equorum*	1	1	1.27	86.08
515F_023	*Methylobacterium hispanicum*	1	1	1.27	87.34
515F_033	*Ralstonia* sp.	1	1	1.27	88.61
515F_054	*Deinococcus* sp.	1	1	1.27	89.87
515F_061	*Streptococcus infantis*	1	1	1.27	91.14
515F_063	*Lactococcus* sp.	1	1	1.27	92.41
515F_113	*Paucibacter* sp.	1	1	1.27	93.67
515F_122	*Pseudomonas stutzeri*	1	1	1.27	94.94
515F_163	*Aeromonas caviae*	1	1	1.27	96.20
515F_178	*Enhydrobacter* sp.	1	1	1.27	97.47
515F_233	*Hymenobacter* sp.	1	1	1.27	98.73
515F_257	*Rothia mucilaginosa*	1	1	1.27	100.00

(b) Invasive range (Australia*)*

OTU	Taxon	No. seqs	Seqs/sample	RA (%)	Cumulative RA (%)
515F_004	*Sphingomonas yabuuchiae*	445	29.7	52.73	
515F_009	*Phytoplasma australiense*	86	86.0	10.19	62.91
515F_005	*Escherichia coli*	84	14.0	9.95	72.87
515F_022	Bacteria sp.	57	1.9	6.75	79.62
515F_016	*Staphylococcus equorum*	30	5.0	3.55	83.18
515F_014	Pseudomonadaceae sp.	17	8.5	2.01	85.19
515F_008	*Rhodococcus* sp.	12	4.0	1.42	86.61
515F_043	*Acinetobacter schindleri*	10	3.3	1.18	87.80

[a]Only OTUs with >1.0 relative abundance are presented. For the full list of recovered OTUs and the associated plant part/sampling site data, see the electronic supplementary material

Chapter 6 Supplementary Material

Table S 6.1 Relative abundance (RA) of fungal ITS OTUs[a] recovered from stem tips, stems and roots from healthy (a) and dieback-affected (b) *Parkinsonia aculeata*.

(a) Healthy parkinsonia

OTU	Taxon	No. seqs	Seqs/Sample	RA	Cumulative RA
ITS1F_0017	*Toxicocladosporium rubrigenum*	1591	144.64	14.99%	
ITS1F_0961	Ascomycota sp.	1206	603.00	11.37%	26.36%
ITS1F_0005	*Penicillium spinulosum*	1057	62.18	9.96%	36.32%
ITS1F_0010	*Malassezia restricta*	824	26.58	7.77%	44.09%
ITS1F_1543	*Fusarium graminearum*	533	20.50	5.02%	49.11%
ITS1F_0030	*Cladosporium halotolerans*	345	28.75	3.25%	52.36%
ITS1F_1424	*Fusarium graminearum*	336	16.00	3.17%	55.53%
ITS1F_0373	*Fusarium sublunatum*	225	12.50	2.12%	57.65%
ITS1F_0386	Trichosphaeriales sp.	194	19.40	1.83%	59.48%
ITS1F_1427	Pleosporales sp.	155	11.92	1.46%	60.94%
ITS1F_1164	Ascomycota sp.	151	10.07	1.42%	62.36%
ITS1F_1128	*Fusarium pseudensiforme*	147	73.50	1.39%	63.75%
ITS1F_0110	*Antennariella placitae*	145	36.25	1.37%	65.11%
ITS1F_1608	*Fusarium* sp.	134	16.75	1.26%	66.37%
ITS1F_0104	Dothideomycetes sp.	131	131.00	1.23%	67.61%
ITS1F_0029	Ascomycota sp.	121	20.17	1.14%	68.75%
ITS1F_0011	Ascomycota sp.	110	9.17	1.04%	69.79%

(b) Dieback-affected parkinsonia

OTU	Taxon	No. seqs	Seqs/Sample	RA	Cumulative RA
ITS1F_0005	*Penicillium spinulosum*	5457	227.38	10.20%	
ITS1F_0373	*Fusarium sublunatum*	3578	137.62	6.69%	16.89%
ITS1F_0011	Ascomycota sp.	2792	99.71	5.22%	22.11%
ITS1F_1164	Ascomycota sp.	2362	107.36	4.41%	26.52%
ITS1F_1427	Pleosporales sp.	1875	93.75	3.50%	30.03%
ITS1F_0030	*Cladosporium halotolerans*	1662	75.55	3.11%	33.13%
ITS1F_0093	*Capnodium* sp.	1331	190.14	2.49%	35.62%
ITS1F_1543	*Fusarium graminearum*	1229	55.86	2.30%	37.92%
ITS1F_0027	*Neurospora terricola*	1136	49.39	2.12%	40.04%
ITS1F_0031	*Trichomerium foliicola*	1132	94.33	2.12%	42.16%
ITS1F_0029	Ascomycota sp.	1063	62.53	1.99%	44.14%
ITS1F_0110	*Antennariella placitae*	1029	171.50	1.92%	46.07%
ITS1F_1424	*Fusarium graminearum*	1017	42.38	1.90%	47.97%
ITS1F_0047	*Inocybe* sp.	939	42.68	1.76%	49.72%
ITS1F_1041	Eurotiomycetes sp.	686	98.00	1.28%	51.01%
ITS1F_0026	*Curvularia lunata*	637	27.70	1.19%	52.20%
ITS1F_0010	*Malassezia restricta*	574	20.50	1.07%	53.27%
ITS1F_0119	*Aspergillus parasiticus*	551	39.36	1.03%	54.30%

[a]Only OTUs with >1.0 relative abundance are presented. For the full list of recovered OTUs and the associated plant part/sampling site data, see the electronic supplementary material

Table S 6.2 Relative abundance (RA) of bacterial 16s OTUs[a] recovered from stem tips, stems and roots from healthy (a) and dieback-affected (b) *Parkinsonia aculeata*.

(a) Healthy parkinsonia

OTU	Taxon	No. seqs	Seqs/Sample	RA	Cumulative RA
515F_005	*Escherichia coli*	590	21.07	39.41%	
515F_009	*Phytoplasma australiense*	438	15.64	29.26%	68.67%
515F_004	*Sphingomonas yabuuchiae*	313	11.18	20.91%	89.58%
515F_022	Bacteria sp.	21	0.75	1.40%	90.98%
515F_014	Pseudomonadaceae sp.	16	0.57	1.07%	92.05%

(b) Dieback-affected parkinsonia

OTU	Taxa	No. seqs	Seqs/Sample	RA	Cumulative RA
515F_005	*Escherichia coli*	1503	40.62	49.21%	
515F_004	*Sphingomonas yabuuchiae*	297	8.03	9.72%	58.94%
515F_019	*Sphingomonas wittichii*	100	2.70	3.27%	62.21%
515F_034	*Mycobacterium* sp.	85	2.30	2.78%	65.00%
515F_023	*Methylobacterium hispanicum*	71	1.92	2.32%	67.32%
515F_026	*Bacillus longiquaesitum*	56	1.51	1.83%	69.16%
515F_009	*Phytoplasma australiense*	55	1.49	1.80%	70.96%
515F_022	Bacteria sp.	34	0.92	1.11%	72.07%
515F_044	*Beijerinckiaceae* sp.	33	0.89	1.08%	73.15%
515F_016	*Staphylococcus equorum*	32	0.86	1.05%	74.20%
515F_025	*Paenibacillus amylolyticus*	32	0.86	1.05%	75.25%
515F_035	*Sphingomonas wittichii*	32	0.86	1.05%	76.29%

[a]Only OTUs with >1.0 relative abundance are presented. For the full list of recovered OTUs and the associated plant part/sampling site data, see the electronic supplementary material

Table S 6.3 SIMPER results showing the mean similarity and dissimilarity of the fungal genera within healthy and dieback *Parkinsonia aculeata* samples. Cut-off = 90%

Average similarity = 27.25%	Healthy		
Genera	**Average abundance**	**Contribution %**	**Cumulative %**
Malassezia	2.68	32.98	32.98
Fusarium[b]	1.76	21.77	54.75
Penicillium[c]	1.68	15.80	70.55
Fusarium[a,c]	1.23	8.87	79.41
Phoma[c]	0.81	6.72	86.13
Aspergillus[c]	0.56	2.64	88.77
Toxicocladosporium[a]	0.82	2.14	90.91

Average similarity = 29.06 %	Dieback		
Genera	**Average abundance**	**Contribution %**	**Cumulative %**
Penicillium[c]	3.75	16.29	16.29
Fusarium[a,c]	2.29	13.72	30.01
Malassezia	1.73	12.38	42.40
Fusarium[b]	1.96	8.55	50.95
Aspergillus[c]	1.74	6.80	57.75
Inocybe[a]	1.12	4.82	62.57
Cladosporium[a,c]	1.21	4.60	67.17
Talaromyces[a]	1.16	4.32	71.49
Toxicocladosporium[a]	0.73	3.13	74.62
Neurospora[a,c]	0.72	3.01	77.63
Myrmecridium[a]	0.86	2.60	80.22
Coprinellus[a]	0.68	2.21	82.44
Phoma[c]	1.24	2.18	84.62
Curvularia[a]	0.75	1.72	86.34
Acremonium[a]	0.48	1.53	87.87
Hypocrea[a]	0.47	1.48	89.35
Tomentella[a]	0.47	1.08	90.43

Average dissimilarity = 76.32%	Healthy	Dieback		
Genera	**Average abundance**		**Contribution %**	**Cumulative %**
Penicillium[c]	1.68	3.75	9.43	9.43
Malassezia	2.68	1.73	8.17	17.61
Fusarium[a,c]	1.23	2.29	6.25	31.39
Phoma[c]	0.81	1.24	4.74	36.13
Aspergillus[c]	0.56	1.74	4.67	40.80
Toxicocladosporium[a]	0.82	0.73	4.58	45.38
Cladosporium[c]	0.42	1.21	3.93	49.31
Inocybe[a]	0.41	1.12	3.45	52.76
Talaromyces[a]	0.25	1.16	3.12	55.88
Strelitziana	0.53	0.34	2.41	58.29
Capnodium[c]	0.24	0.55	2.31	60.60
Myrmecridium[a]	0.24	0.86	2.30	62.90
Coprinellus[a]	0.05	0.68	2.20	65.10
Neurospora[a,c]	0.11	0.72	1.96	67.06
Curvularia[a]	0.04	0.75	1.77	68.83
Tomentella[a]	0.22	0.47	1.65	70.48
Hypocrea[a]	0.07	0.47	1.61	72.09
Acremonium[a]	0.07	0.48	1.37	73.46
Chaetomium[a,c]	0.09	0.41	1.12	74.58
Ochrocladosporium	0.11	0.29	1.03	75.61

..Genera	…Average abundance		…Contribution %	…Cumulative %
Phomopsis[c]	0.10	0.16	0.97	76.59
Periconia	0.12	0.24	0.95	77.54
Cercophora[a]	0.10	0.32	0.91	78.45
Falciformispora[a]	0.06	0.30	0.83	79.28
Teratosphaeria[a]	0.08	0.18	0.77	80.05
Alternaria[c]	0.23	0.03	0.71	80.76
Diplodia[c]	0.07	0.13	0.67	81.43
Marasmius	0.06	0.20	0.63	82.06
Purpureocillium[a]	0.02	0.23	0.60	82.66
Cladophialophora[a]	0.06	0.15	0.59	83.25
Cyphellophora	0.08	0.18	0.58	83.83
Mycena[a]	<0.01	0.23	0.58	84.41
Rhizoctonia	0.07	0.14	0.55	84.96
Devriesia[a]	<0.01	0.16	0.55	85.52
Clonostachys[a]	0.05	0.17	0.51	86.03
Paraconiothyrium[a]	0.04	0.16	0.44	86.47
Engyodontium[a]	<0.01	0.21	0.43	86.90
Cryptococcus	0.1	0.03	0.39	87.29
Letendraea[a]	0.02	0.14	0.35	87.65
Phaeosphaeria	0.01	0.13	0.35	87.99
Hypochnicium[a]	<0.01	0.13	0.33	88.32
Cochliobolus[c]	0.06	0.06	0.33	88.65
Savoryella[a]	<0.01	0.15	0.33	88.98
Xylomyces	<0.01	0.13	0.32	89.30
Saccharomyces	0.09	0.03	0.32	89.63
Thanatephorus	<0.01	0.11	0.32	89.95
Mortierella	0.01	0.12	0.29	90.24

[a]Classified as associated with dieback-affected parkinsonia in Fig 6.4

[c]Isolated member/s of genus in culture from Chapter 3 (Steinrucken et al., 2017)

APPENDIX B: DETAILS OF ELECTRONIC SUPPLEMENTARY MATERIAL

The following is a list of electronic supplementary material available through the Google Drive at this address:

http://bit.ly/2osHAZC

Chapter 2

T-RFLP OTU relative abundance data for each taxonomic group studied

- *Ch 2 - Raw Archaea T-RFLP Data.txt*
- *Ch 2 - Raw Bacterial T-RFLP Data.txt*
- *Ch 2 - Raw Fungal T-RFLP Data.txt*

Chapter 3

Contains data submitted to Genbank on all identified fungal isolates

- *Ch 3 - Isolate sequences and annotations.xlsx*

Chapter 4

Contains data submitted to Genbank on all identified oomycete isolates

- *Ch 4 - Isolate sequences and annotations.xlsx*

Chapter 5 and 6

OTU data for 16S and ITS sequences recovered from *Parkinsonia* spp. samples and used in bother or either chapter. Also includes PCR and Illumina primer sequences as used in Chapters 5 and 6 (see detailed protocol in Appendix C).

- *Ch 5 and 6 - 16S OTU data.xlsx*
- *Ch 5 and 6 - ITS1F OTU data.xlsx*
- *Ch 5 and 6 - PCR and Illumina Primers.xlsx*

APPENDIX C: DETAILED PROTOCOL FOR MULTIPLEX DUAL-INDEX ILLUMINA SEQUENCING OF ENDOPHYTE DNA

This protocol was designed and tested by T.V Steinrucken (Western Sydney University) for research in Chapters 5 and 6 of her PhD thesis titled "Investigating the cause of dieback in the invasive plant, *Parkinsonia aculeata*". With work carried out at UC Berkeley in Nov 2015 - April 2016.

Extract DNA from plant tissue

1. Surface sterilise plant parts in 70% ETOH 1 min, 50% NaHCl 3 min, 75% ETOH 30 s, then rinse with ds H_2O and dry with sterile paper towel.
2. Strip or shave bark or skin off using a sterile blade. Shave small pieces of endophytic tissue into a sterile envelope. Freeze dry for 48-72 h, then bead beat on high with 2x 6 mm glass ball bearings in 2 mL screw-cap tubes, until powdered.
3. Extract DNA using the Mobio Soil DNA Isolation Kit as per manufacturer's instructions but elute with 50 µL H_2O (instead of 100 µL Buffer 6).
4. Freeze at -20°C until next use.

Normalise and sort DNA extractions

1. Quantify DNA extractions and normalise samples to approx. 5 ng/uL.
2. Sort normalised samples into wells on a 96-well plate so you know which sample is in which well. Remember to always leave well A1 empty for any controls used in subsequent analyses. Allocate the next 2 wells for controls on every plate (Fig C1).

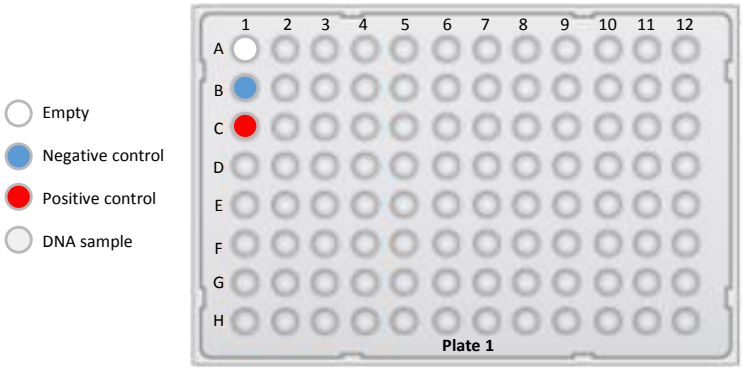


Figure C1. Arrangement of normalised DNA extractions on every 96-well plate. Maintain this arrangement throughout protocol.

First PCR round with Amplicon-Specific Primers

1. Design amplicon-specific primers (Table C1) using the **5'-nex-n5-primer-3'** formula (try maintain at <60 bp per primer) to amplify a region of <350 bp target DNA. NexF and NexR are part of the Nextera Illumina Sequencing Kit and can be found at http://www.illumina.com/products/nextera_dna_library_prep_kit.html.

2. Order 1x FWD and 1x REV amplicon-specific primer for each taxon from IDT (25 nm in tubes is fine). Prepare 10 µM aliquots of each amplicon primer.

Table C1. Primers used for fungal (Toju et al), 16s (Peay & Smith et al) and oomycete (Cooke et al) amplicons

Primer name	Sequence (5'- sequencing primer - 5mer Ns - amplicon specific primer - 3')
Fungal ITS1:	
nexF-N5-ITS1F_KYO1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNCTHGGTCATTTAGAGGAASTAA
nexR-N5-ITS2_KYO2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNTTYRCTRCGTTCTTCATC
Bacterial 16s:	
nexF-N5-515f	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNGTGYCAGCMGCCGCGGTAA
nexR-N5-806r	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNGGACTACNVGGGTWTCTAAT

3. Set up the first PCR reaction for all samples (Tables C3 and C4). I used the Bioline MyTaq PCR kit.

Table C3. First PCR reaction volumes

Reagent	Per reaction
MyTaq Buffer	4.0
Fwd Primer (10 µM)	0.4
Rev Primer (10 µM)	0.4
dsH_2O	12.8
MyTaq Polymerase	0.4
DNA template (5 ng/µL)	2.0
TOTAL	20.0

Table C4. Fist PCR reaction conditions by primer pair

Amplicon	Reaction conditions
ITS1F-ITS2	95°C for 2 min; 35 cycles of 95°C for 20 s, 50°C for 30 s, 72°C for 50s; then 72°C for 10 min
515f-806r	95°C for 2 min; 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30s; then 72°C for 5 min

4. Before filling all plates, test each primer pair on ≈8 endophyte DNA extractions and a positive control (mock community*) using the same conditions as for the first PCR round. Always include a negative control with PCR water.
   *I made 2 mock communities (fungi and bacteria) and ran two positive controls per primer pair for the test run. Control 1 was just the target amplicon mock community, control 2 was the pooled mock communities.
5. Run a 1.5% agarose gel. Analyse gel to confirm desired amplicon size (Fig B2), and to see if controls worked.

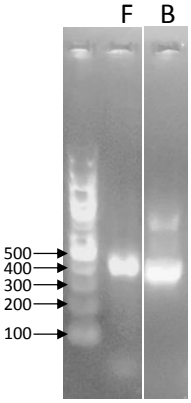


Figure C2. Expected product sizes for Fungal ITS1 (F) and Bacterial 16s (B) PCR products using amplicon-specific primers from Table B1.

6. If PCR products for each primer pair are as expected, repeat reaction for all samples, keeping track of which sample is in each well, which plate has amplicons from which taxon, and the volume of PCR product remaining in each well (important for normalization).
7. Run a 1.5% agarose gel on all plates, testing the first column of each plate (seven samples including mock community, negative control and five DNA samples). Analyse gel to confirm amplification of samples, and to see if controls worked.
8. Run PCR clean-up on all samples.

Quantify & normalise samples

1. Quantify and normalise to about 10 ng/uL.
2. Pool normalised amplicons by samples (i.e. pool fungal and 16s amplicons from sample X in one well) by adding an equal volume (5 µL) of each normalised PCR product to the corresponding well on a new plate.
3. Using the 260/280 measurements, choose the best positive controls from each taxa and combine for your mock community control.
4. Add water (10 µL) to dilute each sample by half to 5 ng/µL. Mix well and centrifuge down.

Choose and order indexing primers

1. Using the **5'-illumina_adaptor-index-nex_overlap-3'** formula, order enough FWD and REV to cover all your samples (Table C5). Randomise index sequence (i5 and i7) list to ensure each base is represented at each sequencing cycle.
2. For 352 samples, I ordered 20 FWD and 20 REV (Thesis, Table S5.1).
3. Prepare 10 µM aliquots of each primer. Test a few pairs on PCR products from reaction above. Freeze until needed.
4. Sort primers on a spreadsheet so that forward and reverse index sequences form a unique combination for each sample.

Table C5. Forward and reverse indexing primers chosen from Toju et al (2016). For details of which forward and reverse primers were used for each sample in the thesis, see the electronic supplementary material (Appendix B)

Forward Primer Name	P5 Adaptor	i5 index	Nex_F overlap
P5-Hamady-nexF_1407	AATGATACGGCGACCACCGAGATCTACAC	CATGCATG	TCGTCGGCAGCGTC
P5-Hamady-nexF_1442	AATGATACGGCGACCACCGAGATCTACAC	AGACCACT	TCGTCGGCAGCGTC
P5-Hamady-nexF_0348	AATGATACGGCGACCACCGAGATCTACAC	AGCAAGCA	TCGTCGGCAGCGTC
P5-Hamady-nexF_0151	AATGATACGGCGACCACCGAGATCTACAC	CTCAAGTG	TCGTCGGCAGCGTC
P5-Hamady-nexF_0835	AATGATACGGCGACCACCGAGATCTACAC	ACCTACGA	TCGTCGGCAGCGTC
P5-Hamady-nexF_1340	AATGATACGGCGACCACCGAGATCTACAC	CGATCGTA	TCGTCGGCAGCGTC
P5-Hamady-nexF_0089	AATGATACGGCGACCACCGAGATCTACAC	GTAGCTTG	TCGTCGGCAGCGTC
P5-Hamady-nexF_0032	AATGATACGGCGACCACCGAGATCTACAC	GCATCGAT	TCGTCGGCAGCGTC
P5-Hamady-nexF_1384	AATGATACGGCGACCACCGAGATCTACAC	TACGATGC	TCGTCGGCAGCGTC
P5-Hamady-nexF_1357	AATGATACGGCGACCACCGAGATCTACAC	ACGTCATG	TCGTCGGCAGCGTC
P5-Hamady-nexF_0312	AATGATACGGCGACCACCGAGATCTACAC	TGCTTCGT	TCGTCGGCAGCGTC
P5-Hamady-nexF_0128	AATGATACGGCGACCACCGAGATCTACAC	AGCAGTAG	TCGTCGGCAGCGTC
P5-Hamady-nexF_0379	AATGATACGGCGACCACCGAGATCTACAC	AACCATGC	TCGTCGGCAGCGTC
P5-Hamady-nexF_0034	AATGATACGGCGACCACCGAGATCTACAC	GTACGTTG	TCGTCGGCAGCGTC
P5-Hamady-nexF_1355	AATGATACGGCGACCACCGAGATCTACAC	TCACAGAC	TCGTCGGCAGCGTC
P5-Hamady-nexF_0163	AATGATACGGCGACCACCGAGATCTACAC	TAGCGCTA	TCGTCGGCAGCGTC
P5-Hamady-nexF_0496	AATGATACGGCGACCACCGAGATCTACAC	ACTCCACT	TCGTCGGCAGCGTC
P5-Hamady-nexF_0704	AATGATACGGCGACCACCGAGATCTACAC	TGTCTCTG	TCGTCGGCAGCGTC
P5-Hamady-nexF_0904	AATGATACGGCGACCACCGAGATCTACAC	GATGAGGT	TCGTCGGCAGCGTC
P5-Hamady-nexF_0690	AATGATACGGCGACCACCGAGATCTACAC	TTAAGCGC	TCGTCGGCAGCGTC

Reverse Primer Name	P7 Adaptor	i7 index	NEX_R overlap
P7-Hamady-nexR_1313	CAAGCAGAAGACGGCATACGAGAT	CTGTACTC	GTCTCGTGGGCTCGG
P7-Hamady-nexR_0012	CAAGCAGAAGACGGCATACGAGAT	CAACCTAG	GTCTCGTGGGCTCGG
P7-Hamady-nexR_0692	CAAGCAGAAGACGGCATACGAGAT	CTAGTGCA	GTCTCGTGGGCTCGG
P7-Hamady-nexR_0742	CAAGCAGAAGACGGCATACGAGAT	CAACTGGT	GTCTCGTGGGCTCGG
P7-Hamady-nexR_0054	CAAGCAGAAGACGGCATACGAGAT	CCATCCTA	GTCTCGTGGGCTCGG
P7-Hamady-nexR_0752	CAAGCAGAAGACGGCATACGAGAT	AGGATCCT	GTCTCGTGGGCTCGG
P7-Hamady-nexR_0009	CAAGCAGAAGACGGCATACGAGAT	CGTTCCTA	GTCTCGTGGGCTCGG
P7-Hamady-nexR_0675	CAAGCAGAAGACGGCATACGAGAT	GTTCTGGA	GTCTCGTGGGCTCGG
P7-Hamady-nexR_0042	CAAGCAGAAGACGGCATACGAGAT	GTGTAGTC	GTCTCGTGGGCTCGG
P7-Hamady-nexR_0743	CAAGCAGAAGACGGCATACGAGAT	GAAGAGGA	GTCTCGTGGGCTCGG
P7-Hamady-nexR_0558	CAAGCAGAAGACGGCATACGAGAT	CACAAGTC	GTCTCGTGGGCTCGG
P7-Hamady-nexR_1439	CAAGCAGAAGACGGCATACGAGAT	AAGGCCAA	GTCTCGTGGGCTCGG
P7-Hamady-nexR_0422	CAAGCAGAAGACGGCATACGAGAT	GAACTCCA	GTCTCGTGGGCTCGG
P7-Hamady-nexR_1357	CAAGCAGAAGACGGCATACGAGAT	CCATGCTT	GTCTCGTGGGCTCGG
P7-Hamady-nexR_0608	CAAGCAGAAGACGGCATACGAGAT	AGACTCTG	GTCTCGTGGGCTCGG
P7-Hamady-nexR_1457	CAAGCAGAAGACGGCATACGAGAT	CCTAATGG	GTCTCGTGGGCTCGG
P7-Hamady-nexR_0779	CAAGCAGAAGACGGCATACGAGAT	CTCTACAC	GTCTCGTGGGCTCGG
P7-Hamady-nexR_0364	CAAGCAGAAGACGGCATACGAGAT	TTCGATGG	GTCTCGTGGGCTCGG
P7-Hamady-nexR_0092	CAAGCAGAAGACGGCATACGAGAT	CGATGGTT	GTCTCGTGGGCTCGG
P7-Hamady-nexR_0319	CAAGCAGAAGACGGCATACGAGAT	CCTACCAT	GTCTCGTGGGCTCGG

Second PCR round with index primers

1. Record which index primer combination represents each sample and mix 1 µL of each forward and reverse primer (10 µM each) into the sample's corresponding well in a new plate. Don't forget to assign a unique primer pair to this control.
2. Run a test-PCR using the exact same conditions you expect to use for the second round (step 3). Include pooled and individual fungal and bacterial amplicons.
3. Analyse PCR on a gel (Fig C3) and adjust and/or re-test varied protocol until you can minimise primer dimers and obtain the right product size.
   NOTE: it's very difficult to avoid primer dimers all together – you can do size-selection after the second round PCR to eliminate smaller, unwanted products, bearing in mind you may lose some yield, despite an increase in DNA quality (260/280).
4. Set up the second PCR reaction (Tables C6 & C7) for all samples using the Barker Hall sequencing plates. Continue to leave well A1 blank for Barker Hall, B1 for a negative control without DNA and C1 for a negative control with DNA but without index primers.

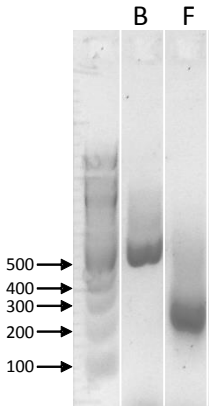


Figure C3. Expected product sizes for Fungal ITS1 (F) and Bacterial 16s (B) PCR products using index primers from Table C2.

Table C6. Second PCR reaction volumes

Reagent	Per reaction
Accumprime Pfx Supermix	16.0
Fwd Primer (10 µM)	0.5
Rev Primer (10 µM)	0.5
dsH_2O	1.0
DNA template (5ng/µL)	2.0
TOTAL	20.0

Table C7. Second PCR reaction conditions

Amplicon	Reaction conditions
Index primers	95°C for 2 min; 8 cycles of 95°C for 20 s, 52°C for 30 s, 72°C for 50s; then 72°C for 10 min

5. Run a gel on all samples in column 1 for each plate. Analyse gel to confirm desired amplicon sizes (add 120 bp to each product from 1st round), and to see if controls worked.
6. Submit PCR products to Barker Hall for "PCR product size selection >200 bp". Quantify and normalise to 10 ng/uL.
7. Create a pool from each plate using 5 µL from each well. Freeze the remaining sample for later use

Bioanalyzer, qPCR, create final library and sequence

1. Submit to Life Sciences Addition Room 255 (Justin Choi jygchoi@berkeley.edu) for Bioanalyzer and Qubit. Verify by qPCR with the P5/P7 primers.
2. Check the results to make sure the right-sized product has been amplified (Fig C4)
3. They will combine your samples equivalently to form your final library and then can submit to QB3 for sequencing on your behalf once you've completed the sequencing forms.

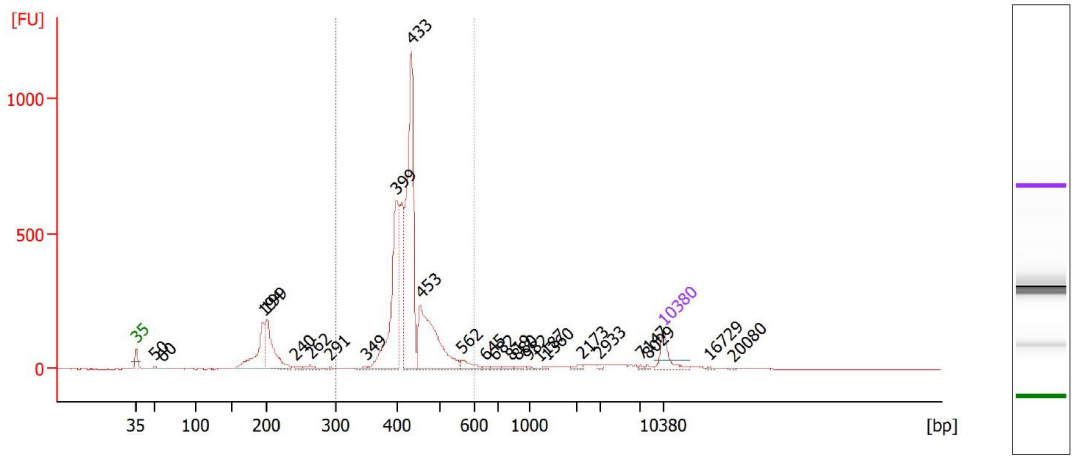


Figure C4. Example of results from Bioanalyzer showing peak sizes between 450 and 500 bp (as expected). Purple and Green peaks are standards.

Congratulations, you're done! Now the analysis begins!

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