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Edaphic and geographic factors underlying the heterogeneous regeneration patterns that woody legumes display following bushfire in Australia

by
Valerie Susanne Densmore

A DISSERTATION

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy
Faculty of Agriculture and Environment
The University of Sydney,

13 April, 2015
DEDICATION

To my darling Nana, a gracious Southern lady whose love, laughter, and wisdom imparted a loveliness beyond all measure.

Mary Louise Smith 1918—2014
# TABLE OF CONTENTS

Dedication ................................................................................................................................. ii

Table of Contents ..................................................................................................................... iii

List of Figures and Tables .......................................................................................................... vi

Acknowledgments ..................................................................................................................... x

Declaration .................................................................................................................................. xii

Abstract ..................................................................................................................................... xiii

Chapter 1 – Adapted to fire and famine? Factors that affect the success of woody legumes in
Australia ...................................................................................................................................... 1

1.1 How fire shapes plant communities .................................................................................... 1

1.2 Woody legumes .................................................................................................................... 9

1.3 Legume seed ....................................................................................................................... 15

1.4 Thesis aim and approach .................................................................................................... 20

Chapter 2 – Geographic and edaphic factors influencing population densities of woody
legumes regenerating following bushfire in Australia.............................................................. 22

Abstract ..................................................................................................................................... 22

2.1 Introduction ....................................................................................................................... 23

2.2 Methods ............................................................................................................................ 27

2.3 Results ................................................................................................................................ 43

2.4 Discussion .......................................................................................................................... 51
Chapter 3 – Woody legume populations across Australia – do optimum temperatures for breaking seed dormancy vary among climate zones?.................................................................58

Abstract........................................................................................................................................58

3.1. Introduction ............................................................................................................................58

3.2. Methods ..................................................................................................................................63

3.3 Results ......................................................................................................................................73

3.4 Discussion ................................................................................................................................80

Chapter 4 – Expression of genes encoding acid phosphatases or carboxylate precursors - methodology ..........................................................................................................................89

Abstract........................................................................................................................................89

4.1 Introduction .............................................................................................................................89

4.2. Methods ..................................................................................................................................92

4.3 Results .....................................................................................................................................105

4.4 Discussion ................................................................................................................................126

Chapter 5 – Expression of genes encoding acid phosphatases or carboxylate precursors – induction by phosphorus deficiency ........................................................................................................131

Abstract........................................................................................................................................131

5.1. Introduction ...........................................................................................................................131

5.2 Methods ..................................................................................................................................134

5.3 Results ....................................................................................................................................143
5.4 Discussion .......................................................................................................................... 159

Chapter 6 – Phos-fire-us: contributing factors and implications of the success of woody legumes to regenerate after fire in SE Australia ........................................................................ 165

6.1 Distribution patterns of Australian woody legumes ................................................................ 165

6.2 Interactions between selected factors and woody legume traits ........................................... 167

6.3 Ecosystem services and implications for future fires ................................................................. 171

6.4 Future directions ................................................................................................................... 175

References ................................................................................................................................... 177
LIST OF FIGURES AND TABLES

Figure 2.1 The location of selected field sites.................................................................30
Table 2.1 The coordinates, climate and elevation of field sites........................................31
Table 2.2 The potential predictors of population density.................................................36
Table 2.3 The range of stem densities used to define categories of population density....40
Figure 2.2 Box-plots of the densities of individuals at each site........................................44
Figure 2.3 Ellipsoidal distances between waypoints..........................................................45
Figure 2.4 Scatter plots between slope and aspect of each site relative to the fire direction...47
Table 2.4 Two potential models produced by ordinal logistic regression.........................48
Table 2.5 Discriminant analysis results for Models 1 and 2..............................................49
Figure 2.5 Modelled functions of population density against each potential predictor......50
Table 2.6 Ranked distribution of each predictor across population densities.....................51
Figure 3.1 Climate classification map of Australia according to temperature and humidity..60
Table 3.1 Species of woody legumes used to compare threshold temperatures to break seed dormancy...........................................................................................................67
Figure 3.2 The two methods used to exposure seeds to heat.............................................70
Figure 3.3 Cues used to determine if seeds had germinated or were viable..........................71
Table 3.2 Average proportion of germination of seeds of Australian woody legumes used in the meta-analysis...........................................................................................................74
Figure 3.4 Grouped results from the meta-analysis..............................................................75
Figure 3.5 Effect of temperature to permit successful germination of woody legume seeds...77

Figure 3.6 Germination success following heat treatment compared across climate zones......78

Figure 3.7 The effect of temperature on germination of seeds from *Acacia* species..............79

Figure 3.8 The effect of increasing temperature to kill seeds of woody legumes....................80

Table 4.1 The four sets of primers designed for PCR.................................................................95

Figure 4.1 Fine roots excavated from eight different species of woody legumes....................97

Table 4.2 Locations of the species of woody legumes used to obtain fine roots...................98

Figure 4.2 Homology of the region used to design degenerate primers to amplify genes encoding secreted acid phosphatase (sAP)...................................................................................107

Figure 4.3 Homology of the region used to design degenerate primers to amplify genes encoding purple acid phosphatase (PAP).............................................................................................108

Figure 4.4 Homology of the region used to design degenerate primers to amplify genes encoding malate dehydrogenase (MDH)................................................................................................109

Figure 4.5 Homology of the region used to design degenerate primers to amplify genes encoding citrate synthase (CS)...............................................................................................................110

Figure 4.6 Gel electrophoresis to test the quantity and quality of total RNA extracted from plant roots..............................................................................................................................................112

Table 4.3 Comparison of key differences between methods to extract total RNA from woody plant tissues....................................................................................................................................................114

Table 4.4 Absorbance values and ratios for total RNA samples extracted from plant roots...115
Figure 4.7 Gel electrophoresis to test the quantity and quality of total RNA extracted from plant roots using different protocols...................................................................................................116

Figure 4.8 Gel electrophoresis to determine the optimum annealing temperature for the four sets of PCR primers..............................................................................................................................118

Figure 4.9 Optimised PCR protocols for five sets of PCR primers.........................................................119

Table 4.5 Quantity and quality of total RNA extracted from roots of six species of woody legumes....................................................................................................................................................121

Figure 4.10 Gel electrophoresis to determine if roots of woody legumes express genes for acid phosphatases...........................................................................................................................................122

Figure 4.11 Homology of the PCR product obtained using PAP primers on cDNA from Acacia dealbata..........................................................................................................................................123

Figure 4.12 Homology of the amino acid sequence from Acacia dealbata.................................................124

Table 4.6 Sequence homologies between Acacia dealbata and five herbaceous legumes.............125

Table 5.1 Nutrient treatments and growth conditions applied to investigate the molecular response to P availability.......................................................................................................................137

Figure 5.1 Concentrations of Bray 1-P from sand following 11 weeks of nutrient treatment..................................................................................................................................................................143

Figure 5.2 Foliar P concentrations and the growth response for woody legumes supplied with different amounts and forms of P........................................................................................................145

Figure 5.3 Excavated roots from two species of woody legumes supplied with different amounts and forms of P.......................................................................................................................146
Figure 5.4 Total RNA extracted from roots of *Goodia lotifolia* and *Acacia obliquinervia*..........147

Figure 5.5 PCR products using cDNA libraries constructed using root extracts from *Goodia lotifolia*.......................................................................................................................................................149

Figure 5.6 PCR products using cDNA libraries constructed using root extracts from *Acacia obliquinervia*...............................................................................................................................................150

Figure 5.7 Homology of the PCR product obtained using PAP primers on cDNA from cultured *Goodia lotifolia*.........................................................................................................................................................153

Figure 5.8 Homology of the putative PAP amino acid sequence from *Goodia lotifolia*........154

Figure 5.9 Homology of the PCR product obtained using sAP primers on cDNA from cultured *Goodia lotifolia*.........................................................................................................................................................155

Figure 5.10 Homology of the putative sAP amino acid sequence from *Goodia lotifolia*........156

Figure 5.11 Comparison of nucleotide and amino acid sequences between putative PAP and sAP from *Goodia lotifolia*.........................................................................................................................................................157

Table 5.2 Sequence homologies compared between *Goodia lotifolia* and herbaceous legumes for putative PAP and sAP nucleotide and amino acid sequences..........................................................158

Appendix 1 Publications used to conduct the meta-analysis.................................................................224

Appendix 2 The germination data for each species examined.............................................................226

Appendix 3 The concentration and purity of total RNA extracted from roots of *Goodia lotifolia* and *Acacia obliquinervia*..........................................................................................................................235
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DECLARATION

This is to certify that:

(i) the thesis comprises only my original work;

(ii) due acknowledgment has been made in the text to all other material used;

(iii) the thesis is less than 80,000 words in length;

(iv) the material presented herein has not been submitted to any other institution in order to obtain a degree.

Valerie Densmore ...................................................................................................

13 April, 2015
ABSTRACT

In Australian temperate zones, woody legumes often display rapid and widespread germination following moderate- to high-intensity bushfires. Resulting population densities are typically highly variable over small distances, producing patchy distributions that are currently unpredictable. Population densities likely reflect environmental variables that influence recruitment in habitats that confer a competitive advantage for woody legumes. To elucidate these variables, my research investigated factors related to soil, landscape, and climate that predict densities of woody legumes after bushfire. Underlying plant traits that potentially facilitate recruitment and competitiveness within the model were then studied.

In February 2009, bushfire burned over 300,000 hectares of forest in central Victoria, Australia. Six species of woody legumes regenerating after this fire were identified growing at two to three stand densities that recurred in patchy distribution patterns across the burned area. Chemical and physical properties of the soil at each site were measured, and topographical and climate variables were analysed using Geographic Information Systems. Of fifty potential factors measured, ordinal logistic regression and discriminant analysis indicated the most accurate model contained five factors. Slope, solar incidence, and soil pH and electrical conductivity comprised four factors which together suggested fire severity influenced densities of recruited populations. Phosphorus (P) availability was also significant. Therefore, subsequent experiments investigated optimum temperatures required for seed germination and the ability of woody legumes to acquire P.

Woody legumes produce hard seeds that require heat to break their physical dormancy before germination. A meta-analysis was undertaken to test the hypothesis that woody legume species from tropical, arid and temperate climate zones have different optimum temperature
thresholds to break seed dormancy. The analysis revealed significantly different optimum temperatures of arid versus temperate species. To test the hypothesis empirically, a germination study involving multiple species from three climate zones was done. The data confirmed that species from arid climates could germinate following exposure to temperatures below 60 °C, while species from temperate or tropical regions required exposure to temperatures above 60 °C to germinate. Furthermore, the response patterns to different temperatures corresponded to conditions that occur within the respective climate zones. In particular, species in temperate climates responded preferentially to high heat exposure. This suggested a mechanism for fire severity to influence population densities through seed germination.

Australian soil is typically acidic and highly weathered, and the majority of P is occluded by organic matter, clay minerals, and metallic cations. To acquire P, plants may exude phosphatases to hydrolyse organic phosphate esters or carboxylates to chelate minerals to which P is adsorbed. Prior to this study, it was unknown whether Australian woody legumes expressed genes for either phosphatases or carboxylate precursors, and the sequences of these genes in woody legumes were unknown. In addition, woody legume roots contain high concentrations of phenolic and polysaccharide compounds that prevented the isolation of RNA using modern, conventional methods. Thus, a method was identified and tested to isolate purified RNA, and PCR primers were designed based on comparative homology between related genes in herbaceous legumes. Of the six species of woody legumes investigated, one species was found to express a sequence homologous to secreted phosphatases known to be sensitive to P availability.

To determine if limited P availability would induce genes for phosphatases or carboxylate precursors, different amounts and forms of P were supplied to woody legumes grown in a
glasshouse. One species expressed two putative phosphatases, although the expression appeared to be constitutive rather than induced by P deficiency. This suggested that woody legumes have the capacity to express phosphatases that might be exuded into the rhizosphere to facilitate acquisition of P.

The research presented in this thesis demonstrates that woody legumes exhibit traits that interact with fire severity and P availability to facilitate establishing high-density populations. It describes the novel discovery of putative phosphatase genes expressed by woody legume roots. The importance of the taxa investigated to aid regeneration of forested ecosystems impacted by bushfire and how this information may assist fire and land management decisions is discussed.
Chapter 1 – Adapted to fire and famine? Factors that affect the success of woody legumes in Australia

Australia is a fire-prone continent that typically has nutrient-poor soils (Webb 1969; Murphy et al. 2013). Consequently, many Australian plants exhibit characteristics that help them adapt to both recurrent fire and scarce nutrients (Beadle 1966; Keeley et al. 2011). Succession or assemblages of flora may facilitate the recovery of plant communities exposed to repeated fire. Australian woody legumes have a range of fire-responsive traits that may function to mitigate some consequences of fire (e.g., loss of soil by erosion, reduction in vegetation cover, altered availability of soil nutrients) and aid the rapid recovery of plant community functioning. Understanding the factors that trigger or enable widespread recruitment of woody legumes will help elucidate the ecosystem processes that enable resilience following a disturbance and is the central aim of this thesis.

1.1 How fire shapes plant communities

1.1.1 Fire properties

Fire is an integral part of most Australian ecosystems, but patterns of recurring fire, called the fire regime, differ among ecosystems (Murphy et al. 2013). Consequently, plant communities are adapted to a particular fire regime, which includes the frequency of return, season of burning, extent, and intensity (Gill 1981; Whelan 1995). The primary consequence of these components that jointly define a fire regime determines the quantity and flammability of available fuel (Bradstock 2010), which affects the production of heat. From the perspective of altering plant communities, heat exposure removes senescent vegetation, can change the structure of different strata, alter cellular metabolism that can lead to plant mortality (Whelan 1995), may aid in seed germination (Hamly 1932; Hanna 1984) and can release bound nutrients
Thus, fire can rejuvenate plant communities provided the amount and timing of heat exposure occurs in a characteristic cycle.

Sufficient heat will induce chemical decomposition of fuel and the volatilisation of flammable gases, called pyrolysis (Whelan 1995; Scarff & Westoby 2006), and allow a fire to become self-perpetuating (Vines 1981). Active fires are often described by their intensity \( I \) (kW m\(^{-1}\)) as the product of heat production \( H \) (J kg\(^{-1}\)), fuel consumption \( \omega \) (kg m\(^{-2}\)) and the rate of spread \( R \) (m s\(^{-1}\)) at the firefront (Byram 1959). Categories of fire intensity indicate the behaviour of a fire and fuel combustion at the firefront (Christensen et al. 1981). However, this metric does not consider how long a fire resides in an area or cumulative heat exposure, which affects both how much biomass burns and damage to unconsumed biomass and is denoted as fire severity (Keeley 2009).

The quantity of available fuel and its chemical composition are key factors determining fire behaviour and severity. Climate and soil nutrient status significantly affect the species composition and productivity of a community (Cheney 1981; Christensen et al. 1981; Bond & Keeley 2005; Sankaran et al. 2008; Bradstock 2010; Duff et al. 2013). Limited concentrations of phosphorus (P) and nitrogen (N) in soil can attenuate optimal growth and net primary productivity of ecosystems (Beadle 1966; Raghothama 1999; Vitousek et al. 2002; Vance et al. 2003; Sankaran et al. 2008; Lambers et al. 2010; Power et al. 2010). Biomass accumulation also balances plant productivity against the rate of decomposition, which is slower in xeric and nutrient-poor communities (Boerner 1982; O’Connell & Menage 1983; Hendriksen 1990; Northup et al. 1998; Hättenschwiler & Vitousek 2000; Orians & Milewski 2007; Cattanio et al. 2008; Hoorens et al. 2010). In addition, plants will undergo secondary metabolism and increase the synthesis of flammable phenolic compounds when subjected to environmental stressors.
common to Australian communities, including excess light, low temperatures, drought and P
limitation (Specht 1981; Vines 1981; Dickinson & Kirkpatrick 1985; Dixon & Paiva 1995;
Northup et al. 1998; Grace & Logan 2000; Hättenschwiler & Vitousek 2000; Close &
McArthur 2002; Tharayil et al. 2011).

Dry fuels can be classified as fine or coarse, depending on whether the diameter of dead
organic matter is less or greater than 6 mm, respectively. Most fires will readily consume dry,
fine fuels, which can include litterfall, cured grass, leaves, twigs and bark. Coarse fuels, like
branches, may burn when exposed to sufficient heat (Walker 1981; Wilgen & Richardson 1985;
Gould et al. 2008). Several factors affect the flammability of fuels, but moisture levels are
critical, because energy is required to evaporate water from fuel, and the fuel-derived water
vapour and relative humidity both affect the radiation efficiency of flames (Vines 1981;
Dickinson & Kirkpatrick 1985; Whelan 1995; Catchpole 2002; Gould et al. 2008).

Fuels are typically dispersed heterogeneously across a landscape, following patterns of
vegetation and topography (Whelan 1995; Catchpole 2002). Topography influences moisture
and light availability, which affect the type of vegetation and how much fuel is
produced (Attiwill et al. 1996; O’Brien et al. 2000; Bradstock 2010). Topography thus indirectly
and directly helps create heterogeneous patterns of fire severity. Aspects that receive more solar
radiation will increase ambient fuel temperatures and decrease fuel moisture. Depressions may
allow water to pool and increase fuel moisture. Steeper slopes increase the angle of flames,
bringing them closer to adjacent fuel and augmenting their radiation efficiency (Cheney 1981;
Vines 1981). Topography can also affect the speed and direction of wind that provides
ventilation and may redistribute heat to facilitate or hinder ignition (Cheney 1981; Catchpole
2002; Gould et al. 2008). Although severe fire conditions can ameliorate some direct effects of
topography, fuel type still influences fire intensity and behaviour (Cruz et al. 2012). Altogether, heterogeneous landscape features and plant compositions produce a mosaic of fire severities that influence the recovery of communities following fire (Christensen et al. 1981; Whelan 1995).

Most energy released during a fire is lost by convection through the air column or radiation, and soil absorbs only around 5% of the total heat output (Raison 1979; Gill 1981; Humphreys & Craig 1981; Whelan 1995). However, soil may retain residual heat for several hours after fire (Romanyà et al. 1994). The insulating capacity of a soil contributes significantly to heat penetration and retention, thus bulk density, moisture content, heat conductivity, and the initial temperature are all influential (Raison 1979; Gill 1981; Humphreys & Craig 1981; Vines 1981; Whelan 1995). Soil moisture can facilitate heat conduction up to 100 °C, when latent heat is used for evaporation. Thus, variable distribution of water in the soil profile prevents uniform heating of the solum, promoting the formation of heterogeneous microsites (Beadle 1940; Raison 1979; Whelan 1995; Choromanska & DeLuca 2002).

Fire oxidises organic matter to produce alkaline, cation-rich ash. As discussed in Chapter 2, the severity of fire determines physical and chemical properties of ash that affect several soil properties, including bulk density, moisture content, pH and electrical conductivity (Tomkins et al. 1991; Escuey et al. 2010; Hinojosa et al. 2012; Pereira et al. 2012; Yusiharni & Gilkes 2012a; Bodi et al. 2014). In addition, heat from fire can directly affect physical, chemical, and biotic features of soil, including soil particle size, availability of some essential nutrients, enzyme activities, and the composition of soil microbial and fungal communities (Raison 1979; Capogna et al. 2009; Yusiharni & Gilkes 2012c; b).
Fire also alters availability of N and P, as described in more detail in Chapters 2 and 4, respectively. The volatilisation temperatures of most essential macronutrients except N are above 500 °C (Raison et al. 1985), thus ash deposited on the soil surface is typically nutrient-rich and potentially at risk of erosion when the ground is exposed after fire (Raison et al. 1985; Bolan 1991; Islam et al. 2000; Escudey et al. 2010). Thus, rapid recolonisation by plants is important to cover bare soil and modulate soil properties to help retain ash and newly-available nutrients (Adams & Attiwill 1984; Guinto et al. 1999; Hirobe et al. 2003; Certini 2005).

1.1.2 Fire regime and plant communities

Fire alters the distribution of biomass, nutrients, and resources creating a heterogeneous landscape that influences how the resulting community develops (Raison 1979; Ashton 1981; Christensen et al. 1981; Whelan 1995; Rodríguez et al. 2009; Siegwart Collier & Mallik 2010). Fires may have a greater impact when subsequent climate conditions are unfavourable for plant regeneration (Christensen et al. 1981; Walker 1981; Nieuwenhuis 1987; Whelan 1995; Bellingham & Sparrow 2000; Williams et al. 2004). Plants that regenerate rapidly help stabilise soil, modify light and nutrient availability, provide new growth for herbivores and possibly attract pollinators (Whelan 1995; Certini 2005; Dong et al. 2014). Thus, the distribution of colonisers helps create a mosaic of biotic and abiotic conditions that may facilitate or inhibit regeneration of subsequent flora (Christensen et al. 1981; Noble & Slatyer 1981).

The Australian flora found in fire-prone areas have anatomical, physiological and life-history traits that are acclimatised to a particular fire regime (Gill 1981; Noble & Slatyer 1981; Morrison et al. 1995; Keeley et al. 2011). To tolerate fire, a plant may use height, thick bark or the insulation properties of soil to shield critical tissues from lethal temperatures (Vines 1981; Whelan 1995; Bellingham & Sparrow 2000), build protected bud and energy reserves that allow
them to resprout (Ashton 1981; Whelan 1995; Holmes & Cowling 1997), or produce
propagules that use fire to trigger germination (Gill 1981; Whelan 1995; Hodgkinson 2002). The
methods used for recovery of plant populations after fire are broadly categorised as resprouting
or seeding, and their success depend on the characteristics and historical context of a fire and
the developmental stage of a plant (Gill 1981; Noble & Slatyer 1981).

Sprouting following injury is not specifically an adaptation to fire, and many more species
will resprout following a low-intensity fire compared to medium-intensity fire (Christensen et al.
1981; Whelan 1995; Bradshaw et al. 2011; Keeley et al. 2011). Resprouting enables plants to
continue occupying a site where fires are frequent enough that subsequent generations may be
killed before they reach fecundity (Noble & Slatyer 1981; Nieuwenhuis 1987; Bellingham &
Sparrow 2000). ‘Sprouters’ allocate resources to store necessary reserves and form a protective
layer over dormant buds, reducing resources for rapid stem growth and seed production and
potentially limiting genetic diversity in the local population (Whelan 1995; Bellingham &
Sparrow 2000; Verdú 2000). If fire or other disturbances occur too frequently, reserves can be
depleted enough to cause mortality (Whelan et al. 2002), but resprouting otherwise represents a
rapid method to restore aboveground biomass after fire (Bellingham & Sparrow 2000).

‘Seeders’ may be obligate or facultative depending respectively on whether they lack
adventitious buds and protective tissues, or their capacity to resprout is limited by their height
or ability to set seed (Whelan 1995; Hodgkinson 2002). Plants that regenerate from seed after
fire often allocate more resources to rapidly accumulate above-ground biomass and to produce
many seeds (Gill 1981; Whelan 1995; Keeley & Fotheringham 2000; Verdú 2000; Saura-Mas et
al. 2010), but short fire intervals may deplete the seed bank (Whelan et al. 2002; Auld &
Denham 2006). Seeders that grow quickly can intercept more light if they are not competing
with neighbouring resprouters (Nieuwenhuis 1987). Plant communities with a fire regime of less frequent, more severe fires typically contain a greater proportion of seeders (Nieuwenhuis 1987; Bellingham & Sparrow 2000; Keeley & Fotheringham 2000; Scarff & Westoby 2006; Siegwart Collier & Mallik 2010).

To ensure that seeds survive intense fires, seeders may retain seeds in protective woody fruits in the canopy, called bradyspory, and release the seeds only when the branch dies or a fire cue stimulates a synchronised release (Gill 1981; Whelan 1995). Alternatively, seeders may store seeds in the soil. For example, woody legumes regularly release seeds that develop physical dormancy when their seedcoat dries and hardens, providing some protection against fire and predation (Gill 1981; Whelan 1995; Verdú 2000; Keeley et al. 2011). Either method of seed storage potentially supplies a large seed-bank that produces a flush of germination following fire, when newly-available nutrients, moisture and light facilitate rapid growth (Specht 1981; Nieuwenhuis 1987; Whelan 1995; Verdú 2000; Saura-Mas et al. 2010).

1.1.3 Fire and woody legumes

Studies investigating how fire affects a particular plant community often comment that woody legume species regenerate profusely following unplanned fire, which can help restore N volatilised during the fire if the species are N-fixers (Harshbarger et al. 1975; Shea et al. 1979; Adams & Attiwill 1984; Boring et al. 1988; Islam et al. 2000; Newland & DeLuca 2000; da Silva & Batalha 2008). Many woody legumes can grow rapidly and produce seed within 2–5 years following moderate or high intensity fires (Monk et al. 1981; Hansen et al. 1987). Large stands of woody legumes have been shown to decrease soil erosion and bulk density and increase the organic matter, carbon, and N content of degraded soil (Dong et al. 2014). Thus woody legumes have the capacity to help restore communities affected by severe fire. However, fire
does not stimulate homogeneous distributions of legumes to regenerate, even within the same plant community (Guinto et al. 2000; Hodgkinson 2002; Hirobe et al. 2003; Warton & Wardle 2003). Soil characteristics including temperatures reached during fire, moisture content, texture and nutrient content along with seed availability have been separately hypothesized to underlie the heterogeneous distribution of legumes after fire (Clark 1988; Auld & Bradstock 1996; Crews 1999; Islam et al. 2000; Warton & Wardle 2003; Houlton et al. 2008). Considering such variable causes, a comprehensive study is needed to examine which biotic and abiotic factors influence legume regeneration following severe fire.

Despite uncertainty concerning the mechanisms underlying the patchy distribution of woody legumes, most studies indicate a moderate-intensity fire is a minimal requirement to induce widespread germination and recruitment of woody legumes. Therefore, low-intensity fires that produce a thicket of woody legumes may indicate the occurrence of local hot spots within the fire (Christensen et al. 1981). A requirement for greater heat exposure to germinate may explain why annual fires in subtropical Australia recruited fewer acacias than burning every 2–4 years when more fuel had accumulated (Guinto et al. 2000). Australian *Acacia* species were also found interspersed with other plant communities or were recruited at higher densities on rocky soils that may have been drier and produced more heat during the fire (Hodgkinson 2002; Warton & Wardle 2003). However, acacias recruited in deep moist soil grew more vigorously and had greater reproductive fecundity (Warton & Wardle 2003). Concentrations of mineral-N and extractable-P showed a heterogeneous pattern in soil following fire, particularly in plots containing leguminous shrubs (Rodríguez et al. 2009). Thus, temperatures produced during fire may not be the only causal factor underlying the patchy distribution of woody legumes.
It is evident that several biotic and abiotic features of the environment modulate the distribution of woody legumes after fire or other disturbances. The research presented in this thesis initially classifies patterns of legume distribution after bushfire and identifies key extrinsic factors in soil or the landscape that shape these patterns. The research then investigates intrinsic controls related to the identified factors affecting woody legume distribution. Therefore, the remainder of this chapter expounds on the current understanding of two important control processes – nutrient acquisition and seed germination.

1.2 Woody legumes

1.2.1 The Fabaceae

Legumes belong to the family Fabaceae or Leguminosae (former name used herein), which is the third-largest family of angiosperms, containing approximately 650 genera and 18,000 species worldwide (Crews 1999; Sprent 2007). Fabaceae species also represent a significant component of the Australian flora and inhabit almost every terrestrial ecosystem on the continent (Robinson 2003; CHAH 2013). Legumes are characterised by their fruit, comprising seeds that mature within a pod that splits down two sides when ripe (Wohlmuth 2001). The life form of legumes range from small annual herbs, vines, and woody shrubs to understorey and canopy trees (Rundel 1989). Although all legume flowers have five petals, their arrangement can be classified into distinct categories, which have been used to divide the Fabaceae into three subfamilies: the Caesalpinioideae, Papilionoideae and Mimosoideae (Crews 1999; Vitousek et al. 2002; Sprent 2007). Recent molecular analyses suggest the Caesalpinioideae subfamily evolved just prior to the Papilionoideae, while the Mimosoideae subfamily branched from the Caesalpinioideae and was the last to evolve (Lavin et al. 2005; Sprent 2007).
The 18th International Botanical Congress found the term FABOIDEAE was a suitable alternative to PAPILIONOIDEAE. In addition, the term ‘LEGUMINOSAE’ was still found an acceptable substitute for ‘FABACEAE’ (2011), but the latter term is more common and will be used throughout this thesis. To avoid confusion between FABACEAE and FABOIDEAE, PAPILIONOIDEAE will also be used herein.

Australia has around 19 genera and 85 species of CAESALPINIOIDEAE legumes (Robinson 2003). The CAESALPINIOIDEAE subfamily diversifies primarily in pantropical regions, where most species are woody (Lavin et al. 2005), but some herbaceous species occur in temperate zones (Crews 1999; Sprent 2007). Australian Cassia/Senna species often have an arid distribution and dominate shrub communities on sandy plains and rocky hillsides (Randell 1970).

Australian PAPILIONOIDEAE (or FABOIDEAE) comprise over 1100 species in 136 genera, including the endemic Bossiaceae and Mirbelieae (Robinson 2003). Annual or perennial herbs from the PAPILIONOIDEAE occur worldwide and are probably the most widely known legumes (Crews 1999). Tree and shrub species of the PAPILIONOIDEAE grow primarily on nutrient-poor soils and in semi-arid environments, particularly in tropical, subtropical and Australian temperate zones (Rundel 1989; Sprent 1995; Crews 1999; Robinson 2003) and in the wet tropics (Crews 1999; Vitousek et al. 2002; Sprent 2007).

The MIMOSOIDEAE contribute 17 genera containing at least 960 species, most of which are in the genus Acacia, the most speciose genus in Australia (Robinson 2003; Brockwell et al. 2005; Orians & Milewski 2007). Thus, most species of MIMOSOIDEAE are woody legumes (Rundel 1989; Robinson 2003). Acacia species are distributed across many habitats, including dry and wet sclerophyll, coastal scrub, savanna, rainforest, and arid regions (Hodgkinson 2002; Miller et al.
Acacias frequently grow in dry, nutrient-poor soils, where they establish a deep tap root before producing much other biomass (Sprent 2007), allowing them to reach scarce moisture and dominate arid zones (Lavin et al. 2005). The Mimosoideae subfamily also occurs in tropical and temperate Australia, particularly following unplanned fire (Shea et al. 1979; Adams & Attiwill 1984; Guinto et al. 2000; Islam et al. 2000; Hodgkinson 2002; Warton & Wardle 2003).

1.2.2 Acquisition of nitrogen and phosphorus

Many Fabaceae species can form symbioses with rhizobial bacteria that increase their access to N, but legume distributions worldwide do not correspond to concentrations of available N (Crews 1999; Houlton et al. 2008). Temperate ecosystems that have N-poor soils typically have few to no climax legume species, whereas many tropical rainforests with N-rich soil have leguminous trees dominating the canopy (Crews 1999; Houlton et al. 2008). However, the distributions of woody legumes in the Northern Hemisphere (Rundel 1989; Houlton et al. 2008) and Australia (Hodgkinson 2002; Adams et al. 2010) superficially correspond to regions with P-limited soils.

Although dinitrogen (N\textsubscript{2}) comprises around 78% of the atmosphere, plants cannot use N\textsubscript{2} until it is reduced or ‘fixed’ into NH\textsubscript{4}\textsuperscript{+} or further oxidized to NO\textsubscript{3}\textsuperscript{-} (Ross 1992; Marschner 1995; Dakora & Phillips 2002). Legumes are the largest family of plants to host N-fixing symbiotic bacteria inside nodules located predominately on shallow fibrous roots (Ross 1992; Brockwell et al. 2005). The legumes provide carbohydrates and conditions optimised for the key enzyme, nitrogenase, to fix N\textsubscript{2} efficiently. In turn, the bacteroids transfer over 90% of fixed NH\textsubscript{3} to the host. This symbiosis, called biological nitrogen fixation (BNF) has the highest capability of any natural process to amend soil stores with inorganic N, and may add up to 50 kg N ha\textsuperscript{-1} yr\textsuperscript{-1}. 
However, to fix one mole of N requires approximately 960 kJ of energy and a large quantity of P (Ross 1992; Marschner 1995; Turner et al. 2008). Therefore, legume species typically exhibit BNF only when sufficient N is not available from the soil, such as when N is volatilised during fire (Vitousek et al. 2002; Menge et al. 2008).

Biological nitrogen fixation is not universal across the FABACEAE subfamilies. Over 90% of the PAPILIONOIDEAE and MIMOSOIDEAE species examined were found to nodulate, but less than 25% of CAESALPINIOIDEAE examined produced nodules (Crews 1999; Sprent 2007; Adams et al. 2010). Nitrogenase activity is most efficient in the temperature range of 20–25 ºC, and low soil temperatures require a greater amount of enzyme and energy to fix N at rates akin to BNF in warm soil (Lawrie 1981; Brockwell et al. 2005; Houlton et al. 2008; Adams et al. 2010). The energetic demands of BNF are met using ATP, representing a large demand for P, particularly in temperate climates.

Phosphorus is among the least available essential nutrients in the highly weathered soils that typify Australia (Beadle 1962; Turner & Lambert 1986; Adams et al. 1989; Hodgkinson & Oxley 1990; Stewart et al. 1990; Adams 1992; Lambers et al. 2010). As described further in Chapter 4, legumes may modify their root structures into cluster roots or form symbioses with mycorrhizal fungi to acquire limited P (Bolan 1991; Pate 1994; Adams et al. 2002; Vance et al. 2003; Lambers et al. 2010; Power et al. 2010). Using cluster roots rather than mycorrhizae may be species-specific (Warcup 1980; Weisskopf et al. 2006a; Lambers et al. 2013) or be used when P limitation is particularly severe (Vance et al. 2003; Lambers et al. 2010).

To dissociate or mineralise P ions into a form available to plants, cluster roots and mycorrhizae release organic acids, carboxylates and cations, or phosphatases (Bolan 1991; Abel et al. 2002; Lambers et al. 2013). Organic acids provide a ligand exchange that chelates metal
cations to release inorganic or organic sources of P (Raghothama 1999; Abel et al. 2002; Vance et al. 2003; Power et al. 2010). Carboxylates function similarly to organic acids but require cations to be co-released to maintain a balanced charge (Lambers et al. 2006). Phosphatases mineralise organic phosphate esters to release inorganic P that is more readily available to plants (Abel et al. 2002; Vance et al. 2003; Lambers et al. 2006). Phosphatase production rates are significantly higher in soils beneath N-fixing plants (Houlton et al. 2008), which may give woody legumes a competitive advantage when growing in P-limited soils.

Limited P favours production of leaves that have a high mass per unit area and are tough, or sclerophyllous (Beadle 1962; Farrell & Ashton 1978; Crews et al. 1995). Foliar P is also recycled from senescing leaves in considerable proportions, with some species withdrawing 50-80% (Wright & Cannon 2001). Scleromorphic structures require less foliar P and are thought to deter herbivores and reduce mechanical damage, thus increasing their potential lifespan (Lambers et al. 2010; Meers et al. 2010). Seeds are a stronger sink than leaves because P is not recycled back to the parent plant, however, plants may produce fewer seeds to compensate when available P is low (Raghothama 1999; Lambers et al. 2006; Cramer & Midgley 2009). Phosphorus limitation may also induce the phenylpropanoid pathway, or secondary metabolism, which allows plants to produce phenolics like flavonoids and isoflavonoids (Dixon & Paiva 1995; Grace & Logan 2000; Vance et al. 2003). Altered carbohydrate metabolism produced higher phenolic concentrations in Phaseolus vulgaris (Juszczuk et al. 2004) and increased tannin levels in Acacia anistrocarpa and Senna notabilis (Islam et al. 2000). However, legumes produce isoflavonoids even without exposure to apparent stress factors, and the PAPILIONOIDEAE subfamily in particular accumulates considerable amounts in healthy seeds, roots and shoots (Dakora & Phillips 1996).
1.2.3 Distributions of woody legumes

The Fabaceae is considered an ecologically successful family, having achieved a worldwide distribution that includes clear dominance in some regions including rainforest canopies in tropical lowlands and the overstorey of many arid ecosystems (Crews 1999). Legumes either evolved in the humid tropics (Lavin et al. 2005) or adjacent semi-arid habitats and quickly colonised the mesic lowlands (Sprent 2007). Woody legumes exhibit several additional attributes that may facilitate adaptation to varied environmental conditions (Farrell & Ashton 1978; Rundel 1989). For example, woody legumes may adapt their root systems to obtain more P (Adams et al. 2002; Houlton et al. 2008), which often becomes scarce in warm wet soils (LeBauer & Treseder 2008). Thus, an advantage that may underlie the considerable amount of biomass that legumes accumulate when growing on flooded or leached soils in lowland tropical ecosystems may be related to P acquisition (Rundel 1989). Similarly, following bushfire, woody legumes have used BNF to potentially support their widespread post-fire establishment and contribute to restoring fire-affected communities (Harshbarger et al. 1975; Shea et al. 1979; Adams & Attiwill 1984; Boring et al. 1988; Islam et al. 2000; Newland & DeLuca 2000; Forrester et al. 2007; da Silva & Batalha 2008).

The distribution of woody legumes may not be solely or directly reliant on nutrient availability. As precipitation decreases, legumes reduce their stature and become more deciduous (Rundel 1989; Adams et al. 2010). Low moisture restricts BNF (Hansen et al. 1987; Sprent 1995; Brockwell et al. 2005) and P acquisition (Islam et al. 2000; Lambers et al. 2010). Thus Acacia species in arid climates exhibit slow growth patterns and longevity (Crisp & Lange 1976; Lange & Purdie 1976) and grow an extensive taproot system before producing much foliage (Sprent 2007). As with non-legumes, woody legumes can adapt their osmotic
potential and perhaps even modify their xylem structure to use available water more efficiently (Clemens & Jones 1978; Rundel 1989). Furthermore, species of *Acacia* from arid environments use modified chlorophyll-containing stems, or phyllodes, that follow a diurnal series of positions to maximise their solar incidence and reduce water lost through stomata (Farrell & Ashton 1978; Rundel 1989). Overall, these adaptations have allowed acacias to comprise 13% of the flora in arid Australia (Rundel 1989).

Temperate climate zones have a smaller proportion of legumes, but the diversity of legume species remains equivalent across latitudes (Rundel 1989). In the Northern Hemisphere, woody legumes flourish following a disturbance in temperate ecosystems but are succeeded when the canopy closes (Crews 1999; Vitousek et al. 2002), possibly due to BNF becoming unfeasible after secondary succession (Houlton et al. 2008; Menge et al. 2008). However, in Australia, woody legumes are prevalent in areas with a Mediterranean-type climate (Brockwell et al. 2005; Sprent 2007; Adams et al. 2010) and temperate ecosystems regardless of the canopy (Adams & Attiwill 1984; Robinson 2003). Woody legumes may be more competitive in temperate zones due to soils that have become depleted in P or, as Australia is an extremely dry landmass, adaptations that serve arid legumes might translate well to temperate regions (Adams et al. 2010).

### 1.3 Legume seed

Most Australian woody legumes produce seeds that are dormant to maintain their viability as they are predominately stored in the soil (Morrison et al. 1992; Bell 1999). Although woody legumes exhibit a flush of germination following bushfires (Purdie 1977b; Shea et al. 1979; Bradstock & Auld 1995; Rawson et al. 2012), fire intervals can be several years to several decades (Murphy et al. 2013).
1.3.1 Achieving dormancy

Legumes are not unique in using seed dormancy to prevent untimely germination, although other plant families may use different methods to safeguard their embryos (Baskin & Baskin 1998d). Seeds of woody legumes are characteristically impermeable to water due to a hard seed coat, or testa, that must be breached to permit germination (Baskin & Baskin 1998d). This physical dormancy is considered orthodox because legume seeds may dry to 0.5% moisture before losing viability, unlike recalcitrant seeds that must retain 30-65% moisture (Baskin & Baskin 1998a).

The testa of Fabaceae seeds contains three strata that facilitate seed survival in unfavourable conditions. The two innermost cell layers have anatomical features that help control the drying and ripening process (Hyde 1954; Scott et al. 1962; Rolston 1978; De Padua Teixeira et al. 2004), cushion the embryo (Hamly 1932), and provide a food source after seeds have germinated (Hamly 1932; Scott et al. 1962; Rolston 1978; Lush & Evans 1980). The malpighian or epidermal cells that construct the outer palisade layer have apical ends that taper to a blunt cone or ‘cap’ comprised of suberin, a waxy substance common to cork (Hamly 1932; Hyde 1954; Scott et al. 1962; Tran & Cavanagh 1980; Morrison et al. 1992). The caps are tightly coherent, and exposure to acids, ammonia, acetone, dyes, and ultrasonic vibration has indicated the malpighian cap is ultimately responsible to maintain impermeability to water (Hamly 1932; Scott et al. 1962; Rolston 1978; Rangaswamy & Nandakumar 1985; Manning & Van Staden 1987).

Although the majority of the testa presents a homogeneous hydrophobic surface, one longitudinal edge is more permeable to water due to the hilum and strophiole. The hilum is a depression where the aril or stalk attaching the seed to its pod was connected (Lush & Evans
In PAPILIONOIDEAE species, the hilum is fissured and opens in low humidity to facilitate dehydration of the seed until it reaches hygroscopic equilibrium (Hyde 1954; Rolston 1978; Lush & Evans 1980; Rangaswamy & Nandakumar 1985). Full water impermeability isn’t achieved until legume seeds contain only 10-15% moisture (Hyde 1954; Lush & Evans 1980; Morrison et al. 1992), and some species undergo the drying process after the pods have released the seeds. The relative humidity during this period can affect whether legume seeds achieve dormancy, particularly for PAPILIONOIDEAE species (Hyde 1954; Van Staden et al. 1989; Morrison et al. 1992). Therefore certain circumstances may allow legumes to germinate without a dormancy-breaking cue (Hyde 1954; Marbach & Mayer 1974; Van Staden et al. 1989; Baskin & Baskin 1998c), and non-dormant seeds are more susceptible to fire (Morrison et al. 1992).

The strophiole or lens lies beside the hilum and permits initial water entry through a cleft when dormancy is broken (Hamly 1932; Hyde 1954; Tran & Cavanagh 1980; Hanna 1984; Morrison et al. 1998). This cleft is important to control the rate and direction of imbibition, as absorption of water through other portions of the testa has been shown to increase the number of malformed seedlings and decrease their survival rate (Manning & Van Staden 1987).

1.3.2 Germination

Legume seeds are a food source for ants, birds, and other fauna, and it has been argued that chemical or mechanical scarification represent a natural mechanism to break dormancy of legume seeds (Baskin & Baskin 2000). Of several acidic, alkaline, and hydrophobic compounds tested, only concentrated sulphuric acid increased the percentage of germination for two Australian species of woody legumes (Hamly 1932; Rangaswamy & Nandakumar 1985; Manning & Van Staden 1987; Reichman et al. 2007), and the testa was damaged enough to
reduce seedling viability (Manning & Van Staden 1987). The digestive enzymes of birds and ruminants have not been successful to increase the percentage of legume seeds that germinate (Ibañez & Passera 1997; Letnic et al. 2000), but a portion of the testa may be cracked, pierced, or otherwise removed while the legume fruit or seed is being eaten. Being deposited on the soil surface in fermenting faecal matter may break dormancy of legume seeds by providing additional heat for the seeds (Baskin & Baskin 1998c).

Mechanical scarification using threshing has commonly been used to allow otherwise dormant seeds from herbaceous legumes to imbibe water. Numerous studies have also used mechanical scarification with sandpaper to provide a positive control or test seeds for viability (Tran & Cavanagh 1980; Auld & O'Connell 1991; Morrison et al. 1998; Letnic et al. 2000; Campbell & Clarke 2006; Van Klinken et al. 2006). Although scarification has been likened to ants dragging the seeds over coarse ground, it makes little evolutionary sense that seeds which developed physical dormancy would have their dormancy broken by events that don't cue an optimal time to germinate (Baskin & Baskin 2000).

Australian woody legumes typically exhibit a flush of germination following a bushfire (Shea et al. 1979; Clark 1988), indicating that one or more fire cues are sufficient triggers. Studies have investigated heat, smoke and nitrates separately and together, and the data repeatedly indicates only heat is necessary or sufficient to break legume seed dormancy (Cushwa et al. 1968; Tieu et al. 2001; Williams et al. 2003; Williams et al. 2005; Campbell & Clarke 2006; Rawson et al. 2012). Heat has been applied using both wet and dry methods, because buried legume seeds may be in moist soil during fire (Beadle 1940; Harshbarger et al. 1975; Shea et al. 1979; Portlock et al. 1990; Williams et al. 2003). Germination of woody legume seeds has also been investigated following exposure to fire (Williams et al. 2004), fluctuating temperatures (Preece 1971), solar
radiation (Van Klinken et al. 2006) and soil inundation (Lange & Purdie 1976). Not all Australian species of woody legumes are found in environments that are fire-prone, so signals indicating adventitious conditions for recruitment are likely to vary between climate zones.

The Australian Bureau of Meteorology has subdivided the Australian continent into three climate zones using temperature and humidity properties averaged over 1961–1990 (Bureau of Meteorology 2005), and native legume species can be found across all these areas. As detailed more extensively in Chapter 3, most studies investigating dormancy of woody legumes have used single species or discrete communities, and have been overwhelmingly focused on temperate climates. The optimum temperatures reported in most studies correspond to cues achieved only by moderate to high-intensity fire (i.e., Auld & Bradstock 1996). However, some data for species from tropical and arid zones have suggested moisture and solar radiation may provide sufficient cues to break seed dormancy (i.e., Lange & Purdie 1976; Van Klinken et al. 2006). Altogether, the existing data are too limited to determine whether lower temperature thresholds may be sufficient to permit germination of species from arid or tropical climates.

1.3.3 Dispersal

Most Australian woody legumes produce diaspores that have an elaiosome or aril attached to the seed (Sweedman & Brand 2006) that facilitates myrmecochory, or dispersal by ants (Berg 1975; Davidson & Morton 1984; Auld 1986a; Hughes & Westoby 1990; Jurado et al. 1991; Hughes & Westoby 1992; Campbell & Clarke 2006; Meers et al. 2010). Some arid species of woody legumes retain diaspores on the parent plant that display large yellow or red elaisomes, which promotes dispersal by birds in addition to ants (Davidson & Morton 1984; Letnic et al. 2000). Legume seeds may also be released ballistically prior to dispersal by ants (Berg 1975; Malo 2004), or be dispersed within their fruits through water (O'Dowd & Gill 1986). The
majority of myrmecochoric seeds are dispersed less than 3 m from their source, depending on the size and territorial behaviour of dispersing ants (Andersen 1988; Hughes & Westoby 1992). Ballistic or avian dispersal can increase the distance to 5 m or more (Letnic et al. 2000; Malo 2004).

Once on the ground, both seed and soil particle size may influence vertical dispersal down the soil profile, (Baskin & Baskin 1998b), but rainfall was sufficient to bury *Acacia suaveolens* seeds in sandstone soil (Auld 1986a). Seeds that reach an ant nest are buried an average of 0—3 cm, and nest-cached seeds may contribute to a clumped recruitment of woody legumes (Shea et al. 1979; Hughes & Westoby 1992). However, genetic analysis of a tropical leguminous shrub indicated numerous individuals were contributing to a widely-shared genepool (Andersson et al. 2007), and that a high population density arises when multiple seed shadows overlap within a 3 m distance. Former high-density populations promote future high-density populations (Harms et al. 2001; Zhang et al. 2013), suggesting factors in addition to seed dispersal help promote aggregated populations of woody legumes to become established (Yamada et al. 2013).

### 1.4 Thesis aim and approach

Woody legumes exhibit several characteristics that may facilitate adaptation to a variety of ecosystems. These traits may enable species from temperate climates to demonstrate rapid establishment and growth and to aid the resilience of communities following moderate to high-severity bushfires. However, woody legumes exhibit heterogeneous distributions of population densities after bushfire, and the factors contributing to these dispersal patterns have not been investigated. The aim of this thesis is to analyse extrinsic factors and associated intrinsic mechanisms that facilitate the establishment of high population densities of woody legumes following bushfire. In Chapter 2, landscape and edaphic factors that shape recurrent
distribution patterns for several species of woody legumes are identified. The optimum temperatures to break seed dormancy without causing significant mortality are investigated for woody legume species from three different climate zones in Chapter 3. Molecular mechanisms in woody legume roots that can facilitate the acquisition of limited P are also studied, beginning with the technical approach to isolating genes of interest in Chapter 4. Chapter 5 extends the molecular study by investigating whether the form and concentration of P available affects expression of select genes in woody legume roots. Although varied, these approaches will help elucidate mechanisms that can facilitate recovery of Australian plant communities following bushfire.
Chapter 2 – Geographic and edaphic factors influencing population densities of woody legumes regenerating following bushfire in Australia

Abstract

In Australian temperate zones, tree and shrub species from the family LEGUMINOSAE often display rapid and widespread germination following moderate- to high-intensity bushfires. Resulting population densities are typically highly variable over small distances producing patchy distributions that are currently unpredictable. Population densities likely reflect environmental variables that influence recruitment in habitats that confer a competitive advantage for woody legumes. A severe bushfire in that burned wet and dry sclerophyll forests in central Victoria, Australia in February 2009 provided an opportunity to investigate which edaphic, landscape or climate factors best predicted population densities of woody legumes after bushfire. In this chapter, recurring density ranges that could be categorised as low, medium, or high population densities were found to recur for six species of woody legumes in a patchy pattern across an area of 120,000 ha. Sites identified while deriving recurrent population densities were analysed for fifty factors that included chemical and physical properties of soil and topographical and climate variables determined using Geographic Information Systems. Ordinal logistic regression and discriminant analysis were used to develop a model that predicted population densities of woody legumes with an accuracy of 49% for all species combined, and 63—94% when species were analysed separately. The model identified five factors including slope, solar incidence, soil pH, electrical conductivity and phosphorus availability at 5—10 cm depth in the soil. This study supported the hypothesis that variable population densities following fire reflect a common set of factors that influences multiple species of woody legumes. Sites containing high-density populations had the steepest slopes, lowest solar incidence, high cation concentrations and low availability of P, although the soils were also the
least acidic. These factors likely represented a niche habitat rather than an environment that would support growth and productivity for a broad array of species.

2.1 Introduction

2.1.1 Background

Defining what factors constitute a suitable habitat for woody legumes has proved difficult. Woody legumes are distributed in Australia and worldwide across a wide range of habitats, including mesic, tropical and subtropical ecosystems, arid tropical regions, Mediterranean climates, and cooler temperate regions (Rundel 1989; Crews 1999; Adams et al. 2010). Investigating the recruitment of woody legumes following a disturbance, such as fire, provides the opportunity to model soil and landscape factors that best predict high population densities of woody legumes.

In temperate zones in Australia, woody legumes can regenerate profusely following fire. Woody legume populations defined at the local scale of a plant community often demonstrate variable stand densities (Clark et al. 1999; Guinto et al. 2000; Hodgkinson 2002; Warton & Wardle 2003). This mosaic pattern is frequently repeated at a regional scale across the forest and woodland portions of a firezone. Although the availability of viable seed is a rate-limiting factor, variable population densities may also reflect the occurrence of appropriate germination cues, suitable habitat, and competitive advantage (Harms et al. 2001; Freckleton & Watkinson 2002; Nathan & Casagrandi 2004; Yamada et al. 2013). Fire provides a cue for germination, but fire behaviour may influence the distribution of suitable habitat that enables successful recruitment of woody legumes (Christensen et al. 1981; Clark 1988; Bradstock & Auld 1995; Rodríguez et al. 2009). Comprehensive studies examining variables that may jointly influence legume recruitment are lacking.
Fire affects several physical properties of soil in a temperature-dependent fashion (Tomkins et al. 1991; Capogna et al. 2009; Yusiharni & Gilkes 2012c). Higher temperatures can dehydroxylate some clay minerals, including kaolinite and gibbsite, to create finer soil particles that increase bulk density and alter the moisture capacity of soil (Yusiharni & Gilkes 2012b). Soil organic matter begins to oxidise above 200 °C, while temperatures greater than 450 °C produce inorganic carbonates, and complete combustion above 580 °C produces more alkaline inorganic oxides (Escudey et al. 2010; Bodi et al. 2014). Ash texture becomes finer as combustion progresses, and fine ash particles may obstruct soil pores to increase bulk density and decrease water retention. Coarser ash produced at lower temperatures can increase soil porosity and water-holding ability (Raison 1979; Christensen et al. 1981; Boerner 1982; Whelan 1995; Certini 2005; Stoof et al. 2010) but also melt hydrophobic compounds and redistribute them into a water-repellent layer (Humphreys & Craig 1981; Boerner 1982; Whelan 1995; Stoof et al. 2010; Bodi et al. 2014). Soil and ash texture influences the incorporation of alkaline, cation-rich ash into soil, subsequently increasing soil pH and electrical conductivity (Raison et al. 1985; Yusiharni & Gilkes 2012b; Bodi et al. 2014). Therefore, heterogeneous fuels that affect fire severity can produce a mosaic of physical soil conditions (Christensen et al. 1981; Humphreys & Craig 1981; Boerner 1982; Raison et al. 1985; Whelan 1995; Pereira et al. 2012).

Heat from fire can significantly alter N content of soil. Nitrogen volatilises around 200 °C, a temperature that occurs during flaming combustion (Whelan 1995; Guinto et al. 1999). Therefore, the amount of litter that burns during a fire is close to being linearly proportional to the amount of N lost from surface fuel (Boerner 1982; Raison et al. 1985). Soil is a good insulator, thus high fire intensities are required to volatilise N from organic matter within soil (Beadle 1940; Shea et al. 1979; Williams et al. 2004). However, elevated soil temperatures may still be lethal to soil microbes, and microbial activity determines how much soil N is mineralised.
to \(\text{NH}_4^+\), nitrified to \(\text{NO}_3^-\), denitrified and lost to the atmosphere or immobilised when taken up by microbes (Raison 1979; Adams & Attiwill 1986; Weston & Attiwill 1990; Ross 1992; Fenn et al. 1993; Guinto et al. 1999; Newland & DeLuca 2000; Choromanska & DeLuca 2002; Hirobe et al. 2003; Certini 2005; Lambers et al. 2006; Ford et al. 2007; Durán et al. 2009; Busse et al. 2010; Rivas et al. 2012). As soil moisture facilitates heat conduction, the variable distribution of soil water ultimately promotes a heterogeneous distribution of available soil N after fire (Beadle 1940; Raison 1979; Whelan 1995; Choromanska & DeLuca 2002; Busse et al. 2010).

Most Australian species of woody legumes have the capacity to establish symbioses with rhizobia to access atmospheric N through BNF (Vitousek et al. 2002; Brockwell et al. 2005; Sprent 2007). Woody legumes may use BNF to acquire supplemental N after fire (Harshbarger et al. 1975; Shea et al. 1979; Adams & Attiwill 1984; Boring et al. 1988; Islam et al. 2000; Newland & DeLuca 2000; da Silva & Batalha 2008), but the high demand for energy and P, and the requirement for adequate moisture and environmental temperatures within an optimal range make N acquisition from soil more preferable (Rundel 1989; Ross 1992; Marschner 1995; Crews 1999; Vitousek et al. 2002; Forrester et al. 2007; Sprent 2007; Houlton et al. 2008; Adams et al. 2010). However, nitrogen-fixing species have been found to produce phosphatases, enzymes used to liberate organic P, at a three-fold greater rate than non-nitrogen-fixing species (Houlton et al. 2008). In addition, suitable conditions for BNF and the availability of P partially reflect the global distributions of woody legumes (Crews 1999; Houlton et al. 2008).

As described in more detail in Chapter 4, the acidic, highly weathered soils typically found in Australia are depleted in P, but fire can effectively increase the availability of P in soil (Beadle
1966; LeBauer & Treseder 2008; Lambers et al. 2010). Oxides of iron (Fe) and aluminium (Al) complex P in acidic soils (Crews et al. 1995; Lambers et al. 2006; Lambers et al. 2010), but alkaline ash can increase soil pH and release complexed P (Humphreys & Craig 1981; Raison et al. 1985; Adams & Pate 1992; Romanyà et al. 1994; Abel et al. 2002; Certini 2005; Rodríguez et al. 2009; Escudey et al. 2010). Considerable amounts of P are also held in organic matter and must be mineralised to an inorganic form to be available to plants (Duff et al. 1994; Abel et al. 2002; Daufresne et al. 2005). Phosphorus can generally withstand temperatures above 500 °C, so when fire oxidises organic matter, P is deposited on the soil surface in nutrient-rich ash (Raison 1979; Boerner 1982; Raison et al. 1985; Certini 2005; da Silva & Batalha 2008). However, the solubility of inorganic P decreases in the alkaline, cation-rich ash P (Bolan 1991; Romanyà et al. 1994; Certini 2005; Hinojosa et al. 2012; Pereira et al. 2012). Inorganic P is also relatively immobile in dry soil (Bolan 1991; Lambers et al. 2006), thus the characteristics of ash affect distribution of P in the solum. However, woody legumes may redistribute water from deeper soil layers to aid P diffusion (Lambers et al. 2006; Sprent 2007).

On 7 February 2009, the Kinglake fire complex, which comprised the Kilmore East and Murrindindi Mill fires on ‘Black Saturday’, burned around 400,000 ha in Victoria, Australia (Cruz et al. 2012). A considerable portion of this area had not experienced a bushfire since 1939. The fires occurred during a record-breaking heat-wave and high winds and coincided with a decade-long drought (Cai et al. 2009; Mullen 2009). The fires promoted considerable recruitment of woody legumes, which included species that had not been observed in the area for decades (personal communication from I. Maher, Kinglake National Park). This fire event provided a rare opportunity to investigate how multiple species of woody legumes were distributed following a single recruitment event and what factors may have interacted to determine variable population densities.
2.1.2 Aim

The objective of the study presented in this chapter was to evaluate which edaphic and geographic factors best predict post-fire population densities of woody legumes. The aim was to test the following hypotheses: (i) population densities of woody legumes recruited following a single bushfire event vary on a local scale; (ii) edaphic and geographic variables that predict population densities would have a common effect on multiple species of woody legumes; (iii) factors that affect P availability contribute to the distributions of woody legumes.

2.2 Methods

2.2.1 Study area

Geology and soils

The Kinglake fire complex circumscribed the general location of all the sites selected for this study. Sampling sites were distributed across the Black Range State Forest (BRSF), Marysville State Forest (MSF), Yarra Ranges National Park (YRNP), and Kinglake National Park (KNP) in central Victoria (VIC), Australia (Fig. 2.1). The Great Dividing Range (GDR) encompasses all four parks and state forests. In this region, the GDR has an east-west orientation characterised by a series of plateaus and ridges separated by numerous gaps, creating a diverse geography and geology (Ollier 1995; Joyce et al. 2003). Upper Devonian volcanic and granitic complexes have formed several cauldron structures, including the Acheron and Cerebean cauldrons, which comprise the iron-rich Marysville Igneous Complex. The Black Range pluton is granitic and intrudes on the northwestern edge of the Acheron cauldron (Marsden 1973; Cas et al. 2003). The complexes are interspersed by folded marine sediments from the Lower Devonian/Silurian known as the Murrindindi Supergroup. These sediments are typically siltstone and some sandstone with mudstone formations, but limestone also occurs in shallower depressions.
(Marsden 1973; VandenBerg 2003). Along the western margin of the Murrindindi Supergroup lies the Kinglake Surface, which forms a low-relief plateau of folded sedimentary rocks from the Silurian or Lower Devonian (Rowan 1990; Joyce et al. 2003).

The variable geology in the region means the parent materials and soils differ between the four parks and state forests. Soils from the BRSF are primarily derived from intrusive granodiorite forming a friable reddish brown gradational soil with a silt loam to very fine sandy loam texture (Rowan 1990; Joyce et al. 2003). The MSF also has friable red gradational soils that are fine sandy to silt loams, but they are iron-rich and overlay rhyodacite as well as toscanite and porphyrite (Rowan 1990; Cas et al. 2003; Joyce et al. 2003). The YRNP has yellow brown-mottled duplex soil with a silt loam texture that is less well-drained over alluvial mudstone and siltstone (VandenBerg 1973; Rowan 1990; Joyce et al. 2003). Finally, the KNP has yellow tenosols with a silt loam texture overlaying mudstones, siltstones, shales and sandstones (Jeffery 1981; Joyce et al. 2003). The BRSF, YRNP, and MSF have mull litter layers and a more developed organic layer than in KNP, where an organic layer was mostly absent following the 2009 fires. The steepest slopes are about 45° and occur in the BRSF and KNP; the sites sampled in MSF are close to 20° and in YRNP are undulating and less than 10°(Rowan 1990).

Climate, vegetation, and fire history

Temperature and rainfall in the study region are mainly influenced by southwesterly air flows. These carry warm moist air from Port Phillip Bay that cools with rising elevation, causing precipitation particularly on southwest-facing slopes (Bureau of Meteorology 2008; Risbey et al. 2009). However, as the region has a series of ridgelines running north-south, the variable topography creates a heterogeneous pattern of temperature and rainfall at the landscape level.
Average annual rainfall varies between 1170–1660 mm, and average temperature ranges relative to elevation between 15.8–19.0 °C (Table 2.1).

The vegetation of the region corresponds to elevation and rainfall, thus showing considerable variation over small distances. Gullies and foothills have open-forests dominated by Brown Stringybark (*Eucalyptus baxteri* (Benth.) Maiden & Blakely ex J. M. Black), Narrow-leaf Peppermint (*E. radiata* Sieber ex DC), Broad-leaf Peppermint (*E. dives* Schauer), Messmate (*E. obliqua* L'Hér), and Long-leaf Box (*E. goniocalyx* F. Muell. ex Miq.) (SCA 1976; Attiwill et al. 1996). Lower slopes have closed forests containing Messmate and Candlebark (*E. rubida* H. Deane & Maiden) or tall open forests with dominant stands of Mountain Ash (*E. regnans* F. Muell.) when rainfall exceeds 1200 mm (SCA 1976; Vertessy et al. 2001). Upper slopes have tall, open forests of Mountain Ash, but these become dominated by Alpine Ash (*E. delegatensis* R. T. Baker) above 1000 m elevation (SCA 1976; Attiwill et al. 1996).

All of the sites selected for sampling were burnt in the ‘Black Friday’ bushfire in 1939. Since that time, fires have occurred in several parts of the region, but existing maps and data suggest that all but one of the sites selected in this study had not been burnt for 70 years. A bushfire in 1954 burnt the sites located in KNP.
Figure 2.1 The location of field sites (stars, n = 46) selected within the Kinglake fire complex boundary (dark grey shaded area) in Victoria, Australia. Population density classes of woody legumes are indicated by colour: red represent high-, green represent medium-, and blue represent low-density populations. Top right inset displays sites for *Acacia leprosa*; bottom left inset contains sites for *A. lanigera*.

Table 2.1 The sampling sites (n = 46) found in the Black Range State Forest (BRSF), Marysville State Forest (MSF), Yarra Ranges National Park (YRNP), and Kinglake National Park (KNP). Abbreviations are A: *Acacia*; G: *Goodia*. Coordinates and elevation obtained using a hand-held global positioning system. Climate data derived from nearest relevant climate stations listed on Bureau of Meteorology climate data online: [http://www.bom.gov.au/climate/data/](http://www.bom.gov.au/climate/data/)

<table>
<thead>
<tr>
<th>Park/State Forest</th>
<th>Latitude range</th>
<th>Longitude range</th>
<th>Elevation range (m)</th>
<th>Mean annual rainfall (mm)</th>
<th>Mean maximum temperature (°C)</th>
<th>Number of sites</th>
<th>Species represented</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRSF</td>
<td>-37°23’12.7”</td>
<td>145°36’18.3”</td>
<td>596-</td>
<td>1373</td>
<td>16</td>
<td>21</td>
<td><em>A. dealbata, A. leprosa,</em></td>
</tr>
<tr>
<td></td>
<td>-37°28’55.2”</td>
<td>145°39’29.6”</td>
<td>932</td>
<td></td>
<td></td>
<td></td>
<td><em>A. obliquinervia, G. lotifolia</em></td>
</tr>
<tr>
<td>MSF</td>
<td>-37°32’00.2”</td>
<td>145°42’17.1”</td>
<td>391-</td>
<td>1657</td>
<td>17</td>
<td>12</td>
<td><em>A. dealbata, A. myrtifolia</em></td>
</tr>
<tr>
<td></td>
<td>-37°33’26.6”</td>
<td>145°43’12.4”</td>
<td>559</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YRNP</td>
<td>-37°37’41.5”</td>
<td>145°35’36.7”</td>
<td>190-</td>
<td>1170</td>
<td>19</td>
<td>2</td>
<td><em>G. lotifolia</em></td>
</tr>
<tr>
<td></td>
<td>-37°37’48.6”</td>
<td>145°35’41.6”</td>
<td>237</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KNP</td>
<td>-37°34’44.2”</td>
<td>145°21’21.0”</td>
<td>183-</td>
<td>685—1201</td>
<td>17</td>
<td>11</td>
<td><em>A. lanigera, A. leprosa</em></td>
</tr>
<tr>
<td></td>
<td>-37°34’55.2”</td>
<td>145°21’41.6”</td>
<td>527</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.2.2 Species selection

Six species of woody legumes were used in this study (Table 2.1). Species included Silver Wattle (*Acacia dealbata* Link), Mountain Hickory Wattle (*A. obliquinervia* Tindale), Myrtle Wattle (*A. myrtifolia* (Sm.) Willd.), Cinnamon Wattle (*A. leprosa* Sieber ex DC.), Woolly Wattle (*A. lanigera* A. Cunn.), and Golden-tip (*Goodia lotifolia* Salisb.). Of the acacias, *A. dealbata* is the longest-lived and the only bipinnate species (Tame 1992). The MIMOSOIDEAE subfamily of FABACEAE contains all the species of *Acacia*, and *G. lotifolia* is in the PAPILIONOIDEAE subfamily.

2.2.3 Site selection

*Determining population densities*

To determine the population density of each species, the total number of stems was counted within 4 m$^2$ plots. Temporary plots (2 × 2 m, n = 5 per site) were spaced approximately 5 m apart and the number of individual stems within the boundaries were counted using a digital hand tally (Compass Instruments; Thomas Scientific, Swedesborough NJ). The placement of plots was non-random and targeted particular areas to estimate varying population densities. The counts were used to create box-plots, and the distribution and spread were used to delineate ‘low’ and ‘high’ density categories for three species, and ‘low’, ‘medium’, and ‘high’ density categories for three species. Designated sites were assigned a waypoint (wp) using a hand-held global positioning system (GPS; Garmin eTracker; Olathe, KS, USA) to allow sites to be located again without placing a physical marker. Elevation for each site was also determined using the GPS. Altogether, 46 sites were selected to represent three replicates of the two to three density classes delineated for the six woody legume species under investigation.
Distance measurements

Clusters of waypoints representing different density categories were found for many species. The distances between paired or clustered waypoints were calculated using Geoscience Australia’s online calculator (http://www.ga.gov.au/geodesy/datums/vincenty_inverse.jsp used 26/03/2013). This uses the latitude and longitude values of two points in Vincenty’s Inverse Formula to calculate the ellipsoidal distance (Vincenty 1975).

Differences in population density were calculated for each pair of waypoints for a species. Pearson Product Moment was used to test whether ellipsoidal distance between waypoints was significantly correlated to the difference in population density.

2.2.4 Edaphic variables

Thirty-two edaphic variables were measured or calculated for each site (Table 2.2). These included physical and chemical properties, and nutrient ratios between soil carbon (C), nitrogen (N), and available phosphorus (P). To measure soil physical and chemical properties, a steel soil corer, 5 cm diameter and approximately 12 cm length, was used to collect soil samples between February and April 2012. Engraved marks on the side of the corer delineated 2, 4, and 10 cm and one end was sharpened to aid penetration of the soil. At each waypoint, three new temporary 2 × 2 m plots were set up approximately 5 m apart, and four soil samples from 0—2, 2—5, and 5—10 cm depth were taken near the corners. Samples from each depth were bulked and sieved to collect the 2 mm fraction before being stored in air-tight plastic bags and cooled or refrigerated at 4ºC until analysis. From the first plot established at each waypoint, an additional set of soil samples from the three depths were collected to measure bulk density and for particle size analysis. Thus for every species, at least
three replicate samples were used to measure physical and chemical properties for soil from each given population density.

Soil samples collected to measure bulk density were dried at 105 °C for 72 h and weighed. These samples were then bulked to the 0—10 cm depth for each waypoint, sieved to 2 mm, and used for particle size analysis. Briefly, 30 g of dried soil was mixed for 72 h with 50 mL 5% sodium metahexaphosphate, pH 8.5 in approximately 500 mL distilled water (dH₂O). Volumes were brought to 1 L in a graduated cylinder, and the temperature of the solution was recorded at 24 °C, indicating the time for fine sand to settle would be 4 min 16 s (Bowman & Hutka 2002). Hydrometer measurements were taken at time 0, 4 min 16 s to determine the weight of sand, and 8 h to determine the weight of silt. A blank of 50 mL 5% sodium metahexaphosphate in 950 mL dH₂O was also measured at 0 min and 8 h, and the blank was subtracted from samples to find adjusted hydrometer readings, which were used to calculate fraction weights (Bowman & Hutka 2002).

Gravimetric water content of bulked soil samples was measured using approximately 15 g of fresh (field moist) sieved soil that was dried at 105 °C for 72 h and reweighed. Fresh sieved soil was suspended in 1:5 dilution with dH₂O to determine pH and electrical conductivity. The remaining soil was dried at 40 °C for 72 hrs, and 30 g of each sample was used to measure water-holding capacity. Briefly, soil was placed in a plexiglass tube that had one end covered using Whatman number 1 filter paper. Tubes were filled with dH₂O that was allowed to drip through until the soil was saturated. Tubes were weighed and dried at 105 °C with daily weight checks until oven-dry weights were constant.

To measure total N, C, P, and available P, approximately 25 g soil was ground and the 5 µm fraction collected. A CNS analyser (Variomax Elementar Analysensysteme, Hanau, Germany)
was used to determine total N and C by combustion of 750—900 mg ground soil. Available P was measured using the standard Bray 1-P method (Menage & Pridmore 1973). For this analysis, 1 g of ground soil was vortexed for 1 min in 7 mL 0.03M NH₄F at room temperature before being centrifuged for 15 min at 3000 rpm. To induce the molybdenum blue colour, 0.5 mL of supernatant was reacted with 2mL colorimetric reagent (0.011M ascorbic acid, 2mM ammonium molybdate and 0.18mM potassium antimonyl tartrate) and allowed to stand 30 min at room temperature before absorbance at 882 nm was measured using a UV spectrophotometer. Total P of soil samples collected from 0—2 cm depth was measured commercially by CSBP Soil and Plant Laboratory (Bibra Lake, WA, Australia) by digestion with sulfuric acid-potassium-copper sulphate and measured colourmetrically at 880 nm (Allen & Jeffery 1990).

2.2.5 Geographic variables

Eighteen geographic and climate variables were calculated for each site using a handheld global positioning system (GPS) and geographic information systems (GIS; Table 2.2). A digital elevation model (DEM; 1 arc second (30 m) Shuttle Radar Topographic Mission) created in 2010 and covering the study area was obtained from the National Elevation Data Framework Portal (Geoscience Australia) (http://nedf.ga.gov.au/geoportal/catalog/main/home.page). The metadata regarding the creation of this raster dataset can be viewed at http://nedf.ga.gov.au/geoportal/catalog/search/viewMetadata.page?uuid=9&option=view.

The DEM and ArcMap version 10 (ESRI, Redlands, CA, USA) were used to analyse aspect and slope; the latter using a Z factor adjusted to 1.17 x 10⁻⁵ to convert m of elevation to GDA94 projection of latitude and longitude coordinates at 37º S latitude. Watershed was also
determined using flow direction and snap pour point, which was calculated assuming a flow accumulation weight of 1 and snap distance of 0. Topographic wetness index (TWI) was calculated using the formula \( \ln(\text{watershed}/\tan[\text{slope}]) \). A fire severity map of the 2009 fires was provided in raster format by K. Tolhurst, University of Melbourne. The direction the fire was travelling when it burned sites was determined according to Bradstock & Price (2009).

Data from several local climate stations (Bureau of Meteorology) were used to calculate average annual and 2009 quarterly rainfall, maximum temperature averages for the wettest month and annum, and total annual and quarterly solar incidence in 2009. Co-kriging was then used to derive values for these climate variables at each waypoint. The resolution of the derived climate variables was established by the DEM at a 30 m minimum. Direct and total solar incidence for the DEM raster were also calculated by ArcMap over the year beginning 09/02/2009 and ending 08/02/2010. All values were then extracted by point using the waypoint coordinates.

**Table 2.2** A list of the fifty variables analysed as potential predictors of population density for woody legumes regenerating after bushfire. This set of variables was measured or derived as indicated for each waypoint in turn. Numbers after the variable name indicate sampling depth. Detailed methodology provided for each variable in the Methods section. EC: electrical conductivity; \( \text{H}_2\text{O} \): water; N: nitrogen; C: carbon; C:N: ratio of C to N; P: phosphorus; N:P: ratio of N to Bray 1-P; N:P\(_T\): ratio of N to total P; GPS: handheld global positioning system; TWI: topographic wetness index; avg: average; max: maximum; min: minimum; wet: within the wettest month; quarter: refers to 3-month period in a year.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Type</th>
<th>How obtained</th>
<th>Relevance</th>
</tr>
</thead>
<tbody>
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<td>pH, 0—2</td>
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<td>measured</td>
<td>nutrient availability</td>
</tr>
<tr>
<td>pH, 2—5</td>
<td>edaphic</td>
<td>measured</td>
<td>nutrient availability</td>
</tr>
<tr>
<td>pH, 5—10</td>
<td>edaphic</td>
<td>measured</td>
<td>nutrient availability</td>
</tr>
<tr>
<td>Parameter</td>
<td>Measured Property</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>-------------------</td>
<td>----------------------------------</td>
<td></td>
</tr>
<tr>
<td>EC ($\mu$S cm$^{-1}$), 0—2</td>
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<td>nutrient availability</td>
<td></td>
</tr>
<tr>
<td>EC ($\mu$S cm$^{-1}$), 2—5</td>
<td>edaphic measured</td>
<td>nutrient availability</td>
<td></td>
</tr>
<tr>
<td>EC ($\mu$S cm$^{-1}$), 5—10</td>
<td>edaphic measured</td>
<td>nutrient availability</td>
<td></td>
</tr>
<tr>
<td>Bulk density, 0—2</td>
<td>edaphic measured</td>
<td>root respiration</td>
<td></td>
</tr>
<tr>
<td>Bulk density, 2—5</td>
<td>edaphic measured</td>
<td>root respiration</td>
<td></td>
</tr>
<tr>
<td>Bulk density, 5—10</td>
<td>edaphic measured</td>
<td>root respiration</td>
<td></td>
</tr>
<tr>
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<td>water availability</td>
<td></td>
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<tr>
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<td>water availability</td>
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<tr>
<td>$H_2O$-holding capacity, 5—10</td>
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<td></td>
</tr>
<tr>
<td>Sand (%)</td>
<td>edaphic measured</td>
<td>drainage and nutrients</td>
<td></td>
</tr>
<tr>
<td>Silt (%)</td>
<td>edaphic measured</td>
<td>drainage and nutrients</td>
<td></td>
</tr>
<tr>
<td>Clay (%)</td>
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<td>drainage and nutrients</td>
<td></td>
</tr>
<tr>
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<td>essential nutrient</td>
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</tr>
<tr>
<td>Total N (mg g$^{-1}$), 2—5</td>
<td>edaphic measured</td>
<td>essential nutrient</td>
<td></td>
</tr>
<tr>
<td>Total N (mg g$^{-1}$), 5—10</td>
<td>edaphic measured</td>
<td>essential nutrient</td>
<td></td>
</tr>
<tr>
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<td>soil health indicator</td>
<td></td>
</tr>
<tr>
<td>Total C (mg g$^{-1}$), 2—5</td>
<td>edaphic measured</td>
<td>soil health indicator</td>
<td></td>
</tr>
<tr>
<td>Total C (mg g$^{-1}$), 5—10</td>
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<td>soil health indicator</td>
<td></td>
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<td>C:N, 2—5</td>
<td>edaphic measured</td>
<td>nutrient cycling</td>
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</tr>
<tr>
<td>C:N, 5—10</td>
<td>edaphic measured</td>
<td>nutrient cycling</td>
<td></td>
</tr>
<tr>
<td>Total P ($\mu$g kg$^{-1}$), 0—2</td>
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<td>essential nutrient</td>
<td></td>
</tr>
<tr>
<td>Bray 1-P ($\mu$g kg$^{-1}$), 5—10</td>
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<td>essential nutrient</td>
<td></td>
</tr>
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<td>nutrient balance</td>
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<td>N:P, 2—5</td>
<td>edaphic measured</td>
<td>nutrient balance</td>
<td></td>
</tr>
<tr>
<td>N:P, 5—10</td>
<td>edaphic measured</td>
<td>nutrient balance</td>
<td></td>
</tr>
<tr>
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<td>nutrient balance</td>
<td></td>
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<td>Fire severity class</td>
<td>landscape provided</td>
<td>fire impact indicator</td>
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<td>landscape GPS</td>
<td>affects climate</td>
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<tr>
<td>Slope</td>
<td>landscape GIS</td>
<td>fire intensity</td>
<td></td>
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<tr>
<td>Aspect</td>
<td>landscape GIS</td>
<td>climate, fire intensity</td>
<td></td>
</tr>
<tr>
<td>Watershed</td>
<td>landscape GIS</td>
<td>water availability</td>
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</tr>
<tr>
<td>TWI</td>
<td>landscape GIS calculation</td>
<td>water availability</td>
<td></td>
</tr>
</tbody>
</table>
2.2.6 Data analysis

Six species of woody legumes were identified growing at two to three population densities that recurred within each species, but differed substantially among species. To combine all six species in a single regression model, the average counts per m$^2$ for each site could be used in a Poisson regression or used to create ordinal categories for use in an ordinal logistic regression (Agresti 2002). The Poisson regression ignored the possibility that each species demonstrated a different range of counts due to species-level variations rather than external factors and therefore failed to produce a model applicable to all six species. Instead, ordinal logistic regression (OLR) was used to distinguish what factors would predict population densities of woody legumes after bushfire.

The OLR used to determine the variables that best predicted population density was a proportional odds (PO) model based on cumulative probabilities originally developed by
McCullagh (1980). The model can be stated as follows, for a response variable $j$ having levels 1, 2, ..., $k$:

$$\text{prob}(Y \geq j | X) = \frac{1}{1 + \exp[-(\alpha + X\beta)]} \quad (2.1)$$

where $Y$ is the event (e.g. high population density) and $X$ is each predictor. Graphing the relationship between $X$ and $Y$ gives the intercept ($\alpha$) and the slope, or regression coefficient ($\beta$). This model assumes $\beta$ is independent of $j$, thus including $\beta$ in this model connects the probabilities for varying $j$, and facilitates parsimonious modelling of the distribution of $Y$.

The effects of a PO model can be interpreted using the odds ratios. The odds ratio can be calculated from $\beta$ (equation 2.2) to indicate how a 1-unit increase of the predictor affects the odds that population density will be high (i.e., $Y = 1$).

$$\text{prob}(Y = 1 | X = x) = e^{\beta} \quad (2.2)$$

The sign of $\beta$ indicates whether a predictor has a direct or inverse relationship to increasing population density.

The PO model was chosen because it allowed the response variable to be classified into ordered categories (low < medium < high) without specifying the intervals between them (McCullagh 1980; Norusis 2012). Although the relative densities for each category were computed, the absolute numbers varied between species, such that the highest density recorded for one species equated numerically to the lowest density for another species (Table 2.3). Grouping them categorically normalised these differences, allowing multiple species to be included in the analysis.
Table 2.3 The range of stem densities that was used to define ‘low’, ‘medium’ and ‘high’ categories of population density for each species. Medium density categories were assigned a range equal in size to corresponding low density categories. Three species exhibited population densities that only permitted low and high categories to be assigned (see Fig 2.2). A: *Acacia*; G: *Goodia*; n/a: not applicable.

<table>
<thead>
<tr>
<th>Population Density (stems m(^{-1}))</th>
<th><em>A. dealbata</em></th>
<th><em>G. lotifolia</em></th>
<th><em>A. obliquinervia</em></th>
<th><em>A. lanigera</em></th>
<th><em>A. leprosa</em></th>
<th><em>A. myrtifolia</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>0—7</td>
<td>0—2</td>
<td>0—10</td>
<td>0—5</td>
<td>0—7</td>
<td>0—11</td>
</tr>
<tr>
<td>Medium</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>5—10</td>
<td>7—15</td>
<td>11—22</td>
</tr>
<tr>
<td>High</td>
<td>≥8</td>
<td>≥3</td>
<td>≥10</td>
<td>≥10</td>
<td>≥15</td>
<td>≥23</td>
</tr>
</tbody>
</table>
Using logistic regression allowed the inclusion of multiple predictors covering a range of spatial scales, thus increasing overall predictive power. However, the model assumes the response variable, Y, behaves linearly with all the predictors, X, although many continuous predictors often do not show linearity (Harrell Jr. 2001; Hosmer et al. 2013). In this study, the edaphic and geographic variables were all continuous predictors that did not show linearity when graphed against the categories of population density. To address this, a nonparametric regression spline that used alternating least squares with a spline ordinal level (CATREG function in SPSS) was applied to optimally scale each continuous variable except pH, which was smoothed using functional intervals defined for Australian soils (Rayment & Lyons 2011). CATREG uses two interior knots placed at the terciles to join piecewise polynomials that transform set intervals of the data, and the flexibility this permits creates a better fit than a single transformation function applied over the entire dataset (Ramsay 1988). Incorporating alternating least squares allows this method to be iterative and automated (Breiman & Friedman 1985).

The edaphic and geographic variables chosen as possible predictors of population density of woody legumes totalled 48. These included physical and chemical traits of soil at three depths; ratios between N and C or P; fire severity, geographic and climate features derived using a 30 m digital elevation model; and elevation measured using a hand-held GPS. To reduce the number of possible predictors to be tested and minimise the chance of collinearity confounding the results (Harrell Jr. 2001; Agresti 2002; Hosmer et al. 2013), correlations between transformed variables were examined. An upper threshold of 0.5 was used to determine what combinations of variables should be avoided in subsequent tests. Stepwise OLR with a logit link function was done using the set of uncorrelated variables, and the importance of each covariable was assessed using the P-value of its Wald statistic. Variables
were sequentially eliminated according to the largest $P$-values, and variables that had been initially excluded to avoid confounding the results were added back once their correlated variables had been removed. During this process, model fit was determined using chi-square significance, Pearson and Deviance goodness-of-fit measures, and the test of parallel lines.

Akaike’s information criterion (AIC) offers another method to test the parsimony of potential models using the maximised log likelihood ($L$):

$$
AIC = 2k - 2L
$$

where $k$ is the number of factors. Models with lower AIC values are considered to have a better fit, thus fewer factors may improve AIC values. However, AIC is intended to compare models testing a discrete number of prespecified factors (Harrell Jr. 2001). A backwards stepwise method was used to generate the models in this study, thus AIC values were not considered.

Discriminant analysis was used to determine how accurately the categories of population density could be distinguished using the potential models. Similar to OLR, discriminant analysis assumes a linear relationship between the response variable and the predictors. To satisfy this assumption, the data-set that had been smoothed using regression splines for OLR was also used for this test. To cross-classify the analysis, subsets of data were removed before repeating the analysis, called a ‘leave-one-out’ classification. This secondary analysis was done in lieu of using uncategorised sites to test the models, and it gives some indication of their accuracy to predict population density.

This process produced multiple potential models. To compare their fitness, discriminant analysis was done using cases from all species and again using individual species separately. Predicted versus actual classification was examined, including a leave-one-out step to provide
further cross-validation. Variables comprising the model that provided the best classification both overall and at the species-level were chosen as predictors for use in this study. All the analyses were performed using the software SPSS Statistics v.20 (IBM).

2.3 Results

2.3.1 Distribution patterns of woody legumes

The species of woody legumes regenerating after the 2009 Kinglake fire complex displayed a recurring series of population densities that could be grouped into two or three categories (Fig. 2.2). The ranges of counts used to calculate population density showed considerable disparity between species (Table 2.3), but the respective counts were found repeatedly at multiple locations (waypoints) for each species.

For each species investigated, population densities changed considerably over short distances. Ellipsoidal distances calculated between clustered waypoints using Vincenty’s Inverse Formula showed the typical range of separation was 25—300 m (Fig. 2.3A). The distance between waypoints was not significantly correlated to the net change in population densities at those waypoints ($P=0.334$), suggesting a punctate rather than gradual pattern of change (Fig. 2.3B). The distances between different categories of population density was not significantly different for the three species that could be assigned a medium density category (Fig. 2.3C).

The distribution of waypoints across the landscape varied among species. Sites with *A. leprosa* were the most widely dispersed across the sampling region, whereas sites with *A. lanigera* or *A. myrtifolia* were clustered within a single forest community.
Figure 2.2 Box-plots showing densities of individuals found at each waypoint (site). Each waypoint contained one of the six species studied, and was subsequently grouped into categories representing low, medium, or high population density for that species.
Figure 2.3 Ellipsoidal distances between waypoints for the three categories of population densities using Vicente’s Formula. (A) Pairs demonstrating the shortest distances for each species. (B) Correlation of the distances between all waypoints for each species compared to difference in number of individuals counted in a 2 m² area at those waypoints. (C) Mean and SE of distances between three different categories of population density A: Acacia; G: Goodia.
2.3.2 Potential models predicting population density

Ordinal logistic regression identified two possible models to predict relative population densities of woody legumes regenerating following bushfire (Table 2.4). Models were initially evaluated using goodness of fit (Model fit), Pearson’s and Deviance values, and Nagelkerke’s $R^2$. Model 1 had a higher $R^2$ value than Model 2.

To select the most accurate set of predictors, both models were tested using discriminant analysis (Table 2.5). Greater accuracy was more apparent when species were tested independently rather than together. However, Model 1 correctly categorised more sites than Model 2 regardless of whether all species were combined or each species was examined separately (Table 2.5). When only two categories of population density were tested, accurate predictions occurred more frequently. Although medium density populations were assigned low-density status more often than high density, inaccurate predictions did not follow a clear pattern across all species within either model.

Both models had four predictors in common, including total solar incidence in 2009, soil pH, electrical conductivity (EC), and Bray P values all measured at 5—10 cm depth (Table 2.4). Slope was the only variable that differed between them. Slope showed a direct relationship to population density, but the slope angle was not related to the aspect facing the approaching fire (Fig. 2.4). Interactions between copredictors were tested, but they did not improve the fit of either model. Regression coefficients ($\beta$, Table 2.4) for three of the four common predictors were similar between the models. However, pH at 5—10 cm depth had a larger $\beta$ value in Model 1 compared to Model 2 (Table 2.4). Positive or negative values for $\beta$ indicated whether predictors influenced population density directly or inversely. The sign of
$\beta$ most accurately reflected distribution curves for high and low density populations, and the placement of medium density curves had less influence on both the sign and value of $\beta$(Table 2.4, Fig. 2.5).

Most of the predictors did not demonstrate a sequential distribution of values according to population densities. The medium density population only had intermediate values for pH, and the remaining predictors distributed intermediate values equally between low and high density populations (Table 2.6).

Figure 2.4 Scatter plots between slope and aspect of each site relative to the direction the fire was travelling when the site burned. Scatter plots are shown for all densities combined (A) and separately for low (B), medium (C) and high (D) density populations. 3: aspect faced the approaching fire, 2: aspect parallel to approaching fire, 1: aspect faced away from approaching fire.
Table 2.4 Two potential models produced by ordinal logistic regression. Model fit and Log-likelihoods indicate goodness of fit. Regression coefficients, significance, confidence intervals and Wald statistics are provided for each predictor/covariable in each model. Odds ratio = $e^{\beta}$. Numbers included with dependents/covariables indicate soil depth where measurement occurred. The number associated with pH, Bray P and EC represents 5—10 cm depth in soil. CI: confidence interval; Fit: Goodness of fit; LL: Log likelihood; LR: Likelihood ratio.

<table>
<thead>
<tr>
<th>Model</th>
<th>Dependents/ covariables</th>
<th>Regression coefficient ($\beta$)</th>
<th>Odds ratio</th>
<th>SE</th>
<th>$P$</th>
<th>Lower CI</th>
<th>Upper CI</th>
<th>Wald</th>
<th>Fit indicators</th>
<th>Fit values</th>
<th>df</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pH3</td>
<td>1.169</td>
<td>0.31</td>
<td>0.31</td>
<td>&lt;0.01</td>
<td>0.556</td>
<td>1.783</td>
<td>12.76</td>
<td>Pearson</td>
<td>5</td>
<td>201</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>Bray P3</td>
<td>-0.526</td>
<td>1.69</td>
<td>0.19</td>
<td>&lt;0.01</td>
<td>-0.905</td>
<td>-0.148</td>
<td>6.79</td>
<td>Deviance</td>
<td>201</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Solar hrs</td>
<td>-0.293</td>
<td>1.34</td>
<td>0.13</td>
<td>0.03</td>
<td>-0.551</td>
<td>-0.035</td>
<td>4.54</td>
<td>LL</td>
<td>-121.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC3</td>
<td>0.293</td>
<td>0.75</td>
<td>0.13</td>
<td>0.03</td>
<td>0.032</td>
<td>0.555</td>
<td>4.42</td>
<td>LR</td>
<td>23.2</td>
<td>5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>Slope</td>
<td>0.373</td>
<td>0.69</td>
<td>0.12</td>
<td>&lt;0.01</td>
<td>0.139</td>
<td>0.606</td>
<td>8.92</td>
<td>Nagelkerke $R^2$</td>
<td>0.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>pH3</td>
<td>0.727</td>
<td>0.48</td>
<td>0.27</td>
<td>&lt;0.01</td>
<td>0.199</td>
<td>1.255</td>
<td>0.20</td>
<td>Pearson</td>
<td>5</td>
<td>156</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>Bray P3</td>
<td>-0.431</td>
<td>1.54</td>
<td>0.18</td>
<td>0.02</td>
<td>-0.788</td>
<td>-0.074</td>
<td>-0.79</td>
<td>Deviance</td>
<td>156</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Solar hrs</td>
<td>-0.274</td>
<td>1.32</td>
<td>0.12</td>
<td>0.03</td>
<td>-0.517</td>
<td>-0.030</td>
<td>-0.52</td>
<td>LL</td>
<td>-107.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC3</td>
<td>0.248</td>
<td>0.78</td>
<td>0.13</td>
<td>0.05</td>
<td>-0.004</td>
<td>0.500</td>
<td>-0.003</td>
<td>LR</td>
<td>12.31</td>
<td>4</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nagelkerke $R^2$</td>
<td>0.10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.5 Accuracies of population densities predicted with discriminant analyses on all species combined and each species separately using Models 1 or 2 (see Table 2.4). The overall percentage correct and correct classification for each category is indicated in bold. Leave-one-out classification was done for cross-validation, and these values are shown as the second number after the forward slash. The upper table shows combined species and the three species with a medium-density category. The lower table has the three species without a medium-density category. The percentage incorrectly classified for each category of population density is also shown in regular font style.

Cat: category; O: overall; L: low; M: medium; H: high; A: *Acacia*; G: *Goodia*.

<table>
<thead>
<tr>
<th>Model</th>
<th>Cat</th>
<th>All species combined</th>
<th><em>A. lanigera</em></th>
<th><em>A. leprosa</em></th>
<th><em>A. myrtifolia</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>O</td>
<td>49.3/43.5</td>
<td>66.7/37</td>
<td>80/66.7</td>
<td>63/37</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>35/35 35/35 30/30</td>
<td>44/22 11/22 44/56</td>
<td>78/56 0/0 22/44</td>
<td>44/22 56/67 0/11</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>30/33 63/47 7/20</td>
<td>22/44 78/56 0/0</td>
<td>8/8 75/75 17/17</td>
<td>22/44 78/33 0/22</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>18/24 26/26 56/50</td>
<td>22/67 0/0 78/33</td>
<td>11/33 0/0 89/67</td>
<td>33/44 0/0 67/56</td>
</tr>
<tr>
<td>2</td>
<td>O</td>
<td>40.6/37.7</td>
<td>48.1/25.9</td>
<td>60/23.3</td>
<td>59.3/33.3</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>31/24 39/44 30/31</td>
<td>22/0 44/44 33/56</td>
<td>67/22 11/33 22/44</td>
<td>33/0 44/67 22/33</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>23/23 47/47 30/30</td>
<td>0/0 78/78 22/22</td>
<td>25/25 33/17 42/58</td>
<td>22/33 78/56 22/11</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>20/20 33/33 46/46</td>
<td>22/67 33/33 44/0</td>
<td>11/22 0/44 89/33</td>
<td>11/22 22/33 67/44</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Model</th>
<th>Cat</th>
<th><em>A. dealbata</em></th>
<th><em>G. lotifolia</em></th>
<th><em>A. obliquinervia</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>O</td>
<td>94.4/94.4</td>
<td>77.8/61.1</td>
<td>77.8/55.6</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>89/89 11/11</td>
<td>67/56 33/44</td>
<td>67/56 33/44</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>0/0 100/100</td>
<td>11/33 89/67</td>
<td>11/44 89/56</td>
</tr>
<tr>
<td>2</td>
<td>O</td>
<td>88.9/83.3</td>
<td>66.7/22.2</td>
<td>66.7/61.1</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>78/78 22/22</td>
<td>89/22 11/78</td>
<td>67/56 33/44</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>0/11 100/89</td>
<td>56/78 44/22</td>
<td>33/33 67/67</td>
</tr>
</tbody>
</table>
Figure 2.5 Modelled functions of population density against each potential predictor found using ordinal logistic regression. Separate curves for three categories of population density show the cumulative percentage of values falling within intervals determined by categorical regression using alternative least squares (CATREG, SPSS). (A) pH intervals at 5—10 cm, (B) EC intervals at 5—10 cm, (C) Bray 1-P intervals at 5—10 cm, (D) Total solar hour intervals for 02/2009—02/2010, (E) Slope intervals.
Table 2.6 Ranked distribution of each predictor across high, medium and low population densities. *Green, up-arrows* indicate highest values; *red, down-arrows* indicate lowest values; and *blue, left-right arrows* indicate intermediate values. The number associated with pH, Bray P and EC represents 5—10 cm depth in soil.

<table>
<thead>
<tr>
<th>Predictors</th>
<th>High density</th>
<th>Medium density</th>
<th>Low density</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH3</td>
<td>↑</td>
<td>←</td>
<td>↓</td>
</tr>
<tr>
<td>EC3</td>
<td>←</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Bray P3</td>
<td>←</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Slope</td>
<td>↑</td>
<td>↓</td>
<td>←</td>
</tr>
<tr>
<td>Solar hrs, 02/09—02/10</td>
<td>↓</td>
<td>↑</td>
<td>←</td>
</tr>
</tbody>
</table>

2.4 Discussion

2.4.1 Post-fire distribution of woody legumes

This study confirmed the hypothesis that woody legumes recruited after a single fire event establish stand densities that vary within a population yet recur at local and larger scales. The six species investigated were recruited within a 120,000 ha area, and population densities showed considerable disparity among the six species. However, each species demonstrated recurrent stand densities, allowing these population densities to be classified into reliable and recognisable categories.

Each species demonstrated different categories of population density adjacent to one another and spaced less than 200 m apart. Four of the six species repeated this pattern at sites located in distinct forest communities spaced tens of kilometres apart. The ellipsoidal distance separating neighbouring stands did not correlate to the difference in their population densities, nor were the same groups always adjacent when three categories were possible. Although the frequency that different population densities
occurred varied between species, low-density populations were located more often than high-density populations for all species except *Goodia latifolia*. However, the differences in frequencies were only between 10—30%, thus medium and high-density populations were found almost as often as low-density populations. The mosaic pattern of population densities indicated a disjunct distribution of habitat that was recapitulated in the distribution curves of the five potential predictors.

According to the models selected, the factors influencing population density included solar incidence, slope, soil pH, electrical conductivity (EC) and available P in the soil at 5—10 cm depth. Values for four of these predictors did not follow a continuum corresponding to population density, reiterating the discrete pattern of stand densities that was observed. Studies investigating aggregated distribution patterns in other woody species have also reported that the configurations of population densities neither follow obvious habitat boundaries nor correspond to any single predictor (Clark et al. 1999; Condit et al. 2000; Harms et al. 2001; Hyatt et al. 2003; Suzuki et al. 2013; Yamada et al. 2013; Zhang et al. 2013). Instead, successful recruitment likely reflects multiple biotic and abiotic factors that influence seed availability, seedling establishment, growth, fecundity, and mortality (Weiner & Thomas 1986; Harms et al. 2001; Yamada et al. 2013; Zhang et al. 2013).

2.4.2 Interpreting the model

If the distribution curves of the five predictors were viewed as a composite, the putative habitats would likely constitute a niche rather than broadly optimal environment for growth and productivity. Sites containing high-density populations had the steepest slopes, lowest solar incidence, higher cation concentrations and low availability of P, although the soils were also the least acidic. This corroborates a previous study that found slopes with lower solar irradiation had the highest population densities of two
Acacia species (Page et al. 2011). The present model extended those findings by incorporating pH, EC, and available P at equivalent positions in the soil profile. These edaphic factors were important to differentiate habitats, because high P availability and strongly acidic soils clearly distinguished low-density from high-density sites. That soil depth was consistent across all three edaphic factors suggests a cooperative influence on population density, potentially by affecting P availability and subsequent acquisition.

Most of the P present in Australian soils requires chemical modification to become available to plants. As the time interval between fires lengthens, an increasing amount of P is bound in organic matter that must be mineralised to release inorganic P. Nitrogen-fixing species may be able to secrete phosphatases to mineralise organic P (Houlton et al. 2008), but the efficacy of different phosphatases depends on soil pH (Duff et al. 1994). Soil pH also affects whether cations chelate organic or inorganic P, making it inaccessible to phosphatases or plants, respectively (Lambers et al. 2006; Escudey et al. 2010; Hinojosa et al. 2012). Some legume species were found to exude carboxylates to displace P from cations (Neumann & Römheld 1999; Rengel 2002; Ryan et al. 2012; Lambers et al. 2013), thus woody legumes may have the capacity to acquire P bound in organic matter or occluded by soil minerals.

Severe fires produce ash that has higher contents of metallic cations (e.g., Na, Mg, K, Ca) that elevate EC (Weston & Atiwill 1990; Yusiharni & Gilkes 2012b), and increase pH since metallic oxides are particularly alkaline (Humphreys & Craig 1981; Certini 2005; Escudey et al. 2010). The buffering capacity of soil and the incorporation of ash into the soil profile largely determine whether a fire alters pH and EC below the upper soil layers (Escudey et al. 2010; Hinojosa et al. 2012; Badía et al. 2014), but it is unlikely that fire would affect soil pH and EC at 5—10 cm but not at 0—5 cm depth. Sites with high-density populations had the highest soil pH and intermediate EC, but these edaphic
factors were measured at 5—10 cm depth, while pH and EC in the top 5 cm were not included in the model. Total carbon (C) and N in soil were also not selected for the model, although fire severity can influence the concentrations of soil C and N (Raison et al. 1985; Homann et al. 2011; Yusiharni & Gilkes 2012c). Stands of woody legumes on degraded soil or sand-dunes have been associated with higher EC but lower soil pH and bulk density and higher total C and N and available P (Munoz-Valles et al. 2011; Dong et al. 2014). Thus, it is possible that high-density populations of woody legumes ameliorated differences in soil C, N, pH, EC, bulk density and available P in soil, thereby obscuring the contribution of these edaphic factors to influence the recruitment of woody legumes. Nevertheless, soil pH, EC, and available P at 5—10 cm depth were selectively found to contribute to the model, and the distribution curves for pH and available P contrast with the reported effects of woody legumes on soil. Altogether, this suggests the edaphic factors included in the model reflect conditions before rather than after the fire and subsequent recruitment of woody legumes. The joint effect of soil pH, EC and extractable P to influence P availability warrants further investigation of methods woody legumes use to acquire P, which is undertaken in Chapters 4 and 5 of this thesis.

Fuel, topographic, and microclimate variability can cause fires to burn in a heterogeneous pattern that might correspond to the disjunct stand densities that were found (Cheney 1981; Walker 1981; Whelan 1995). Steeper slopes can either increase or decrease the rate of spread and fireline intensity depending respectively on the flames being closer to unburnt fuel upslope or further from unburnt fuel downslope (Byram 1959; Cheney 1981). When slope was removed from the model, medium and high-density categories were incorrectly exchanged more frequently. Fire travelled up steep slopes more frequently for sites containing medium-density populations and down steep slopes more frequently for sites with high-density populations. This may suggest that more sites
with medium-density stands experienced greater fireline intensities than sites with high-density stands. Severe fires allow more heat to penetrate into the soil and might expose seeds in upper soil layers to high, and perhaps lethal temperatures (Auld & O’Connell 1991; Bell 1999). However, fireline intensity does not dictate cumulative heat exposure (Keeley 2009), and fire severity measured using Landsat images and normalised burn ratios (Cruz et al. 2012) did not contribute to the model. The sites were primarily assigned to the top two categories of severity, which included 75-100% or 25-75% crown scorch, thus variations in fire severity that could be ecologically meaningful might have been obscured by this broad classification range. Consequently, the proportion of woody legume seeds that germinate after exposure to temperatures approximating mild, moderate or severe fires was investigated in a subsequent chapter.

The inclusion of solar incidence in this model may reflect an influence on plant aggregation or survival. Understorey species were found aggregated more frequently than canopy species, and this trend was emphasised when species were shade tolerant or light-demanding (Zhang et al. 2013). The woody legume species included in this study are understorey species and are reported to persist some years after the canopy has closed (Adams & Attiwill 1984; Pfautsch et al. 2009b). In addition, some woody legume species were found to have greater survival rates in moderate rather than full sunlight (Valladares et al. 2003). High light availability may also have increased competition with non-leguminous species, accelerating mortality and reducing stand densities of woody legumes (Grant & Loneragan 2001).

Several variables that affect soil moisture did not contribute to the model, nor did annual temperatures, rainfall, aspect or elevation. Temperature and rainfall were estimated for the sites using co-Kriging, and it is plausible the available resolution was not fine enough to detect differences between sites in close proximity. When waypoints were
spaced only marginally more than the 30 m minimum resolution, calculated rainfall was usually within 0.5 mm while maximum and minimum temperatures were identical. A contribution from aspect was likely encapsulated within the total solar incidence, which was included in the model. Soil moisture has been implicated to influence distributions of multiple woody species, but a clear influence on population density has not yet been established. For example, three species of Neotropical woody legumes were significantly associated with alluvial, stream-valley, or swampy soils, but population densities at these sites were not reported (Clark et al. 1999). Six dipterocarp species demonstrated greater growth and fecundity on poorly-drained soils, but growth was significantly correlated to mortality rates rather than high population densities (Yamada et al. 2013). In addition, soil moisture facilitated species diversity rather than aggregation in arid Australia (Capon 2005). That indicators of soil moisture did not contribute significantly to the model may reflect that adequate moisture was available, or the ability of Australian woody legumes to adapt their root structure and water-use efficiency (Clemens & Jones 1978; Rundel 1989; Sprent 2007).

2.4.3 Accuracy of the model

The model containing five factors predicted population densities that varied between 63 to 94% accuracy for the six different species of woody legumes investigated. This supported the hypothesis that variable population densities following fire reflect a common set of factors that influence multiple species of woody legumes. The model more accurately predicted medium and high density than low density stands irrespective of considering the species separately or together. The availability of viable seed is one factor that could explain this pattern (Condit et al. 2000; Andersson et al. 2007; Delerue et al. 2013; Joubert et al. 2013; Zhang et al. 2013). Seed availability was impossible to quantify after the fire, but a broad range of temperatures that facilitate germination
would allow seeds dispersed more widely in the soil profile to germinate. This hypothesis is addressed using a germination experiment in Chapter 3.

Biotic factors, including allometric measurements that might indicate self-thinning (Weiner & Thomas 1986), were not included in this study due to practical constraints. The rate of self-thinning may be independent of initial stand densities (Li et al. 2013), thus variables to elucidate intraspecific competition were not likely to increase the accuracy of the model. Interspecific competition is likely to be important in determining stand densities but would depend on habitat variables. Therefore, identifying the variables that predict high-density populations represents an important first step that can facilitate the subsequent investigation of mechanisms by which these variables exert influence, including interspecific competition. Consequently, the present study focussed on abiotic variables delineating post-fire habitats that supported high-density populations of woody legumes. The resulting data provide a basis for future investigation testing how the selected factors separately or together affect the competitive ability of woody legumes.
Chapter 3 – Woody legume populations across Australia – do optimum temperatures for breaking seed dormancy vary among climate zones?

Abstract

Native tree and shrub legumes grow in almost every terrestrial ecosystem in Australia. Most Australian woody legumes produce physically-dormant seeds that require heat to break their dormancy before moisture can trigger germination. There is strong consensus that a threshold temperature between 80—90 °C is required to break dormancy. However, some field observations and experiments suggest that lower threshold temperatures may apply for species endemic to arid or tropical zones. In this chapter, a meta-analysis approach is used to test temperature cues for breaking dormancy of seeds from native Australian woody legumes. Results suggested that species from arid climates require lower temperatures to break seed dormancy than species from temperate climates. This hypothesis was tested further in the laboratory. Germination rates following cool (50 °C), moderate (75 °C), or hot (90 °C) dry or wet heat treatment were compared among species from arid, tropical and temperate climates. Seed of species from arid regions germinated more readily after treatment at lower temperatures than species from temperate or tropical climates. Collectively, the results show that optimum temperatures for breaking dormancy of woody legume seeds vary with climate. This characteristic is one of many underpinning distributions of woody legumes across Australia.

3.1 Introduction

3.1.1 Background

Germination is a precarious time for plants because young seedlings are particularly vulnerable to environmental conditions, including extremes of temperature and moisture. Conversely, germination under favourable conditions of temperature and moisture can
promote seedling establishment. It is not surprising that many angiosperm seeds possess traits that impose barriers to germination, inferring a state of dormancy (Baskin & Baskin 1998b; Finch-Savage & Leubner-Metzger 2006). The cues that break dormancy are often distinct from those that promote germination, when seeds imbibe water and swell until breaking free of the seed coat (Finch-Savage & Leubner-Metzger 2006). Separating the cues to break dormancy from those that promote water imbibition safeguards seeds from germinating when circumstances are only briefly suitable (Vleeshouwers et al. 1995).

The FABACEAE family contributes nearly 2145 species of woody legumes from 174 genera to Australia (Robinson 2003). Native legume species can be found in all climate zones delineated by temperature and humidity (Fig. 3.1) (Rundel 1989; Bureau of Meteorology 2005; Adams et al. 2010). Despite this broad distribution and the obvious importance of woody legumes as keystone species in many Australian ecosystems, there is little known about dormancy-breaking cues relative to local climatic conditions. However, a diversity of habitats is likely to provide a range of signals to indicate when conditions are optimal for legume seedlings to survive.
Figure 3.1 Climate classification map of Australia according to temperature and humidity data collected between 1961–1990. Key zones are defined according to the summer and winter conditions. Maps and accompanying descriptions are from the Bureau of Meteorology (2005).

Legume seeds dry during development, and a hard seed-coat, or testa, must be breached to permit water imbibition, giving the seeds physical dormancy (Finch-Savage & Leubner-Metzger 2006). The testa begins to harden when legume seeds reach 20—25% moisture, but full water impermeability does not occur until seeds dry to less than 10—15% moisture (Hyde 1954; Lush & Evans 1980; Morrison et al. 1992). Physically dormant seeds are considered ‘orthodox’ if they can be dried to as little as 0.5% moisture before losing viability, whereas ‘recalcitrant’ physical dormancy requires moisture contents greater than 30% (Baskin & Baskin 1998a). The testa of legume seeds retains some water permeability when seeds contain 30% moisture (Hyde 1954; Lush & Evans 1980; Morrison et al. 1992), thus it is arguable that legumes have orthodox physical dormancy.
The strophiole or lens lies along one longitudinal edge of the testa and permits initial water entry through a cleft when dormancy is broken (Hamly 1932; Hyde 1954; Tran & Cavanagh 1980; Hanna 1984; Morrison et al. 1998). Mechanically scarifying the testa is often used to encourage water imbibition, but this has been shown to increase the number of malformed seedlings and decrease their survival rate (Manning & Van Staden 1987). Scarification in natural conditions is not likely to cue the optimum time to germinate (Baskin & Baskin 2000).

The hard seeds produced by woody legumes typically require heat to break dormancy. Under natural conditions, solar radiation or fire can provide the necessary temperatures, but heating patterns differ between these two cues. While a typical summer day can elevate soil temperatures near the surface for several hours, temperatures typically do not exceed 60°C (Hagon 1971; Auld & Bradstock 1996; Van Klinken et al. 2006). Moderate or high-intensity fires can heat upper soil layers between 60—120 °C, but these temperatures are reached for only minutes during the passage of a fire unless a large mass of fuel causes localised burning for several hours (Beadle 1940; Raison 1979; Shea et al. 1979; Auld 1986b; Bradstock & Auld 1995). However, maximum temperature has been demonstrated to be more influential than duration of heat exposure for breaking dormancy of woody legume seeds (Auld & O'Connell 1991). Although fire produces smoke that contains germination-promoting chemicals (Bell et al. 1995) and may augment soil nitrates, these fire-related cues have shown little promise to break dormancy of woody legume seeds (Purdie 1977b; Tieu et al. 2001; Williams et al. 2003; Auld & Denham 2006; Rawson et al. 2012).

Mineral soil retards heat conduction to the extent that temperatures decrease exponentially with depth (Beadle 1940; Bradstock & Auld 1995; Auld & Bradstock 1996; Williams et al. 2004). Soil moisture can increase the rate of heat conduction but
constrains maximum temperatures while latent heat is used to evaporate soil water (Beadle 1940; Portlock et al. 1990; Whelan 1995). Some studies have found lower temperatures are required to break dormancy of legume seeds when wet heat is applied (Cushwa et al. 1968; Portlock et al. 1990; Williams et al. 2004), but long incubations in boiling water are mostly detrimental to seed viability (Bell 1994; Bell & Williams 1998).

Most studies investigating dormancy of woody legumes have used single species or discrete communities, and have been overwhelmingly focused on temperate climates. The proportion (%) of seeds that germinate serves as the standard measure that a treatment can effectively break dormancy (Vleeshouwers et al. 1995; Finch-Savage & Leubner-Metzger 2006). Studies of species from temperate climates often report optimal germination after exposure to 90 °C or greater, while numerous studies have reported an optimum of 65–85 °C (Purdie 1977a; b; Shea et al. 1979; Portlock et al. 1990; Auld & O'Connell 1991; Bell 1994; Bell et al. 1995; Tieu et al. 2001). The preponderant representation of species from temperate climates previously supported the conclusion that 80–90 °C was the optimum temperature range to break seed dormancy for woody legumes (Segelquist 1971; Martin et al. 1975; Auld & O'Connell 1991). However, moderate (65–85 °C) to low (<65 °C) temperature thresholds have been reported as sufficient to break legume seed dormancy in species from humid tropics (Farrell & Ashton 1978; Hopkins & Graham 1984; Van Klinken et al. 2006) and the arid interior (Lange & Purdie 1976). Altogether, the existing data does not clarify the optimum conditions to prompt germination of Australian woody legumes from arid or tropical climates.

Although many studies have investigated legume seeds and their germination characteristics, comparisons of the requirements for breaking seed dormancy among legume species from different climate zones in Australia have not been conducted and are
rarely discussed. A direct empirical comparison is needed to address the question whether woody legumes have adapted their dormancy to conditions in their local environment. Such data will help elucidate mechanisms underlying the broad distribution patterns of these species in Australia.

3.1.2 Aim

The aim of the study presented in this chapter is to determine whether woody legume species have adapted their dormancy traits to regional conditions. A meta-analysis of existing literature was used first to determine whether woody legume species exhibit germination preferences that are clearly aligned to climate zones. To further test this hypothesis in the laboratory, conditions required to break seed dormancy were compared for a number of woody legume species with geographic ranges in different climate zones.

3.2 Methods

3.2.1 Meta-analysis

*Literature selection*

The preliminary inclusion criterion for literature was for studies to investigate woody legume species native to Australia. Papers investigating Australian natives growing as introduced species on other continents were excluded. Herbaceous species, whether native or introduced, were also excluded as outside the scope of this meta-analysis. A literature search was conducted using Thomson Reuters Web of Knowledge across all databases. Search topics included the topic “germination” and “Australia” as the location in addition to the topics: “legume”, “pea”, “bean”, “Acacia”, “Bossiaea”, “Cassia”, “Daviesia”, “Dillwynia”, “Gompholobium”, “Hardenbergia”, “Hovea”, “Kennedia”, “Mirbelia”, “Pultenaea”, and “Senna”. Both articles and reviews published in peer-reviewed journals were examined. Initial results totalled 315 articles, and examination of
the abstracts allowed papers focussing on herbaceous species to be discarded. From the remaining articles, those that used scarification rather than heat to stimulate germination were excluded to focus exclusively on effects of heat and moisture to break seed dormancy. Articles that did not report the temperature used to break seed dormancy were also excluded. This left 36 articles to be considered in the meta-analysis (Appendix 1). All articles used included some form of test for seed viability. Articles reported results as the proportion of viable seeds that germinated, and the results included the number of seeds per species tested (n) but variance, standard error, or standard deviation was not reported in several papers Consequently, the data used for the meta-analysis in this chapter was the mean proportion of viable seeds that germinated, but the variance could not be incorporated into the analysis.

*Data compilation*

The selected literature included several diverse approaches to exposing dormant seeds to heat. These included heated water, cabinets set to fluctuating temperatures, burial in the field with accompanying temperature probes and exposed to solar radiation or intentional fire, covered with soil substrate beneath controlled fires in a laboratory setting, and baked in ovens either with or without soil substrate that was dry, moist, or wet to field capacity. Maximum temperatures ranged from 40 to over 120 °C, and length of exposure to heat also varied from seconds to days. Therefore, data were grouped to maximise homogeneous treatment methods while maintaining sufficient numbers to allow statistical comparison.

To compile the data, categories were created for heat application method, temperature range, and exposure period. Data was grouped according to wet or dry application of heat, because some species require lower temperatures to break seed dormancy when moist heat is applied (Cushwa et al. 1968; Martin et al. 1975; Portlock et al. 1990). Seeds
enclosed within soil substrates that had been moistened or wet were considered to have received a “wet” heat application. Temperature categories included low heat (<65 °C), moderate heat of 65–85 °C, and high heat (>85 °C). These divisions all approximated a 20-25 °C range, because included studies used temperatures between 40–120 °C. To facilitate statistical analysis of contrasts between groups, exposure periods were divided into five categories, including 0.5—10 min, 11—30 min, 31—60 min, 61—720 min, and >720 min. The final group mostly comprised species treated with temperatures that fluctuated every 12 h between maximums and minimums designated to represent daily soil temperatures.

The species investigated were also grouped according to their climate zone of origin: temperate, arid, or tropical according to ranges of temperature and humidity (Bureau of Meteorology 2005). This particular mapping system was chosen because it included both temperature and water availability and could be simplified into three germination scenarios: hot/warm humid summer (hereafter referred to as “Hot Humid”; HH), hot dry summer (“Hot Dry”; HD), or warm/mild summer (“Warm”; W). The Australian Virtual Herbarium (AVH) mapping service was used to determine where the majority of records were distributed for each species. Overlapping the AVH records with the Bureau of Meteorology map allowed each species to be assigned a climate zone. Although each climate zone included representative species from each subfamily of the FABACEAE, dividing species into these groups reduced sample sizes sufficiently to hamper statistical analysis, thus subfamily categories were not used.

Statistical analysis – meta-analysis

The proportion (as %) of viable seeds that germinated successfully from each climate zone were compared using generalised estimating equations (GEE) with a negative binomial log link. Exchangeable, independent, and unstructured data for the working
correlation matrix were all modelled separately to compare the quasi likelihood under independence model criterion (QIC), and the exchangeable data structure was chosen because it produced the lowest QIC. Species were used as the repeated measure. The interaction between climate zone, temperature category, time category, and application was tested, and contrasts composed of pairwise comparisons were conducted with a sequential Sidak correction for multiple comparisons. A \( P \) value less than 0.05 was considered to be significant, and all statistical analyses were done using SPSS version 20 (IBM).

3.2.2 Germination studies

*Selecting and sourcing seed*

Species chosen to represent each climate zone included at least two representatives from each legume subfamily, the CAESALPINIOIDEAE, PAPILIONOIDEAE, and MIMOSOIDEAE. Climate zones corresponded to the meta-analysis (Fig. 3.1) (Bureau of Meteorology 2005). Species were selected to include a broad range of habitats and geographical locations within each climate zone. Habitats were determined using online herbariums from NSW, WA, QLD, and the Commonwealth of Australia (WAH 1998; Trust 1999; Hyland et al. 2010; ABRS 2011; CHAH 2013). Species chosen to represent the hot humid (tropical) or hot dry (arid) regions totalled 9 and 12, respectively, and 27 species were chosen to represent the warm (temperate) climate zone. Seeds were purchased from the Australian Seed Company (Hazelbrook, NSW), Nindethana (Albany, WA), Kimseed (Osborne Park, WA), Goulburn Broken Indigenous Seedbank (Dookie, VIC) and AustraHort Seed Merchants (Cleveland, QLD). Origin and collection dates were provided for most species and used to confirm the seeds were obtained from the intended climate zone and were less than 10 years old. Table 3.1 lists all the species of woody legumes and their respective habitats used in this experiment.
Table 3.1 Species of woody legumes used to compare threshold temperatures to break seed dormancy. Abbreviations – MIM: MIMOSOIDEAE; CAES: CAESALPINIOIDEAE; PAP: PAPILIONOIDEAE; HH: Hot Humid; HD: Hot Dry; W: Warm; Rf: rainforest; Scl: sclerophyll; OF: open forest; <_m: elevation above sea level; MF: montane forest; VT: vine thicket; CF: closed forest; C: coastal; Wd: woodland; Shrub: shrubland TF: tall forest; MV: mountain valley; He: heath; JF: Jarrah forest (WAH 1998; Trust 1999; Hyland et al. 2010; ABRS 2011; CHAH 2013).

<table>
<thead>
<tr>
<th>#</th>
<th>Climate zone</th>
<th>Sub-Family</th>
<th>Species</th>
<th>Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HH</td>
<td>MIM</td>
<td><em>Acacia aulacocarpa</em></td>
<td>Dry Rf, Wet Scl</td>
</tr>
<tr>
<td>2</td>
<td>HH</td>
<td>MIM</td>
<td><em>Acacia cinnamnata</em></td>
<td>Rf margin, OF, &lt;800m</td>
</tr>
<tr>
<td>3</td>
<td>HH</td>
<td>MIM</td>
<td><em>Acacia crassicarpa</em></td>
<td>C, OF, Rf margin</td>
</tr>
<tr>
<td>4</td>
<td>HH</td>
<td>MIM</td>
<td><em>Acacia flavescens</em></td>
<td>OF, MF, Rf margin, &lt;1000m</td>
</tr>
<tr>
<td>5</td>
<td>HH</td>
<td>MIM</td>
<td><em>Acacia holosericea</em></td>
<td>OF, MF, VT, &lt;900m</td>
</tr>
<tr>
<td>6</td>
<td>HH</td>
<td>MIM</td>
<td><em>Acacia mangium</em></td>
<td>Rf, Disturbed, &lt;750m</td>
</tr>
<tr>
<td>7</td>
<td>HH</td>
<td>MIM</td>
<td><em>Acacia shirleyi</em></td>
<td>MF, Dry scrub, 100-600m</td>
</tr>
<tr>
<td>8</td>
<td>HH</td>
<td>CAES</td>
<td><em>Bauhinia cunninghamii</em></td>
<td>OF, MF, CF, &lt;500m</td>
</tr>
<tr>
<td>9</td>
<td>HH</td>
<td>PAP</td>
<td><em>Sesbania formosa</em></td>
<td>Rf, CF, &lt;100m</td>
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<tr>
<td>10</td>
<td>HD</td>
<td>MIM</td>
<td><em>Acacia aneura</em></td>
<td>OF, Wd, Shrub</td>
</tr>
<tr>
<td>11</td>
<td>HD</td>
<td>MIM</td>
<td><em>Acacia burkittii</em></td>
<td>Wd, Shrub</td>
</tr>
<tr>
<td>12</td>
<td>HD</td>
<td>MIM</td>
<td><em>Acacia murrayana</em></td>
<td>Wd, Scrub, Mallee</td>
</tr>
<tr>
<td>13</td>
<td>HD</td>
<td>MIM</td>
<td><em>Acacia ramulosa</em></td>
<td>C, Wd, Shrub</td>
</tr>
<tr>
<td>14</td>
<td>HD</td>
<td>MIM</td>
<td><em>Acacia papyrocarpa</em></td>
<td>C, Wd, Shrub</td>
</tr>
<tr>
<td>15</td>
<td>HD</td>
<td>MIM</td>
<td><em>Acacia tenuissima</em></td>
<td>Scree, Ranges</td>
</tr>
<tr>
<td>16</td>
<td>HD</td>
<td>MIM</td>
<td><em>Acacia victoriae</em></td>
<td>Dry creek beds, flats</td>
</tr>
<tr>
<td>17</td>
<td>HD</td>
<td>PAP</td>
<td><em>Bossiaea walkeri</em></td>
<td>Mallee</td>
</tr>
<tr>
<td>18</td>
<td>HD</td>
<td>CAES</td>
<td><em>Senna artemisoides ssp filifolia</em></td>
<td>Plains, Mallee</td>
</tr>
<tr>
<td>19</td>
<td>HD</td>
<td>CAES</td>
<td><em>Lysiphyllum gilvum</em></td>
<td>Dry creek beds, Flats</td>
</tr>
<tr>
<td>20</td>
<td>HD</td>
<td>PAP</td>
<td><em>Daviesia ulicifolia</em></td>
<td>Dry Scl, Sand dunes</td>
</tr>
<tr>
<td>21</td>
<td>HD</td>
<td>CAES</td>
<td><em>Senna notabilis</em></td>
<td>Rocky hills, Desert</td>
</tr>
<tr>
<td>22</td>
<td>W</td>
<td>MIM</td>
<td><em>Acacia alata</em></td>
<td>Rocky hills, Salt pans</td>
</tr>
<tr>
<td>23</td>
<td>W</td>
<td>MIM</td>
<td><em>Acacia dealbata</em></td>
<td>MF, nr streams</td>
</tr>
<tr>
<td>24</td>
<td>W</td>
<td>MIM</td>
<td><em>Acacia longifolia var soporae</em></td>
<td>C, Sand dunes</td>
</tr>
<tr>
<td></td>
<td>Key</td>
<td>Location</td>
<td>Species</td>
<td>Characteristics</td>
</tr>
<tr>
<td>---</td>
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<td>----------</td>
<td>--------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>4</td>
<td>W</td>
<td>MIM</td>
<td><em>Acacia mearnsii</em></td>
<td>C, OF, Cleared areas</td>
</tr>
<tr>
<td>5</td>
<td>W</td>
<td>MIM</td>
<td><em>Acacia melanoxylon</em></td>
<td>C, TF, MV, rain&gt;600mm</td>
</tr>
<tr>
<td>6</td>
<td>W</td>
<td>MIM</td>
<td><em>Acacia myrtifolia</em></td>
<td>C He, low OF, Mallee</td>
</tr>
<tr>
<td>7</td>
<td>W</td>
<td>MIM</td>
<td><em>Acacia obliquinervia</em></td>
<td>MF, 500-1700m</td>
</tr>
<tr>
<td>8</td>
<td>W</td>
<td>MIM</td>
<td><em>Acacia oxycedrus</em></td>
<td>Dry Sel, Dry He</td>
</tr>
<tr>
<td>9</td>
<td>W</td>
<td>MIM</td>
<td><em>Acacia pentadenia</em></td>
<td>Swampy</td>
</tr>
<tr>
<td>10</td>
<td>W</td>
<td>MIM</td>
<td><em>Acacia suaveolens</em></td>
<td>He, Wd</td>
</tr>
<tr>
<td>11</td>
<td>W</td>
<td>MIM</td>
<td><em>Acacia urophylla</em></td>
<td>Creeklines</td>
</tr>
<tr>
<td>12</td>
<td>W</td>
<td>MIM</td>
<td><em>Acacia verniciflora</em></td>
<td>Dry OF, MF</td>
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<tr>
<td>13</td>
<td>W</td>
<td>PAP</td>
<td><em>Bossiaea foliosa</em></td>
<td>high MF, nr streams</td>
</tr>
<tr>
<td>14</td>
<td>W</td>
<td>PAP</td>
<td><em>Daviesia cordata</em></td>
<td>JF</td>
</tr>
<tr>
<td>15</td>
<td>W</td>
<td>PAP</td>
<td><em>Dillwynia glaberrima</em></td>
<td>OF</td>
</tr>
<tr>
<td>16</td>
<td>W</td>
<td>PAP</td>
<td><em>Glycine clandestina</em></td>
<td>Moist, Sheltered</td>
</tr>
<tr>
<td>17</td>
<td>W</td>
<td>PAP</td>
<td><em>Gompholobium marginatum</em></td>
<td>C, JF, Mallee</td>
</tr>
<tr>
<td>18</td>
<td>W</td>
<td>PAP</td>
<td><em>Gompholobium tomentosum</em></td>
<td>C</td>
</tr>
<tr>
<td>19</td>
<td>W</td>
<td>PAP</td>
<td><em>Goodia lotifolia</em></td>
<td>MF, Disturbed</td>
</tr>
<tr>
<td>20</td>
<td>W</td>
<td>PAP</td>
<td><em>Hovea ellipitica</em></td>
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</tr>
<tr>
<td>21</td>
<td>W</td>
<td>PAP</td>
<td><em>Mirbelia dilatata</em></td>
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<tr>
<td>22</td>
<td>W</td>
<td>PAP</td>
<td><em>Kennedia prostrata</em></td>
<td>He, Wd</td>
</tr>
<tr>
<td>23</td>
<td>W</td>
<td>PAP</td>
<td><em>Kennedia rubicundra</em></td>
<td>F, Wd, C</td>
</tr>
<tr>
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<td>W</td>
<td>MIM</td>
<td><em>Paraserianthes lophantha</em></td>
<td>Swamps</td>
</tr>
<tr>
<td>25</td>
<td>W</td>
<td>PAP</td>
<td><em>Pultenaea stricta</em></td>
<td>Wet He</td>
</tr>
<tr>
<td>26</td>
<td>W</td>
<td>CAES</td>
<td><em>Senna barclayana</em></td>
<td>OF, Floodplains</td>
</tr>
<tr>
<td>27</td>
<td>W</td>
<td>CAES</td>
<td><em>Senna odorata</em></td>
<td>Rf margin, Sel</td>
</tr>
</tbody>
</table>
Before treating seeds with heat, seed coats were sanitised to minimise fungal or bacterial colonies growing in the germination dishes. All seeds were placed in mesh strainers and immersed in 1% sodium hypochlorite for 4 min, rinsed with distilled water (dH$_2$O) for 2 min, immersed in 70% ethanol for 30s, and rinsed again in dH$_2$O for 2 min. Seeds were spread evenly over a Petri dish and allowed to air-dry uncovered at room temperature before subsequent treatment.

Seeds were subjected to a total of 16 heat applications that were completed for each species in random order within a single day. Heat treatments were applied conjointly to four species, including two from the W climate zone and one each from HH and HD climate zones. The heat treatments included 25 °C to act as a control, and 50, 75, and 90 °C temperatures, each applied for 10 or 60 min using either wet or dry heat. For the wet heat treatment, seeds were placed in mesh strainers and suspended in a water bath. For the dry heat treatment, seeds were placed onto light-coloured, shallow ceramic crucibles (Fig. 3.2) and incubated in a dry oven for the required time at the specified temperature. Sample sizes were 10-25 seeds per treatment, depending on seed availability. Replications were not done for individual species, because the number of species tested made this logistically impossible. In addition, the comparison of germination was made between climate zones represented by multiple species. Thus individual species were considered to act as replicates for the climate zone.
Figure 3.2 The two methods used to exposure seeds to heat. (A) To apply wet heat, seeds were placed in a mesh strainer and suspended into a water bath. (B) To apply dry heat, seeds were placed onto light-coloured, shallow ceramic crucibles and heated in a dry oven.

When seeds were removed from the heat source, they were allowed to cool to room temperature within the strainer or crucible. Cooled seeds were spread over a 60 mm Petri dish containing a Whatman 1 filter paper soaked with 2 mL 50 µM CuSO₄ to discourage bacterial and fungal growth. Dishes were sealed using Parafilm M sealing film (Bernis Company Inc, Chicago, USA) to prevent evaporation. Dishes were kept at 20 °C for 4 weeks in commercial incubator, except for approximately 1h per day when they were examined for signs of germination. A 4-week period was chosen to maintain consistency with other germination studies (e.g., Hodgkinson & Oxley 1990; Auld & O’Connell 1991; Bell et al. 1995; Morrison et al. 1998). Placement of dishes within the incubator was changed randomly on a daily basis to minimise any positional effects due to unequal conditions. Seeds were considered to have germinated when the radicle had visibly penetrated the seed coat (Fig. 3.3A), and germinated seeds were removed from the Petri dish. Additional 50 µM CuSO₄ was then added as needed to the Petri dish to keep any remaining seeds moist. Although sanitary methods and 50 µM CuSO₄ were used, fungal
growth occurred in nearly every Petri dish after the first or second week. Despite the fungal growth, seeds continued to germinate. Fungal growth was not considered to have selectively altered the outcome of percentage germination for any species or climate region because all species experienced fungal growth in almost every dish, including controls, and the percentage of germination remained low in control dishes. After 4 weeks, remaining seeds were nicked using a razor blade and soaked in 1% tetrazolium dye (Sigma Australia) and incubated in the dark for 48 h at 20 °C as detailed by Williams (2003). Seeds were then cut through the midline and examined for staining. A pink colour throughout the embryo indicated a viable seed, whereas a non-viable seed remained white or cream-coloured (Fig. 3.3B). When only the surface tissue near the nick was pink, this was deemed to be evidence of a bacterial or fungal infection, and seeds were considered non-viable.

**Figure 3.3** Examples of visual cues used to determine if seeds had germinated or were viable. (A) When the radicle had visibly penetrated the seed testa (arrow), seeds were considered to have germinated. (B) Following a 48-h exposure to 1% tetrazolium dye, viable seeds were stained pink. Non-viable seeds remained white or cream-coloured.
The proportion (%) of viable seeds that germinated was the primary measure of breaking seed dormancy. In many treatments, some seeds became soft and rotten without germinating. This suggested the seeds were either non-viable prior to treatment or the heat application killed the seed. To distinguish between these possibilities, the number of rotten seeds found in the respective control treatment was considered to represent the average number of non-viable seeds in each treatment group, and this number was subtracted from the number of rotten seeds per treatment to give the adjusted germination percentage. Any remaining rotten seeds were considered to have been viable and killed by the application of heat. Although the estimation of viable seed may have been underestimated, limited numbers of seed for several species precluded testing for viability before the germination experiment was conducted. Testing for viability after the experiment has been previously by several groups (Auld 1986b; Grice & Westoby 1987; Auld & O’Connell 1991; Letnic et al. 2000; Williams et al. 2003; Campbell & Clarke 2006; Van Klinken et al. 2006), thus it was considered to be an acceptable method for use in this experiment.

The proportions of viable seeds from each climate zone that germinated successfully were compared using GEE with a negative binomial log link and exchangeable data structure. A significant interaction between climate zone, application method, exposure time and temperature was detected using GEE. As a result, pairwise comparisons were done using a sequential Sidak correction for multiple comparisons. Comparisons within climate zones across temperatures are shown in a separate figure from comparisons across climate zones within temperatures. A P value less than 0.05 was considered significant, and all analyses were done using SPSS version 20 (IBM).
3.3 Results

3.3.1 Meta-analysis

Considerably more data investigating optimal temperature thresholds to break seed dormancy has been published for temperate (W) than tropical (HH) or arid species (HD) of woody legumes. Table 3.2 illustrates that the HH and HD zones typically had low sample sizes, particularly within the wet-heat treatment group. Generalised estimating equations (GEE) showed a four-way interaction between temperature, wet or dry heat treatment, length of exposure to heat, and climate zone significantly affected the proportion of seeds that germinated ($P < 0.01$, 36 df, Wald Chi-Square = 4.14$^{11}$).

Pairwise comparisons of the available data suggested that different climate zones demonstrated distinct response patterns to increasing temperatures and exposure times (Table 3.2). The lowest temperature and shortest exposure to permit optimal germination for species from W zones was 65—85 °C for 0.5—10 min, regardless of wet or dry heat application. If dry heat was applied, species from HD climates showed optimal treatment thresholds at temperatures less than 65 °C for several hours. When exposed to wet heat, temperatures fluctuating daily with 65—85 °C maximums were optimal for species from HD zones. Significant differences were not detected between groups for species from HH climates. However, the highest average proportion of germination occurred when HH species were treated with dry heat at 65—85 °C for 30—60 min, or less than 65 °C wet heat for 30—60 min.
Table 3.2 Mean± standard error and n for germination of seeds of Australian woody legumes at all time and temperature ranges and wet and dry heat applications used in the meta-analysis. Means were calculated using every relevant mean proportion (n) of viable seeds that germinated reported in each study as listed in Appendix 3.1. The interaction between climate zone, application method, exposure time (T1—T5), and temperature was investigated using GEE, and significant differences were detected using pairwise comparisons. Significant differences between groups within each climate zone and application method are indicated by different letters. HH: Hot Humid; HD: Hot Dry; W: Warm; m: minutes; n/a: no data available.

<table>
<thead>
<tr>
<th>Climate zone</th>
<th>Application method</th>
<th>Temp (°C)</th>
<th>0.5—10 m (T1)</th>
<th>11—30 m (T2)</th>
<th>31—60 m (T3)</th>
<th>61—720 m (T4)</th>
<th>&gt;720 m (T5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HH Dry</td>
<td>&lt;65</td>
<td>5.9 ± 0.7</td>
<td>n/a</td>
<td>14</td>
<td>n/a</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>HH Dry</td>
<td>65—85</td>
<td>8.9 ± 2.3</td>
<td>n/a</td>
<td>12</td>
<td>n/a</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>HH Dry</td>
<td>&gt;85</td>
<td>3.4 ± 1.6</td>
<td>n/a</td>
<td>12</td>
<td>n/a</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>HH Wet</td>
<td>&lt;65</td>
<td>27.7 ± 10.6</td>
<td>n/a</td>
<td>4</td>
<td>n/a</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>HH Wet</td>
<td>65—85</td>
<td>16.3 ± 5.4</td>
<td>n/a</td>
<td>4</td>
<td>n/a</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>HH Wet</td>
<td>&gt;85</td>
<td>65.0 ± 0.0</td>
<td>n/a</td>
<td>1</td>
<td>n/a</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>HD Dry</td>
<td>&lt;65</td>
<td>n/a</td>
<td>0</td>
<td>n/a</td>
<td>0</td>
<td>66.7 ± 6.0</td>
<td>69</td>
</tr>
<tr>
<td>HD Dry</td>
<td>65—85</td>
<td>3.0 ± 0.0</td>
<td>n/a</td>
<td>1</td>
<td>n/a</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>HD Dry</td>
<td>&gt;85</td>
<td>9.3 ± 3.3</td>
<td>28.3 ± 24.4</td>
<td>3</td>
<td>0.0 ± 0.0</td>
<td>2</td>
<td>33.0 ± 0.0</td>
</tr>
<tr>
<td>HD Wet</td>
<td>&lt;65</td>
<td>35.0 ± 0.0</td>
<td>n/a</td>
<td>1</td>
<td>n/a</td>
<td>0</td>
<td>25.3 ± 10.6</td>
</tr>
<tr>
<td>HD Wet</td>
<td>65—85</td>
<td>25.5 ± 12.4</td>
<td>n/a</td>
<td>2</td>
<td>n/a</td>
<td>0</td>
<td>51.0 ± 7.7</td>
</tr>
<tr>
<td>HD Wet</td>
<td>&gt;85</td>
<td>39.7 ± 14.8</td>
<td>9</td>
<td>3</td>
<td>n/a</td>
<td>0</td>
<td>57.0 ± 6.4</td>
</tr>
<tr>
<td>W Dry</td>
<td>&lt;65</td>
<td>33.1 ± 4.1</td>
<td>260</td>
<td>33.2 ± 4.7</td>
<td>73</td>
<td>32.3 ± 4.7</td>
<td>80</td>
</tr>
<tr>
<td>W Dry</td>
<td>65—85</td>
<td>63.7 ± 5.3</td>
<td>147</td>
<td>69.9 ± 4.4</td>
<td>44</td>
<td>78.5 ± 3.7</td>
<td>38</td>
</tr>
<tr>
<td>W Dry</td>
<td>&gt;85</td>
<td>43.0 ± 3.0</td>
<td>231</td>
<td>46.9 ± 3.9</td>
<td>83</td>
<td>38.9 ± 4.0</td>
<td>74</td>
</tr>
<tr>
<td>W Wet</td>
<td>&lt;65</td>
<td>22.1 ± 1.4</td>
<td>20</td>
<td>33.0 ± 0.0</td>
<td>4</td>
<td>41.0 ± 0.0</td>
<td>2</td>
</tr>
<tr>
<td>W Wet</td>
<td>65—85</td>
<td>59.1 ± 3.1</td>
<td>20</td>
<td>69.3 ± 0.0</td>
<td>3</td>
<td>85.0 ± 0.0</td>
<td>1</td>
</tr>
<tr>
<td>W Wet</td>
<td>&gt;85</td>
<td>45.6 ± 4.2</td>
<td>211</td>
<td>76.0 ± 3.9</td>
<td>8</td>
<td>27.5 ± 12.4</td>
<td>2</td>
</tr>
</tbody>
</table>
The results for species from each climate zone treated with dry heat did not change when data was grouped across all exposure times to increase sample sizes (Fig. 3.4). Comparisons between climate zones suggested that temperatures less than 65 °C applied as dry heat to HD species were equally effective as 65—85 °C dry heat applied to W species. When wet heat was used, significant differences were not detected between HD and W climate zones, and species from HH regions only showed a significantly lower percentage germination at temperatures of 65—85 °C (Fig. 3.4).

![Figure 3.4](image)

**Figure 3.4** Grouped results from a meta-analysis of threshold temperatures to break dormancy of seeds from Australian woody legumes. Mean percentage (± standard error) of viable seed germinating following exposure to dry (A) or wet (B) heat application at three temperature ranges. Pairwise comparisons were done between temperature ranges and climate zones (see Fig. 3.1) for germination responses averaged across all exposure times. Bars with different letters are significantly different ($P<0.05$) from each other. HH: Hot Humid; HD: Hot Dry; W: Warm
3.3.2. Germination study

Dry heat and optimal temperature thresholds

Climate zone, application method, exposure time, and temperature interacted significantly to alter the proportions of woody legume seeds that germinated ($P<0.01$, 35 df, Wald Chi-Square 7498.5). Woody legume species from the three climate zones responded differently to both increasing temperature and the method used to apply heat. When dry heat was applied, significant differences were not detected using pairwise comparisons for species from HH or HD climates. However, the highest values for HH species were obtained after incubation at 90 °C for 10 or 60 min. Species from HD zones showed the highest mean values after dry heat at 75 °C for 10 min or 90 °C for 60 min. A significant optimum threshold for species from W climates was 75 °C for 10 or 60-min incubations, although exposure to 90 °C produced the highest means (Fig. 3.5A, C). The patterns for all three climate zones reiterated what had been found for the meta-analysis (Table 3.2).

Wet heat and optimal temperature thresholds

The duration of exposure to wet heat altered the overall threshold temperature for species from HD and HH climates (Fig. 3.5B, D). Species from HD zones responded best to incubation at 75 °C for 10 min, but a 60 min exposure reduced their threshold temperature to 50 °C. Statistically, the threshold for HH species was 50 °C for 10 min, but no threshold was detected for the 60-min incubations. Mean values for HH species exposed to wet heat were greatest at 75 °C for either 10 or 60 min. Species from W climates showed optimum germination after incubation at 75 °C for either 10 or 60 min (Fig. 3.5B, D). Direct comparisons between climate zones only detected a significant difference when wet heat was applied for 60 min (Fig. 3.6).
Figure 3.5 Effect of temperature in breaking dormancy as measured by germination success of woody legume seeds from three different climate zones. Mean percentage (± standard error) of viable seed germinating following exposure to dry (A, C) or wet (B, D) heat for 10 (A, B) or 60 (C, D) min. Bars that have different letters within each climate zone are significantly different ($P<0.05$) from each other. HH: Hot Humid; HD: Hot Dry; W: Warm.
Figure 3.6 Germination success following heat treatment of dormant woody legume seeds compared across climate zones. Note that comparisons of different temperatures within climate zones are shown in Fig. 3.5. Mean percentage (± standard error) of viable seed germinating following exposure to dry (A, C) or wet (B, D) heat for 10 (A, B) or 60 (C, D) min. Bars that have different letters were significantly different (P<0.05) from each other according to GEE pairwise comparisons with a sequential Sidak correction. 

Response of Acacia species

To test whether the inclusion of species from PAPILIONOIDEAE and/or CAESALPINIOIDEAE altered the results, GEE was done on the germination data from Acacia species. Acacias demonstrated similar temperature thresholds to what was detected for all woody legumes species combined (Fig. 3.7). Acacias from HH climates showed a significantly greater percentage of germination when 60-min exposure to 75°C dry heat was compared to 50 °C dry heat (Fig. 3.7C).
Figure 3.7 The effect of temperature on germination of seeds from *Acacia* species from three different climate zones. Mean percentage (± standard error) of viable seed germinating following exposure to dry (A, C) or wet (B, D) heat for 10 (A, B) or 60 (C, D) min. Bars that have different letters were significantly different (P<0.05) from each other. HH: Hot Humid; HD: Hot Dry; W: Warm.

*Lethal temperatures*

A significant four-way interaction was found when the proportion of viable seeds that rotted following exposure to increasing temperatures were compared (P<0.01, 36 df, Wald Chi-Square 6.04e12). Pairwise comparisons detected a significant effect only for W species incubated at 90 °C wet heat for 60 min. Exposure to 90 °C wet heat produced higher means for HH and HD species after 10 or 60-min, but pairwise comparisons showed that these effects were not significant (P>0.05; Fig. 3.8).
Figure 3.8 The effect of increasing temperature to kill seeds of woody legumes from three different climate zones. Mean percentage (± standard error) of viable seed killed following exposure to dry (A, C) or wet (B, D) heat for 10 (A, B) or 60 (C, D) min. Bars that have different letters were significantly different \((P<0.05)\) from each other according to pairwise comparisons done using GEE. HH: Hot Humid; HD: Hot Dry; W: Warm.

3.4 Discussion

3.4.1. Climate zones and minimum temperatures for optimal germination

Results from the meta-analysis and germination study supported the hypothesis that the minimum temperatures to break seed dormancy and maximise the percentage of germination vary between climate zones defined by temperature and humidity. In addition, the germination study largely confirmed what was found in the meta-analysis, despite the latter having groups with small sample sizes or missing data. A central finding from both studies was that species from HD climates generally exhibited lower minimum
temperatures to optimise germination than species from W climates, and species from HH climates showed minimal to no response to treatment with dry heat, unless only species of *Acacia* were considered.

Temperatures between 65—85 °C were sufficient to permit an optimal percentage of germination for W species in most cases examined in this study. In the germination experiment, exposure to 90 °C was equally effective as 75 °C providing the duration was short. Long incubations in a 90 °C water bath significantly increased seed mortality, and temperatures above 85 °C in the meta-analysis were generally less effective to permit germination compared to the 65—85 °C temperature range. These results concur with data published for many temperate legume species (e.g., Auld & O'Connell 1991; Morrison *et al*. 1992; Bell 1999).

To heat soil below 1 cm depth above 65 °C requires moderate to high intensity fires or a long flame residence (Beadle 1940; Shea *et al*. 1979; Cheney 1981; Auld 1986b; Bradstock & Auld 1995; Williams *et al*. 2004). As seeds from temperate woody legume species occur predominately in the top 6 cm of soil (Shea *et al*. 1979; Auld 1986b; Bradstock & Auld 1995; Auld & Denham 2006), they are likely to require considerable combustion of fuel to heat the soil enough to break seed dormancy (Christensen *et al*. 1981; Clark 1988; Auld & O'Connell 1991; Bradstock & Auld 1995; Crews 1999; Orians & Milewski 2007). Furthermore, the greatest number of seedlings of temperate species emerge from soil depths between 2—5 cm, suggesting that seeds in the top 2 cm of soil, which can reach temperatures over 90 °C during severe fires (see Beadle 1940; Shea *et al*. 1979), experience mortality when conditions are otherwise suited for wide-spread germination of woody legumes (Shea *et al*. 1979; Auld 1986b; Bradstock & Auld 1995; Auld & Denham 2006). Overall, this suggests that woody legume species from temperate
climates have adaptations that promote their persistence in areas that experience infrequent, high-intensity fires.

Species from HD climates generally exhibited lower minimum temperatures to maximise germination than W species. The meta-analysis suggested exposure to dry heat below 65 °C for several hours, or wet heat fluctuating cyclically to 65—85 °C for several days was optimum, although small sample sizes limited interpretation of these results. Dry heat was statistically ineffectual for HD species in the germination study, but the exposure time was limited to 60 min, whereas 61—720 min incubations characterised the optimum germination following dry heat in the meta-analysis. Notably, HD species responded well to cool to moderate wet heat applied for longer durations in both the germination study and meta-analysis. The highest mean values in the meta-analysis occurred when HD species were subjected to wet heat fluctuating every 12 h between 65—85 °C and 18—25 °C in a regime intended to replicate heat from solar radiation. Similarly, 50 °C was statistically optimum for the 60-min incubation in a water bath in the germination experiment.

Lower temperatures during a burn occur deeper in the soil profile or if fires are low intensity (see Bradstock & Auld 1995; Williams et al. 2004). Alternatively, solar radiation can heat upper soil horizons to 60 °C when vegetation is absent (Hagon 1971; Auld & Bradstock 1996; Van Klinken et al. 2006). Long exposure to a moderate temperature implicates solar radiation as a primary source of heat. The requirement that HD seeds be heated in wet conditions to break dormancy indicates moisture availability is an important cue for these species. Thus, seeds from species living in a hot dry environment deposited in open areas and lying near the soil surface would be cued to germinate by substantial precipitation, which might not correspond with a fire event (Orscheg & Enright 2011). Corresponding to this, a previous study found wide-spread germination
of seeds of western myall (*Acacia sowdenii*) in arid Australia after inundation (Lange & Purdie 1976). A limited or negligible response to dry heat at cooler temperatures would help ensure dormancy was only broken when sufficient moisture was present. Species from HD climates also did not demonstrate significant mortality when exposed to high temperatures of dry heat. Together, these data suggest that solar radiation following soil inundation may provide sufficient heat to break seed dormancy of species from hot and dry or arid climates.

Species from HH climates showed equivalent germination across the range of temperatures applied using dry heat. However, neither the meta-analysis nor germination study tested exposures longer than 60-min duration, which was necessary for HD species to respond to dry heat. Short-term exposure to wet heat suggested that 50 °C was the minimum temperature for optimal germination, but the highest mean proportion of germination occurred following exposure to over 85 °C in both the meta-analysis and germination study. For longer incubations, 65—85 °C stimulated the most germination of HH species, but the effect was not significant.

A more distinct pattern of germination responses became apparent when only species of *Acacia* from HH climates were analysed. A minimum temperature of 75 °C maximised germination when wet heat was applied for either duration or dry heat was applied for 60 min. The HH group originally included one species each from the CAESALPINIOIDEAE and PAPILIONOIDEAE subfamilies. Testa anatomy of PAPILIONOIDEAE species includes a counter-palisade that typically opens when the relative humidity is lower than the moisture content of the seed to facilitate seed drying, but gradually increasing relative humidity could cause PAPILIONOIDEAE seeds to lose physical dormancy (Hyde 1954; Rolston 1978). Accordingly, a study found that lower minimum temperatures permitted
an optimum percentage of seeds from a species of PAPILIONOIDEAE to break their
dormancy compared to species of Acacia in tropical climates (Hopkins & Graham 1984).

3.4.2 Disturbance regimes and germination responses

Many species of woody legumes produce seeds that can remain dormant in the soil for
years to decades (Morrison et al. 1992), and both wet and dry applications of heat have
relevance to field conditions. While fire is a primary source of the heat needed to break
dormancy (Shea et al. 1979; Auld 1986b; Bradstock & Auld 1995; Williams et al. 2004),
solar radiation can also significantly raise soil temperatures (Auld & Bradstock 1996).
Solar radiation coupled with rainfall can provide heat under moist or wet conditions, but
unplanned fires are most common during periods when the soil profile lacks moisture.
Some authors have reported that the minimum temperature required to break dormancy
may be further reduced if there is abundant soil moisture (Cushwa et al. 1968; Martin et
al. 1975; Portlock et al. 1990), while others have found no difference between wet and dry
heat applications (Harshbarger et al. 1975; Hodgkinson & Oxley 1990). Due to the
uncertainty in the literature, both wet and dry heat treatments were compared in the
germination study.

Location in the soil profile affects the temperatures experienced by woody legume
seeds. Most woody legume seeds have either unassisted dispersal or are myrmecochores,
dispersed by ants that are attracted to the lipid-rich aril or elaiosome attached to the seed
(Berg 1975; Hughes & Westoby 1990; Jurado et al. 1991; Rodgerson 1998). Arils may be
removed by ants from seeds while still aboveground or within the nest, and seeds from
both arid and temperate legumes can be found on ant mounds (Davidson & Morton
1984; Hughes & Westoby 1992; Campbell & Clarke 2006). This suggests dispersal
patterns are potentially similar for species from both climate regions. It does not appear
that seeds from legume species found in arid climates are simply buried deeper in the soil profile than seeds from temperate regions.

A recent pyrogeographic model of Australia illustrated that typical frequency and intensity of fires vary between climatic regions (Murphy et al. 2013). Temperate (W) regions have a median fire intensity equivalent to moderate to high fire intensities able to scorch or burn the crown layer (Cheney 1981; Christensen et al. 1981). Tropical (HH) and arid (HD) regions have median fire intensities ranging from low to moderate, however the median interval between fires is much shorter for HH compared to HD climate zones (Table 3.7). Thus the gradient for fire intensity is likely to be W>HH and HD, and for frequency is HH >W and HD.

Table 3.7 Median fire intensities and intervals for different climate zones in Australia. All upper and lower thresholds for each vegetation community under typical conditions were used to calculate medians. Data adapted from Murphy et al. (2013).

<table>
<thead>
<tr>
<th>Climate zone</th>
<th>Median fire intensity (kW m⁻¹)</th>
<th>Median fire interval (yr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>1000–5000</td>
<td>20–100</td>
</tr>
<tr>
<td>HH</td>
<td>100–1000</td>
<td>5–20</td>
</tr>
<tr>
<td>HD</td>
<td>100–1000</td>
<td>20–100</td>
</tr>
</tbody>
</table>

The model by Murphy et al. (2013) agrees with other studies that found many temperate sclerophyll communities in Australia accumulate enough biomass to fuel moderate-intensity fires every 5 to 10 years (Walker 1981; Gould et al. 2008; Penman & York 2010), but other factors such as moisture availability increases return intervals of
intense fires in this climate zone from 20 to 100 years (Keeley & Fotheringham 2000; Hodgkinson 2002; Murphy et al. 2013). Corresponding to an extended fire interval, most woody legume species growing in temperate regions complete their life-cycles within 20 years (Hansen et al. 1987; Crews 1999; Grant et al. 2007). This temporal convergence theoretically allows woody legumes to fully replenish the soil seedbank before the next fire cue. Fires capable of heating the upper soil layers to 90 °C are also likely to decrease the canopy cover, thereby temporarily increasing light availability for seedlings (Christensen et al. 1981). By combusting litter and woody debris, fire also deposits nutrient-rich ash into the soil (Christensen et al. 1981; Humphreys & Craig 1981; Whelan 1995; Certini 2005; Escudery et al. 2010). The greater light availability and concentrations of soil nutrients after a moderate or high-intensity fire can help create an environment conducive to developing plants. A positive relationship has also been found between increasing fire intensity and the numbers of woody legumes that germinate after fire (Williams et al. 2004).

Optimal minimum temperatures for HD species suggest that either fire or solar radiation can provide the heat needed to break seed dormancy. The hummock grasslands and acacia shrublands common to arid (HD) communities create less contiguous fuel layers than tropical or temperate regions (Russell-Smith et al. 2007; Murphy et al. 2013). Arid climates dominated by *Acacia* species often experience fire intensities less than 100 kW m⁻¹ at 20–100-yr intervals (Murphy et al. 2013). These fires may generate insufficient heat to break dormancy of seeds, particularly if the upper soil layers are dry. Inundation followed by solar radiation would reach the minimum temperatures required (Hagon 1971; Auld & Bradstock 1996; Van Klinken et al. 2006) and provide the moist environment required for germination. Arid regions that experience higher flood frequency have also shown a greater floral diversity, indicating that land inundation
creates favourable conditions for many arid species to germinate (Westbrooke et al. 2005; Capon & Brock 2006). However, precipitation events large enough to inundate arid zones are infrequent and both seasonally and temporally unpredictable (Capon 2005). That species from the HD climate zone could germinate equally well following 75 °C dry heat and 50 °C wet heat suggests a mechanism to compensate for infrequent heat and moisture cues. Some arid species have been recorded to live for over 100 yrs (Crisp & Lange 1976; Lange & Purdie 1976), which extends well beyond the predicted frequency of fire in arid climate zones and the El-Nino Southern Oscillation, which is tied to flooding events (Westbrooke et al. 2005).

Compared to species from W climates, few studies have investigated woody legume species from HH climates in Australia. A provocative study found that seeds buried in areas without canopy cover had a significantly greater percentage of germination than seeds buried under either a tree canopy or shadecloth (Van Klinken et al. 2006). This may correspond to observations that recruitment in tropical rainforests often correspond to disturbances that create a gap in the canopy (Hubbell et al. 1999; Wright et al. 2003). The data presented in this chapter suggests that the minimum temperatures to maximise germination of species from HH climates would require greater soil heating than solar radiation could provide. While it remains possible that species from rainforest environments in HH climate zones could use canopy gaps to promote recruitment, further studies specifically tailored to address this hypothesis are needed.

This study supports the hypothesis that seed dormancy of Australian woody legumes has co-evolved with climate. Woody legumes include *Acacia*, the most specious genus in Australia, and are broadly distributed across nearly all terrestrial ecosystems on the continent (Miller et al. 2003; Orians & Milewski 2007). Identifying potential mechanisms underlying the distribution patterns of this taxon helps elucidate the processes that
structure communities in different climate zones and may further efforts to conserve species or communities facing a changing climate.
Chapter 4 – Expression of genes encoding acid phosphatases or carboxylate precursors – methodology

Abstract

The ancient and highly-weathered soils typical in Australia have low levels of available P, an essential nutrient for plants. Additional P may be sequestered in soil by organic matter, clay minerals and metallic cations, but to acquire this P requires the hydrolysis of organic phosphate esters or the chelation of minerals. Some species of herbaceous legumes have been shown to exude phosphatases or carboxylates to make recalcitrant forms of P available, but it was unknown whether Australian woody legumes express genes encoding secreted forms of phosphatases or carboxylates. In addition, the sequences of these potential genes in woody legumes were unknown. In this chapter, a method was identified to isolate purified RNA from woody legume roots that contain high concentrations of phenolic and polysaccharide compounds that hinder conventional, modern methods of RNA isolation. PCR primers were designed based on comparative homology between related genes in herbaceous legumes. Six species of woody legumes were investigated, and one species was found to express a sequence homologous to purple acid phosphatase (PAP). Furthermore, this sequence showed homology to PAPs known to be sensitive to P availability and secreted into the rhizosphere.

4.1 Introduction

4.1.1 Background

Phosphorus (P) represents one of the most essential and least available nutrients for plants. Plants incorporate P into many bio-molecules, including nucleotides, metabolites in the sucrose synthesis pathway, and all compounds involved in the Calvin cycle.
Consequently, P constitutes 0.3–0.5% of plant dry matter during the vegetative phase (Raghothama 1999). Such high usage causes 30—80% of P in most ecosystems to be held in organic monoesters including phytic acid that must be mineralised to be accessible to plants (Adams 1992; Abel et al. 2002).

In soil, metal cations and inorganic compounds can sequester P, tempered by soil development and pH. Most P in young or developing soil is held by calcium apatite minerals that slowly dissolve to release orthophosphate (P$_i$) as PO$_4^{3-}$ or H$_2$PO$_4^-$, which is the form that organisms preferentially take up. As clay soil ages, secondary silicates dissolve to release Fe and Al oxides that will surround and occlude P (Crews et al. 1995; Lambers et al. 2010). Soil acidification promotes P forming complexes with Fe and Al (Lambers et al. 2006). Thus, the concentrations plants require are often 1000-fold greater than the amount of P available, even in fertile soils (Duff et al. 1994; Raghothama 1999). Australia typically has acidic weathered soils, causing P to often be the limiting nutrient for growth (Beadle 1966).

When available P$_i$ is scarce, many plants use exudates to directly or indirectly enhance concentrations of P$_i$. Exudates can originate from cluster roots, densely-packed clusters of small lateral roots with copious root hairs that effectively mine small soil patches for P (Watt & Evans 1999; Adams et al. 2002; Vance et al. 2003; Lambers et al. 2010; Power et al. 2010). As an alternative to cluster roots, plants may form symbiotic relationships with mycorrhizal fungi, which secrete exudates from extensive hyphal networks that expand the surface area and decrease the distance P must diffuse in soil to be acquired (Bolan 1991; Pate 1994; Lambers et al. 2010). Mycorrhizal associations represent the most common method that plants use to facilitate P$_i$ acquisition. However, when P-limitation is severe, cluster roots may be used to a greater extent than mycorrhizal symbioses (Pate
Both the form of P present in soil and the age of roots may stimulate plants to vary the exudates produced. Carboxylates or organic acids are used to release P chelated by metal cations. Carboxylates can also solubilise organic forms of P ($P_o$) that remain unavailable to plants without the action of phosphatases to mineralise $P_o$ to $P_i$ (Vance et al. 2003). The stage of root development typically affects which carboxylate is exuded by cluster roots (Raghothama 1999; Vance et al. 2003; Weisskopf et al. 2006b). Developing or juvenile cluster roots exude malate, and mature cluster roots exude citrate and protons before they senesce and become relatively quiescent (Weisskopf et al. 2006b). Citrate has been shown to solubilise $P_i$ more effectively than malate (Power et al. 2010). Protons accompanying citrate exudation lower the pH within mature cluster roots to around 4, which attenuates bacteria mineralising the carboxylates as an energy source (Weisskopf et al. 2006a). Malate and citrate also function to alleviate Al-toxicity, and their release may aid acid phosphatases to hydrolyse organic forms of P (Vance et al. 2003).

Phosphatases are an important group of enzymes that increase P-availability by hydrolysing phosphoric acid monoesters into $P_i$. Phosphatases function within an optimal pH range, which classifies the enzymes as alkaline or acid phosphatases (Duff et al. 1994). Cluster roots typically exude acid phosphatases (APase), which are suited to the protonated rhizosphere and can mineralise various forms of $P_o$. Alkaline phosphatases are typically substrate-specific (Duff et al. 1994; Vance et al. 2003). Purple acid phosphatases (PAPs) comprise the largest group of plant APases and have a binuclear site containing Fe and either manganese (Mn) or zinc (Zn) (Duff et al. 1994; Olczak et al. 2003). Soil phosphate levels were shown to regulate a transcript encoding a large homodimeric purple acid phosphatase (PAP) in *Arabidopsis thaliana* (Wang et al. 2011).
Roots of *Lupinus luteus* express abundant transcripts for a PAP that is structurally similar to a PAP in *Phaseolus vulgaris* (Olczak et al. 2003). Phosphorus deficiency has also been shown to upregulate expression of a secreted acid phosphatase (sAP) in proteoid roots of *Lupinus albus* (Miller et al. 2001; Wasaki et al. 2003).

Several genes encoding APases and precursors to carboxylates have been sequenced for herbaceous legumes (Neumann & Römheld 1999; Wasaki et al. 1999; Liang et al. 2010), but no related genes have been sequenced for any Australian species of woody legume. The only woody legume to have any P-acquiring genes sequenced is *Sesbania rostrata* (Bremek. & Oberm.), a woody legume native to Africa (Aono et al. 2001). Root exudates from woody legumes have also been found to have carboxylase activity (Power et al. 2010; He et al. 2012). Immunological studies suggest the major isoforms of plant APases evolved from a common ancestral gene (Duff et al. 1994). Therefore, regions showing high homology between different species of herbaceous legumes and *S. rostrata* might also retain homology with Australian woody legumes.

4.1.2 Aim

The aim of this study was to refine and describe a methodology to determine whether woody legume roots expressed genes for either APases or carboxylate precursors.

4.2. Methods

4.2.1 Gene targets and primer design

To maximise the possibility that homology between herbaceous species would extend to woody species, the mRNA sequences of interest were compared between representatives from multiple clades of the FABACEAE family (Wink & Mohamed 2003). The two APase mRNA sequences targeted included a secreted APase (sAP) and purple
APase (PAP). The sAP corresponded to *Lupinus albus* secreted acid phosphatase 2 (*LASAP2*, GenBank accession # AB037887.1), which is induced in cluster roots by P deficiency and encodes an amino acid sequence identical to a known sAP protein (Wasaki et al. 2003). The PAP corresponded to *Phaseolus vulgaris* purple acid phosphatase 3 (*PvPAP3*, GenBank accession # FJ464333), which is induced in roots by P deficiency and was shown to increase the mineralisation of nucleic acids (dNTPs) supplied to the rhizosphere (Neumann & Römheld 1999; Liang et al. 2012b).

To investigate whether roots of woody legumes utilise carboxylates, two mRNA sequences were targeted that encode enzyme precursors for citrate and malate. Both the citrate synthase (CS, GenBank accession # AB057662) and malate dehydrogenase (MDH, GenBank accession #AJ295348) corresponded to mRNA sequences expressed in the roots of *S. rostrata* (Aono et al. 2001).

To identify conserved regions within each gene of interest, homology between the selected mRNA sequence and its counterparts in other species from the FABACEAE taxa were compared using the BLASTn (Basic Local Alignment Search Tool for nucleotides) function available on the National Center for Biotechnology Information (NCBI) website (blast.ncbi.nlm.nih.gov) (Oburger et al. 2011). The program was optimised for megablast, which only selects sequences with high similarity to the query sequence. Aligned sequences were subsequently refined to include the most homologous representatives from a minimum of three different clades. Alignment between these sequences was investigated in a pairwise fashion using BLASTn, and the results were used to align all sequences together. Homology across a minimum of four sequences from three clades was assessed manually, and nucleotides were individually marked as being conserved 100%, 75% or less than 75%.
To confirm whether PCR products displayed homology to known nucleotide and amino acid sequences, BLASTn and BLASTp from NCBI were used, respectively. The amino acid translation of the PCR product was obtained using the open reading frame (ORF) finder function in NCBI (ncbi.nlm.nih.gov/gorf/gorf.html, accessed 03/04/2014). The translated amino acid sequence was also examined for domains conserved across protein families using the conserved domain database from NCBI (ncbi.nlm.nih.gov/cdd).

The NCBI Primer-Blast tool (ncbi.nlm.nih.gov/tools/primer-blast/) was used to find candidate primers targeted to highly homologous regions for each mRNA sequence of interest (Nuruzzaman et al. 2006). A range was designated for each relevant accession number to only include the portion of the sequence that had been previously aligned between multiple clades. Potential primers were assessed according to homology of the target sequence, the percentage of guanidine and cytosine nucleotides, the melting point, and the product length, respectively. The primers that best maximised these four criteria were chosen. Any nucleotides within the primer sequence that were not 100% homologous were designed to have a degenerate sequence as dictated by the number of nucleotide variants at the respective position. Degenerate primers were created by providing a mix of sequences containing different designated nucleotides at specified positions (D'Esposito et al. 1994). Once designed, primers were obtained from Integrated DNA Technologies (IDT; Baulkham Hills, NSW). Positive controls included primers (provided by N. Wilson, University of Sydney) targeted to ribosomal 18s. Additional primers targeted to tubulin 6 or EF 1a (provided by J. Risopatron, University of Sydney) were also used as positive controls. Table 4.1 details primer sequences and specifications and projected product lengths.
Table 4.1 The four sets of primers designed to amplify product for two APases, two enzyme precursors for organic acids, and a positive control. All sequences are listed in the 5’ to 3’ direction. SAP: secreted acid phosphatase; PAP: purple acid phosphatase; CS: citrate synthase; MDH: malate dehydrogenase; T6: tubulin 6; 18s: ribosomal 18s; F: forward; R: reverse; Tm: melting point; %GC: % guanine/cytosine bases; nt: nucleotide; T: thymine; C: cytosine; A: adenosine; G: guanine; M: A or C; H: A, C or T; R: A or G; W: A or T; K: G or T; S: C or G; Y: C or T; D: A, G or T; n/a: no product expected.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Tm</th>
<th>%GC</th>
<th>RNA product (nt)</th>
<th>DNA product (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAP-F</td>
<td>5’-TCA ACC TTG GAT MTG GAC HGC HGG-3’</td>
<td>61.2</td>
<td>54.8</td>
<td>163</td>
<td>n/a</td>
</tr>
<tr>
<td>SAP-R</td>
<td>5’-TRW GCW GRK SCT CTY TTG AYW CAA T-3’</td>
<td>57.4</td>
<td>42.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAP-F</td>
<td>5’-AYA GRG GWR ATG YHR ARG CMC A-3’</td>
<td>58.0</td>
<td>49.2</td>
<td>316</td>
<td>951</td>
</tr>
<tr>
<td>PAP-R</td>
<td>5’-TCT TSD GTR TYM CCA TGA TGC CCA-3’</td>
<td>59.9</td>
<td>49.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS-F</td>
<td>5’-KGG TGT CAT GGC CCT YCA GGT-3’</td>
<td>62.7</td>
<td>61.9</td>
<td>476</td>
<td>2942</td>
</tr>
<tr>
<td>CS-R</td>
<td>5’-CAA WTG TTY WGY ACT WAT GTT-3’</td>
<td>46.7</td>
<td>28.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDH-F</td>
<td>5’-TGC HGG RCA RAT YGG RTA TGC T-3’</td>
<td>64.1</td>
<td>51.5</td>
<td>206</td>
<td>338/344</td>
</tr>
<tr>
<td>MDH-R</td>
<td>5’-ACS CCR GTG CAT GCC TCA ACC-3’</td>
<td>59.7</td>
<td>64.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T6-F</td>
<td>5’-GCT GGT GGA AAA TGC TGA TG-3’</td>
<td>57.7</td>
<td>50.0</td>
<td>163</td>
<td>n/a</td>
</tr>
<tr>
<td>T6-R</td>
<td>5’-CTA ACC CGG GAA CCT AAG AC-3’</td>
<td>57.0</td>
<td>55.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EF1a-F</td>
<td>5’-CAC GCT CTT CTT GCT TCC AC-3’</td>
<td>57.7</td>
<td>50.0</td>
<td>70</td>
<td>n/a</td>
</tr>
<tr>
<td>EF1a-R</td>
<td>5’-TGG TGG CAT CCA TCT TGT TA-3’</td>
<td>56.8</td>
<td>45.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18s-F</td>
<td>5’-ATACGTGCAACAAACCACCC-3’</td>
<td>52.5</td>
<td>47.1</td>
<td>303</td>
<td>303</td>
</tr>
<tr>
<td>18s-R</td>
<td>5’-CTACCTCCCCGTGTA-3’</td>
<td>53.8</td>
<td>62.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.2.2 Constructing cDNA libraries

Tissue collection

To determine which protocol yielded optimal mRNA, sample tissues were collected from several species of *Acacia* growing in New South Wales (NSW). The species used initially was *A. longifolia* var. *sophorae* ((Labill.) Benth.) collected from Collaroy Beach, NSW, with the sandy substrate facilitating the excavation of fine roots. Using gloved hands, the sand was gently brushed away from points of contact between supine stems and the sand until connecting roots were located (Fig. 4.1). Sand was then brushed away along the length of the root until fine roots less than 1 mm diameter were uncovered. These were excavated as thoroughly as possible without breaking their connection to the larger root. Secateurs were used to excise sections containing fine roots, which were immediately placed into 100% ethanol and stored on dry ice. Samples remained on dry ice until they were transported to the laboratory, where they were stored at -20°C until analysis. Similar excavation techniques were later used to collect fine roots from *A. suaveolens* ((Sm.) Willd.) growing in Ku-ring-gai Chase National Park, NSW, except soil was brushed away beginning at the base of the main stem (Fig. 4.1). Fine roots from potted specimens of *Vicia faba* (L.) and *Glycine max* (L.) and from uncultivated *Erharta erecta* (Lam.) growing in Alexandria, NSW were also used as positive controls.

Fine roots were collected from the field from the six species of woody legumes regenerating after the Black Saturday fires in low density populations, as described in Chapter 2. Table 4.2 lists each species and respective coordinates (waypoint) from which it was collected. To ensure adequate sample sizes for mRNA extraction, fine roots were collected from three individuals of each species and bulked into a single sample per
waypoint. Before excision, roots were verified to be growing from the stem of the target species (Fig. 4.1).

**Figure 4.1** Fine roots excavated from eight different species of woody legumes. Roots were exposed and verified to be connected to the stem of each species of woody legume (*white arrowheads*) before being excised and placed in cold 100% ethanol. (A) *Acacia longifolia var. sophorae*, (B) *A. suaveolens*, (C) *A. dealbata*, (D) *A. myrtifolia*, (E) *A. obliquinervia*, (F) *A. leprosa*, (G) *A. lanigera*, (H) *Goodia lotifolia*. 

97
Table 4.2 Locations of the species used to investigate whether fine roots of woody legumes express genes for APases or carboxylate precursors. Coordinates are in WGA 84 projection (see Chapter 2). SF: State Forest, NP: National Park.

<table>
<thead>
<tr>
<th>Waypoint</th>
<th>Species</th>
<th>Latitude</th>
<th>Longitude</th>
<th>National Park/State Forest</th>
</tr>
</thead>
<tbody>
<tr>
<td>126</td>
<td><em>Acacia dealbata</em></td>
<td>-37°32'15.7&quot;</td>
<td>145°42'28.4&quot;</td>
<td>Marysville SF</td>
</tr>
<tr>
<td>121</td>
<td><em>A. dealbata</em></td>
<td>-37°23'12.7&quot;</td>
<td>145°36'58.6&quot;</td>
<td>Black Range SF</td>
</tr>
<tr>
<td>97</td>
<td><em>A. lanigera</em></td>
<td>-37°34'51.7&quot;</td>
<td>145°21'36.2&quot;</td>
<td>Kinglake NP</td>
</tr>
<tr>
<td>117</td>
<td><em>A. leprosa</em></td>
<td>-37°23'57.4&quot;</td>
<td>145°36'28.6&quot;</td>
<td>Black Range SF</td>
</tr>
<tr>
<td>near 97</td>
<td><em>A. leprosa</em></td>
<td>-37°34'51.7&quot;</td>
<td>145°21'36.2&quot;</td>
<td>Kinglake NP</td>
</tr>
<tr>
<td>68</td>
<td><em>A. myrtifolia</em></td>
<td>-37°33'13.8&quot;</td>
<td>145°42'18.3&quot;</td>
<td>Marysville SF</td>
</tr>
<tr>
<td>84</td>
<td><em>A. obliquinervia</em></td>
<td>-37°25'53.4&quot;</td>
<td>145°39'19.1&quot;</td>
<td>Black Range SF</td>
</tr>
<tr>
<td>87</td>
<td><em>A. obliquinervia</em></td>
<td>-37°24'58.8&quot;</td>
<td>145°38'30.7&quot;</td>
<td>Black Range SF</td>
</tr>
<tr>
<td>115</td>
<td><em>Goodia lotifolia</em></td>
<td>-37°25'07.5&quot;</td>
<td>145°38'25.2&quot;</td>
<td>Black Range SF</td>
</tr>
</tbody>
</table>

_Nucleotide extraction – commercial reagents_

Two different methods using reagents from Bioline (Alexandria, NSW) were initially tested to extract total RNA from legume roots. Roots from *A. longifolia* var. *sophorae* were processed using the Isolate Plant RNA kit according to the manufacturer’s instructions. Briefly, roots were homogenised in 450 µL lysis buffer APR in a 1.5 mL Eppendorf tube using a microtube pestle (Scientific Specialties Inc; Lodi, CA). The tube was centrifuged at 13,000 rpm for 1 min and supernatant transferred to a PR1 spin column in a 2 mL collection tube. After centrifugation at 13,000 rpm for 2 min, the filtrate was mixed with 1 volume of 70% ethanol and transferred to a PR2 spin column in a collection tube. This
was centrifuged as before and the column washed with first 500 µL buffer APR and then 650 µL buffer BPR with intervening centrifugations at 13,000 rpm for 1 min. The product was eluted using 50 µL RNase-free water.

Roots from *G. max* and *A. longifolia* var. *sophorae* were processed for total RNA extraction using the TriSure reagent from Bioline. Tissue was homogenised in liquid N using a mortar and pestle that had previously been baked overnight at 200 °C to eliminate RNase contamination. Ground roots were suspended in 1 mL TriSure in 1.5 mL Eppendorf tubes with a stainless steel bead and further homogenised using a TissueLyser (Qiagen; Doncaster, VIC) set at 25 beats per second (bps) for 6 min. After centrifugation at 12,000 x g for 10 min at 4 °C, the supernatant was incubated at room temperature for 5 min before adding 200 µL chloroform and vortexing for 15 s. The mixture was incubated at room temp for 3 min and centrifuged at 2500 x g for 20 min at 4 °C. The aqueous phase was transferred to a clean tube, leaving the interphase behind, and 500 µL of -20 °C isopropyl alcohol added. The sample was incubated at room temp for 10 min before centrifugation at 2500 x g for 20 min at 4 °C. The pellet was washed once with 1 mL 75% ethanol before recentrifugation at 7500 x g for 5 min at 4 °C. The pellet was then air-dried for 10 min and dissolved in 25 µL autoclaved dH₂O, which had been treated overnight with 1% diethylpyrocarbonate (DEPC-treated water).

**Nucleotide extraction – CTAB methods**

Multiple published protocols (i.e., Chang *et al.* 1993; Rubio-Pina & Zapata-Perez 2011; Zamboni *et al.* 2008) using CTAB (hexadeclytrimethylammoniumbromide) and PVPP (polyvinylpolypyrrolidone) were trialled to find an effective method to extract total RNA from woody legume roots. For all three methods, roots previously stored at -20 °C were transferred to liquid N, ground to powder using a baked mortar and pestle, and the cold
powder placed in a 2 mL Eppendorf tube containing an extraction buffer. All aqueous reagents were prepared using DEPC-treated water.

The first method tested was described by Rubio-Piña and Zapata-Pérez (2011). This protocol used an extraction buffer containing 2% CTAB (w/v) and 2% PVPP (w/v) in a buffer of 100 mM Tris, pH 8.0; 20 mM EDTA and 1.4 M NaCl to which 10% β-mercaptoethanol (β-ME; v/v) was added immediately prior to use. Ground roots were vortexed for 30 s in 1 mL of extraction buffer and then incubated at 65 °C for 10 min on a thermomixer (Eppendorf, North Ryde, NSW). To this mixture, 800 µL chloroform:isoamyl alcohol (24:1) was added, vortexed for 30 s, and then centrifuged at 4,400 rpm for 10 min at 4 °C. The supernatant was added to 400 µL phenol and 400 µL chloroform:isoamyl alcohol, vortexed for 30 s, and centrifuged at 10,000 rpm for 10 min. An equal volume of chloroform:isoamyl alcohol was added to the supernatant and vortexed and centrifuged as before. To precipitate the nucleotides, the supernatant was added to one-third its volume of 8 M LiCl, and the sample was stored at -20 °C overnight. The following day, the sample was centrifuged at 10,000 rpm for 20 min, and the pellet washed sequentially with 1 mL 100% and 70% ethanol with a 5 min centrifugation at 10,000 rpm following each wash. The pellet was briefly air-dried before being dissolved in 250 µL DEPC-treated water, and 125 µL each of phenol and chloroform:isoamyl alcohol were added to the sample before mixing with a vortex for 30 s. The sample was centrifuged at 10,000 rpm for 3 min, and the supernatant was purified by adding an equal volume of chloroform:isoamyl alcohol and vortexing and centrifuging as before. To precipitate RNA, 0.1 volume of 3 M sodium acetate and 2 volumes of 100% ethanol were added to the aqueous phase, and the sample was incubated at -20 °C for at least 2 h. After centrifugation at 13,000 rpm for 15 min, the pellet was washed in 70% ethanol and centrifuged at 10,000 rpm for 5 min before being air-dried. The pellet
was then resuspended in 30 µL DEPC-treated water, and the sample was tested for RNA concentration and purity.

The second protocol (Chang et al. 1993) tested eliminated the use of phenol. The extraction buffer contained more EDTA (25 mM) and NaCl (2 M) and less β-ME (2%) than the first method tested (Rubio-Pina & Zapata-Perez 2011). Ground roots were incubated in 1 mL extraction buffer for 60 min at 65 °C in a thermomixer. A 900 µL volume of chloroform:isoamyl alcohol was added to the sample, followed by mixing with a vortex for 30 s and centrifugation at 10,000 rpm for 10 min. The supernatant was washed twice more using 1 mL chloroform:isoamyl alcohol with the vortex and centrifugation steps as before. Nucleotides were precipitated using 0.25 volume of 10 M LiCl and incubated at -20 °C overnight. Centrifugation at 10,000 rpm for 20 min was used to form a pellet that was then dissolved in 500 µL of SSTE buffer (1 M NaCl, 0.5% SDS, 10 mM Tris, 1 mM EDTA, pH 8.0). The sample was washed using an equal volume of chloroform:isoamyl alcohol followed by vortexing and centrifugation as before. To precipitate RNA, 0.1 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol were added to the sample, which was then stored at -10 °C for 2.5 h. After centrifugation at 13,000 rpm for 20 min, the pellet was washed sequentially with 100% and 70% ethanol with 3 min centrifugation at 10,000 rpm after each wash. The pellet was resuspended using 25 µL DEPC-treated water before being tested for RNA concentration and purity.

The third protocol (Zamboni et al. 2008) tested used the same extraction buffer as the method described by Chang et al. (1993). The fine root powder was initially incubated using the thermomixer as before. This method was unsuccessful, so subsequent tests used a water bath to incubate samples at 65 °C for 15 min interspersed with brief mixing using a vortex (8—10 repeats). To this mixture, 1 mL chloroform:isoamyl alcohol (24:1)
was added, mixed using a vortex and centrifuged at 13,000 x g for 15 min at room temperature. An equal volume of chloroform:isoamyl alcohol was added to the supernatant and centrifuged as before. To precipitate RNA, the supernatant was added to 0.3 equivalent volume of 8 M LiCl, mixed by inverting the tube, and stored overnight at 0 °C. This temperature was achieved and maintained by placing the tube on ice and refrigerated at 4 °C. After centrifugation at 15,500 x g for 35 min, the pellet was dissolved in 500 µL resuspension buffer (1 M NaCl, 0.5% SDS, 10 mM Tris-HCl, 1 mM EDTA; pH 8). The suspension was extracted once with an equal volume of chloroform:isoamyl alcohol and centrifuged at 15,000 x g for 10 min. The supernatant was mixed with two volumes of ice-cold 100% ethanol, and the sample was stored at -80 °C for at least 30 min to precipitate RNA. The tube was centrifuged at 17,000 x g for 20 min, and the pellet was air-dried for 10 min before being resuspended in 20—100 µL DEPC-treated water. The sample was then tested for RNA concentration and purity.

To remove DNA contamination, total RNA samples were digested with RQ1 DNase (Promega; Alexandria, NSW) following the manufacturer's instructions. Digestion mixtures were incubated in a 37 °C water bath for 1 h. To terminate the digestion, 1 µL of Stop solution was added, and samples were incubated in a 65 °C water bath for 10 min. Samples were purified using the Isolate II RNA Micro Clean-up kit (Bioline) according to the manufacturer's instructions. Briefly, samples were brought to a 100 µL volume with DEPC-treated water, before adding 100 µL of clean-up buffer. Samples were mixed using a vortex for 5 s, briefly centrifuged at 1000 x g, and 200 µL of solution was transferred to columns placed in 2 mL collection tubes. Samples were centrifuged for 30 s at 11,000 x g, columns were placed in fresh collection tubes, and 400 µL of buffer RW2 was added to each column. Samples were centrifuged as before and flow-through
discarded before 200 µL of buffer RW2 was added to each column. Samples were centrifuged at 11,000 x g for 2 min to dry the membrane, and columns were transferred to a fresh tube. RNA was eluted with 10 µL DEPC-treated water and centrifuged for 30 s at 11,000 x g.

The Tetro cDNA Synthesis kit from Bioline was used to construct cDNA libraries from total RNA extractions. A priming premix was prepared on ice containing 7 µL total RNA, 1 µL Oligo dT primer, 1 µL 10 mM dNTP mix, and 1 µL DEPC-treated water. This was incubated in a water bath at 70 ºC for 5 min and chilled on ice for 2 min before 10 µL reaction mix (4 µL 5x RT buffer, 1 µL RNase inhibitor, 1 µL Tetro reverse transcriptase, 4 µL DEPC-treated water) was added with gentle pipetting to mix. Tubes were incubated in a water bath at 45 ºC for 70 min, and the reaction was terminated by incubating tubes in a 85 ºC water bath for 5 min before chilling on ice.

Genomic DNA was extracted from root samples using Bioline’s PowerSoil DNA Isolation kit according to the manufacturer’s instructions. Briefly, roots were ground in liquid N using a mortar and pestle. Samples were incubated in a water bath at 65 ºC for 15 min in 60 µL solution C1 before a further homogenisation step using steel beads and the TissueLyser for 10 min at 25 bps. Tubes were centrifuged at 10,000 x g for 30 s, and 250 µL solution C2 was added to the supernatant, which was mixed using a vortex and incubated on ice for 5 min. Tubes were centrifuged for 1 min at 10,000 x g and 200 µL solution C3 was added to 600 µL of supernatant before a second incubation on ice. Tubes were centrifuged as before, and 750 µL supernatant was transferred to a collection tube containing 1.2 mL solution C4. Tubes were briefly vortexed, and 675 µL of solution was added onto a spin filter and centrifuged as before. This was repeated until all solution had passed through the filter and flow-through had been discarded. To wash filters, 500
µL solution C5 was added before centrifugation at 10,000 x g for 30 s, flow-through discarded, and tubes recentrifuged for 1 min at 10,000 x g. The spin filter was placed in a clean collection tube, and 100 µL of solution C6 was added to the centre of the membrane. Tubes were centrifuged at 10,000 x g for 30 s to elute the DNA.

A nanodrop spectrophotometer (ThermoFisher Scientific; Scoresby, VIC) was used to measure RNA or DNA concentration and purity for all samples. Nucleotide concentration and purity were confirmed using gel electrophoresis on 1.5% agarose gels in 1x TAE (40 mM Tris acetate, 1 mM EDTA pH 8.0, 1.14% glacial acetic acid) containing 2% ethidium bromide (Sigma).

4.2.3 Polymerase Chain Reaction

Polymerase chain reaction (PCR) procedures for each set of primers were optimised using genomic DNA samples from *G. max* and *A. longifolia var. sophorae*. The polymerase enzyme used initially was MangoTaq (Bioline) that is supplied with a buffer that does not contain MgCl$_2$, allowing MgCl$_2$ concentrations to be adjusted easily. For PAP primers, MgCl$_2$ concentrations were 2.5 mM, the annealing temperature was 59 °C, and extension time was 40 s. For MDH primers, MgCl$_2$ concentrations were 1.5 mM, the annealing temperature was 56 °C, and extension time was 40 s. For CS primers, MgCl$_2$ concentrations were 1.5 mM, the annealing temperature was 52.1 °C and extension time was 120 s. The optimum annealing temperature for each set of primers was determined using temperature gradients. All PCRs began with a hot start at 95 °C for 5 min and concluded with a 10 min extension at 72 °C.

The polymerase enzyme used to perform PCR on cDNA samples was MyTaq (Bioline), which was supplied with a buffer containing 1.5 mM MgCl$_2$ and dNTPs. For MDH primers, a touchdown PCR method was used beginning at 72 °C and decreased by
1 °C each cycle until reaching 56 °C, when 17 additional cycles were completed at 56 °C. The annealing time was 15 s per cycle, and the extension time was 10 s per cycle. For PAP primers, the annealing parameters were 56 °C for 15 s, and the extension time was 10 s. To obtain a product, however, this PCR procedure was run twice: first with cDNA samples and then with a 1:10 dilution of the initial products. Two sequential PCRs were also conducted for the SAP primers using first the cDNA samples followed by a 1:100 dilution of the initial products. The annealing parameters for the SAP primers were 50.4 °C for 15 s, and the extension time was 10 s per cycle. The Tub 6 primers were used according to the same dual PCR protocol and parameters as those used for the SAP primers. Both the 18s and EF1a primers used a 56 °C annealing for 10 s with a 10 s extension time.

All PCR products were initially tested by gel electrophoresis before samples were sequenced to confirm homogeneity with target sequences. Gels were made using 2% agarose in 1X TAE containing 2% ethidium bromide. Bands were visualised using a ChemiDoc XRS+ system (BioRad; Gladesville, NSW). PCR products were commercially sequenced by Macrogen (Seoul, Republic of Korea).

4.3 Results

4.3.1 Homology

Using the basic local alignment search tool for nucleotides (BLASTn) from NCBI, conserved mRNA sequences encoding sAP, PAP, MDH or CS were found from multiple clades of legumes. Primers designed using Primer Blast (NCBI) were chosen to encapsulate sequences having high homology between at least three different legume clades, aligned in Figs. 4.2 through 4.5. The primers included degenerate nucleotides at each position where the sequences varied. However, the average percentage of identical
sequences for all the primers was 78%. Only 5 of 180 nucleotide positions showed less than 75% homology between the aligned sequences (Figs. 4.2–4.5).
Figure 4.2 Homology of the region used to design degenerate primers to amplify genes encoding secreted acid phosphatase. Four mRNA sequences from three different species of legumes were compared. Nucleotide positions at the beginning and end of each sequence are indicated, as are the number of nucleotides between the primers for each sequence. Clades for each species are indicated in parentheses. AB037887.1: *Lupinus albus* (Genistaeae) *LASAP2*; AB023385: *L. albus* (Genistaeae) *LASAP1*; AB023386: *Glycine max* (Phaseoleae) APase; EF415885: *Sesbania rostrata* (Robinieae) mRNA sequence; nt: nucleotide; M: A or C; H: A, C or T; W: A or T; R: A or G; S: C or G; Y: C or T; | = complete homology across all four genes; / = 75% homology (1 gene has a different nt); . = 50% or less homology.
**Figure 4.3** Homology of the region used to design degenerate primers to amplify genes encoding purple acid phosphatase. Sequences for mRNA were compared between four different species of legumes. Nucleotide positions at the beginning and end of each sequence are indicated, as are the number of nucleotides between the primers for each sequence. NM_001248618: *Glycine max* (Phaseoleae) PAP; XM_003530928: *G. max* (Phaseoleae) PAP 17-like; FJ464333: *Phaseolus vulgaris* (Phaseoleae) *PvPAP3*; XM3635771: *Medicago truncatula* (Trifolieae) PAP; AJ617676.1: *Lupinus luteus* (Genisteae) acPase3; nt: nucleotide; Y: C or T; R: A or G; W: A or T; H: A, C or T; M: A or C; K: G or T; S: C or G; | = complete homology across all four genes; / = 75% homology (1 gene has a different nt); . = 50% or less homology.
<table>
<thead>
<tr>
<th>primers</th>
<th>TGCHGGRCARATYGGRTATGCT</th>
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</tr>
</thead>
<tbody>
<tr>
<td>AF459646 84</td>
<td>GGTGCTGCGGACAAATCGGGTATGCT</td>
<td>n~GATGCAGTTGAGGCATGCACTGGGGTCAATA 302</td>
</tr>
<tr>
<td>AJ295348.1 13</td>
<td>GACTGTGCTGGGCAAATCGGATATGCT</td>
<td>n~GATGTGGTTGAGGCATGCACTGGGGTCAATA 231</td>
</tr>
<tr>
<td>AJ299059.1 99</td>
<td>GGTGCTGCAGGGCAAATTGGGTATGCT</td>
<td>n~GATGTGGTTGAGGCATGCACTGGGGTCAATA 317</td>
</tr>
<tr>
<td>NM_001249732 85</td>
<td>GGAGCTGCAGGGCAAATTGGGTATGCT</td>
<td>n~GATGTGGTTGAGGCATGCACTGGGGTCAATA 303</td>
</tr>
</tbody>
</table>

**Figure 4.4** Homology of the region used to design degenerate primers to amplify genes encoding malate dehydrogenase. Sequences for mRNA were compared between four different species of legumes. Nucleotide positions at the beginning and end of each sequence are indicated, as are the number of nucleotides between the primers for each sequence. AF459646: *Lupinus albus* (Genistae) *LAMDH2*; AJ295348.1: *Sesbania rostrata* (Robinieae) MDH; AJ299059.1: *Cicer arietinum* (Ciceraeae) cytosolic MDH; NM_001249732: *Glycine max* (Phaseoleae) cytosolic MDH; nt: nucleotide; H: A, C or T; R: A or G; Y: C or T; S: C or G; | = complete homology across all four genes; / = 75% homology; . = 50% or less homology.
<table>
<thead>
<tr>
<th>primers</th>
<th>KGGTGTCATGGCCCTYCACGGT</th>
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<tr>
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</tr>
<tr>
<td>XM_003531407</td>
<td>705 TCACATGGGTGTCATGGCCCTCCAGGTGCAAA<del>424 nt</del>CTCCTAACATTAGTACAGAACAATTGTCTGACTACA 1197</td>
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<tr>
<td>XM_007149042</td>
<td>722 ACAACTGGGTGTCATGGCCCTCCAGGTGCAAA<del>425 nt</del>CTCCTAACATTAGTACAGAACAATTGTCTGACTACA 1214</td>
<td></td>
</tr>
<tr>
<td>XM_004488408</td>
<td>731 AGTACTGGGTGTCATGGCCCTCCAGGTGCAAA<del>426 nt</del>CTCCTAACATTAGTACAGAACAATTGTCTGACTACA 1223</td>
<td></td>
</tr>
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</table>

**Figure 4.5** Homology of the region used to design degenerate primers to amplify genes encoding citrate synthase. Sequences for mRNA were compared between four different species of legumes. Nucleotide positions at the beginning and end of each sequence are indicated, as are the number of nucleotides between the primers for each sequence. AB057662: *Sesbania rostrata* (Robinieae) *SrCS*; XM_003531407: *Glycine max* (Phaseoleae) CS, mitochondrial-like; XM_007149042: *Phaseolus vulgaris* (Phaseoleae) hypothetical protein; XM_004488408: *Cicer arietinum* (Cicereae) CS, mitochondrial-like; nt: nucleotide; K: G or T; Y: C or T; W: A or T; -: missing nt | = complete homology across all four genes; /= 75% homology; .= 50% or less homology.
4.3.2 mRNA extraction

Two RNA-extraction products from Bioline were initially trialled to extract total RNA from roots of woody legumes. Roots and leaves from a grass and two herbaceous legumes were used as alternative tissue types. For woody legume tissue, the Isolate Plant RNA kit did not successfully extract total RNA that could be detected using either spectro-photometry (Table 4.3) or gel electrophoresis (Fig. 4.6). Spectrophotometer results for woody legume tissue processed using the TriSure reagent suggested total RNA was present, but the 260:230 ratio indicated significant impurities. Total RNA suitable for downstream application was successfully extracted from the roots of the herbaceous legume, *Glycine max*, using TriSure (Fig. 4.6B), suggesting one or more compounds in the woody tissue interfered with the protocol.
Figure 4.6 Gel electrophoresis to test the quantity and quality of total RNA extracted from plant roots. (A) RNA extracted using Isolate Plant RNA kit. (B) RNA extracted using TriSure reagent. *Als*: *A. longifolia* var. *sophorae*. ; b: blank lane; I: hyperladder I; *Gm*: *Glycine max*. Size of bands in basepairs for hyperladder I is shown in far left image, obtained from the manufacturer’s website (www.bioline.com.au).

Three methods using 2% CTAB and 2% PVPP were tested to extract total RNA from tissues with high polysaccharide and polyphenol content. Table 4.3 outlines the key differences between these protocols. Total RNA from roots of *Acacia longifolia* var. *sophorae* was not detected when the method from Rubio-Piña & Zapata-Pérez (2011) was used (Fig. 4.7A, Table 4.4). The method described by Chang *et al.* (1993) did not produce detectable RNA from roots from either *A. longifolia* var. *sophorae* or the grass *Ehrharta*
erecta (Fig. 4.7B, Table 4.4). Total RNA from roots of *A. longifolia* var. *sophorae* or leaves of the herbaceous legume, *Vicia faba*, was also not detected when a heating block was used during the extraction step described in Zamboni *et al.* (2008; Fig. 4.7C, Table 4.4). However, when the extraction step was done using a water bath, total RNA was successfully extracted from both *A. longifolia* var. *sophorae* and *V. faba* (Fig. 4.7D-E, Table 4.4).
Table 4.3 Comparison of key differences between methods using 2% CTAB and 2% PVPP to extract total RNA from woody plant tissues. Only differences between the three protocols are listed. β-ME: β-mercaptoethanol; temp: temperature; SSTE: 1M NaCl, 0.5 % SDS, 10 mM Tris, 1 mM EDTA, pH 8; EtOH: ethanol.

<table>
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<tr>
<th>Source of method</th>
<th>Extraction buffer</th>
<th>Extraction temp / time / method</th>
<th>Median centrifugation speed</th>
<th>Phenol step</th>
<th>1st precipitation reagent / temp</th>
<th>Solubilisation buffer</th>
<th>2nd precipitation reagent / temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rubio-Piña &amp; Zapata-Pérez 2011</td>
<td>1.4 M NaCl, 20mM EDTA, 10% β-ME</td>
<td>65 °C / 10 min / heating block</td>
<td>10,000 rpm (~9500 x g)</td>
<td>Yes</td>
<td>0.3 vol 8 M LiCl / -20 °C</td>
<td>DEPC-treated water</td>
<td>0.1 vol 3 M sodium acetate, 2 vol 100% EtOH / -20 °C</td>
</tr>
<tr>
<td>Chang et al. 1993</td>
<td>2 M NaCl, 25 mM EDTA, 5% β-ME</td>
<td>65 °C / 10 min / heating block</td>
<td>10,000 rpm (~9500 x g)</td>
<td>No</td>
<td>0.25 vol 10 M LiCl / -20 °C</td>
<td>SSTE</td>
<td>0.1 vol 3 M sodium acetate, 2.5 vol 100% EtOH / -20 °C</td>
</tr>
<tr>
<td>Zamboni et al. 2008</td>
<td>2 M NaCl, 25 mM EDTA, 5% β-ME</td>
<td>65 °C/15 min with vortex 8-10 times / water bath</td>
<td>13,000 x g</td>
<td>No</td>
<td>0.3 vol 8 M LiCl / 0 °C</td>
<td>SSTE</td>
<td>2 vol cold EtOH / -80°C</td>
</tr>
</tbody>
</table>
Table 4.4 Absorbance values and ratios for total RNA samples extracted from plant roots. Methods included a commercially-produced kit and reagent and three different published methods using 2% CTAB. Measurements were obtained using a Nanodrop spectrophotometer. A: absorbance; nm: nanometer.

<table>
<thead>
<tr>
<th>Species</th>
<th>Method</th>
<th>Source</th>
<th>RNA concentration (ng µL⁻¹)</th>
<th>A₂₆₀ nm</th>
<th>A₂₈₀ nm</th>
<th>260:280</th>
<th>260:230</th>
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</thead>
<tbody>
<tr>
<td><em>Acacia longifolia var. sophorae</em></td>
<td>Isolate Plant RNA kit</td>
<td>Bioline</td>
<td>8</td>
<td>0.4</td>
<td>0.25</td>
<td>1.59</td>
<td>0.06</td>
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<tr>
<td><em>A. longifolia var. sophorae</em></td>
<td>TriSure</td>
<td>Bioline</td>
<td>509</td>
<td>12.73</td>
<td>9.43</td>
<td>1.35</td>
<td>0.27</td>
</tr>
<tr>
<td><em>Glycine max</em></td>
<td>TriSure</td>
<td>Bioline</td>
<td>1342.5</td>
<td>33.56</td>
<td>16.64</td>
<td>2.02</td>
<td>1.55</td>
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<td><em>A. longifolia var. sophorae</em></td>
<td>CTAB</td>
<td>Rubio-Piña &amp; Zapata-Pérez 2011</td>
<td>1.35</td>
<td>0.07</td>
<td>0.04</td>
<td>1.54</td>
<td>0.07</td>
</tr>
<tr>
<td><em>A. longifolia var. sophorae</em></td>
<td>CTAB</td>
<td>Chang <em>et al.</em> 1993</td>
<td>1.4</td>
<td>0.03</td>
<td>0.01</td>
<td>4.95</td>
<td>0.09</td>
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<tr>
<td><em>Ehrharta erecta</em></td>
<td>CTAB</td>
<td>Chang <em>et al.</em> 1993</td>
<td>1.1</td>
<td>0.03</td>
<td>0.02</td>
<td>1.44</td>
<td>0.1</td>
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<tr>
<td><em>Vicia faba</em> leaves</td>
<td>CTAB/heating block</td>
<td>Zamboni <em>et al.</em> 2008</td>
<td>0.4</td>
<td>0.01</td>
<td>0.01</td>
<td>0.86</td>
<td>0.07</td>
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<td><em>V. faba</em> leaves</td>
<td>CTAB/water bath</td>
<td>Zamboni <em>et al.</em> 2008</td>
<td>15.1</td>
<td>0.38</td>
<td>0.19</td>
<td>1.95</td>
<td>1.78</td>
</tr>
<tr>
<td><em>A. longifolia var. sophorae</em></td>
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<td>Zamboni <em>et al.</em> 2008</td>
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<td>2.00</td>
<td>1.00</td>
<td>2.01</td>
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Figure 4.7 Gel electrophoresis to test the quantity and quality of total RNA extracted from plant roots using different protocols. (A) RNA extracted using protocol described by Rubio-Piña and Zapata-Pérez (2011). (B) RNA extracted using protocol described by Cheng et al. (1993). C-E) RNA extracted using protocol described by Zamboni et al. (2008) with the extraction done using a heating block (C) or water bath (D-E). (F) RNA extracted from roots of woody legumes collected from the field. I: hyperladder I; b: blank; As: Acacia suaveolens; Als: A. longifolia var. sophorae; Ee: Ehrharta erecta; Vf: Vicia faba leaves. Numbers correspond to each waypoint described in Table 4.2. Sizes of bands in basepairs for hyperladder I are shown in Fig. 4.6.
4.3.3 PCR

PCR optimisation with genomic DNA

Genomic DNA from *Glycine max* was used to optimise the PCR protocols for each set of primers. Temperature gradients using primers for secreted acid phosphatase (sAP) did not produce a specific product, as expected for genomic DNA (Fig. 4.8 C, Table 4.1). Primers for citrate synthase (CS) produced multiple bands, but none were of the expected size, and increasing the annealing temperature did not improve the results (Fig. 4.8A, Table 4.1).

Temperature gradients using primers for purple acid phosphatase (PAP) and malate dehydrogenase (MDH) produced smears, suggesting more stringent conditions were required (Fig. 4.8B, C, Table 4.1). For PAP, a faint band of the expected size was detected when the annealing temperature was 57 °C. Increasing the annealing temperature by 2 °C and the concentration of MgCl₂ by 0.5 mM produced a more distinct band of the expected size without an accompanying smear for *G. max*, although no band was found using genomic DNA from *Acacia longifolia* var. *sophorae* (Fig. 4.9). For MDH, an appropriately-sized band within a smear was darker at an annealing temperature of 49.7 °C compared to 57 °C. Subsequent tests using MDH primers on genomic DNA extracted from *G. max* demonstrated an annealing temperature of 56 °C was optimum if the PCR mixture was transferred from ice directly to 94 °C at the beginning of the protocol, called a ‘hot start.’ However, a faint smear without a band was visible for genomic DNA from *A. longifolia* var. *sophorae* (Fig. 4.9). Primers for 18s as a positive control indicated the DNA from *A. longifolia* var. *sophorae* was suitable for PCR (Fig. 4.9)
Figure 4.8 Gel electrophoresis to determine the optimum annealing temperature for the four sets of PCR primers. PCR was done using genomic DNA extracted from *Glycine max* roots. Temperatures for each PCR reaction are given. A (-) indicates no DNA was included in the reaction as a negative control. CS: citrate synthase; b: blank; I: hyperladder I; PAP: purple acid phosphatase; IV: hyperladder IV; MDH: malate dehydrogenase; SAP: secreted acid phosphatase. Sizes of bands in basepairs for hyperladder IV are shown on the far right, obtained from the manufacturer's website ([www.bioline.com.au](http://www.bioline.com.au)). Sizes of bands in basepairs for hyperladder I are shown in Fig. 4.6.
Figure 4.9 Optimised PCR protocols for five sets of PCR primers. PCR was done using genomic DNA (A) or cDNA libraries transcribed from mRNA (B) extracted from roots. I: hyperladder I; b: blank; PAP: purple acid phosphatase; MDH: malate dehydrogenase; IV: hyperladder IV; 18s: ribosomal 18s as a positive control; SAP: secreted acid phosphatase; CS: citrate synthase; Gm: Glycine max; Alr: Acacia longifolia var. sophorae; -: no DNA as a negative control. Size of the bands in basepairs for hyperladders I and IV are shown in Figs. 4.6 and 4.8, respectively.

**PCR on woody legumes regenerating after Black Saturday fires**

Total RNA was extracted from roots of six species of woody legumes regenerating after the Black Saturday fires (Figs. 4.1 and 4.6F, Tables 4.2 and 4.5). The cDNA transcribed from this RNA was tested using primers for EF1a and were found to be sufficient for subsequent PCR (Fig. 4.10A). PCR protocols developed using DNA from G. max only resulted in products of the expected size for PAP primers for two species of woody legumes, A. dealbata and A. obliquinervia (Fig. 4.10B, Table 4.1). After sequencing, the PCR product from A. dealbata demonstrated homology to mRNA sequences for PAP from herbaceous legumes (Fig. 4.11, Table 4.6).
The amino acid sequence translated from the mRNA sequence also demonstrated homology to known and putative PAP proteins from herbaceous legumes (Fig. 4.12, Table 4.6). Analysing the amino acid sequence with the eukaryote KOG database from NCBI revealed homology with a purple (tartrate-resistant) acid phosphatase domain (E-value: 598e-33). Repeating the analysis using the conserved domain database from NCBI indicated the sequence contained a conserved domain related to Homo sapiens acid phosphatase 5 (E-value: 3.61e-15), which belongs to the metallophosphatase superfamily that also includes PAP (Fig. 4.12). Together, the homology suggested that the PCR product obtained from roots of A. dealbata encodes a purple acid phosphatase.
Table 4.5 Quantity and quality of total RNA extracted from roots of six species of woody legumes regenerating after the “Black Saturday” fires. Two measurements, divided by a forward slash, are given for several samples. The first measurement was taken after the initial extraction, and the second was taken after treating samples with RQ1 DNase and re-purification with the Isolate II Micro RNA kit. A: absorbance.

<table>
<thead>
<tr>
<th>Waypoint</th>
<th>Species</th>
<th>RNA concentration (ng µL⁻¹)</th>
<th>A260</th>
<th>A280</th>
<th>260:280</th>
<th>260:230</th>
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<tbody>
<tr>
<td>126</td>
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<td>0.409/0.321</td>
<td>0.191/0.168</td>
<td>2.14/1.91</td>
<td>1.15/1.87</td>
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<td>8.5/9.2</td>
<td>0.212/0.230</td>
<td>0.101/0.123</td>
<td>2.09/1.87</td>
<td>1.38/0.77</td>
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<td>0.340/0.295</td>
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<td>2.02/1.92</td>
<td>0.76/0.67</td>
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<td>97</td>
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<td>6.3/4.6</td>
<td>0.156/0.116</td>
<td>0.075/0.059</td>
<td>2.07/1.95</td>
<td>0.99/0.12</td>
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<tr>
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<tr>
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<td><em>A. obliquinervia</em></td>
<td>17.5</td>
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<td>0.181/0.086</td>
<td>1.91/1.83</td>
<td>0.81/0.49</td>
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</table>
Figure 4.10 Gel electrophoresis to determine if roots of woody legumes express genes for acid phosphatases. PCR was done using cDNA transcribed from total RNA extracted from roots of woody legumes species identified by waypoints described in Table 4.2. b: blank; IV: hyperladder IV; -: no cDNA; nr: near 97; P: purple acid phosphatase; S: secreted acid phosphatase; 100: ladders incrementing by 100 basepairs (bp) from 100—1000 bp. Size of the bands in basepairs for hyperladder IV are shown in Fig. 4.8.
<table>
<thead>
<tr>
<th>Accession</th>
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</tr>
</thead>
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<td>XM004513281</td>
<td>TTAGGAGGCTAAGACGAGAAATAGAGATGCTATGCTGAGATCATTAGTTGAGATCAGAGCTAG</td>
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</table>

**Figure 4.11** Homology of the PCR product obtained using purple acid phosphatase (PAP) primers on cDNA from *Acacia dealbata* sampled at waypoint 126. Comparisons were made with mRNA sequences encoding known or putative PAPs from five species of herbaceous legumes, identified by GenBank accession numbers. Green shading indicates the herbaceous species has nucleotides identical to *A. dealbata*. Blue shading indicates the nucleotide is conserved across all the herbaceous species but differs from that sequenced for *A. dealbata*. Numbers indicate position within the sequence. FJ464333: *Phaseolus vulgaris* PvPAP3; AJ617676: *Lupinus latens* acPase3; XM003635771: *Medicago truncatula* PAP; XM003530928: *Glycine max* PAP 17-like isoform 1; XM004513281: *Cicer arietinum* PAP 17-like isoform X1; -: skipped nucleotide.
**Figure 4.12** Homology of the amino acid sequence from *Acacia dealbata*. Amino acid sequence corresponds to the largest open reading frame translated by the NCBI website. Comparisons were made with amino acid sequences for known or putative PAP from five species of herbaceous legumes, identified by GenBank reference sequences. A conserved domain analysis was also conducted. Green shading indicates the herbaceous species has amino acids identical to *A. dealbata*. Blue shading indicates the amino acid is conserved across all the herbaceous species but differs from that sequenced for *A. dealbata*. Red shading indicates an identical amino acid with the consensus sequence for the metallophosphatase conserved domain cd07378. Numbers indicate position within the sequence. ACO25293: *Phaseolus vulgaris* PvPAP3; CAE85073: *Lupinus luteus* acPase3; XP003635819: *Medicago truncatula* PAP; XP003530976: *Glycine max* PAP 17-like isoform 1; XP004513338: *Cicer arietinum* PAP 17-like isoform X1; CDD: cd07378: metallophosphatase domain for acid phosphatase 5 (*Homo sapiens*); -: skipped amino acid.
Table 4.6 Sequence homologies between *Acacia dealbata* and five herbaceous legumes. Both mRNA and amino acid sequences were compared between *A. dealbata* and each of the five other legume species. The percentage of identical nucleotides or amino acids is shown. The identifiers for each species refer to GenBank accession numbers. aa: amino acid.

<table>
<thead>
<tr>
<th>Species</th>
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<th><em>Phaseolus vulgaris</em></th>
<th><em>Medicago truncatula</em></th>
<th><em>Glycine max</em></th>
<th><em>Lupinus luteus</em></th>
<th><em>Cicer arietinum</em></th>
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<td>XM003635771/</td>
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<td>XP003635819</td>
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<tr>
<td>aa sequence homology</td>
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<td>83%</td>
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<td></td>
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<td>63%</td>
<td>62%</td>
<td>83%</td>
<td>75%</td>
<td>73%</td>
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</table>
4.4 Discussion

4.4.1 Woody legume roots express a putative phosphatase

Gene products

This study reports the novel finding that an Australian species of woody legume can express a gene in its roots that is highly homologous to a known acid phosphatase. *Acacia dealbata* roots expressed an mRNA sequence that exhibited over 70% homology to genes encoding known or putative purple acid phosphatases (PAPs) in several herbaceous species of legumes. These homologous PAPs included *PrPAP3*, which is induced in *Phaseolus vulgaris* roots by phosphate deficiency and increases phosphatase activity in the rhizosphere (Liang et al. 2012b). The regulation or activity for homologous sequences from the other herbaceous species has not been published.

The amino acid sequence translated from the mRNA expressed by *A. dealbata* demonstrated over 60% homology to PAP proteins from several herbaceous legumes. The largest open reading frame from the amino acid sequence also contained a domain common to metal-binding sites within the metallophosphatase superfamily and found in mammalian acid phosphatase 5. This represents a key finding, because mammalian type 5 acid phosphatases are homologous to plant-derived small PAPs that can be induced by phosphate deficiency and exuded into the rhizosphere (Olczak et al. 2003). The cumulative homology suggests *A. dealbata* roots can express a gene encoding a small plant PAP. Furthermore, it is possible that phosphate deficiency induces this gene, and the gene product is exuded into the rhizosphere.

Several other species of woody legumes were investigated to determine if their roots expressed putative phosphatases or carboxylates. Two PCR products were isolated from *A. obliquinervia* roots using primers against PAP. The sequenced products did not show homology to any putative phosphatases in GenBank (NCBI) for the FABACEAE taxa. No
PCR products were found when the other six species of woody legumes were tested using primers targeted against PAP, secreted acid phosphatase (sAP), malate dehydrogenase (MDH) or citrate synthase (CS). However, the lack of PCR products may reflect unsuitable primers for these species, rather than a lack of enzyme-related genes.

**PCR primers**

Genomes have not been sequenced for any species of Australian woody legume. Consequently, the probable gene sequences encoding phosphatases or carboxylates were inferred from taxa most related to the woody legume species of interest. This is a reputable assumption given purple acid phosphatases contain metal-binding sites that are highly conserved between plant families (Olczak et al. 2003). The two known sAPs cloned from *Lupinus albus* demonstrate some homology with PAP from other species (Wasaki et al. 2000), decreasing the likelihood that a specific sAP could be cloned from woody legume tissues. Isoforms of CS and MDH function in plant metabolic pathways (La Cognata et al. 1996; Foyer et al. 2009). Thus, homology was compared to genes upregulated by phosphate deficiency and encoding products that were secreted into the rhizosphere (Aono et al. 2001; Uhde-Stone et al. 2003).

For each gene of interest, related sequences from at least three different FABACEAE clades were compared to determine if conserved sequences could be used to design primers for woody legume species. The range of homology was 67—81% for PAP, 88—92% for CS, 70—85% for sAP, and 85—94% for MDH. Therefore, a considerable number of nucleotides were identical across multiple herbaceous species from different clades. Although the high percentages of homology facilitated the design of PCR primers, the genes were not identical. Thus, each primer included degenerate sequences. Degenerate primers contain a mixture of sequences that are identical except at specified positions where nucleotides are substituted. These primers are used when the target gene
has an unknown sequence, and homology between related genes indicates more than one possible nucleotide within an otherwise identical, or evolutionarily conserved, sequence.

The degenerate primers for both PAP and MDH successfully amplified specific products when genomic DNA and cDNA from *Glycine max* were used as positive controls. The primers for CS generated a range of products from genomic DNA that were likely non-specific, as increasing the stringency of the PCR protocol failed to isolate a single product. No products using the primers for CS were amplified from cDNA. The primers for sAP included boundaries between exons and introns, precluding the ability to optimise the PCR protocol for sAP using genomic DNA. A specific product was amplified from cDNA extracted from *G. max* using the primers for sAP. The MDH and sAP primers failed to amplify any specific products using cDNA from any woody legume species. Consequently, it is unclear whether the primers were unsuitable for woody legumes, homologous genes were absent, or if the environmental conditions precluded expression of these genes.

4.4.2 RNA extraction methods

Identifying mRNA sequences present in samples is an invaluable tool to determine how the tissues have been interacting with their environment. Consequently, several kits and reagents are commercially available to extract mRNA from a wide range of tissues. However, large amounts of polysaccharides and phenolics or other secondary compounds in tissues have been reported to inhibit successful isolation of purified RNA from several woody plants (Fang et al. 1992; Chang et al. 1993; Salzman et al. 1999; Kiefer et al. 2000; Zamboni et al. 2008; Rubio-Pina & Zapata-Perez 2011). Woody legume species demonstrated similar recalcitrance for RNA extraction from their roots. Thus, a primary challenge that needed to be overcome before proceeding with further studies was to identify a protocol that successfully obtained purified RNA from woody legume roots.
The principle chemicals used to isolate nucleic acids from phenolic compounds and polysaccharides include vinyl-pyrrolidone polymers and high salt concentrations, respectively, prior to ethanol precipitation (Fang et al. 1992; Salzman et al. 1999). In this study, three methods were trialled that all used 2% CTAB as a detergent and 2% polyvinylpolypyrrolidone (PVPP) to bind phenolic compounds. β-mercaptoethanol was added to inhibit RNase enzymes. This extraction buffer contained a high salt content to increase the solubility of nucleotides, and two of the methods tested also used a high salt content in the resolubilisation buffer (Chang et al. 1993; Zamboni et al. 2008). The protocol that proved successful had adapted a prior method (Kiefer et al. 2000) by increasing centrifugation speeds, more frequent mixing with a vortex during the initial incubation and using a water bath to incubate samples in the extraction buffer (Zamboni et al. 2008). If a heating block was used in lieu of a water bath, the method was not successful. This indicated that a crucial component of the method entailed uniform heating during the initial extraction when phenolic compounds were bound by PVPP.

Although sufficient mRNA was extracted to permit translation into cDNA libraries, the concentration and purity of the RNA samples were not ideal. A 260:280 ratio below 1.8 indicates proteins, and a 260:230 ratio dropping from 1.5 suggests phenolic compounds or guanidine salts are present (Nanodrop.com 2008). The choice of vinylpyrrolidone polymer may have attenuated the concentrations and purity of the mRNA isolated. The initial protocol trialled used polyvinylpolypyrrolidone (PVPP) to bind phenolic compounds (Rubio-Pina & Zapata-Perez 2011). PVPP is relatively insoluble, thus other protocols substituted PVPP with polyvinylpyrrolidone (PVP) (Chang et al. 1993; Zamboni et al. 2008). To maintain uniformity, PVPP was used throughout this study, but it had a tendency to precipitate out of the detergent solution. Thus, PVPP may
have been less effective for binding phenolic compounds, and it is advisable to use PVP in the extraction buffer.

Cleaning up the RNA using the commercially available Isolate II micro RNA clean up kit further reduced the 260:230 ratio. The low concentration of RNA produced may have reduced the absorbance at 260 nm to such an extent that the 260:230 ratio was artificially low. The ability to successfully transcribe cDNA from the RNA extracts with low 260:230 ratios supports the possibility that RNA concentration, rather than purity, was responsible for low ratios.

Despite the challenges inherent in isolating mRNA from woody tissue and designing PCR primers without knowing the gene sequences, a putative PAP was isolated from roots of *A. dealbata*. This novel discovery provides a foundation to investigate whether P availability regulates the expression of PAP in woody legumes, as detailed in Chapter 5.
Chapter 5 – Expression of genes encoding acid phosphatases or carboxylate precursors – induction by phosphorus deficiency

Abstract

In the previous chapter, a sequence homologous to a secreted form of purple acid phosphatase (PAP) was isolated from woody legume roots. The expression of some forms of phosphatases and carboxylates in herbaceous legumes has been shown to be regulated by P availability. Therefore, the experiment in this chapter was designed to test whether limited P availability would induce genes for phosphatases or carboxylate precursors in woody legumes. Two species of woody legumes were grown under glasshouse conditions and supplied different amounts and forms of P for eleven weeks. Fine roots were collected to isolate RNA, which was examined for the presence or absence of genes homologous to phosphatases or carboxylates. One species expressed two putative phosphatases, including a sequence homologous to that found in chapter 4 and a novel sequence. The expression of both putative phosphatases appeared to be constitutive rather than induced by P deficiency. This suggested that woody legumes have the capacity to express phosphatases that might be exuded into the rhizosphere to facilitate acquisition of P. Although P deficiency did not induce expression of these genes, P availability may still regulate the levels of their expression.

5.1. Introduction

5.1.1 Background

Both geochemical and biological reactions regulate the availability of phosphorus (P) in soils (see Chapter 4). Weathered geological substrates release clay minerals and Fe and Al oxides that adsorb P, particularly in acidic soils (Crews et al. 1995; Vance et al. 2003). Weathering may also release calcium carbonate, which readily binds P in alkaline soil
Phosphorous is an essential component of nucleic acids, and plants use P to construct cell membranes, regulate metabolic processes, and in energy transfer (Duff et al. 1994). As a result, the two main forms of organic P in soil are nucleic acids and phytic acid (Abel et al. 2002). These compounds are phosphate esters that can adsorb to inorganic soil components and become unavailable for plant uptake (McGill & Cole 1981; Lambers et al. 2006).

Legumes require P-rich molecules to supply the high energy demands inherent to biological nitrogen fixation (BNF) (Ross 1992; Marschner 1995). Accordingly, P deficiency may attenuate BNF in ecosystems that are N-limited due to recent fire (Guinto et al. 2000; Turner et al. 2008; Vitousek et al. 2010). Although fire increases concentrations of inorganic P due to the presence of P in ash and upper soil layers, fire also releases metallic cations that can occlude a significant amount of P (Weston & Attiwill 1990; Adams 1992). Nevertheless, N-fixing species have been found to improve the rate of vegetation recovery after fire (Certini 2005). As described in Chapter 4, utilising root exudates like carboxylates or phosphatases would facilitate woody legumes to acquire the P necessary for BNF.

Carboxylates, or the related organic acids, function to displace phosphates bound to metallic cations (Raghothama 1999; Abel et al. 2002). Legumes typically exude citrate and malate, depending on the species of legume and the age of the root (Ohwaki & Hirata 1992; Neumann & Römheld 1999; Raghothama 1999; Peñaloza et al. 2002; Rengel 2002; Ryan et al. 2012; Lambers et al. 2013). The enzymes citrate synthase or malate dehydrogenase are required to synthesise these carboxylates (Raghothama 1999), and P-deficiency increased expression of these enzymes in *Lupinus albus* (Rengel 2002). Iron-
deficiency or aluminium toxicity may also increase expression of citrate synthase (Vance et al. 2003; Lambers et al. 2013). Other species may alter enzyme activity rather than expression to affect biosynthesis of carboxylates (Neumann & Römheld 1999). In addition, various species of herbaceous legumes exude different quantities of citrate and malate in response to P-deficiency (Ohwaki & Hirata 1992). A few Australian species of *Acacia* and several fynbos species of woody legumes are known to produce cluster roots associated with carboxylate exudation (Power et al. 2010; He et al. 2012). In Chapter 4, however, PCR products corresponding to citrate synthase or malate dehydrogenase were not found from roots of six woody legume species. Additional investigation is required to determine whether these genes could be induced by limited P or P-deficiency.

Phosphatases encompass a large family of enzymes that function to hydrolyse organic P. Substrate specificity and kinetic activity varies among phosphatases (Duff et al. 1994; Rengel 2002; Zhu et al. 2005). For example, phytases are required to hydrolyse phytic acids (Vance et al. 2003; Lambers et al. 2006). Conversely, the largest known family of phosphatases, purple acid phosphatases (PAPs), can often hydrolyse several different phosphate esters with varying kinetic activity (Olczak et al. 2003), but they are relatively ineffectual for phytic acids (Lambers et al. 2013). In addition, three secreted acid phosphatases (sAPs) that are not substrate-specific have been isolated from *Lupinus albus* (Wasaki et al. 1999; Wasaki et al. 2000; Miller et al. 2001). Thus to access P from different phosphate esters, plants often exude more than one phosphatase into the rhizosphere (Rengel 2002; Lambers et al. 2013).

Several legume species will express phosphatase genes in their roots when the plants experience P-deficiency, but P-limitation is not always required to induce phosphatase expression. Phosphorus-deficiency upregulates expression of some phosphatases that are constitutively expressed in roots of *Lupinus luteus, L. albus* and *Medicago truncatula* (Miller et
al. 2001; Xiao et al. 2006a; Lu et al. 2008). Alternatively, P-deficiency induces expression of other phosphatases in roots of *L. albus*, and *Phaseolus vulgaris* (Wasaki et al. 1999; Wasaki et al. 2000; Tian et al. 2007; Liang et al. 2010; Liang et al. 2012b). Plants detect P-deficiency by concentrations of inorganic P in the cytosol or xylem rather than P availability in soil (Duff et al. 1991; Burleigh & Harrison 1999; Abel et al. 2002). Lower thresholds of P concentrations used to modulate gene expression vary between phosphatases, even within the same plant (Nilsson et al. 2010; Liang et al. 2012a). In Chapter 4, it was found that *Acacia dealbata* was the only woody legume species that expressed a putative PAP in root tissues. However, the study did not address the possibility that the other species can express root phosphatases but P availability was sufficient to down-regulate expression.

5.1.2 Aim

The aim of this study was to determine whether P-deficiency would induce expression of genes encoding putative phosphatases or enzymes to synthesise carboxylates. To this end, the following hypotheses were tested: (i) Woody legume species other than *A. dealbata* will express genes for putative phosphatase or carboxylate precursors in root tissues; and (ii) limited availability of P will induce expression of genes for putative phosphatase or carboxylate precursors in woody legume roots.

5.2 Methods

5.2.1 Glasshouse pot culture

To establish whether phosphate deficiency induces the expression of genes encoding acid phosphatases or carboxylates, woody legumes were grown in a glasshouse under controlled conditions. Two concentrations of an organic and inorganic source of P were provided to plants in nutrient solution. Plants were grown in pot culture for up to 11
weeks prior to their roots being collected for molecular analysis. After harvesting of roots, soil and leaf tissues were analysed for P content as proof of differences in availability of P and subsequent plant uptake.

Species selection and seed germination

Two species of woody legumes that had been investigated in the field experiment (Chapter 2) were selected due to seed availability. These included *Acacia obliquinervia*, and *Goodia lotifolia*. Prior to heat treatment, seed coats were sanitised as described in Chapter 3. Between 85—100 seeds were treated for each species. Heat treatment to break seed dormancy was chosen based on seed germination trials reported in Chapter 3. Thus, *Acacia obliquinervia* seeds were heated in a 75 °C water bath for 60 min, and *Goodia lotifolia* seeds were heated in a 90 °C dry oven for 60 min. Following heat application, seeds were placed in Petri dishes containing Whatman #1 filter paper moistened with 2 mL 100 μM CuSO\textsubscript{4} and stored in a dark cabinet at 20 °C. Dishes were monitored daily and germinated seeds were removed.

Growth conditions

Plants were grown in 18 mm diameter plastic pots containing approximately 2 kg washed sand. Prior to any nutrient application, the pH of the sandy soil varied between 6.8—6.9, electrical conductivity was between 192—200 μS cm\textsuperscript{-1}, and extractable P measured by Bray 1-P (see below) was below detectable limits. A Chux cloth (Clorox Australia, Padstow NSW) was used to line the inside of each pot to prevent loss of sand, and each pot was placed on an individual plastic circular base. Four to five germinated seeds were evenly spaced within each pot and planted at a depth of approximately 2 cm deep.
The glasshouse was maintained at 25 ± 2 °C during the day and 19 °C at night. Humidity and light were dependent on ambient conditions. Pots were rotated randomly every week over a 3.7 m² bench to minimise effects due to possible variations in temperature, humidity, or light availability. Twice per week, the height from the substrate surface to the top of each plant was measured using a ruler. Plants were grown for 11 weeks following the emergence of seedlings.

Nutrient addition and analyses

Ten pots were established for each species, allowing five P treatments to be administered in duplicate for both species (Table 5.1). These P treatments included no additional P (hereafter referred to as ‘Control’), 10 or 50 mg kg⁻¹ of organic inositol phosphate (C₆H₁₈O₂₄P₆; ‘OP’) and 10 or 50 mg kg⁻¹ of inorganic orthophosphate (KH₂PO₄; ‘IP’). Previous studies have found woody legumes show a positive growth response to P concentrations from 5.5—24 mg kg⁻¹, but concentrations of P much above 50 mg kg⁻¹ are detrimental to growth (Hingston et al. 1982; Islam et al. 2000; Aono et al. 2001; Pang et al. 2010). Nutrients were supplied as a 10% strength Hoagland’s solution (200 µM MgSO₄, 500 µM KNO₃, 500 µM Ca(NO₃)₂, 50 µM Fe-Na-EDTA, 5µM H₃BO₃, 0.2 µM MnCl₂, 0.15µM ZnSO₄, 32 nM CuSO₄, 12nM Na₂MoO₄, 21nM CoCl₂), which was administered in a 250 mL volume twice per week with three to four days between applications beginning one week after seedling emergence. The day prior to nutrient addition, pots were watered to capacity and allowed to drain freely to prevent accumulation of nutrients from previous applications. After the plants were harvested, samples of sand from each pot were analysed for available P using the Bray 1-P test as described in Chapter 2 (Menage & Pridmore 1973).
Table 5.1 Nutrient treatments and growth conditions applied to two species of woody legumes being investigated for the molecular response to varying phosphorus availability. The average number of plants per pot indicates the number of plants at the end of the experiment. *A*: *Acacia*; *G*: *Goodia*; P: phosphorus; Avg: average

<table>
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<tr>
<th>Species</th>
<th>P type</th>
<th>chemical</th>
<th>mg kg(^{-1})</th>
<th>Name</th>
<th># of pots</th>
<th>Avg plants per pot</th>
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</thead>
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<td>5</td>
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<td>IP10</td>
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<td>OP10</td>
<td>2</td>
<td>3</td>
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<tr>
<td><em>A. obliquinervia</em></td>
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<td>C(<em>6)H(</em>{18})O(_{24})P(_6)</td>
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<td>OP50</td>
<td>2</td>
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<td>3</td>
</tr>
<tr>
<td><em>G. lotifolia</em></td>
<td>KH(_2)PO(_4)</td>
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<td>50</td>
<td>OP50</td>
<td>2</td>
<td>4</td>
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</tr>
</tbody>
</table>

**Foliar chemistry**

After excision, the shoots from all plants were weighed individually before being dried at 40 °C for 72 h and reweighed. Leaves from each species and treatment group were removed, bulked and ground using a ball mill. Foliar samples were analysed for total N and C using a CNS analyser (Variomax Elementar Analysensysteme, Hanau, Germany). For total P, samples were digested with nitric acid using an Ethos-1 microwave
digester (Milestone Inc, Shelton CT). Digests were analysed for total P using a Varian Vista-Pro simultaneous ICP-OES (Agilent Technologies Inc, Palo Alto, CA).

5.2.2 Constructing cDNA libraries

Tissue collection and RNA extraction

Total RNA was extracted using the protocol described in Chapter 4 (i.e., Zamboni et al. 2008). Over a four-day period, one plant representing each species and each treatment was collected daily and assigned a number, which was used to identify the sample throughout the extraction and PCR process.

While still attached to the stem, roots were carefully removed from the pot and rinsed with distilled water to remove any remaining sand. The whole root system was separated from the stem and transferred immediately to a ceramic mortar containing liquid N. Roots were ground to fine powder using a ceramic pestle, and the powder transferred to 1 mL of RNase-free extraction buffer that was warmed to 65 °C and contained 2% CTAB, 2% PVPP, and 2% β-mercaptoethanol (see Chapter 4). Each plant was ground in a separate mortar and pestle, which were washed and heated overnight at 200 °C to denature any residual nucleic acids or RNase before being used again. Total RNA was extracted, and the final pellet was dissolved in 30 µL DEPC-treated water. RNA concentration and purity was measured using a Nanodrop spectrophotometer and electrophoresis through a 1.5% agarose gel. Root specimens that provided marginal amounts of total RNA were substituted with roots from additional plants sampled one week after initial sampling. The nutrient regimen continued through this additional week.

As described in Chapter 4, total RNA was treated to remove DNA contamination before being cleaned and confirmed to be free from DNA contamination using PCR.
The concentration and purity of cDNA was tested using a Nanodrop spectrophotometer.

5.2.3 PCR

PCR was done using primers for purple acid phosphatase (PAP), secreted acid phosphatase (sAP), malate dehydrogenase (MDH) and tubulin 6 (Tub6) as described in Chapter 4. For *G. lotifolia*, cDNA libraries from four plants from each treatment were tested. For *A. obliquinervia*, cDNA libraries from four plants were used for the Control and 50 mg kg\(^{-1}\) orthophosphate treatments, and three plants were used from the remaining P treatments.

A touchdown protocol was used with the primers for MDH. MyTaq polymerase and its accompanying buffer were used with 1 µL cDNA sample. The protocol began with a hot start at 95 °C for 5 min and the cycles included 95 °C for 30 s, the annealing temperature for 15 s, and an extension at 72 °C for 10 s. The annealing temperature began at 72 °C and decreased by 1 °C with each successive cycle until 56 °C was reached. An additional 18 cycles were then run with a 56 °C annealing temperature. After all cycles were completed, samples were incubated at 72 °C for a final 10 min before being cooled and stored at -20 °C.

For both PAP and sAP primers, the PCR programs were run twice. The first run used MyTaq polymerase and provided buffer with 1 µL of cDNA sample. The second run used a 1:10 dilution of the initial PCR product with fresh polymerase and buffer. All protocols began with a hot start at 94 °C for 5 min. The PAP program used 36 cycles of 95 °C for 30 s, 56 °C for 15 s, and 72 °C for 10 s. A final 10 min extension at 72 °C completed the program. The sAP program used 36 cycles of 95 °C for 30 s, 50.4 °C for 15 s, and 72 °C for 10 s, with a final 10 min extension at 72 °C.
For Tub6, the PCR programs were also run twice. The first run used MyTaq polymerase with the provided buffer and 1 µL of cDNA sample. The second run used a 1:100 dilution of the initial PCR product with fresh polymerase and buffer. The protocols began with a hot start at 95 °C for 5 min. The program then used 36 cycles of 95 °C for 30 s, 56 °C for 15 s, and 72 °C for 10 s. A final 10 min extension at 72 °C completed the program.

At least one negative control containing PCR primers but no added cDNA was included with each PCR program. Where dilutions were used, dilutions of the negative controls were also included to ensure samples were free of contaminating DNA. Enough MyTaq polymerase, buffer and nuclease-free water for all the PCR reactions were made up and then aliquoted into each PCR tube containing primers and cDNA. All primers were used at a 10 µM concentration and premixed before an aliquot was transferred into each PCR reaction tube. All PCR reactions were done at a 10 µL volume except the final select reactions used for sequence analysis.

PCR products were separated using electrophoresis with a 2% agarose gel containing ethidium bromide in 1x TAE running buffer. Any bands were visualised using a ChemiDoc XRS+ system (BioRad; Gladesville, NSW).

PCR products were sequenced commercially by Macrogen (Seoul, Republic of Korea). PCR samples to be sequenced were initially isolated using a 2% agarose gel containing ethidium bromide and electrophoresis in 1X TAE buffer. The bands were visualised using UV light, and 26 gauge needles were inserted once into each band of interest to collect a sample of the PCR product. A subsequent 30 µL volume PCR was run for PAP, MDH, and sAP samples using the protocols described, except the cDNA sample was supplied from the needles (Bjourson & Cooper 1992).
The PCR products for sequencing were precipitated using 0.1 volume of 3 M sodium acetate, pH 5.2, and 2 volumes of 100% ethanol and an overnight incubation at -20 °C. Tubes were centrifuged at 12000 x g for 20 min, the supernatant was removed, and the pellet was washed with 500 µL of 70% ethanol. Samples were centrifuged for 10 min at 12000 x g, supernatant was discarded, and pellets were allowed to air-dry for 5 min. Samples were resuspended in 20 µL DEPC-treated water, and the concentration was checked using a Nanodrop spectrophotometer.

To confirm whether PCR products displayed homology to known sequences, the basic local alignment search tool (BLAST) for nucleotides (BLASTn) and proteins (BLASTx) from NCBI (blast.ncbi.nlm.nih.gov) were used, respectively. BLASTx translates the nucleotide sequence to amino acids to compare protein sequences.

For the putative PAP nucleotide sequence isolated from *Goodia lotifolia* plant 6 (GlP6), homology was compared to: PAP5 from *Phaseolus vulgaris* (GQ891043), PvPAP3 from *P. vulgaris* (FJ464333), PAP 17-like isofrom 2 from *Glycine max* (N0M001255621), a PAP from *Medicago truncatula* (XM003631096), acPase3 from *Lupinus lutens* (AJ617676), PAP 17-like variant 1 from *Glycine max* (XM003530928), PAP 17-like variant from *Cicer arietinum* (X1XM004513281), and the putative PAP from *Acacia dealbata* identified in Chapter 4 (Wp126).

For the putative sAP nucleotide sequence isolated from *Goodia lotifolia* plant 6 (GlS6), homology was compared to: Lasap2 from *Lupinus albus* (AB037887), LaSAP from *Lupinus albus* (AF309552), Lasap1 from *Lupinus albus* (AB023385), Acpase2 from *Lupinus lutens* (AJ505579), a PAP precursor from *Phaseolus vulgaris* (AJ001270), a phytase from *Glycine max* (NM001253997), a PAP-like sequence from *Cicer arietinum* (XM004506271), PAP14 from *Glycine max* (JN967626), and MtPAP1 from *Medicago truncatula* (AY804257).
The translated amino acid sequence was also examined for domains conserved across protein families using BLASTx and the conserved domain database from NCBI (ncbi.nlm.nih.gov/cdd). The translated amino acid sequence for the putative PAP from Goodia lotifolia (GlP6) was compared to: PAP 17-like isoform from Cicer arietinum (X1XP004513338), PAP 17-like isoform 1 from Glycine max (XP003530976), acPase3 from Lupinus luteus (CAE85073), a PAP from Medicago truncatula (XP003631144), PAP5 from Phaseolus vulgaris (ADK56125), PAP3 from Phaseolus vulgaris (ACO25293), an uncharacterised protein from Glycine max (NP001242550), the putative PAP isolated from Acacia dealbata in Chapter 4 (Wp126), and a consensus sequence for a conserved domain of metallophosphatase ACP5 (CDD:cd07378).

The translated amino acid sequence for the putative sAP from Goodia lotifolia (Gls6) was compared to: LASAP2 from Lupinus albus (BAA977745), LASAP from L. albus (AAK51700), a PAP-like sequence from Cicer arietinum (XP004506328), PAP 14 from Glycine max (AFH08750), MTPAP1 from Medicago truncatula (AAX20028), a putative APase from Lupinus luteus (CAD44185), a phytase from Glycine max (NP001240926), a PAP precursor from Phaseolus vulgaris (CAA04644), and a consensus sequence for conserved domain of metallophosphatase PAP (CDD:cd00839).

5.2.4 Statistical analysis

Differences in height and weight and soil concentrations of P among treatments were analysed using two-way analysis of variance. Tukey’s post-hoc tests were used when an overall effect was detected. The two species were analysed separately, and tests of normality were conducted. Any nested effects due to pots were not tested, as the pot of origin was not recorded at the time of tissue collection. A $P$ value less than 0.05 was considered significant. All the analyses were performed using the software SPSS Statistics v.20 (IBM).
5.3 Results

5.3.1 Phosphorus concentration and type – growth and tissue chemistry

Concentrations of extractable P in sand from pots differed significantly according to the quantity but not the form of P supplied (Fig. 5.1). Extractable P was less than 0.5 µg g\(^{-1}\) sand in the group that was not supplied P (Control). At both concentrations, the addition of inorganic P (IP) increased the concentration of extractable P in sand to a greater extent than organic P (OP; Fig. 5.1).

![Bray 1-P (µg kg\(^{-1}\) soil)](image)

**Figure 5.1** Concentrations of extractable P in sand following 11 weeks of nutrient treatment. Woody legumes were supplied with no added P (Control) and 10 or 50 mg kg\(^{-1}\) of organic P (OP) or inorganic P (IP). Each bar represents the mean and standard error from four pots. Different letters indicate significant differences between treatments.

Due to production of small amounts of biomass, leaves for each species were bulked according to treatment group prior to foliar P analysis, precluding subsequent statistical analysis. Fig 5.2A shows overall foliar P concentrations to suggest possible effects of different types and concentrations of P for *Goodia lotifolia* and *Acacia obliquinervia*.
Supplying 10 mg kg$^{-1}$ of IP (IP10) increased mean dry weight of *Goodia lotifolia*, but dry weight for this species was highly variable and statistical differences were not detected (Fig. 5.2B). For *Acacia obliquinervia*, neither the amount nor form of P supplied significantly altered above-ground weight compared to Control over the 11 wk period (Fig. 5.2B). Dry weight of shoots tended to be lower for the plants receiving 50 mg kg$^{-1}$ of either form of P, but no significant effects were detected (Fig. 5.2B). The amount of type of P supplied did not significantly alter height for either species (Fig 5.2C).
Figure 5.2 Foliar P concentration and growth of woody legumes supplied with different amounts and forms of P. (A) Total foliar P expressed as percentage of foliar weight, (B) oven-dry weight, and (C) final height of Goodia lotifolia or Acacia obliquinervia. Each bar represents the mean and standard error.
**Figure 5.3** Representative pictures showing excavated roots from two species of woody legumes supplied with different amounts and forms of P. *Acacia obliquinervia* and *Goodia lotifolia* were supplied with no added P (control) and 10 or 50 mg kg\(^{-1}\) of organic P (OP) or inorganic P (IP). The entire plants that produced the roots are shown as insets.
Figure 5.4 Total RNA extracted from roots of *Goodia lotifolia* and *Acacia obliquinervia* supplied with different amounts and forms of P. Total RNA was extracted from 51 plants, alternating between species and treatment groups in random order, and plants were subsequently identified by number, corresponding to ID number in Appendix 5.1. Extracts from *G. lotifolia* are indicated by an asterix. Hyperladder IV from Bioline was used as the basepair ladder. The far right of the image shows band sizes, taken from the manufacturer's website (www.bioline.com.au). IV: hyperladder IV.
5.3.2. PCR results

**RNA extraction**

The amount or type of P supplied did not alter the quantity of roots produced by either species of woody legume (Fig. 5.3). *G. lotifolia* plants often appeared to produce larger volumes of roots than *A. obliquinervia* plants, regardless of above-ground size (Fig. 5.3, insets). The darker bands and lack of smearing in Fig. 5.4 indicated the yield of total RNA generally had a higher quantity and quality from *G. lotifolia* than *A. obliquinervia* (see also Appendix 3.

**Gene induction**

When cDNA libraries constructed using root extracts from *G. lotifolia* were tested, PCR products were obtained using all three primer sets (Fig. 5.5). These included primers targeting malate dehydrogenase (MDH), purple acid phosphatase (PAP), and secreted acid phosphatase (sAP). PCR products obtained using any set of primers were not restricted to plants receiving a specific amount or type of P. The use of Tubulin 6 (Tub) as a positive control indicated that the quality of cDNA transcribed was not adequate for PCR for all individuals of *G. lotifolia* tested.

PCR using PAP primers amplified a product from a single individual of *A. obliquinervia* that had been growth with 50 mg kg⁻¹ inositol phosphate (Fig. 5.6). However, distinct bands were not observed when primers targeting sAP were used. Some plants produced a smear when MDH primers were used, but subsequent attempts to produce a distinct band, indicating a specific product, were not successful (data not shown). Results using Tub primers suggested that the quality of cDNA transcribed using RNA from several plants of *A. obliquinervia* were not adequate for PCR.
Figure 5.5 PCR products using 20 cDNA libraries constructed using root extracts from *Goodia lotifolia*. Four PCR reactions were conducted for each plant, designed by the sequence ‘M P S T’. Hyperladder IV was used as the basepair ladder. Band sizes are shown in Fig 5.4. A ‘-‘ symbol following the primer designation indicates a negative control without added cDNA. M: malate dehydrogenase; P: purple acid phosphatase; S: secreted acid phosphatase; T: tubulin 6.
Figure 5.6 PCR products using 17 cDNA libraries constructed using root extracts from *Acacia obliquinervia*. Four PCR reactions were conducted for each plant, designed by the sequence ‘M P S T’. Hyperladder IV was used as the basepair ladder. Band sizes are shown in Fig. 5.4. A '-' symbol following the primer designation indicates primers without added cDNA. b: blank lane; M: malate dehydrogenase; P: purple acid phosphatase; S: secreted acid phosphatase; T: tubulin 6.
Homology

The nucleotide sequence amplified using PAP primers on cDNA from *G. lotifolia* demonstrated 69—86% homology to nucleotide sequences encoding known or putative PAPs from several herbaceous legumes (Fig. 5.7, Table 5.2). The corresponding amino acid sequences from the known or putative PAPs showed 62—82% homology to the amino acid sequence translated from *G. lotifolia*’s putative PAP (Fig. 5.8, Table 5.2). Considerable homology was also found between putative PAPs for *G. lotifolia* and *Acacia dealbata* (Chapter 4). The putative PAP from *G. lotifolia* contained a conserved domain related to *Homo sapiens* acid phosphatase 5 (E-value: 1.52e-4), which belongs to the metallophosphatase superfamily that also includes PAP (Fig. 5.8).

The nucleotide sequence amplified using sAP primers on cDNA from *G. lotifolia* demonstrated 77—85% homology to nucleotide sequences encoding known sAPs and PAPs and a putative PAP and phytase from several herbaceous legumes (Fig. 5.9, Table 5.2). The corresponding amino acid sequences from these known or putative acid phosphatases showed 59—78% homology to the amino acid sequence translated from the putative sAP from *G. lotifolia* (Fig. 5.10, Table 5.2). The sequence encoding the putative sAP from *G. lotifolia* did not contain any conserved domains.

The PAPs homologous to the putative sAP were different than the PAPs homologous to the putative PAP from *G. lotifolia*. Similarity between the sAP and PAP nucleotide sequences was 26% and occurred as scattered rather than contiguous blocks of nucleotides (Fig. 5.11). Only 4% of the amino acid sequences were homologous between the putative sAP and PAP from *G. lotifolia* (Fig. 5.11).
The nucleotide sequence amplified using MDH primers on cDNA from *G. lotifolia* did not show homology to any known or putative MDH sequences from any FABACEAE species. In addition, the nucleotide sequence amplified using PAP primers on cDNA from *Acacia obliquinervia* did not show any homology to known or putative acid phosphatase sequences from any FABACEAE species.
Figure 5.7 Homology of the PCR product obtained using purple acid phosphatase (PAP) primers on cDNA from Goodia lotifolia grown in a pot-culture.

Comparisons were made with mRNA sequences encoding known or putative PAPs from five species of herbaceous legumes, identified by GenBank accession numbers, as detailed in 5.2.3. Green shading indicates the herbaceous species has an identical nucleotide to that for G. lotifolia. Blue shading indicates the nucleotide is conserved across all the herbaceous species but differs from that sequenced for G. lotifolia. Numbers indicate position within the sequence.

- : gap introduced in sequence to maximise alignment.
Figure 5.8 Homology of the amino acid sequence translated from the putative PAP from Goodia lotifolia. The nucleotide sequence was translated and compared to amino acid sequences from the Fabaceae taxid using NCBI’s blastx. Comparisons with known or putative PAPs are shown for five species of herbaceous legumes, identified by GenBank reference sequences as given in 5.2.3. Green shading indicates the herbaceous species has an identical amino acid to that for G. lotifolia. Blue shading indicates the amino acid is conserved across all the herbaceous species but differs from that sequenced for G. lotifolia. Red shading indicates the amino acid conserved with the consensus sequence for metallophosphatase conserved domain. Numbers indicate position within the sequence. -: gap introduced into sequence to maximise alignment; *: stop codon.
Figure 5.9 Homology of the PCR product obtained using secreted acid phosphatase (sAP) primers on cDNA from Goodia lotifolia grown in pot-culture. Comparisons were made with mRNA sequences encoding known or putative sAPs from six species of herbaceous legumes, identified by GenBank accession numbers as detailed in 5.2.3. Green shading indicates the herbaceous species has an identical nucleotide to that for G. lotifolia. Blue shading indicates the nucleotide is conserved across all the herbaceous species but differs from that sequenced for G. lotifolia. Numbers indicate position within the sequence. -: gap introduced into sequence to maximise alignment.
Figure 5.10 Homology of the amino acid sequence translated from the putative sAP from Goodia lotifolia. The nucleotide sequence was translated and compared to amino acid sequences from the Fabaceae taxid using blastx from NCBI. Comparisons with known or putative PAPs are shown for four species of herbaceous legumes, identified by GenBank reference sequences as detailed in 5.2.3. Green shading indicates the herbaceous species has an identical amino acid to that for G. lotifolia. Blue shading indicates the amino acid is conserved across all the herbaceous species but differs from that sequenced for G. lotifolia. Red shading indicates the amino acid is conserved with consensus sequence for metallophosphatase conserved domain. Numbers indicate position within the sequence. G1S6: -: gap introduced into sequence to maximise alignment; *: stop codon.
Figure 5.11 Comparison of nucleotide and amino acid sequences between putative PAP and sAP from Goodia lotifolia plant 6. Green shading indicates an identical nucleotide or amino acid. Numbers indicate position within the sequence. G1P6: G. lotifolia PAP product from plant 6; G1S6: G. lotifolia sAP product from plant 6; -: gap introduced into sequence to maximise alignment; *: stop codon.
Table 5.2 Sequence homologies between putative PAP or sAP from *Goodia lotifolia* and sequences from herbaceous legumes. Both nucleotide and amino acid sequences were compared among *G. lotifolia* and each of the legume species. The percentage of identical nucleotides or amino acids is shown. The accession numbers for each species refer to GenBank. #: number; nt: nucleotide; aa: amino acid.

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<th>Species</th>
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<th>Cicer arietinum nt/aa</th>
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5.4 Discussion

5.4.1. Phosphatase expression in woody legume roots

*Putative phosphatases expressed in woody legume roots*

Roots of *Goodia lotifolia* expressed two putative phosphatases when plants were grown under nutrient-controlled conditions in a pot-culture. The two PCR products included a putative PAP that displayed homology with known or putative PAPs from herbaceous legumes and to the putative PAP isolated from roots of *A. dealbata* in Chapter 4. This homology included both nucleotide and translated amino acid sequences. The second PCR product from *G. lotifolia* was homologous with known sAPs and some PAPs from herbaceous legumes. Neither the nucleotide nor amino acid sequences were homologous between the putative sAP and putative PAP from *G. lotifolia*. Discovering these two PCR products supported the first hypothesis that multiple species of woody legumes would express genes encoding putative phosphatases or carboxylate precursors.

The putative PAP from roots of *G. lotifolia* showed the most homology to low molecular weight PAPs that included PvPAP5 and PvPAP3 from *Phaseolus vulgaris* and PAP 17-like isoforms. Low molecular weight, or small, PAPs are more closely related to mammalian than fungal PAPs, and typically include the conserved AcP5 metallophosphatase domain (cd07378) to encode the metal-binding portion of these PAPs (del Pozo et al. 1999; Olczak et al. 2003; Lu et al. 2008). The putative PAP from *G. lotifolia* contained this conserved domain, indicating the sequence encoded a metal-binding portion and was a small PAP.

Purple acid phosphatases constitute the largest family of acid phosphatases, and they consequently exhibit a broad range of functions, regulation, and substrate specificities (Olczak et al. 2003). The sequence isolated from roots of *G. lotifolia* exhibited homology
to a group of PAPs that are commonly found to be expressed in plant roots, contain sequences that signal for the proteins to be secreted, and are upregulated by P-deficiency (Zhu et al. 2005; Lu et al. 2008; Liang et al. 2010; Liang et al. 2012b). Substrate specificity was only characterised for PvPAP3, which exhibited the greatest activity on nucleotides and very little activity with phytic acid (Liang et al. 2010). The other homologous PAP from *Phaseolus vulgaris*, PvPAP5, demonstrated little activity on nucleotides, but it was not tested with other substrates (Liang et al. 2012b). Substrate specificity is unknown for the other PAPs found to be homologous with the sequence from *G. lotifolia*. Therefore, the putative PAP from *G. lotifolia* may be secreted from roots in response to P-deficiency, but it is unclear whether the protein would demonstrate significant phosphatase activity on nucleotides or a different phosphate ester.

The putative sAP from *G. lotifolia* was homologous to the three LASAPs isolated from *Lupinus albus* and other, closely-related PAPs (Wasaki et al. 2003; Xiao et al. 2006a). The homologous sequences included the conserved PAP metallophosphatase domain (ed00839) found within the same metallophosphatase superfamily that contains the AcP5 domain identified in the putative PAP from *G. lotifolia*(NCBI 2000). A conserved domain search using the putative sAP from *G. lotifolia* did not find significant homology with this conserved domain, but detecting homology may have been hampered by the short sequence for sAP available for comparison.

Secreted acid phosphatases from *Lupinus albus* are closely related to a group of secreted PAPs (Wasaki et al. 2003; Xiao et al. 2006b), therefore sAPs may constitute a type of PAP (Xiao et al. 2006a). The sAPs and related PAPs that have been characterised were found to be expressed in root tissues, were upregulated by P-deficiency, and contained sequences that targeted the proteins for exudation into the rhizosphere (Miller
et al. 2001; Olczak & Wątorek 2003; Wasaki et al. 2003; Xiao et al. 2006a). Substrate specificity was quite broad and included nucleotides and multiple components of the citric acid cycle (Miller et al. 2001) and phytate (Xiao et al. 2006b). Thus, the sequence from *G. lotifolia* may encode a sAP with broad specificity that roots exude into the rhizosphere when plants experience P-deficiency.

**Low phosphorus supply and phosphatase expression**

Contrary to the second hypothesis, low P supply did not induce the expression of putative phosphatases in roots from either *Goodia lotifolia* or *Acacia obliquinervia*. PCR products amplified using primers that targeted PAP or MDH were found in roots of *G. lotifolia* regardless of the amount of inorganic or organic P supplied. The MDH primers also amplified PCR products across all treatment groups for *A. obliquinervia*. Primers targeting sAP or PAP amplified products in a single sample from *G. lotifolia* or *A. obliquinervia*, respectively. Although the putative PAPs and sAP were not found to be induced by the treatments used in this study, it is possible P availability modulates the degree to which these genes are expressed.

Phosphorus deficiency is not always required to induce genes encoding exuded phosphatases. Species including *Brassica napus*, *Arabidopsis thaliana*, *Medicago truncatula* and *Lupinus luteus* expressed PAP genes in their roots when supplied with adequate concentrations of inorganic P, although P-deficiency increased PAP gene expression (Olczak & Wątorek 2003; Zhu et al. 2005; Xiao et al. 2006a; Lu et al. 2008). Conversely, roots from *P. vulgaris* expressed multiple exuded PAPs only when plants were P-deficient (Tian et al. 2007; Liang et al. 2010; Liang et al. 2012b). Roots of *L. albus* expressed two exuded sAPs only when plants were P-deficient (Wasaki et al. 2000; Miller et al. 2001). *L.*
albus roots expressed a third sAP constitutively, but this phosphatase was postulated to be retained within the root tissue (Wasaki et al. 1999; Miller et al. 2001).

Phosphorus treatment alone did not alter whether a putative PAP was expressed in roots of *G. lotifolia*. This putative PAP showed the most homology to PAP 17-like isoforms, related to the constitutively-expressed PAPs in *B. napus* and *A. thaliana* (Lu et al. 2008). Nevertheless, the PAP-17s and putative PAP from *G. lotifolia* are closely related to the PAPs from *P. vulgaris* that are induced by P-deficiency (Miller et al. 2001; Wasaki et al. 2003; Lu et al. 2008). Thus, it is possible that roots of *G. lotifolia* express a PAP constitutively, but P-deficiency enhances its expression. Additional studies using quantitative methods are necessary to test this hypothesis. The PCR products amplified using PAP primers on cDNA from *A. obliquinervia* or using MDH primers on either species did not show homology to known PAPs or MDHs. Therefore, the levels or forms of P supplied in this study were not suitable to induce expression of these genes.

The putative sAP from *G. lotifolia* showed equivalent homology to sAPs from *L. albus* that were induced by P-deficiency (Miller et al. 2001; Wasaki et al. 2003) and to PAPs from *L. luteus* and *M. truncatula* that were constitutively expressed (Olczak & Wątorek 2003; Xiao et al. 2006b). Only a single plant that received no additional P clearly expressed the potential sAP in *G. lotifolia*. Expression in other plants may have been below detectable limits, because this sAP product required two consecutive PCR programs to become visible by gel electrophoresis. Alternatively, sAP expression in *L. albus* is higher in proteoid roots than normal roots (Miller et al. 2001; Wasaki et al. 2003), thus utilising the entire root mass to extract mRNA may have diluted the contents of sAP.

5.4.2. Growth response of woody legumes to P supply
The organic and inorganic P supplied to *G. lotifolia* and *A. obliquinervia* was provided at concentrations reported to support optimum stem growth or promote P-deficiency (Hingston et al. 1982; Islam et al. 2000; Aono et al. 2001; Pang et al. 2010). Resulting foliar concentrations of P and biomass differed between the two species, but neither showed a clear correspondence to P supply. Foliar P concentrations in *A. obliquinervia* corresponded to extractable P, although the no differences were seen between the two concentrations of inorganic P. Aboveground biomass of *A. obliquinervia* was highly variable within each treatment group and did not corresponded to foliar P concentrations.

For *G. lotifolia*, the quantity of inorganic P supplied demonstrated an apparent direct relationship to foliar P concentration and aboveground dry mass. Conversely, organic P appeared to decrease the amount of foliar P, even compared to no supplemental P. This pattern suggests *G. lotifolia* utilises foliar P reserves when P is available only in an organic form. More extensive roots or root exudates to facilitate acquisition of P might account for the lower foliar P that was observed.

Phosphorus deficiency has often been shown to alter growth patterns in plants. Many herbaceous species increase the root to shoot ratio of mass or length when P is limiting (Föhse et al. 1988; Tjellström et al. 2008). Roots may be lengthened, cluster roots may form, and root architecture may shift to place roots in shallower soil horizons (Adams & Pate 1992; Abel et al. 2002; Lambers et al. 2006). Overall growth may be slowed in P-deficient plants (Hingston et al. 1982; Islam et al. 2000; Aono et al. 2001; Pang et al. 2010). Alternatively, some P-deficient legume species may change leaf size or number rather than overall mass (Fernandez & Ascencio 1994). Plants may also conserve P using galactolipids to replace phospholipids in cell membranes, or by withdrawing P from older
and senescing leaves (Wright & Cannon 2001; Tjellström et al. 2008; Lambers et al. 2010). As a result, the foliar P concentrations, rather than growth measurements, serve as a better indicator of P limitation.

This study presents the novel finding that a second species of woody legume expressed two putative phosphatases in root tissue. Phosphorus deficiency did not induce expression of these genes. Whether, P availability modulates their expression is worth investigating in future studies.
Chapter 6 – Phos-fire-us: contributing factors and implications of the success of woody legumes to regenerate after fire in south eastern Australia

The Australian continent is characteristically fire-prone, dry, and contains some of the oldest and most weathered soils in the world. Expressing traits that enable survival despite recurring fire, limited moisture and nutrient deficiencies has enabled some plant families to thrive in Australia. These plant families often coexist in plant communities potentially by occupying different ecological niches (Pfautsch et al. 2009a; Campbell et al. 2012). Woody legumes represent one of the most successful taxa in Australia, contributing the most speciose genus to the flora and occupying almost every terrestrial ecosystem on the continent (Hnatiuk & Maslin 1988; Rundel 1989; Orians & Milewski 2007; González-Orozco et al. 2011). In addition, woody legumes can regenerate rapidly in southern Australian forests following bushfire (Shea et al. 1979; Bell & Koch 1980; Auld & Denham 2006). The results presented in this thesis suggest that traits purported to facilitate the broad dispersal of woody legumes across Australia may also enable their recruitment after severe fire. How these characteristics affect productivity and fuel-loading in fire-affected communities provides grounds for further discussion and research.

6.1 Distribution patterns of Australian woody legumes

Although woody legumes exhibit a flush of recruitment following bushfire in Australian temperate regions, the stand densities of woody legumes exhibit a mosaic pattern across the burned landscape (Hodgkinson 2002; Warton & Wardle 2003; Auld & Denham 2006). This thesis has confirmed the patchy distribution pattern and found that stand densities for six species of woody legumes recurred with sufficient frequency to permit categorisation. Stands from different categories often clustered together, but the distances separating them suggested the factors influencing population density exhibited
a discrete distribution rather than forming a continuum. Habitat boundaries were not readily discernible, similar to observations for several Neotropical woody species (Harms et al. 2001). This aligned with the concept that recruitment reflects the influence of multiple factors on germination, establishment, growth, fecundity and mortality (Nathan & Casagrandi 2004; Yamada et al. 2013). Sites where ‘high-density’ populations recurred were likely to contain a confluence of factors optimised to facilitate recruitment of woody legumes following bushfire.

The woody legume species investigated in this study regenerated following a particularly severe bushfire (Cruz et al. 2012), although the tall wet sclerophyll forests that burned typically experience the highest intensities of fire regimes in Australia (Ashton 1981; Christensen et al. 1981; Murphy et al. 2013). The six species investigated also grow in regions with considerably different fire regimes, as do numerous other species of woody legumes (Rundel 1989; Sprent 1995; Costermans 1996; Murphy et al. 2013; Bui et al. 2014). Thus, a high fire severity is not necessary to recruit woody legumes, but low-intensity fires are typically considered insufficient to promote the regeneration of high-density populations of woody legumes (Christensen et al. 1981; Auld & O'Connell 1991).

Fire severity depends on fire intensity, the proportion of fuel consumed and residence time, which together determine the cumulative amount of heat produced within an area (Brooks et al. 2004; Keeley 2009). Heat is necessary to break the dormancy of legume seeds, but successful recruitment of woody legumes could also reflect the availability of soil nutrients, light or water, which can be altered according to fire severity. Although low, medium and high-density populations were all recruited following a severe fire, variable stand densities might reflect a heterogeneous pattern of fire severity. The three edaphic factors contributing to the most accurate model could be influenced by fire severity, but
all three factors occurred at 5—10 cm depths, while factors in upper soil layers were not selected. It is possible the lower range of fire severities were still high enough that edaphic differences could only be detected deeper in the soil, or edaphic factors at 5—10 cm depth could reflect conditions prior to the fire (Bui et al. 2014). Regardless, the selected edaphic factors would influence the acquisition of P. In addition, the greater heat associated with more severe fire could have directly affected the number of woody legume seeds that became permeable to water or experienced lethal temperatures.

6.2 Interactions between selected factors and woody legume traits

6.2.1 Heat and dormant seed

Woody legumes produce physically dormant seeds that require heat exposure to make the seed-coat permeable to water. Exposure to 90 °C wet heat was lethal to seeds for the greatest number of species. Although low or moderate-intensity fires typically heat soil to 90 °C above 2 cm depth (Auld 1986b; Bradstock & Auld 1995; Penman & Towerton 2008), high-intensity or severe fires can produce 90 °C temperatures at 5 cm or greater depths (Beadle 1940; Shea et al. 1979; Williams et al. 2004). If high fire severity killed woody legume seeds through a wider range of the soil profile, this would constrain the recruitment of high population densities.

Several factors suggested that sites with medium-density populations may have experienced the greatest fire severities. When fire severities were calculated post-hoc (Cruz et al. 2012), some low and high-density sites were assigned lower categories of fire severity than those found on medium-density sites. Medium-density sites more frequently had aspects that enabled the fire to travel upslope. In addition, several soil characteristics measured at medium-density sites could have resulted from greater fire severity, including higher bulk density, lower water-holding capacity, and less total nitrogen (N) and carbon.
(C) (Cheney 1981; Tomkins et al. 1991; Romanyà et al. 1994; Homann et al. 2011). Three species were not assigned a medium-density category, which might indicate a higher heat tolerance of their seed (Ooi et al. 2014) or that fire severity did not influence the establishment of medium-density populations.

Temperature thresholds to break seed dormancy or increase seed mortality varied among species from different climate regions in a manner that facilitated rational interpretation. Arid-zone species showed the lowest thresholds to germinate or kill seed when wet heat was applied. The temperature thresholds corresponded to either solar radiation affecting the upper soil layers (Hagon 1971; Zeng et al. 2005; Van Klinken et al. 2006; Penman & Towerton 2008; Ooi et al. 2014) or low-intensity fires common to arid ecosystems in Australia (Murphy et al. 2013). Seeds from tropical species had higher mortality rates when exposed to 90 °C in the absence of moisture or 90 °C wet heat for long periods, corresponding to the low-intensity fires typical for the wet tropics (Murphy et al. 2013). Temperate-zone species produced seeds with the highest temperature thresholds for both mortality and breaking seed dormancy, and high-intensity fires occur more frequently in temperate regions (Murphy et al. 2013). Altogether, these patterns suggest that seed traits for woody legumes are tailored to the climate zone the species inhabit (Moreira & Pausas 2012; Bui et al. 2014), which would facilitate both broad dispersal patterns across Australia and germination success following severe fire in temperate areas.

6.2.2 Nutrient acquisition

A large proportion of woody legume species can support biological nitrogen fixation (BNF). Nitrogen stored in organic matter volatilises to the atmosphere during combustion, thus a severe fire can significantly reduce the amount of N available to
recruited plants (Raison 1979). Woody legumes regenerating after bushfire in Australian forests typically use BNF to acquire additional N when soil N concentrations are low (Hingston et al. 1982; Adams & Attiwill 1984; Hansen et al. 1987; Pfautsch et al. 2009b). Woody legumes from several Mediterranean ecosystems were also found to have significantly higher foliar N concentrations compared to non-leguminous neighbours, indicating woody legumes use adaptations to acquire N (Stock & Verboom 2012).

Regardless, adaptations to acquire N did not appear to underlie the distribution of high-density populations in this study, and global distributions of woody legumes do not correspond to N-limited soils. The global pattern has been attributed to the high levels of energy and phosphorus (P) required for BNF (Crews 1999; Vitousek et al. 2002; Houlton et al. 2008; Menge et al. 2008). Similarly, this study found low P availability was a predictor for sites containing high-density populations of woody legumes.

Australian woody legumes may use mycorrhizal associations or form cluster roots (Adams et al. 2002; Adams et al. 2010) that may exude carboxylates, organic acids or phosphatases to increase P acquisition (Bolan 1991; Duff et al. 1994; Lambers et al. 2006). Roots of several herbaceous legume species have been shown to express genes encoding acid phosphatases (Miller et al. 2001; Wasaki et al. 2003; Xiao et al. 2006a; Liang et al. 2012a), but this study is the first to demonstrate that two woody Australian species from the MIMOSOIDEAE and PAPILIONOIDEAE subfamilies can also express phosphatase genes in their roots. The sequences of these putative phosphatases exhibited a high homology to phosphatases known to be secreted into the rhizosphere. The capacity to produce and exude phosphatases without requiring mycorrhizae represents another trait that would facilitate broad dispersal across varied ecosystems.
Acid phosphatases are most efficient within a specified pH range, and soil pH was a contributing factor to the model developed in this study. In addition, soil pH was a nominated factor influencing assemblages of *Acacia* species in the southeastern temperate zone of Australia (Bui et al. 2014). Soil moisture is also necessary to allow diffusion of P, and drought conditions have been shown to attenuate the usual effects of added P on woody legume growth (Islam et al. 2000). Although factors affecting soil moisture were not found to predict high-density populations, the forests in this study typically receive greater than 1200 mm of rainfall annually. Furthermore, *Acacia* seedlings can change their xylem structure to alter xylem conductivity (Clemens & Jones 1978), similar to obligate seeders from Mediterranean climates (Vilagrosa et al. 2014). *Acacia* species can also establish long taproots to access water deep below dry sandy soils (Sprent 2007). Despite the ability to adapt to low water availability, precipitation contributes significantly to assemblages, or phytogeographical regions, of *Acacia* species (Hnatiuk & Maslin 1988; González-Orozco et al. 2013), suggesting species are physiologically adapted to regional precipitation, akin to seed dormancy thresholds being adapted to fire regimes (Hyde 1954; Ooi et al. 2014). Several Australian species of woody legumes are restricted to particular geochemical conditions where the species exhibit dominance, indicating the species have adapted to local or regional conditions (Bui et al. 2014). The dominance of woody legumes across numerous Australian ecosystems, however, suggests the taxa can successfully adapt or exapt traits related to fire regime, water availability, and soil nutrient status, and this ability is likely to contribute to the establishment of high population densities following bushfire.
6.3 Ecosystem services and implications for future fires

Several factors influence the propensity of a plant community to burn, and these determinants may have acute or chronic effects and varied impact over time (Mutch 1970). Although senescent woody legumes can directly supply a fuel source, they may indirectly contribute to conditions that create a more flammable community long after the legumes are no longer apparent. Considering the dependence of most legume seed for heat to break seed dormancy, this may represent a positive feedback cycle to promote their continued recruitment (Bond & Keeley 2005; Moreira et al. 2014). However, experiments that directly measure if woody legumes alter the overall flammability of a community are rare.

6.3.1 Woody legumes and productivity

Woody legumes can establish within 6 months following bushfire, and many species are able to set seed within 2—4 years after fire (Monk et al. 1981; Clark 1988; Guinto et al. 2000; Auld & Denham 2006). Moreover, some *Acacia* species may maintain a residual seedbank in the soil following even a moderate to high-intensity bushfire, reducing the risk that two fires in quick succession would cause local extinction of the species (Auld & Denham 2006). The rapid establishment of woody legumes may partially ameliorate the effects of fire on soil N, pH and bulk density; may be instrumental to reduce the risks of soil erosion and plant pathogens and may provide habitat and food sources to local fauna (Christensen et al. 1981; Adams & Attiwill 1984; O'Connell 1986; Musil 1993; Letnic et al. 2000; Certini 2005; Lindenmayer et al. 2009; Barton et al. 2014; Dong et al. 2014).

Thus, it is plausible that sizeable populations of woody legumes may boost productivity of regenerating communities.
Nitrogen limitation has been found to attenuate net primary productivity of many ecosystems worldwide (Vitousek et al. 2002). Most Australian genera of woody legumes can undertake BNF, but multiple factors influence whether woody legumes significantly augment soil N in the communities where they grow (Lawrie 1981; Hansen et al. 1987; Guinto et al. 2000; Brockwell et al. 2005; Forrester et al. 2007; Houlton et al. 2008; Durán et al. 2010). Woody legumes retain a significant amount of ‘fixed’ N in tissues that must senesce and decompose to contribute N to the soil some years after the woody legumes become established (Adams & Attiwill 1984; Hansen et al. 1987; Forrester et al. 2007; Turner et al. 2008). Although a high foliar N may encourage herbivores to selectively graze woody legumes, a third or more of foliar compounds may represent non-protein N, and woody legumes typically contain phenolic compounds that can reduce the palatability of their leaves (Langenheim 1994; Islam et al. 2000; Vitousek et al. 2002; Orians & Milewski 2007). However, foliar phenolics can bind proteins, slowing decomposition and N mineralisation, help prevent leaching of soil N and P, and promote fungal-based food webs that may retain more nutrients in the soil compared to bacteria (O'Connell 1986; Fox et al. 1990; Northup et al. 1998; Cattanio et al. 2008; Hoorens et al. 2010; Tharayil et al. 2011). Thus, the overall effect of a woody legume understorey is to increase concentrations of soil N.

The concentration of inorganic P in many Australian soils are low enough to attenuate optimal growth unless plants adopt strategies to acquire P and use it efficiently (Beadle 1966; Raghothama 1999; Vance et al. 2003; Lambers et al. 2010). Although fire mineralises organic forms of P to increase inorganic P concentrations, P availability is only elevated for a few years and only in the top few centimetres of soil (Tomkins et al. 1991; Adams 1992; Romanyà et al. 1994). In plant communities that have not been burnt for 6—10 years, most soil P is bound in organic forms, and both foliar P levels and
protein synthesis decrease significantly (Adams 1992; Romanyà et al. 1994; Ferwerda et al. 2006; Rodríguez et al. 2009; Durán et al. 2010; Lambers et al. 2010). Woody legumes have been found to increase soil phosphatase, which may help increase soil levels of inorganic P and boost net productivity (O'Connell 1986; Houlton et al. 2008). In addition, leaf area, number of leaves, stem length, and internode distance were found to increase when soil P was higher (Beadle 1966; Vitousek 1982; Kondracka & Rychter 1997). Thus, more available P might promote more ventilated fuel, but P may also affect inherent flammability.

The chemical composition of fuel affects its flammability and rate of decomposition (Boerner 1982). If fuel is P-rich, it may catalyse cellulose to form char that can form a surface barrier to heating and increase the temperatures required to oxidise the fuel (Scarff & Westoby 2008). In addition, adequate P availability may decrease the production of flammable oils, terpenes or phenolic compounds directly or indirectly through decreased N-limitation (Specht 1981; Langenheim 1994; Dixon & Paiva 1995; Northup et al. 1998; King et al. 2006; Ormeño et al. 2009). Decomposition rates are typically lower for plant matter from nutrient-poor soils, facilitating higher fuel loads (Northup et al. 1998; Hättenschwiler & Vitousek 2000; Cattanio et al. 2008; Hoorens et al. 2010). However, moisture availability is also a key factor influencing both fuel production and litter decomposition (Gill 1981; Walker 1981; Bradstock 2010). Thus the high fuel loads and intense fires typical for wet sclerophyll forests likely result more from moisture availability than the presence of a legume understorey (Ashton 1981; Christensen et al. 1981; Penman & York 2010).

6.3.2 Woody legumes as fuel
Woody legumes display growth habits that include short sprawling shrubs, trees taller than 10 m, and a range of intermediate heights. Despite this variability, Australian woody legumes typically form an understorey layer, although *Acacia* species can constitute the dominant strata in some arid zones (Randell 1970; Grant et al. 2007; Pfautsch et al. 2009b; Page et al. 2011). Mid-strata legumes may provide a bridge to carry fire upwards and allow it to scorch or burn the overstorey. This hazard increases when short-lived woody legumes begin to senesce and provide elevated fuel until the overstorey achieves enough height to provide a practical fire-break (Grant et al. 2007). Many *Acacia* species growing in wet or dry sclerophyll tend to senesce when the overstorey canopy provides considerable shade. However, fire history does not significantly alter the overall biomass of subtropical *Acacia* species (Guinto et al. 2000; Grant et al. 2007; Pfautsch et al. 2009b).

Morphology that alters ventilation nor fuel diameter also influences flammability in addition to fuel chemistry (Mutch 1970; Dickinson & Kirkpatrick 1985). Many woody legume genera produce foliage that has high energy levels and a small specific area that promotes aeration of the fuel (Dickinson & Kirkpatrick 1985; Wink & Mohamed 2003; Meers et al. 2010). Some arid-zone legume species were found to vary their growth forms according to the surrounding landscape, such that mid-strata individuals growing in a woodland were multi-stemmed and tall (Page et al. 2011). In addition, all subfamilies of the FABACEAE produce terpenes that can increase the rate of combustion, maximum flame height and rate of spread, although they do not appear to increase average flame temperature or the percentage of biomass burned (Langenheim 1994; Wink & Mohamed 2003; Ormeño et al. 2009). These characteristics of flammability reflect specific attributes that leaves from Tasmanian species of *Acacia* displayed, regardless if they were fresh or dried (Dickinson & Kirkpatrick 1985). Furthermore, the woody legumes producing foliar terpenes occurred predominately within the mid-strata layer. Recent studies using the
Mediterranean woody legume, *Ulex parviflorus*, found that plants growing in communities with frequent fires produced stems that ignited more rapidly and released more heat than plants growing where fire was infrequent (Pausas et al. 2012). Moreover, the authors found a genetic basis to the increased flammability, suggesting this woody legume species adapted its flammability to the fire regime (Moreira et al. 2014). Woody legumes in Australian temperate zones typically require at least moderate-intensity fires to permit germination (Auld & O’Connell 1991), thus promoting flammable communities could benefit the persistence of these species.

The results from this study indicate that Australian woody legumes in temperate regions express multiple traits that facilitate their regeneration following severe bushfire. Optimum threshold temperatures to break seed dormancy are suited to high-intensity fires in temperate zones, whereas threshold temperatures for arid-zone species are adapted to less severe fires or solar radiation before periodic land inundation. Moreover, woody legumes have the capacity to produce phosphatase genes that may facilitate a competitive advantage for these species when available P is limited. Contributions from woody legumes may play a restorative role following bushfire and promote resilience of plant communities to recurrent fire. Understanding these processes can help support decisions about the management of fire or land restoration.

6.4 Future directions

The data presented in this thesis proffers several additional avenues of research. The model presented in Chapter 2 was developed *post-hoc* and provides an opportunity to test the model when bushfires occur in the future. In addition, the identification of different population densities that are accompanied by soil analyses would allow future studies to examine if the population size of woody legumes significantly affects net primary
productivity. From Chapter 3, the differences in optimum threshold temperatures between species from arid and temperate climates prompts the question whether the testa of these species exhibit physical or physiological differences. Further studies could investigate if a genetic component underlies the different threshold temperatures, throwing light on whether physical dormancy characteristics are adapted to a particular climate. The molecular work in Chapters 4 and 5 opens a large area of future studies, including the identification of homologous genes in other woody legume species and quantitative measurements of gene expression. The related proteins may also be isolated and sequenced to provide additional information about the production and use of phosphatases by woody legumes. These all represent important questions to be addressed, particularly in light of future climate variability, because woody legumes may play a central role in establishing or maintaining the resilience of ecosystems to the increased risk of future fire (Bradstock 2010).
References


Tame T. (1992) *Acacias of Southeast Australia*. Kangaroo Press Pty Ltd, Kenthurst, NSW.


**Appendix 1** Publications including number of species, method to apply heat and climate zones used to conduct the meta-analysis. HH: Hot Humid; HD: Hot Dry; W: Warm

<table>
<thead>
<tr>
<th>Reference</th>
<th>Species (n)</th>
<th>Climate zone</th>
<th>Method of heat application</th>
</tr>
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<td>Wet</td>
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<td>Wet</td>
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Appendix 2 The germination data for each species examined in the germination experiment. Mean proportion of viable seed is given in addition to the n per species in each treatment. m: minutes.

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Appendix 3 The concentration and purity of total RNA extracted from roots of *Goodia lotifolia* and *Acacia obliquinervia* plants supplied with no added P (control) or 10 or 50 mg kg$^{-1}$ of organic P (OP) or inorganic P (IP). A: absorbance.

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