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Seed morphology, dormancy and germination of South-West Australian Ericaceae

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Seed morphology, dormancy and germination of South-West Australian Ericaceae

This thesis is presented in fulfilment to the requirements for the
Degree of Master of Biological Science

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School of Science
2018



Abstract

The Ericaceae in South West Australia contains species with difficult to germinate seeds, including many species with deep intractable dormancy. A better understanding of seed biology and species specific dormancy, and germination mechanisms is required to overcome these difficulties. Land clearing, salinity and disease has resulted in over 125 species within 15 genera being listed as rare, highly restricted, threatened and endangered (Western Australian Herbarium 1998–). The present study examined the seed biology of eight species of Ericaceae native to Western Australia, exploring fruit and seed morphology, dormancy and germination. Cold and warm stratification was used in combination with gibberellic acid to classify dormancy. Among the two distinct fruit types that occur within the Ericaceae separate patterns of dormancy were found. Seeds held within a dehiscent capsule were found to possess non-deep and intermediate physiological dormancy whilst those within an indehiscent drupe possessed physiological and morphophysiological dormancy. Oxygen and nitric oxide enriched atmospheres, removal of seeds from endocarps and propagation from cuttings provided potential avenues for the propagation of study species.

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Declaration

I certify that this thesis does not, to the best of my knowledge and belief:

- i. Incorporate without acknowledgment any material previously submitted for a degree or diploma in any institution of higher education;
- ii. Contain any material previously published or written by another person except where due reference is made in the text of this thesis; or
- iii. Contain any defamatory material



Table of Contents

Abstract	i
Acknowledgments	ii
Declaration	ii
Table of Contents	iii
List of Tables	v
List of Figures	vi
Chapter 1 <i>Introduction and literature review</i>	
1.1 Introduction	1
1.2 Germination response and dormancy types within Ericaceae.....	10
1.3 Australian Ericaceae.....	11
1.4 Aims	16
Chapter 2 <i>General methods</i>	18
2.1 Introduction	18
2.2 Species selection and collection sites.....	18
2.3 Collection and processing.....	20
2.4 Sterilization	20
2.5 Germination tests.....	20
Chapter 3 <i>Fruit, seed and embryo morphology of eight Ericaceae from South-West Australia: implications for viability, dormancy and germination</i>	
3.1 Introduction	21
3.2. Methods	23
3.3. Results	27
3.4 Discussion	44
3.5 Conclusions	48
Chapter 4 <i>Dormancy class and germination in eight species of South-West Australian Ericaceae</i>	
4.1 Introduction	50
4.2 Methods	53

4.3 Results	58
4.4 Discussion	62
4.5 Conclusions	66
<i>Chapter 5 Techniques for the rapid propagation of intractable South-West Australian Ericaceae</i>	
5.1 Introduction	68
5.2 Methods	69
5.3 Results	74
5.4 Discussion	80
5.5 Conclusions	83
<i>Chapter 6 Conclusions</i>	85
References	92
Appendix	105

List of Tables

Table 1.1 A dichotomous key of seed dormancy, re-drawn from Baskin and Baskin (2014).....	4
Table 1.2 The hierarchical system of dormancy classification by Baskin and Baskin (2014).....	7
Table 1.3 Effective treatments for previously studied taxa of Ericaceae in South-West Australia.....	14
Table 2.1 Species, fruit type and collection information for study taxa	19
Table 3.1 Sample size for morphological characteristic measurements	23
Table 3.2 Key characteristics of Ericaceae fruit and seed.....	29
Table 3.3 Mean viability (\pm SE) as determined by cut test for fresh (<3 month old) seeds of <i>Andersonia heterophylla</i> and <i>Lysinema pentapetalum</i>	38
Table 3.4 Estimated impact of seed predation in fresh (<3 month old) drupes of <i>Conostephium pendulum</i>	42
Table 4.1 Study species, stratification conditions and treatment combinations applied to intact endocarps and extracted seeds of drupaceous taxa	53
Table 4.2 Germination of extracted Ericaceae seeds incubated in total darkness at 21°C for 30 days.	58
Table 4.3 Maximum germination percentages (G) and germination times (t_1 and t_{50}) as a mean \pm SD for fresh seed (< 3 months).	60
Table 5.1 Embryo to seed ratios calculated for fresh seed and pre-germinating <i>Croninia kingiana</i> seed.	77
Table 5.2 Assessment of Ericaceae cuttings after 6 months.	77
Table 5.3 Germination (% of initial viability), time until first germination (t_1) and time until 50% germination (t_{50}) of pre-treated Ericaceae seed incubated in an oxygen enriched atmosphere.....	79

List of Figures

Figure 1.1 Non-deep physiological dormancy types as proposed by Baskin and Baskin, 2004.....	6
Figure 1.2 A diagrammatic representation of the loss/induction of dormancy and the stimulation of germination, re-produced from Merritt <i>et al.</i> (2007).....	13
Figure 2.1 Map of collection sites in South-west Australia	19
Figure 3.1 Select features of <i>Andersonia heterophylla</i> capsules and seeds.	30
Figure 3.2 Select features of <i>Astroloma xerophyllum</i> endocarps and seeds..	31
Figure 3.3 Select features of <i>Astroloma serratifolium</i> endocarps and seeds.....	32
Figure 3.4 Select features of <i>Conostephium minus</i> endocarps and seeds..	33
Figure 3.5 Select features of <i>Conostephium pendulum</i> endocarps and seeds.	34
Figure 3.6 Select features of <i>Croninia kingiana</i> endocarps and seeds.....	35
Figure 3.7 Select features of <i>Leucopogon polymorphus</i> endocarps and seeds.	36
Figure 3.8 Select features of <i>Lysinema pentapetalum</i> capsules and seeds.....	37
Figure 3.9 Mean number of fully formed locules per fresh (<3 months old) endocarps.	40
Figure 3.10 Average percentage of fresh (<3 month old) endocarps (\pm SE) containing at least one seed	41
Figure 3.11 Mean number of seeds per fresh (<3 month old) endocarp (\pm SE) as determined by cut tests.....	42
Figure 3.12 Moisture content of dry imbibed endocarps after 24 hours at room temperature.	43
Figure 3.13 Water uptake of <i>Andersonia heterophylla</i> and <i>Lysinema pentapetalum</i> ...	43
Figure 4.1 Climate data from 1981 to 2010 from the Australian Bureau of Meteorology weather station at Hyden, Western Australia.....	55
Figure 4.2 Climate data from 1996 to 2017 from the Australian Bureau of Meteorology weather station at Gingin, Western Australia.	55
Figure 4.3 Germination of extracted Ericaceae seeds (\pm SE) incubated in darkness at 21°C for 30 days.	59
Figure 4.4 Cumulative germination (%) of <i>Lactuca sativa</i> (lettuce) incubated at 21°C in total darkness	59
Figure 4.5 Mean germination (%) for <i>Andersonia heterophylla</i> and <i>Lysinema pentapetalum</i> seeds treated with gibberellic acid.	61

Figure 4.6 Germination (% of initial viability) of pre-treated <i>Lysinema pentapetalum</i> seeds (\pm SE) incubated in constant darkness for 30 days (21°C).....	61
Figure 5.1 Donor dish method of exposing endocarps/seeds to generated gases.....	71
Figure 5.2 Select features of the method used to propagate Ericaceae from cuttings.....	73
Figure 5.3 Moisture content of 3 month old endocarps (\pm SE) before imbibition and after 24hrs imbibition with DI water or DI water and nitric oxide.	75
Figure 5.4 Comparison of germinating and non-germinating embryos of <i>Croninia kingiana</i> seeds taken from oxygen enrichment trials.....	76
Figure 5.5 Assessment of Ericaceae cuttings..	78

Chapter 1

Introduction and literature review

1.1 Introduction

The Ericaceae is a widely dispersed plant family (Judd and Kron, 1993; Stevens *et al.* 2004) with an estimated 4426 species in 129 genera (Schwery *et al.* 2015). Species occur in a range of climates from tropical montane to temperate and arctic with the highest diversity found in the northern hemisphere, particularly in the tropical montane zone (Schwery *et al.* 2015). Species are also abundant in lowland heathlands and shrublands (Specht, 1979; Read, 1996; Schwery *et al.* 2015) with high species richness in Cape fynbos (Brown, Kotze and Botha, 1993), Californian chaparral (Keeley, 1984; Davis *et al.* 1989) and Australian heathlands (Pate and Beard, 1982; Schwery *et al.* 2015; Western Australian Herbarium 1998–). Typically preferring acidic environments (Marrs and Bannister, 1978; Schwery *et al.* 2015) the Ericaceae make up a significant proportion of species diversity in the South-West of Western Australia (SWWA) (Western Australian Herbarium 1998–) where their fungal symbiosis (Read, 1996; Cairney and Ashford, 2002; Selosse *et al.* 2007) and replenishing seedbank (Meney *et al.* 1994) allow them to thrive in the nutrient poor, fire-prone environment (Cairney and Ashford, 2002).

The family displays a range of growth forms, leaf types (Stevens *et al.* 2004; Schwery *et al.* 2015), fire response strategies (Roche *et al.* 1997), dispersal mechanisms (Quinn *et al.* 2003) and seed types (Kron *et al.* 2002). Ericaceae occur as ever-green shrubs although species within *Arbutus* (Hickey and King, 1997) and *Richea* (Wagstaff *et al.* 2010) can occur as trees. Leaves tend to be simple and leathery and are usually alternate, but can be either opposite or whorled without stipules (Hickey and King, 1997; Kron *et al.* 2002; Stevens, 2017-). Flowers are usually regular and bisexual with a calyx consisting of 4 or 5 persistent, united sepals and a corolla having 4 or 5 united petals forming an urn-, bell- or salver-shape (Hickey and King, 1997; Kron *et al.* 2002; Stevens, 2017-). Flowers are valuable to horticulture where *ex situ* collections can be established and species within *Arbutus*, *Azalea*, *Erica*, *Pieris* and *Rhododendron* are known for being significant garden plants (Hickey and King, 1997). Fruit are multilocular and form either loculicidal capsules or drupes with woody, indehiscent endocarps (Kron *et al.* 2002; Quinn *et al.* 2003). Capsular-fruited species are obligate re-seeders while drupe-fruited species have the capacity to re-sprout after fire (Roche *et al.* 1997). Endocarps and capsules carry multiple small seeds (Kron *et al.* 2002; Turner *et al.* 2009) which display orthodox storage behaviour (Royal Botanic Gardens Kew, 2015) and complex dormancy (Turner *et al.* 2009; Hancock, 2014).

The Ericaceae is divided into 8 subfamilies and 20 tribes (Kron *et al.* 2002). All native Australian taxa are classified in the sub-family Epacridoideae Am. (Ericaceae Juss.) (Kron *et al.* 2002; Puente-Lelièvre *et al.* 2016). Epacridoideae is comprised of 7 tribes, of which Styphelieae is the most widely distributed and contains the majority of WA Ericaceae with ~350 species in 22 genera (Puente-Lelièvre *et al.* 2016). The sub-family is currently undergoing revision (Hislop and Puente-Lelièvre, 2017) and the genus *Styphelia* is being expanded to include all those taxa currently in *Astroloma* and 108 taxa within *Leucopogon* (Hislop and Puente-Lelièvre, 2017).

In Western Australia the Ericaceae is represented by 20 genera (Western Australian Herbarium 1998–) with the vast majority of species occurring within *Leucopogon* (Ooi *et al.* 2006; Hislop, 2008; Hislop and Puente-Lelièvre 2017). Currently, 187 species of Ericaceae occur in the SWWA floristic region (Western Australian Herbarium 1998–), 84% of which are endemic (Beard, Chapman and Gioia, 2000). Land clearing, salinity and disease has resulted in 125 species of Ericaceae being listed as rare, highly restricted, threatened and endangered (Western Australian Herbarium 1998–). These anthropogenic factors are expected to increase in Australia (Ooi, Auld and Denham, 2012; Andrich and Imberger, 2013) and as such there will be a greater demand for ex situ collections of species for use in horticulture and restoration. Propagation and rehabilitation of a number of Western Australian species has been problematic (Ralph, 2003; Dempster, 2017 Pers. Comm.) due to complex dormancy and a lack of well-defined germination protocols able to produce germination on demand (Ralph, 2003; Hancock, 2014). Negligible levels of germination make many species impractical for horticulture and generally no more than a third of the species of the Perth Plain are available as seedlings at a given time (Hancock, 2014).

Other than having a significant role in understory vegetation throughout Australia, the Ericaceae have significant horticultural potential as they produce large quantities of flowers that possess unique shapes and characteristics not seen in other species. Australian Ericaceae have a reputation for producing difficult-to-germinate seeds (Ralph, 2003; Merritt *et al.* 2007; Hancock, 2014) and include many species with deep intractable dormancy. As such, the Ericaceae are underrepresented within horticulture and restoration (Pike, 2017 Pers. Comm.). Prolonged periods of stratification at specific temperatures are required to elicit minimal germination (Roche *et al.* 1997), making most methods impractical for use in horticulture. Propagation from seed is the best source material for restoration and rehabilitation outcomes (Turner and Merritt, 2009; Hancock,

2014) and the intractability associated with Ericaceae seeds (Hancock, 2014) limits the ability to replace lost biodiversity in restored sites. A better understanding of species specific morphology, dormancy and germination will provide insight into the mechanisms that restrict germination and how best to resolve issues associated with propagation of Ericaceae from seeds.

Seed dormancy

Dormancy is defined as the absence of germination of viable seeds under otherwise favourable conditions of temperature, moisture and light (Murdoch and Ellis, 1992; Hilhorst, 2007) and is characterized by low metabolic activity and a lack of sensitivity to growth promoting signals (Graeber *et al.* 2012). Dormancy is crucial in determining plant fitness because it prevents seeds from germinating into conditions unfavourable for sustained growth and development (Donohue *et al.* 2005; Huang *et al.* 2010; Graeber *et al.* 2012; Krasuska *et al.* 2015).

Dormancy is a natural yet highly complex phenomenon that has evolved in response to the diverse range of conditions to seeds are exposed. Seed dormancy is established during seed maturation as storage compounds accumulate prior to the onset of metabolic quiescence (Holdsworth *et al.* 2008; Graeber *et al.* 2012). The environment can directly affect seed growth and maturation and therefore dormancy (Bewley *et al.* 2006). Environmental conditions may affect dormancy establishment directly by regulating the ability to accumulate dry matter, or indirectly by limiting the ability of the mother plant to supply raw material to seeds (Bewley *et al.* 2006). Not all seeds are dormant at dispersal. Phytohormones fed to the developing seed determine the degree of dormancy (Finch-Savage and Leubner-Metzger, 2006) and as such, the onset of dormancy is affected by climatic and environmental conditions during seed maturation, as well as conditions of the parent plant and the seed (Bewley *et al.* 2006). After seeds disperse dormancy is mediated either hormonally or physically and can require individual events or a sequence thereof to alleviate (Baskin and Baskin, 2014).

Classification of dormancy

Five classes of dormancy are recognised and they are characterized by the physiological and morphological state of the embryo, and physical barriers to water uptake by the pericarp or seed coat (Baskin and Baskin, 2014; Tables 1.1-1.2). The major classes of dormancy are: physical dormancy, determined by a water impermeable palisade cells; physiological dormancy, maintained through hormonal control of embryo growth; and

morphological dormancy, characterised by an immature embryo at the time of dispersal which grows prior to germination (Baskin and Baskin, 2014). Interactions between physiological dormancy and physical or morphological dormancy occur and are termed combinational and morphophysiological dormancy respectively. Dormancy is further subdivided based on the temperatures at which dormancy is alleviated, the temperatures required for germination, the response to exogenous gibberellic acid (GA) and the time course of radical and shoot emergence (Baskin and Baskin, 2014). Baskin and Baskin, (2014) produced a dichotomous key for the classification of seed dormancy which is presented in Table 1.1. The specific characteristics of each dormancy class, and the subdivisions therein, are presented in Table 1.2.

Table 1.1 A dichotomous key of seed dormancy, re-drawn from Baskin and Baskin (2014).

1. Embryo differentiated and fully developed.....	2
2. Seeds imbibed water.....	3
3. Root emergence occurs within about 4wk (usually a few days).....	4
4. After root emergence, shoot emergence occurs within a few days.....	NONDORMANT
4. After root emergence, shoot emergence is delayed 3-4wk or more.....	PHYSIOLOGICAL EPICOTYL DORMANCY
3. Root emergence requires more than 4wk 5	
5. After root emergence, shoot emergence occurs within a few days.....	PHYSIOLOGICAL REGULAR DORMANCY
5. After root emergence, shoot emergence is delayed 3-4wk or more.....	PHYSIOLOGICAL EPICOTYL DORMANCY
2. Seeds do not imbibe water.....	6
6. Scarified seeds become fully imbibed (usually in 1 day) and germinate within about 4wk (usually in a few days).....	PHYSICAL DORMANCY
6. Scarified seeds become fully imbibed (usually within 1 day) but do not germinate within about 4 wk.....	COMBINATIONAL DORMANCY
1. Embryo undifferentiated or if differentiated it is underdeveloped.....	7
7. Embryo not differentiated	8
8. After seed dispersal, embryo differentiated and grows in imbibed seed.....	9
9. Seeds germinate within about 4wk.....	MORPHOLOGICAL DORMANCY
9. Seeds do not germinate within about 4wk.....	MORPHOPHYSIOLOGICAL DORMANCY
8. After seed dispersal, embryo never differentiated into a shoot-root axis.....	10
10. Seeds germinate within about 4 wk.....	SPECIALIZED MORPHOLOGICAL DORMANCY
10. Seeds do not germinate within about 4wk.....	SPECIALIZED MORPHOPHYSIOLOGICAL DORMANCY
7. Embryo differentiated but underdeveloped.....	11
11. After seeds are placed on a moist substrate, the embryos grow, and seeds germinate within about 4wk.....	MORPHOLOGICAL DORMANCY
11. After seeds are placed on a moist substrate, the embryo does not grow, and seeds do not germinate within about 4wk.....	MORPHOPHYSIOLOGICAL DORMANCY

Physical dormancy

Physical dormancy is imposed by one or more water impermeable palisade cells surrounding seeds and can be measured directly through analysis of imbibition (Baskin and Baskin, 2014; Table 1.1). Physically dormant seeds or seeds have a developed embryo (radical and cotyledons can be distinguished) (Table 1.1) and germination is restricted by a lack of water availability (Baskin and Baskin, 2014). Germination requires water impermeable palisade cells (Table 1.2) be made permeable, usually through scarification or heat shock (Baskin and Baskin, 2014). Once physical dormancy has been alleviated it cannot be reinstated as permeated layers cannot be made impermeable. Once physical dormancy has been alleviated seeds tend to germinate rapidly (Table 1.1) unless a component of physiological dormancy is also present.

Morphological dormancy is determined by the presence of a differentiated, underdeveloped (Martin, 1946) embryo at the time of dispersal that grows prior to germination (Baskin and Baskin, 2004, 2014). Seeds that germinate within 4 weeks, in which the embryo can be seen to grow prior to germination, typically have morphological dormancy (Table 1.1). If embryo growth occurs and seeds take longer than 4 weeks to germinate then there is likely to be a physiological component of dormancy that must be alleviated prior to, during or post- embryo growth.

Physiological dormancy

Physiological dormancy is dormancy imposed by concentrations of phytohormones gibberellic acid and abscisic acid within the embryo (Finch-Savage and Leubner-Metzger, 2006). Physiological dormancy is divided into three levels; non-deep, intermediate and deep (Baskin and Baskin, 2004; Table 1.1-1.2). Characteristics used to classify physiological dormancy include the delay of germination, and the effect of scarification, after ripening and embryo excision (Table 1.1-1.2). Non-deep physiologically dormant seeds are responsive to exogenously applied gibberellic acid and, depending on the species, require warm or cold stratification to break dormancy (Baskin and Baskin, 2004; Table 1.2). Non-deep physiological dormancy is further divided (Table 1.2) based on the temperatures at which seeds will germinate once dormancy is alleviated (Figure 1.1). Intermediate and deep physiologically dormant seeds require cold stratification to alleviate dormancy (Table 1.2), and deep physiologically

dormant seeds are unresponsive to exogenous gibberellic acid (Finkelstein *et al.* 2008; Table 1.2).

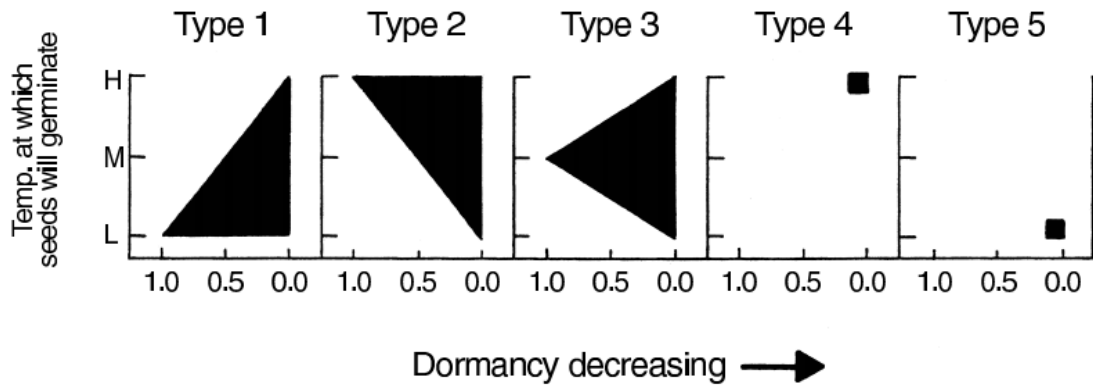


Figure 1.1 Non-deep physiological dormancy types as proposed by Baskin and Baskin, (2004).

Morphophysiological dormancy

Morphophysiological dormancy is imposed by hormonal regulation of germination and embryo growth. Seeds possess an underdeveloped embryo at the time of dispersal and have been noted to respond to a diverse range of conditions (Table 1.2). Successive seasons of stratification have been noted to alleviate morphophysiological dormant seeds (Baskin and Baskin, 2014). Depending on the locality of species and the depth of physiological dormancy, seeds may require cold and/or warm temperatures and have dormancy overcome by exogenous gibberellic acid (Table 1.2).

Combinational dormancy

Combinational dormancy is defined as a physiologically dormant embryo which has a water impermeable coat (Baskin and Baskin, 2004; 2014). Seeds in this class usually express non-deep physiological dormancy (types 1 and 2). In non-deep physiologically dormant seeds the excised embryos produce normal seedlings and gibberellic acid overcomes dormancy. Specific temperatures required to alleviate physiological dormancy depend on the historical range of the species and dry-after ripening may reduce the depth of dormancy. Scarification may promote germination as there are one or more water impermeable layers of palisade cells that restrict water uptake.

Table 1.2 The hierarchical system of dormancy classification by Baskin and Baskin (2014).

Dormancy class ¹	Subdivisions ¹	Characteristics ¹
Physical dormancy	Needs subdivision ¹	One or more water impermeable layers of palisade cells in the seed or fruit coat
Morphological dormancy		Immature embryos that grow prior to germination Does not include seeds with undifferentiated embryos
Physiological dormancy	Non- deep • Types 1-5 ²	Excised embryo produces normal seedling GA overcomes dormancy Cold (0-10°C) or warm (>15°C) breaks dormancy Seeds may after ripen in dry storage Scarification may promote germination
	Intermediate	Excised embryo produces normal seedling GA overcomes dormancy in some, but not all, species 2-3 months of cold stratification breaks dormancy Dry storage can shorten the stratification period
	Deep	Excised embryo produces abnormal seedling GA does not overcome dormancy 3-4 months of cold stratification breaks dormancy
Morphophysiological dormancy	Non-deep simple	Warm or cold stratification breaks dormancy Embryo growth requires warm stratification GA ₃ overcomes dormancy
	Intermediate simple	Warm + cold stratification breaks dormancy Embryo growth requires warm stratification GA ₃ overcomes dormancy
	Deep simple	Warm + cold stratification breaks dormancy Embryo growth requires warm stratification GA ₃ may overcome dormancy
	Deep simple epicotyl	Warm + cold stratification breaks dormancy Embryo growth requires warm stratification GA ₃ may overcome dormancy
	Deep simple double	Cold + warm + cold stratification breaks dormancy Embryo growth requires warm stratification Effect of GA ₃ is poorly understood
	Non-deep complex	Warm stratification + Cold stratification breaks dormancy Embryo growth requires cold stratification GA ₃ may overcome dormancy
	Intermediate complex	Cold stratification breaks dormancy Embryo growth requires cold stratification GA ₃ may overcome dormancy
	Deep complex	Cold stratification breaks dormancy Embryo growth requires cold stratification GA ₃ does not overcome dormancy
Combinational dormancy	Non-deep • Types 1- 2 ²	Excised embryo produces normal seedling GA overcomes dormancy Cold (0-10°C) or warm (>15°C) breaks dormancy Seeds may after ripen in dry storage Scarification may promote germination One or more water impermeable layers of palisade cells in the seed or fruit coat

Considerations for seeds with physiological dormancy

The level of physiological dormancy present in seeds cannot be directly assessed, but can be indirectly measured through germination tests (Graeber *et al.* 2012). The alleviation of dormancy requires specific environmental conditions and as such, the germination sensitivity of seeds changes continuously as a function of variable ambient conditions (Finch-Savage and Leubner-Metzger, 2006). As the depth of dormancy decreases in response to environmental conditions of temperature and moisture, sensitivity to the germination environment increases, and the window of conditions at which a seed will germinate widens (Baker *et al.* 2005; Figure 1.2). At a given time dormancy of a seed population is expressed across a spectrum and cannot be measured by the germination of a single seed (Vleeshouwers *et al.* 1995). Therefore, a definitive definition of the dormancy state does not exist, but rather there are different germination sensitivities that may fall within range of what can be supplied by the natural environment (Vleeshouwers *et al.* 1995; Graeber *et al.* 2012). Thus, as dormancy is alleviated within a population seeds with shallower dormancy are able to germinate at a greater range of conditions. If germination stimulating conditions are not provided, seeds with physiological dormancy may re-enter dormancy (Baker *et al.* 2005). Therefore, a lack of germination for viable seed does not necessarily imply that seeds are dormant, but rather that germination stimulating conditions different to those that alleviate dormancy may be required.

Physiological dormancy is the most conserved dormancy type (Baskin and Baskin, 2004; Finch-Savage & Leubner-Metzger 2006; Graeber *et al.* 2012) and is well represented within the Ericaceae (Baskin and Baskin, 2014; Table 1.2). Certain Ericaceae are known to exhibit a morphological component of dormancy (Ooi *et al.* 2006; Turner *et al.* 2009; Baskin and Baskin, 2014; Table 1.2) in combination with physiological dormancy. There are also known interactions between seed covering materials and dormancy (Turner *et al.* 2009), termed mechanical and chemical dormancy, that do not fall within the realm of combinational dormancy (Table 1.2). Fruit and seed coats may restrict germination by direct chemical inhibition, indirect inhibition through the binding of oxygen or mechanically by physically restricting growth and oxygen (Baskin and Baskin, 2014).

Mechanical and chemical dormancy were excluded from Baskin and Baskin, (2004) classification system and Nikolaeva (2004) agreed that ‘... exclusion of mechanical and chemical dormancy from the Nikolaeva classification scheme is probably correct’ (Baskin and Baskin, 2008). The rationale for their exclusion was that once primary

dormancy is alleviated seeds are able to overcome any mechanical or chemical constraint to germination (Baskin and Baskin, 2014). Thus, mechanical and chemical dormancy are an aspect of physiological dormancy, rather than a class of their own (Baskin and Baskin, 2014).

Induction and release of dormancy

Several plant hormones are involved in the onset, maintenance and release of seed dormancy (Finch-Savage and Leubner-Metzger, 2006; Finkelstein *et al.* 2008; Nambara *et al.* 2010; Graeber *et al.* 2012). Abscisic acid and gibberellic acid have crucial and antagonistic roles in dormancy and germination (Debeaujon and Koornneef, 2000; Thompson *et al.* 2000; Qin and Zeevaart, 2002; Okamoto *et al.* 2006; 2010; Finkelstein *et al.* 2008; Nambara *et al.* 2010; Graeber *et al.* 2012), with the balance between abscisic acid and gibberellic acid regulating induction and release of dormancy and the initiation of germination (Graeber *et al.* 2012). Abscisic acid has been demonstrated as a positive regulator of dormancy in a diverse group of species including Asterids (*Nicotiana* spp; Petruzzelli *et al.* 2003), Monocots (*Zea mays*; McCarty, 1995), Rosids (*Arabidopsis*: Nambara and Marionpoll, 2005) and Gymnosperms (*Pinus monticola*; Feurtado *et al.* 2004, *Pseudotsuga menziesii*; Corbineau *et al.* 2002). Transgenic lines of these species show that abscisic acid induces primary dormancy and inhibits germination (Graeber *et al.* 2012). Abscisic acid inhibitors demonstrated that the maintenance of dormancy in imbibed seeds requires *de novo* synthesis of abscisic acid (Debeaujon and Koornneef, 2000; Grappin *et al.* 2000; Ali-Rachedi *et al.* 2004; Nambara *et al.* 2010). Therefore, seasonal stratification conditions that reduce or increase the *de novo* synthesis of abscisic acid would facilitate a reduction or induction of dormancy for soil-stored seed (Baker *et al.* 2005; Merritt *et al.* 2007; Figure 1.2).

Dormancy release and germination

The mechanisms controlling dormancy release are poorly understood (Graeber *et al.* 2012). The process of dormancy release by ambient conditions of temperature and moisture is called stratification (Graeber *et al.* 2012) and the mechanism by which stratification releases dormancy is largely unknown (Graeber *et al.* 2012). Stratification is the main factor controlling morphological and physiological dormancy release (Ooi *et al.* 2006; Baskin and Baskin, 2014). Cold temperatures have been shown to increase GA₃ production and sensitivity through the induction of gibberellic acid biosynthesis genes in *Arabidopsis thaliana* (Yamauchi *et al.* 2004; Finch-Savage and Leubner-Metzger, 2006)

and alternating temperatures have been shown to reduce abscisic acid biosynthesis and signalling in *Cynara cardunculus* L. (Huarte *et al.* 2014). The effect of temperature and moisture on the metabolic pathways of dormancy control depends on the historical climate of the species and the dormancy and germination requirements that have evolved *in situ* (Baskin and Baskin, 2014). Typically, imbibition at warm temperatures releases dormancy in winter annual species, while cold temperatures release dormancy in summer annual species (Graeber *et al.* 2012). In climates with limited rainfall stratification may only occur when soil moisture increases (Merritt *et al.* 2007) and this may determine the conditions required for dormancy alleviation.

1.2 Germination response and dormancy types of species within Ericaceae

Ericaceae are known to vary in their response to germination enhancing treatments (Vera, 1997; Cruz *et al.* 2003; Baskin and Baskin, 2014) due to species possessing different mechanisms of dormancy or being non-dormant. Research shows a positive response to storage (Valbuena and Vera, 2002) and smoke (Cruz *et al.* 2003; Bargmann, Måren and Vandvik, 2014), a negligible impact of scarification (Oliva, Leidi and Valdés, 2009), and a variable response to light (Pons, 1989; Obeso and Vera, 1996; Pons, 2000) and temperature (Warm <15°C; Gonzalez, 1995; Obeso and Vera, 1996. Cold, <10°C; Aparicio, 1995; Valbuena and Vera, 2002; Jurado, Márquez-Linares and Flores, 2011).

Species' responsiveness to temperature correlates with temperatures *in situ* (Baskin and Baskin, 2014). Control groups of *Erica tetralix* L and *Calluna vulgaris* L germinated in high numbers due to natural cold stratification *in situ* (Bargmann, Måren and Vandvik, 2014). Cold stratification (4°C for 30 days) broke dormancy in *Arctostaphylos pungens* Kunth (Jurado *et al.* 2011) and *Erica andervalensis* Cabezudo and Rivera E (Aparicio, 1995), both of which occur where temperatures are cool during periods of rainfall (*WeatherSpark*; SMA, Spain). *Erica andervalensis* was shown to have no response to cold dry treatment (Oliva, Leidi and Valdés, 2009), emphasizing the requirement for water in dormancy release via cold temperatures. Fire cues of smoke and nitrogenous compounds have been shown to influence dormancy and germination in numerous Ericaceae. Smoke and nitrogen increased germination for *Erica australis* although results were variable at the interpopulation, intrapopulation and intraindividual levels (Cruz *et al.* 2003). Brown, Kotze and Botha, (1993) found that 26 of the 40 *Erica* species studied had a significantly positive response to plant derived smoke.

Taxa within the subfamily Ericoideae (e.g. *Erica* and *Calluna*) respond to treatments in a manner consistent with physiological dormancy (Brown, Kotze and Botha, 1993; Aparicio, 1995; Vera, 1997; Cruz *et al.* 2003; Jurado *et al.* 2011; Bargmann, Måren and Vandvik, 2014). Ericoideae produce small seeds that do not possess an indehiscent endocarp (McGuire and Kron, 2005) and seeds of most species germinate readily (Brown, Kotze and Botha, 1993; Aparicio, 1995; Vera, 1997; Cruz *et al.* 2003; Jurado *et al.* 2011; Bargmann, Måren and Vandvik, 2014). Techniques for the rapid propagation of non-Australian Ericaceae are available (Brown, Kotze and Botha, 1993; Aparicio, 1995; Jurado *et al.* 2011) and suggest that the endocarp of Australian taxa may be responsible for the difficulties associated with on-demand propagation. The comparison of germination response between Australian and non-Australian Ericaceae further highlights the importance of local climatic patterns and seed morphology in determining the dormancy and germination requirements of species.

1.3 Australian Ericaceae

Many Australian Ericaceae (Dixon *et al.* 1995; Roche *et al.* 1997; Cochrane *et al.* 2002; Allan *et al.* 2004) have unknown dormancy mechanisms (Ooi *et al.* 2006) although, it has been suggested that physiological and/or morphological mechanisms control dormancy in species from fire-prone ecosystems (Schatral *et al.* 1997; Keeley and Fotheringham, 1998; Bell, 1999; Tieu and Egerton-Warburton, 2000; Allan *et al.* 2004; Ooi *et al.* 2006). Studies into Australian Ericaceae have previously sought to produce germination in numerous species (Dixon *et al.* 1995; Roche *et al.* 1997; Cochrane *et al.* 2002; Allan *et al.* 2004) however, with the established classification system for seed dormancy (Baskin and Baskin, 2004; 2014) the focus has shifted to classifying dormancy and its interactions with fire prone environments (Tieu *et al.* 2001; O'Brien and Johnston, 2004; Ooi *et al.* 2004; Ooi *et al.* 2006; Turner *et al.* 2009).

Seed of some Ericaceae are formed within fleshy drupes or woody capsules (Quinn *et al.* 2003). Freshly dispersed drupes or capsules tend to not respond to direct fire cues (Dixon *et al.* 1995; Roche *et al.* 1997; Tieu *et al.* 2001; Ooi *et al.* 2006) however, germination of species in response to fire cues has been noted *in situ* and after a period of burial (Ooi *et al.* 2006). There is also a growing body of evidence (Keeley, 1987; Davis *et al.* 1989; Dixon *et al.* 1995; Roche *et al.* 1997; Keeley and Fotheringham, 1998; Clarke *et al.* 2000; van Staden *et al.* 2000; Ooi *et al.* 2006) that suggests the passage of fire does not break primary dormancy in many species from fire-prone environments. Soil stored seeds

experience diurnal and seasonal temperature fluctuations and physical deterioration, both of which would facilitate physiological changes over time and increase germination sensitivity. Therefore, a number of processes influence dormancy and germination in the inter-fire period and occur within a single season or across multiple seasons.

The South-West of Western Australia

In SWWA rainfall increases after summer (Merritt *et al.* 2007). Thus, stratification would occur in autumn and early winter with a release of dormancy between January and July (Merritt *et al.* 2007; Figure 1.2). Dormancy of many physiologically dormant species would be alleviated by warm temperatures over the summer and autumn months. Ericaceae species from fire-prone ecosystems can have germination stimulated by chemicals found in smoke and the post-fire environment (South Africa; Brown, Kotze and Botha, 1993, California; Keeley, 1984, Australia; Dixon, Roche and Pate, 1995). Germination may be stimulated by the passage of fire or the build-up of soil stored chemicals from previous years which become available as soil moisture increases. This has been demonstrated for many species in Australia (Meney *et al.* 1994; Dixon *et al.* 1995; Baker *et al.* 2005), and in SWWA fire based germination stimulants occur in the summer months (Figure 1.2). Therefore, germination of species in SWWA would occur between May and July.

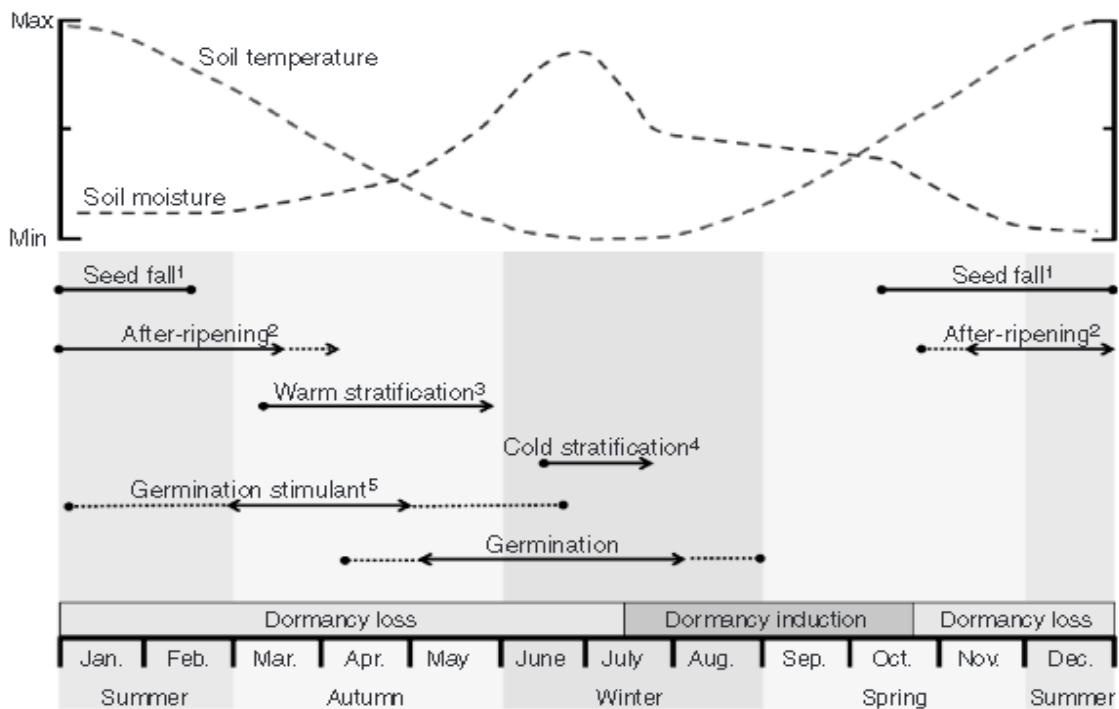


Figure 1.2 A diagrammatic representation of the loss/induction of dormancy and the stimulation of germination, reproduced from Merritt *et al.* (2007). Dormancy loss/induction, may occur over numerous years until seeds receive germination conditions, or until seeds lose viability. ¹Seed fall is the entry of seeds into the soil seedbank. ²After-ripening is the loss of dormancy of seeds in a dry state. ³Warm stratification is defined as dormancy alleviation of moist seeds at temperature >15°C. ⁴Cold stratification is the alleviation of dormancy in moist seeds at temperature <10°C. ⁵In SWWA the predominant germination stimulant is smoke.

Dormancy and germination have been studied in fewer than 15% of SWWA Ericaceae (Table 1.3). Total germination percentage is often low and treatments time consuming. Species vary in response to storage, smoke, gibberellic acid (GA₃) and scarification (Dixon and Nielsen, 1992; Dixon *et al.* 1995; Roche *et al.* 1997; Cochrane *et al.* 2002; Allan *et al.* 2004) with prolonged soil storage and smoke treatments increasing germination (Roche *et al.* 1997; Dixon and Nielsen, 1992; Cochrane *et al.* 2002; Table 1.3). Information on the morphology of seeds and their dormancy and germination requirements is available as grey literature. Detailed fruit and seed morphological accounts of Australian Ericaceae are scarce (Dixon and Nielsen, 1992; Norman and Koch, 2008; Turner *et al.* 2009) and the commercial confidence (Hancock, 2015 Pers. Comm.) surrounding germination protocols of SWWA Ericaceae (Hancock, 2014) suggests much of this information is hard to come by. Similarly, a lack of incentive to publish null data (Dixon, 2017 Pers. Comm.) suggests previously trialed treatments are not documented within the scientific literature.

Species of *Andersonia* (Cochrane *et al.* 2002) have been shown to respond to treatments in a reasonably time efficient manner and reliable propagation from seeds is possible for some species. For seeds contained within an endocarp the dormancy and germination requirements are more complex (Turner *et al.* 2009). The low proportion of studied Ericaceae (Table 1.3) means there is a lack of information on morphological characteristics and how they interact with dormancy and germination. Dormancy and germination studies in Ericaceae commonly document seed length and weight (Dixon, Roche and Pate, 1995; Roche, Dixon and Pate, 1997) however, this information fails to encapsulate the variation in endocarp and seed characteristics that impact dormancy and germination. Furthermore, the dormancy class of numerous studied Ericaceae in SWWA has not been inferred (Dixon, Roche and Pate, 1995; Roche, Dixon and Pate, 1997; Cochrane *et al.* 2002; Allan *et al.* 2004). As such, studies have typically treated the drupaceous Ericaceae in a manner similar to capsular species (Dixon *et al.* 1995; Roche *et al.* 1997) which compounds the intractability associated with the Ericaceae as a whole.

Table 1.3 Effective treatments for previously studied taxa of Ericaceae in South-West Australia

Genus	# Species ¹	# Species Studied	Responsive
<i>Acrotriche</i>	8	1 ^{2, 10}	Soil storage + Smoke.
<i>Andersonia</i>	49	14 ^{2, 3, 5, 10, 12}	Smoke, Soil storage, GA ₃ .
<i>Astroloma</i>	28	7 ^{2, 3, 6, 9, 10, 12}	Smoke + Soil storage, GA ₃
<i>Conostephium</i>	14	2 ^{3, 8}	Smoke + Soil storage
<i>Croninia</i>	1	1 ⁸	Smoke + Soil storage.
<i>Leucopogon</i>	220	8 ^{2, 3, 4, 7, 10, 11, 12}	Smoke + Soil storage.
<i>Lysinema</i>	6	1 ^{3, 12}	Smoke + Soil storage
<i>Styphelia</i>	9	1 ^{2, 3, 7, 10}	NA

[1]Western Australian Herbarium (1998–) [2] Roche *et al.* (1997) [3] Dixon and Nielssen, (1992) [4] Tieu *et al.* (2001), [5] Cochrane *et al.* (2002), [6], Turner *et al.* (2009) [7] Norman and Koch (2008) [8] Dixon, Roche and Pate,(1995) [9] Merritt *et al.* (2007), [10] Bell, (1999), [11] Allan *et al.* (2004), [12] Meney, Nielssen and Dixon (1994).

Treatment with smoke products affects the germination of some Australian Ericaceae however, species response appears to require a period of seed aging in soil or specific incubation conditions (Dixon, Roche and Pate, 1995; Roche, Dixon and Pate, 1997; Bell, 1999). The effect of soil storage and the associated decline in physical integrity (Roche *et al.* 1997) suggests multiple factors influence dormancy alleviation and that endocarps of drupaceous Ericaceae play a significant role in the dormancy mechanism. This is supported by the germination of non-drupaceous Australian

Ericaceae (Roche *et al.* 1997; Dixon and Nielssen, 1992; Cochrane *et al.* 2002) in the absence of prolonged soil storage. The variation in dispersal mechanism and seed type among Australian Ericaceae has a direct effect on the conditions required to alleviate dormancy and stimulate germination (Dixon, Roche and Pate, (1995). An interaction between dormancy and endocarp in SWWA Ericaceae was first noted in Dixon, Roche and Pate (1995), who concluded that smoke products affected small seeds and those lacking a woody endocarp.

The lack of germination in scarification treatments (Dixon and Neilson, 1992) suggests that dormancy of SWWA Ericaceae is not controlled by an impermeable seed coat which is supported by water uptake (Ooi *et al.* 2006) and moisture content (Turner *et al.* 2009) analysis in some, but not all, species. The role of the endocarp was demonstrated for *Astroloma xerophyllum* (Turner *et al.* 2009), in which extraction of seeds from endocarps produced a significant increase in germination. This provided evidence for a chemical or mechanical aspect of physiological or morphophysiological dormancy in SWWA Ericaceae, highlighting the significance of endocarp restriction in their germination biology.

A better understanding of species-specific fruit and seed morphology, and how they interact with dormancy and germination, is required if species are to be properly represented in horticulture and restored sites. Currently, methods for the germination of Ericaceae seeds are available however, the timeframe until germination is great and the total germination percentages low. Thus, the available methods of germination are not widely applied within industry as they are not able to produce 'on demand' germination in a manner that is cost and labour effective.

Research question

The Ericaceae are an intractable group in terms of their seed dormancy and their ability to be propagated in a manner useful to horticulture and restoration. Therefore, the overarching research question that must be addressed is:

- Why are SWWA Ericaceae so difficult to propagate from seed?

There are a number of avenues that require investigation, including the effects of light, cold stratification, single temperature stratification, the effect of endocarp removal, the mechanism by which endocarps restrict germination and methods by which germination can be produced in a commercially viable timeframe,

defined here as 3 months. This period (3 months) was chosen as it allows for seed collected in summer (December-February) to be processed before being treated and sown in the germination window (June-August) (Merritt *et al.* 2007) of the same year.

Light has been shown to affect germination of numerous species of Ericaceae (Pons, 1989; Obeso and Vera, 1996; Pons, 2000) but the effect of varying light regimes has not been widely studied in Australian Ericaceae. The effect of single temperature cold stratification has also received little attention in Australian species. Single temperature warm stratification was effective for *Andersonia* (Cochrane *et al.* 2002) and, while it is likely that SWWA Ericaceae require warm stratification to alleviate dormancy, the soil stored seed bank receives both cold (0-10°C) and warm (>15°C) stratification conditions for a significant portion of the year (Merritt *et al.* 2007; Figure 1.2). Turner *et al.* (2009) inferred that endocarps restrict germination of *Astroloma xerophyllum* by maintaining an anoxic environment within locules however, however these methods are yet to be trialed in other Ericaceae and the mechanism by which endocarps restrict germination requires further study.

The use of treatments outside of varying stratification regimes and smoke products is not common. With the success of oxygen enrichment (Turner *et al.* 2009) it is feasible that oxygen donors may prove successful within the family. Applications of hydrogen peroxide (Lui *et al.* 2010; Lui *et al.* 2011) and nitric oxide (Giba *et al.* 1995; Keeley and Fotheringham, 1997; Batak *et al.* 2002; Renata and Agnieszka, 2006) have increased germination in some species, including *Arabidopsis thaliana* and *Vaccinium myrtillus*, and may prove useful in the propagation of Ericaceae from seeds.

1.4 Aims

The aim of the research is:

- To investigate why the Ericaceae in the South-west of Western Australia are such an intractable group to propagate from seed.

To unravel the dormancy and germination mechanisms of SWWA Ericaceae and design on demand germination protocols the following questions will be addressed in Chapters 3-5:

- Chapter 3: What fruit, seed and embryo characteristics exist among study taxa and how do they affect dormancy classification?
- Chapter 4: What dormancy class and germination inhibiting factors delay germination in eight study taxa?
- Chapter 5: How can intact endocarps or true seeds be made to germinate within three months?

Scope

The scope of research is limited to the timeframe of a masters by research (18 months) and the quantity of seed that can be collected by a small team within that timeframe. The present study did not seek to establish temperature optima for study taxa, but rather selected single temperature treatments that reflect the average yearly and average minimum temperatures at collection sites.

Significance

On a theoretical level, the study was designed to establish a baseline of information on the fruit, seed and embryo morphologies expressed among study taxa and determine how they affect dormancy classification and germination. This will benefit those working with intractable Ericaceae and help make better use of collection opportunities and seed, increasing the capability to meet the requirements of industry. The intended significance of the study, at a practical level, is to make the Ericaceae more available in horticulture and restoration. By confirming the mechanisms of dormancy involved it should be possible to design and test treatments able to produce germination within a commercially viable timeframe (less than 3 months).

Chapter 2

General methods

2.1 Introduction

Methods that were applied consistently throughout Chapters 3, 4 and 5 are presented in this chapter. Collection methods were applied consistently between species and all fruits and seeds were processed and stored as described below. Where processing, sterilization and germination protocol methods differ from those described in this chapter the deviations are outlined in the respective chapter.

2.2 Species selection and collection sites

Eight species of Ericaceae were selected for study. The sponsors of the study (The Wildflower Society of Western Australia; Kulin and Northern Suburbs Branches) were interested in establishing a number of Ericaceae from their local area (Kulin Shire, Western Australia). To reduce travel costs species that occur in both the Kulin and Perth region were selected. The difficulties associated with germination of Australian Ericaceae are found primarily within drupaceous taxa and species selection reflected this by focusing on 6 drupe-fruited and 2 capsular-fruited species. Species selection was also designed to reflect genera or species that had previously been studied to allow for comparison of methods and data. Finally, selection was based on the species in which sufficient seed could be collected. Three study taxa; *Andersonia heterophylla*, *Astroloma serratifolium* and *Conostephium minus* were only collected in the second year of study (2016). This affected the duration of incubation for these species in Chapter 4 and 5, only allowing for 26 weeks of incubation for these species (Appendix 2, 3 and 4) as opposed to 52 weeks for other study taxa.

Table 2.1 Species, fruit type and collection information for study taxa

Species	Fruit type	Years collected	Collection Site	GPS
<i>Andersonia heterophylla</i> Sond.	Capsule	2016	Gnangara-Moore River State Forest	31°49'6.22"S 115°51'2.28"E
<i>Astroloma serratifolium</i> Druce	Drupe	2016	Pederah Nature Reserve	32°38'27.38"S 118°40'40.52"E
<i>Astroloma xerophyllum</i> Sond.	Drupe	2015-16	Gnangara-Moore River State Forest	31°44'10.48"S 115°56'25.84"E,
<i>Conostephium minus</i> Lindl.	Drupe	2016	Gnangara-Moore River State Forest	31°22'34.08"S 115°42'47.65"E
<i>Conostephium pendulum</i> Benth.	Drupe	2015-16	Gnangara-Moore River State Forest	31°22'34.08"S 115°42'47.65"E
<i>Croninia kingiana</i> F.Muell	Drupe	2015-16	Gnangara-Moore River State Forest	31°44'10.48"S 115°56'25.84"E,
<i>Leucopogon polymorphus</i> Sond.	Drupe	2015-16	Landsdale Conservation Park	31°44'10.48"S 115°56'25.84"E,
<i>Lysinema pentapetalum</i> R. Br.	Capsule	2015-16	Landsdale Conservation Park	31°44'14.62"S 115°56'19.73"E

Seed dispersal of study taxa occurs in spring (September-November) to summer (December-February) (Merritt *et al.* 2007). Seeds were collected approximately 250km South-East of Perth and 50km North of Perth (Table 2.1), under license SW017584 from Department of Biodiversity, Conservation and Attractions.

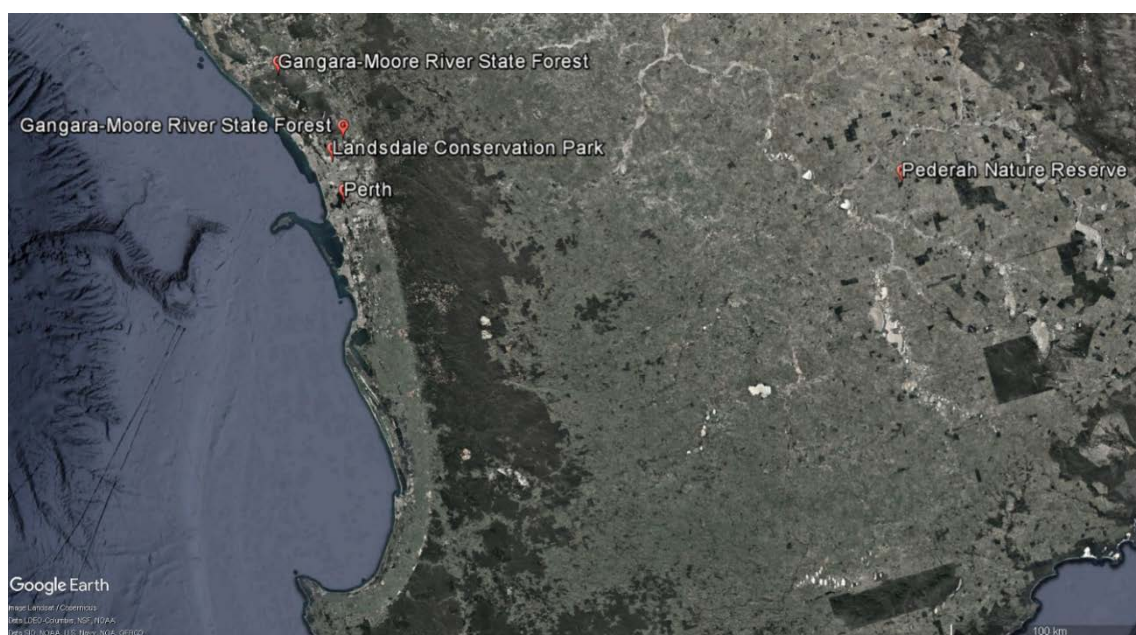


Figure 2.1 Map of collection sites in the South-west of Western Australia

2.3 Collection and processing

A combination of bagging and hand picking were used to collect fruit and seed from all eight study taxa. White nylon netting was sewn into bags and cable ties were used to seal the base of each bag around fruit bearing branches. As dispersal began, fruits and opening capsules were also handpicked. No more than 20% of seed dispersed from a single population was taken in any one round of collection.

Fruit and seed were stored at ambient room temperature (22-24°C) and relative humidity (~50%) for 1-3 months prior to experimentation. Fruits and seeds of each species were sorted for quality in trays where heavily predated, destroyed seeds were removed. Heavily damaged seeds were evident as part of the endocarp would with crumble to nothing, or had obvious holes throughout. Paper bags were used to store seeds post-sorting, ensuring moisture could not accumulate.

Fleshy mesocarp and exocarp material was removed from fruits of *Astroloma serratifolium*, *Astroloma xerophyllum*, *Conostephium minus* and *Conostephium pendulum* before experimentation. Fruits were soaked in distilled water while being agitated on a magnetic stirrer and heated to 40°C for three hours (adapted from Turner *et al.* 2009). Excess mesocarp was then removed by hand before air drying endocarps on paper towels for several days.

2.4 Sterilization

All samples were imbibed in treatment solutions for 24 hours before being surface sterilized in a 4% solution of sodium hypochlorite for five minutes (Turner *et al.* 2009) and rinsed thoroughly under running distilled water for 60 seconds (Cochrane *et al.* 2002).

2.5 Germination tests

Sterilized seeds were placed into Petri dishes containing three distilled water saturated Whatman No.1 filter papers, sprayed with a 5gl⁻¹ solution of Mancoz EB fungicide. Dishes were sealed with parafilm and placed in temperature controlled incubators at 21°C in constant light or darkness. Petri dishes in darkness treatments were wrapped in aluminium foil and kept in dark incubators. Incubation temperatures were monitored regularly with thermometers and shelves rotated within incubators randomly. Petri dishes were inspected three times a week for 52 weeks to re-moisten filter papers as required. Germinated seeds (determined by >2mm radical protrusion) were counted and removed.

Chapter 3

Fruit, seed and embryo morphology of eight Ericaceae from South-West Australia: implications for viability, dormancy and germination.

3.1 Introduction

The number of studies into Ericaceae in Western Australia are few (Dixon, Roche and Pate; 1995, Roche, Dixon and Pate, 1997; Cochrane *et al.* 2002; Norman and Koch, 2008; Turner *et al.* 2009) and as a consequence there is limited information available on the characteristics of fruit, seed and embryos. This information is crucial as it allows for confirmation of dormancy class and can be useful to those seeking to establish *ex situ* seed collections and produce plants for horticulture and restoration. Therefore, the question addressed was: what fruit, seed and embryo characteristics exist among study taxa and how do they affect dormancy and germination?

Detailed morphological aspects of Ericaceae fruits and seeds are presented in Dixon and Nielsen (1995), Tieu and Egerton-Warburton, (2000), Norman and Koch, (2008) and Turner *et al.* (2009). The study of Ericaceae seed biology stands to benefit from further application of this methodology as it facilitates a greater understanding of how fruit and seed characteristics affect dormancy and germination. Furthermore, the accumulation of photographic and numeric data on fruit and seed characteristics increases the ability of managers and practitioners to understand the outcomes of research.

Fruits and seeds of Ericaceae occur in a range of shapes and sizes and can be grouped into loculicidal, dehiscent capsules or multilocular, uniovulate drupes that possess woody, indehiscent endocarps (Kron *et al.* 2002). The former release seeds directly into the environment while the latter provide prolonged protection from fire, desiccation and endozoochory. The variation in dispersal mechanism correlates with differing dormancy types within the family (Dixon, Roche and Pate, 1995; Dixon, 2017 Pers. Comm.; Table 1.1). To better understand the impacts of these dispersal mechanisms on dormancy and germination a study of six drupe-fruited and two capsular-fruited Ericaceae was conducted.

This study investigates the role of endocarps in imposing dormancy and seeks to establish a baseline of information on fruit and seed characteristics. The study tests to see if morphological dormancy may occur and confirms whether physical dormancy is present within the 8 study taxa. For seeds within an endocarp it is likely that endocarp water permeability differs between species, while providing variable levels of protection from desiccation, fire, seed predation and endozoochory. Therefore, the hypothesis tested was: differences in fruit morphology between species impact dormancy classification and viability.

Aims

The aims of this study were to (a) provide a detailed photographic and descriptive account of seed and fruit morphology, (b) define embryo type and identify differences between study taxa, and (c) determine the impact of fruit and seed morphology on water uptake, moisture content and viability. Results of these analyses will provide a detailed account of seed morphology in study taxa, providing information crucial for the development of germination protocols for Ericaceae in the South-west of Western Australia.

3.2. Methods

The aims will be accomplished by documenting fruit, seed and embryo morphology through photographs and numeric accounts of fruit and seed characteristics. Eight species of Ericaceae will be examined using cut tests (Ooi *et al.* 2004), tetrazolium tests (Ooi *et al.* 2004) and non-destructive x-ray screening (Turner *et al.* 2009). The increase in moisture content for imbibed fruit will be quantified (Ooi *et al.* 2006; Turner *et al.* 2009) to determine if physical dormancy is present within study taxa. Likewise, the developmental state of embryos will be assessed for fresh seed to determine if morphological dormancy might occur within the family.

General methods

Study species, collection sites and general methods were conducted as per Chapter 2.

Morphological characteristics

Seeds were extracted by hand from both capsules and drupes. Forceps were used to remove seeds from partially opened capsules of *Andersonia heterophylla* and *Lysinema pentapetalum*. Endocarps of drupaceous species were opened using a combination of a vice, secateurs and a scalpel. The number of locules, numbers of viable and non-viable seeds and presence of seed predators were recorded.

Table 3.1 Sample size for morphological characteristic measurements

Sample size	Endocarp length/weight	Seed length/weight	X-ray analysis	Tetrazolium test	Cut test	Embryo length
Endocarps and seed	90	30	75	10	90	10
Capsular seed	NA	120	NA	10	30	30

Endocarp and seed weight

The endocarps and seeds of all species were weighed on a Shimadzu AUX320 balance correct to four decimal places. Seeds of the capsular fruited species *Andersonia heterophylla* and *Lysinema pentapetalum* were weighed in three replicates of 40 seeds and mean seed weight and standard error calculated. In drupaceous species, endocarp weight was determined using three replicates of 30 unopened endocarps, then seeds were extracted and weighed in three replicates of 10.

Endocarp, seed and embryo length

The morphology of fruits and seeds were observed using a Leica M205 C dissecting microscope, photographed with a Leica DFC450 digital camera and analysed with Leica Application Suite © software to obtain all length data which was truthed using digital callipers.

Seeds were imbibed on moist filter paper for 5 minutes before being placed under a dissecting microscope. Embryos were removed and embryo length was determined for 30 seeds of *Andersonia heterophylla* and *Lysinema pentapetalum* and 10 seeds of all other study taxa. Mean embryo length and standard error was calculated and compared to mean seed length to determine the embryo to seed ratio.

Embryo to seed ratio was determined as:

$$\text{Embryo to seed ratio} = \frac{\text{mean embryo length}}{\text{mean seed length}}$$

Fruit morphology and seed viability of drupaceous species was assessed using non-destructive x-ray screening (Turner *et al.* 2009), cut tests (Ooi *et al.* 2004) and tetrazolium tests (Ooi *et al.* 2004).

X-ray screening

X-ray analysis was performed on three replicates of 25 endocarps using a Faxitron X-Ray Specimen Radiography System (Turner *et al.* 2009) at the Biodiversity Conservation Centre, Kings Park. Endocarps were exposed to 18kv for 10 seconds. Images were analysed for locule number and seed fill. Empty locules showed up as black in x-rays while filled locules were white. Separation between seed and locule wall, separation between embryo and endosperm and inconsistency's of density within seeds were used to determine viability. An endocarp with one potentially viable seed is considered viable, as opposed to an endocarp with no seeds, and an endocarp containing more than one seed is considered to have greater viability as the diaspore and germination unit has a greater potential to produce seedlings. The dosages of radiation used here have previously been shown to have no impact on the germination of other species (Turner, 2017 Pers. Comm.) and the use of x-rays to determine viability is well studied, having been shown to closely resemble results of germination testing (Al-Turki and Baskin, 2016).

Cut test

A cut test (Ooi et al. 2004) was used to determine the viability of seeds and confirm viability data collected from x-ray and tetrazolium analyses. Three replicates of 30 endocarps were cut laterally and scored visually to determine the number of locules, number of seed predators, number of viable seeds (firm, moist and white endosperm) and the number of non-viable seeds (Shriveled, dry, brown or mushy). Mean seed fill and standard error

(Table 3.3) were calculated based on the number of filled locules and empty locules per endocarp. Where seed predators were recorded the effect of predation on seed fill was determined by classing each predator as a seed and calculating seed numbers accordingly (Table 3.4).

Seed fill for each endocarp was determined as:

$$\text{Seed fill} = \text{number of seeds} / \text{number of locules}$$

Tetrazolium test

Viability of firm white seeds was assessed using a standard tetrazolium test following the methods of Turner *et al.* (2009). Ten seeds were cut longitudinally, placed cut side down on filter paper soaked with 1% tetrazolium and incubated at 30°C for 48hr before assessing the staining pattern. Embryos that stained red to any degree were considered viable.

Moisture content and water uptake

The moisture contents (MC) of endocarps was determined by weighing endocarps using three replicates of 0.3g. Initial MC was measured by drying endocarps for 17hrs at 103°C and reweighing (Turner *et al.* 2009). Increase in MC was determined by weighing endocarps imbibed in distilled water for 24hr before oven drying (17hrs at 103°C) and reweighing.

Fruit and seed moisture content (MC) was determined gravimetrically on an oven dry mass (DW) basis using the equation:

$$\% \text{ increase in moisture content} = [(W_f - W_d) / W_d] \times 100$$

Where W_f and W_d are the masses of fresh and dried endocarps, respectively.

A sample of 0.3g of endocarps was not dried and dissected post-imbibition to examine the relative softness of the endosperm (Ooi, Auld and Whelan, 2006), which indicates whether water was reaching seeds through the endocarp (Ooi, Auld and Whelan, 2006).

For capsular-fruited species *Andersonia heterophylla* and *Lysinema pentapetalum* oven drying was forgone as seeds were small and could not be collected in sufficient quantity to warrant the use of 0.9g of seed per species. Instead, water uptake was measured using methods adapted from Ooi, Auld and Whelan, (2006). Seeds were imbibed on three discs of Whatman No 1 filter paper soaked in distilled water at room temperature and humidity. Three replicates of eight seeds were used for both species. After five minutes seeds were blotted dry and weighed, time 0. Seeds were removed, blotted dry and re-weighed at 4, 8, 12, 16 and 24 hours and the uptake of water calculated as the percentage increase in mass (Figure 3.13).

Percentage increase in mass (water uptake) was determined as

$$\% \text{ increase in mass} = [(W_i - W_d) / W_d] \times 100$$

Where W_i and W_d are the mass of imbibed and dry respectively.

Analysis of morphological data

Graphs were generated using Sigma Plot V11. All data were analysed using SPSS 22. ANOVA and a post-hoc Tukey's test were used to determine of statistical differences between treatment ($p = 0.05$). The normality and equal variance assumptions of an ANOVA were confirmed via the Shapiro-Wilks test and the Levene test. For the viability of capsular species (Table 3.3) the basic assumptions of ANOVA were not fulfilled and a Mann-Whitney non-parametric test was used.

3.3. Results

Andersonia heterophylla Sond.

Seeds ellipsoid, elliptical in outline, reddish-brown, 1.92 ± 0.02 mm (mean \pm SD) long, mass 1.29 ± 0.06 mg, surface rugose. Embryo developed 1.14 ± 0.06 mm long.

Astroloma xerophyllum (DC.) Sond.

Drupe leathery, white to red, exocarp thin, mesocarp thin. Endocarp stony, globose, circular in outline, dark brown to black, 3.7 ± 0.09 mm (mean \pm SD) long, mass 22.2 ± 0.06 mg, (5)-6 locular (mean \pm SE; 5.45 ± 0.09), (2)-3 seeds (mean \pm SE; 2.23 ± 0.1). Seeds 2.73 ± 0.01 mm long, mass 1.29 ± 0.06 mg. Embryo underdeveloped 1.36 ± 0.05 mm long.

Astroloma serratifolium (DC.) Druce

Drupe leathery, reddish-brown, exocarp thin, mesocarp thin. Endocarp stony, ellipsoid, elliptical in outline, light brown-tan, 6.46 ± 0.05 mm (mean \pm SD) long, mass 43.16 ± 0.69 mg, (1)-2 locular (mean \pm SE; 2.47 ± 0.13), (1)-2 seeds (mean \pm SE; 1.56 ± 0.13). Seeds ellipsoid, elliptical in outline, yellowish-white, 3.49 ± 0.06 mm long, mass 1.5 ± 0.14 mg. Embryo underdeveloped, 1.43 ± 0.02 mm long.

Conostephium minus Lindl.

Drupe fleshy, exocarp green, thin and hairy, mesocarp thin; endocarp stony, globose, light brown-tan, 4.98 ± 0.06 mm (mean \pm SD) long, mass 26.4 ± 0.1 mg, (2)-3 locular (mean \pm SD; 3.06 ± 0.11), (1)-2 seeds (mean \pm SD; 1.39 ± 0.12). Seeds ellipsoid, elliptical in outline, yellowish-white, 2.50 ± 0.07 mm long, mass 0.98 ± 0.01 mg. Embryo underdeveloped, 0.52 ± 0.02 mm long.

Conostephium pendulum Benth.

Drupe fleshy, exocarp green and thin, mesocarp well-developed at maturity with the surface reticulate or conspicuously rugose on dried specimens, mesocarp often absent; endocarp stony, turbinate, light brown-tan, 8.51 ± 0.06 (mean \pm SD) long, mass 102.46 ± 7.67 mg, (3)-4 locular (mean \pm SD; 3.06 ± 0.11), (0)-1 seeds (mean \pm SD; 0.68 ± 0.07). Seeds ellipsoid, elliptical in outline, 3.79 ± 0.03 mm long, mass 2.63 ± 0.13 mg. Embryos underdeveloped 1.02 ± 0.02 mm long.

Croninia kingiana (F.Muell.) J.M.Powell

Drupe densely hairy, yellow to tan, lacking mesocarp or exocarp. Endocarp thin and papery, cylindrical in shape, tapering at the proximal end, apex relatively broad, produced into slightly raised lobes leaving the style in a pronounced depression, 4.4 ± 0.07 mm (mean \pm SD) long, mass 16.24 ± 0.6 mg, (4)-5 locular (mean \pm SD; 4.74 ± 0.06) (0)-1 seeds (mean \pm SD; 0.91 ± 0.14). Seeds ellipsoid, elliptical in outline 3.87 ± 0.09 mm long, mass 1.495 ± 0.03 mg. Embryos underdeveloped 0.96 ± 0.01 mm long.

Leucopogon polymorphus Sond.

Drupe lignified, light brown, lacking mesocarp or exocarp. Endocarp thin, cylindrical in shape, apex relatively broad, produced into slightly raised lobes leaving the style in a pronounced depression, 2.34 ± 0.04 mm (mean \pm SD) long, mass 2.99 ± 0.045 mg, (4)-5 locular (mean \pm SD; 4.19 ± 0.13) (1)-2 seeds (mean \pm SD; 1.15 ± 0.13). Seeds ellipsoid, elliptical in outline 1.65 ± 0.22 mm long, mass 0.73 ± 0.03 mg. Embryos underdeveloped 0.49 ± 0.02 mm long.

Lysinema pentapetalum R.Br.

Seeds ellipsoid, elliptical in outline, dark to light brown 1.89 ± 0.07 mm (mean \pm SD) long, mass 0.242 ± 0.001 mg. Embryo developed 1.21 ± 0.06 mm long.

Table 3.2 Key characteristics of fruit and seed (\pm SE) of two capsular-fruited and six drupe-fruited Ericaceae collected in November-December from the South-west of Western Australia

Species	Endocarp length (mm) (mean)	Endocarp weight (mg) (mean)	Seed length (mm) (mean)	Seed weight (mg) (mean)	Endocarps with at least one seed (%) (mean)	Seeds per capsule/endocarp (mean)	Number of locules (mean)	Seed fill/ Viability	Embryo length (mm)	E/S ratio
<i>Andersonia heterophylla</i>	NA	NA	1.92 \pm 0.02	0.14 \pm 0.01	NA	4.2 \pm 0.76	NA	0.7 \pm 0.1	1.14 \pm 0.06	0.59 \pm 0.02
<i>Astroloma xerophyllum</i>	3.7 \pm 0.09	22.2 \pm 0.6	2.73 \pm 0.01	1.29 \pm 0.06	89.3 \pm 3.1	2.23 \pm 0.1	5.45 \pm 0.09	0.46 \pm 0.04	1.36 \pm 0.05	0.50 \pm 0.06
<i>Astroloma serratifolium</i>	6.46 \pm 0.05	43.16 \pm 0.69	3.49 \pm 0.06	1.5 \pm 0.14	86.7 \pm 0.27	1.56 \pm 0.13	2.47 \pm 0.13	0.85 \pm 0.09	1.43 \pm 0.02	0.32 \pm 0.01
<i>Conostephium pendulum</i>	8.51 \pm 0.06	102.46 \pm 7.67	3.79 \pm 0.03	2.63 \pm 0.13	47.78 \pm 0.07	0.68 \pm 0.07	3.06 \pm 0.11	0.23 \pm 0.07	1.02 \pm 0.02	0.27 \pm 0.01
<i>Conostephium minus</i>	4.98 \pm 0.06	26.4 \pm 0.1	2.50 \pm 0.07	0.98 \pm 0.01	81.94 \pm 0.88	1.39 \pm 0.12	4.31 \pm 0.12	0.34 \pm 0.03	0.52 \pm 0.02	0.21 \pm 0.01
<i>Croninia kingiana</i>	4.4 \pm 0.07	16.24 \pm 0.6	3.87 \pm 0.09	1.49 \pm 0.03	42.2 \pm 0.39	0.91 \pm 0.14	4.74 \pm 0.06	0.17 \pm 0.03	0.96 \pm 0.01	0.25 \pm 0.01
<i>Leucopogon polymorphus</i>	2.34 \pm 0.04	2.99 \pm 0.04	1.65 \pm 0.22	0.73 \pm 0.03	65.2 \pm 0.18	1.15 \pm 0.13	4.19 \pm 0.13	0.35 \pm 0.06	0.49 \pm 0.02	0.30 \pm 0.01
<i>Lysinema pentapetalum</i>	NA	NA	1.89 \pm 0.07	0.24 \pm 0.01	NA	25.6 \pm 4.83	NA	0.90 \pm 0.14	1.21 \pm 0.01	0.64 \pm 0.02

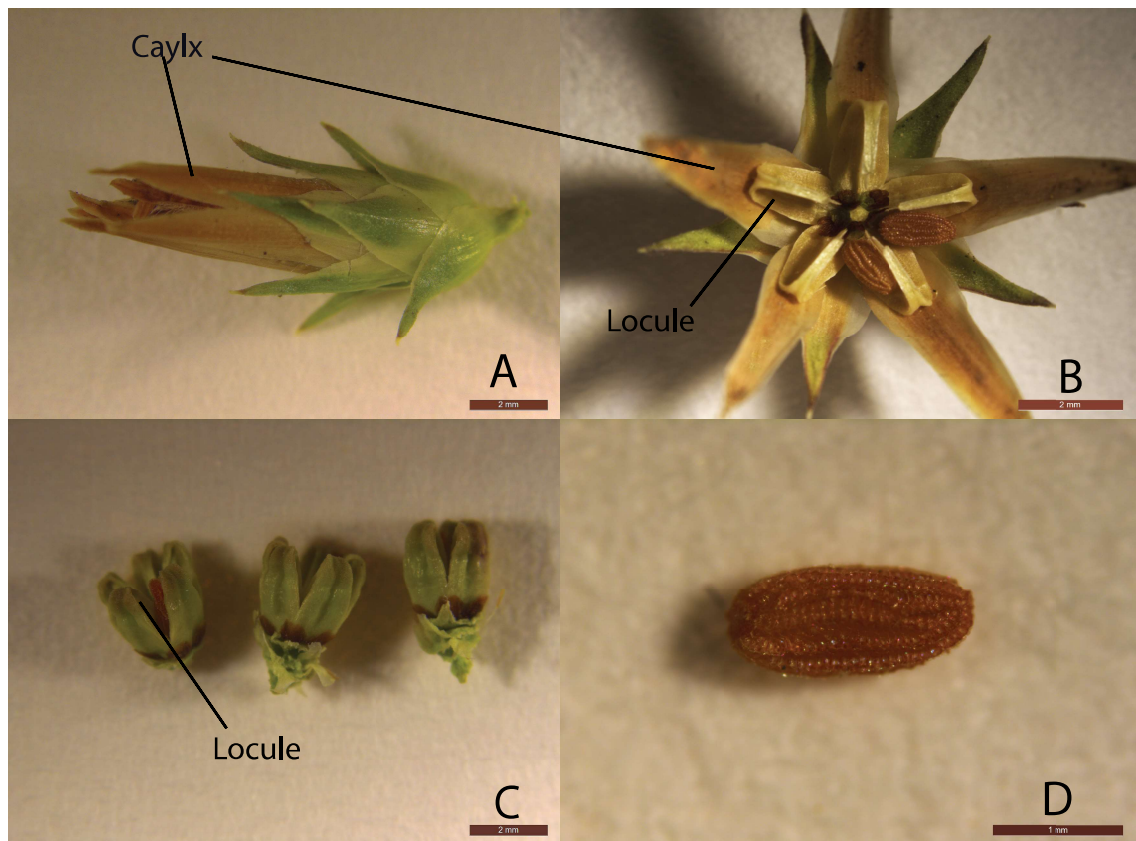


Figure 3.1 Select characteristics of *Andersonia heterophylla* capsule and seed. A, fresh fruit containing capsule, line = 2mm; B, opened fruit containing open capsule, two seeds and three empty locules, line = 2mm; C, three unopened capsules, line = 2mm; D, viable seed, line = 1mm.

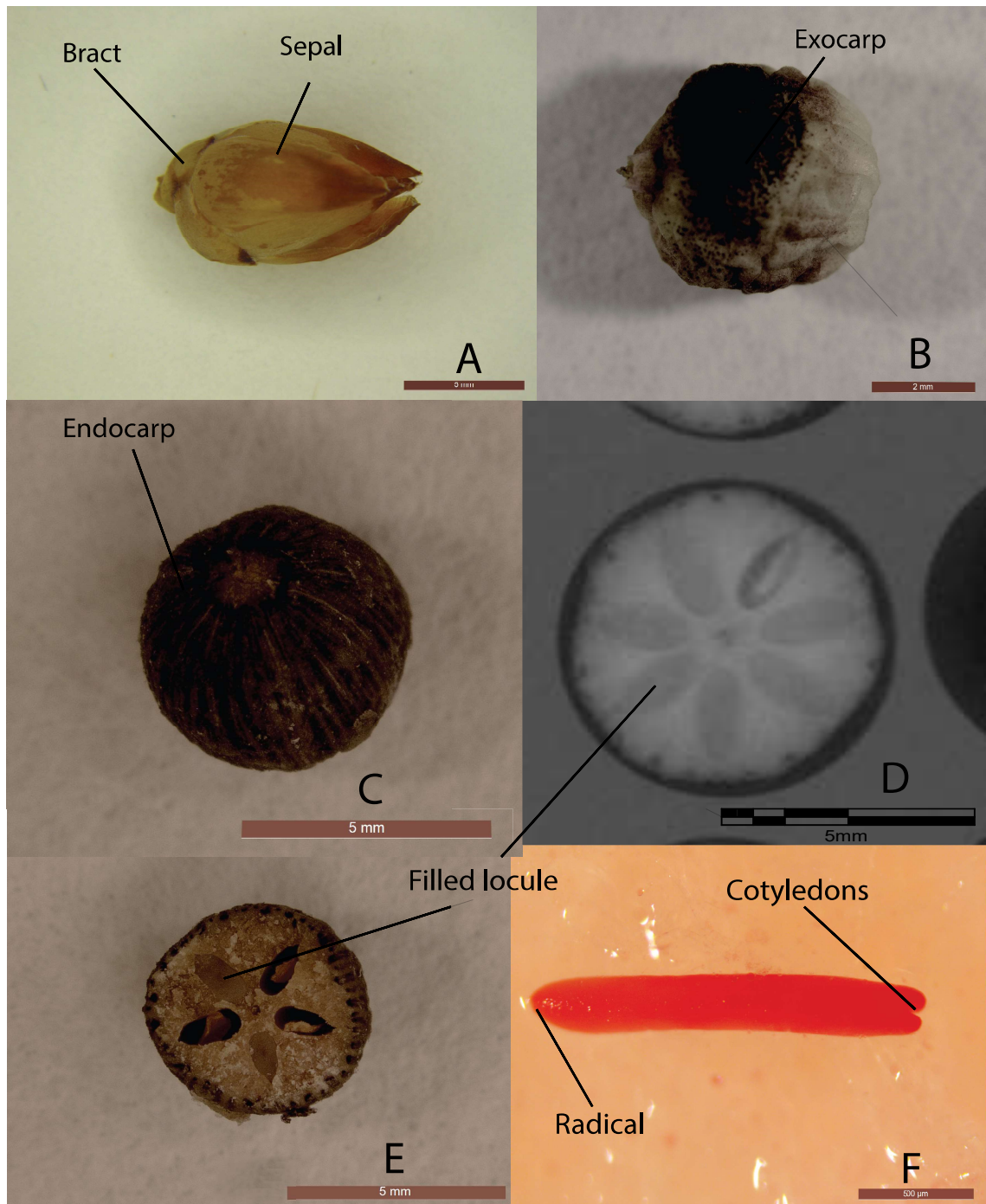


Figure 3.2 Select characteristics of *Astroloma xerophyllum* endocarps. A, fresh diaspore showing endocarp encased in bracts and sepals, line = 5mm; B, whole drupe with exocarp and mesocarp surrounding the endocarp, line = 2mm; C, fresh endocarp after removal of exocarp and mesocarp, line = 5mm; D, x-ray of endocarp showing 7 locules, 6 filled and 1 un-filled, line = 5mm; E, cross-section of fresh endocarp showing five locules containing two viable and three non-viable seeds, line = 5mm; F, linear, underdeveloped, differentiated embryo used in tetrazolium staining, line = 500 μ m.

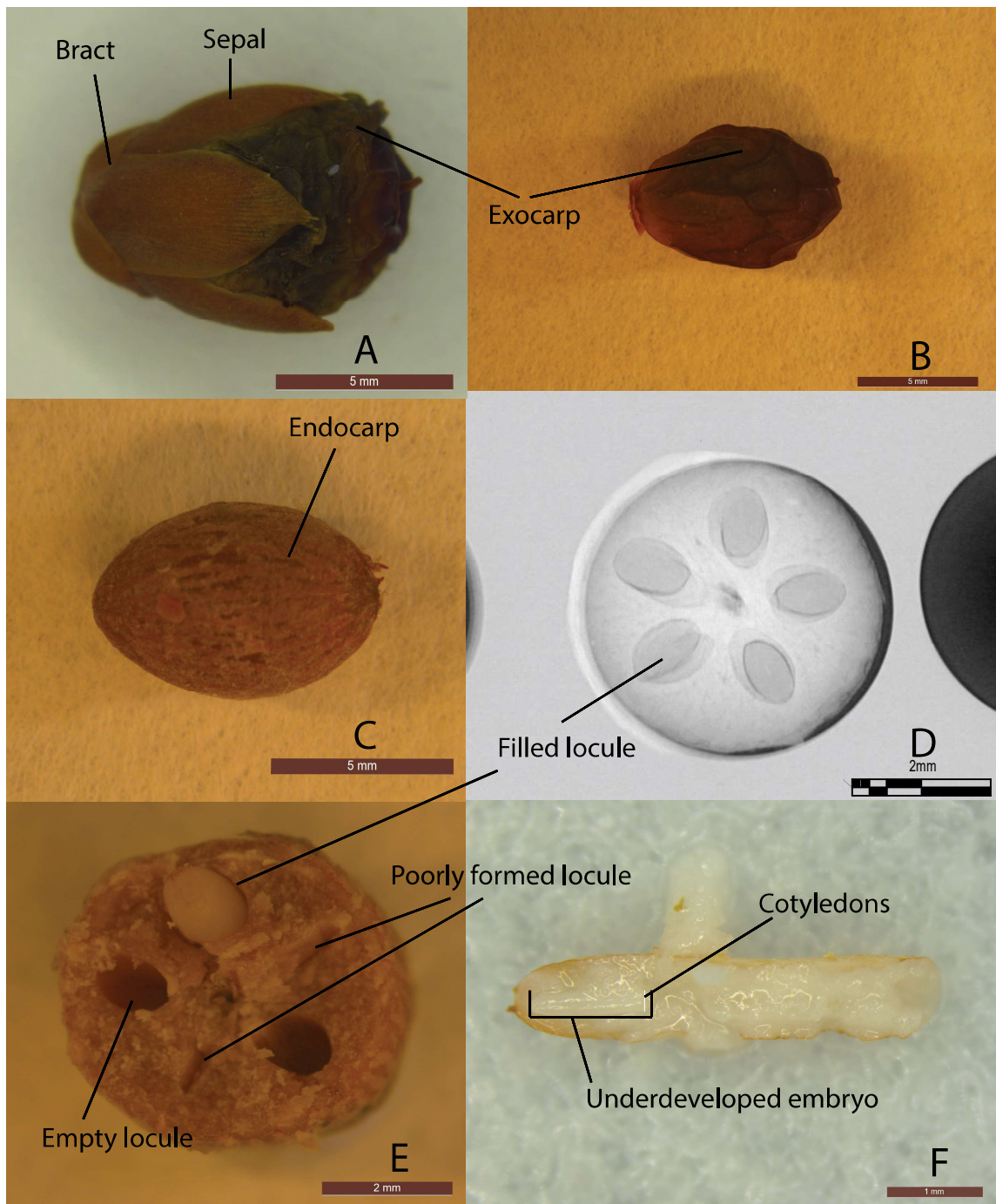


Figure 3.3 Select characteristics of *Astroloma serratifolium endocarps*. A, Fresh diaspore with whole drupe and attached bracts and sepals, line = 5mm; B, whole drupe with exocarp,mesocarp and endocarp, line = 5mm; C, whole endocarp after removal of exocarp and mesocarp material, line = 5mm; D, x-ray showing whole endocarp and 5 filled locules, line = 2mm;E, cross section of endocarp showing 5 locules, one filled, two empty and two poorlyformed, line = 2mm; F, extracted seed dissected to reveal underdeveloped, linear, differentiated embryo, line = 1mm.

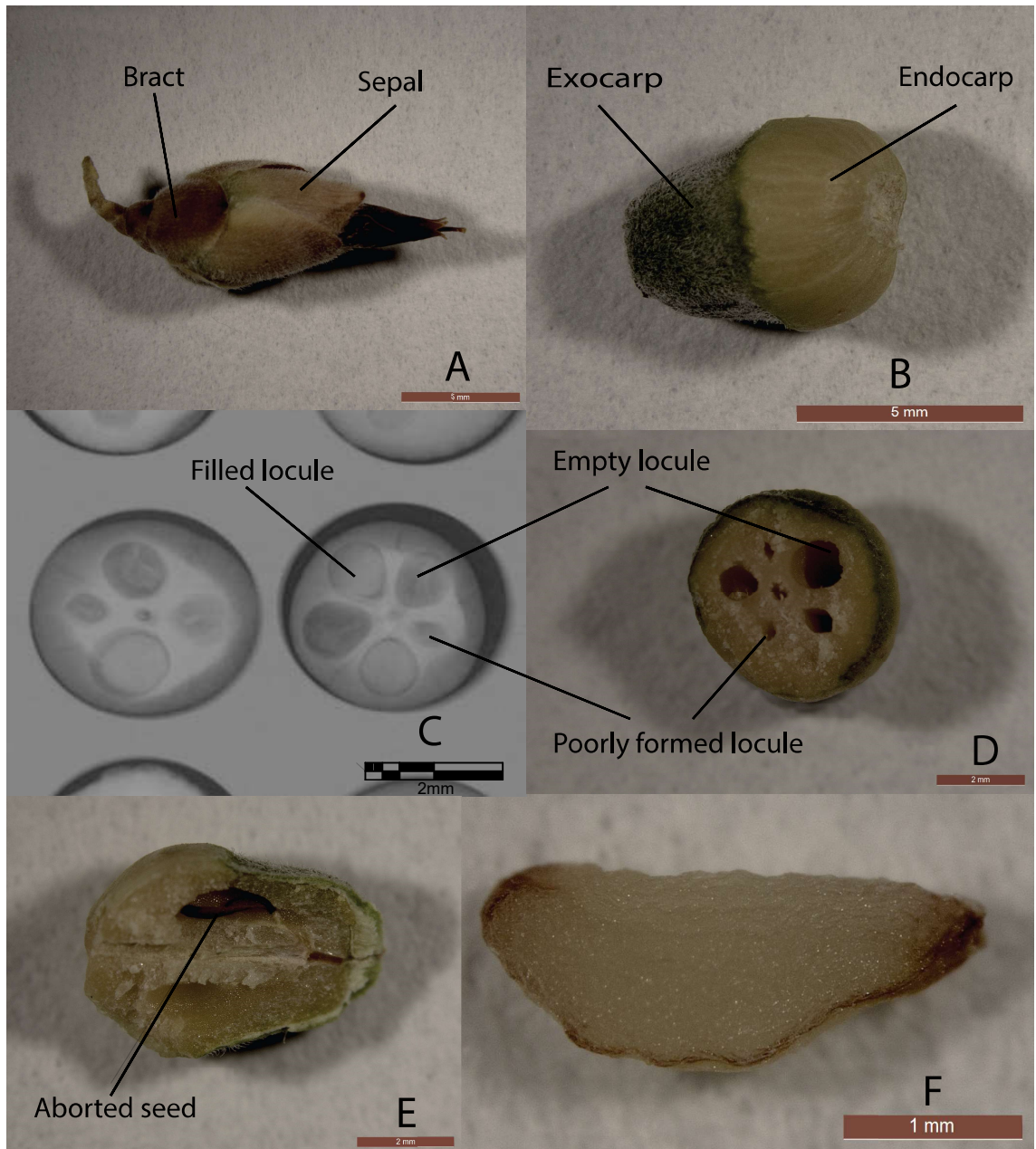


Figure 3.4 Selected characteristics of *Conostephium minus* endocarp and seed. A, intact diaspore showing endocarp contained within bracts and sepals, line = 2mm; B, intact endocarp possessing exocarp and mesocarp material, line = 5mm; C, x-ray of two endocarps showing filled, unfilled and poorly formed locules, line = 2mm; D, cross-section of endocarp showing 5 locules at varying stages of formation, line = 2mm; E, dissected endocarp showing one locule containing an aborted seed, line = 2mm; F, freshly extracted seed in the dry state, line = 1mm.

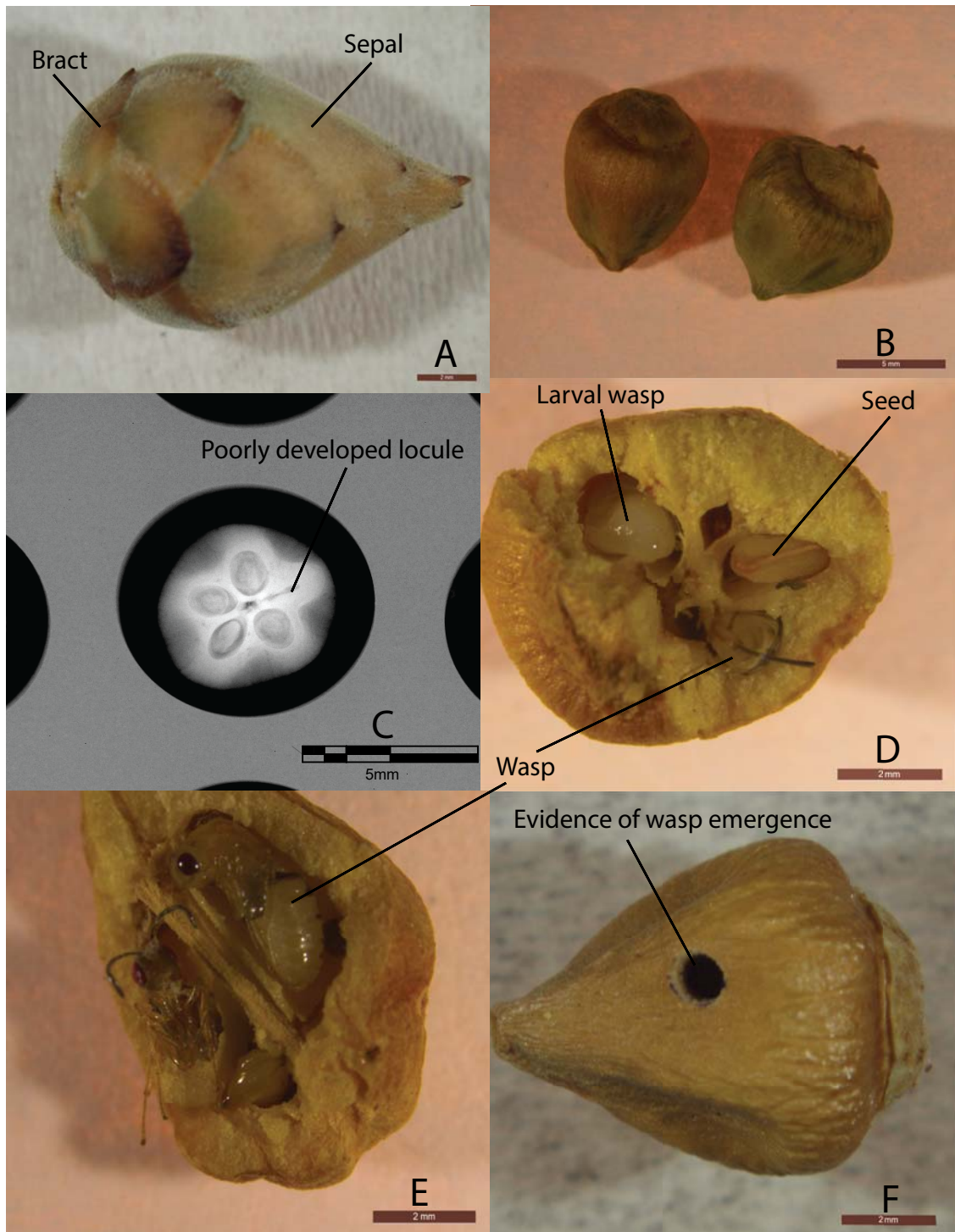


Figure 3.5 Select characteristics of *Conostephium pendulum*. A, intact diaspore showing endocarp contained within bracts and sepals, line = 2mm; B two fresh, intact endocarps, line = 5mm; C, x-ray showing 5 locules, 4 filled and 1 poorly formed, line = 5mm; D, top-down view of partially dissected endocarp showing 3 locules containing either a seed, a wasp larvae or a maturing wasp, line = 2mm; E, dissected endocarp showing two maturing wasps, line = 2mm; F, endocarp collected from forest floor showing suspected emergence wound created by wasp, line = 2mm.

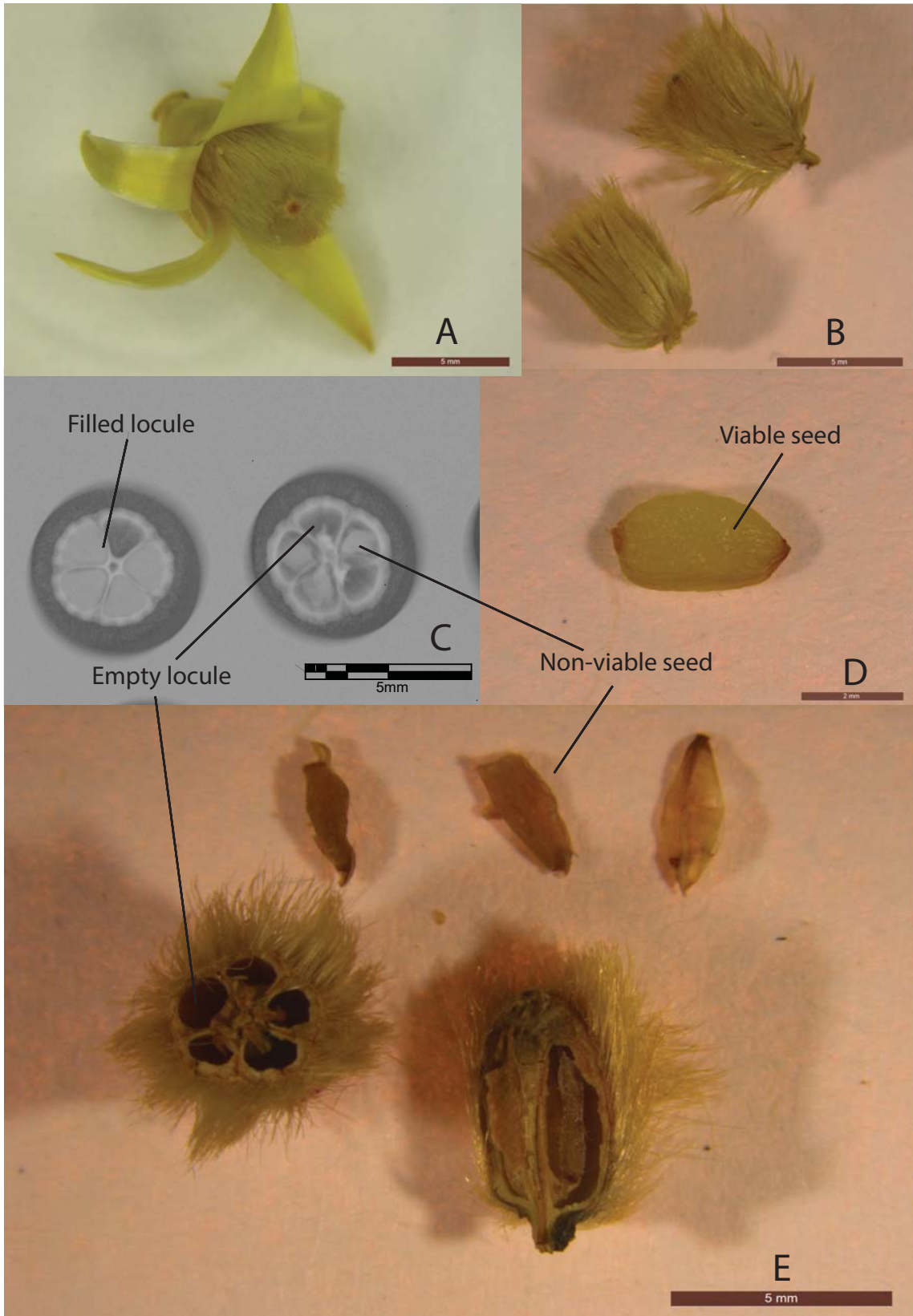


Figure 3.6 Select characteristics of *Croninia kingiana* endocarps and seeds. A, intact diaspore showing endocarp contained within bracts and sepals, line = 5mm; B two intact endocarps, line = 5mm; C, x-ray of two endocarps showing filled, empty and poorly formed locules, line = 5mm; D, intact, viable seed; line = 2mm; E, disected endocarps showing emptylocules and non-viable seeds, line = 5mm.

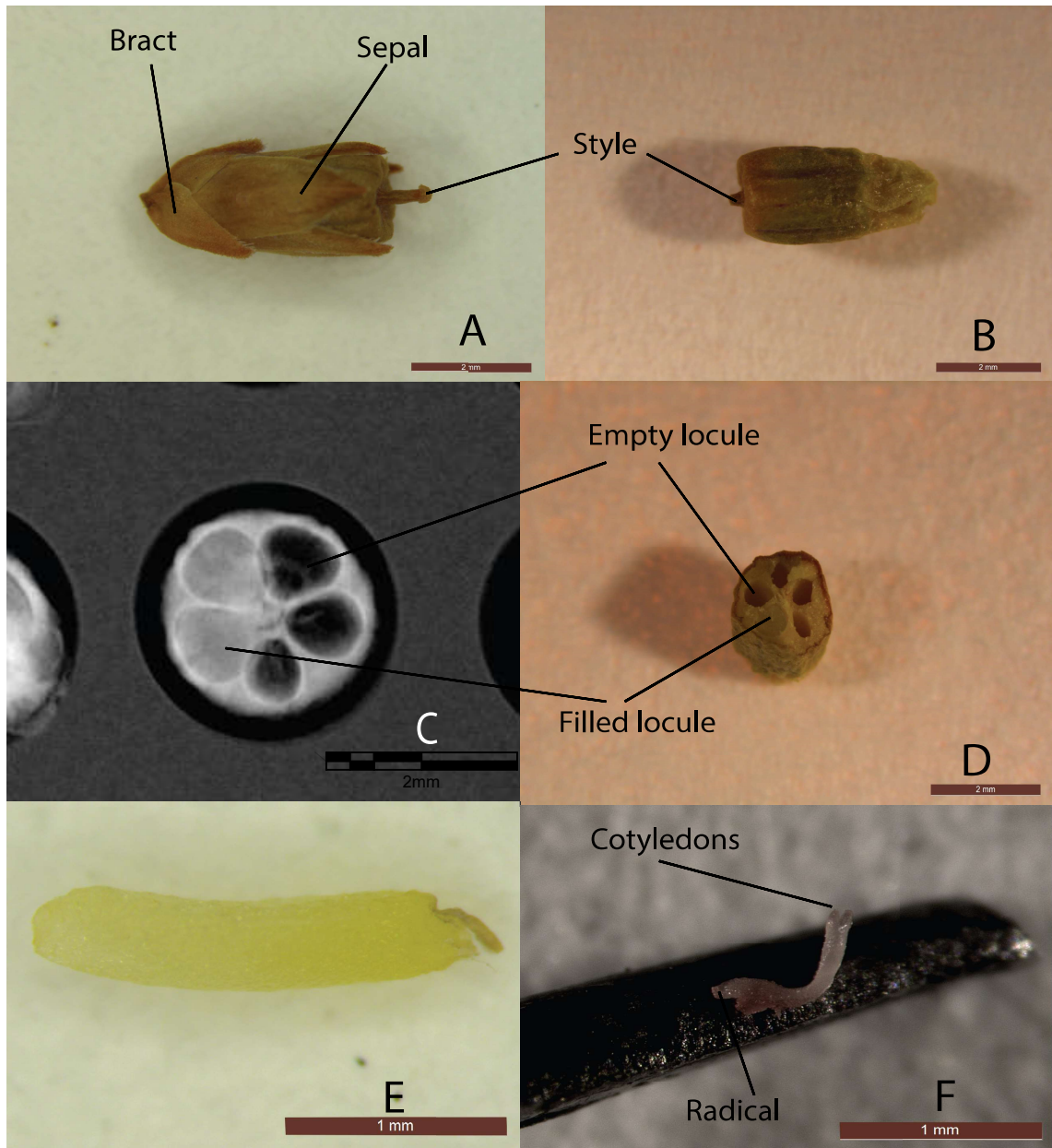


Figure 3.7 Select characteristics of *Leucopogon polymorphus* endocarps and seeds. A, intact diaspore showing endocarp contained within bracts and sepals, line = 2mm; B, intact endocarp, line = 2mm; C, x-ray showing 5 locules, 2 filled and 3 unfilled, line = 2mm; D, cross section of endocarp showing 5 locules, 1 filled and 4 un-filled, line = 2mm; E, freshly extracted seed, line = 1mm; F, extracted embryo stained for viability, line = 1mm.

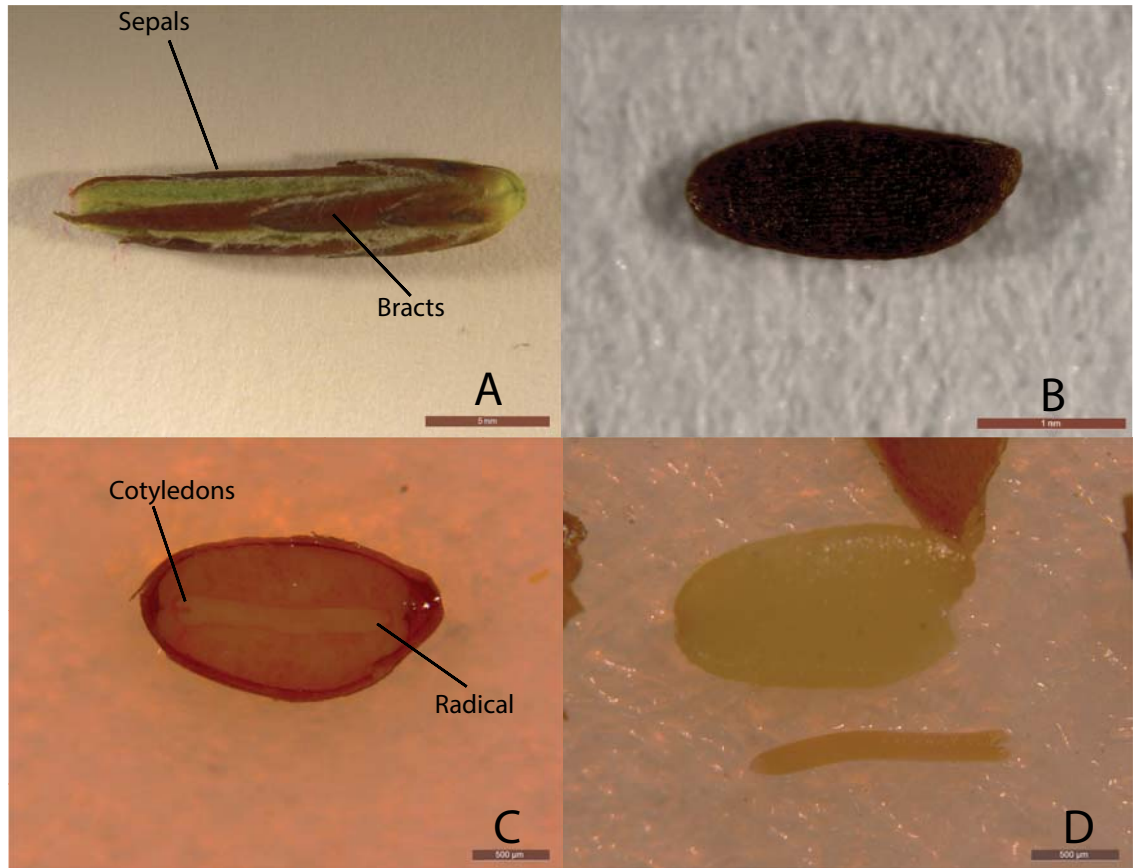


Figure 3.8 Select characteristics of *Lysinema pentapetalum* capsule and seed. A, intact capsule, line = 5mm; whole, freshly extract seed, line = 1mm; C, partially dissected seed showing developed, differentiated embryo, line = 500um; dissected seed and extracted embryo stained with tetrazolium, line = 500um.

Characteristics of endocarps, seeds and embryos

Capsules of *Andersonia heterophylla* held, on average, 4.2 ± 0.76 seeds which is significantly lower ($p < 0.05$) than capsules of *Lysinema pentapetalum* which held 25.6 ± 4.83 seeds (mean \pm SE). *Andersonia heterophylla* and *Lysinema pentapetalum* had a mean seed weight of 0.0142 ± 0.01 mg and 0.0242 ± 0.01 mg respectively and seed length (Table 3.2) did not differ significantly ($p > 0.05$). Seeds of both species were endospermic and possessed linear, differentiated embryos. Embryos were smaller than those of drupaceous species, but filled seeds more completely (Table 3.2; Figure 3.8C) with *Andersonia heterophylla* having an embryo to seed ratio of 0.59 ± 0.02 and *Lysinema pentapetalum* 0.64 ± 0.02 .

The smallest endocarps were those of *Leucopogon polymorphus* (2.34 ± 0.04 mm, 2.99 ± 0.045 mg), while the largest occurred in *Conostephium pendulum* (8.51 ± 0.06 mm, 102.46 ± 7.67 mg). Larger endocarps contained larger seeds (Table 3.2). Seeds were endospermic, containing a linear, differentiated embryo (Figure 3.2F; Figure 3.3F; Figure 3.7F; Table 3.2). Drupaceous species did not have an embryo to seed ratio greater than 0.50 (Table 3.2) and could be considered rudimentary or underdeveloped.

Seed viability, endocarp morphology and seed fill

Seeds of capsular-fruited species did not stain well, although in some cases a degree of staining was evident. Non-viable seed could be scored because of the presence of degraded endosperm. Viability did not differ significantly between these species ($p > 0.05$). Approximately 90% of *Lysinema pentapetalum* seed was viable at the time of dispersal. *Andersonia heterophylla* (Table 3.3) had an initial viability estimated at 70% and both species had an initial viability greater than drupaceous species (Table 3.3).

Table 3.3 Mean viability \pm SE as determined by cut test for fresh (<3 month old) seeds of *Andersonia heterophylla* and *Lysinema pentapetalum*.

	Viability
<i>Andersonia heterophylla</i>	70 ± 1^a
<i>Lysinema pentapetalum</i>	90 ± 1.4^a

Columns with the same letter are not significantly different as per the Mann-Whitney non-parametric test.

Average number of locules (Figure 3.9) and seeds per endocarp (Figure 3.11) were found to vary between species, with no study species having more than two seeds per endocarp on average (Figure 3.10). The percentage of endocarps containing one or more seeds (endocarp viability) was similar between study taxa in the same genera (Figure 3.10). *Astroloma xerophyllum* was found to have the highest proportion of endocarps containing at least one viable seed ($89.3 \pm 3.1\%$). *Croninia kingiana* had the lowest proportion of endocarps with at least one seed ($42.2 \pm 0.39\%$), followed by *Conostephium pendulum* ($47.78 \pm 0.07\%$) and *Leucopogon polymorphus* ($65.2 \pm 0.18\%$), however once *Conostephium pendulum* endocarp viability was corrected to account for predation ($82.2 \pm 1.46\%$) it was similar to both *Astroloma* and *Conostephium minus*.

Seeds within intact endocarps of *Conostephium pendulum* were predated by a species of wasp in the genus *Megatistigmus*. Predators were noted in all collections of *Conostephium pendulum* and were observed in three separate populations approximately 50km from one another. When the number of endocarps containing at least one seed and the mean seed number per endocarp are adjusted to account for seed predators the values were found to increase substantially in both cases (Table 3.4).

Moisture content and water uptake

Intact fruits of all study taxa increased in moisture content (Figure 3.12) after 24 hours imbibition although this was only statistically significant for *Leucopogon polymorphus* ($p < 0.05$). The endosperm was noted to be significantly softer than that of non-imbibed seeds (Ooi *et al*, 2006), indicating water had passed beyond the pericarp and entered the seed. Over 24 hours imbibition both *Andersonia heterophylla* and *Lysinema pentapetalum* increased in mass with the majority of water uptake occurring in the first 4 hours (Figure 3.13).

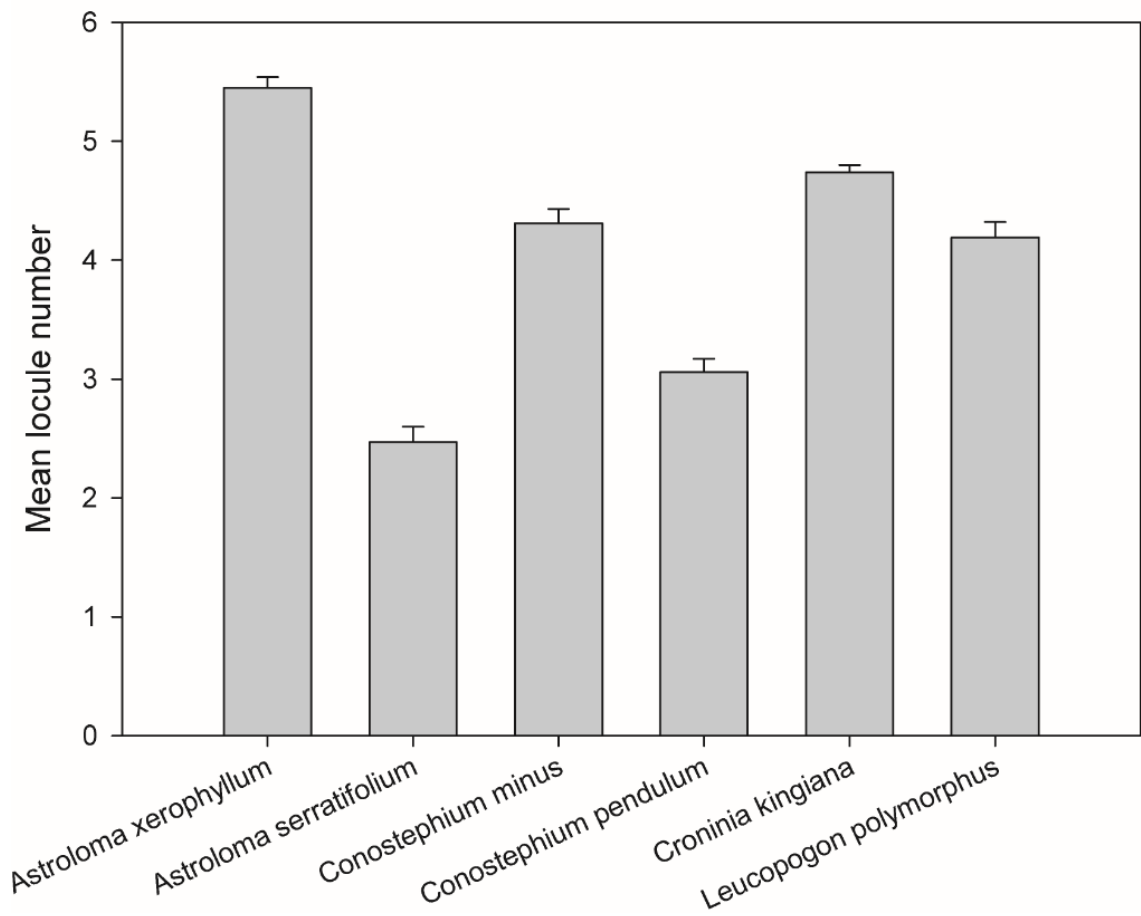


Figure 3.9 Mean \pm SE number of fully formed locules per fresh (<3 months old) endocarp as determined by x-ray analysis.

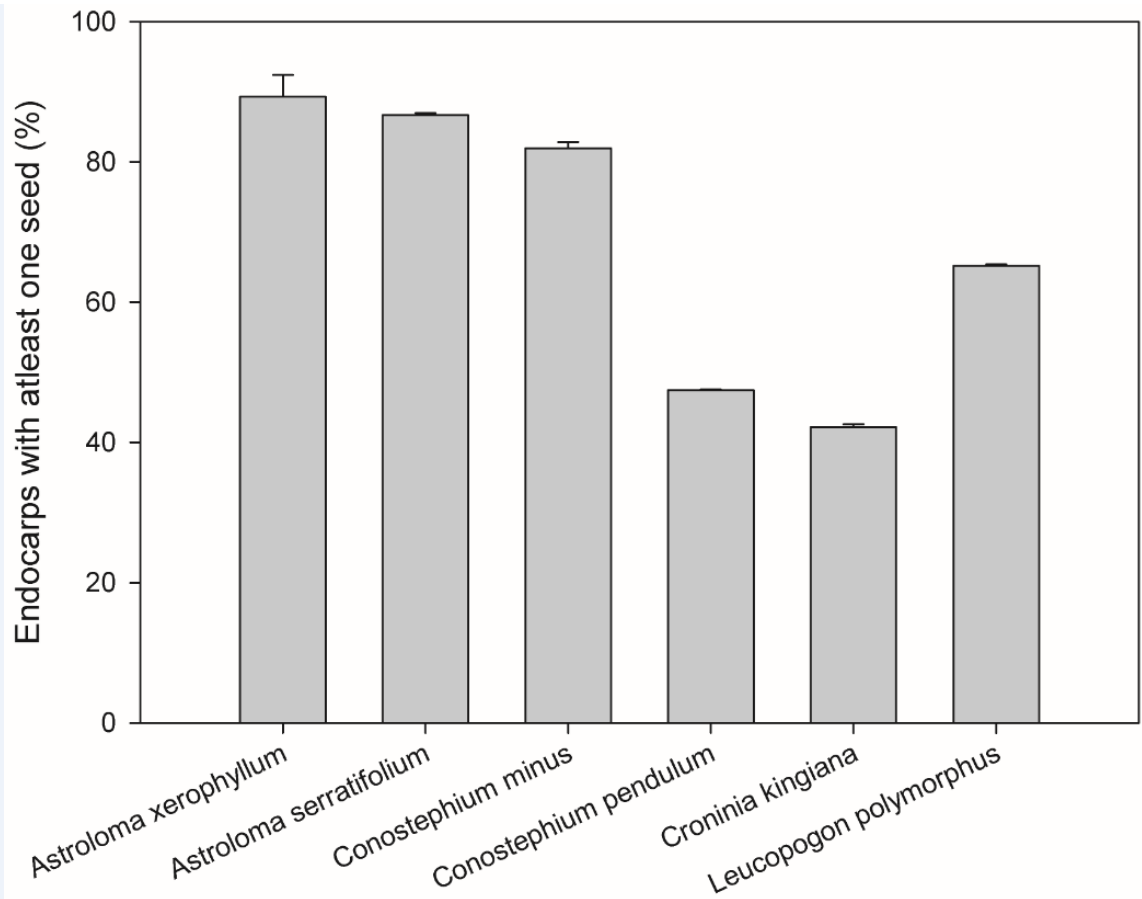


Figure 3.10 Mean \pm SE percentage of fresh (<3 month old) endocarps containing at least one seed as determined by cut tests.

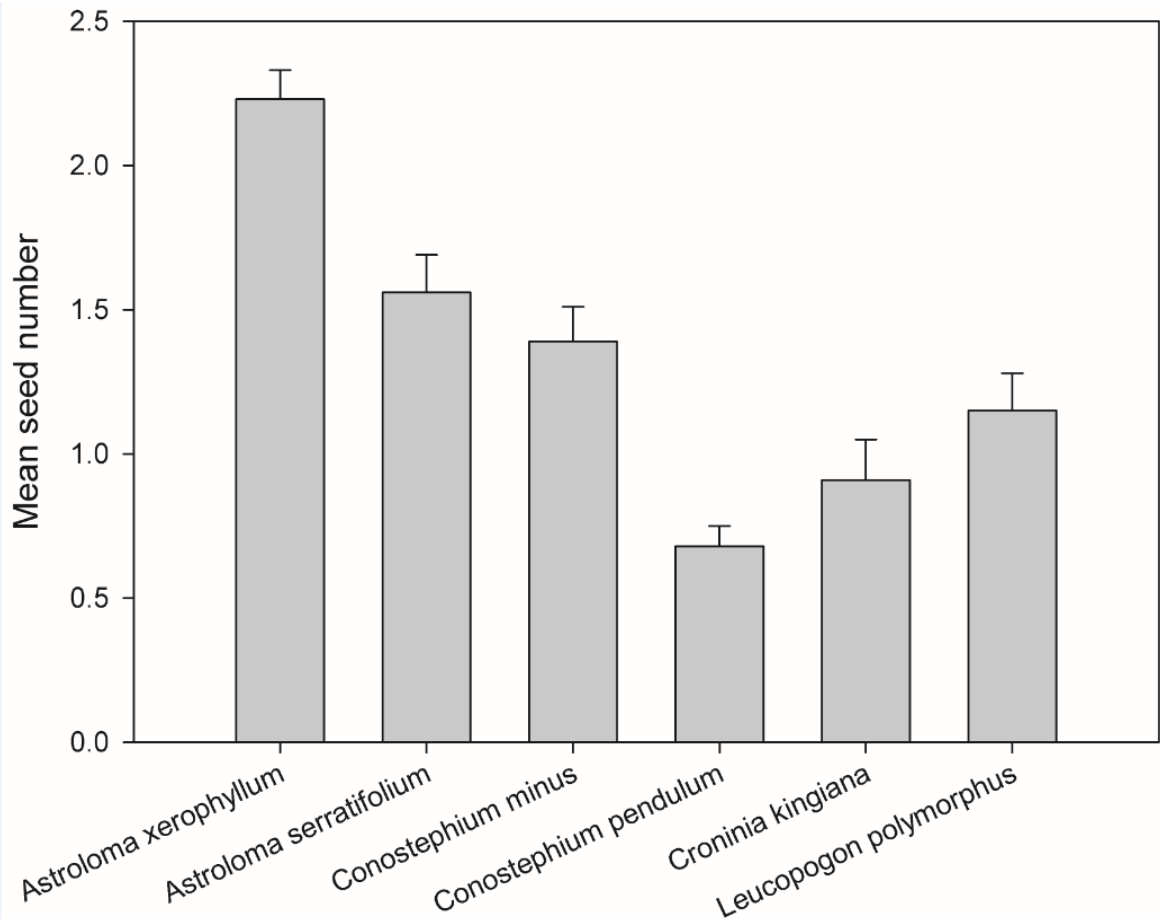


Figure 3.11 Mean \pm SE number of seeds per fresh (<3 month old) endocarp as determined by cut tests.

Table 3.4 Estimated impact of seed predation in fresh (<3 month old) drupes of *Conostephium pendulum*. Columns with differing letters are significantly different ($p < 0.05$).

Mean seed number \pm SE		Percentage (\pm SE) of endocarps containing at least one seed (%)	
Predators	No predators	Predators	No predators
0.69 \pm 0.07 ^a	1.44 \pm 0.11 ^b	47.78 \pm 2.95 ^a	82.22 \pm 1.46 ^b

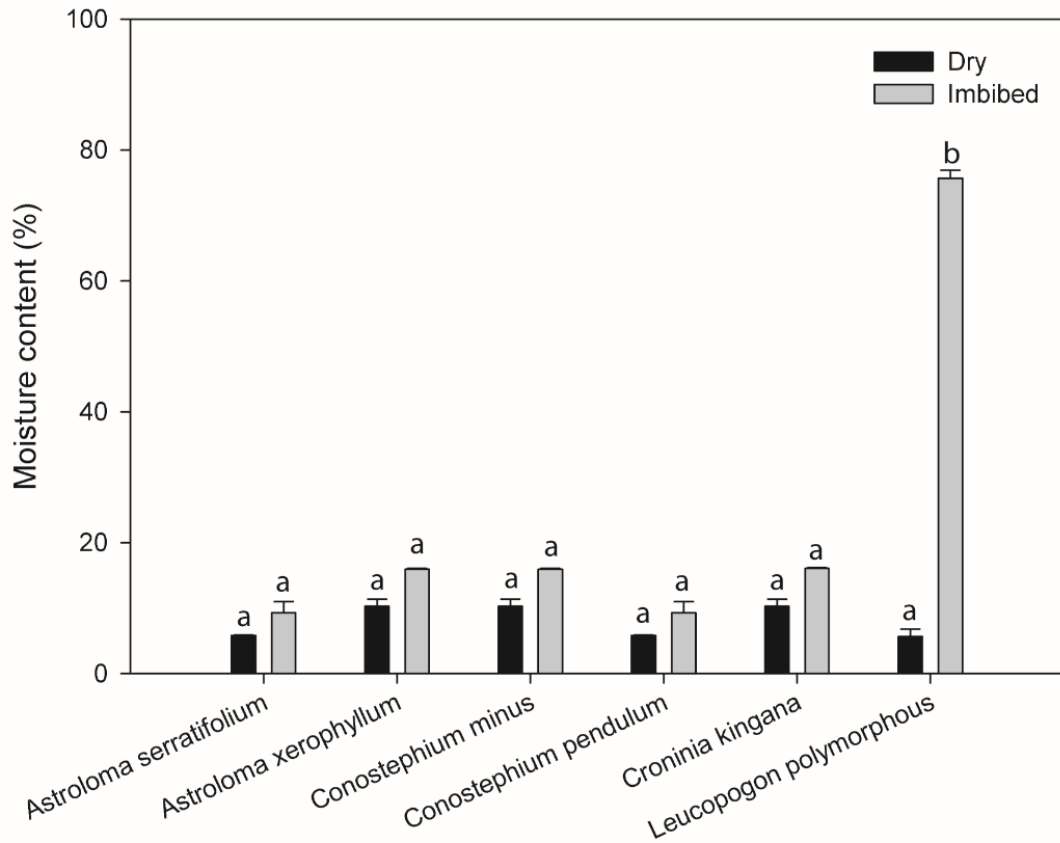


Figure 3.12 Moisture content of dry imbibed endocarps after 24 hours at room temperature. Treatments with the same letter are not significantly different ($P < 0.05$)

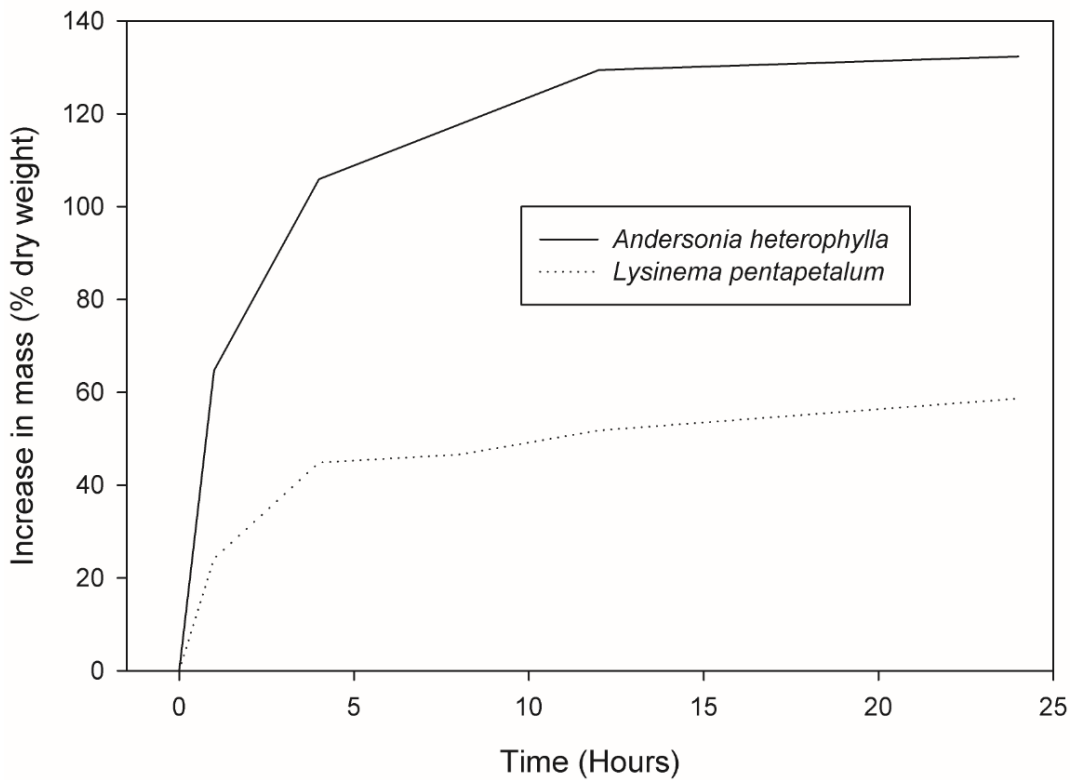


Figure 3.13 Water uptake of *Andersonia heterophylla* and *Lysinema pentapetalum*.

3.4 Discussion

This study found differing morphologies among study taxa which suggested a spectrum of requirements for physiological and morphological dormancy alleviation, requiring a range of approaches for species in *Astroloma*, *Andersonia*, *Conostephium*, *Croninia*, *Leucopogon* and *Lysinema*.

Endocarp morphology and seed viability

A key finding was that drupaceous fruits display variability in the number of locules and viable seeds per endocarp (Table 3.2). Considerable infrageneric variation of floral locule number has been observed in some taxa within Styphelieae (Powell *et al.* 1997; Powell, 1998; Taaffe *et al.* 2001; Kron *et al.* 2002; Hislop and Chapman, 2007; Hislop, 2013; Hislop *et al.* 2013) however, specific accounts of fruit locule and seed numbers are not widely available (e.g. as in Turner *et al.* 2009). Taxonomic literature documents the range of locules observed in flowers (Powell *et al.* 1997; Powell, 1998; Taaffe *et al.* 2001; Kron *et al.* 2002; Hislop and Chapman, 2007; Hislop, 2013; Hislop *et al.* 2013) but mean floral locule number is not typically provided. A comparison of mean floral and fruit locule number may provide insight into the success of pollination, seed development and the timing of embryo abortion on the mother plant. It is generally accepted that a developing embryo or seed controls the rate and sustenance of cell division in the surrounding fruit tissues (Gillaspy, Ben-David and Gruissem, 1993). Observations in tomato's (Varga and Bruinsma, 1986; Gillaspy, Ben-David and Gruissem, 1993) support this as the number of fertilized ovules generally determines the initial rate of cell division (Varga and Bruinsma, 1986; Gillaspy, Ben-David and Gruissem, 1993). Cut test and x-ray analysis of drupaceous Ericaceae (Figure 3.3D-E; 3.4C-D; 3.5C-D) shows a range of locule development within individual endocarps. In the absence of a viable seed locules appear to develop poorly and variably (Figure 3.3D-E; 3.4C-D; 3.5C-D). Whether a portion of embryos are aborted prior to substantial locule formation is unclear. A significant disparity between floral and fruit locule number may indicate the success of pollination events and how much seed is lost prior to observable locule expansion.

Tetrazolium tests were found to be a poor indicator of viability (e.g. *Leucopogon polymorphus*, Figure 3.7F; *Lysinema pentapetalum*, Figure 3.8D) within study taxa although, staining was evident in a portion of taxa (e.g. *Astroloma xerophyllum*; Figure 3.2F). Low metabolic activity in dormant seeds likely resulted in the minimal degree of staining observed here. This is a similar result to that found in studies of *Leucopogon* (Ooi, Auld and Whelan, 2004), which concluded that cut tests are a better estimate of viability.

Species determined to have higher potential viability (Figure 3.10-3.11) among the drupaceous Ericaceae were those within *Astroloma* and *Conostephium minus*. It is important to recognise that when dealing with multi-seeded fruits, viability as used here refers to the potential of the germination unit to produce seedlings, and not the specific viability of a single seed. *Conostephium pendulum*, *Croninia kingiana* and *Leucopogon polymorphus* had the lowest proportion of viable seed per endocarp. *Croninia kingiana* and *Leucopogon polymorphus* had thinner endocarp walls which were weaker and less lignified than other study taxa. This variation in morphology will likely impact longevity within the soil and the conditions required for dormancy alleviation and germination.

Conostephium pendulum was found to interact closely with a species of native wasp in the genus *Megastigmus* (Hymenoptera: Chalcidoidea: Torymidae) (Figure 3.5, see; Bouček, 1988). Species within *Megastigmus* form galls in a range of plant species (Bouček, 1988) and are common seed destroyers, however their use of Ericaceae as larval host plants was previously un-documented (Table 1 in Doganlar and Hassan, 2010).

Little is known about the reproductive biology of SWWA Ericaceae. Species of *Conostephium* have been shown to be buzz pollinated (Houston and Ladd, 2002) however, the specificity of frequency required for buzz pollination makes it unlikely that *Megastigmus sp* would have a role in the pollination of *Conostephium pendulum*. The method by which larvae are deposited within endocarps is yet unknown. Adults likely interact with flowers or pericarp early in their development, depositing an egg which develops within locules (Figure 3.5D), and this is supported by a lack of entry wounds in developing drupes. The resulting larvae consumes the seed as it develops (Figure 3.5E), prior to emergence from the endocarp through a small hole it creates (Figure 3.5F). The impact of this relationship on seed viability within endocarps is substantial (Table 3.4) and explains the low estimate of seed fill (Table 3.2). A cut test of *Conostephium pendulum* endocarps determined that 48% of fresh (<3 month old) endocarps contain wasp larvae (data not shown), and the presence of larvae was noted in three populations approximately 50km from one another. In the absence of seed predator's estimates of viability within endocarps increased substantially (Table 3.4) and were within the expected range for study taxa possessing thick, lignified endocarps.

The trend seen here (Figure 2.10-2.11) suggests endocarp morphology has a functional effect on seed development and viability. Thinner, less lignified endocarps of *Croninia kingiana* had a significantly lower proportion of viable seeds compared to other study

species. It is possible that thinner, less lignified endocarp walls make seeds more susceptible to desiccation on the mother plant. Impermeable, hard or thick seed coats provide greater protection against fluctuations in humidity and temperature which could damage seeds (Christiansen *et al.* 1960; Christiansen and Justus, 1963; Mayne *et al.* 1969; Halloin, 1986; Mohamed-Yasseen *et al.* 1994) and reduce viability. In thicker, more lignified endocarps of *Astroloma* and *Conostephium*, estimates of viability are consistently higher in the absence of seed predators (Table 3.2; Table 3.4). The higher level of protection afforded to species within these genera will likely have impacts on their longevity within the soil seed bank, their ability to withstand predation and the breadth of conditions required to alleviate dormancy and stimulate germination.

Extracting seeds of *Astroloma xerophyllum* from endocarps, or incubating endocarps in an oxygen enriched atmosphere (Turner *et al.* 2009), produces germination within three months. Observations on endocarp morphology suggest that these methods (Turner *et al.* 2009) will be less effective in *Astroloma serratifolium* and study taxa in *Conostephium*, which have thicker endocarp walls. Thus, study taxa possessing thicker, more lignified endocarps are likely to prove more intractable, taking longer to respond to dormancy and germination cues. While study taxa with thicker endocarps possessed higher viability, it cannot be concluded that thinner endocarp walls directly correlate with a reduction in seed viability and longevity. Future research using a greater sample size of species would be required to determine the functional effect of endocarp morphology on seed viability, longevity and dormancy and germination.

Seed and embryo characteristics

Seeds of capsular and drupaceous Ericaceae are endospermic (Figure 3.6D; Figure 3.7E; Figure 3.8C-D) with a linear, differentiated embryo (Figure 3.2F; Figure 3.3F; Figure 3.7F; Figure 3.8C-D; Martin, 1946). Drupaceous species possess an underdeveloped embryo (Martin, 1946) at the time of dispersal which generally shows a degree of differentiation. In comparison, seeds of *Andersonia heterophylla* and *Lysinema pentapetalum* showed little variation in embryo development, with embryos of fresh seeds being consistently developed (Martin, 1946; Table 3.2).

Embryo development in capsular and drupaceous seed provides insight into the potential dormancy mechanisms present within the Ericaceae. The requirement for embryo growth prior to germination is the sole criterion for morphological dormancy (Baskin and Baskin, 1998, 2004 and 2014). Embryos of drupaceous study taxa did not fill more than half the seed ($E/S < 0.50$) (Table 3.2; Figure 3.2F; Figure 3.3F; Figure 3.7E-F), while the

E: S ratio of capsular seeded species was consistently > 0.50 (Table 3.2; Figure 3.8C-D), indicating that morphological dormancy may be present in drupaceous study taxa and is likely absent in capsular-fruited taxa. *Astroloma xerophyllum* (Turner *et al.* 2009) was found to have embryos that grew prior to germination (E/S of 0.44) however, *Leptecophylla tameiameiae* (Ericaceae) (Baskin *et al.* 2005) had an E/S ratio of 0.75 and embryos did not grow prior to germination. Therefore, morphological dormancy is not a definitive characteristic found in drupe-fruited species. This combination of evidence indicates that drupaceous species may possess a degree of morphological dormancy. This would have a significant impact on dormancy classification, alleviation and germination, requiring treatment conditions different from taxa in which the embryo runs the full length of the seed.

These results provide an example of dormancy as it occurs along a spectrum, with sympatric species and individual seeds having variable levels of morphological development and dormancy types. Very little is known about the phenology of Ericaceae seed in and how embryos develop during seed maturation. Most Australian species possess a narrow window of collection opportunity and it can be difficult to establish collections of comparable maturity (Hay and Probert, 2013). Seed viability is known to be significantly affected by maturity at collection (Hay and Smith, 2003), and collection methods significantly affected the germination response of *Zieria arborescens* (Rutaceae) (Frith, Offord, and Martyn, 2009). Seed biology of Australian species, and in particular the Ericaceae, stands to benefit greatly from a study assessing the development of seeds on the mother plant and the development of embryos post dispersal. Future studies of genera characterised by species with deep and intractable seed dormancy should examine the impact of collection timing on the morphology, viability, dormancy and germination of seeds.

Water uptake and moisture content

A clear finding here is that endocarps (Figure 3.12) and seeds (Figure 3.13) of the studied Ericaceae readily take up water. Therefore, physical dormancy as defined by Baskin and Baskin (2004) is not present in study taxa, and this is supported by a lack of findings of physical dormancy within the Ericaceae (Baskin and Baskin, 2014). Visual inspection as per Ooi, Auld and Whelan, (2006) determined that a portion of water reached seeds within endocarps. Endocarps increase in mass during imbibition, however seeds contained within receive a slightly lower portion of water than they would if not contained within the endocarp (Merritt *et al.* 2007; Turner *et al.* 2009). The limitations to water uptake imposed by intact endocarps could limit the ability of seeds to germinate however, it has been shown that the

endocarp degrades over a year of soil storage (Roche, Dixon and Pate, 1997) which would increase the availability of water. The significant decline of endocarp integrity (Roche *et al.* 1997) and the germination restricting ability of the endocarp (Turner *et al.* 2009) suggests that the endocarp plays a primary role in the dormancy and germination process. Turner *et al.* (2009) concluded that *Astroloma xerophyllum* seeds possessed morphophysiological dormancy which was overcome rapidly once seeds were extracted from the endocarp. *In situ*, alleviation of morphophysiological dormancy would provide seeds with the growth potential required to overcome mechanical restraints to germination imposed by the endocarp. As endocarp integrity of soil stored seed declines (Roche *et al.* 1997) the growth potential required for germination would decrease. Therefore, it is likely that the mechanisms of dormancy and germination within drupe-fruited Ericaceae are not exclusively morphophysiological, but rather a combination of requirements for dormancy alleviation and endocarp degradation.

3.5 Conclusions

The present study has provided a detailed photographic account of characteristics found in *Astroloma*, *Andersonia*, *Conostephium*, *Croninia*, *Leucopogon* and *Lysinema*. Results highlight the importance of documenting fruit and seed morphology as sufficient documentation allows for determination of dormancy mechanisms and can increase the *ad hoc* use of research outcomes. Most studies neglect to document seed morphology in sufficient detail, and the discipline stands to benefit greatly from an increased focus on fruit and seed characteristics that directly relate to dormancy classification and alleviation.

Seed predation of *Conostephium pendulum* by native wasps in the genus *Megastigmus* was documented for the first time and found to be highly significant. The role of wasps in the reproductive biology of *Conostephium pendulum*, and the role of *Conostephium pendulum* in the reproductive biology of wasps, is poorly understood. To better understand the reproductive ecology of vegetation in the South-west of Western Australia a greater understanding of pollination events and seed phenology of the Ericaceae is required with particular importance given to species within *Conostephium*.

Study of moisture content and fruit, seed and embryo characteristics indicates interactions between the endocarp, physiological and morphological dormancy likely drive the intractability associated with drupaceous Ericaceae. Water uptake and an increase in moisture content was identified for all study taxa, suggesting endocarps restrict germination

or impose dormancy chemically or mechanically, rather than physically. Morphological immaturity in drupaceous taxa makes morphological dormancy a likely class within these species. Further investigation into the effects of stratification, germination stimulants, endocarps and light is required to better understand the dormancy class and germination mechanisms present within study taxa.

Chapter 4

Dormancy class and germination in eight species of South-West Australian Ericaceae

4.1 Introduction

The majority of Australian Ericaceae have unconfirmed dormancy classes (Ooi *et al.* 2006) as studies (outlined in Chapter 1) predominantly occurred prior to the establishment of the current classification system (Baskin and Baskin, 2004). The high number of treatments shown to elicit at least some germination within the family suggests complex and interacting dormancy mechanisms have evolved in response to a range of environmental factors. It is likely that these interacting factors drive the intractable nature of dormancy within Australian Ericaceae. The characteristics of fruits and seeds (Chapter 3) support the idea that multiple dormancy mechanisms occur within the family. The focus of this chapter is to identify dormancy classes for the eight study taxa and determine whether mesocarp, endocarp and the presence of light inhibits germination.

Species of *Andersonia*, *Astroloma*, *Conostephium*, *Leucopogon* and *Lysinema* produce seeds which mature in spring (September to November) and germinate in late autumn (April-May) and winter (June-August) (Meney and Dixon 1988; Merritt *et al.* 2007). Germination has been noted to take several seasons (Dixon *et al.* 1995; Roche *et al.* 1997; Merritt *et al.* 2007) and previous work has demonstrated a suppression of germination by endocarps (Turner *et al.* 2009), a significant decline in endocarp integrity during soil storage (Roche *et al.* 1997) and an alleviation of dormancy by soil storage (Dixon *et al.* 1995; Roche *et al.* 1997).

Based on previous research (Ooi *et al.* 2006; Turner *et al.* 2009) and measurements of embryo size in Chapter 3, it is expected that drupaceous species show physiological or morphophysiological dormancy, while capsular species have physiological dormancy. If dormancy is alleviated, seeds may not germinate in the absence of germination stimulants and in Australia the predominant germination stimulant is smoke (Dixon *et al.* 1995). Smoke has been studied in Australian Ericaceae however, the germination stimulating effect is variable among species (Dixon and Nielssen, 1992; Dixon *et al.* 1995; Roche *et al.* 1997). The effect of light (Merritt *et al.* 2004) and endocarp removal (Turner *et al.* 2009) has been studied in drupaceous Ericaceae. Light had no effect on the germination of *Astroloma xerophyllum* seeds contained within endocarps (Merritt *et al.* 2004), while

extracting seeds from endocarps significantly increased germination (Turner *et al.* 2009). The inhibition of germination by endocarps requires further investigation (Turner *et al.* 2009) and the effect of constant light or dark conditions has not been studied in capsular-fruited Ericaceae.

The hypothesis tested in this chapter was that endocarps restrict germination of drupe-fruited species while light inhibits germination of capsular-fruited species. The aims were to assess the dormancy class of study taxa and to determine if endocarps, and the presence of light inhibit, germination of seeds stratified at warm temperatures. These aims were investigated through the following questions:

1) Does prolonged warm or cold stratification break dormancy? Dormancy alleviation by cold or warm stratification is useful in identifying dormancy class, level and type (Baskin and Baskin, 2004) since breaking dormancy of seeds possessing deeper levels of physiological dormancy generally requires periods of cold stratification.

2) Does imbibition of smoke water post cold stratification or prior to warm stratification stimulate germination? Aerosol smoke and smoke water have been shown to increase germination of drupaceous and capsular fruited species in the lab and *in situ* (as described in Chapter 1). Smoke is well known as a germination stimulant in Australia, however the role of smoke in dormancy and germination of Ericaceae is poorly understood.

3) Does application of gibberellic acid stimulate germination? Gibberellic acid is well known for the role it plays in dormancy and germination processes. Exogenous application of gibberellic acid can produce germination depending on the sensitivity and concentration of abscisic acid within seeds. Thus, application of gibberellic acid is widely used as a pre-treatment for germinating seeds and is one of the indicative factors used to classify dormancy (Baskin and Baskin, 2004). Germination of seeds possessing non-deep and intermediate physiological dormancy may be stimulated by gibberellic acid (Chapter 1; Baskin and Baskin, 2004).

4) Do endocarps restrict germination and, if so is the restriction mechanical or chemical? Turner *et al.* (2009) suggested that endocarps may restrict germination of *Astroloma xerophyllum* by maintaining an anoxic environment within locules. The mechanism by which anoxia is established is not understood and may be grounded in oxygen impermeability of endocarps or the binding of oxygen by aromatic glycosides. It

is also possible that endocarps or fruit material restrict germination with a chemical inhibitor.

5) How does light impact germination in capsular-fruited species *Andersonia heterophylla* and *Lysinema pentapetalum*? Seed of capsular fruited species such as these are released directly into the environment. Hence, it is possible that light is a significant factor impacting germination of seeds on the soil surface.

These questions are addressed in a series of experiments designed to determine the level of physiological or morphophysiological dormancy present and to consider the role of the endocarp and light in restricting germination. Answers to these questions will help to explain why SWWA Ericaceae are so difficult to propagate and it should lead to the design of improved germination protocols.

4.2 Methods

General methods and experiment design

Study species, collection sites and general methods are given in Chapter 2. Cold and warm stratification conditions were selected to reflect mean minimum and maximum annual temperatures. Climate data were obtained from the Australian Bureau of Meteorology's weather stations closest stations to collection sites (Gingin and Hyden) (Figures 4.1 and 4.2). These data were supplemented with soil temperature data (Appendix 1) obtained from the Ozflux monitoring tower, Gingin (Silberstein, 2015), which measures daily max. and min. temperatures. These max. and min. temperatures were averaged to obtain yearly max. and min. average temperature. Cold and warm stratification temperatures were selected as 4°C and 21°C respectively as they reflect the mean max and minimum temperatures at collection sites, are significantly below and above the threshold for warm and cold stratification and they are commonly used temperatures in propagation.

While the germination window for study species is June-August (Figure 1.2), species within Ericaceae are known to persist within the soil for multiple seasons (Roche *et al*, 1997). Thus, temperatures representing the entire year were selected. Differences in temperature between collection sites were not analysed in the present study. Figures 4.1 and 4.2 show that there is a difference in annual climate conditions between sites however, a comparison between climatic conditions was beyond the scope of the present study.

Limitations to collection efforts resulted in three study taxa that were not collected in the first year of study (Table 2.1) and as such, these species were incubated post-treatment for 26 weeks (Table 4.1) due to time constraints. Species investigated, treatments applied and the duration of stratification are presented in Table 4.1. Unless stated, final germination was expressed as a cumulative percentage of germinating seeds or fruit per Petri dish and is otherwise presented as a percentage of the initial viability (Table 3.2).

Table 4.1 Study species, stratification conditions and treatment combinations applied to intact endocarps and extracted seeds of drupaceous taxa in Experiment 1 and Experiment 2.

Species ⁶	Control	Cold ⁴	GA	SW	Seed extraction ⁵
<i>Andersonia heterophylla</i>	X ¹	X ¹	X ¹	X ¹	NA
<i>Astroloma xerophyllum</i>	X ²	X ²	X ²	X ²	X ³
<i>Astroloma serratifolium</i>	X ¹	X ¹	X ¹	X ¹	X ³
<i>Conostephium pendulum</i>	X ²	X ²	X ²	X ²	X ³
<i>Conostephium minus</i>	X ¹	X ¹	X ¹	X ¹	X ³
<i>Croninia kingiana</i>	X ²	X ²	X ²	X ²	X ³
<i>Leucopogon polymorphus</i>	X ²	X ²	X ²	X ²	X ³
<i>Lysinema pentapetalum</i>	X ²	X ²	X ²	X ²	NA

¹Treatments ran for 26 weeks. ²Treatments ran for 52 weeks. ³Treatments ran for 30 days. ⁴Cold stratification periods were 30, 60, and 90 days after which seeds were transferred to 21°C. ⁵All 16 treatment combinations were carried out for intact endocarps and true seeds. ⁶ Cold stratification and combinative GA+SW was not applied to extracted seeds.

Germination experiments

Experiment 1: Effect of constant temperature, cold and warm stratification, smoke water and gibberellic acid on germination

The temperature requirements for germination and how these interact with germination stimulants were investigated using a factorial experiment with the factors being two temperatures, smoke and gibberellic acid (Appendix 2). Three replicates of 30 endocarps or seeds were used in each experiment.

Warm stratification trials were conducted on endocarps and seeds imbibed for 24 hours in distilled water, 1:10 v/v smoke water or 1gl⁻¹ gibberellic acid. Smoke water solutions were made fresh for each trial from a 100% stock solution of commercially available Smokemaster regen. A 1000p.p.m solution of gibberellic acid was made fresh from 90% pure gibberellic acid. Imbibed seeds were sterilized as described and placed in 90mm Petri dishes containing filter paper moistened with DI water. Petri dishes were placed in temperature controlled incubators set at 21°C with constant light. Three time periods of cold stratification at 4°C were trialed. Seeds were imbibed in distilled water, sterilized and incubated in darkness 4°C for 30, 60 or 90 days before treating seeds with distilled water, 1:10 v/v smoke water or 1gl⁻¹ gibberellic acid and re-sterilized. After the cold stratification period Petri dishes were transferred to a temperature controlled incubator set to 21°C, constant light. Germination was scored weekly for 52 weeks, removing seeds as they germinated (Appendix 2).

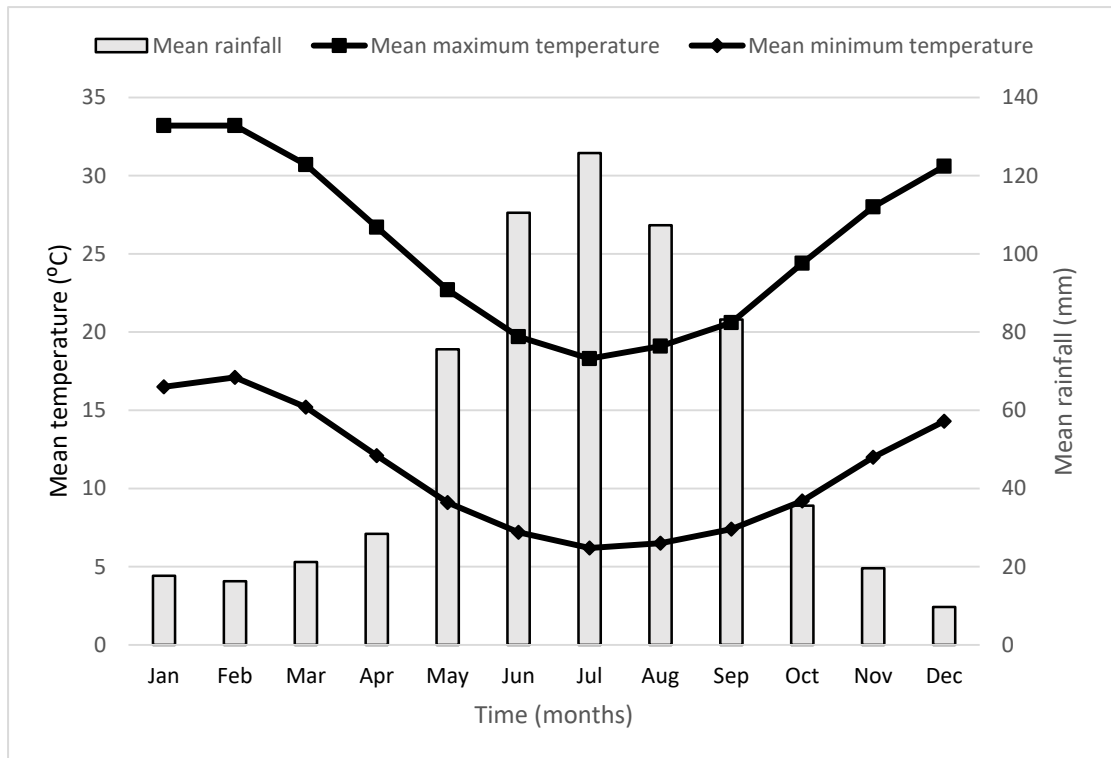


Figure 4.1 Climate data from 1981 to 2010 from the Australian Bureau of Meteorology weather station at Hyden, Western Australia. Mean monthly minimum (◆) and maximum (■) temperatures and mean month rainfall are shown.

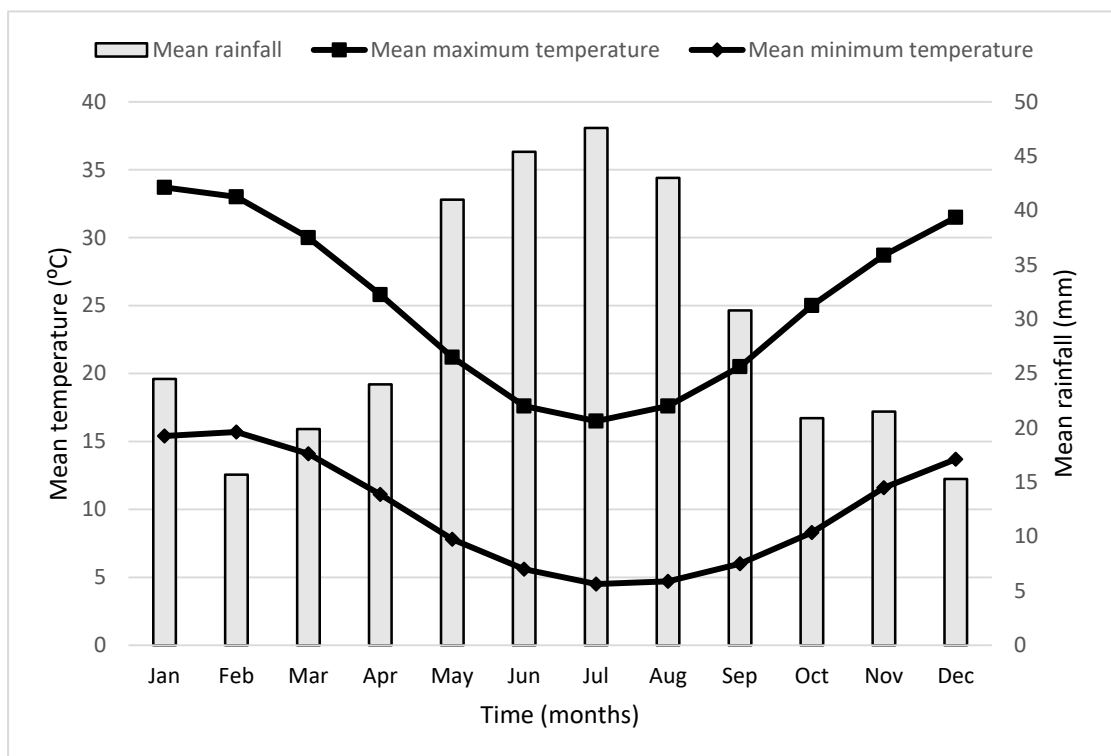


Figure 4.2 Climate data from 1996 to 2017 from the Australian Bureau of Meteorology weather station at Gingin, Western Australia. Mean monthly minimum (◆) and maximum (■) temperatures and mean month rainfall are shown.

Experiment 2: Effect of endocarp removal on germination in drupe-fruited species

The germination restricting effect of endocarps was investigated by examining the difference in germination between extracted seeds and those contained within an endocarp. Seeds held within drupaceous fruit were removed by hand after opening fruits with a vice and a scalpel. Seeds were then treated with DI water, 1: 10v/v smoke water or 1g l^{-1} gibberellic acid, sterilized and incubated in total darkness at 21°C for 30 days (Table 4.1; Figure 4.3). Three replicates of 10 seeds were used for each trial and germinated seeds counted and removed daily.

*Experiment 3: Effect of endocarp and mesocarp material on germination in *Lactuca sativa**

To investigate whether germination inhibiting chemicals were present in pericarps the germination of *Lactuca sativa* was scored in the presence of leachate from exocarp and mesocarp. The fleshy mesocarp of 100 fruits of *Astroloma xerophyllum* was removed by soaking drupes for 3 hours in 200mL of DI water heated to 40°C and agitated on a magnetic stirrer. Endocarps were removed and the remaining solution used to saturate filter papers. Similarly 100 endocarps left over from seed extraction were crushed in a mortar and pestle and added to 200ml of DI water heated to 40°C and agitated on a magnetic stirrer for 3 hours. Both solutions were left in darkness for 24 hours and agitated before use. Seeds of *Lactuca sativa* (lettuce) were sown onto four pieces of filter paper moistened with either solution (Datta *et al.* 1970) and incubated in dark-interrupted with light at 21°C . Controls were sown onto filter paper moistened with DI water and germination scored after 48hr (Figure 4.4).

Experiment 4: Effect of light on germination in capsular fruited species

Seed of capsular-fruited species likely require dark conditions for germination as seeds on the soil surface are unlikely to survive (Egley, 1990; Sheldon, 1974). To determine if light inhibits germination the germination of capsular-fruited species was scored in two light regimes. The effect of light on germination of capsular seeded species *Andersonia heterophylla* and *Lysinema pentapetalum* was explored through two treatments, constant light or darkness interrupted with brief light for the purpose of scoring. This design used two factorial combinations, light and dark-interrupted, each of which were tested in three replicates of 30 seeds requiring 180 seeds per species.

Gibberellic acid was the only pre-treatment found to produce reliable germination in these species and thus, light experiments were conducted on seeds pre-treated with 1g l^{-1} gibberellic acid. Seeds were imbibed in gibberellic acid for 24 hours before being sterilized and placed onto filter paper in Petri dishes sealed with parafilm. Petri dishes were incubated at 21°C in either constant light or dark interrupted with light. Germination in both treatments was scored weekly, ensuring seeds were exposed to as little light as possible. Germination was scored weekly for 12 weeks and germinating seeds removed (Table 4.3; Figure 4.5).

*Experiment 5: Effect of long term incubation in constant light on germination of *Lysinema pentapetalum**

To determine if light increases the depth of dormancy or inhibits germination seeds of *Lysinema pentapetalum* stratified for 52 weeks in light (*Experiment 1*) were transferred to darkness and the rate of germination compared to fresh seeds germinated at the same conditions. Non-germinating seeds of *Lysinema pentapetalum* from control and cold stratification treatments in *Experiment 1* were combined and transferred to three 150mm Petri dishes containing sterilised quartz sand ($n = 120$) and either 1g l^{-1} gibberellic acid, 1:10v/v smoke water or a combination of 1g l^{-1} gibberellic and 1:10v/v smoke water. Petri dishes were incubated in darkness at 21°C , germination scored daily and germinating seeds removed (Figure 4.6).

Data analysis

Germination data were analysed using final germination percentages for each treatment. Graphs were generated using Sigma Plot V11. All statistics were calculated using SPSS 22. ANOVA and a post-hoc Tukey's test was used to determine statistical differences between treatment effects ($p < 0.05$). Normality of variables was checked with the Shapiro-Wilks test ($p > 0.05$) and homogeneity of variances determined through the Levene test ($p > 0.05$).

4.3 Results

Experiment 1: Effect of constant temperature cold and warm stratification, smoke water and gibberellic acid on germination

Endocarps did not germinate in any treatment even after 52 weeks of incubation (Appendix 2). *Andersonia heterophylla* and *Lysinema pentapetalum* seeds stratified at cold or warm temperatures did not germinate in control or smoke water treatments. *Andersonia heterophylla* and *Lysinema pentapetalum* germinated after treatment with 1gl^{-1} gibberellic acid (*Experiment 4*; Table 4.3, Figure 4.5).

Experiment 2: Effect of endocarp removal on germination in drupe-fruited species

Once extracted from endocarps seeds of *Astroloma xerophyllum*, *Astroloma serratifolium* and *Croninia kingiana* germinated readily after 30 days incubation at 21°C in total darkness (Figure 4.3, Table 4.2). Controls of *Astroloma* and *Croninia* species germinated, as did smoke water and gibberellic acid trials, with gibberellic acid alone producing the highest level of germination (Table 4.2, Figure 4.3). *Conostephium minus* and *Conostephium pendulum* did not germinate over the trial period and seeds of *Leucopogon polymorphus* proved to be too small and delicate to extract in sufficient numbers for experiments. At the conclusion of the experiments it was observed that the embryos of *Conostephium pendulum* had grown (Appendix 3) compared to those of fresh seeds (Table 3.2).

Table 4.2 Percentage germination \pm SE of extracted Ericaceae seeds incubated in total darkness at 21°C for 30 days.

Species	Maximum germination (%)	Sig
<i>Astroloma xerophyllum</i>	96 ± 1.05	0.001
<i>Astroloma serratifolium</i>	86 ± 1.05	0.008
<i>Conostephium minus</i>	0	NS
<i>Conostephium pendulum</i>	0	NS
<i>Croninia kingiana</i>	96 ± 1.05	0.003

Maximum germination for all species was achieved with gibberellic acid. Difference is significant at $P = 0.05$. NS, not significant.

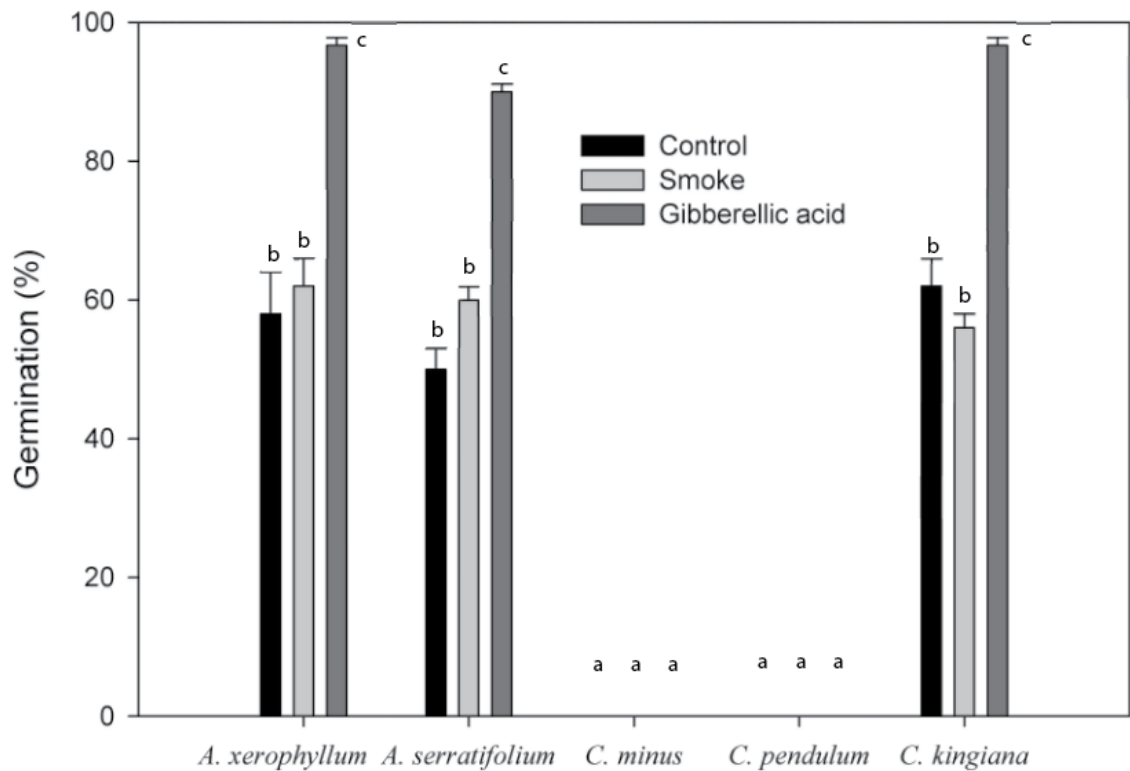


Figure 4.3 Percentage germination \pm SE of extracted Ericaceae seeds incubated in darkness at 21°C for 30 days. Treatments with the same letter are not significantly different ($P < 0.05$)

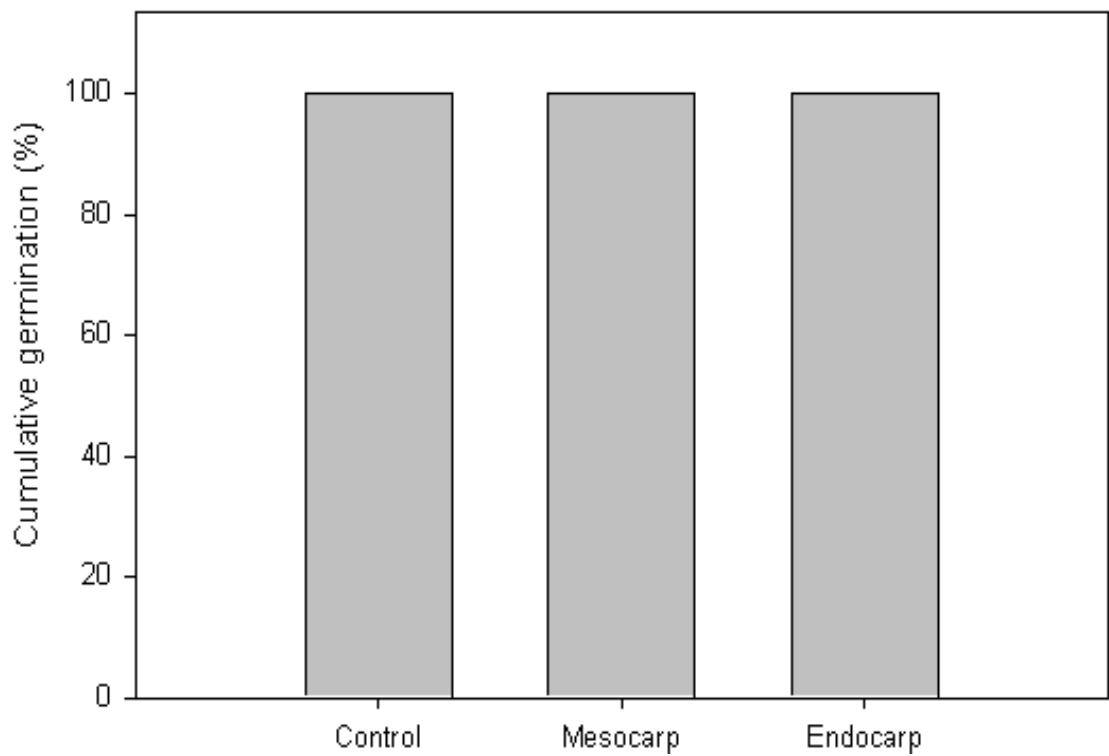


Figure 4.4 Cumulative germination percentage of *Lactuca sativa* (lettuce) incubated at 21°C in total darkness for 48 hours with a solution made using endocarp and mesocarp material.

Experiment 3: Effect of endocarp and mesocarp material on germination in Lactuca sativa.

Total germination (%) of *Lactuca sativa* seeds was not affected by the presence of exocarp or mesocarp material (Figure 4.4). Seeds of *Lactuca sativa* germinated to 100% regardless of the presence of mesocarp or exocarp material, or when incubated with distilled water.

Experiment 4: the effect of light on germination in capsular fruited species.

When treated with gibberellic acid and incubated in constant light germination of *Andersonia heterophylla* and *Lysinema pentapetalum* was significantly reduced compared to total darkness (Figure 4.5). In darkness *Lysinema pentapetalum* germinated to $90.12 \pm 2.31\%$ while *Andersonia heterophylla* germinated to $23.33 \pm 3.46\%$ (Table 4.3). Adjusting for viability (Table 3.2) increases the total germination percentages of *Andersonia heterophylla* and *Lysinema pentapetalum* to 28.57% and 100% respectively.

Table 4.3 Maximum germination percentages (G) and germination times (t_1 and t_{50}) as a mean \pm SD for fresh seed (< 3 months).

Species	Treatment	G (%)	t_1 (days)	t_{50} (days)
<i>Lysinema pentapetalum</i>	Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Cold Stratification	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Smoke	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Gibberellic Acid	90.12 ± 2.31	15 ± 2	23 ± 2
<i>Andersonia heterophylla</i>	Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Cold Stratification	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Smoke	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Gibberellic Acid	23.33 ± 3.46	15 ± 1	NA

Cold stratification encompasses three time periods (30, 60 and 90 days). t_1 is the time between treatment and first germination; t_{50} is the time in which germination percentages to reach 50%.

Experiment 5: Effect of long term incubation in constant light on germination of Lysinema pentapetalum

Seeds of *Lysinema pentapetalum* incubated for 52 weeks in constant light (*Experiment 1*) germinated within 21 days after being transferred to quartz sand treated with gibberellic acid in darkness. No germination was produced in treatments containing smoke water (Figure 4.6).

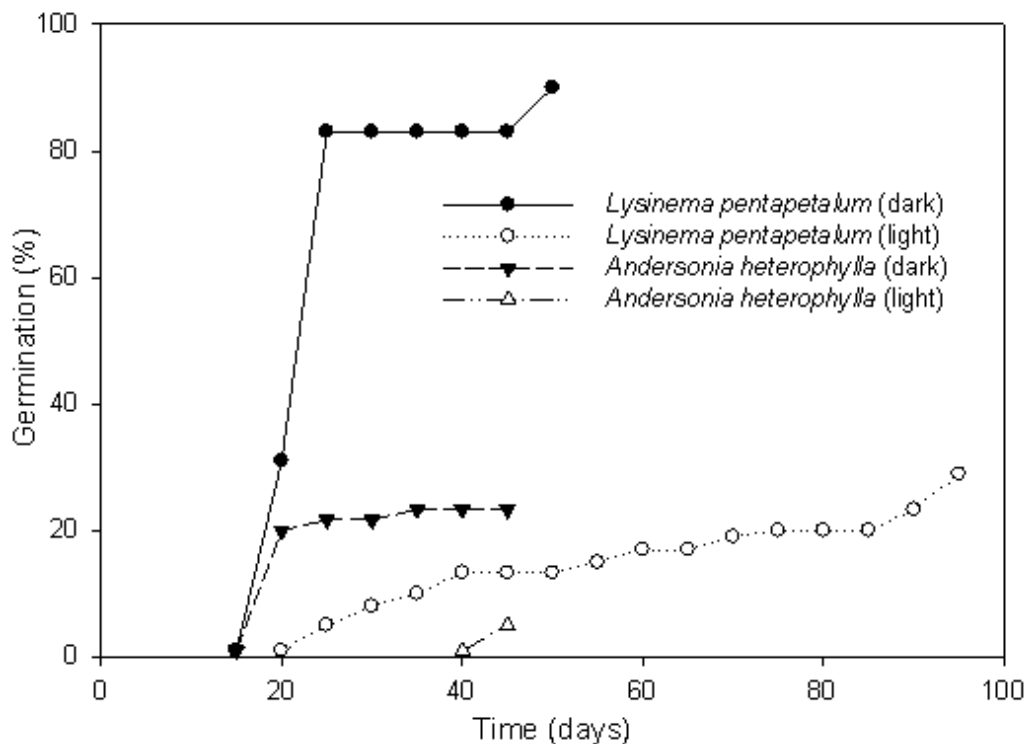


Figure 4.5 Mean germination (%) for *Andersonia heterophylla* and *Lysinema pentapetalum* seeds treated with gibberellic acid. Lines terminate where maximum germination was achieved.

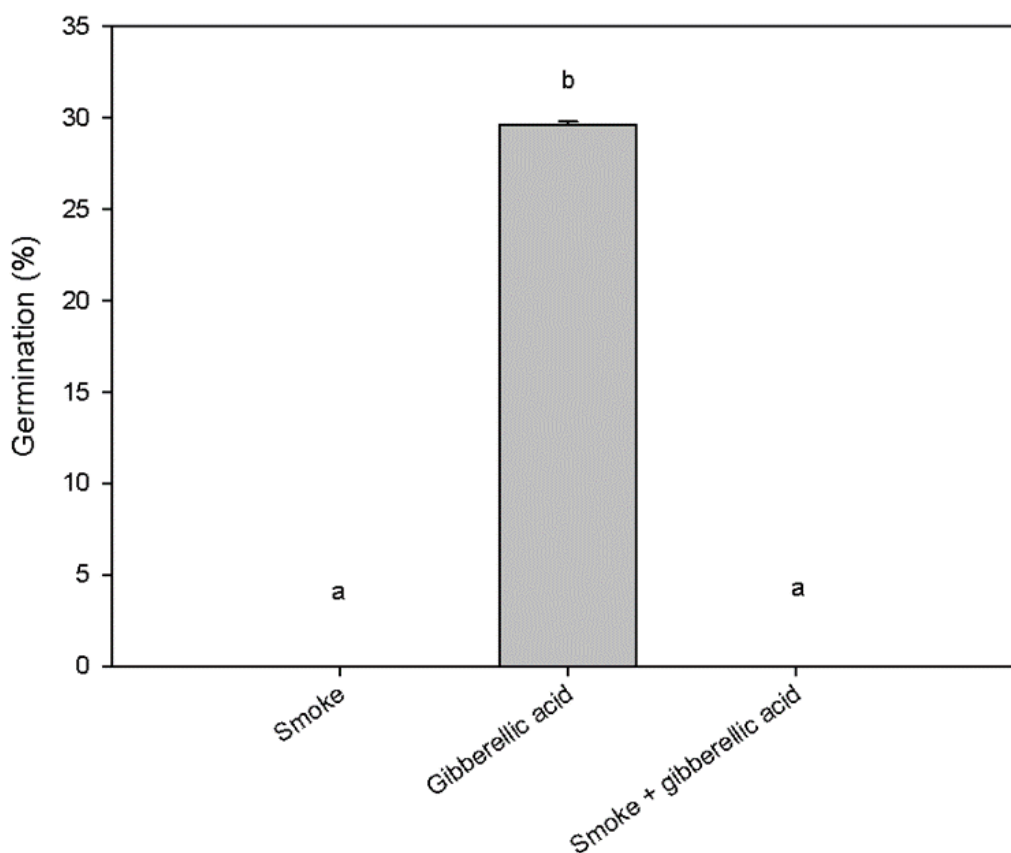


Figure 4.6 Germination (% of initial viability) \pm SE of pre-treated *Lysinema pentapetalum* seeds incubated in constant darkness for 30 days (21°C). Treatments with the same letter are not significantly different ($P < 0.05$)

4.4 Discussion

To investigate why Ericaceae in the South-West of Western Australia are such an intractable group to propagate from seed, the present study sought to determine the class of dormancy in study taxa and investigate factors that inhibit germination. Germination restricting phenomena were identified for all study taxa with the endocarp and light negatively effecting germination of drupe- and capsular-fruited species respectively.

Capsular species

Andersonia heterophylla and *Lysinema pentapetalum* behaved according to Baskin and Baskin's, (2004) definition of physiologically dormant seeds. Seeds of both species take up water readily (Figure 3.13), indicating that seed coat layers do not physically restrict water uptake required for germination. *Andersonia heterophylla* and *Lysinema pentapetalum* possessed developed embryos at the time of dispersal (Table 3.2), ruling out the presence of morphological dormancy (Baskin and Baskin, 2004). While fresh seeds (<3months post-collection) with no prior treatment did not respond to cold or warm stratification in the presence or absence of smoke water, fresh seeds germinated within 30 days once treated with gibberellic acid (Table 4.3; Figure 4.5). This response to gibberellic acid (Table 4.3) is typical of seeds expressing non-deep or intermediate physiological dormancy (Baskin and Baskin, 2004), which have dormancy broken by either cold (<10°C) or warm (>15°C) stratification and have germination stimulated by gibberellic acid.

Lack of germination in warm stratification, cold stratification and smoke water treatments could have been caused by a range of factors. In *Cynara cardunculus*, a species with physiological dormancy, it has been shown that fluctuating temperatures turn off abscisic acid synthesis and reduce abscisic acid signalling (Huarte *et al.* 2014) leading to the alleviation of dormancy and allowing endogenous gibberellic acid to stimulate germination (Graeber *et al.* 2012). Therefore, alternating temperatures may be required to alleviate dormancy in *Andersonia heterophylla* and *Lysinema pentapetalum*. Seeds transferred to quartz sand after 52 weeks incubation did not germinate in the presence of smoke water but germinated readily with gibberellic acid alone (Figure 4.6).

This suggests that the 10% solution of smoke water produced from Smokemaster Regen may be too high for small-seeded capsular-fruited species and previous work using Regen 2000[®] (Adkins and Peters, 2001) supports this. Therefore, consideration of concentration and source of smoke water is required when applying smoke products to capsular-fruited Ericaceae.

Direct florescent light suppressed germination of *Andersonia heterophylla* and *Lysinema pentapetalum*. Seeds treated with gibberellic acid and then subjected to constant light did not germinate as well as gibberellic acid treated seeds left in darkness (Figure 4.5). Seeds incubated for 52 weeks in constant light and DI water germinated readily once treated with gibberellic acid and incubated in darkness interrupted with light (Figure 4.6), indicating that light does not increase the depth of dormancy but suppresses germination. Future research could investigate whether diurnally alternating temperatures, or a different constant temperature alleviates physiological dormancy in the presence of different light regimes and various concentrations and applications of smoke.

Previous studies (Dixon *et al.* 1995; Cochrane *et al.* 2002) found similar germination responses in species of *Andersonia* and *Lysinema* however, neither of these investigations used fresh seed and this may have influenced their results. *Lysinema ciliatum* was shown to respond to aerosol smoke when sown in glasshouse conditions (Dixon *et al.* 1995). The response to smoke under greenhouse conditions supports the hypothesis that dormancy alleviation of *Lysinema* species requires alternating diurnal and seasonal temperatures or a different application of smoke. Cochrane *et al.* (2002) produced maximum germination of *Andersonia* species with gibberellic acid (10–500mg l⁻¹) and this is similar to findings for *Andersonia heterophylla* (Table 4.3).

Incubating seeds with a solution of gibberellic acid provides a reliable means for propagating both *Andersonia heterophylla* and *Lysinema pentapetalum*. Germination of *Lysinema pentapetalum* approached 100% (Table 4.3; Figure 4.5). *Andersonia heterophylla* germination was significantly lower than that of *Lysinema pentapetalum* indicating higher levels of, or greater sensitivity to, abscisic acid that could not be overcome by exogenously applied gibberellic acid (Finkelstein, Reeves, Ariizumi and Steber, 2008). This suggests that seeds of *Andersonia heterophylla* are likely to possess intermediate physiological dormancy while those of *Lysinema pentapetalum* possess non-deep physiological dormancy.

To better understand the germination ecology of capsular seeded Ericaceae a method for geminating seeds in the absence of exogenous gibberellic acid is required. Including the effects of after-ripening within a study exploring the effects of alternating temperatures, wet-dry cycles and photo-period would further advance our understanding of these species, allow for greater analysis of studies that have used aged seed and facilitate the development of consistently effective germination protocols.

Drupaceous Ericaceae

It is clear that the endocarp plays a significant role in the dormancy and germination mechanisms of drupaceous Ericaceae. Seeds contained within an endocarp are dormant at dispersal, as confirmed by a lack of germination in cold and warm stratification treatments regardless of the pre-treatment (Appendix 2). Underdeveloped embryos (Table 3.2) that grow prior to germination (Ooi, Auld and Whelan, 2006; Turner *et al.* 2009) indicate that seeds within endocarps possess morphophysiological dormancy. According to Baskin and Baskin (2004) morphophysiological dormancy requires various combinations of warm and cold stratification to alleviate (Table 1.2). Findings for *Astroloma xerophyllum* (Turner *et al.* 2009), *Acrotriche patula*, *Astroloma pallidum* and multiple *Leucopogon* species (Roche, Dixon and Pate, 1997) suggest a requirement of alternating temperatures with germination increasing after long term incubation at temperatures representative of field conditions. A lack of germination from intact endocarps in the present study was likely caused by a need for diurnally and seasonally alternating temperatures during stratification.

Baskin and Baskin, (2005) concluded that drupaceous fruit of *Leptecophylla tameiameia* (Ericaceae) possessed seeds with deep physiological dormancy. Thus, a morphological component of dormancy only occurs in some drupaceous Ericaceae however, underdeveloped embryos (Chapter 3) and a lack of germination from intact endocarps in the present study suggests that study taxa likely possess morphophysiological dormancy. This is further supported by observations of substantially larger embryos in seeds of study taxa extracted prior to and during germination (Appendix 3).

Extracted seeds of *Astroloma xerophyllum*, *Astroloma serratifolium* and *Croninia kingiana* germinated in control, gibberellic acid and smoke water treatments at a constant 21°C (Figure 4.3). This suggests that study species exhibit non-deep morphophysiological dormancy which corroborates the results of previous research into *Astroloma*

xerophyllum (Turner *et al.* 2009). Neither *Conostephium minus* nor *Conostephium pendulum* germinated under treatment conditions once extracted from endocarps. Based on the ideas of Baskin and Baskin, (2005) and the role of gibberellic acid in classifying dormancy (Baskin and Baskin, 2004), it is likely that study taxa within *Conostephium* possess intermediate or deep physiological dormancy in combination with morphological dormancy.

The method by which endocarps either impose dormancy or inhibit germination is poorly understood. Likewise, the relative importance of seed dormancy and endocarp associated factors in delaying germination of study taxa are unknown. Germination of control groups (Figure 4.3) suggests that endocarps may have a primary role in maintaining dormancy. Stratification may alleviate dormancy of seeds within endocarps and allow the seeds to overcome mechanical constraint to germination however, whether growth potential can overcome mechanical constraint without substantial endocarp weakening requires further investigation. Work in *Persoonia* (Chia, 2016) has demonstrated that the endocarp is a barrier to germination, and that the restriction of germination may be imposed via mechanical restraint, limitation to water uptake or a restriction of oxygen availability. It cannot be concluded at present whether the endocarp of drupaceous Ericaceae restricts germination by via a single mechanism or multiple and further work is required to better understand the relationship between germination, mechanical restraint, water availability and oxygen. It has been demonstrated (Turner *et al.*, 2009; Chia, 2016) that while the endocarp allows water to enter, the increase in moisture content of intact endocarps is significantly less than that of naked seeds. While water can penetrate the endocarp it is likely that oxygen can as well although the degree of oxygen and water availability may be less than that required to increase metabolism to the point where seeds can overcome mechanical restraint imposed by the endocarp. The rapid germination response of extracted seeds suggests that alleviation of endocarp restricting factors may be required prior to dormancy alleviation, which potentially takes place once oxygen becomes available. The requirement for alternating temperatures in dormancy alleviation may relate to a requirement for endocarp weakening or the breakdown of inhibitory compounds, rather than purely an alleviation of physiological dormancy.

Incubating seeds of *Lactuca sativa* with leachate from endocarp and mesocarp material of drupaceous Ericaceae had no effect on total germination (Figure 4.6). This indicates that endocarps do not prevent germination by direct chemical inhibition and this is consistent with the conclusions of Turner et al. (2009), who suggested that endocarps impose anoxia on seeds contained within locules. It is plausible that restriction of oxygen by the endocarp limits the ability of embryos to metabolise and grow, requiring weathering, fire or disturbance to increase oxygen availability before dormancy alleviation can occur. Oxygen restriction by seed coat or pericarp material has been suggested as a germination inhibiting mechanism for both *Acer pseudoplatanus* L. (Dungely and Pinfield, 1980) and *Beta vulgaris* L. (Coumans, Cme and Gaspar, 1976) and it is possible that endocarps physically exclude oxygen from reaching seeds. Phenolic compounds within seed covering materials have also been shown to bind oxygen (Cme and Tissaoui, 1973; Coumans et al. 1976) thus depriving seeds of oxygen. *Eremophila maculata* (Ker Gawl.) F.Muell. and *Eremophila resinosa* (Endl.) F.Muell.(Myoporaceae), two Australian species that produce indehiscent endocarps, have been found to contain significant levels of aromatic glycosides (Richmond and Ghisalberti, 1994) that bind oxygen. Seeds of these species germinate readily from intact endocarps after an extended period of weathering in situ (Richmond and Ghisalberti, 1994) indicating that phenolic compounds may be leached or progressively broken down. This provides a possible explanation for the germination response of drupe-fruited Ericaceae *in situ* after extended periods of soil storage (Roche et al. 1997; Tieu et al. 2001; Ooi et al. 2004; Chapter 1).

The results of seed extraction experiments (Figure 4.3) suggest that once oxygen becomes available morphophysiological dormancy is alleviated by warm stratification. As drupe-fruited species possess the ability to re-sprout after fire, germination from the soil seedbank is not an obligate requirement. Therefore, multiple mechanisms of delaying germination ensure seeds persist in the soil seedbank, facilitating recruitment over successive years and allowing seeds to respond to a range of conditions. The delay of germination by oxygen restriction makes sense in light of *in situ* observations of drupaceous Ericaceae germinating in disturbed areas in the absence of fire (Dixon Pers. Comm, 2016). Ecologically, a mechanism to germinate in the inter-fire period would greatly benefit recruitment as time between fires can be substantial (Gill and McCarthy, 1998). Further work into the effect of oxygen on germination stimulation of intact endocarps and extracted seeds is required to better understand how primary dormancy is alleviated in seeds both *in situ* and *ex situ*.

4.5 Conclusions

Seeds of *Andersonia heterophylla* and *Lysinema pentapetalum* possess physiological dormancy and have germination inhibited by light. Dormancy of seeds held within endocarps is more complex and involves the interaction of the endocarp and physiological and morphological mechanisms of dormancy. Intact endocarps behave as though seeds possess morphophysiological dormancy however, once removed seeds of study taxa in *Astroloma* and *Croninia* germinate as though they were non-dormant or possessed a very shallow physiological or morphophysiological dormancy. The rapid germination response post- extraction from endocarps indicates that once oxygen becomes available dormancy is rapidly alleviated by warm stratification. Thus, dormancy alleviation by stratification may not be the only requirement for germination and seeds potentially require endocarps to be degraded to release anoxia within locules.

Removing seeds from endocarps has been shown as a viable method for germinating *Astroloma xerophyllum* (Turner *et al.* 2009) and these results (Figure 3.3) confirm this for *Astroloma xerophyllum*, *Astroloma serratifolium* and *Croninia kingiana*. A method for propagating capsular-fruited species with gibberellic acid was determined and the germination inhibitory effect of light on two capsular Ericaceae was demonstrated. Germination of intact endocarps was not stimulated in the present study, suggesting that single temperature stratification is ineffective. Results suggest that the mechanism by which intact endocarps restrict germination is by limiting embryo metabolism through reduced oxygen availability. Thus, fluctuating temperatures may be required to facilitate a breakdown of endocarp contained phenolics as well as to alleviate dormancy to a point in which seeds can overcome germination restriction via anoxia and mechanical constraint.

Germination of intact endocarps under field conditions or laboratory equivalents will always be a lengthy process due to interactions between the dormancy state of the embryo and restrictions imposed by the endocarp. By moving away from replicating field conditions *ex situ* it would be possible to design germination protocols able to elicit germination in a shorter time frame, allowing on demand propagation of species within the Ericaceae. A study of dormancy alleviation by alternating temperatures and oxygen enrichment in these species is required as both methods have proven effective for *Astroloma xerophyllum* (Turner *et al.* 2009).

Chapter 5

Techniques for the rapid propagation of intractable South-West Australian Ericaceae

5.1 Introduction

Species of Ericaceae in the South-West of Western Australia display multiple dormancy types (Chapter 4). Seeds of *Andersonia heterophylla* and *Lysinema pentapetalum* possess non-deep or intermediate physiological dormancy and have germination inhibited in the presence of light (Chapter 4). Germination of these species can be induced with gibberellic acid and thus, propagation from seed in a commercially viable timeframe of three months (Chapter 1) is possible. Dormancy of seeds within an indehiscent drupe is more complex as endocarp restriction interacts with dormancy alleviation to delay germination (Chapter 4). Methods of germinating drupaceous Ericaceae seeds are available (Dixon *et al.* 1995; Roche *et al.* 1997; Cochrane *et al.* 2002; Turner *et al.* 2009; Chapter 4) however, the requirement for alleviation of interacting dormancy mechanisms makes these methods time consuming (up to 540 days). Therefore, currently available methods are not useful to industry as they are time and labour intensive while producing low germination. As such, members of the Australian Ericaceae are not widely used in horticulture and restoration (McLean *et al.* 1994; Hancock, 2014). This study addressed this issue by exploring methods to propagate Ericaceae within a commercially viable timeframe, defined here as 3 months (see Chapter 1).

Turner *et al.* (2009) demonstrated the effectiveness of oxygen enrichment and endocarp removal on germination in *Astroloma xerophyllum*. Endocarp removal was shown to increase germination of *Astroloma xerophyllum*, *Astroloma serratifolium* and *Croninia kingiana* (Chapter 4) and the use of oxygen enrichment as a germination stimulant has not been explored within other Ericaceae. Nitric oxide (NO) and hydrogen peroxide (H₂O₂) are naturally occurring compounds that affect dormancy and germination in a range of species (Nitric oxide: *Vaccinium myrtillus*; Giba *et al.* 1995, *Mallus domestica* Borkh; Keeley and Fotheringham, 1997, *Arabidopsis thaliana*; Batak *et al.* 2002, *Emmenanthe penduliflora*; Renata and Agnieszka, 2006. Hydrogen peroxide: *Arabidopsis thaliana*; Lui *et al.* 2010, *Amaranthus retroflexus*; Lui *et al.* 2011, *Helianthus annuus*; Lui *et al.* 2011). The use of nitric oxide and hydrogen peroxide in Australian Ericaceae has not been documented but could break dormancy, stimulate germination and increase the rate of water uptake of intact endocarps.

Owing to a lack of commercially viable germination protocols, tip cuttings have traditionally been used to provide Ericaceae tube stock for restoration (McLean *et al.* 1994). While propagation from seed is the best source material for restoration outcomes (Turner and Merritt, 2009; Hancock, 2014), tip cuttings provide a method for the propagation of difficult-to-germinate species. The ability of Ericaceae to strike from cuttings is variable (Dempster, 2017 Pers. Comm.) with some commercial growers reporting strike rates as low as 10% (McLean, 1994). As such, the potential of cuttings to produce material for translocation requires further investigation. The Wildflower Society's Northern Suburbs Branch has established *Acrotriche cordata*, *Astroloma sp* and *Brachyloma preissii* from cuttings, providing a means for the present study to assess the health of cuttings taken from wild individuals and those established in the nursery.

Restoration of SWWA Ericaceae stands to benefit from the production of on demand methods for the germination and propagation of species. This study aimed to determine the effects of oxygen, nitric oxide and hydrogen peroxide on the germination of 6 drupaceous Ericaceae and also assesses the ability of *Acrotriche cordata*, *Astroloma sp*, *Brachyloma preissii*, *Leucopogon polymorphus* and *Lysinema pentapetalum* to produce roots from cuttings.

5.2 Methods

General methods

Study species, collection sites and general methods were conducted as per Chapter 2. Stratification temperatures were selected as per Chapter 4.

Germination experiments

Experiment 1: Effect of oxygen enrichment on germination from intact endocarps

Intact endocarps were soaked in solutions of DI water, 1g^l⁻¹ gibberellic acid, 1: 10 v/v smoke water or a combination of gibberellic acid and smoke water for 48 hours before being surface sterilized (Turner *et al.* 2009). To ensure endocarp exposure to the atmosphere, the lid of each Petri dish was penetrated with nine >5mm holes using a soldering iron. Petri dishes were sealed with parafilm and placed into zip lock polyethylene bags flooded with industrial grade 100% oxygen or ambient air and

incubated in darkness at 21°C. Each bag was re-flooded with O₂ or air weekly and germination was monitored weekly for 12 weeks.

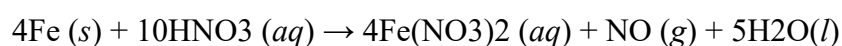
Experiment 2: Effect of nitric oxide treatment on germination from intact endocarps

Intact endocarps were placed into replicate Petri dishes containing filter paper moistened with DI water, 1g^l⁻¹ gibberellic acid or 1:10v/v smoke water and exposed to nitric oxide gas in a donor dish method adapted from Bethke *et al.* (2006a) (Figure 5.1). Petri dishes containing endocarps were placed within a sealable 3.5L polyethylene box and a glass Petri dish containing 1g of iron filings was placed in the corner of the box below a 9mm hole. The contents was flushed with pure (99%) nitrogen gas (N). A 400mg^l⁻¹ concentration of nitric oxide was produced by introducing 5ml of a 6M solution of nitric acid to the Petri dish containing 1g of iron filings and the box sealed and left in a fume hood for 24 hours. Endocarps were then surface sterilized and placed into replicate Petri dishes. Petri dishes were sealed with parafilm and transferred to an incubator set at 21°C. Treatment combinations and raw data are given in Appendix 4.

Experiment 2.1: Effect of nitric oxide treatment versus surface sterilization

Endocarps of *Astroloma xerophyllum* were prepared as per *Experiment 2* in a parallel experiment designed to test the effect of the nitric oxide treatment on fungi. Two treatment groups and a control were imbibed in deionised water over 24 hours. Treatment groups were imbibed in a nitric oxide rich environment. One treatment was subjected to surface sterilization as described in Chapter 2 after 24 hours imbibition while the other was incubated after nitric oxide treatment with no surface sterilization. The control group was not subjected to nitric oxide treatment or surface sterilization. Both treatments were incubated at 21°C in darkness and monitored qualitatively weekly for the presence of fungi over 6 weeks.

The concentration of nitric oxide produced was determined using the equation:



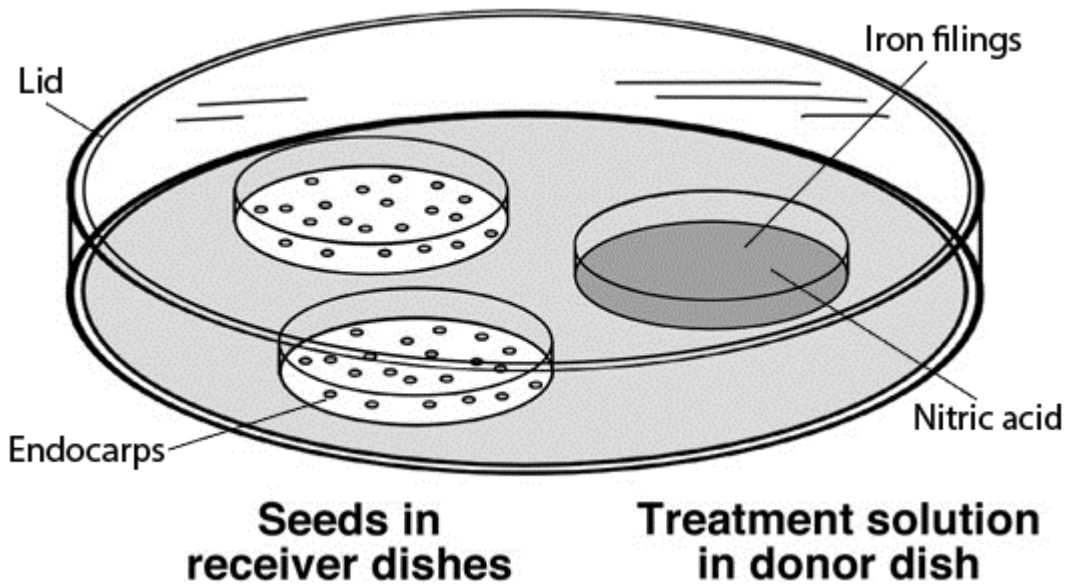


Figure 5.1 Donor dish method of exposing endocarps/seeds to generated gases.

Experiment 3: Effect of nitric oxide treatment on moisture content

Intact endocarps of *Astroloma xerophyllum*, *Astroloma serratifolium*, *Conostephium minus*, *Conostephium pendulum*, *Croninia kingiana* and *Leucopogon polymorphus* were used to determine the effect nitric oxide on the rate of imbibition (Figure 5.3). Three replicate Petri dishes containing 0.3g of fresh (< 3 month old) endocarps were placed inside the donor dish set up (Figure 5.1) and allowed to imbibe deionised water for 24 hours. Endocarps were weighted before and after imbibition. Moisture content of dry, 24 hour imbibed and 24 hour nitric oxide imbibed endocarps was determined gravimetrically on an oven dry mass (DW) basis using the equation:

$$\% \text{ increase in moisture content} = [(Wf - Wd) \div Wd] \times 100$$

Where Wf and Wd are the masses of fresh and dried seeds, respectively.

Experiment 4: Effect of imbibition in hydrogen peroxide on germination from intact endocarps

Intact endocarps were placed onto filter paper moistened with a 1% solution of hydrogen peroxide and imbibed for 24 hours before being surface sterilized. Smoke water and gibberellic acid was added to Petri dishes to test the combined effect of these germination stimulants with hydrogen peroxide imbibed endocarps. Treatment combinations and raw data are given in Appendix 5.

Experiment 5: Embryo growth of pre-germinating seeds

Where possible endocarps were removed from oxygen enrichment trials (when endocarps split after the conclusion of experiments) and seeds extracted to examine embryo growth. Where at least 10 pre-germinating seeds could be obtained the embryo to seed ratio was calculated as previously described and analysed to determine if embryos grow prior to germination.

Experiment 6: Success of propagation from cuttings

Individuals of *Acrotriche cordata* (Labill.) R.Br and *Brachyloma preissii* Sond at the Wildflower Society's northern suburbs branch nursery have previously had cuttings taken from them. This process was replicated for these plants as well as wild specimens of *Leucopogon polymorphus* and *Lysinema pentapetalum*. Fresh growth, noticeable by its soft texture and light green colouring, was cut from established plants of *Acrotriche cordata* and *Brachyloma preissii* and a wild specimen of *Leucopogon polymorphus*. Material taken from *Lysinema pentapetalum* did not share the characteristics of fresh growth present in *Acrotriche cordata*, *Brachyloma preissii* and *Leucopogon polymorphus*. Leaf material was stripped from the bottom 2cm of the stem. Each cutting was dipped in a solution of Clonex Purple containing 3g/L of the rooting hormone indole butyric acid (Figure 5.2F). Cuttings were potted (Figure 5.2D) and sprayed with a 5g/L solution of Mancozeb fungicide before being thoroughly watered and capped with a bottle (Figure 5.2E). The status of cuttings was assessed on the 17/11/2016 and again on the 17/04/2017. Cuttings were visually inspected and considered healthy if the leaves were still green and turgid and no rotting was evident on stems. The cuttings were then removed and scored for the presence of roots (Table 5.1).

Data analysis

Germination data were analysed using final germination percentages for each treatment and the t_1 (time until first germination) and t_{50} values (time until 50% of final germination). Where stated germination is presented as a percentage of the initial percentage of endocarps with at least one seed (Table 3.2) and is otherwise expressed as total germination. Graphs were generated using sigma plot V11. All statistics were calculated using SPSS 22. ANOVA and a post-hoc Tukey's test was used to determine the degree of statistical significance between treatment effects ($p < 0.05$). Normality of variables was calculated with the Shapiro-Wilks test ($p > 0.05$) and homogeneity of variances determined through the Levene test ($p > 0.05$).



Figure 5.2 Select features of the method used to propagate Ericaceae from cuttings. A, *Brachyloma preissii* established in 2009; B, *Acrotriche cordata* established in 2006; C, New growth cuttings taken from *Brachyloma preissii*; D, Cuttings of *Acrotriche cordata* and *Brachyloma preissii* after being stripped, dipped in root gel and potted; E, plastic soft drink bottles were used to ensure cuttings were kept moist until roots begin to grow; F, Clonex – purple rooting gel used for all cuttings.

5.3 Results

Experiment 1: Effect of oxygen enrichment on germination from intact endocarps

The use of oxygen in combination with gibberellic acid increased germination in intact endocarps of *Astroloma xerophyllum*, *Croninia kingiana* and *Leucopogon polymorphus* (Table 5.3). While germination of *Astroloma xerophyllum* and *Croninia kingiana* was low that of *Leucopogon polymorphus* was above 50% and differed significantly from non-oxygenated controls ($p < 0.05$). Treatments including gibberellic acid significantly increased germination ($p < 0.05$) of *Astroloma xerophyllum* and *Leucopogon polymorphus* but not *Croninia kingiana* (Table 5.3). Treatments excluding gibberellic acid did not produce germination.

Maximum germination for *Leucopogon polymorphus* was achieved with gibberellic acid alone ($66.46 \pm 4.51\%$) and the presence of smoke water produced a slightly lower response ($61.35 \pm 1.4\%$). Time until first germination was longer in the presence of smoke water however, time until 50% germination was shorter. *Astroloma xerophyllum* and *Croninia kingiana* had a greater total germination in the presence of smoke water and the time until first germination was reduced in the presence of smoke water for both species.

Experiments were left to run after being concluded at three months. No further germination was noted for endocarps that had not previously split, however multiple seeds continued to germinate from endocarps of *Croninia kingiana* that had already split. Seeds within split endocarps were extracted and used to examine embryo growth (*Experiment 5*). After 120 days of incubation $1.33 \pm 0.12\%$ of *Conostephium minus* endocarps germinated in oxygen + gibberellic acid + smoke water, but not in any other treatment. *Conostephium minus* seeds that germinated had observably larger embryos than those of fresh seed (Table 2.3). No further germination was recorded for any other species after the conclusion of *experiment 1*.

Experiment 2: Effect of nitric oxide treatment on germination from intact endocarps

Treatment with nitric oxide (Appendix 4) did not increase germination. Once experiments had been concluded it was discovered that 4 endocarps ($< 5\%$) of *Astroloma xerophyllum* split upon drying to release seeds.

Experiment 2.1: Effect of nitric oxide treatment versus surface sterilization

After 6 weeks incubation control groups of *Astroloma xerophyllum* had succumbed heavily to fungi. Those treated with nitric oxide or surface sterilized showed no evidence of fungi after 6 weeks.

Experiment 3: Effect of nitric oxide treatment on moisture content

Imbibition in DI water within a nitric oxide enriched atmosphere significantly (ANOVA, $\alpha = 0.05$) increased imbibition of intact endocarps of study species, excluding *Leucopogon polymorphus*, over 24 hours (Figure 5.3).

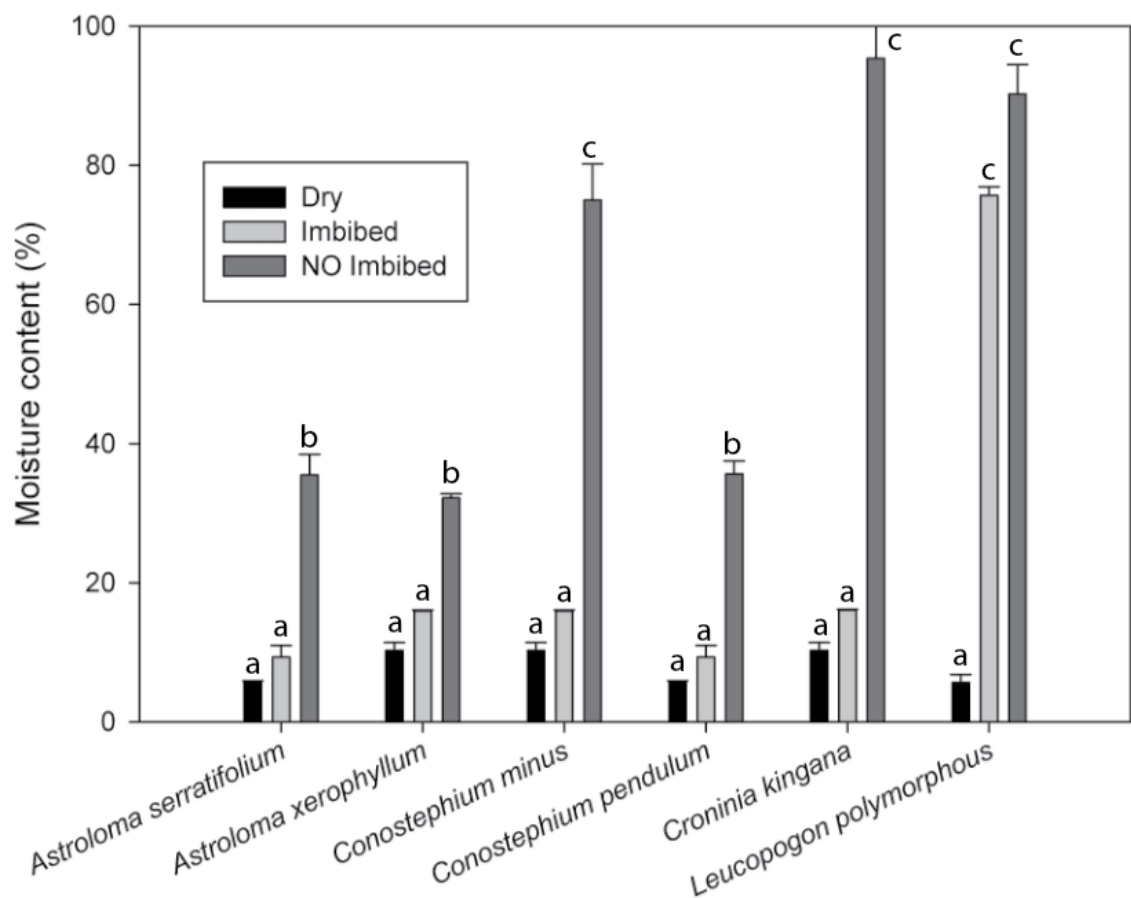


Figure 5.3 Moisture content of 3 month old endocarps (\pm SE) before imbibition and after 24hrs imbibition with deionised water or deionised water and nitric oxide. Treatments with the same letter are not significantly different (ANOVA, $p < 0.05$).

Experiment 4: Effect of imbibition in hydrogen peroxide on germination from intact endocarps

Treatment with 1: 100v/v hydrogen peroxide (Appendix 5) failed to increase germination regardless of treatment with cold stratification, smoke water or gibberellic acid.

Experiment 5: Embryo growth of pre-germinating seeds of Croninia kingiana

Embryo growth was confirmed for *Croninia kingiana* and concluded to be significant when comparing fresh (Table 3.2) and germinating seeds (Table 5.1; Figure 5.4).



Figure 5.4 Comparison of germinating (top) and non-germinating (bottom) seeds of *Croninia kingiana* taken from oxygen enrichment trials. Seeds were dissected to reveal embryos before being stained with 1% tetrazolium to show embryos more clearly. Line=1mm.

Table 5.1 Embryo to seed ratios calculated for fresh seed and pre-germinating *Croninia kingiana* seed. Seed and embryo length of intact endocarps imbibed for 24 hours was determined as per Table 3.2. Seed and embryo length of seed taken from O₂ trials was determined using 10 seeds. Numbers in a column followed by the same letter are not significantly different ($p>0.05$).

Treatment	Seed length (<i>S</i>) (mm) (mean ±SE)	Embryo length (<i>E</i>) (mm) (mean ±SE)	<i>E/S</i>
Seeds extracted from intact endocarps	3.87±0.09 _a	0.96±0.01 _a	0.25±0.01 _a
Seeds extracted from O ₂ enrichment trials	3.91±0.02 _a	3.56±0.21 _b	0.91±0.06 _b

Experiment 6: Success of propagation from cuttings

After 6 months cuttings of *Leucopogon polymorphus* (Figure 5.5A), *Brachyloma preissii* (Figure 5.5C) and *Astroloma sp* (Figure 5.5D) were viable. *Lysinema pentapetalum* (Figure 5.5A) and *Leucopogon polymorphus* (Figure 5.5B) had not produced roots. *Brachyloma preissii* cuttings were seen to have some roots although they were not present on all cuttings and were small. *Lysinema pentapetalum* cuttings had begun to rot at the base and leaves had withered while those of *Leucopogon polymorphus* remained green with no evidence of stem rotting. Cuttings of *Acrotriche cordata* (Figure 5.5E) were the most successful with all cuttings planted in November (2016) having roots in April (2017). Numerous cuttings of *Acrotriche cordata* taken on 28/02/2015 had been successfully propagated and were doing well on 13/04/2017 (Figure 5.5F).

Table 5.2 Assessment of Ericaceae cuttings after 6 months. Cuttings were determined to be viable if stems had not begun to rot and if leaves were still green.

Species	Cuttings viable ¹	Roots present
<i>Acrotriche cordata</i>	Yes	Yes
<i>Astroloma sp</i>	Yes	Yes
<i>Brachyloma preissii</i>	Yes	Yes/No
<i>Leucopogon polymorphus</i>	Yes	No
<i>Lysinema pentapetalum</i>	No	No



Figure 5.5 Assessment of Ericaceae cuttings. A, *Lysinema pentapetalum* (6 months old); B, *Leucopogon polymorphus* (6 months old); C, *Brachyloma preissii* (6 months old); D, *Astroloma sp* (6 months old); E, *Acrotriche cordata* (6 months old); F, *Acrotriche cordata* (22 months old).

Table 5.3 Germination (% of endocarps with at least one seed), time until first germination (t_1) and time until 50% germination (t_{50}) of pre-treated Ericaceae seed incubated in an oxygen enriched atmosphere. Whole endocarps were incubated in total darkness at 21°C, except for when germination was scored (weekly) over 12 weeks. Significant $\alpha = 0.05$, NA = not assessed. Germination was not produced in ambient air treatments (data not shown).

Species	Treatment	G (%)	Sig	t_1 (days)	t_{50} (days)
<i>Astroloma xerophyllum</i>	Control	0	-	NA	NA
	SW	0	-	NA	NA
	GA	4.97 ± 0.39	0.017	71 ± 1.04	NA
	SW + GA	8.71 ± 0.39	0.001	68 ± 0.34	NA
<i>Astroloma serratifolium</i>	Control	0.0	NA	NA	NA
	SW	0.0	NA	NA	NA
	GA	0.0	NA	NA	NA
	SW + GA	0.0	NA	NA	NA
<i>Conostephium pendulum</i>	Control	0.0	NA	NA	NA
	SW	0.0	NA	NA	NA
	GA	0.0	NA	NA	NA
	SW + GA	0.0	NA	NA	NA
<i>Conostephium minus</i>	Control	0.0	NA	NA	NA
	SW	0.0	NA	NA	NA
	GA	0.0	NA	NA	NA
	SW + GA	0.0	NA	NA	NA
<i>Croninia kingiana</i>	Control	0	-	NA	NA
	SW	0	-	NA	NA
	GA	7.94 ± 2.51	0.061	59 ± 2.13	NA
	SW + GA	15.87 ± 1.25	0.054	42 ± 0.06	NA
<i>Leucopogon polymorphus</i>	Control	0	-	NA	NA
	SW	0	-	NA	NA
	GA	66.46 ± 4.51	0.001	19 ± 0.88	35 ± 0.67
	SW + GA	61.35 ± 1.4	0.002	21 ± 0.57	32 ± 1.73

5.4 Discussion

In an attempt to produce germination and propagate study taxa the present set of experiments assessed the effect of oxygen, nitric oxide and hydrogen peroxide on germination from intact endocarps and explored the ability of five Ericaceae to be propagated from tip cuttings.

Incubating endocarps in an oxygen enriched atmosphere provides a viable means for producing on demand germination for some taxa of Ericaceae however, gibberellic acid was required to produce germination in *Experiment 1*. Endocarps of *Astroloma xerophyllum* stratified at alternating temperatures have germination stimulated by gibberellic acid (Turner *et al.* 2009) and at constant temperature gibberellic acid was not able to produce germination in drupaceous study taxa (Chapter 4). Alternating temperatures often terminate dormancy by turning off abscisic acid synthesis and reducing abscisic acid signalling (Huarte *et al.* 2014), but do not stimulate gibberellic acid synthesis or signalling (Roberto *et al.* 2014). This explains why gibberellic acid is able to produce germination in endocarps incubated at alternating temperatures but not constant temperature. Incubating seeds in an oxygen enriched atmosphere increased germination at single temperatures in the presence of gibberellic acid, indicating that oxygen enrichment removes the requirement for a reduction in abscisic acid via alternating temperature. Thus, it is likely that oxygen enrichment increased seed metabolism, facilitating a reduction in endogenous abscisic acid and gibberellic acid which resulted in the alleviation of dormancy. A lack of germination in treatments lacking gibberellic acid suggests that endogenous gibberellic acid was not synthesised and therefore, required exogenously for germination to take place (Table 5.3).

A possible explanation for why oxygen treatments failed to produce germination in *Astroloma serratifolium*, *Conostephium minus* and *Conostephium pendulum* is the presence of observably thicker endocarp walls (Chapter 3). Restriction of oxygen by seed-covering structures has been proposed as a barrier to germination for several species (Turner *et al.* 2009), including *Acer pseudoplatanus* L. (Dungely & Pinfield, 1980) and *Beta vulgaris* L. (Coumans, Côme & Gaspar, 1976). Ericaceae with relatively thick endocarps may require a longer period of incubation at increased O₂, or a period of weathering to degrade endocarps and leach oxygen binding compounds. *Eremophila maculata* and *Eremophila resinosa*, two Australian species that produce fruit similar to drupes of the Ericaceae, contain high levels of aromatic glycosides and germinate readily

after a period of soil storage (Richmond and Ghisalberti, 1994). Drupaceous Ericaceae respond to prolonged soil storage in a similar manner to *Eremophila*, and the factor may be the result of removing oxygen binding compounds and/or breakdown of endocarp. Future research employing similar methods to those of Richmond and Ghisalberti, (1994) could determine if there are inhibitory compounds within the fruit of drupaceous Ericaceae, and whether thick endocarp walls correlate with a greater quantity of inhibitory compounds.

Morphological dormancy has been observed in Australian Ericaceae that produce drupaceous fruits (Ooi *et al.* 2006; Turner *et al.* 2009). Observations in seed extraction trials (Chapter 4; Appendix 3) corroborate this as a degree of embryo growth could be seen prior to germination in *Astroloma serratifolium*, *Astroloma xerophyllum*, *Conostephium minus*, *Conostephium pendulum* and *Croninia kingiana*. Substantial embryo growth was confirmed for seeds of *Croninia kingiana* taken from oxygen enrichment trials (Table 5.1; Figure 5.4) and this confirms the presence of morphological dormancy in this species. The combination of prior research (Ooi *et al.* 2006; Turner *et al.* 2009), confirmation of embryo growth in *Croninia kingiana* and observations of embryo growth in Chapter 4 provides evidence for morphological dormancy in drupaceous taxa of Australian Ericaceae. Therefore, the likely dormancy class in drupaceous Ericaceae in Australia is morphological, with certain species also possessing physiological dormancy.

Nitric oxide and Hydrogen peroxide

Application of nitric oxide or hydrogen peroxide had no effect on germination. Nitric oxide mediates abscisic acid catabolism (Liu *et al.* 2009) which is required to decrease levels of abscisic acid and this has been shown to play a crucial role in dormancy break of *Arabidopsis* (Batak *et al.* 2002, Bethke *et al.* 2004, 2006a, b), Barley (Bethke *et al.* 2004) and *Lactuca* (Beligni and lamattina, 2000). Gibberellic acid and abscisic acid are both under the regulation of hydrogen peroxide in *Arabidopsis thaliana* (L.) Heynh (Liu *et al.* 2010) and *Amaranthus retroflexus* L (Liu *et al.* 2011). A marked increase in super oxide anions and hydrogen peroxide accumulation was noted for dormant *Helianthus annuus* L. seeds treated with hydrogen cyanide gas (Oracz *et al.* 2009). The role of endogenously produced hydrogen peroxide (Chance *et al.* 1973; Puntarulo, Sánchez and Boveris, 1988; Puntarulo *et al.* 1991; Ma, Wan and Shen, 2007) and nitric oxide (Beligni and

lamattina, 2000; Batak *et al.* 2002; Bethke *et al.* 2004; Liu *et al.* 2009) in dormancy break and germination of seeds is well documented. Their exogenous application may not break dormancy or stimulate germination of study taxa due to a lack of required mechanisms that proceed a marked increase of endogenous hydrogen peroxide and nitric oxide. It is also possible that nitric oxide and hydrogen peroxide failed to produce germination in study taxa due to anoxia within locules which restricted dormancy alleviation and germination. The methods and concentrations used may have been too high or low and an examination of concentrations, treatment times and methods of application could give insight into the roles in dormancy and germination in Ericaceae.

The methods used here for application of nitric oxide to intact endocarps had a significant effect on total imbibition over 24 hours (Figure 5.3). The significant increase in the rate of imbibition may have resulted in the splitting of <5% of *Astroloma xerophyllum* endocarps observed post-conclusion of the experiment. This highlights the potential of nitric oxide in germination studies of species with intractable dormancy and treatment with nitric oxide may provide an avenue for alleviation of physical dormancy in species with a water impermeable seed coat. The mechanism by which nitric oxide mediated an increase in total imbibition of intact fruits is yet unknown. The role of nitric oxide in plants is better understood and provides potential explanation for the observed affect on water uptake. Root-nitric oxide is known to have a key regulatory role in the humic substance (HS)- mediated promoting action of plant development (Mora *et al.* 2015). HS action is known to have a direct affect on water uptake via root systems (See: Mora *et al.*, 2015 Figure 15.1). Studies have shown that hormonal-related events observed in roots treated with different HS types could be triggered by HS-mediated enchantment of root NO production (Mora *et al.*, 2015). While the observed increase in water uptake of intact fruits occurred in the absence of an organic layer, it is possible that the interaction between nitric oxide and plant hormones caused the increased rate of imbibition.

Fungal attack in nitric oxide treatments was significantly reduced. Treatments with nitric oxide, in the absence of sterilization techniques, did not suffer any fungal attack while control groups lacking nitric oxide treatment or surface sterilization succumbed heavily to fungi. The mode of action for sterilization by nitric oxide demonstrated here is unknown. Possible explanations are that increased nitric oxide reduced ambient oxygen to levels that effectively surface sterilized endocarps. It is also possible that the increased rate of imbibition observed in endocarps also occurred in fungi. The resulting expansion may have ruptured cell walls causing cell death. The effect on the rate of imbibition and the observed sterilization ability of the nitric oxide application used here provide further evidence for the potential of nitric oxide as a pre-treatment for seeds.

The method of nitric oxide application employed in this study enabled pre-treatment of a large quantity of seed and, compared to traditional sterilization techniques was more efficient. Once a better understanding of the impact of this method on seed viability and germination is established it may prove successful as a pre-treatment to accelerate water uptake and reduce fungal infection.

Cuttings

Propagation of Ericaceae from cuttings is possible and provides a viable method for producing tube stock for translocation in rehabilitation efforts. *Lysinema pentapetalum* was the only species assessed that did not produce at least some roots after 6 months. This result puts emphasis on the need to use fresh growth as cutting material for propagation as *Lysinema pentapetalum* was the only species for which fresh growth material could not be acquired. The time period of establishment for material assessed in this study was at least 6 months, with *Acrotriche cordata* and *Brachyloma preissii* having the most success in this period. *Acrotriche cordata* cuttings established on 28 February 2015 were relatively small approximately 26 months later however, roots were well developed (Figure 4.4F). Therefore, the methods of propagating plants from cuttings demonstrated here were not able to produce tube stock in a commercially viable time-frame as previously defined. The application of mycorrhizal fungi to soils is likely to increase strike rates and the health of cuttings (McLean *et al.* 1994). The results of this chapter indicate that propagation through cuttings is a viable method that can be applied within the Ericaceae although the time period for establishment is long. A study exploring various rooting hormones, soil inoculation techniques, soil types and plant material could reduce the time until cuttings are able to be translocated.

5.5 Conclusions

Dormancy within drupaceous Ericaceae is difficult to alleviate due to interactions between the endocarp and dormancy. Incubating intact endocarps in oxygen enriched atmospheres provides a viable means to germinate seeds of *Astroloma xerophyllum*, *Croninia kingiana* and *Leucopogon polymorphus* from endocarps within 3 months. The positive germination response of these species supports the idea that endocarps restrict germination or maintain dormancy by creating an anoxic environment within locules. Hydrogen peroxide failed to produce germination from intact endocarps. Nitric oxide also failed to produce germination however, the rate of imbibition was significantly increased after 24 hours exposure to a nitric oxide rich atmosphere. Therefore, the use of nitric oxide as a pre-treatment should be subject to further investigation with a focus on various application methods and concentrations.

Propagation of Ericaceae from fresh growth tip cuttings was successful however, the presence of roots was variable 6 months after cuttings were potted. Observations of 22 month old cuttings suggest that the time period for establishment is long however, further research investigating different methods may reduce the time period until plants can be translocated. Cuttings provide a viable way forward for the propagation of specific species although efforts should continue to focus on increasing levels of germination. Propagation from seed will always be a better source material for restoration efforts and the results of oxygen enrichment trials in the present study suggest that dormancy can be alleviated in a timeframe suitable to produce ‘on demand’ germination required for industry.

The results of this chapter suggest that by moving away from the replication of field conditions in germination protocols, it is possible to shorten the time until germination for drupaceous Ericaceae. Future research should consider not only oxygen enrichment trials but various applications of chemicals shown to have a role in dormancy and germination, but not necessarily a role in *in situ* germination processes.

Chapter 6

Conclusions

The aim of this study was to investigate why the Ericaceae in the South-west of Western Australia are an intractable group to propagate from seed. To explore this problem three questions were addressed in a series of experiments designed to document fruit and seed characteristics (Chapter 3), determine dormancy class and identify germination restricting factors (Chapter 4), and investigate methods for propagating plants over a three month period (Chapter 5).

Dormancy classification and the restriction of germination

Germination restricting factors were found to occur in all study taxa (Chapter 4). The capsular-fruited species examined possess physiological dormancy and have germination inhibited in the presence of constant light. Drupe-fruited species did not germinate under any of the conditions tested at standard atmospheric conditions however, seeds germinated from intact endocarps after a period in an environment of elevated oxygen or after extraction from the endocarp. An immature embryo that was seen to grow during incubation suggests drupe-fruited Ericaceae possess morphophysiological dormancy. It is probable that physiological dormancy in these species manifests through hormonal control and as a mechanical or chemical block to germination that further regulates endogenous hormones (Baskin and Baskin. 2014). Germination at increased ambient oxygen (Chapter 5) or at standard levels of oxygen made freely available by the removal of the endocarp (Chapter 4) support the idea that physiological dormancy is imposed on seeds within the endocarp via anoxia within locules (Turner *et al.* 2009) although, the present study cannot conclude the mode of action.

Endocarps and seeds were found to take up water readily and further morphological analysis determined that water is available to embryos (Chapter 3). Thus, physical dormancy is not present within study taxa. However, observations of endocarp characteristics suggest a functional effect of endocarps in the maintenance of viability and dormancy (Chapter 3). To conclude whether endocarp morphology has a functional effect of viability and longevity within the soil-seed bank a study utilizing a large sample size is required. While the present study did not seek to determine the mechanism by which endocarps prevent germination, it is clear they have a significant role and previous work (Turner *et al.*, 2009) suggests a maintenance of anoxia within locules via oxygen

binding phenolics imposes dormancy (Richmond and Ghisalberti, 1994). Removing the endocarp or increasing ambient levels of oxygen increased germination and these methods provide avenues for the propagation of drupe-fruited taxa from seed. No other methods trialled in the present study were able to produce ‘on demand’ germination within the allotted three month period. Treatment with nitric oxide (Chapter 5) had a significant effect on the rate of imbibition of intact endocarps, suggesting a period of time under nitric oxide rich atmospheres could accelerate dormancy loss however, germination was not increased in the present study. This treatment may also prove successful in increasing the rate of imbibition of other species with and should be subjected to further investigation. .

The identification of light as a germination inhibitor in some capsular-fruited taxa (Chapter 4) suggests the intractability associated with propagation of these species is grounded in insufficient understanding of the requirements for germination, rather than complex dormancy mechanisms. By incubating seeds of *Lysinema pentapetalum* in darkness interrupted with brief light the present study was able to produce germination percentages approaching 100% in the presence of gibberellic acid (Chapter 4). This was not the case for *Andersonia heterophylla* and germination in light or darkness was below 25% for all treatment conditions, although greater in darkness. This suggests a deeper level of physiological dormancy and further study focusing on establishing temperature optima for this species is likely to produce greater germination percentages. A 10% solution of smoke water produced from commercially available Smokemaster Regen did not stimulate germination in small seeds of capsular-fruited study taxa (Chapter 4) and previous work suggests 10% v/v may be too high a concentration (Adkins and Peters, 2001). Adkins and Peters, (2001) found Regen 2000[®] to be toxic at higher concentrations, with germination decreasing at concentrations above 2%v/v. Extracted seeds of drupe-fruited taxa germinated at this concentration of smoke water however, the germination of small-seeded capsular-fruited Ericaceae was 0% in smoke water treatments. The findings here suggest that light and the source, concentration and method of application of smoke products (smoke water versus aerosol smoke) have a role in the intractability of germination of capsular-fruited Ericaceae. Thus, careful consideration must be applied on a species by species basis when selecting germination protocols.

Dormancy alleviation in drupaceous taxa is restricted by the endocarp and interactions between dormancy mechanisms makes drupe-fruited taxa difficult to propagate from seed. The minimal germination of drupaceous Ericaceae in response to the range of applied treatments (See Chapter 1) can be explained by chemical or mechanical manifestations of physiological dormancy, which limit the ability for morphophysiological dormancy to be alleviated. A period of soil storage (Dixon *et al.* 1995; Roche *et al.* 1997) or incubation at alternating temperatures (Ooi *et al.* 2006; Turner *et al.* 2009) alleviates dormancy in drupe-fruited Ericaceae. Presumably, soil storage and prolonged incubation facilitate weathering of the endocarp, while alternating temperature cycles alleviate morphophysiological dormancy and allow seeds to overcome further physiological blocks to germination.

Perhaps there exists a period of anoxia within locules where dormancy cannot begin to be alleviated. Weathering of the endocarp could leach oxygen binding compounds or reduce mechanical exclusion of oxygen, increasing oxygen availability to seeds. Pre-germinating drupes of Ericaceae have been noted to split a number of days prior to germination, indicating that embryo growth and seed expansion may further increase oxygen availability by rupturing the endocarp. Anoxia within locules that limits the dormancy alleviation and germination processes via reduced respiration would prolong the dormancy alleviation period, requiring a progressive reduction in anoxia coupled with the alleviation of morphophysiological dormancy before germination can take place. The interaction between limits upon respiration and the dormancy alleviation process likely drives the intractability associated with *ex situ* germination of drupe-fruited Ericaceae.

Once seeds were extracted from endocarps germination increased substantially for all species except those within *Conostephium*. Heat shock, scarification and endocarp cracking (Dixon and Nielssen, 1992) produced minimal germination for other drupaceous species within the Ericaceae, suggesting oxygen restriction via oxidation rather than the physical exclusion of oxygen via the endocarp. This explains why treatments designed to permeate the endocarp have failed (Dixon and Nielssen, 1992), and those involving soil storage (Dixon *et al.* 1995; Roche *et al.* 1997) or prolonged incubation (Ooi *et al.* 2006; Turner *et al.* 2009) are more successful. Increasing ambient oxygen produced germination from intact endocarps within three months, indicating a finite quantity of phenolics within endocarps that can only

bind standard ambient levels of oxygen. Observations of endocarp morphology (Chapter 3) and the lack of germination for *Astroloma serratifolium*, *Conostephium minus* and *Conostephium pendulum* under oxygen enrichment trials suggest that thicker endocarps possess a greater quantity of phenolics and were able to bind greater quantities of oxygen, continuing to restrict dormancy alleviation and germination over the treatment period.

Oxygen enrichment was highly successful in *Leucopogon polymorphus* however, the effect of an enriched oxygen atmosphere on Ericaceae seedlings is unknown. Furthermore, the oxygen enrichment methods used here produced low germination in *Astroloma xerophyllum* and *Croninia kingiana* and null germination in other study taxa. Future research on the growth and vigour of seedlings germinated in oxygen enriched atmosphere would provide a crucial answer as to whether oxygen enrichment is a viable treatment for propagation from endocarps. By reducing the quantity of oxygen used in enrichment trials it may be possible to reduce any negative effects associated with germination under these conditions. Combining treatments that speed the loss of oxygen binding compounds with an oxygen enriched atmosphere may make this treatment more successful in responsive taxa and produce germination in taxa that did not germinate in the present study.

The relationship between Australian Ericaceae, the fire-prone environment and climate change

The distinct dispersal and dormancy mechanisms present within Australian Ericaceae are directly related to the strategy of coping with fire. Capsular-fruited species are re-seeders and drupe-fruited species are re-sprouters (Roche *et al.* 1997). As such, capsular-fruited species have evolved the capacity to germinate within a single year, possessing non-deep and intermediate physiological dormancy that can be alleviated and reinstated over successive years. The ability to germinate in a single year is well suited to species that obligately regenerate from the soil seedbank post-fire as it ensures post-fire recruitment. Once physiological dormancy is alleviated by seasonal temperature, germination can be stimulated by products stored in the soil or the addition of new stimulants from fire, disturbance and seasonal change in temperature. In contrast, drupaceous species have the ability to re-sprout post-disturbance and there is not an obligate requirement for recruitment from the soil seedbank. As such, drupaceous Ericaceae have evolved a dormancy mechanism that requires a period of time within the soil before dormancy alleviation can begin to take place, allowing

germination over multiple years depending on the physical and climatic conditions *in situ*. This ensures that seeds do not germinate within a single year, but rather require multiple seasons to leach oxygen binding compounds and allow morphophysiological dormancy to be alleviated.

Germination of extracted seeds in control groups suggests that physiological dormancy is rapidly alleviated when oxygen becomes freely available. The exception is species within *Conostephium* which did not germinate under any treatments after seed extraction, indicating a deeper level of morphophysiological dormancy that was not alleviated in the present study. For seeds contained within an endocarp it is likely that anoxia limits respiration, reducing the rate of dormancy alleviation via catabolism and synthesis of endogenous hormones. To conclude the effect of anoxia on the rate of catabolism and synthesis of hormones further research is required to quantify the rate of change in hormones of seeds incubated at various levels of anoxia, and this could be concluded by applying the methods of Richmond and Ghisalberti, (1994) for quantifying abscisic acid. The mechanism by which endocarps restrict germination also requires further study. Exploring the germination of seeds at various levels of anoxia would determine if this is the mechanism of germination restriction. Quantification and analysis of phenolics contained within endocarps or seeds following the Folin-Ciocalteu method (Prodanov *et al.* 2013; Pantelić *et al.* 2016) would confirm if anoxia is imposed by oxidation, or if germination is otherwise limited chemically or mechanically.

Under natural conditions weathering would breakdown and leach oxygen binding compounds, making oxygen gradually more available and allowing dormancy alleviation to occur at a slower rate than that of extracted seeds. It is likely that seeds possess a shallow physiological dormancy that restricts germination until oxygen becomes available. Once oxygen is available and dormancy is alleviated seeds possess the growth potential to overcome mechanical constraints imposed by the endocarp. What is not clear is whether a significant decline in endocarp integrity is required before seeds are able to split the endocarp. What is clear is that weathering likely plays a crucial role in dormancy alleviation of drupe-fruited taxa. Conditions of weathering would determine the rate of loss of oxygen binding phenolics, which determine the timing and degree of respiration that allows germination or maintains dormancy. Therefore, the expected impacts of climate change on local weather

patterns in the South-west of Western Australia are likely to go beyond changes in stratification temperatures which effect dormancy alleviation. While a decline in rainfall will ultimately limit the period of stratification that result in dormancy alleviation, the requirement for weathering means a decline in rainfall will prolong the period prior to dormancy alleviation in drupe-fruited Ericaceae. The expected outcome is a prolonged dormancy period for these species, which may result in a reduction in the size of the soil seedbank and reduced seedling recruitment.

The Ericaceae in the South-west of Western Australia are a fascinating, albeit difficult group to work with. Their complex dormancy and germination mechanisms make them well suited to the harsh Australian conditions but limit their usefulness in horticulture and restoration. A lack of published morphological characteristics and interacting and complex dormancy mechanisms has facilitated the intractability associated with propagation of Ericaceae from seed. *Ex situ* germination of capsular-fruited Ericaceae was not found to be overly difficult in the present study. Germination in darkness after pre-treatment with gibberellic acid produced sufficient seedlings of *Andersonia heterophylla* and *Lysinema pentapetalum*. This suggests inadequate documentation of morphological characteristics has allowed capsular-fruited species to be associated with difficult-to-germinate drupe-fruited taxa.

To meet the requirements of restoration, germination studies of Australian Ericaceae must move away from replicating near-natural conditions

The complex dormancy and germination biology of Australian Ericaceae suggests that this group will always be difficult to propagate from seed. Interactions between morphological and physiological dormancy make germination under near natural conditions time consuming. Further investigation of treatments able to produce germination rapidly under altered ambient conditions will reduce the timeframe until germination however, these treatments tend to be labour intensive and require further study before they can be applied consistently. Alternatively to germination protocols, there exists avenues to be explored in an attempt to propagate intractable Ericaceae from seed without the need for prolonged treatments. Previous work has consistently demonstrated the effectiveness of soil storage. Through careful acquisition and management of topsoil it is possible for restored sites to produce a similar density and species richness of Ericaceae that existed in the pre-disturbed site (Rokich *et al.* 2000; Stevens *et al.* 2016). While methods of properly managing top-soil

are typically resource and labour intensive the benefits are extensive (Stevens *et al.* 2016). Genetic provenance of produced plants is identical to the pre-disturbed site as seeds were sourced *in situ*. The cost of managing top-soil is also mitigated by a reduction in the costs associated with the propagation and translocation of plants in restoration. Furthermore, the collection of fruit from the soil seed bank could provide material that has had anoxia and seed dormancy alleviated under natural conditions, requiring basic seed treatments prior to broadcasting seed.

While the present study began to unravel the complex seed biology of Australian Ericaceae, further research is required if this valuable group is to be conserved *in situ* and successfully propagated *ex situ*. Careful consideration of stratification conditions, light regimes and germination stimulants are likely to produce sufficient quantities of capsular-fruited Ericaceae from seed, allowing species to be sufficiently represented in *ex situ* collections. The mechanisms involved in the dormancy and germination of drupe-fruited taxa are much more complex however, avenues for their propagation are available. Fresh tip-cuttings are able to provide tube stock for restoration efforts however, the establishment time for material is great and requires further study before plants can be produced consistently. Seed material will always be the better option for restoration outcomes and focus should remain on establishing consistent germination protocols. The effects of seed extraction and oxygen enrichment should be considered for further investigation, with an emphasis on streamlining these methods to make them more efficient and able to be applied on the scale required for horticultural and restoration operations.

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Appendix 2 Total germination percentages (G) and germination times (t0 and t50) as a mean \pm SD.

Species	Treatment	G (%)	t0 (days)	t50 (days)
<i>Astroloma serratifolium</i>	Control (DI water, 21°C)	0.0	NA	NA
	Cold Stratification (4°C)	0.0	NA	NA
	Smoke (10%)	0.0	NA	NA
	Gibberellic acid (1000ppm)	0.0	NA	NA
<i>Astroloma xerophyllum</i>	Control (DI water, 21°C)	0.0	NA	NA
	Cold Stratification (4°C)	0.0	NA	NA
	Smoke (10%)	0.0	NA	NA
	Gibberellic acid (1000ppm)	0.0	NA	NA
<i>Conostephium minus</i>	Control (DI water, 21°C)	0.0	NA	NA
	Cold Stratification (4°C)	0.0	NA	NA
	Smoke (10%)	0.0	NA	NA
	Gibberellic acid (1000ppm)	0.0	NA	NA
<i>Conostephium pendulum</i>	Control (DI water, 21°C)	0.0	NA	NA
	Cold Stratification (4°C)	0.0	NA	NA
	Smoke (10%)	0.0	NA	NA
	Gibberellic acid (1000ppm)	0.0	NA	NA
<i>Croninia kingiana</i>	Control (DI water, 21°C)	0.0	NA	NA
	Cold Stratification (4°C)	0.0	NA	NA
	Smoke (10%)	0.0	NA	NA
	Gibberellic acid (1000ppm)	0.0	NA	NA
<i>Leucopogon polymorphus</i>	Control (DI water, 21°C)	0.0	NA	NA
	Cold Stratification (4°C)	0.0	NA	NA
	Smoke (10%)	0.0	NA	NA
	Gibberellic acid (1000ppm)	0.0	NA	NA

Cold stratification encompasses three time periods (30, 60 and 90 days). t0 is the time between the treatment and first germination; t50 is the time needed for germination percentages to reach 50% of total germination.



Appendix 3 *Conostephium pendulum* seed from seed extraction experiments (Chapter 3). Seeds did not germinate over the trial period (30 days) however embryos were seen to grow.

Appendix 4 Germination of intact endocarps treated with 400mg⁻¹ nitric oxide (NO)

Species	Treatment	G (%)	t0 (days)	t50 (days)
<i>Astroloma serratifolium</i>	Control	0.0 ± 0.0	NA	NA
	NOx	0.0 ± 0.0	NA	NA
	NOx + SW	0.0 ± 0.0	NA	NA
	NOx + GA	0.0 ± 0.0	NA	NA
<i>Astroloma xerophyllum</i>	Control	0.0 ± 0.0	NA	NA
	NOx	0.0 ± 0.0	NA	NA
	NOx + SW	0.0 ± 0.0	NA	NA
	NOx + GA	0.0 ± 0.0	NA	NA
<i>Conostephium minus</i>	Control	0.0 ± 0.0	NA	NA
	NOx	0.0 ± 0.0	NA	NA
	NOx + SW	0.0 ± 0.0	NA	NA
	NOx + GA	0.0 ± 0.0	NA	NA
<i>Conostephium pendulum</i>	Control	0.0 ± 0.0	NA	NA
	NOx	0.0 ± 0.0	NA	NA
	NOx + SW	0.0 ± 0.0	NA	NA
	NOx + GA	0.0 ± 0.0	NA	NA
<i>Croninia kingiana</i>	Control	0.0 ± 0.0	NA	NA
	NOx	0.0 ± 0.0	NA	NA
	NOx + SW	0.0 ± 0.0	NA	NA
	NOx + GA	0.0 ± 0.0	NA	NA
<i>Leucopogon polymorphus</i>	Control	0.0 ± 0.0	NA	NA
	NOx	0.0 ± 0.0	NA	NA
	NOx + SW	0.0 ± 0.0	NA	NA
	NOx + GA	0.0 ± 0.0	NA	NA

Pre-treatments were DI water (control), 1: 10 smoke water (SW), 1000p.p.m gibberellic acid (GA) or a combination of the two (SW + GA). Treatments ran for 52 weeks except for *Astroloma serratifolium* and *Conostephium minus* which were incubated for 26 weeks.

Appendix 5 Germination of endocarps treated with 1% hydrogen peroxide (H₂O₂) for 24 hours

Species	Treatment	G (%)	t0 (days)	t50 (days)
<i>Astroloma serratifolium</i>	Control	0.0 ± 0.0	NA	NA
	H ₂ O ₂	0.0 ± 0.0	NA	NA
	H ₂ O ₂ + GA	0.0 ± 0.0	NA	NA
	H ₂ O ₂ + SW	0.0 ± 0.0	NA	NA
<i>Astroloma xerophyllum</i>	Control	0.0 ± 0.0	NA	NA
	H ₂ O ₂	0.0 ± 0.0	NA	NA
	H ₂ O ₂ + GA	0.0 ± 0.0	NA	NA
	H ₂ O ₂ + SW	0.0 ± 0.0	NA	NA
<i>Conostephium minus</i>	Control	0.0 ± 0.0	NA	NA
	H ₂ O ₂	0.0 ± 0.0	NA	NA
	H ₂ O ₂ + GA	0.0 ± 0.0	NA	NA
	H ₂ O ₂ + SW	0.0 ± 0.0	NA	NA
<i>Conostephium pendulum</i>	Control	0.0 ± 0.0	NA	NA
	H ₂ O ₂	0.0 ± 0.0	NA	NA
	H ₂ O ₂ + GA	0.0 ± 0.0	NA	NA
	H ₂ O ₂ + SW	0.0 ± 0.0	NA	NA
<i>Croninia kingiana</i>	Control	0.0 ± 0.0	NA	NA
	H ₂ O ₂	0.0 ± 0.0	NA	NA
	H ₂ O ₂ + GA	0.0 ± 0.0	NA	NA
	H ₂ O ₂ + SW	0.0 ± 0.0	NA	NA
<i>Leucopogon polymorphus</i>	Control	0.0 ± 0.0	NA	NA
	H ₂ O ₂	0.0 ± 0.0	NA	NA
	H ₂ O ₂ + GA	0.0 ± 0.0	NA	NA
	H ₂ O ₂ + SW	0.0 ± 0.0	NA	NA

Pre-treatments were DI water (control), 1: 10 smoke water (SW), 1000p.p.m gibberellic acid (GA) or a combination of the two (SW + GA). Treatments ran for 52 weeks except for *Astroloma serratifolium* and *Conostephium minus* which were incubated for 26 weeks.