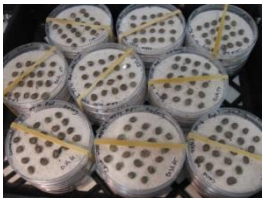
Ecology, seed dormancy and germination biology of *Persoonia longifolia* for use in land restoration and horticulture.

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This thesis is presented for the degree of Doctor of Philosophy

The University of Western Australia

School of Plant Biology

2016

Abstract/summary

Persoonia longifolia R. Br. is a common species found in the jarrah forest of Western Australia that has proven to be difficult to germinate and return to restored areas. This study aimed to better understand this species, by investigating its phenology in the natural environment, the factors affecting the dormancy and germination of seed, and factors affecting re-establishment on areas restored after bauxite mining.

The phenology study found that vegetative growth occurred during the summer months with flowering and fruiting occurring concurrently. Fruits mature from July to September and germination occurs in late winter/early spring from fruit that is at least 1 year old and with poor seedling survival in the natural bush (10%) during the first 12 months. Following fire, *P. longifolia* plants resprout prolifically in the next growing season, although there is very little fruit production in the first year following fire. Fruit is not produced until at least the second year, and then seed requires at least another year in the soil seed bank before germination commences (3 years post fire). A better understanding of the phenology will assist land managers, seed collectors and other stakeholders to make more informed decisions pertaining to the management of this species in its natural environment.

Investigations into the key mechanisms driving dormancy break and germination of *P. longifolia*, was undertaken through burial trials and laboratory germination trials. The dispersal unit was found to be water permeable and germination was greatest when the seed was extracted from the woody endocarp. Soil burial resulted in *in situ* germination of 41.6 % in forest soils and 63.7 % in nursery soils, after 36 months burial. *Ex situ* germination was cyclic and was greatest after 30 months burial (45.4% in nursery burials and 24.3% in field burials). Greatest germination occurred in the laboratory wet/dry trials when the summer season was long (20 weeks), had fluctuating temperatures (30/50 °C), with two long wet cycles (7 days) and winters at fluctuating 10/20 °C. Stratification trials showed that best germination occurred with 12 weeks warm (including 2 weeks wet) followed by 6 weeks cold stratification. Manipulation of the summer watering regime in a nursery trial, initially resulted in significantly more germination occurring in the treatment with four summer wet cycles than any other treatment (natural rainfall, no rainfall or two wet cycles). However, after the second summer there was no significant difference between natural rainfall or four summer wet

cycles and these two treatments resulted in significantly more germination than no rainfall or two wet cycles.

Initial investigations into the use of seeds on restored areas of the mine site found that <1 % of seeds buried or scattered emerged. However, if seeds were cued through burial in surrounding forest, retrieved and sown on restored areas emergence increased to 24 %. Significantly more seeds germinated when buried (14.6 %) compared to those scattered on the soil surface (2.7 %). Survival of seedlings planted at 2-3 weeks of age on to the restored area was initially less than seedlings planted at 12 months of age. However, 20 months after planting, there were no longer any significant differences in survival between both age classes and those seedlings planted when younger were significantly taller (29.0 $\pm$ 2.9 cm) than those that were planted at 12 months of age (4.7 $\pm$ 0.3 cm). Use of "onion bag" guards improved survival from 58.1 ± 4.0 % (no guard) to 70.8 ± 3.4 % (onion bag). While the use of shade cloth guards did not significantly improve survival, these did significantly increase mean plant height after 32 months growth (22 cm compared with 7.2 cm for no guard).

This 6 year study has significantly expanded our knowledge of the phenology and germination biology of *P. longifolia* and makes a significant contribution to the restoration approaches used for reassembling jarrah forest plant communities following disturbances. The various experiments undertaken here indicate how complex natural germination systems can be. Closely monitoring these environmental conditions provides direction for laboratory germination trials which can then be used to clearly identify those factors that break seed dormancy. Clearly, temperatures, moisture levels and the timing of these are all important for regulating seed dormancy for *P. longifolia* during the summer months.

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Acknowledgements

Six years of study and the preparation of a document, such as this, were not undertaken lightly and were not achieved by one person alone, particularly when that study is undertaken 220 km from the University campus. There are many people who have contributed along the way and my thanks go out to all of them.

First and foremost, I would like to thank my primary supervisor Dr Shane Turner who was always available when required, managed to review and return things back to me in an exceptionally timely manner, and who was able to assist with issues that arose from undertaking my study remotely. Your insights, encouragements, guidance and assistance were invaluable.

To my second supervisor, Dr John Koch, thank you for chauffeuring me around the mine site, discussions regarding the direction of my research, and continuing to review papers and thesis chapters whilst enjoying your retirement.

Thank you to the University of Western Australia for awarding me an APA/UWA Top Up scholarship which enabled me to focus on my study. Thanks also go to MERIWA for providing me with a partial scholarship through three of the 6 years of my study.

Thank you to Alcoa of Australia Limited for financial support which allowed this project to be undertaken. In addition I would like to thank all the personnel at Alcoa of Australia Limited (including Dr John Koch, Dr Matthew Dawes, Rod McGregor, Heidi Dougherty, Esther Cromer and Mike Muller and the nursery staff) for their support on the ground, including chauffeuring me around the mine site and assisting me at Marrinup Nursery.

Similar thanks goes to South32 Worsley Alumina (Worsley) for financial contributions to the Project and on site assistance. In particular, thanks go to Brad Stokes, Steve Vlahos, Trudy Worthington, Paul Bullock and Renae Kaciuba.

To the staff at Kings Park that made my life easier and those that assisted me with my experiments, thank you. Studying remotely resulted in some challenges that these people allowed me to overcome – thank you Annette Johnson, Anna Williams, Jessica

Stingemore, Arielle Fontaine, Clare Coultard and Susan Whiteley for looking after my experiments for me and assisting with administration issues.

Thank you to Adrian and Jacquie South who allowed me to access *Persoonia longifolia* plants on their property that had been burnt in a wildfire over a 3 year period.

Thank you to Rohan Sadler for your patience and your assistance with the statistical components of this thesis.

Finally, a big thank you to my family. To my long suffering husband Frank – I don't know how you do it but I do know I could not have done it without you. You provided me with some very practical solutions to some technical difficulties, you were my sounding board for many theories and ideas, and most of all you were there for me throughout the whole experience. To my children, Daniel and Jack who had to listen to many a discussion about *Persoonia longifolia* at the dinner table and at times were shuffled here and there in order for me to undertaken my study. I hope one day you understand the importance of education and why I was keen to continue to learn.

To my parents and parents-in-law, Des and Bev O'Connell and Steve and Jenny Chia, thank you for all the babysitting, field work assistance, PhD related discussions and "hotel accommodation" you provided!

If I have missed anyone it was not intentional and your input to my work was just as important as all of the people outlined above.

DECLARATION FOR THESES CONTAINING PUBLISHED WORK AND/OR WORK PREPARED FOR PUBLICATION

The examination of the thesis is an examination of the work of the student. The work must have been substantially conducted by the student during enrolment in the degree.

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The publications arising from this thesis are original work undertaken by the student (Kerryn Chia) with guidance from her two of her supervisors (Dr Shane Turner and Dr John Koch), with guidance in relation to the statistical component of the papers from Dr Rohan Sadler, and expert guidance into the presentation of the data as papers by Dr Carol Baskin.

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Signature: (Dr Shane Turner for Dr Carol Baskin)..... [redacted]

Statement of Candidate Contribution

This thesis contains published work and/or work prepared for publication, which has been co-authored. The bibliographical details of this work are outlined below.

One of the chapters from this thesis is published. The published document is included in Appendix 1 and the full citation is provided below.

Chapter Two

Chia KA, Koch JM, Sadler R and Turner SR (Chia et al., 2015) Developmental phenology of *Persoonia longifolia* (Proteaceae) and the impact of fire on these events. Australian Journal of Botany **63,** 415-425.

Contributions – KAC 50%, JMK 10%,, RS 10% , SRT 30%

Two of the chapters from this thesis have been submitted and accepted for publication and the current version of these papers can be found in Appendices 2 and 3.

Chapter Three

Chia, KA, Sadler, R, Turner, SR and Baskin, CC, (Chia et al., 2016b)

Identification of the seasonal conditions required for dormancy break of *Persoonia longifolia* (Proteaceae), a species with a woody indehiscent endocarp. Annals of Botany (in press).

Contributions – KAC 35%, RS 10%, SRT 20%, CCB 35%

Chapter Four

Chia, KA, Koch, JM, and Turner SR (Chia et al., 2016a) Re-establishing the mid-storey tree *Persoonia longifolia* (Proteaceae) in restored forest following bauxite mining in southern Western Australia. Ecological Research (in press)

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Contributions – KAC 60%, JMK 10%, SRT 30%

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Chapter One - An Introduction to a Peculiar Plant

Persoonia longifolia is a common and important component of the jarrah forest of south Western Australia and an investigation into understanding its phenology and germination biology is long overdue. Chapter One of this thesis, provides an introduction to this unique plant, its current economic uses and outlines the overall importance of this study. The aims of the research are also outlined and the study environment defined, all of which provides a detailed introduction to the overall Project and highlights the relevance and value of the research undertaken over the proceeding four chapters.

1.1 The plant and its habitat

Persoonia longifolia R.Br. is a small tree found in jarrah forests and jarrah/marri forests in the south west of Western Australia. It is a member of the Proteaceae family (subfamily Persoonioideae) (Weston, 1994) and is one of 98 species of *Persoonia*, all of which are Australian endemics. *Persoonia* species are found in most climatic zones right across the continent, though they are largely absent from arid regions. In Western Australia, there are 43 *Persoonia* species of which 42 are restricted to the south west between Exmouth and Esperance. One species, *Persoonia falcata*, occurs in the very northern region of the state (Department of Parks and Wildlife, 2015). The Proteaceae is a very ancient family and it is almost certain that the hard leaved plants associated with the family today (e.g. *Banksia* species) are derived from rainforest species. *Persoonioideae* is considered to be the most primitive subfamily within the Proteaceae (Wrigley and Fagg, 1989) consisting of just five genera (*Persoonia, Acidonia, Toronia Garnieria* and *Placospermum*) (Weston and Barker, 2006).

Persoonia longifolia, commonly known as snottygobbles (in Western Australia) or geebung (eastern states), is a small, erect lignotuberous tree between 1 and 5 m tall that is found only in sclerophyll woodland and forest of South West of Western Australia (WA) from Perth to Albany (Department of Parks and Wildlife, 2015, Weston, 1995) (Fig. 1.1). This region of WA experiences a Mediterranean climate with hot dry summers and cool wet winters. It is a common and very distinctive understorey tree species (Fig. 1.2a).

The flowers occur in racemes of up to 30 flowers and are relatively insignificant. They are actinomorphic with four tepals which are yellow in colour, and are free but cohere at the base (Fig. 1.2b). The yellow anthers are fused to the tepals (Bernhardt and Weston, 1996) and become a dried mustard colour as they age. The flowers have a slightly unpleasant body odour smell and are pollinated by both native and introduced bees (Fig. 1.2c) (Weston, 2003).

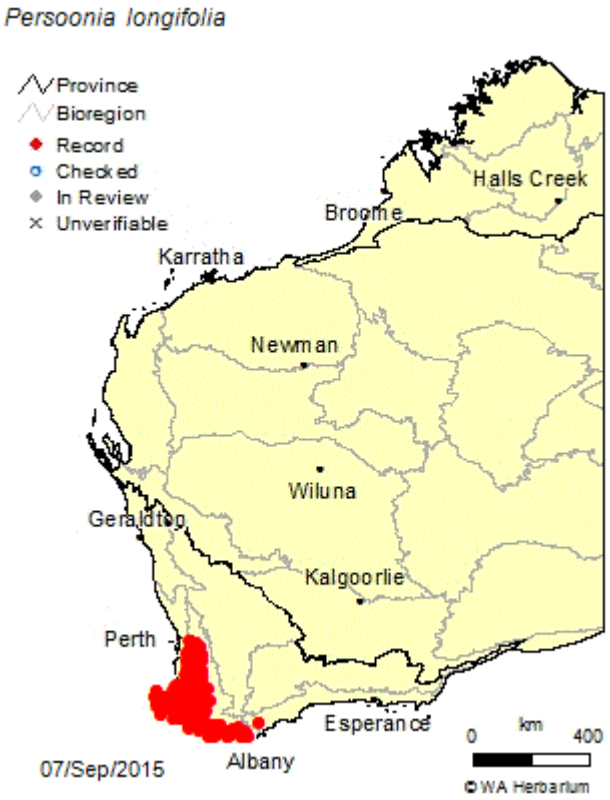

Figure 1.1: Distribution of *Persoonia longifolia.* (Department of Parks and Wildlife, 2015)

The natural dispersal unit produced by *Persoonia longifolia* is a fleshy drupe which is dropped directly onto the ground beneath the tree. It consists of a leathery skin on the fruit which is formed by the exocarp, a fleshy to pulpy middle part of the seed (the mesocarp) and an inner hard stone (endocarp) (Strohschen, 1986). The single crescent shaped embryo (occasionally two), has two cotyledons and is contained within a papery seed coat inside the endocarp. The drupe is consumed by animals and has been reported to be eaten by emus (Mullins et al., 2002), wallabies (J. Stingmore pers comm.) and bobtails (*Tiliqua rugosa*) (observed in the field).

Persoonia longifolia grows in sandy or lateritic soils with low nutrient levels and is highly distinguishable in the forest with its lime green long, leathery, falcate leaves (Fig. 1.2) and flaky bark (Fig. 1.2d). Unlike other members of the Proteaceae family it does not have proteoid roots (Weston, 2003) (a defining characteristic of the entire subfamily). It occurs in jarrah and/or marri forests or woodlands (Marchant et al., 1987) in the Darling range and less commonly on the Swan Coastal Plain.

Figure 1.2: *Persoonia longifolia. (a)* The tree. *(b)* Flowers and buds. *(c)* Pollination by bees. *(d)* Bark. *(e)* Fruits.

Persoonia longifolia grows in an environment that is regularly subjected to fires. Whilst it regularly produces large quantities of seed, it generally recovers from fire by resprouting from the lignotuberous root system or from epicormic buds which are protected by the thick corky bark and is therefore considered to be a resprouter (Grant and Koch, 2007, Weston, 1994, Weston, 2003). Natural germination events are rarely recorded (Abbott, 1984a).

1.2 The importance of Persoonia longifolia

The jarrah forest in the south west of WA is an area that is subject to mining for the production of bauxite. The abundance of *P. longifolia* in the pre-mining environment results in it being considered a priority species for return to restored areas following mining. Around the Dwellingup area of the jarrah forest, it occurs at densities of between 300 to 440 plants per hectare (Mullins et al., 2002, Norman and Koch, 2006). In the Boddington area, further east, the plants occur at lower densities of 185-220

plants per hectare in the natural jarrah forest (B. Stokes pers. comm.) and return to restored areas is negligible.

Persoonia longifolia has, so far, proven to be a difficult species to return to restoration site following mining of the jarrah forest (Norman and Koch, 2005b) and is defined by Alcoa as a recalcitrant species (one that is common in the forest but absent or found in low densities in restored mine sites) (Grant and Koch, 2007, Norman and Koch, 2005b). Mining companies have investigated the effect of direct return topsoil (top soil removed from an area to be mined and immediately placed on an area to be restored), stockpiled topsoil and broadcast seeds in order to return *P. longifolia* to restored areas. In all cases, the density of *P. longifolia* in restoration, is significantly less, around 43 plants per hectare in the Dwellingup area (Mullins et al., 2002), than that of the natural vegetation (~400 plants/ha). At Boddington *P. longifolia* is altogether absent from restored areas (B. Stokes pers. comm.).

Persoonia longifolia is also used by the cut flower trade as a filler species in floral arrangements (Weston, 2003). Whilst the flower is not significant, the foliage is often used and is also exported overseas (B. Long pers. comm.). Currently, foliage is wild sourced from the forest as propagation of this species has proven exceptionally difficult to date. In 1993, 798,558 (or 98%) of *P. longifolia* stems used in floral arrangements were picked from the jarrah forest (Orchard, 1995) placing significant pressure on natural populations.

The shape and size of the tree ensures that there is also potential for this species to be used in the native horticultural industry. Aesthetically it is very appealing with its graceful symmetry, textured bark, lime green weeping leaves and relatively small compact stature. It was first introduced into cultivation in the UK in 1850 but is rarely seen in Australian gardens today (Wrigley and Fagg, 1989). It is likely to be marketable as a water wise feature plant suitable for small courtyard or cottage style gardens. Once established, it is relatively hardy and would be an ideal garden species to cope with the current water restrictions facing Western Australia and the ever shrinking suburban back yard.

Persoonia longifolia is also considered an important plant to the Aboriginal community. The fruits of this species are edible and they were used as a food source for the

Aboriginal people. The common name, Geebung, is a derivative of the Aboriginal word, jibbong, used to describe the fruit of some species (Wrigley and Fagg, 1989). The bark was also used as a poultice for skin complaints and infused to make a tea for stomach upsets (from discussions with the Aboriginal community in Collie, WA).

1.3 The aims of this project

Given its relative abundance and importance to various economic activities, *P. longifolia* is a poorly understood species. The aim of this research program was to further extend the scientific knowledge surrounding *Persoonia longifolia* by:

- Examining the phenology of *P. longifolia* to better understand flowering, fruiting and vegetative growing stages, natural germination events and the impact of seasonal conditions and fire on these stages in its life cycle.
- Investigating factors affecting dormancy and germination in this species through field, nursery and laboratory trials.
- Assessing circumstances affecting re-establishment of *P. longifolia* on areas restored after bauxite mining.

1.4 The study environment

The research contained within this thesis was undertaken in three study areas, Cordering (Area 1), Boddington (Area 2) and Dwellingup (Area 3) in the south-west of Western Australia (Fig. 1.3).

All three areas are located within the jarrah forest of the south western Botanical province (Beard, 1990) and experience a Mediterranean climate with cool wet winters and hot dry summers. The South West Botanical Province has been classified as one of the world's biodiversity hotspots (an area with an exceptional concentration of species and experiencing exceptional habitat loss) (Myers et al., 2000) and is the only one currently recognised in Australia.

Cordering (Study Area 1)

Cordering is the southerly most study area (33.55°S, 116.60°E). It is located approximately 230 km south east of Perth. Studies were undertaken in Haddleton Nature Reserve and private bushland near the corner of Moodiarrup West Road and

Gibbs Road. Nursery burial trials were undertaken at Capercup also located within Study Area 1.

The area experiences an average annual rainfall of 510 mm most of which occurs from May to September (Table 1.1). The hottest months of the year are January and February when the mean maximum temperature is 30.7 °C but can reach 40 °C. During the summer months air temperatures can drop to 13.4 °C. The coldest month of the year is July which has a mean monthly maximum of 16.2 °C and a mean monthly minimum of 3.7 °C. Frosts are common during the winter months.

Cordering is located within the southern jarrah forest of the South-west Botanical Province (Menzies Botanical Subdistrict) (Beard, 1990). The vegetation in this area is mixed, open woodland consisting of *Eucalyptus marginata* up to approximately 15 m in height with other trees such as *Banksia attenuata*, *B. grandis* and *Corymbia calophylla* also contributing to the overstorey. There is a lower layer of smaller midstorey trees such as *P. longifolia, P. elliptica*, and *Acacia* spp. (Beard, 1990).

Boddington (Study Area 2)

South32 Worsley Alumina (referred to from here on as Worsley) Boddington Bauxite Mine is located 136 km south east of Perth on the Quindanning-Boddington Road (32.95°S, 116.49°E).

Boddington experiences higher rainfall than Cordering with an annual average rainfall of 709.2 mm (Table 1.1), most of which occurs during the winter months. The hottest months of the year at Boddington are also January and February with an average maximum temperature of 32 °C but temperatures can reach 45 °C on occasions. During the coldest months of the year July and August temperatures can drop below 1 °C and an average maximum day time temperature at this time of the year is approximately 16 °C.

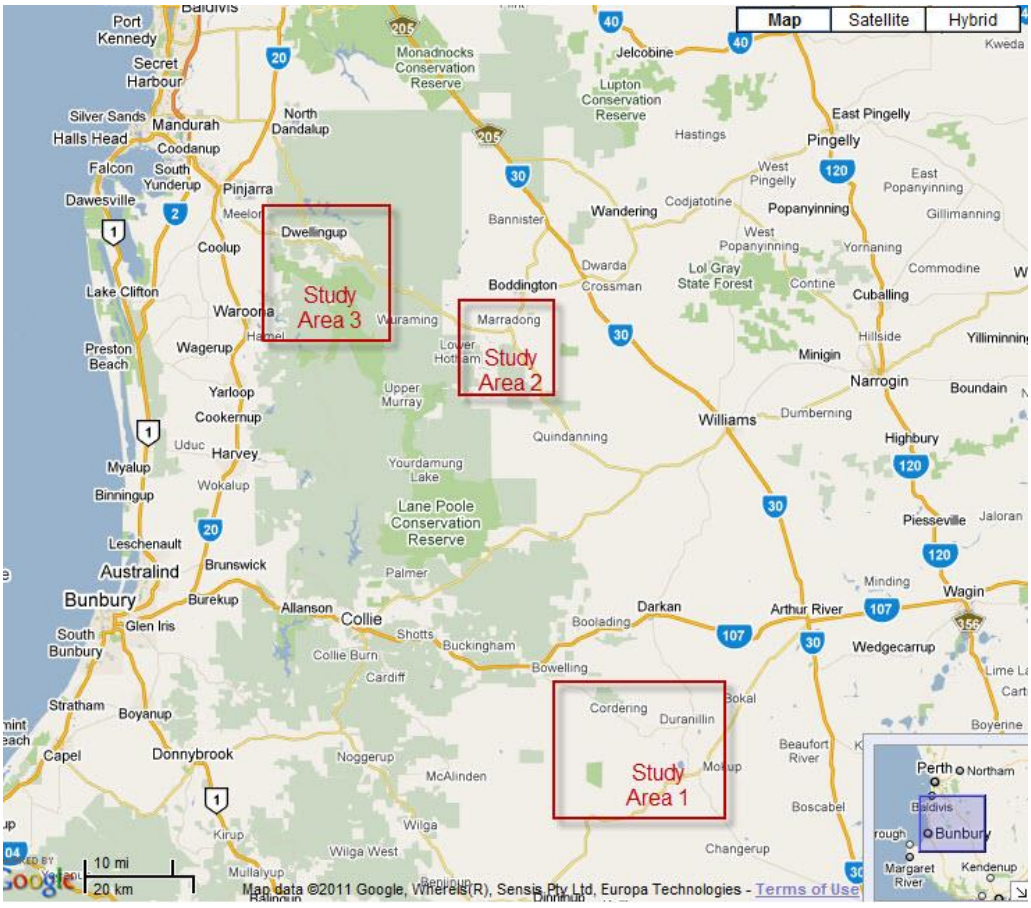

Figure 1.3: Location of study areas. (Source: Google Maps, 2010)

Both Boddington and Dwellingup (Study Area 3) fall within the Northern Jarrah Forest subregion of the South West Botanical Province (Dale Botanical Subdistrict) (Beard, 1990). Vegetation in the areas of interest at Boddington consists largely of open forests of *E. marginata* or *E. marginata-C. calophylla* on the mid to lower slopes of the landscapes. Other tree species present include *B. grandis*, and *Allocasuarina fraseriana* (E.M. Mattiske and Associates, 1996).

Dwellingup (Study Area 3)

Dwellingup is the northerly most study area and contains the Huntly mine site operated by Alcoa of Australia Limited. (hereafter referred to as Alcoa). The Huntly mine site is located 90 km south of Perth (32.63°S, 116.03°E).

It experiences the highest average annual rainfall of 1215.7 mm for the three areas included in this study (Table 1.1). Mean maximum temperatures range from 29.6 °C in January to 15.0 °C in July and mean minima range from 14.5 °C in February to 4.0 °C

in July. Summer maxima can reach up to 43 °C and summer minima can get down to 6.0 °C. In winter (July) temperatures can range from -4.0 °C to 23.1 °C.

Dwellingup is also located within the Dale Botanical Subdistrict (Beard, 1990) but is located further west than Boddington. Vegetation in this area reflects the high rainfall and is composed largely of tall mixed jarrah (*E. marginata)*-marri (*C. calophylla*) forests up to 30 m in height or open forest of *E. marginata – B. grandis – A. fraseriana* with scattered understorey (Mattiske Consulting Pty Ltd, 2001). The area has been extensively logged so that many of the largest trees are absent and have been replaced by regrowth trees. The understorey consists of many small shrubs from the Proteaceae, Myrtaceae and Fabaceae families (Grant and Koch, 2007).

1.5 Thesis structure

In order to manage large scale propagation of a species under varying field conditions it is necessary to determine what controls seed dormancy and germination. This requires detailed information on the plant's ecology and conditions in its natural environment and an understanding of the interactions between these conditions and the seed germination ecology.

Chapter two of this thesis investigates the phenology of *P. longifolia* with the aim of understanding environmental factors that affect vegetative growth, flowering, fruiting, seed maturation, *in situ* recruitment and seedling survival. Understanding the plant in its natural environment provides directions on laboratory and nursery based experimentation. This chapter has been published as a paper in the Australian Journal of Botany and is included in Appendix 1.

Chapter three considers seed biology and dormancy. In particular, it investigates the role of the endocarp in seed dormancy, the use of burial to alleviate seed dormancy and simulation of seasonal conditions in the laboratory in order to determine the drivers of seed dormancy. This chapter is currently being prepared for submission to the Australian Journal of Botany and is presented in its current draft format in Appendix 2.

Chapter four examines the use of *P. longifolia* seeds and seedlings in a restored mine environment and the most appropriate manner in which to plant seedlings on restored former mining pits to maximise survival and growth. This chapter has been prepared as a paper and submitted to the journal “Ecological Restoration". It is presented in the submitted form in Appendix 3.

Finally, Chapter five draws together all of the information in the preceding chapters and puts together a prescriptive approach to germinating seeds for those practitioners that are required to return seedlings to the restored areas of the jarrah forest. It is also anticipated that the methodology outlined here can be used by those in the horticultural and floricultural industries to germinate *P. longifolia* seeds for use in these industries.

Table 1.1: Summary climatic and weather data for the three study areas included in this thesis.

Area	*Statistic*	*Jan*	*Feb*	*Mar*	*Apr*	*May*	*Jun*	*Jul*	*Aug*	*Sept*	*Oct*	*Nov*	*Dec*	*Total*
Cordering*	Mean Rainfall (mm) (51 years)	21.3	15.0	18.1	31.8	62.2	82.7	85.2	69.1	53.7	33.2	25.1	13.4	510.8
	Mean Monthly Maximum Temperature (°C) (14 years)	30.8	30.9	28.4	23.8	20.0	17.3	16.3	17.3	18.4	21.9	25.3	28.5	
	Mean Monthly Minimum Temperature (°C) (14 years)	13.6	14.0	12.0	8.9	6.2	4.4	3.8	4.8	5.7	7.6	10.0	11.5	
	2010	1.4	0.2	76.9	26	36.4	27	62	29.3	10.6	19.7	25.8	5	320.3
	2011	68.0	0.6	0.8	38.1	41	94.5	89.3	73.0	58.2	46.8	50.4	64.2	624.9
	2012	9.8	17.0	0.0	13.5	35.9	104.0	28.4	53.8	90.6	13.2	48.6	63.6	478.4
Boddington#	Mean Rainfall (mm) (32 years)	13.1	14.6	18.5	37.3	87.8	118.2	128.3	109.3	84.4	41.0	30.8	17.6	700.7
	Mean Monthly Maximum Temperature (°C) (17 years)	32.1	31.9	29.0	24.5	20.0	17.0	15.8	16.6	18.4	22.5	27.4	30.3	
	Mean Monthly Minimum Temperature (°C) (17 years)	14.3	14.7	12.6	9.6	6.4	4.6	3.9	4.1	4.8	6.4	9.5	12.0	
	2010	0.0	0.0	22	13.6	49.8	66.5	96.5	53.0	19.9	19.5	9.0	7.7	357.5
	2011	61.5	0.5	0.0	30.7	50.5	136.5	94.0	111.0	80.7	53.3	58.8	96	773.5
	2012	16.0	5.0	0.0	27.8	65.5	132.9	21.5	92.3	110.0	16.3	67.0	54.0	608.3
Dwellingup+	Mean Rainfall (mm) (24 years)	15.8	12.1	27.1	56.8	139.9	206.3	227.4	193.7	159.0	64.4	52.2	21.5	1176.1
	Mean Monthly Maximum Temperature (°C) (59 years)	29.7	29.7	27.0	22.5	18.5	16.0	15.0	15.7	17.3	20.1	23.7	27.3	
	Mean Monthly Minimum Temperature (°C) (59 years)	14.3	14.6	13.0	10.3	7.7	6.5	5.5	5.5	6.5	78.0	10.4	12.6	
	2010	1.5	3.4	36.6	64.3	75.6	106.4	165.7	82.0	40.2	35.6	11.2	7.4	629.5
	2011	52.0	0.0	0.0	44.1	74.9	226.2	201.5	210.6	154.4	42.8	121.2	93.0	1220.7
	2012	9.4	9.3	0.0	98.6	108.8	224.5	38.4	207.4	195.6	42.0	122.2	38.6	1094.8

*Rainfall Data (mm) for Capercup (nearest rainfall station to Cordering) and temperatures from Collie East (nearest temperature data) Australian Bureau of Meteorology station; #Boddington rainfall data from onsite observations. Temperature information from Wandering Australian Bureau of Meteorology station; +Rainfall data from Alcoa of Australia and temperatures from Dwellingup Australian Bureau of Meteorology station.

Chapter Two - Developmental Phenology of *Persoonia longifolia* and the Impact of Fire

Phenology is the study of the timing of recurring seasonal biological events such as vegetative growth, flowering and seed dispersal (Forrest and Miller-Rushing, 2010). The phenology of *Persoonia longifolia* has not been studied in detail to date and previous investigations into the germination biology of this species have been based on methodologies used for other species that occur within the jarrah forests of southern Western Australia with limited success. A more holistic approach centred upon understanding the ecology of this species and the timing of key lifecycle events is required in order to decode the key drivers of dormancy loss in this difficult to germinate species. A study of the phenology of *P. longifolia* is critical to understanding how this plant operates in its natural environment and consequently to facilitate the development of effective methods of breaking seed dormancy and to promote germination that better reflects the key conditions that seeds naturally experience.

To develop a detailed understanding of the phenology of this species Chapter Two outlines investigations into the timing of vegetative growth, flowering, fruiting, seed dispersal and germination events over a three-year period across several different locations. The aim of these detailed *in situ* investigations is to better direct the germination experiments described in Chapter Three. Chapter Two also looks at the impact of fire on these events with the aim of assisting land managers in decision making associated with managing populations of *Persoonia longifolia*.

2.0 Abstract

Understanding the phenology of *P. longifolia* is vital for developing methods for returning this plant to restored areas. To date there have been only a few brief studies detailing the ecology and phenology of this plant. This study investigated in detail different aspects of the phenology of *P. longifolia* over a three year period. Most vegetative growth occurs during the summer months with flowering and fruiting occurring concurrently. Fruits mature from July through to September at which time it is dropped to the forest floor. Germination occurs in late winter/early spring from fruit that is at least one year old and with poor seedling survival in the natural bush (10 %) during the first 12 months. However, if the seedling survives the first year, the changes

of survival long term are high. Following fire, *P. longifolia* plants will resprout prolifically in the next growing season, although there is very little fruit production in the first year following fire. Fruit is not produced until at least the second year, and then seed requires at least another year in the soil seed bank before germination commences (three years post fire).

2.1 Introduction

Whilst there are numerous studies on the phenology of *Persoonia* spp. from eastern Australia (Auld et al., 2007, Bauer et al., 1999, Bauer et al., 2001, Bernhardt and Weston, 1996, Cadzow and Carthew, 2000, Robertson et al., 1996, Rymer et al., 2005, Rymer, 2006), the phenology, general ecology and the impact of fire on *Persoonia longifolia* is currently poorly represented in the scientific literature.

Understanding the ecology and phenology of this species is vital to developing methods of returning this plant to restored areas. Observational studies are required since it is impossible to formulate hypotheses when nothing is known of the systems being studied (Beninger et al., 2012). Baskin and Baskin (2001) also suggest that the first thing to consider when germinating seeds is the phenology of the seed phase of the lifecycle including when seeds mature, when are they dispersed and when they germinate. In addition, by understanding the phenology of *P. longifolia*, it will facilitate better management of wild-harvesting of material for use in the floriculture industry and a greater ability to incorporate this species into the native horticultural industry.

Abbott (1984a) undertook a short term study which included an investigation into the ecology of germination, and early growth and survival (including predation, impact of shading, root competition and presence of litter) of *P. longifolia* seedlings and other jarrah forest species. The initial results in relation to *P. longifolia* were limited by the overall lack of germination in the trial plots. In addition, Abbott's study was undertaken over a period of only one year and recent indications are that fruits require at least 18 months of soil aging prior to germination (Norman and Koch, 2008, Turner et al., 2010).

Abbott (1984a) did note that the seeds of *P. longifolia* take approximately six months to mature and are shed during the winter months (June to September). He indicated that the fruit is then either eaten first by animals or the pericarp rots away. Further studies

indicated that 28 % of *P. longifolia* fruits are removed from plots if not protected by either a cage or insecticide (Abbott and van Heurck, 1985).

Persoonia longifolia is considered to be a slow growing species and is known to resprout after fire (Abbott, 1984a) However, unlike some other resprouting species, *P. longifolia* produces abundant, quantities of viable seed on an annual basis (Abbott, 1984a). This seed however is not persistent in the soil seed bank with most reports indicating that seed viability is significantly reduced after two years (Abbott, 1984b).

Abbott and van Heurck (1988), also investigated the phenology of the closely related *P. elliptica,* another small tree from the jarrah forest of WA. They found that *P. elliptica* flowers in December, with the fruit attaining full size by May the following year and reported large annual variations in the numbers of fruit produced and seedling establishment in the areas surveyed, which they concluded did not appear to be directly related to fire. However, this is contradicted by opportunistic observations made by the same authors of abundant germination of *P. elliptica* seedlings occurring in the winter following a low intensity fire. The survival of these seedlings after a year was poor with seedlings either being eaten or becoming desiccated and dying of stress.

Two detailed phenological studies have been undertaken on the eastern states species, *P. lanceolata* (Auld et al., 2007) and *P. virgata* (Bauer et al., 2001). Unlike *P. longifolia* both these species are obligate seeder species and are killed by fire.

Auld et al (2007) investigated seed dispersal and predation, seedling recruitment, survival and growth after fire and residual seed banks. Fruits of *P. virgata* were eaten by the Swamp Wallaby (*Wallabia bicolor*) and were found to be dormant when extracted from scats. Seedlings emerged 10 months after fire and survival was high. The seedlings did not flower until six years after fire.

Persoonia virgata produces vegetative growth from late spring through to the end of autumn. Floral buds appear during late spring and summer on the new stems and flowering was confined to mid-late summer (January – February). Fruit growth generally occurred when stem production was minimal and takes up to six months to mature (Bauer et al., 2001).

Germination of a species requires an understanding of how that plant operates in its natural environment. By understanding the environmental cues that promote growth, flowering, seed production and germination, investigations into the seed dormancy can become more targeted. Understanding of the phenology of a plant such as *P. longifolia*, which appears to have a long-term regeneration strategy (i.e. seeds that require at least 18 months burial before germination occurs), (Norman and Koch, 2008, Turner et al., 2010), requires a long-term study to look at the patterns of natural germination, growth and flowering, and the occurrence of these events over many years in response to cyclic drought and fire events.

The aim of this study was to describe the phenology of *P. longifolia* in detail over a three year period and to determine how fire and seasonal conditions impact on the flowering, fruiting, vegetative growth and seedling germination and survival of this species across three distinct populations occurring in different parts of its natural range.

2.2 Methods

2.2.1 Study area

This study was undertaken in three different areas (see Section 1.4 for a location map): Cordering; Boddington and Dwellingup.

Eight trees were sampled at Cordering in an area that was located on private property. Half of the bush in this area was burnt in a wildfire in December 2008 and four of the trees were selected from the burnt area and four from the unburnt area (Table 2.1).

At the Dwellingup study site, 15 trees were selected by Alcoa in October 2006. Five of which were located in an unburnt area (last burn is estimated to have been occurred ~ 30 years ago in 1983; J. Koch, pers comm.), five in an area burnt in February 2006 (summer wildfire), and five in an area burnt in November 2006 (prescribed spring burn). These trees were monitored extensively by Alcoa through 2007, and more sporadically in 2008 and 2009. The same trees were used in this study (2010-2012), enabling some comparisons to be made over a longer period (i.e. six years).

The third study area, located at Worsley's bauxite mine near Boddington contained 30 trees. Groups of ten trees were located in three different areas. One area at Boddington

had not been burnt for ~21 years (~1989, S Gunn, pers comm.). It was sampled twice prior to a prescribed burn which occurred in May 2010.

Initially, it was anticipated that a second area would be burnt as part of Worsley's ongoing fire management programme. However this did not occur, so the remaining 20 trees were unburnt throughout the period of the study, 10 of which have not been burnt since 1977/78 and 10 of which were last burnt in 1986/87.

Table 2.1: Location of trees within each site and details on when each area was last burnt.

Study Area	Last Burn	Number of Trees
Cordering	~1988	4
	December 2008	4
Dwellingup	~1977/78	5
	~1986/87	5
	May 2010	5
Boddington	~1977/78	10
	~1986/87	10
	May 2010	10

2.2.2 Vegetative growth, fruiting and flowering

Vegetative growth, fruiting and flowering of the 53 trees in the three areas was measured over a three year period. Measurements were made on a monthly basis commencing in March 2010 through to January 2012, and on a quarterly basis throughout 2012. Sampling was only undertaken in February during the 2011 assessment.

Where possible, four branches on each tree were selected and measured to quantify vegetative growth. Branches were located on the north, south, east and west sides of the tree. In some cases it was not possible to access four branches on a tree (due to the height of the lowest branch) and in this situation only three branches were measured.

Measurements of the branch lengths were made from the node of intersection with the main branch to the apical growing tip. If new branchlets grew on the branch throughout the period of the study these were also measured and included in the entire branch length. This often occurred when insects, particularly tip boring moths (*Ptyssoptera*

tetropa), damaged the growing tip of the branch stimulating a profusion of axillary side shoots.

During monitoring, a record was made of the presence or absence of flower buds, flowers and fruit on each tree. Fruits were counted on the whole tree to obtain an estimate of total seed production. On several occasions fruit production was very high and accurate fruit counts could not be made. In these cases, fruit counts were made up to 2000, after which, the number of fruits on the tree was estimated.

The fruits on the individually marked branches were also separately counted at each assessment time. In addition, a more detailed examination of flowering was undertaken in the 2011/12 flowering season with the number of flowers and buds per stem also recorded for each individually marked branch. These data were used to provide an indication of the percentage of flowers that developed into mature fruit.

This detailed examination of the fruit production of *P. longifolia* aimed to determine if:

- the quantity of fruit produced in any year varies depending on weather conditions;
- the age/size of the tree affects fruit production; and
- fire affects the production of fruit.

General information about the tree, Diameter at Breast Height (DBH at 130cm), health and leaf litter, were also recorded annually.

2.2.3 Germination events

Natural germination events appear to have been rarely observed in *P. longifolia* in previous studies (Abbott, 1984a). It is unknown if this is a result of lack of germination, predation of dispersed fruits, or lack of investigations. This study aimed to investigate:

- what time of year germination events occur;
- the impact of weather conditions on germination events (i.e. do years of high rainfall result in higher numbers of germinants);
- the effect of fire on germination events (if germination occurs, how long after fire does it occur); and
- seedling survival following germination events.

The area beneath the canopy of the trees being assessed was examined at each assessment time for seedlings. Any seedlings that were observed were tagged and the height of these seedlings measured. Survival and height were then measured at each subsequent sampling. Germination events recorded by Alcoa that occurred under the trees at Dwellingup during the period of 2007-2010 were also included in the study.

Similarly resprouting from lignotubers beneath the target plant was measured. Differentiation between seedlings and lignotuberous growth was determined by the presence of the distinctive cotyledons on germinating seedlings.

To monitor germination from fruits of a known age over the duration of the study 20 intact fruits (as they would naturally occur) were placed out on top of the leaf litter in September 2010, under a subset of four trees in each treatment area (i.e burnt or unburnt) in each study site (Cordering, Boddington and Dwellingup). Fruits were placed on the leaf litter as if they had fallen from the tree and were caged using a pre-constructed wire cage pegged to the ground to prevent predation of the fruits and/or seedlings by larger animals such as reptiles or mammals.

The sample size in this instant was constrained by the limited fruit availability in the year of establishment of the experiment (2010). Where possible, fruits were collected from the parent plant, or if insufficient fruits were available from the parent plant, then they were collected from the closest nearby fruiting tree. Twenty fruits were placed outside and adjacent to the cage in order to monitor the impact of predation on potential germinants. All other intact *P. longifolia* fruits and cleaned endocarps were removed from the caged area to ensure that all the fruit under the cages was of a known age.

2.2.4 Data analysis

Initial exploration of the phenology data was undertaken using a linear regression model, which indicated that the time series on both fruit count and vegetation growth were cyclic.

A Linear Mixed Effects model in this instance, is an appropriate framework for quantifying the influence on the response of seed production and changes in vegetation growth of different ecological drivers which in this case included, time since last fire,

time of year, seasonal rainfall and tree size. The data were inspected graphically using plots of residuals and quantile-probability (qq) plots to assess model assumptions (Enright et al., 2011). Fruit count data were $\log_e$ transformed and vegetative growth data were arcsine transformed in order to meet the assumptions of normality.

For each data set, a full model including all main factors (sampling time, time since last burn, tree size, month of assessment, rainfall in the three months preceding the assessment) and their interactions was computed. Rainfall in the three months preceding the assessment was used as this correlates to seasons of rainfall. This model was then reduced by omitting all non-significant interactions (5 % significance level) and comparisons between models was made using maximum likelihood ratio tests in a backwards model-selection procedure (Pinheiro and Bates, 2000). Analysis was undertaken using lme4 package (Bates et al., 2014) in the statistical program R (R Core Team, 2013). Random effects included in the model were year of sampling, site, area within each site and individual trees with a residual for representing variation between individual trees. Comparisons between months and quarters were conducted using Tukey's HSD using the multcomp (Hothorn et al., 2008) and lsmeans packages (Lenth, 2014) in R (R Core Team, 2013).

Seed set data were analysed using a binomial generalised linear mixed effects model (GLMM) with binomial response, to determine whether seed set was influenced by various environmental predictors. Area and sampling site were included as random effects.

2.3 Results

2.3.1 Seasonal conditions

Rainfall was highly variable over the study period. Throughout the southwest of WA, 2010 was the lowest rainfall year on record since records commenced in 1900 (Bureau of Meteorology, 2010). In almost every month of the year, in all three study areas, rainfall was well below average (Table 1.1). Dwellingup had only half its average annual rainfall during 2010. In March 2010 there was a single thunderstorm which accounted for the above average rainfall during that month. The year 2011 was slightly above average in all areas with the autumn months being below average and winter months receiving average rainfall. Rainfall in 2012 was below average in all areas and

this was largely due to the well below average winter rainfall (particularly in July) received.

2.3.2 Age/size classification

Diameter at Breast Height (DBH) was measured for 53 trees in 2011 and 2012 with most trees having a DBH between 5 and 15 cm (Fig. 2.1). The annual change in DBH was small, most likely due to this species being slow growing. The measurements were not considered to be accurate enough to make any assumptions about growth rates or age. *P. longifolia* has very flaky and flexible bark and measurements could be highly variable.

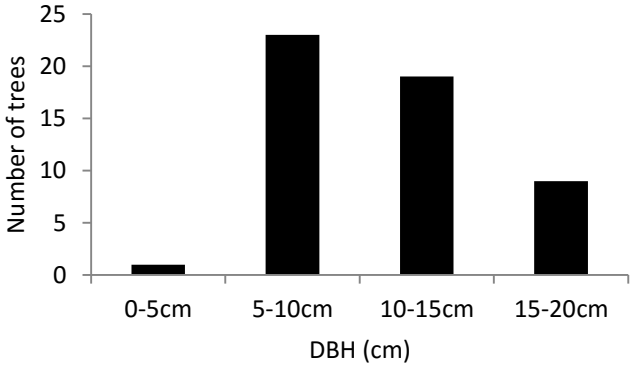

Figure 2.1: Tree Diameter at Breast Height (DBH) for all trees within all three study areas.

2.3.3 Vegetative growth

Most vegetative growth occurred in the summer months with the greatest increase (~6 cm per branch) occurring in November ($P<0.001$) (Fig. 2.2a, b and c). Negative growth recorded in the winter months largely reflects damage inflicted on the trees by birds. During these months, branches were nipped or chewed off by birds resulting in negative changes in vegetative growth. Branches were also damaged by the larvae of the tip boring moth *Ptyssoptera tryphera* which burrows into the apical meristem of the branch causing the apical tip to initially die and then the rest of the branch proceeds to slowly dieback from the tip (Fig. 2.2b). However, the damage caused by the tip boring insects was much less than damage resulting from branches being chewed off by birds such as cockatoos (*Calyptorhynchus banksia naso*) and parrots (*Barnardius zonarius*). During the growth period after the apical meristem was damaged, large numbers of lateral branches characteristically form and grow around the damaged tip.

The model indicated that the principal factors affecting vegetative growth were the month in which the sample was taken (P<0.001) and time since last fire (P<0.001). Although not statistically significant (P=0.057) at a 5 % significance level, rainfall in the three months preceding the assessment is likely to have some influence on vegetative growth. Generally the early and late months of the year were significantly different from the middle months of the year (Fig. 2.2c and d). However, growth in November was significantly greater than all months of the year except October, with the pairwise comparison between October and November being almost significant (P=0.051).

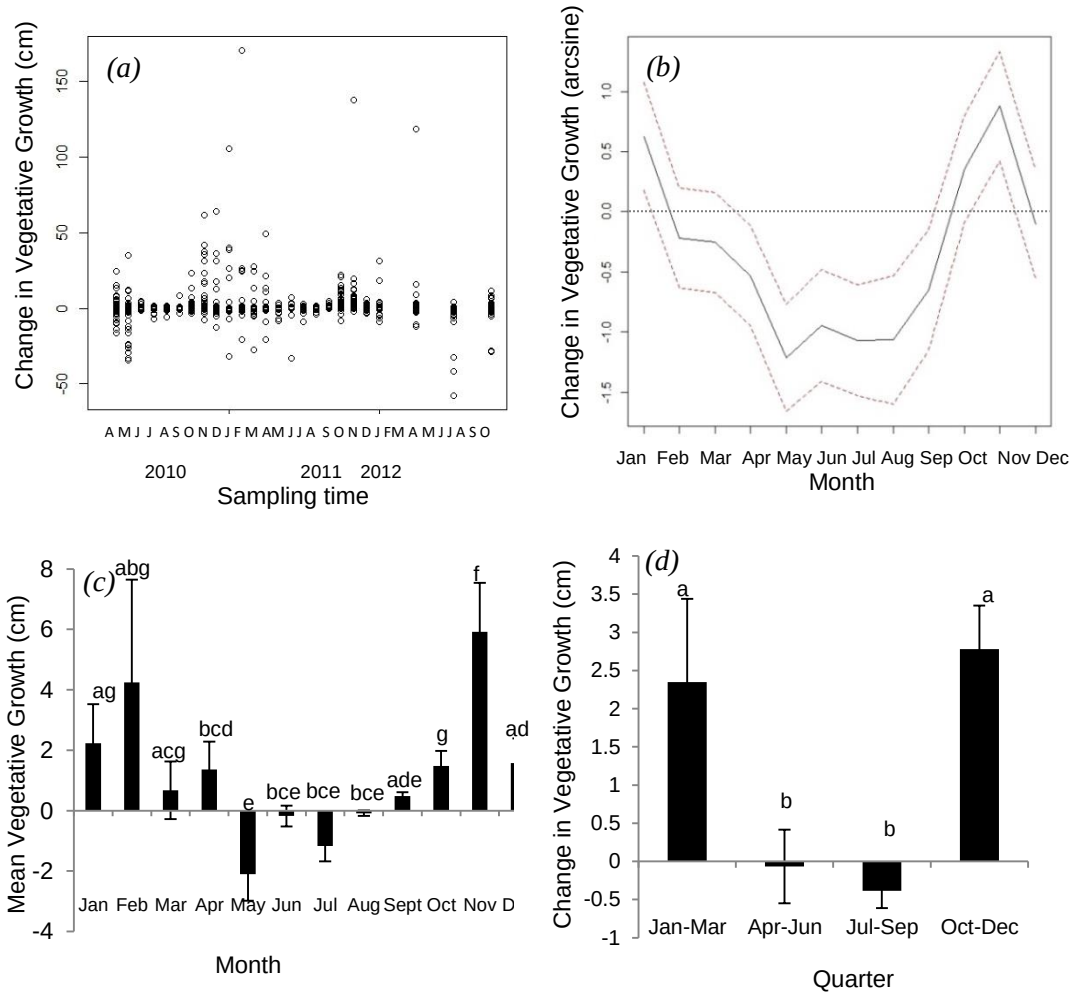

Figure 2.2: Summary of vegetative growth and model estimates**.** *(a)* Actual vegetative growth recorded per tree for each month that sampling was undertaken. *(b)* Vegetative growth estimates generated from the Generalised Linear Mixed Effects model. Dashed lines indicated 95 % confidence intervals. *(c)* Mean change in vegetative growth per tree by month (± SE). Columns with the same letter do not differ significantly from other columns on the same graph. *(d)* Mean change in vegetative growth by quarter

(± SE). Columns with the same letter do not differ significantly from other columns on the same graph.

The relationship between the time since last fire and vegetative growth was negative indicating that vegetative growth was greatest immediately after a fire (Fig. 2.3) with the amount of vegetative growth decreasing in each subsequent year. However this relationship was not simple, as there was also a significant interactive effect between the time since last fire and the quarter in which measurements were made ($P<0.001$) indicating that the growth was seasonal and that the longer it had been since a fire, then less the tree grew during the summer months.

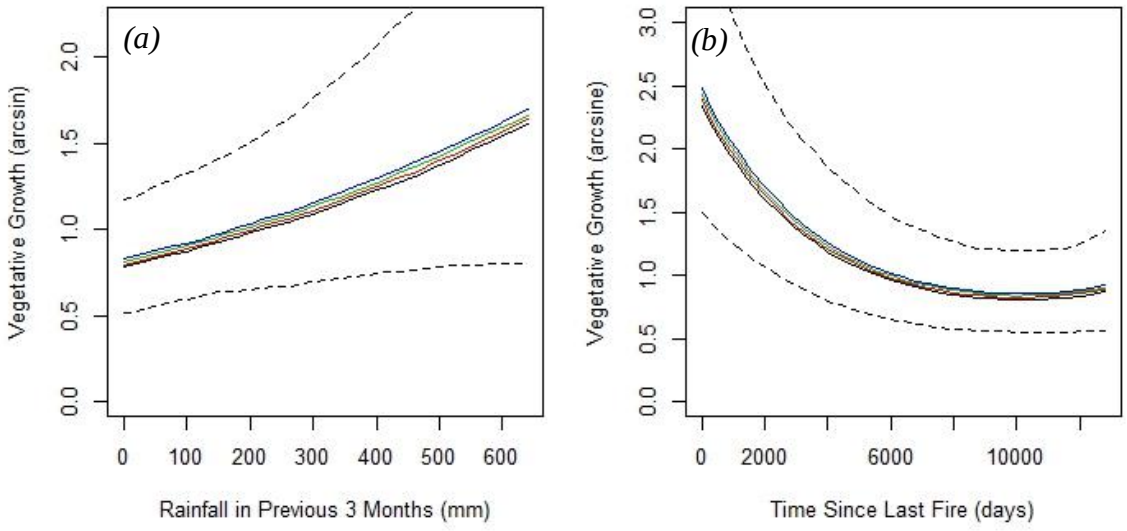

Figure 2.3: Mean estimates of vegetative growth per quarter and the interaction between *(a)* Rainfall in the preceding three months prior to the assessment being undertaken. *(b)* Time since last fire. Quarter 1 is delineated by a black line, quarter 2 by a red line, quarter three by a green line and quarter 4 by a blue line. Only the 95 % confidence interval for quarter one (dashed black line) is shown for clarity.

Lignotuberous growth was also recorded, with 86.8 % of these lignotuberous stems surviving over the period of the study. However, no lignotubers were observed to flower and it is unknown how long it would take for these stems to become reproductive.

2.3.4 Flowering and fruiting

Flowering of *P. longifolia* generally commenced in all areas in October and was finished by January, with peak number of trees flowering in November and December

(Figure 2.4). More trees were recorded with flowers in 2011 than in 2012 highlighting flowering variability between different years.

The estimated mixed effects models indicated that fruit count is significantly affected by the month in which the count is made ($P<0.001$), time since fire ($P<0.001$), and the size of the tree ($P<0.001$).

Fruit count was greatest in the early months of the year (January through to March) following flowering and then declined till fruit drop in July and August (Figure 2.5). This pattern is most evident when examining fruit count by quarters.

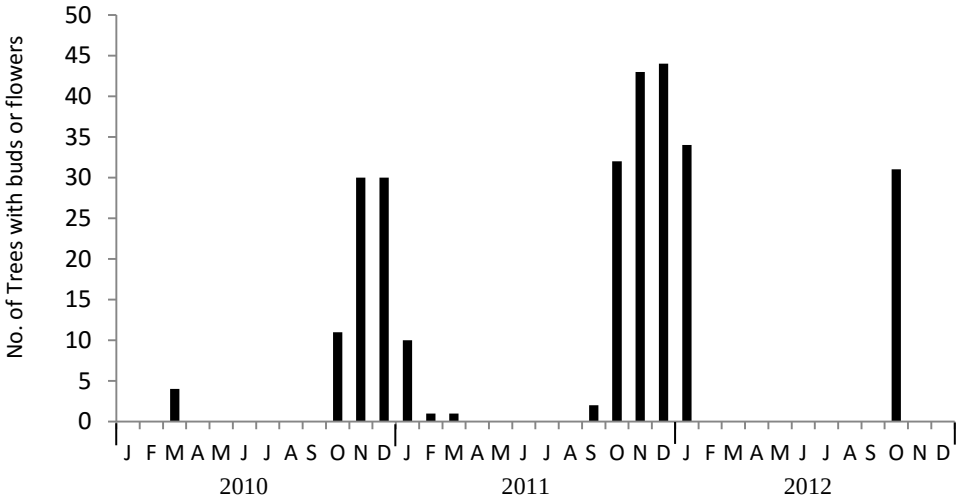

Figure 2.4: Total number of trees assessed with buds or flowers at each sampling time (note that trees were only sampled in January, April, July and October of 2012).

The fruit counts in the early part of the year were significantly greater than the latter months of the year. Those months in the middle of the year did not differ significantly from either the first few months or the last few months (Fig. 2.5c and d)

The interaction between the time since last fire and the quarter in which the fruit count was made was also found to be significant ($P=0.002$). Modelling indicated that a peak in fruit count occurred between 7000 and 8000 days (19 and 21 years) and this peak is most evident in quarter one (January to March) when the majority of the fruit is produced (Fig. 2.6b). Whilst this interaction was found to be significant, the "time since fire" sample size was small with only eight different areas included in this data set. Sampling in areas that had not been burnt for seven to 20 years was not undertaken.

Similarly, estimated models indicated that there was a significant (P=0.008) interaction between the quarter in which the fruit count was recorded and the rainfall preceding the fruit count (P=0.008) meaning that in those years with high rainfall in October, November and December it is likely that there will be greater fruit production in January (Fig. 2.6a).

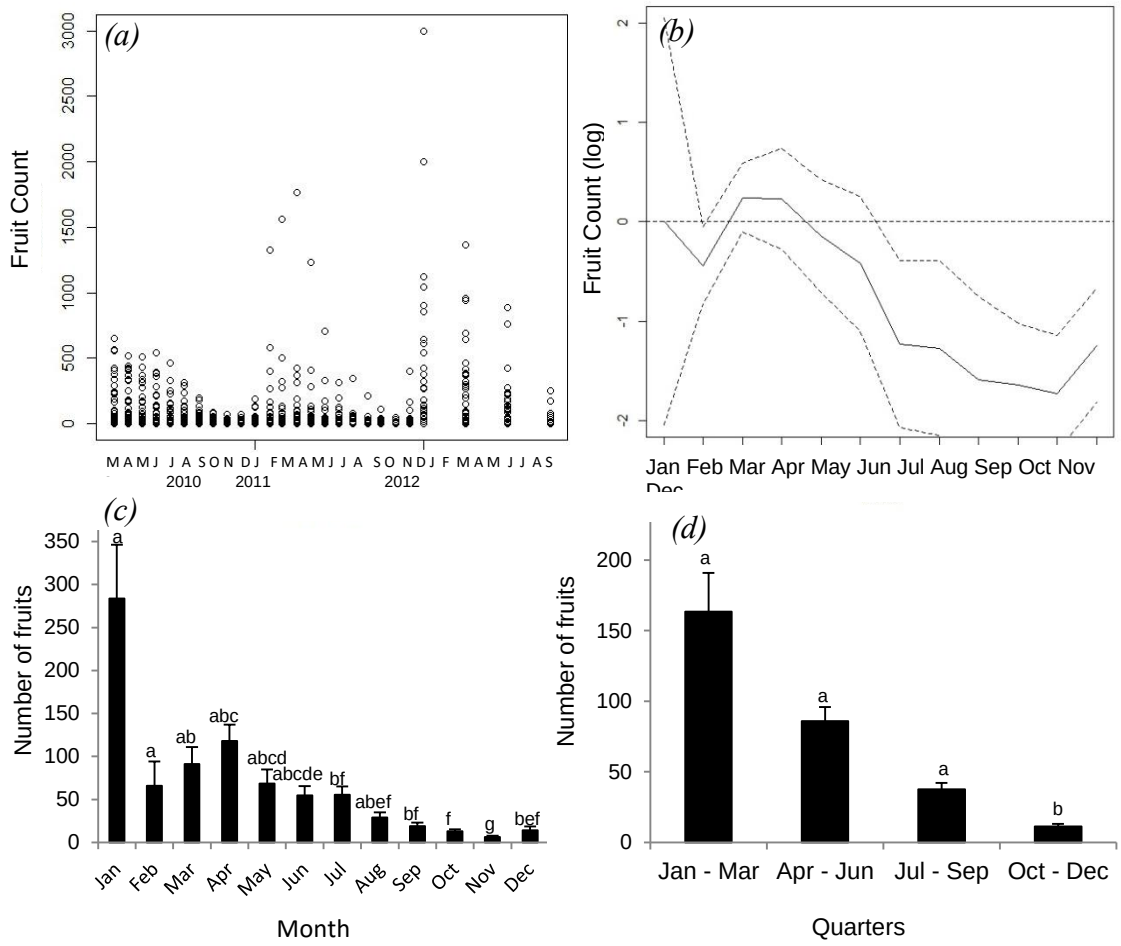

Figure 2.5: Summary of fruit counts and model estimates. *(a)* Actual fruit counts made per tree for each month that sampling was undertaken. *(b)* Fruit count estimates generated from the Generalised Linear Mixed Effects model. Dashed lines indicated 95% confidence intervals. *(c)* Mean fruit count per tree by month (± SE). Columns with the same letters are not significantly different. *(d)* Mean fruit count by quarter (± SE). Columns with the same letters do not differ significantly from other columns on the same graph.

The probability that flowers on a tree developing into mature fruit was not related to season, time since last fire or tree size. However, given that total seed set was clearly related to the number of flowers produced, then expected seed set of each tree was related to the season (P<0.008). The percentage of flowers on the sampled branches that

became mature fruits was 8.0 ± 3.7 % in 2010/11 season (a low rainfall year) and was 9.8 ± 1.6 % in 2011/12 (a higher rainfall year) and this difference was significant ($P<0.001$).

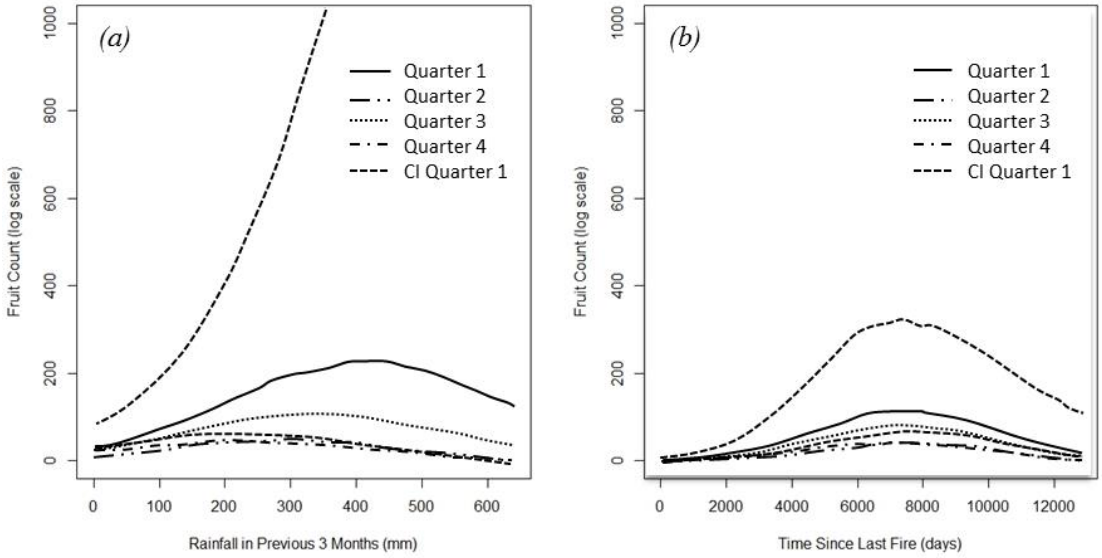

Figure 2.6: Mean estimates of fruit count per quarter and the interactions between *(a)* Rainfall in the preceding three months prior to the assessment being undertaken and *(b)* time since last fire. Only the 95% confidence interval for quarter one (dashed black line) is shown for clarity.

2.3.5 Germination events

From 2006 to 2012, 32 seedlings were observed under the trees being assessed. Of these, 27 were found beneath three of the summer burnt trees in Dwellingup. Germination of most of these seedlings occurred three to four years following fire (Table 2.2). Rainfall in Dwellingup during 2009 (three years post fire) was well above average in August and September (seedlings were observed in September 2009). However, rainfall in 2010 (four years post fire, when a second batch of 10 seedlings germinated) was well below average. A small number of seeds collected from the soil surface and x-rayed after fire were all empty indicating that fire kills soil stored seed. In some cases the endocarp was easily crushed into charcoal and ash.

Table 2.2: Germination following fire in the three different study areas (Note: four additional seedlings were found in unburnt areas).

Location	Year of Fire	Year 1 post fire	Year 2 post fire	Year 3 post fire	Year 4 post fire	Year 5 post fire	Year 6 post fire
Dwellingup	Feb 2006	1	2	10	10	2	2
Boddington	May 2010	1	0	0	NA	NA	NA
Cordering	Dec 2008	0	0	0	0	0	0

Seedlings were mostly observed germinating during late winter/early spring months of August and September (Fig. 2.7). However, one seedling was observed to have germinated in January (following several significant rainfall events – 20 mm at the end of December and 13 mm in early January), with another germinating in April (both of these were found at Boddington).

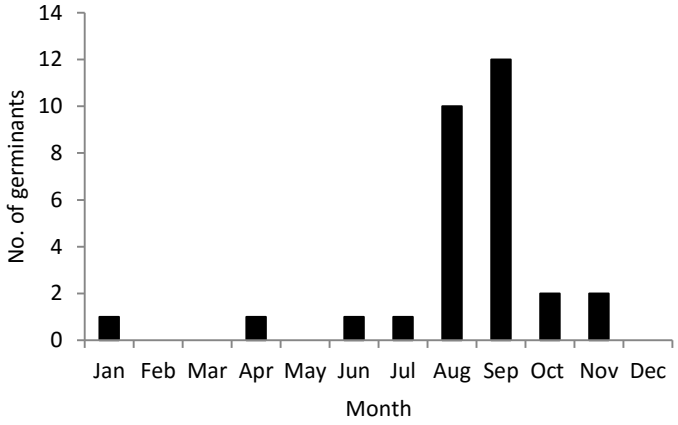

Figure 2.7: Number of seedlings germinating per month. Two seedlings have not been included as it was not possible to determine the month in which they germinated.

Survival of the 32 seedlings was poor with only seven seedlings (21.9%) surviving until the end of the project with three of these first recorded in the very last assessment (October 2012). Three other seedlings were located in unusual locations (somewhat protected areas), namely, two in cracks on top of a log which are unlikely to be able to grow to maturity and one underneath a log. Most seedlings died from lack of water or predation within two to three months of germination. Examples of both desiccation and predation were observed in the field. No seedlings were observed to flower over the course of the study.

None of the fruits of *P. longifolia* placed in cages or adjacent to the cages beneath the trees germinated over the period of this study.

2.4 Discussion

The majority of the *P. longifolia* trees included in this study are likely to be greater than 50 years old. The changes in diameter over the period of the study were very small and difficult to measure indicating that the trees are very slow growing. Flaky, spongy bark also prevented accurate measures. Abbott and van Heurck (1988) estimated the growth of *P. elliptica* over a three year period and the annual diameter growth rate was found to be only 0.11 cm per year. They estimated, based on this data, that trees of 8 cm in diameter would be between 62 and 89 years of age and those trees 20 cm in diameter would be approximately 154-222 years old. Similarly, *Banksia grandis* increases in diameter between 0.19 and 0.27 cm per year and a tree of 20 cm diameter would be between 80-100 years old (Abbott, 1985). Based on these figures and the measurements made for *P. longifolia* trees in this study it is likely that trees greater than 5 cm in diameter (Fig. 2.1) are also likely to be greater than 50 years old.

The growth, development and reproductive cycles of *P. longifolia* are highly seasonal and a generalised phenological outline based on data presented in the present study is shown in Fig. 2.8. Vegetative growth, flowering and fruit set all occur during the summer months with seed maturing in July and dropping to the ground when ripe, from July through to September. Germination occurs during late winter/early spring.

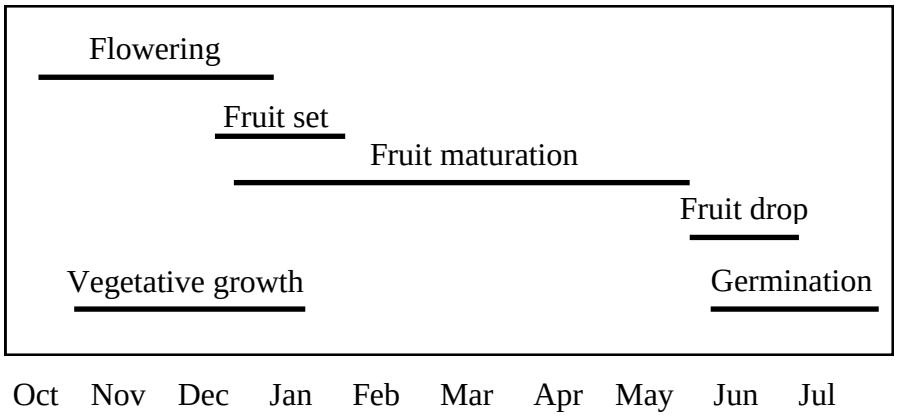

Figure 2.8: Phenology flow diagram for *Persoonia longifolia*.

Flowering commenced in October and was finished by January, with peak flowering occurring in November. This was similar to the flowering times observed for *P. virgata* (Bauer et al., 2001), *P. lanceolata* (Auld et al., 2007), and *P. elliptica* (Abbott and van Heurck, 1988), and corresponds to flowering times for *P. longifolia* recorded by Weston (1995). In comparison *P. juniperina* flowers later in the year

between January and March (Cadzow and Carthew, 2000). Bernhardt and Weston (1996) examined herbarium specimens of all New South Wales (NSW) *Persoonia* species for flowers and found that they largely flower between December and April.

Both native bees and the introduced honey bee were observed to visit the flowers of *P. longifolia* and are likely to be the major pollinators for this species. No other pollinators were observed during this study. Bees and wasps were also observed to be the major foragers on the flowers of eastern states *Persoonia* species (Bernhardt and Weston, 1996, Wallace et al., 2002).

Fruit set occurred in *P. longifolia* from December through to February, and fruits took six to seven months to mature. Mature fruit drop occurred from late July through to September and does not occur all at once, but rather over a two to three month period. *Persoonia elliptica* fruit also takes up to six months to mature and it drops all its fruit by August (Abbott, 1984a, Abbott and van Heurck, 1988). Similarly *P. virgata* seeds take six months to become mature (Bauer et al., 2004).

Mature fruit generally dropped directly to the ground beneath the canopy of the tree, where most fell through the leaf litter to the soil below. Fruits have been observed to be eaten by brush tailed wallabies (*Macropus irma*) and western grey Kangaroos (*M. fuliginosus*) (J. Stingemore pers. comm.) and bobtail lizards (*Tiliqua rugosa*) (pers. obs.). Other animals such as birds and emus have also been attributed to ingesting *P. longifolia* fruits and are likely to facilitate seed dispersal (Mullins et al., 2002). If left untouched by animals, the fleshy mesocarp was observed to rot off when the seeds were in a moist environment and in drier areas, the mesocarp was observed to shrivel, dry and remain attached to the seed.

The number of fruits produced by *P. longifolia* trees was highly variable and differences among individual trees were greater than either differences observed among populations or seasons. This variation in fruit/seed production has been observed for other resprouters from the Proteaceae family such as *Banksia spinulosa* (Carthew, 1993). Factors affecting fruit production in *P. longifolia* included, but were not limited to, the size of the tree, how recently a burn has occurred and the time of year the assessment is made (Section 2.3.4). Abbott (1984a) estimated that *P. longifolia* trees were able to produce in the order of 800 seeds per tree. The results from this present study found

that in a good rainfall year, seed production could vary from as low as zero to greater than 3000 fruits per tree, even within the same population. Abbott and van Heurck (1988) found that *P. elliptica* trees with a diameter less than 11 cm did not fruit over the duration of their study. This was not the case with *P. longifolia* with a tree as small as 4.97 cm in diameter producing flowers (but not fruit) and one of 5.73 cm producing mature fruit over the course of the study.

Fruit set from flower production in *Persoonia* species has been observed to be highly variable. Cadzow and Carthew (2000) found up to 40% of flowers produced fruit in *P. juniperina,* compared with 12-39% in *P. mollis* (Krauss, 1994, Rymer et al., 2005), 24-49% in *P. virgata* (Bauer et al., 2001, Wallace et al., 2002), 67% for *P. rigida* (Trueman and Wallace, 1999), 40-50% for *P. lanceolata* (Rymer et al., 2005) and 10-20% for *P. glaucescens* (Rymer et al., 2005). Of these *Persoonia* species, only *P. juniperina* is a resprouter. By comparison, mean seed set in *P. longifolia* was found to be the lowest of all reported seed set in *Persoonia* species to date at less than 10%. This however corresponds more closely with reported seed set in other resprouter species from the Proteaceae family. For example, only 3% of flowers of *Telopa speciosissima* set seeds (Pyke, 1981), *Banksia spinulosa* has been reported to have only 1-2% of flowers setting seed (Carthew, 1993), whereas *Lambertia formosa* has only 1-5% seed set from flowers produced (Pyke, 1982).

Fruit set observed in *P. longifolia* in this study was variable between trees, and ranged from 0-40%. The probability that flowers became mature fruits was not related to season, fire or tree size. Other factors such as pollination, nutrient availability, or microclimate that were not included in this study may impact on the ability of a flower to become a mature fruit. However, the percentage of fruit set from flowers was greater in 2011/12 (an average rainfall year) than in 2010/11 (a poor rainfall year) and similarly more trees flowered in 2011/12 than in the lower rainfall year of 2010/11. This may have implications for the amount of seed produced if the climate continues to become drier as has already been observed for south-western Western Australia over the past 40 years and which is expected to become more pronounced over the next few decades (Bates et al., 2008).

In 2012, trees across all populations initially set very high numbers of fruits. However, as a possible response to seasonal conditions, most of these small immature fruits were

dropped prior to maturity, most likely as a result of below average rainfall in the early months of the year. Abbott (1984a) observed a similar phenomenon in *P. elliptica* where many of the immature fruits were aborted between March and May. Cadzow and Carthew (2000) found that the number of maturing fruit declined over time until two months after fruit set occurred by which time fruit abortion slowed considerably and remaining fruit proceeded to maturity.

Germination in the natural environment occurred in late winter/early spring from previous season's fruits. Germinants observed in the field regularly had the endocarp still attached to the cotyledons so it was easy to determine that the seedling had developed from either the previous season's fruits or older fruits. In the past, germination events in the field have rarely been observed in *P. longifolia* (J. Koch, pers. comm.).

Conditions for germination appeared to be very narrow with microclimatic variation likely to be highly important. Seeds need to move through the leaf litter and become buried in order to germinate (See results from Burial trials in Section 3.4 and Synthetic Seed Bank trial in Section 4.3) and even when seeds do germinate, survival is poor with desiccation and herbivory accounting for most of the deaths. This is possibly the reason why seedlings are rarely observed in the field. Most previous studies have only been undertaken over short time frames which may not have been sufficient to capture germination events. Unless these studies were undertaken in a year which provided conditions suitable for germination, and an observer is present within a week or two of germination occurring, it is not surprising that seedlings are rarely observed. Abbott (1984a) found that seeds of *P. longifolia* or *P. elliptica* did not germinate at all in their study. Similarly not a single seed placed out under the trees in the present study germinated (over 1200 seeds in total), indicating that there are very specific microclimatic requirements for germination of *P. longifolia* seeds.

Lamont and Bergl (1991) found that *Banksia* seedlings also germinated in a flush and most did not survive. Any that did survive the first wave of mortality were likely to continue to maturity. It appears that *P. longifolia* seedlings are similar to this (see also Chapter 4). Abbott and van Heurck (1988) found *P. elliptica* seedlings to be highly palatable to herbivores during their first year. By the second summer, annual mortality had dropped to 6.9% indicating that the lignotuberous seedlings were no longer

palatable. If the seedling survives the first summer then the chances of survival long term are high.

A resprouter is a plant that resprouts from epicormic buds or from underground lignotubers, corms bulbs or rhizomes after a fire (Bell et al., 1993). *Persoonia longifolia* was observed to resprout from both epicormic buds and lignotubers beneath the ground following a fire, often quite some distance from the parent plant. Resprouting occurred in the growing season following the fire, with the amount of vegetative growth that occurred decreasing over subsequent seasons. Abbott and Van Heurck (1988) indicated that recovery growth of *P. elliptica* began to occur within a few weeks of the fire occurring. However, this was not the case with *P. longifolia* which was not observed to resprout until the following growing season. This may be within a few weeks if the fire occurs in spring, or many months if the fire occurs during late summer or autumn (February – April).

In the first growing and flowering season following a fire, *P. longifolia* trees resprouted and grew prolifically but there was very little fruit production (Fig. 2.9). In the second season following a fire, vegetative growth occurred at a greater rate than was observed in unburnt trees but at a lesser rate than in the first season following fire. Plant resources were also directed into some flowering and seed production by the second season. These seeds take six months to mature and are then released during the third winter (June to August) following the fire before germinating in the following winter, i.e. the fourth winter post-fire assuming an autumn burn. Therefore seedling germination will generally not occur until at least three years post-fire. This is also likely to be dependent on winter rainfall, although this was not investigated. Given that seedling recruitment is slow, seedling survival is highly variable and there is significant phenotypic plasticity in terms of seed set, fire return intervals need to be carefully considered so as to maintain mature populations. Whereas populations may continue to resprout at short fire intervals (less than five years) the long-term replenishment of populations will require fire intervals greater than five years.

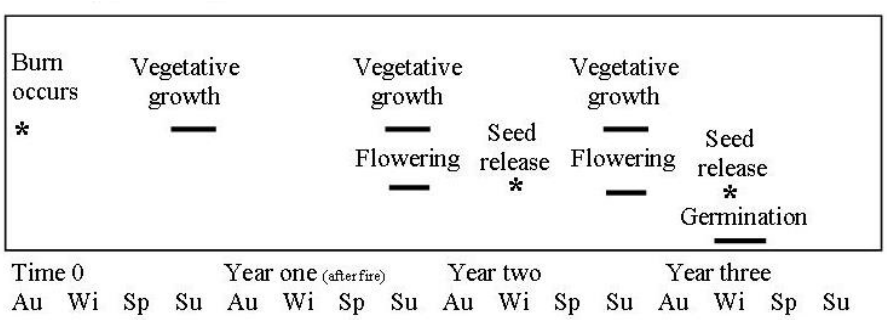

Figure 2.9: Generalised time line of *Persoonia longifolia* phenology following fire.

Some spot flowering was observed at Dwellingup following exceptionally good rainfall in 2007 (one year post fire). This is likely to account for seedlings observed in 2008 and 2009 only two –three years following fire. Lack of germination in Boddington and Cordering could be attributed to the below average rainfall received in the years post fire (2009 and 2010), particularly during the critical months before, or during, seed set and germination.

Model estimates indicated that maximum fruit production in *P. longifolia* occurs between 19 and 21 years following fire. However, there was an absence of data records for trees that had not been burnt between seven and 20 years, which means fruit production may have peaked earlier. Nevertheless, this is the best indication so far of when maximum fruit production after fire is likely to occur.

Fire did not result in the immediate germination of seeds, as is the case with many other plants in the Australian native plants (Bell, 1999, Dixon et al., 1995, Roche et al., 1997a). However, the majority of seedlings observed during this study occurred under trees in burnt areas indicating that germination events in *P. longifolia* are, nevertheless, linked to fire. Fire appears to kill soil stored seed of *P. longifolia.* Seedling recruitment will only occur after burnt trees have resprouted, flowered and set seed, with seed remaining on the ground for at least one year before germination occurs (Fig. 2.9). Observations in the field and in other studies undertaken (see Chapter 3) have shown that seeds need at least one year either on the ground or buried before germination can occur. Therefore, it appears that fire does not break seed dormancy *per se*, but that the post-fire environment creates conditions that are suitable for germination of *P. longifolia* seeds, although this window for *P. longifolia* germination to occur may be several years following a fire event. *Xanthorrhoea* species exhibit a similar phonological response to fire, with flowering and seed production promoted in the post-

fire environment (usually within several months of the fire), although the seeds themselves do not require any fire-related stimulation (i.e. heat or smoke) for germination to occur (Lamont et al., 2004). The post fire environment presents conditions that may be conducive to the success of seedling establishment, including low levels of leaf litter, reduced competition from surrounding plants and, then increased nutrient and moisture availability found in these relatively more recently burnt environments compared with areas that remain unburnt for long periods of time (>10 Years). However, more information is required to determine the underlying seed ecology of *P. longifolia* and the specific drivers of dormancy loss and germination and these are explored in subsequent chapters of this thesis.

Abbott and van Heurck (1988) found that *P. elliptica* seedling establishment was not related to fire and logging and that *P. elliptica* seedlings were largely found in virgin, unburnt forest. However, opportunistic observations identified an abundant germination event of *P. elliptica* seedlings in the winter following a low intensity spring fire.

In the present study, seedlings of *P. longifolia* were observed in areas subjected to summer or autumn burns. No seedlings were observed in the spring burnt area. In comparison, Enright and Lamont (1989) found that autumn burns produce twice as many *Banksia* seedlings as spring burns. Hobbs and Atkins (1990) also found that seedling regeneration in a *Banksia* woodland mainly occurs in autumn-burnt areas. This was attributed to seeds being distributed just prior to the most appropriate time for germination (i.e. winter). This explanation is not applicable to *P. longifolia* seeds, given that they need at least a year in the soil seed bank before germination occurs. However, autumn burns may produce conditions that are more suitable for germination of *P. longifolia* seeds such as reduced leaf litter, competition and soil water repellency (Granged et al., 2011), all of which allow greater moisture penetration during the summer months that the endocarps are on the ground.

Seedling mortality in *P. longifolia* was initially very high with only seedlings germinating in niche habitats surviving past three months. Lignotuberous regrowth survived much better than germinated seedlings.

Enright et al. (2011) found that resprouting trees have higher survival rates when fire intervals are intermediate (ie >5 years and <24 years) and that resprouters are able to

replenish their Total Non-structural Carbohydrates (TNC) within three years. Likewise, in the present study, survival of burnt *P. longifolia* was also high, with 100% of plants showing signs of regeneration following fire. Only one tree died following a burn and was located in the summer burnt area at Dwellingup and died six years post-fire. Death could, therefore, not be directly attributable to the fire in this case. All trees in the burnt areas either resprouted from epicormics buds or via lignotuberous growth around the base of the tree. How often *P. longifolia* trees can be exposed to fire before trees become stressed and exhaust their energy reserves and are, consequently, unable to resprout anymore remains unknown.

Overall, the present study has provided a more detailed understanding of the phenology and the impact of fire on *P. longifolia* which will assist land managers, seed collectors and other stakeholders to make more informed decisions pertaining to the management of this species.

Chapter three - Germination and dormancy in *Persoonia longifolia*

Once a detailed understanding has been obtained of the seed development, maturity and germination phase of *Persoonia longifolia* through a phenology study as outlined in Chapter Two, it is then necessary to identify the likely *in situ* environmental conditions that lead to dormancy break and promote germination and emergence. Such information can then be used to further inform laboratory studies with the aim of identifying the specific drivers of dormancy loss under carefully controlled conditions.

The phenology study in Chapter Two identified late winter/early spring as the time when germination occurs from fruits that are at least one year old. However further investigations into the environmental conditions that the fruits are exposed to in the months preceding germination needs to be carried out in order to clearly identify the sequence of conditions required for dormancy break. In addition, the hard woody endocarp present on the seed has been identified as a potential restriction to germination and detailed studies into its attributes described in Chapter Three help to understand the role of the endocarp in seed dormancy.

Chapter three therefore aims to determine the role of the hard woody endocarp in regulating germination, outlines the environmental conditions the fruits/seeds are exposed to in the natural environment and then replicates these conditions through laboratory based seasonal simulation experiments in order to identify the key drivers of dormancy loss and the conditions that promote germination once seeds have become non-dormant.

3.1 Abstract

The aim of this section was to investigate the key mechanisms driving dormancy break and germination of *P. longifolia*, through a series of field and nursery burial trials, and laboratory germination trials. Fruits, intact endocarps with the lid removed and extracted seeds were all found to be water permeable. Germination of 92.5% occurred in laboratory trials when the endocarp was removed from the seed and this was significantly greater than treatments where the endocarp remained intact (0%) or with the lid removed (4.7-5.9%). Results from a field burial trial indicated that some form of burial was effective for dormancy break. Fruits placed on bare soil did not germinate at

all. An additional burial trial found that during burial in field and nursery soil 41.6% and 63.7% of the endocarps + seeds germinated respectively, after 36 months. *Ex situ* post burial germination was cyclic, with germination only occurring following autumn retrievals and was highest after 30 months burial (45.4% in nursery burials and 24.3% in field burials). Highest germination from seeds contained within intact endocarps occurred in seasonal simulation trials when the dry summer season was long (20 weeks), had fluctuating temperatures (30/50 °C), with two long (7 days) wet cycles and moist winters at 10/20 °C. Stratification trials showed that best germination occurred with 12 weeks warm pre-treatment (including 2 weeks wet) followed by 6 weeks cold stratification. A nursery burial trial, whereby the summer watering regime was manipulated, initially resulted in significantly more germination occurring in the treatment with four summer wet cycles than any other treatment (natural rainfall, no rainfall or two wet cycles). However, after the second summer there was no significant difference between natural rainfall and four summer wet cycles and these two treatments resulted in significantly more germination than no rainfall or two wet cycles. Summer conditions experienced by the endocarps are critical in releasing seed dormancy and allowing the seed to germinate during wet winter months. This study showed that by closely monitoring environmental conditions endocarps are exposed to, dormancy breaking mechanisms can be identified and used to improve germination.

3.2 Introduction

3.2.1 General

Germination of non-dormant seeds occurs when the appropriate set of environmental conditions is within the range of requirements for radicle emergence from the seed (Baskin and Baskin, 2004) and is completed when the structures surrounding the embryo are penetrated by the radicle (Bewley, 1997). These conditions include:

- an adequate supply of water;
- a suitable temperature;
- sufficient oxygen; and
- appropriate light conditions.

The requirement for these conditions is species dependant (Mayer and Poljakoff-Mayber, 1989) and is generally correlated with the optimal conditions required for *in situ* seedling establishment. The period of time when these conditions occur in the

natural environment is often quite limited, restricting the window when germination and emergence take place.

3.2.2 Dormancy

Seed dormancy has been defined as the failure of an intact, viable seed to complete germination under favourable conditions (Bewley, 1997). Vleeshouwers and Bouwmeester (1995) define dormancy as a block or blocks within the seed itself that prevents germination i.e. internal factors rather than external (environmental) factors. Remaining dormant until suitable environmental conditions are available for germination and seedling establishment is considered advantageous for species survival. Postponement of germination by some sort of dormancy mechanism until favourable growing conditions occur, allows the developing seedling the best chance of survival and establishment.

Seed biologists classify dormancy into five main types (Baskin and Baskin, 2014):

- Physiological dormancy (PD) – physiological inhibiting mechanism within the embryo that prevents radicle emergence;
- Morphological dormancy – the embryo is underdeveloped and requires time to grow and mature within the seed in order to germinate;
- Morphophysiological dormancy – seeds with this type of dormancy are underdeveloped but also have a physiological component of dormancy preventing growth and maturation of the embryo within the seed;
- Physical dormancy – seeds have an impermeable layer which prevents water penetration into the seed and therefore germination; and
- Combinational dormancy – the seed coat is water impermeable and the embryo is physiologically dormant

Seeds with PD have a permeable seed coat, a fully developed embryo and an inherent physiological mechanism that inhibits germination (Cook et al., 2008). It is the most widespread dormancy class (Finch-Savage and Leubner, 2006) and *P. longifolia* has previously been categorised as having PD (Norman and Koch, 2006). PD in seeds can occur at three different levels – non deep, intermediate or deep (Baskin and Baskin, 2001, Baskin and Baskin, 2004, Nikolaeva, 1969).

Embryos excised from seeds with non-deep PD will usually grow with the resulting seedlings appearing normal. Non-deep PD can be broken by relatively short periods of cold stratification, a period of after-ripening, exposure to high temperatures (≥15 °C) or exposure to different stimulants such as Gibberellic Acid (GA_3) (Baskin and Baskin, 2001). Embryos extracted from seeds with intermediate PD also grow and result in normal seedlings, though may require several months of cold stratification to break dormancy.

Deep PD is more complicated and whilst extracted embryos may germinate, this process may not occur rapidly, the seedlings may not develop normally and GA_3 will not stimulate germination in the intact dispersal unit.

Seed physiologists also classify PD based on the mechanisms operating in the dispersal unit. PD can work at either an embryo or seed coat level and is referred to as either "embryo" or "coat" (mechanical) dormancy (Finch-Savage and Leubner, 2006, Leubner, 2012). Embryo dormancy is characterised by blocks that inhibit embryo growth and coat/mechanical dormancy is a block that is imposed by the covering layers of the seed such as a hard woody (but still permeable) endocarp or other covering structures. If the block to germination does not come from the embryo then removal from the enclosing seed coverings should result in immediate germination/embryo growth if the appropriate environmental conditions are applied (Werker, 1997).

3.2.3 Seed structure and dormancy in *Persoonia longifolia*

The natural dispersal unit of *Persoonia* species is a drupe, with a fleshy mesocarp and a hard indehiscent endocarp through which a germinating seedling must emerge. The mesocarp is either consumed by animals such as birds (parrots, cockatoos, emus), marsupials (kangaroos, wallabies) or reptiles (bobtail lizards) which, in the process, disperse the remaining endocarp some distance away from the parental plant or, if left on the ground, the mesocarp generally rots and degrades. The embryo inside the endocarp of *Persoonia* species is surrounded by a thin papery testa and is most often a linear bullet shaped embryo with two to nine cotyledons (Weston, 2003). However, the *P. longifolia* embryo differs from most other species within the genus and is recurved with two cotyledons i.e. campylotropous making the embryos quite distinctive and somewhat unusual (Norman and Koch, 2008, Weston, 1994).

A significant amount of research has been conducted on the germination biology and the alleviation of dormancy in *P. longifolia* over the past ten years with generally very limited success. Table 1 outlines the treatments and approaches that have been tested on *P. longifolia* so far and the key findings from these studies to date. Given the diverse range of treatments and approaches utilised, the key drivers regulating seed dormancy and the exact conditions required for germination still remain essentially unknown for this species. Indeed, none of the methods tried are considered to be reliable or successful enough to produce sufficient plant numbers for mine site restoration.

Germination studies have also been undertaken on other species of *Persoonia* (from New South Wales and Queensland). In particular, *P. virgata* and *P. sericea*, two obligate seeder species, have been studied in some detail (Bauer and Johnston, 1999, Bauer et al., 1999, Bauer et al., 2001, Bauer et al., 2004, Ketelhohn et al., 1998) with germination occurring in both species once at least half of the woody endocarp had been removed. Germination studies on *P. pinifolia* also found that removal of the woody endocarp and application of GA_3 enabled germination of these seeds once free from their covering layer (McIntyre, 1969). A small study has also been undertaken on *P. pauciflora,* an endangered seeder species from New South Wales. Unlike *P. virgata*, *P. sericera* and *P. pinifolia* extraction of seeds did not greatly improve germination with only 2% of seeds showing signs of germination (Frith and Offord, 2010).

Whilst endocarp chipping has been investigated for *P. longifolia* with moderate success (Norman and Koch, 2006) there has been no study to date on the removal of most of the endocarp to promote germination. Some investigations into the most effective method of removing the endocarp are required, because unlike the eastern states species which have a linear embryo, the embryo of *P. longifolia* is curved and removal from the endocarp has so far proven highly problematic due to the damage that occurs to the fragile seeds within during the extraction process.

The aim of this part of the study is to investigate the key mechanisms driving dormancy break and germination of *P. longifolia* under natural (*in situ*) conditions, and replicate this under laboratory and nursery conditions to facilitate the development of reliable propagation approaches. This will involve in-depth germination trials which will:

- characterise the endocarp and seed structure and explore the role of the endocarp in the regulation of dormancy in *P. longifolia* seeds (Section 3.3)*;*

- investigate the impacts of natural field burial on *P. longifolia* endocarps (Section 3.4);
- examine the optimal germination requirements for *P. longifolia* seeds once dormancy has been broken (Section 3.5); and
- use the results from the above studies to investigate potential dormancy breaking treatments under both laboratory and nursery conditions to enhance germination for plant production (Section 3.5).

Table 3.1: Germination testing undertaken on *Persoonia longifolia* endocarps, prior to the commencement of this study.

Paper	Treatments	Key Findings and Results
Mullins et al. (2002)	• Mesocarp removal • Endocarp chipping • Hydrogen peroxide soak • Hydrochloric acid soak • Charring • Watering with smoke water • Giberellic Acid (GA_3) • Fed to Kangaroos and emus • Sun dried • Burial vs surface sown	• Positive result of 25% germination to endocarp chipping, smoke treatment and GA_3 and surface sowing. • Endocarp storage resulted in reduced viability. • Unknown if endocarps stored at other temperatures or on the forest floor will lose viability.
Preston et al. (2002)	One or more of the following treatments: • Mechanical scarification • Removal of the seed coat • GA_3 • Kinetin • Smoke Water • Rinsing with tap water • Soaking • Heat shock • Temperature stratification	Only those species which germinated were reported and as there is no reporting for *P. longifolia* it is assumed that there were no germinants with any of the treatments.
Dixon et al.(2002)	• Acid, base and solvent scarification of endocarp • Nicking of endocarp	• Only 5% germination recorded in nicked endocarps. • No germination in other treatments. • Measures made of force to break seed, lid removal and viability following the various treatments in order to determine best method of breaking down endocarp.

Table 3.1 Cont. next page

Table 3.1 Cont.

Paper	Treatments	Key Findings and Results
Norman and Koch (2005b)	• Field broadcast fresh endocarps onto rehabilitation areas • Scarify treatment • Endocarps potted into sand and/or litter with and without the mesocarp	• Mesocarp removed and endocarps buried under 8 cm of leaf litter gave most promising results (14%). • Broadcast of endocarps onto rehabilitation resulted in 0.33% germination. • Suggested trying broadcast on older rehabilitation areas.
Norman and Koch, (2006)	• Hand chipping/machine chipping • GA_3 • Aging (sonification, liquid nitrogen, rapid drying) • Warm temperature stratification (15/30^{o}C) and constant temperature storage	• Highest germination of 13% was obtained in hand chipping, GA_3 and stratification at 15/30 ^{o}C. • Endocarp aging produced negligible germination. • Hand chipping produced best results out of all other endocarp chipping techniques.
Norman and Koch (2008)	• Aging • GA_3 • Hand chipping • *In situ* burial in litter • *In situ* burial in soil	• *In situ* endocarp burial altered dormancy status and improved germination. • Maximum germination of 36.7% was achieved for endocarps chipped and treated with GA_3 and buried for 21 months. • Micrographs showed the natural fracture line on endocarp absent at 12 months but present at 24 months. • Recommend fresh drupes be buried for 18 months and treated with GA_3 following exhumation.
Turner et al. (2010)	• Burial trial – endocarps buried and retrieved at the beginning of summer and beginning of winter • Endocarps germinated *ex situ* with water, GA, Karrikinolide (KAR_1) and smoke water	• Best Germination occurred ex-situ after 18 months *in situ* burial. • Germinated best with KAR_1 (up to 50%).
Turner (unpublished)	• *Ex situ* temperature stratification treatments combined with a range of moisture treatments • Nursery trials with smoke water, water only, and aerosol smoke	• Highest *ex situ* germination occurring in treatment most closely replicating natural temperatures and moisture patterns (45%). • Germination commenced after 18 months soil burial and began as endocarps entered their second winter.

3.3 Role of the endocarp

3.3.1 Introduction

The natural dispersal unit of *Persoonia longifolia* is a drupe, with a fleshy mesocarp and a hard indehiscent endocarp through which a germinating seedling must emerge. The role of the endocarp in seed dormancy of *P. longifolia* is unknown. The challenge in understanding the germination ecology of seeds covered by a hard endocarp is that characteristics of both the endocarp and the embryo may be involved in delaying germination.

Woody indehiscent endocarps have been found to play a role in the dormancy of many south west Australian native species particularly in the Ericaceae family such as *Astroloma xerophylla* (Turner et al., 2009a). A hard woody endocarp may restrict water and/or oxygen movement from the surrounding environment. i.e. the endocarp could be water and/or oxygen impermeable (Li et al., 1999) The hard nature of an endocarp may also physically restrict (as a mechanical barrier) the growth of an embryo and a seed from pushing through the endocarp ("push power") (Leubner, 2012, Orozco-Segovia et al., 2007). Also, the embryo may have some level of PD and warm and/or cold stratification may be required for dormancy break to occur (Baskin et al., 2005, Chen et al., 2007, Chien et al., 2002, Imani et al., 2011, Persson et al., 2006). However, after the PD is broken, the embryo has enough growth potential to overcome the mechanical resistance of the water-permeable endocarp (Nikolaeva, 1969). In some species the endocarp splits into two parts during seed germination, but in others the endocarp opens via a lid-like structure (Hill, 1933, Hill, 1937)

Investigations of *P. sericea* and *P. virgata* indicate that the endocarp plays a role in limiting germination which increased from 0% to 64.6% in *P. sericea* and to 87.5% in *P. virgata* when over half of the endocarp is removed (Bauer and Johnston, 1999). Pricking or removal of the end of the endocarp does not cause any germination and the authors speculate that the endocarp acts as a mechanical barrier to germination. Germination studies on *P. pinifolia* also found that removal of the woody endocarp together with application of GA_3 enables germination of these seeds (McIntyre, 1969). However, seed extraction of *P. pauciflora* and endangered seeder species from New South Wales, resulted in only 2% germination (Frith and Offord, 2010).

Whilst endocarp chipping has been investigated for *P. longifolia* (Mullins et al., 2002, Norman and Koch, 2006, Norman and Koch, 2008), there has been no study to date on the removal of most or all of the endocarp to promote germination. Mullins et al. (2002) found that endocarp chipping at the site of the natural fracture line (i.e. lid removed) produces a significant increase in germination and that this is further enhanced by application of GA_3 and smoke (25%). Norman and Koch (2006) investigated a number of different endocarp chipping methods (hand chipping, penetrometer probe and machine nicking) and found that hand chipping produces higher germination (up to 13%) than any other chipping and aging methods. Further investigations by Norman and Koch (2008) into burial (21 months) prior to chipping the endocarp and treating with GA_3 resulted in 36.7% germination.

These results all indicate that in the *Persoonia* genus the endocarp plays an important role in regulating seed dormancy. The aims of this investigation into the role of the endocarp were to:

- characterise the endocarp and seed structure;
- determine the impact of the woody endocarp on water uptake of the seed of *P. longifolia*;
- investigate the impact of the endocarp on germination of the seed; and
- investigate the role of GA_3 and Karrikinolide (KAR_1) in germination of *P. longifolia* seeds.

3.3.2 Methods

Fruits were collected on 12th September 2011 at Moodiarrup West Road, Cordering Western Australia. Eighteen replicates of 20 freshly collected fruits (including the mesocarp and skin) were weighed.

The mesocarp was removed by "popping" the endocarp from the skin (exocarp). The endocarp was then agitated in a cement mixer with a pectinase solution (10 ml per 10 L of ultrazyme liquid), scrubbed over flywire and rinsed under tap water to remove the remainder of the flesh. The resultant endocarp containing the seed was then left to air dry for 24 hours before being stored at 15% relative humidity (RH) and 15 ± 2 °C in paper envelopes (King Park seed drying room, located in West Perth, Western Australia) until utilised for experimental purposes.

Seed fill and seed viability were tested using an x-ray machine (MX-20 Digital X-ray, Faxitron, USA) and by tetrazolium (TTC) exposure (1% w/v concentration). Three replicates of 20 seeds were x-rayed, and then the lid was removed using side cutters. The seeds were then placed onto germination paper with TTC and incubated at 30 °C in the dark for 48 hours. Seeds were assessed for staining to determine viability.

3.3.2.1 Endocarp and Seed Characteristics

Fifteen intact seed-filled endocarps were each individually weighed. The seed was then extracted by gently squeezing the endocarp longitudinally in an industrial vice to crack open intact endocarps. Each individual seed was weighed separately after extraction from the endocarp. The contribution of the seed was calculated as a percentage of the intact endocarp mass.

To determine the morphological characteristics of endocarps, they were examined using both an x-ray machine (MX-20 Digital X-ray, Faxitron, USA) and a Scanning Electron Microscope (SEM) (JEOL 6000 with an accelerating voltage of 15 kV). Each endocarp was placed on carbon conductive adhesive tape on 13 mm SEM specimen stubs and evaporatively coated with 2 nm of gold for examination by SEM.

3.3.2.2 Seed Imbibition and Moisture Content

Imbibition tests were undertaken at room temperature (22-24 °C) to determine how rapidly the fruits, endocarps and extracted seeds imbibe moisture. When the seeds emerge from the endocarp during germination, a lid or valve is dislodged along a predetermined fracture line (Fig. 3.1). To determine if this lid limits water uptake, imbibition tests were also undertaken on endocarps with the lid carefully removed. The lid was removed using side cutters and the exposed seed was examined to ensure that the seed was not damaged in anyway.

Previous studies (Turner et al., 2010) have shown that burial for 18 months promoted germination in *P. longifolia* endocarps. In order to determine if this is a result of an increased ability of the endocarp or fruit to take up water, imbibition tests were also undertaken on fruit, endocarps and seeds that had been soil buried in a nursery environment for 18 months (September 2011 – March 2013). Fruits and endocarps were placed in mesh bags and buried in clean washed river sand in large deep tubs in

spring (i.e. 2 weeks following collection). They were kept in a nursery environment under shade cloth but were not artificially watered, only receiving natural rainfall which included numerous summer thunderstorms during the two summers the endocarps were buried.

Therefore, imbibition tests were undertaken on the following:

- intact endocarp (not buried);
- endocarp with lid removed (not buried);
- extracted seed with breached testa;
- whole fruit buried for 18 months;
- intact endocarp buried for 18 months;
- endocarp buried for 18 months and lid removed once retrieved; and
- seed extracted from endocarps buried for 18 months.

Three replicates of 20 seeds or endocarps were used for the first four treatments. In those treatments where the endocarp was buried, there was some germination during the period of burial and some seeds were damaged during the cracking process. As a result the numbers of seeds and endocarps in each replicate was variable (8 to 19) in the last three treatments.

Each set of seeds or endocarps was submerged in water in Petri dishes lined with germination papers, and maintained under ambient conditions (22-24 °C) for the duration of the imbibition experiment. Seeds and endocarps were weighed, moistened for 10 minutes then blotted dry and reweighed (Time 0). At 1, 2, 4, 8, 24, 48, and 72 hours replicates were then removed from the Petri dish, blotted dry and reweighed. After 72 hours extracted seeds became soft and began to disintegrate making them difficult to weigh accurately. Therefore at the 72 hour point the final measures were made. The percentage increase in weight was calculated according to Turner et al. (2009a) using the equation:

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

where W_i and W_d are the mass of imbibed and dry (pre-imbibed) seeds respectively.

In addition to the above treatments reverse imbibition studies (i.e. dehydration) were undertaken on fully imbibed endocarps and fully imbibed extracted seeds.

Seeds were extracted from the endocarps and hydrated as previously described for 36 hours to ensure that they were fully imbibed, then allowed to dehydrate naturally by placement onto a dry seed germination paper inside a fresh Petri dish. Endocarps and seeds were weighed at 1, 2, 4, 8, 24, 48 and 96 hours.

Seed moisture content of endocarps and seeds was measured using three replicates of 20 seeds which were weighed then dried for 17 hours at 103 °C then reweighed (International Seed Testing Association, 1999). Moisture content was determined for:

- intact air-dried endocarp;
- hydrated endocarp;
- air dried excised seeds;
- extracted then hydrated seeds;
- seeds extracted from a hydrated endocarp; and
- seeds extracted from endocarps imbibed for 8 weeks.

Seeds and endocarps were hydrated for a period of 96 hours. Seeds derived from hydrated endocarps were quickly extracted and the initial weight was determined as soon as possible after extraction. Seed moisture content was determined gravimetrically on an oven dry mass (DW) basis using the equation:

$$\% \text{ seed Moisture Content (MC)} = [(W_f - W_d)/W_d] \times 100$$

where W_f and W_d are the mass of fresh and dry (pre-imbibed) seeds respectively.

3.3.2.3 Dye Uptake

In order to track water movement through the endocarp and seeds, dye uptake studies were undertaken on both non-buried (control) and buried endocarps. Fruits were collected and cleaned in August 2013 and control endocarps were stored in the Kings Park seed drying room (maintained at 15% RH and 15 $\pm$ 2 °C) over the summer months. Experimental endocarps were buried for five months over summer and were wet four

times during burial to break dormancy (see Section 3.4.2.6 for detailed wetting methodology). These seeds were retrieved in April 2014 for testing.

Initial testing of various stains showed that 1% w/v acid fuchsin was the best stain for observing water movement through the endocarp. All endocarps were placed in the acid fuchsin solution and were retrieved and assessed after 1, 2, 4, 8, 24, 48, 72 and 94 hrs. Endocarps were cracked open using a vice and the movement of dye through the endocarp and seeds was examined under a binocular microscope (Nikon SMZ25 microscope) and visual observations documented with a Nikon P2-FIRL camera attachment. At each retrieval five endocarps from each treatment were examined and the presence or absence of dye was recorded in each of the following categories:

- external staining of endocarp;
- internal staining of endocarp;
- penetration through the micropyle;
- penetration through channels formed by the remnants of the funicular vascular bundles;
- staining of the testa at the micropyle end of the seed;
- staining of the testa at the hilum end of the seed; and
- staining of the seed.

3.3.2.4 Effect of endocarp removal on germination

Germination tests were undertaken in order to determine if the endocarp provides a constraint to germination of *P. longifolia* seeds. To test this hypothesis the following treatments were assessed:

- intact endocarp (control);
- endocarp with the lid removed (Fig. 3.1a) – along the natural fracture lines where the seed naturally emerges using side cutters;
- removal of over 50% of the endocarp by cracking in a vice and prising apart (Fig. 3.1b); and
- naked seed – total removal of the endocarp and testa breached (Fig. 3.1c).

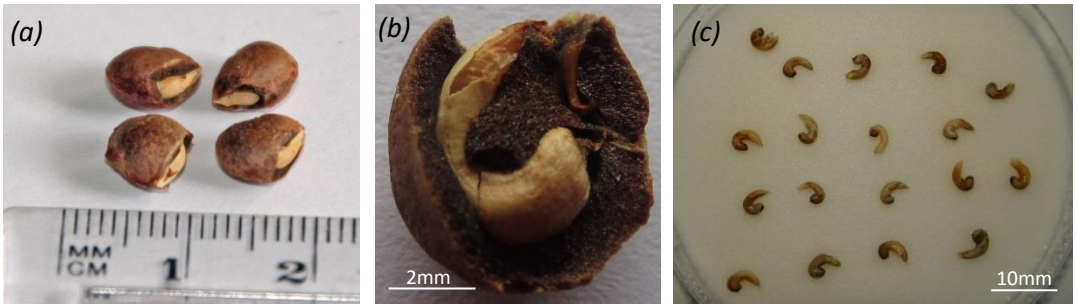

Figure 3.1: Treatments for the endocarp removal germination tests. (a) Lid removed. (b) >50% of the endocarp removed. (c) Naked seeds with testa breached.

A subset of seeds and endocarps in each treatment were also treated with either water, 100 ppm GA_3 or 100 ppb KAR_1 with the exception of those seeds with >50% endocarp removal and naked seeds. Due to significant breakages during the extraction process there were only sufficient seeds to test water and one of the germination stimulants in each of these treatments. KAR_1 was chosen as it was shown to result in an increase in germination in previous studies (S. Turner, pers. comm.). Four replicates of 20 seeds were used for each treatment.

Seeds were surface sterilized in a 2% (w/v) calcium hypochlorite [$Ca(ClO)_2$] solution for 30 minutes (with the exception of naked seeds which were bleached for 15 minutes only, due to concern that the sterilization process may damage the seed). The seeds and endocarps were then rinsed three times in sterile water prior to experimentation (Turner et al., 2009a). Seeds and endocarps were then placed in 90 mm Petri dishes on germination papers (Advantec (Dublin, CA, USA) 84 mm germination papers) and were moistened with either 100 ppm GA_3, 100ppb KAR_1 or water, incubated at 15 °C in light (12 hours light/12 hours dark) and checked weekly for germination for 8 weeks. After this time the endocarps and seeds had significant mould growth and the trial was terminated.

3.3.2.5 Data Analysis

Imbibition and moisture content data were analysed using a linear regression model. The data were inspected graphically using plots of residuals and quantile-probability (qq) plots to assess model assumptions (Enright et al., 2011). Moisture content data were $\log_e$ transformed to satisfy assumptions of normality. The full model with seed

state, burial treatment and interactions was examined. This model was then reduced by omitting all non-significant interactions (5% significance level) in a stepwise manner. Comparisons between the different treatments were made using Tukey's HSD.

Germination data were analysed using a binomial Generalised Linear Mixed Model (GLMM). A full model including the seed state (i.e. seed, 50% of endocarp removed, lid removed or intact endocarp), treatment (GA, KAR_1 or water) and interactions was examined.

Germination rate for endocarp removal was calculated by the method outlined in Preston et al. (2002):

$$GR=\frac{(n_1 xt_1) + (n_2 xt_2) + \ldots (n_x xt_x)}{X_n}$$

where n_1=number of germinants at day of first recording, t_1=weeks from start of first recording and X_n= total number of germinants. The lower the value the faster the germination rate.

Germination rate was analysed using a linear regression model and inspected graphically as described above. Data was transformed using a $\log_e$ transformation to ensure it fitted with the assumptions of normality. A full model including seed state, treatment and interactions was examined. This model was then reduced by omitting all non-significant interactions (5% significance level).

Non-transformed data appears in all figures and tables. All analyses were undertaken in the statistical program R (R Core Team, 2013) using the *lme4*, *mgcv* and *lsmeans* packages (Bates et al., 2014, Lenth, 2014, Wood, 2011).

3.3.3 Results

3.2.3.1 Endocarp and Seed Characteristics

Mean mass of 20 fresh fruits was 20.4 ± 0.3 g and mean mass of a single fruit was 1019.4 ± 12.7 mg. Mean mass of a single intact endocarp was 150.4 ± 5.3 mg. Based on mass, the seed component of the intact endocarp was 11.6 ± 0.2% of the total mass. Cleaned endocarps had a seed fill of 98.3 ± 1.7% and TTC testing indicated that 94.7 ± 3.0% of seeds were metabolically active i.e. viable.

The structure of *P. longifolia* endocarps and seeds are shown in Fig. 3.2. The endocarp has a predefined fracture line at the radicle end of the endocarp (Fig. 3.2a). This fracture line forms a lid (variously referred to as a lid, valve or latch throughout the literature) which is a segment of the endocarp that is dislodged during germination. The fracture line along which the lid is fused to the rest of the endocarp has been found to be rich in suberin (Dixon et al., 2002), a fatty acid polymer that renders the surface impermeable to water. The micropyle and remnants of the funicular vascular bundle remain as open channels in the dormant endocarp (Fig. 3.2c). These could possibly operate as channels for water entry into the seed during the imbibition process

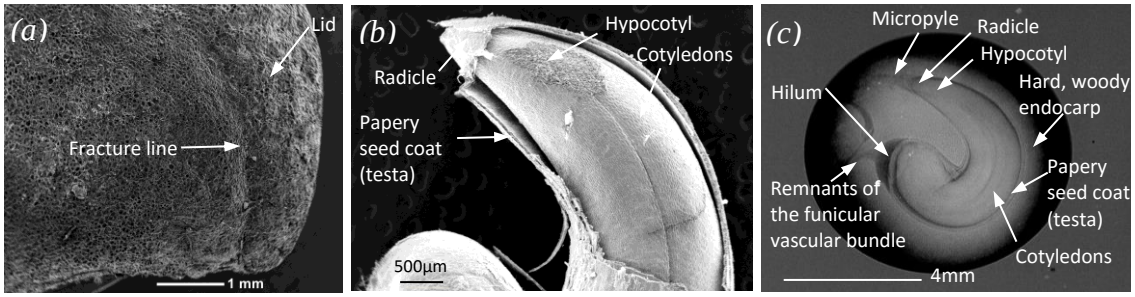

Figure 3.2: Endocarp and seed structure of *Persoonia longifolia*. *(a)* Scanning Electron Microscope (SEM) image of hard woody endocarp showing the pre-defined fracture line and lid. *(b)* SEM image of seed and testa. *(c)* X-ray of seed inside the endocarp.

3.3.3.2 Imbibition and Seed Moisture Content

All seeds and endocarps imbibed water (i.e. increased in mass) to some degree indicating that the seed coverings are permeable to water. There was no significant effect of burial on the ability of seeds or endocarps to imbibe water, however, removal of the seed from the endocarp did result in a significant increase in water uptake (Table 3.2, Fig. 3.3). Both sets of extracted seeds imbibed significantly more water than those

treatments where the seed was retained in the endocarps (Fig. 3.3). Interestingly, endocarps with the lid removed did not imbibe significantly more water than those endocarps where the lid was not removed and this did not vary significantly with either buried or non-buried endocarps.

Table 3.2 Mean percentage increase in mass (gm) + Standard Error (SE) after 72 hours. Levels within each treatment with the same letter are not significantly different.

Treatment	Level	Mean % increase in weight ± SE	P Value
Burial	Buried	22.71 ± 3.23	NS
	Not buried	21.76 ± 3.58	
Reproductive Unit	Fruit	31.33 ± 1.16[a]	<0.001
	Endocarp	15.17 = 0.07[b]	
	Endocarp with lid removed	12.41 ± 0.34[b]	
	Extracted seed	34.80 ± 2.57[a]	

Fruit that had been buried and retrieved increased in mass by 31.3 ± 1.2% (Fig. 3.3) and this increase largely occurred over a period of 48 hours. At least some of this increase is likely to be a result of the fleshy mesocarp rehydrating.

Reverse imbibition showed that imbibed seeds decreased in mass by 37.1 ± 1.3% after 96 hours and fully imbibed endocarps decreased in mass by 15.6 ± 0.2%.

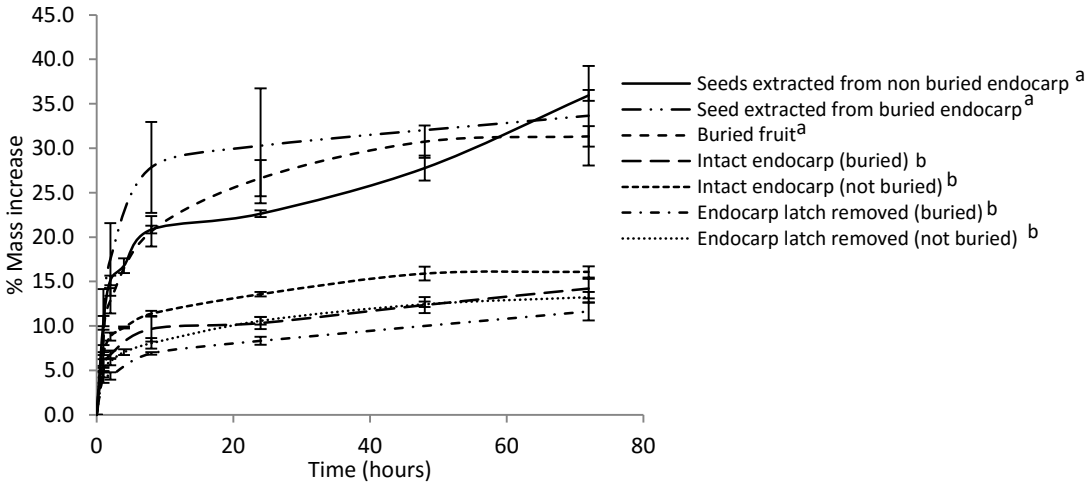

Figure 3.3: Imbibition curves for *Persoonia longifolia* endocarps and seeds immersed in water. Points show mean percentage increase in mass ± SE. Treatments with the same letter do not differ significantly.

The moisture content of hydrated, extracted seeds was significantly higher than that of all other treatments (Fig. 3.4, P<0.001). There was no significant difference in moisture

content of seeds extracted from endocarps imbibed for 8 weeks or 96 hours. Dried endocarps and dried excised seeds had significantly lower moisture content than all other treatments (Fig. 3.4).

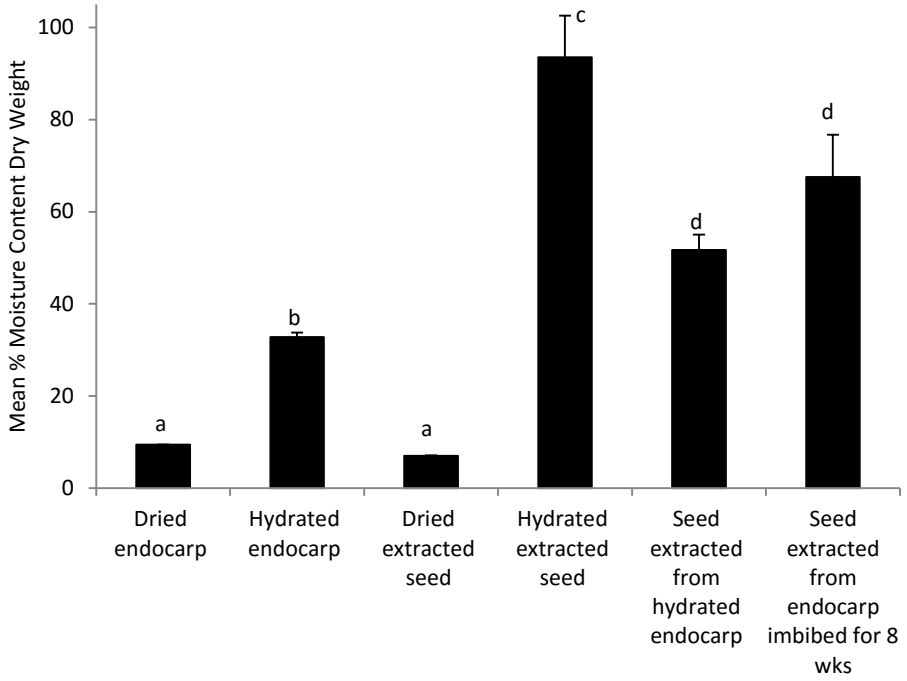

Figure 3.4: Percentage moisture content (on dry weight basis) (mean + SE) of *Persoonia longifolia* fruits, endocarps and seeds after exposure to different treatments. Treatments with the same letters do not differ significantly.

3.3.3.3 Dye Uptake

The external surface of the endocarp of all seeds stained almost immediately when placed in the dye. There was no discernible difference in dye uptake between control endocarps and buried endocarps (non-dormant). At the 8 hour assessment, the dye had penetrated through the micropyle and hilum ends of the seed (Fig. 3.5). Staining of the testa at 8 hours was patchy and generally limited to the areas around the micropyle and channels at the hilum end of the seed. However by 48 hours the testa was generally stained throughout. Interestingly, no dye penetrated the seed at any stage during the trial.

Further experimentation by extracting the seeds from the endocarp and then immersing in the dye solution indicated that dye moved into the seed where a breach in the testa occurred. Extraction of the seed from the endocarp without breaching the testa has

proven extremely difficult and therefore an investigation into dye movement through the intact testa has not been undertaken.

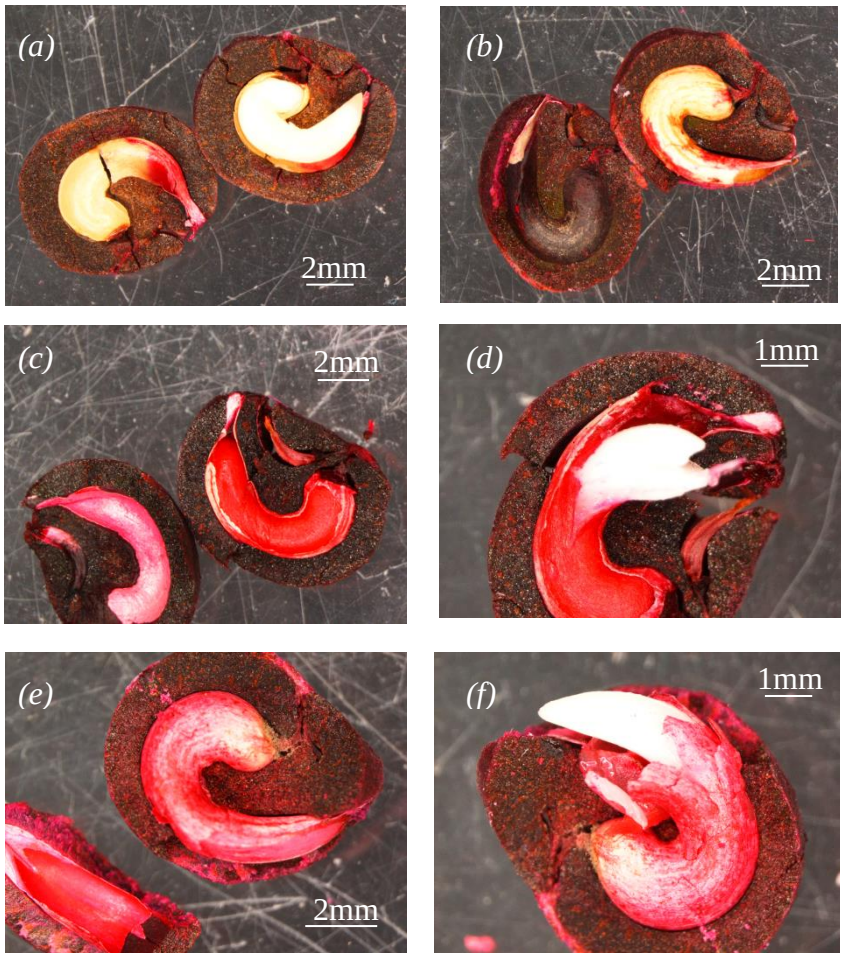

Figure 3.5: Example of dye penetration at: *(a)* 8 hours (control endocarp). *(b)* 8 hours (buried endocarp). *(c)* 48 hours (control endocarp). *(d)* Same endocarp with testa removed and embryo exposed. *(e)* 48 hour (buried endocarp). *(f)* Same endocarp with testa removed and embryo exposed.

3.3.3.4 Effect of Endocarp Removal on Germination

No germination was observed in the control (i.e. intact endocarps) over the 8 weeks of the trial, however, germination was observed in all other treatments (Table 3.3, Fig. 3.6). Endocarp treatment, type of germination stimulant applied to the seed and the interaction between the two, had significant impacts on final germination percentage of *P. longifolia* endocarps and seeds ($P<0.001$ in all three cases). Removal of both the entire endocarp and >50% of the endocarp resulted in significantly higher germination than either removing the lid or leaving the endocarp intact ($P<0.001$). The use of GA_3 resulted in significantly less germination than either KAR_1 or water ($P<0.001$ in both

cases) however this may be a result of GA_3 only being used on seeds with intact endocarps or with only the lid removed where germination was low. There was no significant difference between the use of water or KAR_1 (P=0.700) to promote germination.

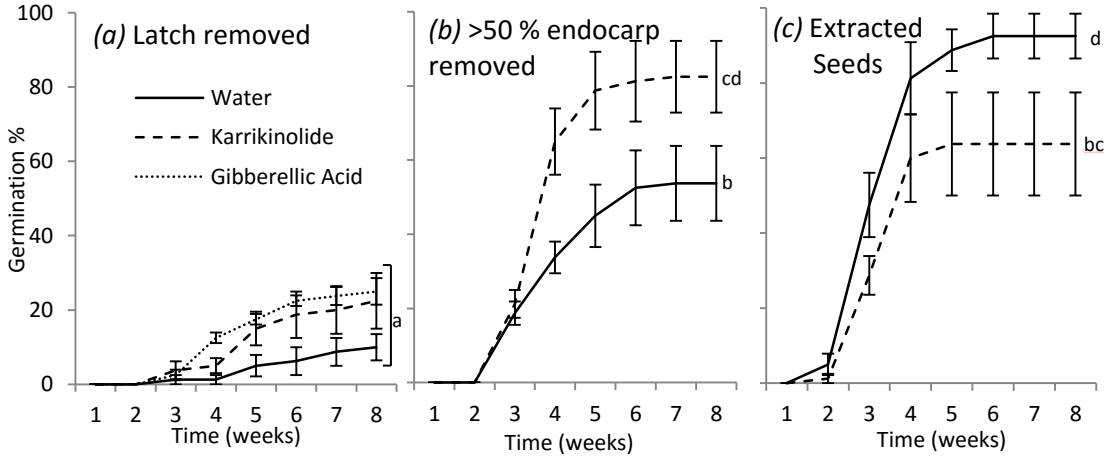

Figure 3.6: Cumulative germination percentage (mean + SE) of *Persoonia longifolia* seeds with varying degrees of endocarp removal; *(a)* Lid removed. *(b)* >50% endocarp removed. *(c)* Naked seed. No germination occurred in seeds from within intact endocarps and therefore the results are not presented here. Treatments with the same letter did not differ significantly in final germination percentage.

Contamination by mould in the germination trials was a significant problem particularly in the treatment seed + KAR_1 and this may have affected the results. However, almost 100% of viable seed germinated in the naked seed + water treatment (seed viability was assessed as 94.7% using a tetrazolium test) and this was significantly higher than all other treatments except >50 % endocarp removal + KAR_1 (P<0.001, Fig. 3.6).

When all the endocarp was removed, or >50% of the endocarp removed, germination commenced in week two with the majority of seeds germinating between weeks three and four (Fig. 3.6). Germination was significantly slower in those seeds in endocarps with the lid removed than either seeds with >50% of endocarp removed or extracted seeds (P<0.001). The germination rate was quickest (i.e. lowest value) in seeds with no endocarp (seeds only) and this slowed (i.e. greater value) with increasing endocarp on the seed (Table 3.3, P<0.001). There was no significant impact of germination stimulant on the germination rate.

Table 3.3: Mean final germination rate of *P. longifolia* seeds and endocarps over 8 weeks (Note: the lower the number the quicker the seeds germinated).

Treatment	Germination Rate
Endocarp only + Water	NA
Endocarp only + KAR_1	NA
Endocarp only +GA	NA
Lid removed + Water	6.0 $\pm$ 0.8
Lid Removed + KAR_1	5.1 $\pm$ 0.2
Lid Removed +GA	4.7 $\pm$ 0.3
>50% endocarp removed + Water*	4.1 $\pm$ 0.3
>50% endocarp removed+KAR_1	4.0 $\pm$ 0.1
Seed + Water	3.6 $\pm$ 0.2
Seed + KAR_1*	3.5 $\pm$ 0.1

*indicates treatments where mould contamination occurred and was likely to have affected the results.

The process of germination in *P. longifolia* seeds is not straightforward. When the seed is extracted from the endocarp (or at least 50% of the endocarp is removed), and the testa is breached, the cotyledons appear to turn green and the hypocotyl extends prior to any radicle extension and development (Fig. 3.7). This process is very slow and can take between 30 and 50 days and can vary quite considerably between the degree of colour change and the amount of cotyledenous or hypocotyl extension that occurs prior to radicle extension (Fig. 3.7b).

3.3.4 Discussion

Extracted seeds, intact endocarps and fruits of *P. longifolia* all imbibed water indicating that they were permeable and that delayed germination was not due to a water impermeable barrier i.e. physical dormancy. Endocarps, however, did not achieve the same degree of water uptake as the naked seeds suggesting the hard endocarp acts as a partial barrier to water uptake. The amount of water uptake for buried endocarps of *P. longifolia* was similar to that observed by Norman and Koch (2008) (10-16%). Interestingly, removal of the lid or burial did not significantly improve water uptake. This degree of water uptake is also similar to that observed in *Astroloma xerophyllum* (a Western Australian species where the seed is also contained in a hard woody endocarp) which mass increase was 38% for extracted seeds but only 16% for intact endocarps after exposure to moisture. African olive seeds (*Olea eropaea* subsp. *cuspidata)* which also have a similar endocarp structure to *P. longifolia* do not show an increase in water uptake when scarified on one end (Cuneo et al., 2010).

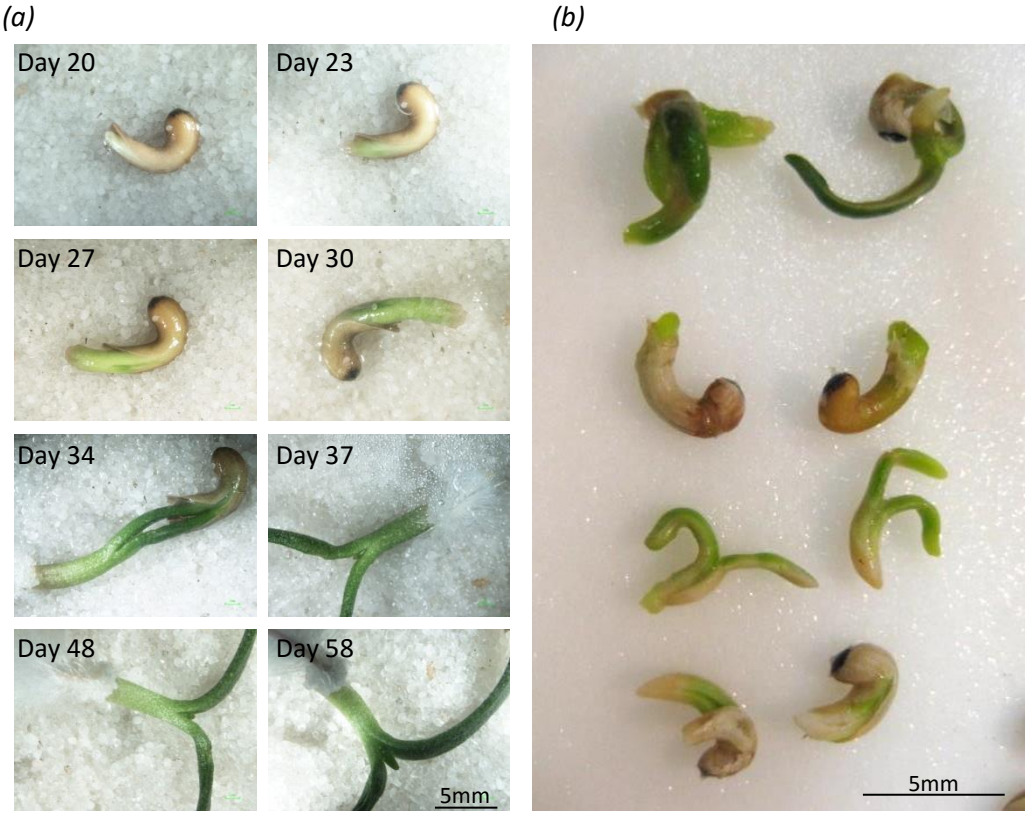

Figure 3.7: Germination of extracted *Persoonia longifolia* seeds. *(a)* The germination process in a single seed. Colour change and hypocotyl extension began on day 23 of the trial and radicle extension commenced day 30. *(b)* Variations of the germination process in a number of seeds, ranging from complete colour change in the cotyledons and very little radicle extension at the top to some radicle extension and some colour change at the bottom.

Dye uptake studies showed, water movement into the endocarp and seed occurred through the channels at either end of the seed and more slowly through the endocarp itself. The dye did not penetrate the seed at any stage however imbibition trials have shown that water does move into the seed through the testa (Fig. 3.4).

Seeds extracted from hydrated endocarps had significantly lower moisture content than seeds hydrated after extraction even when the endocarps were hydrated for a period of 8 weeks prior to the seeds being excised. It would therefore seem reasonable to conclude that whilst the endocarp is permeable, some restriction of water uptake does occur and the seeds within them do not imbibe water to the same degree as extracted seeds. This may impact the growth potential and therefore germination of the seed. However,

dormancy break through burial did not improve water uptake and neither did removal of the lid, two treatments that were clearly shown to promote germination.

Despite issues associated with contamination, a common problem with *Persoonia* species (Bauer and Johnston, 1999, Frith and Offord, 2010, McIntyre, 1969), germination percentages of *P. longifolia* increased when the endocarp was removed, indicating that it is providing some mechanical constraint to germination. Germination of other species of *Persoonia* have also been shown to increase when the seeds are extracted from the endocarp or when the lid is removed (e.g. *P. pinifolia* but only when GA_3 was applied, (McIntyre, 1969, Rintoul and McIntyre, 1975); *P. sericea* and *P. virgata* (Bauer and Johnston, 1999). However, simply filing or scarifying the endocarp does not seem to have the same effect as complete removal of the endocarp or even removal of the endocarp lid in either *P. longifolia* (Norman and Koch, 2006) or *P. elliptica* endocarps (Abbott and van Heurck, 1988). Germination after the endocarp was removed did not occur immediately and took between 2 and 4 weeks to commence indicating that there is some block to germination also occurring in the embryo itself.

Burial of *P. longifolia* endocarps has been shown to improve germination (see Section 3.3) and therefore some other environmental conditions must be releasing dormancy imposed by the hard woody endocarp and embryo. Based on the results from this study, physical dormancy can be excluded as all seeds, endocarps and fruits readily imbibed water. Morphological and morphophysiological dormancy can also be excluded as seeds possess a large well-developed cotyledenous embryo (Fig. 3.1). *Persoonia longifolia* can therefore be classified as having physiologically dormant seeds and thus possesses an embryo with limited growth potential that, when dormant, cannot overcome the constraints of the surrounding endocarp layers. In many plant species the seed covering layers have been shown to present a physical constraint to radicle protrusion (Bewley, 1997, Kelly et al., 1992) and either the embryo growth potential needs to increase to overcome the mechanical constraint and/or the mechanical constraint associated with the seed covering layers needs to be reduced (Finch-Savage and Leubner, 2006).

The germination process in *P. longifolia* is not straight forward with cotyledons changing colour and extending prior to radicle extension (Fig. 3.7). A similar phenomenon was noticed in *P. pauciflora* where two patterns of embryo development

were observed (Frith and Offord, 2010). The first being radicle emergence followed by removal of the testa through growth of the cotyledons (this is the generally accepted definition of germination, C. Baskin pers. comm.). The second pattern of germination in *P. pauciflora* occurs when the testa is breached during the seed extraction process and this resulted in the cotyledons curving outwards and the true leaves developing before radicle development.

In *P. virgata,* the embryos develop through recurvature of the cotyledons and the plumule (or shoot tip) grows upwards. The radicle develops below the cotyledons (Bauer and Johnston, 1999). Similar patterns were observed in *P. pinifolia* (McIntyre, 1969). No indication is given in any of the above references regarding seedling survival in relation to seeds extracted from the endocarp.

Germination of *P. longifolia* from within the endocarp seems to follow a similar pattern to germination of the naked seed, with the hypocotyl and cotyledons extending and pushing the radicle through the endocarp. The hypocotyl and cotyledons then begin to turn green before radicle extension begins to occur (Fig. 3.8). In the example shown in Figure 3.8, radicle extension does not begin to occur until sometime between Day 19 and Day 22 after the germinant has begun to emerge from within the endocarp.

Epicotyl dormancy refers to seeds where there is a delay of approximately 4 weeks in the emergence of the shoot after the radicle has emerged and is a common phenomenon in seeds with morphophysiological dormancy and is also found in seeds with physiological dormancy although is much less reported (Baskin and Baskin, 2014). Radicle dormancy (where dormancy has been broken, the cotyledons extend but the root does not grow) has been found in species including *Hydrophllum macrophyllum* (Baskin and Baskin, 1983), *Hydrophyllum appendiculatum* (Baskin and Baskin, 1985) and *Asarum canadense* (Baskin and Baskin, 1986), however in all these cases radicle dormancy is broken by warm stratification and the radicle emerges prior to any epicotyl or cotylendenous growth.

This study indicates that *P. longifolia* appears to have radicle dormancy with extension of the hypocotyl and growth of the cotyledons occurring before any extension of the radicle. This phenomenon, where the cotyledons turn green prior to root extension is also observed in *Tillandsia recurvata,* an epiphytic bromeliad (Montes-Recinas et al.,

2012). Germinants from extracted seeds of *P. longifolia* were not potted up so it is still unknown if these would produce developmentally normal seedlings.

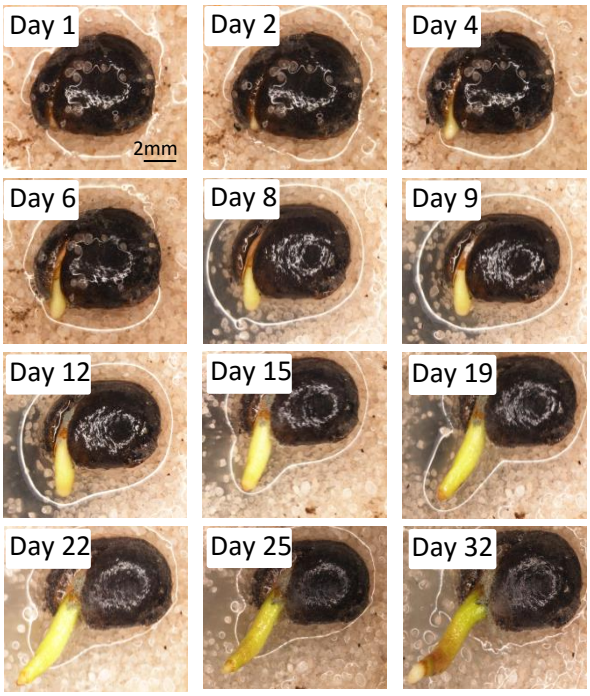

Figure 3.8: Germination process of a seed from within an endocarp showing hypocotyl and cotyledonous extension occurring prior to radicle extension. Day 1 is when the endocarp begins to rupture. Radicle extension does not begin to occur until Day 19 after emergence from the endocarp.

Exposure to the varying temperatures and moisture conditions experienced by *P. longifolia* endocarps when buried may result in either the weakening of the endocarp or a physiological change within the embryo itself which enables the embryo to push through the hard outer seed layers. When buried endocarps have been retrieved over time, it has been noticed that the lid becomes easy to dislodge without any mechanical intervention. Prior to burial it is not possible to remove the lid without some mechanical intervention such as excision with side cutters.

Whilst germination in *P. longifolia* can be promoted through the removal of the endocarp, the removal process is time consuming, not economical and it is currently not known if these seedlings would develop normally, therefore breaking dormancy through different methods needs to be developed. This breaking down of the endocarp occurs naturally during the burial process and includes cracking of the endocarp and loosening of the lid. Although this does not result in an increase in water inflow to the seed (as

seen by the imbibition trials) it may weaken the endocarp sufficiently for the seed to push through, break dormancy within the embryo itself or through a combination of both, rendering seeds more germinable overall.

3.4 The impact of burial on dormancy loss and germination

3.4.1 Introduction

Many species with hard woody fruits from the south western corner of Australia are difficult to germinate due to seed dormancy (Abbott, 1984a, Abbott and van Heurck, 1988, Merritt et al., 2007, Mullins et al., 2002, Norman and Koch, 2008, Turner et al., 2009a, Turner et al., 2010, Turner et al., 2006). These seeds are often described as having physiological dormancy which is caused by a physiological inhibiting mechanism within the embryo that prevents radicle emergence. Structures that cover the embryo, such as the seed coat and indehiscent fruit wall, may also play a key role in suppressing germination (Baskin and Baskin, 2001, Finch-Savage and Leubner, 2006, Leubner, 2012, Tieu and Egerton-Warburton, 2000, Werker, 1997).

Burial has been investigated as a means of alleviating seed dormancy in some Australian species with indehiscent endocarps including *Leucopogon* species (Ooi et al., 2007, Turner et al., 2010), *Astroloma epacris* and *Hibbertia* spp. (Turner et al., 2010), *Leucopogon propinquus* and *Styphelia tenuiflora* (Norman and Koch, 2008), and *Astroloma xerophyllum,* and *Gahnia grandis* (Merritt et al., 2007), with varying degrees of success. Burial trials have also been undertaken on species from other countries around the world such as *Opuntia tomentosa* (Olvera-Carrillo et al., 2009) and *Empetrum hermaphroditum* (Baskin et al. 2002) which also have a hard but permeable seed or fruit coat and which also display problematic germination.

The reasons for the increases in germination of species with indehiscent endocarp following burial are unclear. Fluctuations in environmental conditions such as temperature, moisture and light may alleviate dormancy in soil stored seeds (Tieu and Egerton-Warburton, 2000). However physical changes to the integrity of the hard seed covers may also affect germination of buried seeds (Olvera-Carrillo et al., 2009). Seeds with hard woody endocarps are not necessarily impermeable to water but these covering structures may restrict water uptake to some degree and mechanically restrict radicle

emergence (Olvera-Carrillo et al., 2009, Orozco-Segovia et al., 2007, Tieu and Egerton-Warburton, 2000, Turner et al., 2009a).

Whilst most *P. longifolia* endocarps and seeds are completely burnt during even a mild fire when on the soil surface (K. Chia pers. obs.) it is possible that the removal of leaf litter, decrease in soil water repellency (Granged et al., 2011) or the release of different nutrients to the soil as a result of fire may also assist with germination of soil buried *P. longifolia* seeds.

Temperature is often cited as being the most important environmental factor in alleviating seed dormancy, though cycles of wetting seeds and allowing them to dry out again have also been associated with dormancy release in some species (Baker et al., 2005a, Baskin and Baskin, 1984, Bell, 1999, Hintikka, 1990, Woodall, 2004). Wetting and drying cycles increases germination in *Sida spinosa* and this can be attributed to these cycles increasing the permeability of the seed coat (Baskin and Baskin, 1984). Dormancy release occurs more rapidly in *Actinontus leucocephalus* in warm temperatures when several wetting and drying cycles are used (Baker et al., 2005b). Seeds of *Rumex acetosella* kept moist for 5 years remain dormant. When dried and then remoistened seeds proceed to germinate to levels of 76.7% (Hintikka, 1990). This is seen as an adaptive mechanism to allow the seeds to colonise newly bare rocky outcrops (where soil would rapidly dry out) without any competition. Merritt et al. (2007) indicates that fluctuations in soil moisture closely corresponds with hydration and dehydration in species from south Western Australia and that these fluctuations in seed moisture content are likely to be regular, large and transitory.

Physical changes in the seed coat have also been attributed to dormancy loss in seeds that have been buried. Soil burial of *Opuntia tomentosa*, which has a hard funicular envelope, induces the formation of a germination valve and a water channel which facilitates water uptake (Olvera-Carrillo et al., 2009, Orozco-Segovia et al., 2007). The entire funicular envelope is damaged during the burial process. Similarly, temporal changes to the seed or fruit coat in *Angiozanthos manglesii and Leucopogon propinquus* have been linked to germination responses after seed burial (Tieu and Egerton-Warburton, 2000).

A number of burial trials have been undertaken over many years with *P. longifolia* endocarps with varying results. Initial burial studies were done by Mullins et al. (2002) who found that burial of the endocarps in potting mix did not result in any better germination than those sown on the surface of the soil after a period of seven months. This trial was followed by a more detailed study into burial, retrieval and germination of seeds from within intact *P. longifolia* endocarps by Norman and Koch (2008) who found that burial altered the dormancy status and improved the germination of *P. longifolia* to a maximum of 36.7% after 21 months burial, followed by endocarp chipping and treatment with GA_3 and smoke. Retrieved, treated endocarps were all surface sown onto punnets under glasshouse conditions and assessed regularly for seedling emergence.

Turner et al. (2010) further investigated the impacts of endocarp burial on seed germination of *P. longifolia* and found that germination was greatest (50.8%) from seeds within those endocarps buried for 18 months and treated with KAR_1 prior to incubation in Petri dishes at 15 °C in darkness. It was suggested in this study that endocarps and seeds of *P. longifolia* require either dry after-ripening which occurs during the summer months and moist stratification during the first winter of soil storage or that the endocarps and seeds require an extended period of exposure to moist soil conditions to become germinable.

The variability of the above studies indicates that the germination requirements for *P. longifolia* are not well understood and that further investigation into the processes that occur during soil burial is required. The aims of this burial study are to:

- determine if endocarp burial increases germination in comparison to non-buried endocarps, and if this burial can be replicated in a nursery environment;
- more accurately identify key seasonal changes driving dormancy loss;
- examine the changes in the endocarp over time with burial and compare this with changes to non-buried endocarps and endocarps buried in soil in a nursery environment;
- determine if burial in differing soil conditions impacts on germination; and
- determine if the mesocarp plays a role in preventing germination of *P. longifolia* seeds.

3.4.2 Methods

3.4.2.1 Germination Following Burial Under Different Soil and Leaf Litter Conditions

This experiment was undertaken to determine if different soil and leaf litter conditions impact on dormancy loss of seeds contained in *P. longifolia* endocarps. Two sets of endocarps were buried with the first set of fruits collected from Haddleton Nature Reserve in November 2009. The fleshy mesocarp was left on half of these endocarps (Batch 1) and removed on the other half (Batch 2) to determine if germination was influenced by the presence or absence of the fleshy mesocarp.

The second set of fruits (Batch 3) was collected at Boddington in August 2006. This batch of fruits was cleaned with the surrounding pulpy mesocarp layer removed using pectinex depulping enzymes (Tieu et al., 2008), and the resulting endocarp was dried at 15% RH and 15 °C for 4 weeks, then frozen at -18 °C until utilised for this study (November 2009).

Endocarps were placed in nylon mesh bags and buried the field site at Cordering in November 2009 (beginning of the summer months). The finely woven mesh bags allowed the transfer of water and solutes around the endocarps. The bags were made so that endocarps could be retrieved, assessed and then returned to the burial site on a regular basis. Each replicate was covered with chicken wire and pegged into the ground to prevent the bags being removed by animals, and ensure that leaf litter was kept over the buried bags where required.

Four replicates of 30 endocarps from each batch (Batch 1 – fresh endocarps with mesocarp off; Batch 2 – fresh endocarps, mesocarp on; Batch 3 – frozen endocarps, mesocarp off) were allocated to one of the following treatments:

- placement directly onto bare earth (no leaf litter present) – bags were simply laid on top of the soil surface;
- buried 2 cm under soil surface with no overlaying leaf litter present;
- placement directly on top of leaf litter;
- placement at the leaf litter/soil interface;
- burial 2 cm under soil surface with leaf litter present on top; and

- burial 2 cm beneath an ash bed with no leaf litter present (wild fire occurred in December 2008 approximately one year prior to burial).

Endocarps were retrieved and regularly assessed during the winter months. The assessments were undertaken in March 2010, October 2010, monthly from April to September 2011, and in July 2012. Bags were retrieved, endocarps where the seed had germinated (Fig. 3.9*a*) were removed and the remainder of the endocarps were returned to the bags and placed back in position until the next assessment.

Endocarps were considered to still be viable if the lid on the endocarp was still attached. Those endocarps with no lid were no longer viable and it was assumed that these seeds within the endocarps may have germinated between assessments (Fig. 3.9*b* and *c*). This assumption was based on the premise that in other germination studies undertaken with *P. longifolia,* the lid comes off the endocarp when the seed germinates and that in the time between assessments it is likely that the seeds germinated and then died inside the bag (Fig. 3.9*c*, S. Turner, unpublished data). This may be an over estimate of the germination however other laboratory based trials have shown that weakening of the endocarp is required before germination can occur and therefore detachment of the lid provides a good indication of likely *in situ* germination.

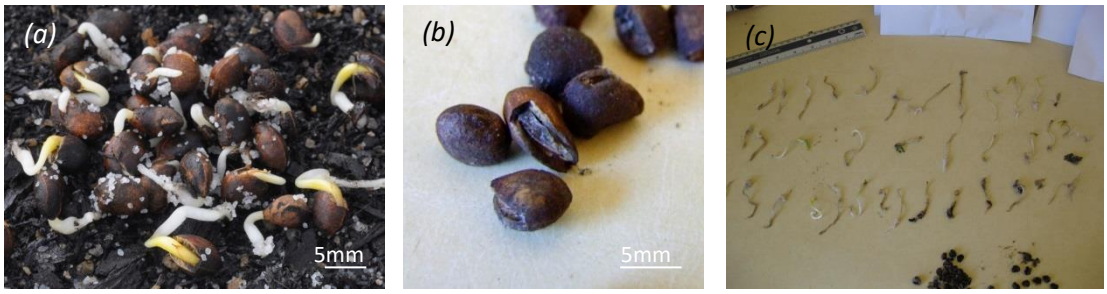

Figure 3.9: Retrieved endocarps *(a)* germinated seedlings found within a retrieved bag (Photo: S. Turner). *(b)* Endocarps without the lid attached after retrieval. These endocarps were assumed to have germinated. *(c)* Germinated seedlings with some seedlings beginning to die before being retrieved.

3.4.2.2 Seed Burial and Retrieval Over an Extended Period of 36 Months

Seed treatment

Fruit was collected on the 3rd September 2010 (batch number VS3059) from the Sotico and Saddleback areas near Boddington. The fruit was cleaned by soaking for 2 days and then scrubbing over a flywire frame to remove the fleshy mesocarp.

Endocarps were placed in nylon mesh bags that allowed soil contact and were buried approximately one month after collection (October 2015). Twenty two bags of 50 endocarps were buried in four randomly selected locations (replicates) within Haddleton Nature Reserve, within an area where mature *P. longifolia* plants naturally occur. Each replicate site (1 m by 0.7 m) was cleared of all litter and then excavated to a depth of 2-3 cm (Fig. 3.10). Bags were buried using the soil previously excavated and leaf litter was respread over the area before it was covered by wire mesh to prevent damage to the seeds or disturbance by animals. Soil moisture and temperature probes (Hobo®) were placed out at each of the burial sites and to log soil moisture and temperature approximately 2-3 cm below the soil surface on an hourly basis. However, the probes failed intermittently resulting in some gaps in data. Data were converted to mean daily maxima and minima temperatures and soil moisture contents.

Figure 3.10: Experimental layout of burial trial. *(a)* Positioning of bags in one replicate. *(b)* the same replicate once bags were buried, leaf litter returned and wire netting placed over the area.

Endocarps were also buried under nursery conditions at Capercup (within Study Area 1 as indicated in Chapter 1). Forty eight bags of 50 endocarps were placed in four replicate plastic tubs (i.e. 12 bags per tub) with clean washed quartz sand. Soil temperature and moisture was recorded in two of the four tubs using soil and temperature probes (Hobo®). Endocarps were kept under 70% shade cloth for the duration of the trial.

A third batch (32 bags of 50 endocarps) was stored at Kings Park in constant temperature and RH (20 °C and 50% RH). These endocarps were used as a control and are referred to as such, from this point onwards.

Seed Retrieval

Endocarps were retrieved from the burial site at various times over a three year period. The retrievals were at approximately six monthly intervals (6, 18, 24, 30 and 36 months) and were timed to occur during autumn (March/April) and spring (September/October). Additional retrievals were made throughout winter (June to August) from the field sites after 9, 16, 17, 19, 20, 21 and 22 months.

At each retrieval, one bag was exhumed from each replicate which was used for germination testing. At six monthly retrievals, an additional bag was retrieved and was used for examination under Scanning Electron Microscopy (SEM), x-rayed for seed fill, and tested for viability.

At each retrieval, endocarps in all bags were scored for *in situ* seed germination. Seeds were considered to still be viable if the lid on the endocarp was still attached. Seeds from empty endocarps with no lid attached were assumed to have germinated as there was evidence of old shrivelled *P. longifolia* seedlings in the bags. Seeds that germinated early in the three years would have been unable to survive in the bags until the next season (Fig. 3.9). Therefore a seed was defined as having germinated if the lid had broken away from the endocarps even if no seedling was attached. Whilst this may provide an overestimate of *in situ* germination, comparison of these numbers with emerging seedlings in later nursery trials indicates that these estimates are reasonably accurate. Endocarps from which the seed had germinated were discarded and the remainder of the endocarps were then placed in a sealed envelope. These were then stored at room temperature for 24 hours prior to additional experimentation.

X-ray analysis

All endocarps exhumed, and that had not germinated, were x-rayed (MX-20 Digital X-ray, Faxitron, USA) for seed fill and those not containing a seed were discarded. In addition, four endocarps from each of replicate retrieved at 6, 12, 18 and 36 months were examined in detail by x-ray analysis to determine if any structural changes had occurred. Ten endocarps from each replicate were then used in the SEM analysis and the remaining endocarps from each replicate were used for viability testing.

Scanning Electron Microscopy

Retrieved endocarps were also examined by SEM to determine the changes that occurred to the endocarp over time and with burial. Endocarps were examined at yearly intervals for each different treatment. For each annual retrieval, the following components of the endocarps were examined:

- surface structure around the lid area;
- surface structure around the micropyle and hilum; and
- internal seed structure – endocarps were cracked open and the micropyle and other channels and cavities inside the endocarps were examined in detail.

For each of these different examinations, one endocarp from each replicate from each treatment was used (i.e. three seeds in total from each replicate). Seeds were placed on carbon conductive adhesive tape on stubs and evaporatively coated with 200 nm of gold. All samples were examined using a JEOL 6000 SEM with an accelerating voltage of 15 kV.

Viability testing

Seed viability was tested using TTC exposure at each six monthly retrieval time as described in Section 3.3.2.

Germination testing

Filled endocarps from field burial and nursery burial where the seed had not germinated *in situ*, were then used in *ex situ* germination tests. Endocarps were surface sterilized, placed into Petri dishes on sterilised silica sand irrigated with either 1µM KAR_1 or distilled water. For each experimental unit, half the endocarps were used for the KAR_1 treatment and half were used for the distilled water treatment.

Petri dishes were sealed with plastic film and incubated in light at 15 °C and checked weekly for germination. Germination was defined as the emergence of the radicle tip from the endocarp. Any germinated seeds observed were removed from the Petri dishes. Endocarps were checked for radicle emergence for a period of three months and then x-rayed for seed fill at the completion of the germination trial.

3.4.2.3 Data Analysis

Soil temperature and moisture data was analysed with a paired t-test using the mean daily maximum and minimum for the field and nursery sites.

Seed fill, viability and germination data were analysed using a binomial Generalised Linear Mixed Model (GLMM) with a logit link function to determine the effects of the different treatment factors. In the first burial trial (i.e. burial under different soil and leaf conditions) the factors included burial treatments and seed batches. In the second burial trial (burial over an extended period of time) the factors included location (nursery, field or stored at Kings Park) and time of retrieval. Interactions between the various factors were included. The model examining the *ex situ* germination data, also included the *ex situ* treatments (KAR_1 or water), the month of retrieval, year of retrieval and all interactions. These models were then reduced by omitting all non-significant interactions (5% significance level) through stepwise variable selection. Comparisons between the different treatments were made using Tukey's HSD. All analyses were undertaken in the statistical program R (R Core Team, 2013), using the *mgcv* and *lsmeans* packages (Bates et al., 2014, Lenth, 2014, Wood, 2011).

3.4.3 Results

3.4.3.1 Germination Following Burial Under Different Soil and Leaf Litter Conditions

Zero inflation (i.e. 0% germination) in the data was associated with endocarps not being buried ($P<0.001$), being located in unburnt soils ($P<0.001$), with leaf litter present ($P=0.012$) or with the mesocarp left on ($P=0.030$).

All three batches of endocarps + seeds germinated significantly better in the ash bed (Fig. 3.11, $P<0.001$) than in any of the other treatments. Seeds within those endocarps

with the mesocarp on germinated at significantly lower levels than those where the mesocarp was removed in all treatments (Fig. 3.11, P<0.001).

Seeds did not germinate when endocarps were placed directly on top of the bare earth. Many of these endocarps weathered poorly and developed unusual cracks and fissures on the endocarp surface. Seeds did not germinate when endocarps were placed between leaf litter and soil (i.e. litter/soil interface) and only two seeds germinated from endocarps placed on top of leaf litter.

Significantly more seeds germinated from endocarps located in the burnt area than in the unburnt areas (Table 3.4, P<0.001) and significantly more seeds germinated from endocarps when no leaf litter was present compared with treatments where endocarps were placed on top of leaf litter or where leaf litter was present (Table 3.4, P<0.001). Both fresh and frozen endocarps with the mesocarp removed resulted in significantly better germination than those treatments where the mesocarp was retained on the endocarps (Table 3.4, P<0.001). In most treatments, germination did not occur until the endocarps had been buried for 18 months. Germination commenced in May and continued through the winter months.

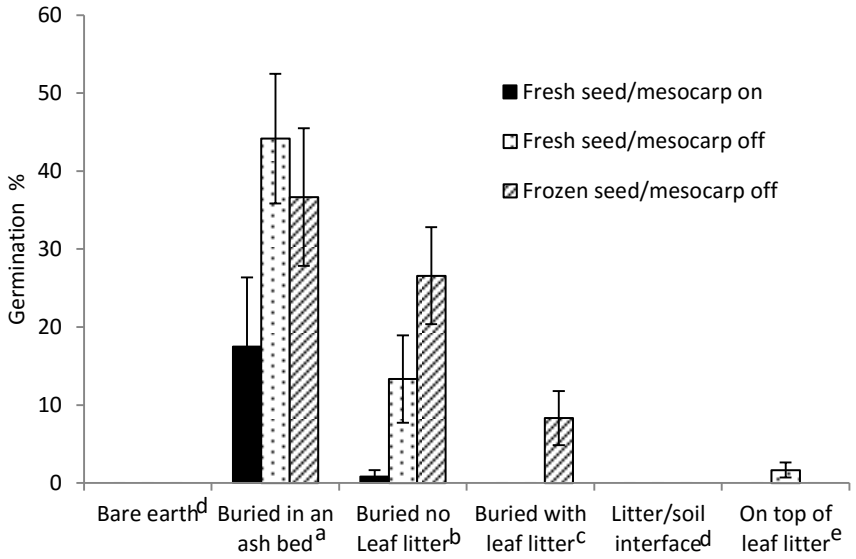

Figure 3.11: Mean maximum germination percentage of seeds from endocarps buried under different soil/litter conditions at Cordering for two and a half years. Treatments with the same superscript letter do not differ significantly.

Table 3.4: Mean percentage germination for *P. longifolia* seeds from endocarps buried at Cordering for two and a half years. Treatments with the same superscript letter do not differ significantly from other treatments within the same factor.

Factor	Treatment	Mean Germination %	P-value
Burnt or Unburnt	Burnt	32.8 ± 5.6^{a}	<0.001
	Unburnt	3.4 ± 1.1^{b}	
Mesocarp on or off	Fresh seed with mesocarp off	9.9 ± 3.7^{a}	<0.001
	Fresh seed with mesocarp on	3.1 ± 1.9^{b}	
	Frozen seed with mesocarp off	11.9 ± 3.5^{a}	
Leaf Litter	No leaf litter	15.4 ± 3.2^{a}	<0.001
	On top of leaf litter	0.6 ± 0.4^{b}	
	Beneath leaf litter	1.4 ± 0.8^{b}	

3.3.3.2 Seed Burial and Retrieval Over an Extended Period of 36 Months

The daily mean maxima and minima temperatures recorded in the soils of the field and nursery environments in which the endocarps were buried, are shown in Fig. 3.12. Generally, the nursery buried endocarps experienced a greater variation in temperatures than field buried endocarps. Maximum temperatures in summer months of the nursery soils were generally higher by 2-3 °C than those in field soils (although this difference was not significant) and winters were significantly lower by 2-3 °C ($P<0.001$).

Similarly, the soils in the nursery experienced slightly different moisture contents than those in the field (Fig. 3.13). Both mean maximum and mean minimum moisture content was significantly higher in the nursery than in the field ($P<0.001$). Both field and nursery soils were exposed to the same rainfall, yet the water penetration in the nursery was generally greater than that of field soils, with the nursery soils having a greater water content following rainfall than the field soils. In addition, nursery soils tended to stay moist for longer.

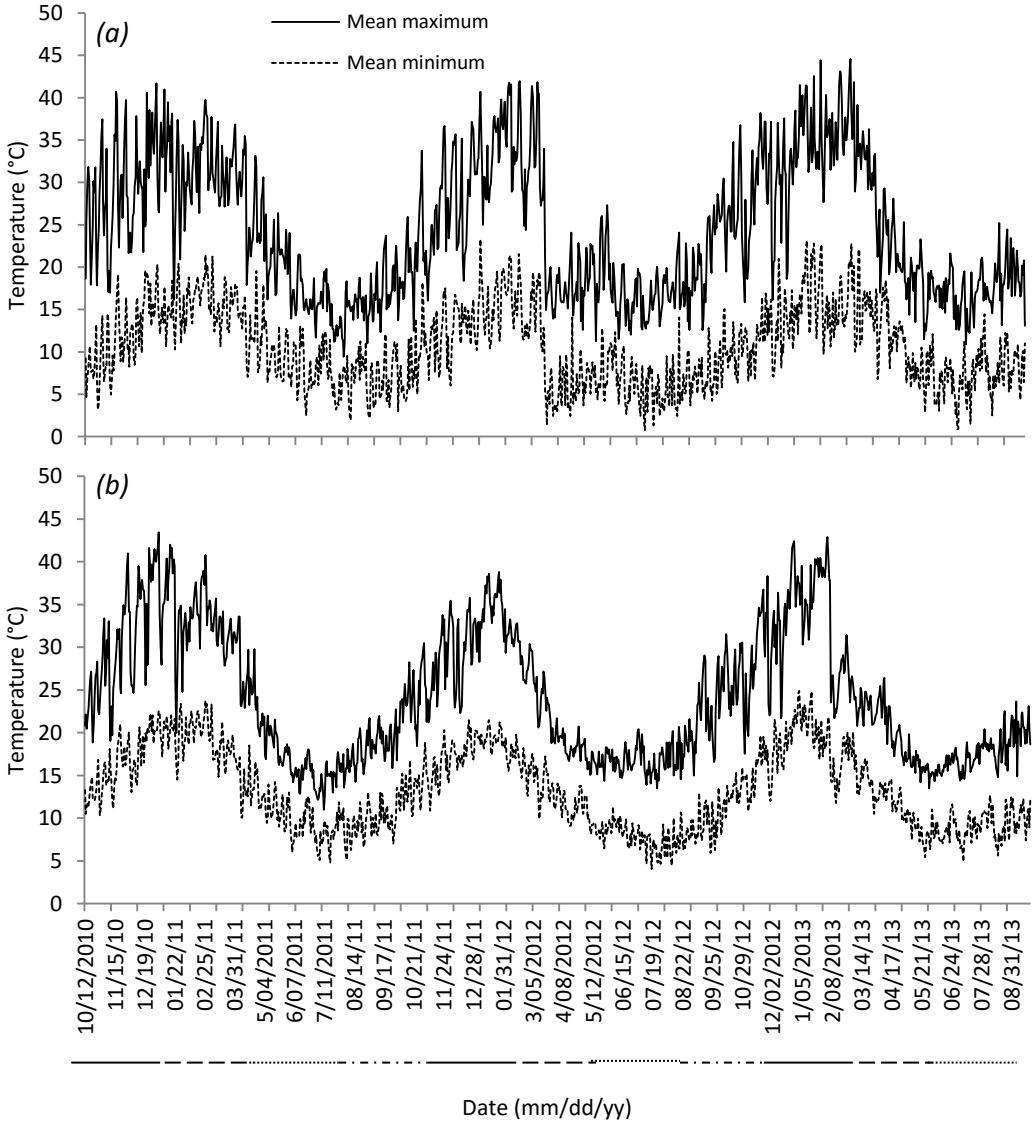

Figure 3.12: Mean minimum and maximum daily temperatures of (a) nursery and (b) field soils in which endocarps were buried. Lines underneath the horizontal axis (Date) indicate seasons – solid line = summer, dashed line = autumn, dotted line = winter and dash/dot line = spring.

The 2010/11 summer had three summer thunderstorms (over 10 ml of rainfall received per event during the summer months of December, January and February, Fig. 3.13). Similarly, the 2011/2012 summer also received three rainfall events over 10 ml and two summer thunderstorms occurred during the 2012/13 summer months.

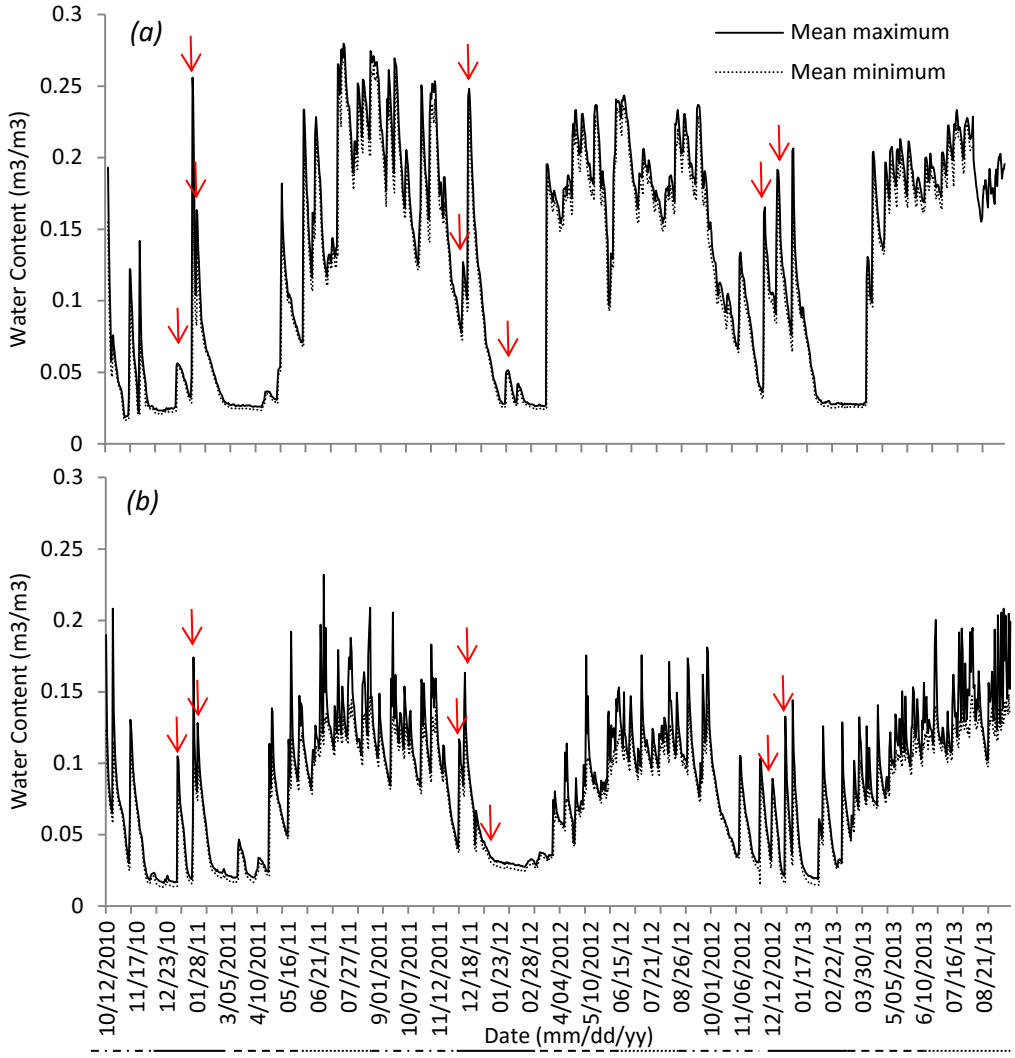

Figure 3.13: Mean minimum and maximum daily water content of (a) nursery and (b) field soil in which endocarps were buried. Red arrows indicate when summer thunderstorms greater than 10mm occurred. Lines underneath horizontal axis (dates) indicate seasons –solid line = summer, dashed line = autumn, dotted line = winter and dash/dot line = spring.

Seed fill of intact endocarps in both the field and nursery treatments declined over time (Table 3.5). There was a significant difference in seed fill at the various times of retrieval ($P<0.001$) and the interaction between time of retrieval and treatment was also significant ($P<0.001$). After three years burial, there was a significant difference between field, nursery and control endocarps with nursery buried endocarps having significantly less seed fill than either field buried ($P<0.001$) or control endocarps ($P=0.045$) (Table 3.5).

Viability of seeds within the retrieved endocarps was variable across the treatments and appeared to increase at 24 months. This is perhaps indicative of the issues associated with using tetrazolium as a measure of viability with these seeds (pers. obs.). However despite these anomalies in the data, field seeds showed a significant decrease in viability from 0 months to three years ($P<0.001$). Nursery seeds declined in viability but not significantly and it was likely to have been as a result of the low numbers of seeds remaining in nursery replicates at the completion of the trial and the high variability associated with these replicates. Control seeds did not decline significantly in viability.

Table 3.5: Mean percentage seed fill and TTC staining of seeds remaining after germinants were removed from the sample (+ SE). Note that for Time 0 the same sample was used for each burial treatment. *=no SE as only one replicate tested. NA= Not Applicable

Treatment	Burial site	Time 0	6 mths	12 mths	18 mths	24 mths	30 mths	36 mths
Seed fill	Field	NA	95.1 ± 1.2	97.1 ± 0.8	93.4 ± 1.3	83.8 ± 4.0	86.1 ± 4.7	83.1 ± 6.4
	Nursery	NA	97.4 ± 0.5	94.6 ± 1.3	88.5 ± 1.3	84.5 ± 3.0	75.2 ± 3.1	68.5 ± 5.4
	Control	93.5 ± 1.9	97.9 ± 0.8	97.8 ± 0.8	98.5 ± 0.5	97.0 ± 0.8	95.8 ± 1.0	94.5 ± 0.9
TTC staining	Field	NA	92.1 ± 1.0	89.0 ± 3.6	60.4 ± 8.6	79.6 ± 8.9	65.2 ± 4.9	57.5 ± 11.9
	Nursery	NA	98.1 ± 1.2	81.0 ± 3.9	55.4 ± 7.1	90.9 ± 9.1	63.3 ± 13.3	33.3*
	Control	90.4 ± 0.5	94.3 ± 1.6	89.3 ± 2.4	81.2 ± 5.0	89.6 ± 2.8	88.8 ± 2.6	NA

It should also be noted that while all levels of staining are reported in the above statistics, the control seeds stained much darker than either of the field or nursery seeds at 30 months.

X-ray analysis of endocarps at each retrieval showed that the fracture line on the endocarps becomes more evident over time in field buried and nursery buried endocarps (Fig. 3.14). Endocarps stored at Kings Park did not develop the fracture line to the same extent as endocarps that had been buried. The fracture line became visible after 12 months burial and is very evident after three years burial. There did not appear to be any change in the various channels within the endocarp itself. SEM analysis also showed the buried endocarps acquiring more cracks and fissures over time than when the endocarps are retained in constant temperature and relative humidity at Kings Park. The lid becomes more defined and the micropyle opening becomes more evident on buried seeds (Appendix 4).

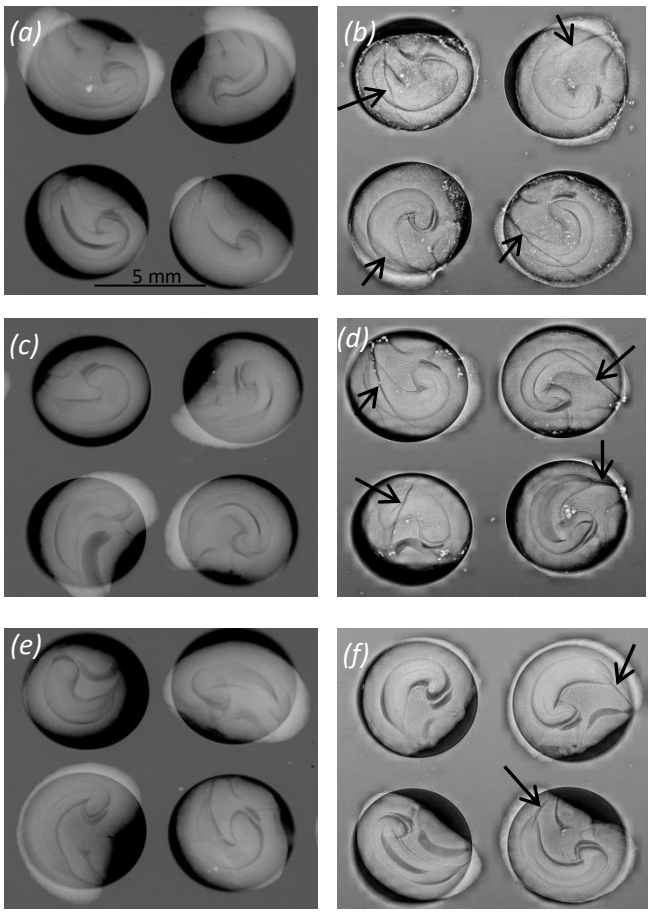

Figure 3.14: X-ray analysis of *Persoonia longifolia* endocarps. The development of the fracture line and lid in the endocarp is indicated with the black arrow. *(a)* Field buried at 6 months. *(b)* Field buried at 36 months. *(c)* Nursery buried at 6 months. *(d)* Nursery buried at 36 months. *(e)* Stored at Kings Park (control) after 6 months. *(f)* Stored at Kings Park (control) after 36 months.

Zero inflation present in the germination data was largely associated with the month in which endocarps were collected, in particularly July and September retrievals. Retrieving endocarps during March resulted in a greater chance of recording germination ($P<0.001$).

In situ seed germination increased over time and was higher in the endocarps retrieved from the nursery than the field at every sampling period (Fig. 3.15, $P<0.001$). Seed germination reached a maximum of 63.7 $\pm$ 6.7% in the nursery and 41.6 $\pm$ 8.1% in the field after three years of burial. No *in situ* germination occurred in those seeds from the control endocarps (dry stored at Kings Park). Seed germination was first recorded at 9 months for field buried endocarps and 12 months for nursery buried endocarps (note

that a retrieval was not conducted at nine months for nursery buried endocarps) indicating that germination commenced in the first winter that endocarps were buried.

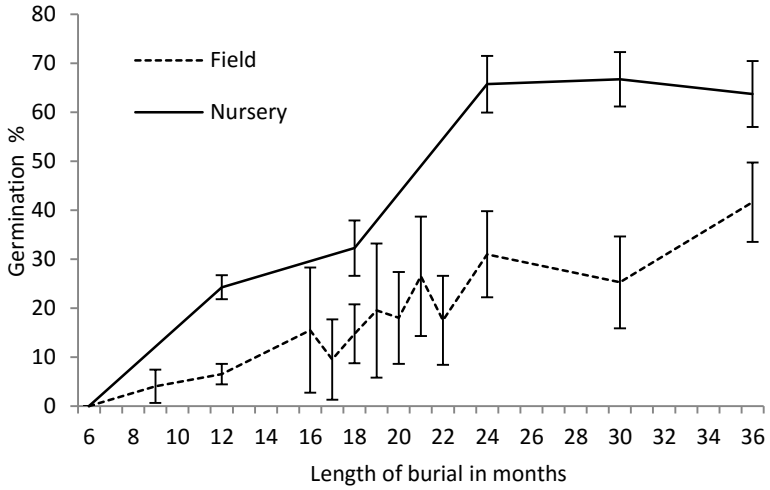

Figure 3.15: *In situ* seed germination of nursery and field buried endocarps. Decreases in germination are a result of different set of bags being retrieved at each retrieval time.

Germination once the endocarps were retrieved and placed into the germination cabinets, commenced after only 6 months soil burial (March retrieval/autumn) in both field and nursery (Fig. 3.16). Germination was cyclic with little or no germination occurring when endocarps were retrieved at the end of winter/beginning of spring (i.e. 12, 24 and 36 months). In each year that retrievals were made, *ex situ* germination peaked in the autumn retrieval (i.e. six, 18 and 30 months) with burial after 30 months resulting in greater germination than in any other retrieval indicating that dormancy had been broken in the largest number of seeds at this time. The interaction between some of the factors included in the analysis was also interesting. Overall seed germination was significantly higher in endocarps buried in the nursery and then treated with water than in any of the other treatments (P=0.040). Interestingly, when the interaction between the month of the year and the *ex situ* treatment was examined there was no significant difference in any month of the year between the use of KAR_1 or water as an *ex situ* treatment.

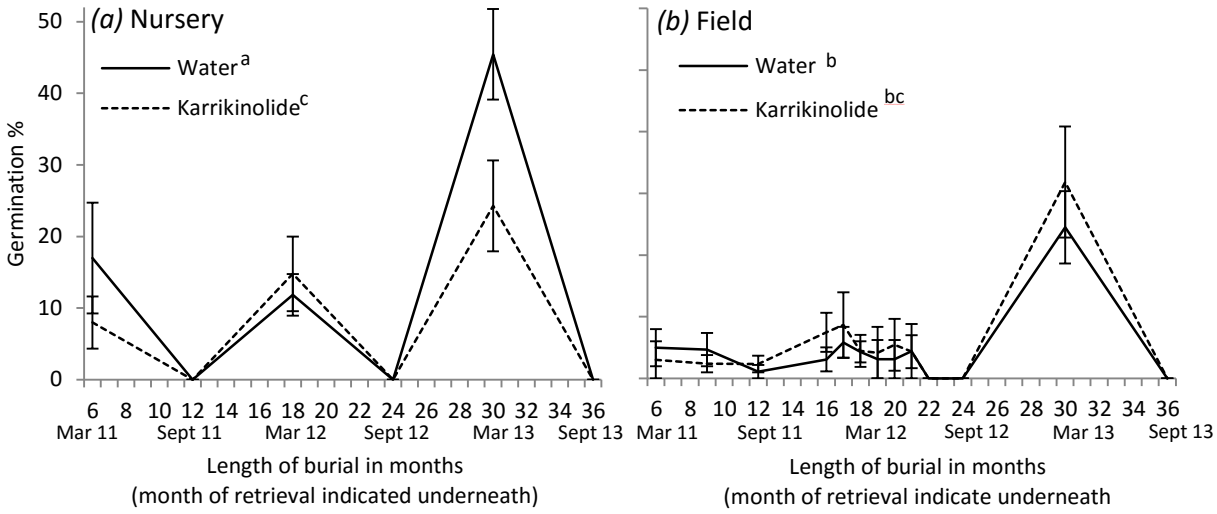

Figure 3.16: Seed germination in laboratory germination trials (i.e. *ex situ* germination) following retrieval at various times of the year. *(a)* Nursery buried endocarps. *(b)* Field buried endocarps. Karrikinolide = *ex situ* treatment with KAR_1; Water = *ex situ* treatment with water. Treatments with the same letter do not differ significantly.

3.4.4 Discussion

Over the years there has been contrasting reports of the effects of burial on germination of *P. longifolia* seeds (Mullins et al., 2002, Norman and Koch, 2008, Turner et al., 2010). This most recent study indicates that a period of burial is required for *P. longifolia* seeds to germinate. In almost all circumstances, in both experiments undertaken, seeds only germinated once burial occurred, indicating that there is some key environmental conditions that endocarps and seeds are exposed to whilst buried, that result in dormancy loss. In the experiment where endocarps were buried under different environmental conditions, seed germination occurred best in the ash bed. The reasons for this are currently not understood and could include nutrient release as a result of the fire, variations and fluctuations in soil temperatures, lack of competition from surrounding vegetation, or the absence of leaf litter which allows moisture to penetrate soil better. Baker et al. (2005b) found moisture content of seeds from fire ephemerals from the south west of Western Australia was higher in seeds buried in burnt areas compared with seeds buried in unburnt areas. Soils in burnt areas of *Eucalyptus* forests have also been shown to lose their water repellency in moderate to high intensity fires (Granged et al., 2011) and this increase in moisture availability may

play an important role in promoting dormancy loss and assisting germination in *P. longifolia* seeds.

Norman and Koch (2008) found that seeds from endocarps buried at the leaf litter/soil interface germinated at the lowest rate when retrieved and sown in a nursery environment when compared with endocarps that were buried 2 cm below the soil surface with all leaf litter removed. Similarly, in this present study germination was best when intact endocarps were buried, or when buried without leaf litter, rather than at the soil/litter interface, on top of the leaf litter or when buried with leaf litter present. It appears that the leaf litter inhibits germination by changing the environmental conditions (such as moisture levels, light availability or nutrient availability) that the endocarps are exposed to, in a manner that suppresses dormancy loss so seeds are less germinable when retrieved.

Endocarps that were not buried or were placed just beneath the leaf litter weathered and cracked, but not in the same way endocarps that were buried beneath the soil weathered and cracked. Endocarps on top of the soil appeared to crack randomly (Fig. 3.17a) whereas endocarps that were buried almost always cracked along the predetermined fracture line (Fig. 3.17b).

In addition to burial, the mesocarp needs to be removed to promote seed germination. If the mesocarp is left on the endocarp, it becomes tough and leathery (Fig. 3.17c) and appears to further inhibit germination. Mesocarp removal under natural conditions is likely to occur either when the fruits are released by the parental plant and ingested by species such as emus, kangaroos and reptiles or through a process of rotting when the fruits move through the wet leaf litter layer.

In the burial and retrieval experiment undertaken over 36 months, seeds within endocarps that were not buried and were stored in constant temperature and relative humidity at Kings Park did not germinate whatsoever at any stage throughout the trial providing further evidence that some form of burial is required to break dormancy. However, seed viability and seed fill remained high in these endocarps for three years showing no significant decline (>90% seed fill after three years). In comparison, seed fill and viability in endocarps that were buried in the field and nursery both steadily

declined over the three years to less than 35% viable for nursery buried endocarps and less than 60% for field buried endocarps.

Figure 3.17: Examples of endocarps retrieved in the burial trial (under different soil conditions). *(a)* Unusual cracks in endocarps left on top of the soil surface and without leaf litter. *(b)* Cracking of the lid when seeds are buried. *(c)* Leathery mesocarp remaining on endocarps at the end of the burial trial.

First observations of *in situ* germination occurred at nine months (winter retrieval) in field buried seeds, and 12 months in nursery buried seeds (although no retrieval was undertaken at nine months for nursery buried seeds) after being buried for one summer. In previous studies (Turner et al., 2010), *in situ* germination in *P. longifolia* was not observed until 18 months after burial and even then, germination was quite low with only 11.5% of seeds germinating compared with 24% germination after 12 months burial in the nursery in this experiment. This suggests that it is not *the length of time* that endocarps are buried for but rather *the environmental conditions that the endocarps and seeds are exposed to* whilst they are buried that is more important for dormancy loss.

On closer examination of rainfall and temperature data in this study and in previous studies (Turner et al., 2010), it became apparent that the environmental conditions experienced by the seeds during the summer months were quite different in each different trial. The first summer that endocarps were buried by Turner et al. (2010) was exceptionally dry with very little summer rainfall occurring (four out of five rainfall events were less than 7 mm and the fifth was only 12.5 mm). By comparison, the first summer of burial in this present study experienced eight rainfall events, two of which were greater than 20 mm (41.8 mm and 27.8 mm) and another two of which were greater than 10 mm (11.8 mm and 14.4 mm). The second summer of Turner et al.'s (2010) trial experienced one large thunderstorm of 43 mm (Bureau of Meteorology, 2015) and the following winter was when *in situ* germination was first recorded.

Norman and Koch (2008) also found seed germination from buried endocarps was highest for those retrieved after burial in the field for 15 to 21 months and then surface sown and kept moist in a nursery. Both summers in the Norman and Koch (2008) study also were very dry with the summer of 2003/2004 having one rainfall event of 8 mm and 2004/2005 experiencing only one decent rainfall event of 7.4 mm.

The difference between *in situ* seed germination that occurred from field buried endocarps compared with nursery buried endocarps in this study is no doubt related to the fact that the endocarps + seeds in the nursery experienced a greater range of temperature and moisture variations than those in the field. Close examination of the temperatures and moisture measurements made during the periods when the summer thunderstorms occurred indicated the soil in the nursery retained the moisture for a longer period of time, dropped below 10 °C and took longer to return to the normal summer soil temperatures and moisture levels; generally about a week compared with field soils which took 2 days to return to "normal" conditions.

Examination of the endocarp through x-ray and SEM analysis shows that the predetermined fracture line becomes more evident over time in those seeds that are buried compared with those seeds that are stored in constant temperature and relative humidity. The summer thunderstorms may result in some weakening of the hard woody endocarp through the rapid wetting and drying/heating and cooling cycles and allow the embryo to develop enough "push power" to push through the hard woody endocarp. Fracturing of the endocarp of *Santalum spicatum* (another Western Australian native species) occurs when the endocarp is rapidly dried after wetting and this is likened to the natural processes that occur during summer thunderstorms (Woodall, 2004). Other species also exhibit this phenomenon. For example, soil burial results in the funicular envelope of *Opuntia tomentosa* becoming damaged which in turn facilitates imbibition and embryo growth (Olvera-Carrillo et al., 2009). Chilling and heating treatments results in increases in germination in teak seeds and is attributed to splitting of the hard endocarp which allowed germination (Rajput and Tiwari, 2001).

Mullins et al. (2002) however, found germination of *P. longifolia* was better from surface sown endocarps than from buried endocarps and occurred during the winter months. In the various combinations of treatments tested by Mullins et al. (2002) the endocarps were continuously kept moist by watering twice daily through both summer

and winter. Therefore the only endocarps that had an opportunity to dry out between waterings may have been those that were surface sown and it is possible that those were the only endocarps that weakened over time.

Leaf litter appeared to prevent moisture getting to the soil (pers. obs.) and it is likely that moisture is an important environmental condition required to break dormancy in *P. longifolia* seeds. The results from these two burial trials suggest that a period of stratification (i.e. a period of moist, incubation) in the summer months is required to break dormancy either through weakening of the endocarp and/or releasing physiological dormancy in the embryo. This period of stratification could be either warm (>15 °C) or cold (<10 °C) (Merritt et al., 2007) and this hypothesis is further examined in Section 3.5.

Once endocarps were retrieved and placed into germination cabinets in the laboratory, germination only occurred from endocarps that had been buried. Once again germination occurred earlier from endocarps buried in this study (i.e. at six months) compared with those endocarps buried in Turner et al. (2010) (10 months).

This *ex situ* germination appears cyclic, with best germination from endocarps retrieved in the autumn months, whilst endocarps retrieved during spring do not result in any germination once in laboratory conditions indicating that either seed dormancy has been reimposed as they were about to enter the harsh summer months or that all seeds that had dormancy broken, had by that point, germinated. Turner et al. (2010) also found cyclic germination in *P. longifolia* with germination only occurring *ex situ* from the 18 month and 30 month retrievals. Seeds in Norman and Koch's (2008) study only germinated in the winter months despite endocarps being kept moist in the nursery over the summer months also indicating cyclic germination. Germination from a cohort of *Hibbertia* seeds can be spread over several years and this was attributed to varying degrees of dormancy within the cohort (Hidayati et al., 2012). Seeds with less dormancy germinate after only one summer whereas seeds with greater dormancy required two or more summers to alleviate dormancy and to germinate.

Gradual dormancy alleviation over summer ensures that seeds will germinate during the cooler and wetter winter months when seedling survival is likely to be greatest. A return to a dormant state during the spring months will result in ungerminated seeds

surviving through the summer and germinating the following winter. Finch-Savage and Leubner (2006) describe dormancy as a moving target continuously reacting to the environment and adjusting the conditions required for germination and this appears to be the case for *P. longifolia.* Whilst cyclic germination was observed for *P. longifolia,* it is still unknown if this cyclic phenomenon is a result of reinstatement of dormancy or as a result of varying degrees of dormancy within the cohort of seeds where all the seeds that have had dormancy broken have germinated and further dormancy breaking conditions are required for germination of the remainder of the seeds.

Both seed viability and seed fill declined over time in nursery and field buried endocarps. Seed fill in nursery buried endocarps declined significantly more than in either field buried or control endocarps. Turner et al. (2010) found that there was minimal decline in viability within the first 18 months of burial, but that a sharp decline in viability occurred between 18 months and 35 months and this was largely attributed to most of the seeds either rotting or germinating in the bags whilst buried. Norman and Koch (2008) found seed viability did not decline in soil buried endocarps but that nursery buried endocarps that were kept constantly moist had significantly lower viability. Based on these results and the results from this current study it appears that moist conditions result in a decline in viability of seeds over time. Given the issues associated with fungal attacks throughout the laboratory trials undertaken it is likely that the presence of moisture exacerbates seed deterioration in *P. longifolia*.

The use of a germination stimulant such as KAR_1 did not have any clear effect on the ability of the seeds to germinate when assessed in this study. This is contrary to the results achieved by Turner et al. (2010) who found that germination was enhanced to some degree by KAR_1 though these results were by no means clear cut. Further investigation is required to determine if germination of *P. longifolia* seeds is smoke responsive as is the case in many other species from fire prone communities (Tieu et al., 2001, Turner et al., 2009b)

3.5 Dormancy break through simulation of seasons

3.5.1 Introduction

Non-dormant seeds cannot germinate unless the appropriate temperature, moisture and light regimes are met. In addition, some seeds may also need quite different temperature and moisture regimes to break dormancy before they are able to germinate at species-specific temperatures. Temperature is often considered to be the most important factor in regulating seed dormancy (Baskin and Baskin, 2001). However, variations in seed moisture through exposure to wetting and drying cycles or periods of moist stratification (particularly during autumn) are also important drivers of dormancy loss as well (Baskin and Baskin, 1995, Baskin et al., 2002, Chen et al., 2007, Hoyle et al., 2008, Merritt et al., 2007, Turner et al., 2006).

The southwest of WA experiences a Mediterranean climate with long, hot, dry summers and cold wet winters. However, while the summer months are generally dry, on average they usually experience a number of intense thunderstorms (with greater than 25 mm of rain) over a three or four month period. Autumn and spring are also distinct seasons with the autumn months being cooler than summer and having more regular, sporadic rainfall events. Similar conditions are experienced in spring.

The role of wetting/drying cycles or stratification in Australian ecosystems has not been investigated in great detail, although a few studies of species from southern Australia are beginning to emerge which indicate that wet/dry cycling in this environment plays an important role in dormancy break and germination of some species. For example, Lush (1984) found that laboratory wetting and drying cycles resulted in a decrease in the time taken for *Clematis microphylla* (a species found in south eastern Australia) seeds to germinate and that this was comparable to the results observed in field weathering experiments. Similarly, Baker et al. (2005b) found that dormancy release in *Actinotus leucocephalus* (a south western Australian species) occurred more rapidly under warm conditions when seeds were sequentially wetted and dried than when stored dry for the same length of time. The authors linked this to the seasonal conditions experienced by this species in autumn when the seeds are exposed to a transition from the dry to wet season through sporadic rainfall events during otherwise warm, dry periods. Several weeks of warm moist conditions resulted in high germination of

Acanthocarpus preissii (another species from south western Australia) and this was also linked to autumn conditions with the onset of regular rainfall events (Turner et al., 2006). Mixed effects of wet/dry cycles were observed in the genus *Hibbertia* with warm stratification increasing germination in some species whilst others (although not all) respond to wet/dry cycles (Hidayati et al., 2012).

Wetting and drying has not only been linked to dormancy alleviation, but is also linked to mechanical changes in the outer coverings of seeds. Woodall (2004) found that wetting and then rapid drying of *Santalum spicatum* nuts resulted in 98% cracking of the endocarp. Laboratory wetting and drying was likened to summer thunderstorms which are likely to crack the endocarp in the natural environment. In this case nuts that were cracked were observed to germinate more readily than those that remained intact (Woodall, 2004).

Results from field and nursery burial trials for *P. longifolia* have indicated that summer rainfall events, and associated temperature drops may be important for breaking seed dormancy (Section 3.2). In contrast to the studies outlined above, logging of soil temperature and moisture for *P. longifolia* burial trials has indicated that often the soil temperature will drop to around 10 °C during the summer months within the 24 hours following a rainfall event, which would equate to short periods of cold stratification. These preliminary burial trials have indicated that germination of up to 24% can occur within 12 months burial providing the required environmental triggers can be met.

At this stage it is unknown if these rainfall events and temperature changes weaken the endocarp sufficiently for the embryo to have enough "push power" to germinate or if they are related to dormancy alleviation within the embryo itself. Moist, warm stratification has been linked to degradation and softening of the endocarp in *Crataegus monogyna* (hawthorn) and cold stratification was linked to dormancy break within the embryo itself (Persson et al., 2006). Anecdotal evidence for *P. longifolia* indicates that the lid (Fig. 3.2) on the endocarp becomes easier to dislodge over time when buried and that a combination of endocarp weakening and enhanced embryo "push power" allows germination to occur. The aim of this section is to replicate environmental conditions observed in burial trials within both laboratory and nursery settings in order to better understand the mechanisms involved in dormancy break within *P. longifolia* seeds. Initial experiments specifically investigated the impact of:

- temperature variations during the winter months;
- wet/dry cycles throughout the summer months including the number of wet/cycles and the length of time seeds are exposed to the wet cycles;
- temperature variations through the summer months both during the wet/dry cycle and during the dry periods of summer; and
- extended summer periods and exposure to autumn temperatures.

Additional experimentation into the specific roles of warm and cold stratification and embryo growth potential aimed to further identify the sequential drivers of dormancy loss within *P. longifolia* seeds and endocarps.

3.5.2 Methods

3.5.2.1 Optimal Winter Temperature For Germination

Fruit was collected in June 2011 and the mesocarp layer removed by fermentation and mechanical means (cleaned using a 0.03% endozyme/0.1% cellulase solution and agitated for 4-5 hours, adapted from Tieu et al. (2008)). Endocarps were then stored in the seed drying room at Kings Park until required. Three replicates of 20 endocarps were tested for seed fill by x-ray analysis and seed viability by TTC tests using methods described in Section 3.3.2.

The trial was conducted using seeds extracted from the endocarps as germination from within the endocarp has proven problematic. Seeds were extracted from the endocarps by soaking in water for 2-4 hours and then cracking the endocarp in a vice before gently extracting the seed. All extracted seeds were checked for damage prior to use in germination trials.

Seeds were surface sterilised in a 2% $Ca(ClO)_2$ solution for 30 minutes and then rinsed three times in sterile water prior to experimentation. Surface sterilized seeds were placed on germination papers in Petri dishes, sealed in plastic film and then placed in various germination cabinets with 12 hours light/12 hours dark.

Seeds were subjected to a number of different incubation temperatures (5, 10, 15, 20, 25, 30 °C and 10/20 °C) to determine the temperature at which optimal germination occurred. For each treatment there were four replicates of 20 seeds with the exception

of 10/20 °C which had three replicates of 20 seeds and one replicate of 12 seeds due to a lack of available seed.

Germination of the seeds was recorded twice a week for 28 days. After 14 days mould developed in many of the dishes. All clean seeds were placed in bleach for 30 seconds and then rinsed before placing them back onto a clean Petri dish. After 28 days, mould once again became an issue and the trial was aborted. Due to the mould issues experienced, it was decided to conduct a second trial to determine the optimal winter temperatures for germination. For this trial endocarps (rather than naked seeds) were used as endocarps appear to be more resilient to mould outbreaks than extracted seeds. Fruits were collected and processed in September 2012 according to the methods described above. A high pressure cleaner instead of agitation was used to remove the mesocarp on this occasion.

Endocarps were buried for 6 months and watered four times over the summer months to break dormancy according to the methods described in Section 3.5.2.6. Endocarps were then retrieved and cracked to remove >50% of the endocarp prior to surface sterilization. The partial endocarp and exposed seed were sterilized in an acidified 1% (w/v) $Ca(ClO)_2$ solution plus Tween 80® after which they were placed under vacuum for 10 minutes, released from vacuum for 10 minutes and vacuumed again for a final 10 minutes. To help minimise fungal contamination, each seed with partial endocarp was placed in its own individual 55 mm Petri dish. Thirty dishes were each incubated in light at 10, 15, 20, 25, 30, 10/20 and 15/25 °C.

Mould was once again present in the trial but did not begin to appear until day 38. The trial was continued, even though mould was present in some dishes, until day 59 after which time there were no more changes in the seeds and mould by this time had become prevalent throughout the experiment. Mould was particularly invasive in the warmer temperatures (20, 25, 30 and 15/25 °C) at around 49 days and may have limited germination after this period.

Germination in both trials was defined as change in colour of cotyledons from white to green (Fig. 3.7) which was assumed to be an indication that metabolic activity associated with germination was occurring. Due to the length of time required for radicle extension and potential for mould to occur in *P. longifolia* seeds, this was considered to be the best means of defining germination for these trials.

3.5.2.2 Simulation of Summer Wet/Dry Cycles to Promote Germination (Wet/Dry Experiment One)

Fruits were collected on 12th September 2011 at Moodiarrup West Road, Cordering, Western Australia. Endocarps were removed from the exocarp (skin) and agitated in a cement mixer for 24 hours with pectinase at a rate of 1 gm/100 L and Cellulase at 1 g/1 L with water at 30 °C (adapted from Tieu et al (2008)). Two handfuls of blue metal were also added to act as a scrubbing agent. Endocarps were then rinsed and scrubbed over a mesh screen to remove any additional mesocarp from the endocarp. Finally the resultant endocarps were dried and stored in the seed drying room at Kings Park until germination tests were established in February 2012.

All endocarps used in the germination tests were x-rayed and any seeds that were empty were removed from the germination trials.

Endocarps were surface sterilized and placed on dry sterilized white silica sand in Petri dishes. Over the following three months endocarps were subjected to different summer "rainfall" events and varying temperatures that may be associated with these intermittent moisture regimes. Four replicates of 20 seeds were used for each treatment.

Summer treatments were conducted over a 12 week period and varied as follows:

- either constant 30 °C all summer or a 30 °C summer with the incorporation of a 4 week period at the extreme summer temperature of 50 °C, simulating the hottest soil temperatures that fruits would naturally be exposed during the summer months;
- two or four wet cycles simulating summer rainfall events;
- summer wet cycles were short (i.e. endocarps wet for 48 hours) or long (i.e. endocarps wet for 7 days) in duration; and
- endocarps were subject to a temperature drop to 10/20 °C for 24 hours during the wet cycle or alternatively were kept at 30 °C during the wet cycle.

Endocarps subject to a summer heat burst were alternately incubated at 30 °C in an alternating 12 hours light and 12 hours dark cabinet for 4 weeks then in darkness at

50 °C temperature (oven) in constant dark for 4 weeks and then back to the 30 °C cabinet for 4 weeks.

Endocarps subjected to a wet cycles were removed from the dry sand and placed on wet germination papers in Petri dishes for either 24 hours or 7 days after which time the endocarps were once again placed on the dry sand and returned to the relevant temperature regime. This treatment aimed to simulate transient-summer rainfall events.

Some endocarps were also subjected to a temperature decreases during the wet cycle. The dishes were incubated at 10/20 °C during the wet cycle described above before being returned to dry at 30 °C. The aim of this treatment was to simulate the conditions endocarps or fruits are exposed to in the natural field environment where soil temperatures drop and then slowly rise in the 24 hours following a rainfall event as seen in the *in-situ* burial trials (Section 3.4.4).

All combinations of the above treatments were included in the experiment with control dishes of constant summer at 30 °C and no wet/dry cycles and a second control with alternating summer temperatures of 30/50 °C and no wet/dry cycles resulting in a total of 18 different treatments.

At the end of the 12 week summer simulation period, all Petri dishes were moved to a constant wet winter cycle at 15 °C for three months and scored for germination weekly (i.e. radicle emergence from the endocarp).

All treatments were cycled through four summers and four winters, over a period of 2 years. Generally each cycle was a 12 week period however in winters three and four germinants were still emerging at the end of the 12 week period so these two winters were extended for up to 16 weeks until germination appeared to have ceased.

3.5.2.3 Winter Temperature Variations to Promote Germination (Wet/Dry Experiment Two)

A second wet/dry trial was conducted with the aim of examining the impacts of variations in winter temperatures on germination following the application of summer dormancy breaking techniques. Based on the results from the first wet/dry trial and

results from a previous unreported laboratory germination trial (S. Turner pers. comm.), the best three treatments were used and combined with various winter temperatures and modifications to the lengths of time that seeds were exposed to summer cycles. Combinations of the following treatments were tested in this experiment:

- summer heat burst of 50 °C as per experiment one.
- Two or four wet cycles simulating summer rainfall events as per experiment one.
- Variations on the length of the summer period (i.e. 12 weeks, 12 weeks + 4 weeks autumn, 16 weeks, or 20 weeks).
- Variations on winter temperatures (15 °C which is the temperature usually associated with germination in seeds from the south west of Western Australia, 20 °C which is the temperature that the best germination results were observed in the Optimum Winter Temperature trial (Section 3.5.2.1), and 10/20 °C which simulates the variations in day time and night time temperatures during the winter months in south west WA).

Fruit was collected on June 26th 2012 and were passed through a coffee depulping machine (Penagos Horizontal Coffee Pulper, Columbia). Endocarps were then put on a wire sieve before being high pressure cleaning to remove the sticky mesocarp. Endocarps were stored in the seed drying room at Kings Park until required.

This experiment was established in May 2013. Endocarps were surface sterilized under vacuum in an acidified 1% (w/v) $Ca(ClO)_2$ solution plus Tween 80®, then rinsed three times with sterile water prior to experimentation. Surface-sterilized endocarps were incubated in Petri dishes on sterilised silica sand irrigated with water according to the treatments described above, incubated at 30 °C light and moved through the various summer regimes. During the winter months dishes were moistened, sealed with plastic film, placed in the relevant temperature cabinets and checked on a weekly basis for germination. Endocarps were cycled through three summer and winter cycles for up to 98 weeks.

3.5.2.4 Cold Stratification During Summer Simulations and Optimal Winter Temperatures to Promote Germination (Wet/Dry Experiment Three)

A final wet/dry trial was established in June 2014 to combine modified treatments from the above two trials and determine the most appropriate laboratory based germination methodology. These combined treatments resulted in the inclusion of a cold stratification treatment during the summer month wet cycles and winter temperatures of 10/20 °C. The three treatments in this trial were:

- 12 week summer period with two long wet cycles at 30 °C and a 4 week period at extreme summer conditions of 50 °C, winter at 10/20 °C;
- 12 week summer period with two long wet cycles at 10 °C and a 4 week period at extreme summer conditions of 50 °C, winter at 10/20 °C;
- An extended summer (24 weeks) with four long wet cycles at 10 °C and an 8 week period at extreme summer conditions of 50 °C, a 24 week winter at 10/20 °C.

Fruits were collected in August 2013 from Moodiarrup West Road and the trial was established in June 2014. Fruits were cleaned, stored, sterilized and plated according to the methods described in Section 3.5.2.3. Four replicates of 20 seeds were used for each treatment.

3.5.2.5 Stratification Experiment

Results from the burial trials (Section 3.4) and the first three wet/dry laboratory experiments indicated periods of stratification are required for *P. longifolia* seeds to germinate from within the endocarp and that it is likely that these periods of stratification are required during the summer months. In order to determine the type of stratification (i.e. warm (>15 °C) or cold (<15 °C)) that these endocarps + seeds require to break dormancy, a stratification experiment was undertaken. In addition to determining the type of stratification, the experiment also aimed to determine the order that stratification needs to occur in order to obtain high germination (i.e. cold then warm stratification or warm then cold stratification).

Fruits were collected in August 2014 from Moodiarrup West Road and the trial was established in December 2014. Fruits were cleaned, stored, sterilized and plated

according to the methods described in Section 3.5.2.3). Four replicates of 20 seeds were used for each treatment. Treatments were:

- Constant temperature - controls kept at constant moist, temperatures of 8, 15 or 20 °C;
- Cold stratification only - a period of cold moist stratification (either 4, 6 or 8 weeks at 8 °C) and then into winter (10/20 °C) cycles (i.e. no warm stratification period);
- Warm incubation only - a period of warm storage (either 6 or 12 weeks) at summer temperatures of 30 °C with two wet/dry cycles (warm stratification) within that dry summer period;
- Cold plus warm incubation - a period of cold stratification (either 4, 6 or 8 weeks wet at 8 °C), then warm storage (12 week summer period at 30 °C with two wet cycles (warm stratification) within the dry summer period) before placing into winter temperatures; and
- Warm incubation plus cold stratification - a period of warm storage (either 6 or 12 weeks with two wet cycles (warm stratification) within the dry summer period), plus 6 weeks cold moist stratification at 8 °C and then moved to winter temperatures.

Once endocarps were placed at winter germination temperatures (10/20 °C) endocarps were checked weekly for radicle emergence.

At the completion of the trial all remaining seeds were removed and air dried before each seed was examined and tested to determine if the lid was easily removed.

3.5.2.6 Nursery Wet/Dry trial

Nursery wet/dry trials were established at the same time (i.e. December 2011) as laboratory wet/dry trials (Section 3.5.2.1) using the same batch of seeds, with the aim of developing practical approaches for germinating *P. longifolia* for restoration practitioners such as Alcoa and Worsley. Cleaned endocarps were placed in large tubs filled with washed white quartz sand and were subjected to one of four treatments during the summer months:

- no water (control);
- natural rainfall only;

- two artificial wet/dry cycles; and
- four artificial wet/dry cycles.

Each treatment had four replicates of 50 endocarps which were buried 2-3 cm below the soil surface. Wet cycles consisted of watering the tubs with 25 ml natural rainfall equivalent, per cycle. Watering was undertaken on cooler days where possible (temperatures <30 °C) and tubs were kept in a shaded nursery environment at all times. All tubs were subject to natural variations in temperatures. A single Tiny Tag data logger (Gemini Data Loggers (UK) Ltd., UK) was included in one tub from each treatment to record soil temperatures approximately 2 cm below the soil surface. Tubs were randomised in the nursery at regular intervals throughout the experiment.

Tubs were moved into natural winter rainfall patterns in April of each year with all treatments receiving natural winter rainfall after this date. Treatments were checked for germinants on a weekly basis throughout the winter months and were placed back in the summer cycle in late November early December of each year. Prior to returning seeds to the summer cycle, the sand from each tub was sifted to count remaining seeds and remove endocarps where the lid was detached and seed was empty. These empty endocarps had either germinated or deteriorated and the seed was no longer present inside the endocarp. Tubs were put through three summers and three winters with the trial completed in October 2014 after germination in the tubs from the 2014 winter was completed.

The sand in the tubs was sifted at the completion of the experiment and all intact seeds were removed from the sand. These seeds were then x-rayed to determine final seed fill.

Given the success from the nursery burial trial for *P. longifolia* it was decided that a similar trial would be undertaken for *P. elliptica* (a species which co-occurs with *P. longifolia* and which is equally difficult to germinate) to determine if a similar germination response could be induced using similar methodology. Fruits were cleaned according to the methods described in Section 3.3.2.5. Four replicates of 50 endocarps were buried 2-3 cm below the soil surface in November 2013 and watered according to the methods described above for the *P. longifolia* nursery trial (i.e. endocarps were placed in tubs filled with white sand) and were treated with four wet cycles in summer,

natural rainfall or no water during summer (control). This trial was undertaken from November 2013 to October 2015 (i.e. two summer and winter cycles).

3.5.2.7 Data Analysis

Germination rates were calculated for the optimal winter temperature for germination experiment according to the methods described in Section 3.3.2.5. Germination rate was analysed using a simple linear model. Data was transformed using a $\log_e$ transformation to ensure it fitted with the assumptions of normality. Non-transformed data appears in all figures and tables.

Germination data for all experiments were analysed using a binomial GLMM with a logit link function. Initial exploration of the data was through a simple model including treatment only. Data was then analysed for zero inflation.

For the laboratory wet/dry trials, full models for each experiment were analysed individually (including length of wet/dry cycle, number of wet dry cycles, temperature during wet/dry cycle, summer temperatures, winter temperatures, length of the summer cycles and all relevant two and three-way interactions).

A full model combining all the germination data after the second winter from the three wet/dry experiments was analysed with an introduced "Trial" factor (experiment three only ran for two winter seasons). All major factors including "Trial" and all two-way interactions were analysed in the model.

Germination data for the stratification trial was analysed in the same manner as for the wet/dry trials. Factors included in the binomial GLMM were the number of weeks seeds were warm treated, number of weeks cold stratified, treatment order (i.e. warm + cold, cold + warm, warm only, cold only or constant wet) and all relevant interactions.

Germination data for the nursery trials was also analysed in the same manner as for the wet/dry trials. Treatment name was the only factor included in the binomial GLMM.

These models were reduced by omitting all non-significant factors and interactions (5% significance level) in a stepwise manner. Comparisons between the different treatments

were made using Tukey's HSD. All analyses were undertaken in the statistical program R (R Core Team, 2013) using the *lme4, mgcv* and *lsmeans* packages (Bates et al., 2014, Lenth, 2014, Wood, 2011).

3.5.3 Results

3.5.3.1 Optimal Winter Temperatures For Germination

Germination of extracted seeds in the optimum temperature trial occurred at all temperatures between 15 and 30 °C (i.e. no germination occurred at 5 or 10 °C). However, the trial was aborted after 28 days due to mould (data not shown). Unfortunately it is around the 28-42 day mark that the majority of germination has previously been observed to occur.

Results from this first trial indicated that the best germination occurred at mid-range winter temperatures between 15 °C (15.4 $\pm$ 6.0%) and 20 °C (19.9 $\pm$ 9.0%), and at the alternating temperatures of 10/20 °C (22.5 $\pm$ 11.3%).

Further investigation into the winter temperatures at which germination occurred was undertaken in a second trial. Some seed colour change was observed in all temperatures in the second trial (Fig. 3.18a). Final germination percentage was significantly higher at 20 °C (76.7 $\pm$ 3.3%) than in all other temperatures and this was followed by 10/20 °C (66.7 $\pm$ 8.8%, $P<0.001$).

The quickest germination (i.e. germination rate where the lower the number the quicker the germination) occurred at the warmer temperatures, with seeds at 15/25 °C germinating the quickest of all seeds (although this was not significantly quicker than either 25 °C or 30 °C, Fig. 3.18b). Germination was significantly slower at 10 °C than at all other temperatures ($P<0.001$) however the final germination percentage at 10 °C was not significantly different from either 30 or 25 °C with all three of these treatments having the lowest final germination percentage (Fig. 3.18).

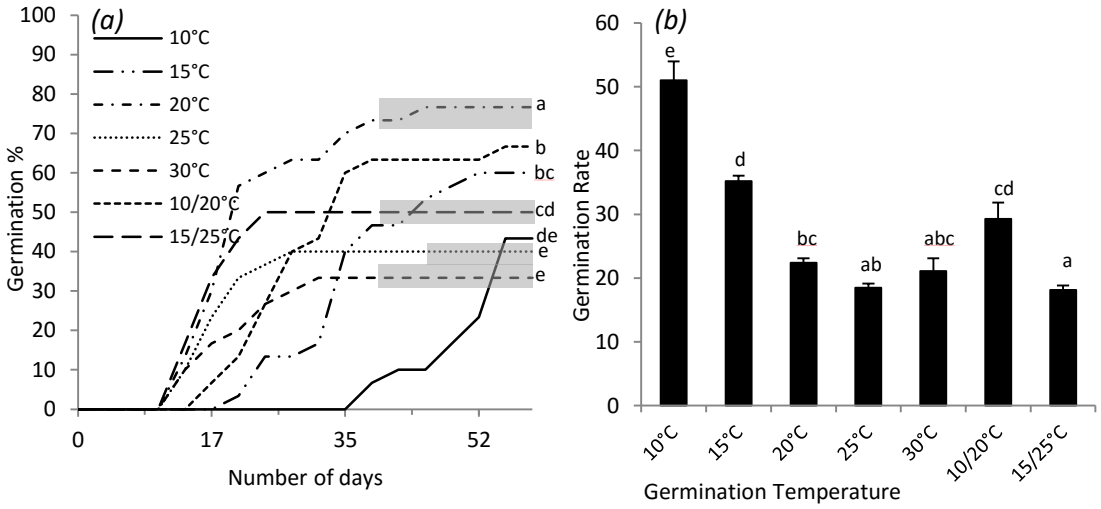

Figure 3.18: (a) Mean cumulative germination percentage at various winter temperatures of seeds contained within buried and cracked endocarps. Grey bars indicate where mould became an issue in each of the different temperatures. (b) Mean germination rate over 59 days (+ SE) (the lower the number the more rapidly germination occurred) at various winter temperatures of extracted seeds.

3.5.3.2 Simulation of Natural Conditions to Promote Germination in the Laboratory (Experiments One, Two and Three)

In the wet/dry experiments one and two, germination after the first winter was very low (<5%). In experiment one, germination after the second winter (i.e. 12 months after commencement of the trial), was still low (≤10%), but had begun to occur in most treatments (i.e. 13 out of 18 treatments) and the majority of germination occurred during the third winter phase (Fig. 3.19). In experiment two, the majority of germination occurred in the second winter (Fig. 3.20). Interestingly, in experiment three similar patterns emerged in the treatments with a 12 week summer (i.e. low germination after the first winter, increasing in the second winter) but for the treatment with an extended summer of 24 weeks, germination began after only 6 weeks at winter temperatures (Fig. 3.19).

The best germination in experiment one (simulations of summer wet/dry cycles) of 62.5 ± 10.1% occurred with two long wet cycles at 10 °C and a constant 30 °C summer temperature (Fig. 3.19d) and this was significantly greater than in all other treatments. No germination occurred in the treatments with four short wet cycles and therefore these results are not presented.

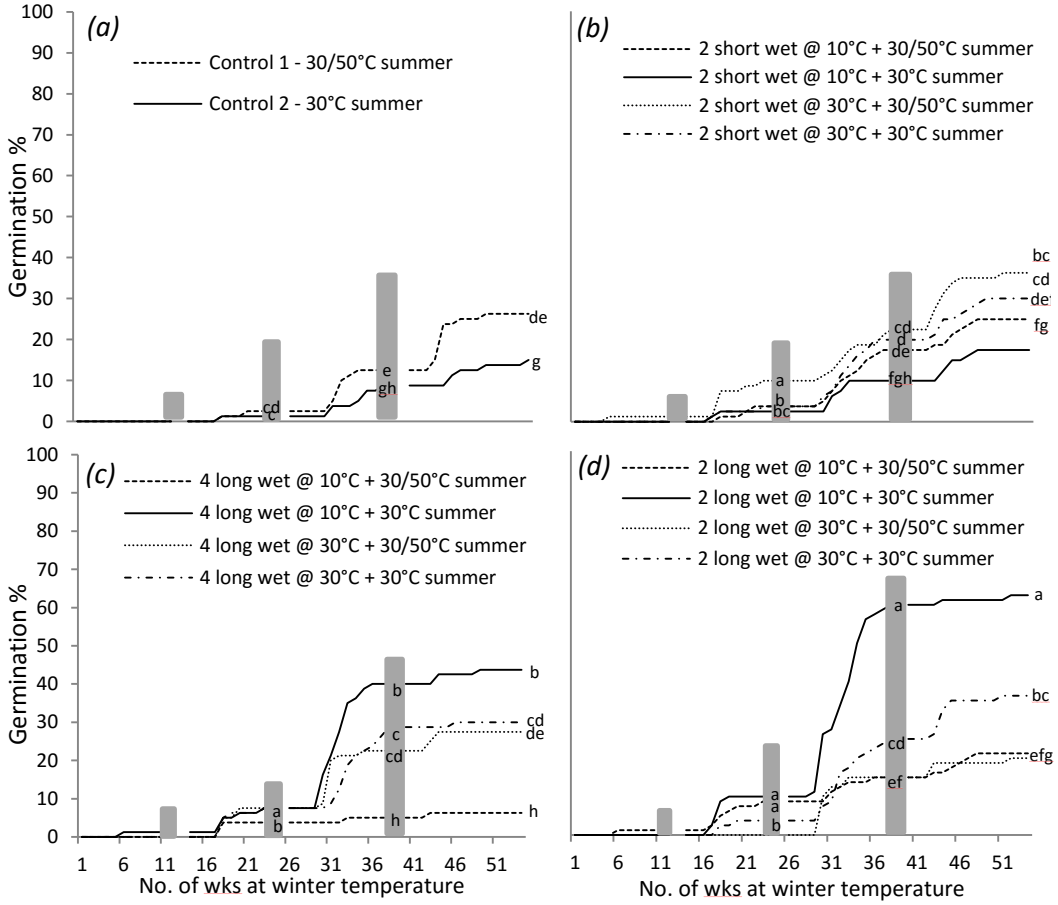

Figure 3.19: Mean cumulative germination percentage from simulation of summer wet/dry cycles (experiment one). *(a)* Control Treatments with no wet/dry cycles during the summer months. *(b)* Two short wet cycles. *(c)* Four long wet cycles. *(d)* Two long wet cycles. Grey shading on all graphs indicates the final three of four summers with the first summer preceding the data shown. Letters within the summer bars indicate significant differences in germination percentages between all treatments at the completion of the preceding winter. No germination occurred in treatments with four short wet cycles so these results are not presented.

In experiment two (simulation of winter temperature variations) the best germination of 75.0 $\pm$ 6.1% occurred in the treatments exposed to a 20 week summer period with a constant summer temperature (30 °C), two long wet cycles and winter temperatures at 10/20 °C (Fig. 3.20f). Treatments at the winter temperature of 20 °C resulted in very poor germination (Fig. 3.20b).

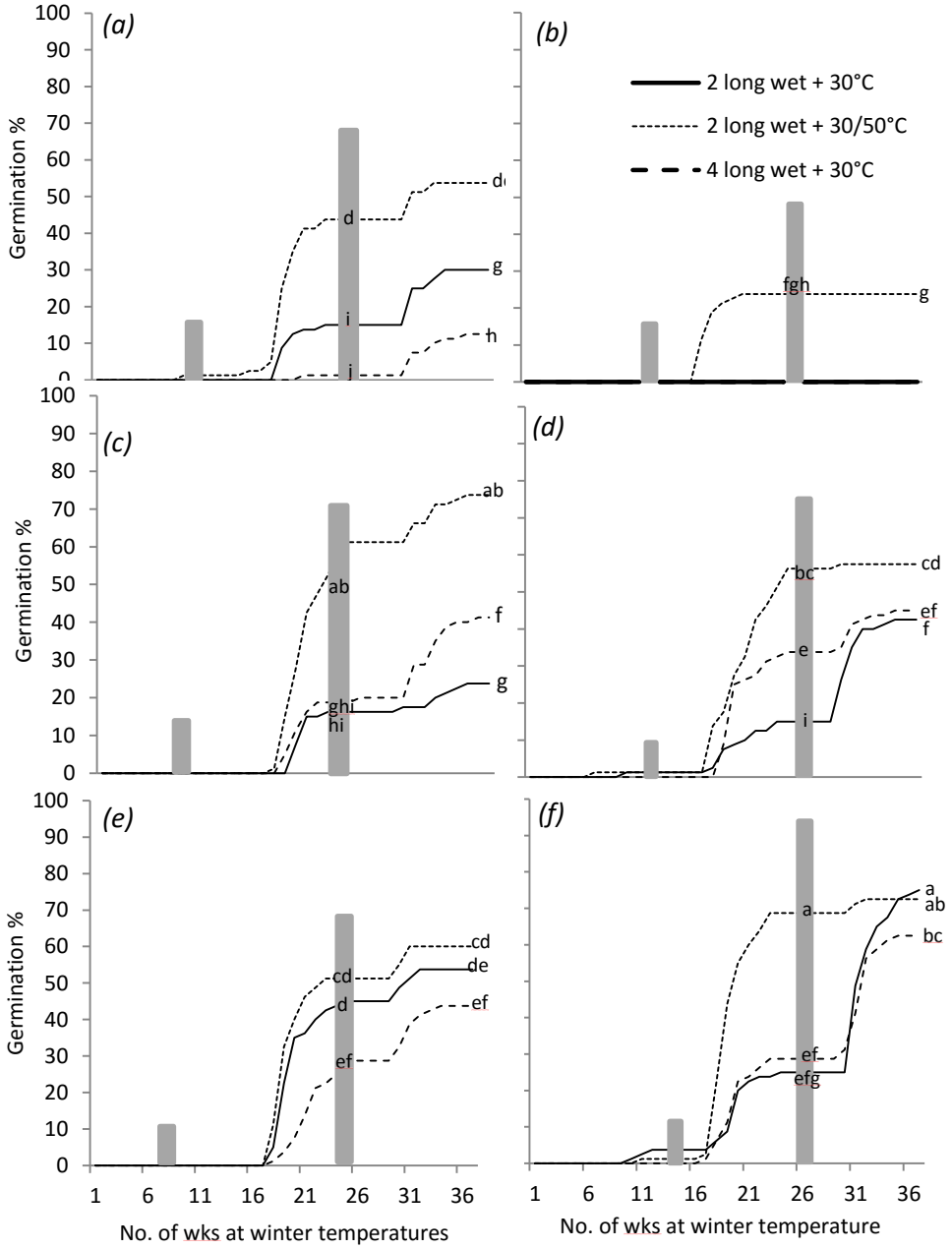

Figure 3.20: Mean cumulative germination percentage from simulation of winter temperature variations (experiment two). *(a)* Winter temperatures of 15 °C (summer 12 weeks). *(b)* Winter temperatures of 20 °C (summer 12 weeks). *(c)* Winter temperatures of 10/20 °C (summer 12 weeks). *(d)* Summer of 16 weeks + winter temperatures of 10/20 °C. *(e)* Summer of 12 weeks + autumn of 4 weeks + winter temperatures of 10/20 °C. *(f)* Summer of 20 weeks + winter temperatures of 10/20 °C. Grey shading on all graphs indicates the final two of three summers with the first summer preceding the data shown. Letters within the summer bars indicate significant differences in germination percentages between all treatments at the completion of the preceding winter.

There was no significant difference in the final germination percentage in any of the three treatments in experiment three (cold stratification during summer wet/dry cycles). However, germination occurred earlier, and after less weeks of being exposed to winter temperatures, from seeds within endocarps that were put through the extended summer treatment than from endocarps in those treatments that only had 12 week summer periods (Fig. 3.21).

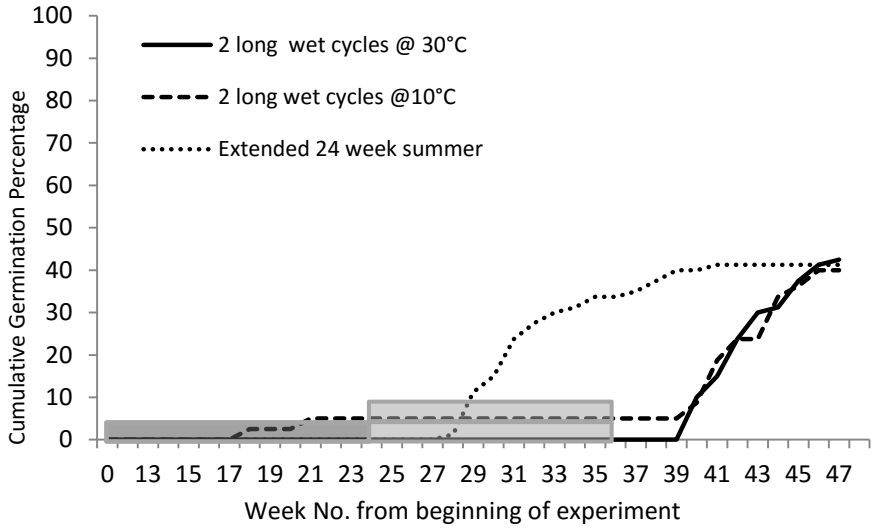

Figure 3.21: Mean cumulative germination percentages in experiment three. Light grey bars indicate the periods at which treatments with 12 weeks summer were exposed to summer conditions. Dark grey bar indicates the weeks that the treatment with 24 weeks summer was exposed to summer conditions. Final germination percentages in each of the treatments were not significantly different.

Detailed examination of the main treatment factors in experiment one indicated that long wet cycles during the summer months resulted in significantly greater germination than short wet cycles (Table 3.6). As a result only long wet cycles were included in subsequent wet/dry experiments.

Significantly more seeds germinated from endocarps exposed to either two or four wet cycles compared with those that experienced dry summers (Control/no wet cycles) (Table 3.6). In experiment one, more seeds germination from endocarps exposed to four wet cycles compared with two wet cycles but this difference was not significant. Conversely, in experiment two, significantly more seeds germinated from endocarps after two wet cycles compared with four (Table 3.7). Wet cycles obviously play a part in breaking dormancy but the exact role is not clearly defined by these results.

The temperature of the endocarps during the summer wet cycles (either 10 °C or 3 0°C) was not significant in either experiment one or three (this factor was not included in experiment two). This factor was investigated based on the differences in soil temperatures between the nursery and the field in the burial trials (Section 3.4) but does not appear to play an important role in dormancy alleviation.

Germination in experiment two occurred best at the winter temperature of 10/20 °C and this was significant in both the second and third winters (Table 3.7). This is the temperature that best equates to the temperatures that the endocarps and seeds experience in their natural environment.

The length of time that the endocarps are exposed to summer temperatures appeared to play a critical role in reducing seed dormancy. The longer the summer period the greater the germination that occurred, with the best germination occurring in the treatments with a 20 week summer (Table 3.7). Germination after the longer summer period was up to 85% in one replicate after only three winters. If the summer period is only 12 weeks long, then a fluctuation in the summer temperature (i.e. 30/50 °C) improves germination significantly ($P<0.001$). This trend (increased germination after fluctuating summer temperatures of 30/50 °C) was also apparent for a 16 week summer and 12 week summer + 4 week autumn but was not significant.

The results related to variations in summer temperatures (i.e. constant 30 °C vs fluctuating 30/50 °C) were not as conclusive with a constant 30 °C summer temperature resulting in significantly greater germination in experiment one (Table 3.6) while the reverse was true for experiment two (Table 3.7). Further investigation into the interactions between summer temperatures and other factors indicated that germination in treatments with 30/50 °C in experiment one was greater than 30 °C, only if there were no wet cycles but this was not significantly different. However, if the wet cycles were long, then germination after exposure to constant 30 °C summer temperatures was significantly greater than at 30/50 °C summer temperatures.

Investigation into the interactions between different treatments in experiment two indicated that a fluctuating summer temperature (30/50 °C) is only important if the summer is short. When all the data from the three experiments were combined and analysed, summer temperature fluctuations had no significant effect unless in combination with other factors.

Table 3.6: Mean percentage seed germination from *P. longifolia* endocarps within the different simulations of summer wet/dry cycles (experiment one). Germination was very low after the first winter and therefore results are not included here. Treatments with the same superscript are not significantly different from other treatments within the same factor and season. NS = Not significant

Factor	Treatment	Germ. % after 2nd winter	P value	Germ. % after 3rd winter	P value	Germ. % after 4th/final winter	P value
Length of wet cycles	No wet cycle	7.5 ± 2.5[a]	<0.001	12.1 ± 3.1	NS	20.6 ± 5.1[b]	0.03
	Short	8.9 ± 1.6[b]		21.5 ± 3.5		27.2 ± 3.3[b]	
	Long	10.8 ± 1.4[c]		30.2 ± 4.1		35.4 ± 4.0[a]	
Number of wet cycles	No wet cycle	7.5 ± 2.5[a]	<0.001	12.1 ± 3.1	NS	20.6 ± 5.1[b]	<0.001
	2 wet cycles	8.5 ± 1.0[b]		25.5 ± 3.6		31.1 ± 3.2[a]	
	4 wet cycles	15.0 ± 2.2[c]		32.1 ± 5.8		35.8 ± 6.3[a]	
Temp. during wet cycles	10 °C	4.53 ± 1.1[a]	0.004	32.8 ± 5.7	NS	35.2 ± 5.3	NS
	30 °C	4.06 ± 1.1[b]		23.3 ± 3.0		30.0 ± 2.8	
Summer Temp.	30/50 °C	4.03 ± 1.0	NS	18.3 ± 1.7[a]	<0.001	26.0 ± 2.2[a]	<0.001
	30 °C only	4.03 ± 1.0		32.1 ± 4.9[b]		34.8 ± 4.5[b]	

Table 3.7: Mean percentage seed germination from *P. longifolia* endocarps in experiment two (simulation of variations in winter temperatures). Germination was very low after the first winter and therefore results are not included here. Treatments with the same superscript are not significantly different from other treatments within the same Factor and Season.

Factor	Treatment	Germ. % after 2nd winter	P-value	Germ. % after 3rd winter	P-value
Number of wet cycles	2 wet cycles	39.2 ± 3.6[a]	<0.003	52.7 ± 3.4[a]	<0.001
	4 wet cycles	26.2 ± 4.7[b]		41.5 ± 5.9[b]	
Summer temperatures	30 °C plus 50 °C	50.8 ± 4.2	NS	56.9 ± 4.9[a]	0.007
	30 °C only	25.3 ± 3.1		44.3 ± 3.7[b]	
Temperature during winter	15 °C	26.7 ± 7.6[a]	<0.001	32.1 ± 7.4[a]	<0.001
	20 °C	22.9 ± 3.6[a]		27.1 ± 4.3[b]	
	10/20 °C	39.3 ± 3.5[b]		57.3 ± 3.1[c]	
Summer length	12 weeks	30.0 ± 4.6[a]	<0.001	38.3 ± 5.0[a]	<0.001
	16 weeks	35.0 ± 6.7[ab]		48.3 ± 6.3[b]	
	12 wks + 4 wks autumn	41.6 ± 5.9[b]		52.5 ± 4.9[b]	
	20 weeks	40.8 ± 7.6[b]		70.8 ± 3.1[c]	

When all three experiments were compared after the second winter phase (experiment three was ended after two winters), the best germination occurred in the treatment with an extended summer of 20 weeks + 30/50 °C summer temperatures + two long wet cycles at 30 °C + 10/20 °C in winter (68.8 ± 4.3%) from experiment two (Fig. 3.20f). This was significantly different from all other treatments except the treatment with a 12 week summer + 30/50 °C fluctuating summer temperatures + two long wet cycles at 30 °C + winter temperatures of 10/20 °C (61.3 ± 9.2%) (Fig. 3.20c).

When experiment one and two were compared after three winters, all of the treatments with the 20 week summer resulted in the best germination (between 65 and 75%). The only other treatment that was significantly better than all others was a 12 week summer + 30/50 °C summer temperatures + two wet cycles + winter temperatures of 10-20 °C (73.8 ± 9.2%).

3.5.3.3 Stratification Experiment

No germination occurred in seeds that were kept constantly wet at 8, 15 or 20 °C. Minimal germination (i.e. <5%) occurred from seeds within those endocarps exposed to warm incubation only, cold stratification only or cold stratification + warm incubation. The only treatment with >5% germination was 12 weeks warm + 6 weeks cold stratification. Germination in this treatment reached 13.8 ± 4.3% (Fig. 3.22). Whilst this is still a relatively low germination percentage it is greater than the germination observed in the first winter in any other laboratory trial undertaken in during the course of this study.

The order of stratification was not significant but the number of weeks of warm summer temperatures and cold stratification were significant ($P<0.001$). Summers with 12 weeks warm (including 2 weeks wet) resulted in significantly higher germination than either no warm summer temperatures or 6 weeks summer temperatures (including 2 weeks wet) ($P=0.003$).

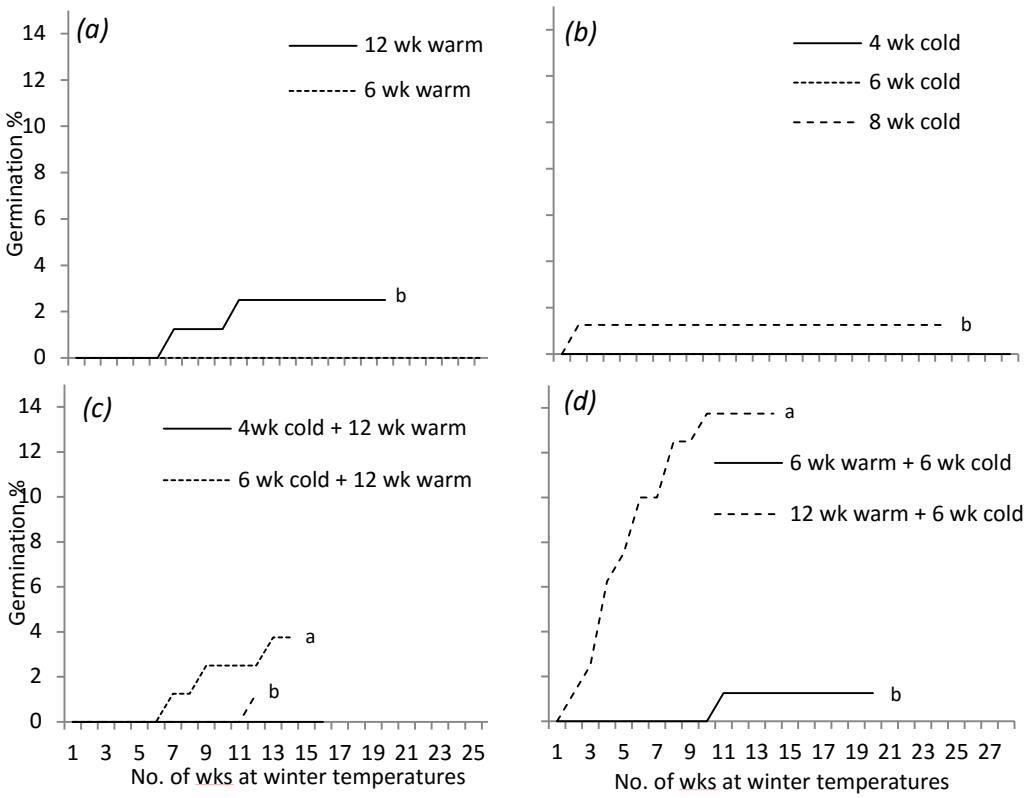

Figure 3.22: Germination of *Persoonia longifolia* seeds from within endocarps exposed to varying combinations of cold and warm stratification. *(a)* Warm stratification only. *(b)* Cold stratification only. *(c)* Cold + warm stratification. *(d)* Warm + cold stratification. No germination occurred in the treatments where endocarps were kept constantly wet and therefore these results are not presented here. Treatments with the same letter do not differ significantly.

Based on the results presented here 6 weeks cold stratification appears to be the optimum number of weeks for cold stratification with significantly greater germination occurring in those treatments compared with either no cold stratification, 4 or 8 weeks cold stratification ($P<0.001$). Results are limited by the low germination numbers and further investigations may provide a better data set if the experiment is continued over more than one winter cycle.

When removing the lids, they were either impossible to remove or dislodged relatively easily making it easy to determine if the lid had weakened in response to the different treatments. In those treatments where lids did pop off easily, significantly more lids came off in those treatments that experienced longer warm periods (i.e. 12 weeks warm

or constant warm at 30 °C) than those that experience shorter warm periods or cold periods (Fig. 3.23).

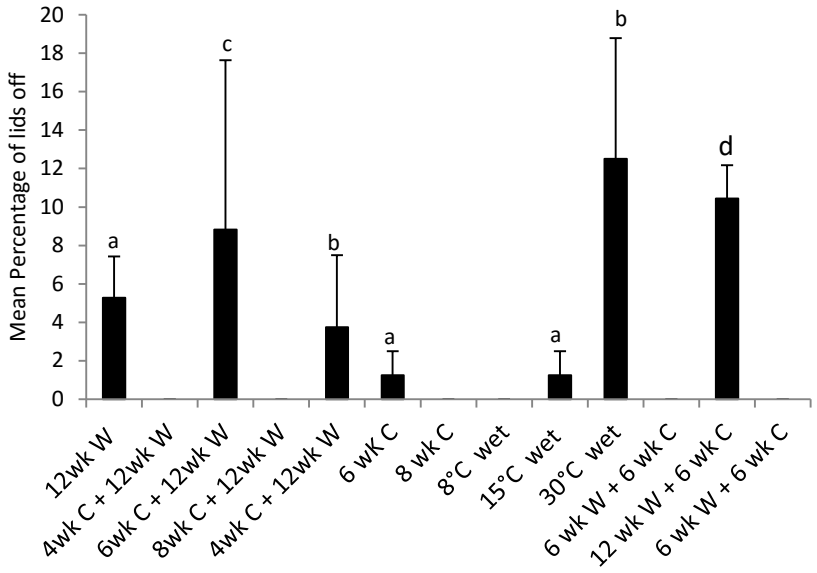

Figure 3.23: Mean percentage of seeds with lids that were readily dislodged at the completion of the stratification trial. W = warm incubation, C = cold stratification. Treatments with the same letters do not differ significantly (P<0.001).

3.5.3.4 Nursery Wet/Dry trials

Natural rainfall during the period that this trial was undertaken is shown in Table 3.8 and temperatures recorded in the tubs on the days the soils were moistened are shown in Table 3.9. On occasions the temperature probes failed to record temperatures and therefore there are some gaps in the data.

Natural rainfall occurred in three main rainfall events in both the first summer (2011/12) and the second summer (2012/13) and was therefore similar to the simulated four wet cycles. In the third summer (2013/14) there was only one rainfall event which occurred and this was not until February 2014.

Seedling emergence commenced once endocarps had been continuously wet for approximately 8 weeks in each of the winter seasons. Seedling emergence was initially greatest in the four wet cycles (i.e. in the first winter, P<0.001), however by the second season there was no significant difference in the final seedling emergence percentage from tubs exposed to four wet cycles and natural rainfall (Fig. 3.24). By the end of the

third winter seedling emergence was greatest for those endocarps that received natural rainfall (Fig. 3.24) Those tubs that received no summer water at all had significantly lower seedling emergence ($P<0.001$) than all other treatments in all winters.

Seed fill at the completion of the trial was greatest in those endocarps that were not watered over the summer months (mean of 9.5 $\pm$ 3.3%) but this was not significantly different from the other three treatments. Mean seed fill for those endocarps that received natural rainfall was 2.0 $\pm$ 1.2%, for two wet cycles was 2.0 $\pm$ 1.1% and for four wet cycles was 2.5 $\pm$ 1.0%.

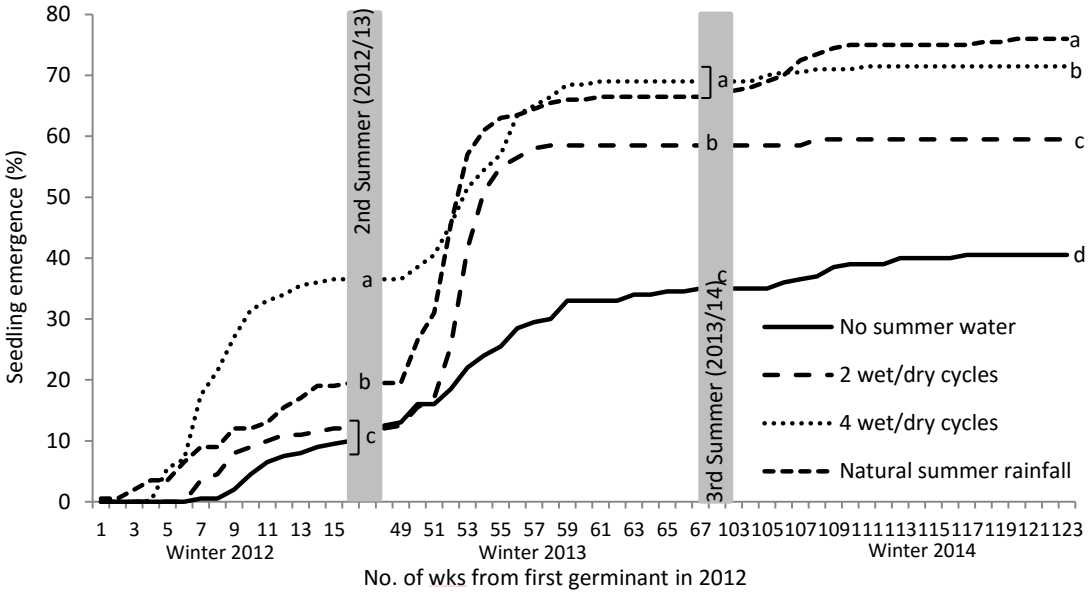

Figure 3.24: Seedling emergence from endocarps buried in the nursery for 3 years and treated with different summer watering regimes. Grey shading indicates summer months. Letters within the grey summer bars indicate significant differences in germination percentages at the completion of the preceding winter.

The second nursery trial with *P. elliptica* endocarps was undertaken from November 2013 to November 2015. Only one main rainfall event occurred during each of the two summers that this trial was undertaken. During the 2013/2014 summer 23 mm was recorded during February and in 2014/2015 18 mm was recorded during March. The remaining summer months were dry or had less than 10 mm of rainfall.

Seedling emergence during the first winter was very low (<3%, Fig. 3.25). In the second winter seedling emergence commenced late May and continued until the end of July. Seedling emergence was greatest when the *P. elliptica* endocarps were exposed

to natural rainfall. There was no significant difference in final seedling emergence in those seeds that received no water or those that were artificially watered.

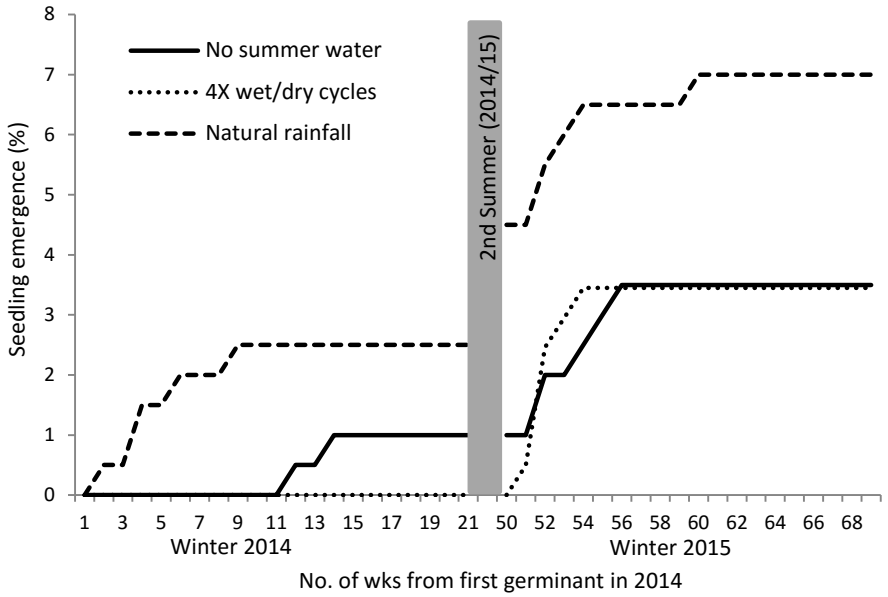

Figure 3.25: Mean cumulative seedling emergence (%) from *Persoonia elliptica* endocarps buried in the nursery for 2 years and treated with different summer watering regimes. Grey shading indicates summer months. Treatments with the same letter do not differ significantly

3.5.4 Discussion

The various experiments undertaken here indicate how complex some natural germination systems can be. Seeds or fruits released from the parent plant are exposed to a variety of different conditions depending on the environment into which they are released. South Western Australia has long hot summers (November to March) which are interspersed with summer thunderstorms. These thunderstorms appear to play an important role in releasing dormancy in *P. longifolia* seeds. Seeds are then exposed to a definitive autumn period of episodic rainfall, followed by a cool wet winter where soil conditions stay more or less constantly moist for up to six months (Merritt et al., 2007, Turner et al., 2006).

Table 3.8: Mean monthly rainfall (mm) occurring at Capercup throughout the period of study and mean monthly temperature data for each treatment.

	Year	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec	Total
Rainfall*	Mean Rainfall (47 yrs)	17.9	15.3	16.8	31.6	62.8	83.5	85.8	69.5	54.0	33.4	25.5	13.2	510.3
	2011	68.0	0.6	0.8	38.1	41	94.5	89.3	73.0	58.2	46.8	50.4	64.2	624.9
	2012	9.8	17.0	0.0	13.5	35.9	104.0	28.4	53.8	90.6	13.2	48.6	63.6	478.4
	2013	6.8	0.2	52.0	29.4	90.2	29.4	73.4	80.2	121.2	20.2	12.4	6.4	521.8
	2014	1.6	27.6	2.2	9.8	114.4	38.2	97.8	62.6	61.8	35	21.2	1.4	473.6
	2015	13.8	0.2	19.4	60.4	30.6	39.0	54.2	52.4	39.0	23.6	6.2		

Table 3.9: Minimum and maximum temperatures recorded by data loggers in each treatment on the days when wetting occurred in summer months. Missing numbers indicate where the temperature probes failed. Numbers in italics indicate the treatments where watering occurred.

Year	Date	2 x wet		4 x wet		Natural rainfall		No rainfall		Air Temperature*	
		Min (°C)	Max (°C)	Min (°C)	Max (°C)	Min (°C)	Max (°C)	Min (°C)	Max (°C)	Min (°C)	Max (°C)
2011/12	23/12/2011			*15.4*	*24.2*	15	34.9	16.9	34.7	16.1	29.2
	6/1/2012			*14.8*	*22.5*	13.3	28.5	17.0	25.4	18.6	25.9
	6/2/2012	*20.6*	*33.3*	*14.7*	*25.7*			14.8	30.2	9.9	27.3
	24/2/2012			*15.5*	*23.1*			17.1	28.5	16.3	28.1
2012/13	20/12/2012	12.5	40.51	*13.6*	*28.2*			13.6	37.0	12.7	33.7
	22/1/2013	*12.3*	*24.9*	*15.0*	*27.6*			13.1	33.0	13.4	33.1
	14/2/2013	19.3	35.8	*17.9*	*28.5*			19.5	37.0	22.1	39.0
	26/2/2013	*15.8*	*27.7*	*16.8*	*26.8*			16.2	36	16.9	33.1
2013/14	20/12/2013	13.3	22.7	*12.6*	*24.1*			13.6	23.1	12.2	22.3
	22/1/2014	*18.0*	*35.5*	*18.5*	*39.3*			18.6	38.8	17.3	35.4
	14/2/2014	15.1	29.5	*14.3*	*32.1*			15.1	30.5	14.6	33.5
	28/2/2014	*13.7*	*25.2*	*13.3*	*27.9*			15.9	32.1	12.7	34.0

*Air temperature data is for Wagin, Australian Bureau of Meteorology station (Bureau of Meteorology, 2015)

In the case of *P. longifolia*, it appears that the summer conditions experienced by the endocarps are critical in releasing seed dormancy and allowing the seed to germinate as winter-like conditions begin to establish. Clearly, temperatures, moisture levels and the timing of these are all important for regulating seed dormancy during the summer months.

A study of 28 jarrah forest species from WA found that greatest germination generally occurred between 10 and 15 °C (Bell and Bellairs, 1992). As a result 15 °C tends to be the temperature at which germination testing for WA species is undertaken (S. Turner pers. comm.). However, as Bell and Bellairs (1992) found in their study this temperature is dependent on the microclimate that the species is found in. If the species was found in an area where water was available into the warmer months then germination tended to occur at higher temperatures.

Initial investigations undertaken here indicated that the best temperatures for germinating *P. longifolia* were the constant temperatures of 15, and 20 °C, and 10/20 °C. Very low temperatures (5 or 10 °C) or very high temperatures (25 and 30 °C) resulted in significantly less germination. Further examination of these winter temperatures in the long-term wet/dry laboratory experiments showed that the fluctuating temperatures of 10/20 °C was the best winter temperature regime for germination of this species. This fluctuation of temperatures is a reflection of the winter temperatures that the endocarps/seeds are exposed to in their natural environment on a daily basis from May through to August (Table 1.1). Assumptions of temperature and moisture regimes based on previous studies in the literature can be misleading and examination of the climatic conditions experienced within these Mediterranean environments could more accurately identify key drivers of dormancy loss.

Germination in *P. longifolia* was significantly improved through the manipulation of temperatures and moisture regimes during the summer months. Most importantly, the seeds require brief periods of wet (or stratification) during the summer months. The environment in which these plants are found is subjected to between one and four summer thunderstorms annually (Fig. 3.13) (Bureau of Meteorology, 2015). The results from the wet/dry trials and the nursery trials indicated that these thunderstorms are one of the key drivers to breaking dormancy in the endocarp and seed. However, the laboratory based wet/dry trials did not conclusively show whether two or four wet

cycles were best, as both were shown in different experiments to be optimal. Nevertheless, it was conclusively shown that the wet cycles needed to be for a longer period of time (i.e. 7 days compared with 24 hours). A small, summer rainfall event, where the seeds are moist for only 24 hours, is not sufficient to promote dormancy loss. Instead the seeds require a significant rainfall event (like that experienced during thunderstorms with >10 mm of rainfall) in order for dormancy to be broken.

Whilst the temperatures were not manipulated in the nursery environment, variations on the summer watering regime under nursery conditions also resulted in improvements to germination. Once again some summer rainfall was shown to be required in order to enhance germination and four wet cycles produced earlier and more germination than two wet cycles or no exposure to any summer moisture. Four wet cycles also produced more germination earlier in the experiment than the natural rainfall although by the completion of the experiment there was no difference between natural rainfall and four wet cycles and this is most likely due to the fact that the natural rainfall events that occurred throughout the experiment were similar to the artificially imposed four wet cycles. Therefore, from a practitioner's viewpoint, using four wet cycles during the summer months is likely to produce more germinants earlier in the process for use in restoration. Indeed, for restoration purposes seedlings could either be removed and potted up as they emerge during the winter months and used as greenstock or alternatively endocarps could be removed from the sand at the end of summer (before germination has commenced) and buried in restoration sites the following autumn as part of other restoration activities.

Mullins et al. (2002) found that seeds that were constantly wet over summer did not germinate unless surface sown. It is likely that those endocarps that were buried did not have a chance to dry out between watering (they were watered twice daily). Staying constantly wet may result in seeds rotting and may explain why no germination occurred from the buried endocarps in that trial. The seed fill from the nursery trials presented in this present study support this theory with those seeds that were not watered at all having the highest seed fill at the completion of the experiment (Table 3.5). Endocarps and naked seeds in the laboratory trials were highly susceptible to fungal infections so keeping them constantly wet and warm during summer is likely to exacerbate this fungal attack and may result in a significant decrease in seed viability.

Summer wet/dry cycles to promote germination may be a phenomenon that is relatively common in species from the South West of WA. For example, Hidayati et al. (2012) used wet/dry cycles to improve germination in three out of four *Hibbertia* species although the wet/dry cycles used in Hidayati et al.'s (2012) trials were by comparison shorter cycles (24 hours). Other studies have also found wet/dry cycling to significantly improve germination in many different species e.g. *Actinobole uliginosum, Goodenia fascicularis, G. cycloptera* and *Velleia glabrata* (Hoyle et al., 2008), and *Actinotus leucocephalus* (Baker et al., 2005b). Wetting and drying of the endocarps and seeds could operate in two ways. The natural wetting and drying, and temperature fluctuations associated with water in the soil, are likely to weaken the endocarp making it easier for the seed to push through the hard, restricting seed coverings. In addition, it is likely that the wetting and drying operates on a physiological level (stratification) allowing the seed to develop the growth potential to emerge from the endocarp once suitable conditions for germination occur. More lids could be dislodged from those endocarps that had been exposed to longer warm treatments compared with those treatments with a short warm treatments or no warm treatments (although this was by no means clear cut) and it may be that warm stratification assists with breaking down the suberin (fatty acid) that binds the lid to the endocarp (Dixon et al., 2002). An experiment was undertaken to attempt to define the effect of stratification on the growth potential of the seed and the effects on its covering layers however this experiment was badly affected by mould and consequently there was no clear outcome from this preliminary trial (data not shown).

Physiological dormancy can be either non-deep, intermediate or deep and it is likely that there is a continuum between the three (Baskin and Baskin, 2014). It can be broken by cold, warm or warm + cold stratification (Baskin and Baskin, 2014). Dormancy in seeds with non-deep physiological dormancy can be broken by warm or cold stratification and excised seeds will germinate and develop into normal seedlings. Seeds with intermediate physiological dormancy require a long period of cold stratification in order to germinate and this can be shortened if the seeds are given a period of warm pre-treatment (either wet or dry) prior to cold stratification. Baskin and Baskin (2014) indicate that at this stage it is unknown if wet or dry warm pre-treatments are equally successful in reducing the length of time that cold stratification is required. Embryos excised from seeds with deep physiological dormancy will germinate but will

not develop into normal seedlings (Nikolaeva, 1969) and dormancy is broken by long periods of warm or cold stratification.

Investigations into the role of stratification in dormancy break of *P. longifolia* showed that warm followed by cold stratification is required but only if the endocarps were exposed to a longer warm period (12 weeks compared with 6 weeks). Although germination was low (<15%) in the stratification trial, it was still greater than germination observed after only one winter in almost all other laboratory trials undertaken to date and in this context are highly encouraging. Norman and Koch (2006) found that a period of warm stratification (15/30 °C for 2 weeks) prior to sowing under greenhouse conditions resulted in significantly better germination than all other treatments. However this treatment only produced 13% germination over two winters. The role of warm stratification and cold stratification in promoting germination through breaking of physiological dormancy is not clearly defined here. Warm stratification has been linked to breaking of physiological dormancy in *Empetrum hermaphroditum* another species with a stony endocarp (Baskin et al., 2002) and Persson et al. (2006) linked warm stratification with weakening of the endocarp and cold stratification with breaking of physiological dormancy within the embryo itself in *Crataegus monogyna.* Further investigations are required to clearly define the roles of warm and cold stratification in promoting germination in *P. longifolia* seeds although anecdotal evidence indicates that warm stratification plays a role in releasing the lid from the endocarp (K. Chia pers. obs.) which may assist germination once the embryos have become less physiologically dormant.

The length of time that endocarps were exposed to summer conditions, and the temperatures that they experienced during those dry periods in summer, were also important. A short summer period of 12 weeks produced less germination than a longer summer period of 20 weeks. Summer temperatures in the jarrah forest soils of 30 °C and above can occur from mid-October through to late March a period of between 19 and 24 weeks (as shown in the burial trials). Once again the laboratory trials reflected the ecological conditions experienced by this species in its natural environment. Hidayati et al.'s (2012) results also indicate that the longer summer period of 16 weeks with wet/dry cycles produced more germinants than the shorter summer periods for *Hibbertia commutata* and *H. huegelii* although no indication is given on the significance of this difference. Likewise, Hoyle et al. (2008) also found a longer period of summer

wet/dry cycling resulted in higher germination in three of four Queensland species investigated (*Actinobole uliginosum, Goodenia cycloptera* and *Velleia glabrata)*. This concept of a longer summer period has not been investigated in great detail for Western Australian species and may be of importance for other native species as well, particularly for those considered to possess deeply dormant seeds (Merritt et al., 2007). The stratification experiment undertaken with *P. longifolia* included a summer of only 12 weeks and only 2 weeks during that period were wet. Germination could potentially be improved with either longer wet periods, more wet periods or a longer summer period and this could be the focus of future investigations.

The effect of fluctuations in the summer temperatures of 30/50 °C was investigated and the effect was variable. If the summer was short then the use of a burst of heat improved germination in comparison to a constant 30 °C summer. Additionally if there were no wet cycles or only two wet cycles then a burst of heat also improved germination.

Merritt et al. (2007) proposed a model of germination timing for Australian flora which provides a useful tool to explore the processes of dormancy loss in native species from Mediterranean environments. The authors indicated that the role of warm stratification had been, at that time, not well explored. Emerging evidence indicated that warm stratification and possibly warm plus cold stratification play an important part in overcoming seed dormancy in species from the south west of WA. The results from the *P. longifolia* dormancy breaking experiments further support this idea.

Merritt et al. (2007) suggests that warm stratification occurs in autumn (between April and May) and in spring (October and November). However, it is also possible that summer thunderstorms provide short periods of warm stratification during the summer months which break dormancy in some species, a fact not considered by these authors.

Figure 3.26 is a modified version of the model developed by Merritt et al. (2007) as it applies to *P. longifolia,* with the major difference being a number of short periods of warm stratification (i.e. moist warm (≥15 °C) conditions) over the summer months. The other variation on Merritt et al.'s (2007) original model is the requirement for a germination stimulant. KAR_1 and GA_3 were used as potential germination stimulants for *P. longifolia* in some of the experiments undertaken here, however the results were

variable and not clear (Section 3.3.3.4 and 3.4.3.2). Additional investigations are required to determine if germination stimulants will further improve germination and particularly whether smoke and smoke products such as KAR_1 enhance germination when applied with effective dormancy breaking treatments at key times i.e. end of the summer temperature regime.

The nursery based trials have shown that burial within the nursery environment (rather than the forest) and manipulation of the various watering regimes can be used to maximise germination over the quickest period of time for *P. longifolia* in order to produce seedlings for use in restoration or horticulture. This approach of manipulating the summer watering regime (i.e. artificially imposing warm stratification) may be transferable between species of *Persoonia* and possibly other species with hard woody endocarps that are readily found in the jarrah forests of Western Australia. Early indications are that burial of endocarps of species such as *P. elliptica* may result in some seed germination but further investigations are required to determine the species specific environmental conditions required to break dormancy. Whilst artificial watering of *P. elliptica* was unsuccessful in promoting germination on this occasion, further experimentation with different procedures may be more successful. Nield et al. (2015) found that *P. elliptica* seed collected from the soil had very low viability with only 13% of endocarps containing a viable seeds and seeds collected from the tree canopy having a viability of only 39%. Therefore, ensuring that buried *P. elliptica* endocarps contain a healthy seed may also result in greater germination, which was not done in this initial experiment. Further investigations into the germination biology of other *Persoonia* species may also assist with utilising these species for different purposes in future using some of the approaches described in this study.

3.6 Conclusion

The seed of *P. longifolia* is recurved, differentiated and fully developed. The seed is encased in a hard woody endocarp and whilst the endocarp is permeable, the rate of water uptake is restricted by its presence. The seed will germinate once removed from the outer covering layers of the dispersed reproductive unit (mesocarp and endocarp) after a period of approximately 30-50 days indicating that the outer endocarp and possibly the testa play a role in dormancy within the reproductive unit. It is therefore concluded that physiological dormancy is present in seeds of *P. longifolia.*

In its natural environment, a complex sequence of environmental conditions are required in order to break dormancy and allow germination to occur. Burial increased germination, although this was dependent on the summer conditions experienced whilst buried. Burial in an environment which allows summer moisture to penetrate the soil (e.g. without leaf litter) was critical. Manipulation of summer watering regimes increased germination from nursery buried endocarps and dormancy can be broken through the use of warm plus cold stratification periods.

The results from the experiments presented in this study indicate that it is likely that *P. longifolia* has intermediate dormancy that is broken by a warm pre-treatment plus cold stratification. In the wet/dry laboratory trials the use of a dry, heat pulse in the summer months increased germination when the summer was short and/or there were no wet periods (warm stratification) during the summer months. In the stratification trials, at least 12 weeks warm (including 2 weeks wet) treatment was required before dormancy could be broken by cold stratification. It would therefore seem that both wet and dry warm pre-treatments can be used to break dormancy although it is still not known if one is more effective than the other. Future studies could continue investigations into determining the optimum warm pre-treatments and its role in dormancy break.

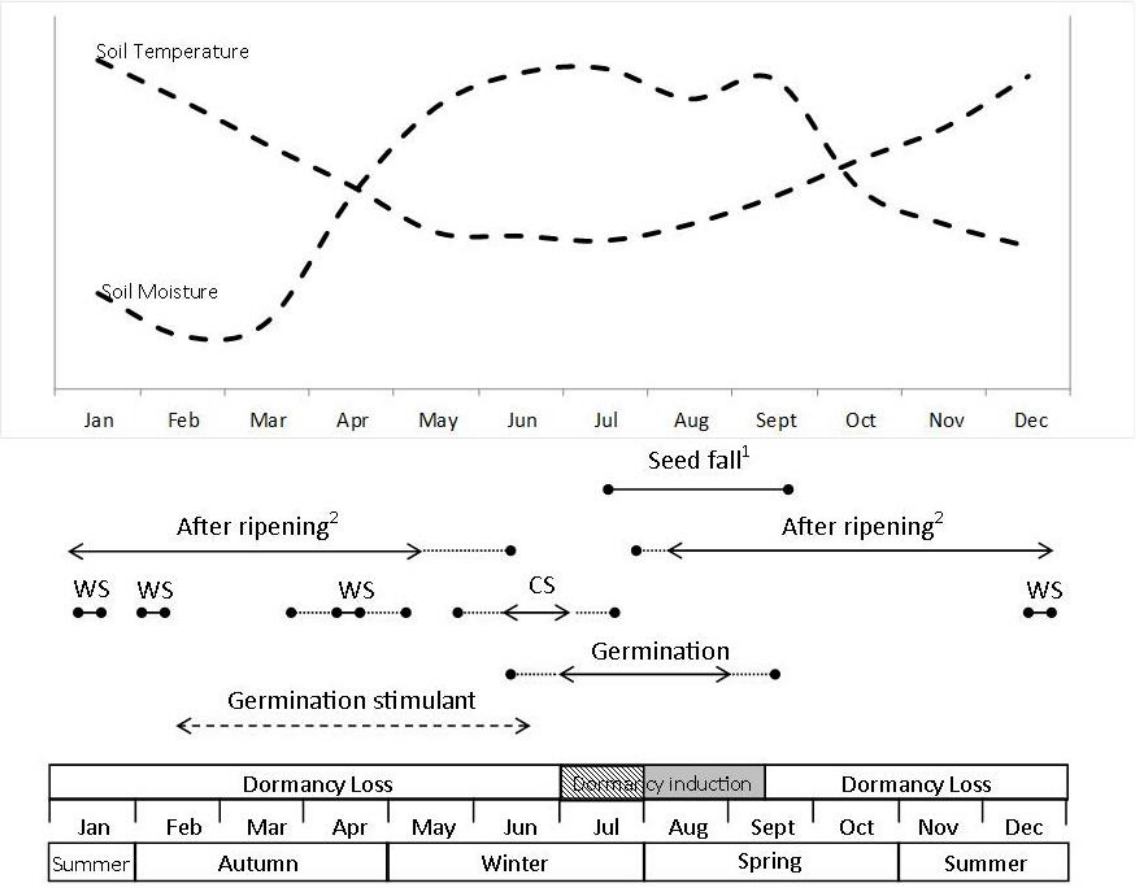

Figure 3.26: Diagrammatic representation of the timing of seed dormancy loss/induction and germination events for seeds of *Persoonia longifolia* released into the soil modified from Merritt et al. (2007). Seeds may experience a number of these dormancy loss/induction cycles before conditions are appropriate for germination. Soil temperature and moisture profiles are indicative only and are based on soil temperatures and moisture levels recorded over the course of this study. CS = Cold stratification (dormancy alleviation of moist seeds at 0-10 °C). WS = Warm stratification (dormancy alleviation of moist seeds at temperatures $\geq$ 15 °C). Solid lines represent the main time periods over which the specified events occur, and the dotted lines the less likely periods when the events may occur. Dashed lines indicated additional events identified in the original model of Merritt et al. (2007), which at this stage are unidentified for *P. longifolia*. Hatched box indicates the period at which dormancy induction can begin to occur depending on environmental conditions experienced. 1. Seed fall is defined as the entry of the seed into the soil seed bank following shedding from the parent plant at maturity. 2. After ripening is defined as dormancy alleviation of seeds in a dry state. After ripening usually occurs most efficiently at warmer temps (eg >15 °C).

In addition, it is still unknown if warm stratification or dry warm pre-treatment assists with breaking down endocarp resistance or is part of the process required to reduce physiological dormancy within the embryo itself. It is possible that the wetting and drying cycles (or extreme heat cycles) result in the breaking down of the suberin which fuses the lid to the rest of the endocarp and generally weakening the endocarp through the development of cracks and fissures as seen in the burial experiments. Cold stratification may increase the growth potential of the embryo providing it with enough expansive force to overcome the mechanical constraints imposed by its covering layers. Further experimentation could be undertaken to define the exact role of warm and cold stratification in the dormancy alleviation process.

Germination in *P. longifolia* is cyclic and this could be a result of either dormancy being reimposed during the spring months or that dormancy break only occurs in part of the seed population in any one season which can have survival value in an uncertain environment. Deep dormancy of some individuals of each seed cohort is a common feature of Australian species to avoid depleting the entire seed bank in the case of a given period of rainfall that promotes germination but is insufficient for seedling establishment (Bell, 1999).

Whilst the internal cues within the seed itself are still not fully understood the results of the investigations undertaken here mean that we are now able to germinate seeds through modification of the soil environment so facilitating the large scale propagation of this species via seeds for use in restoration, horticulture and floriculture.

Chapter Four - Establishing *Persoonia longifolia* in restored jarrah forest following bauxite mining

Understanding the key drivers regulating seed dormancy is critical from a scientific perspective and helps to understand not only the seed ecology of *P. longifolia* but other species with similar germination problems as well. However from a practical restoration perspective it is imperative that this information can be transferred and adapted into either *in situ* germination from directly sown endocarps or the production of a large quantity of seedlings in a nursery environment for planting on restored areas at a later stage. In addition, understanding how these plants perform (survival and growth) once planted out is essential to ensuring they survive in the altered landscape and the conditions that promote germination once seeds have become non dormant.

Chapter Four aims to use the phenology and germination studies outlined in Chapters Two and Three respectively, to direct efforts to return *Persoonia longifolia* to restored areas of former bauxite mines in the jarrah forest of Western Australia. This is undertaken through the use of direct seeding experiments, synthetic seed bank trials, assessment of seedling survival and growth once planted out, and the assessment of the effectiveness of tree guards in maximising selected plant attributes. The ultimate goal is to provide a prescriptive methodology which allows restoration practitioners to return this species successfully to restored areas following the completion of mining activities.

4.1 Abstract

This study aimed to quantify *in situ* emergence of *P. longifolia* seeds on restored areas, investigate the cueing of seeds prior to use in restoration and assess the use of different tree guards to increase seedling survival and health. Initial investigations found that <1% of seeds buried or scattered on restored areas emerged. However, if seeds were cued through burial in surrounding forest, retrieved and sown on restored areas emergence increased to 24%. Significantly more seeds germinated when buried (14.6 %) compared to those scattered on the soil surface (2.7%). Survival of seedlings planted at 2-3 weeks of age was initially less than seedlings planted at 12 months of age. However, 20 months after planting, there were no longer any significant differences in

survival between both age classes and those seedlings planted when younger were significantly taller (29.0 ± 2.9 cm) than those that were planted at 12 months of age (4.7 ± 0.3 cm). Use of "onion bag" guards improved survival from 58.1 ± 4.0% (no guard) to 70.8 ± 3.4% with an onion bag guard. While the use of shade cloth guards did not significantly improve survival, these did significantly increase mean plant height after 32 months growth (22 cm compared with 7.2 cm for no guard). These data demonstrate that consideration needs to be given to specific species requirements in order to improve seedling emergence and survival when attempting to restore recalcitrant species to the post-mining environment.

4.2 Introduction

Persoonia longifolia occurs at a mean density of around 300-440 plants/ha in the Dwellingup area of the northern jarrah forest (Mullins et al., 2002, Norman and Koch, 2008). In the Boddington region, around Worsley's bauxite mine, plants occur at lower densities of 185 to 220 plants/ha (B. Stokes pers. comm.). This species is classified by Alcoa as a recalcitrant species because it is common in the pre-mined forest, is largely absent or found in low densities in restored mine sites and has proven to be exceptionally difficult to propagate using different approaches (Norman and Koch, 2005b).

On restored areas of Alcoa's bauxite mine, *P. longifolia* re-establishes at densities of only 42 plants/ha which is only 10% of their pre-mining abundance (Mullins et al., 2002). Whilst *P. longifolia* occurs more often in those areas restored with direct return topsoil rather than stockpiled topsoil, this difference was not significant and was still relatively low (occurring in 4.8% of the plots in direct return soil and 0.9% of the plots in stockpiled topsoil) (Norman and Koch, 2005b). Mullins et al. (2002) attributed the higher density of seedlings in direct return sites to seed storage in the topsoil but it is also possible that regeneration from the top soil occurs from root fragments removed from the parent plant during the clearing process as *P. longifolia* has been found to regenerate from damaged root portions on road verges (Dewing, 2000). Current aims for restoration of the jarrah forest following bauxite extraction include re-establishing a high botanical diversity that is reflective of the pre-mining environment (Ward et al., 1996). *Persoonia longifolia* is such a common plant in the pre-mining environment, it

is considered an important species to return to the post-mining environment in order to re-establish the forest ecosystem and resilient ecosystem function.

Alcoa has, in the past, included *P. longifolia* fruits in restoration seed mixes which were sown directly on top of the soil surface, As almost no seedlings have been recorded in restoration sites, it has since been removed (Norman and Koch, 2008). To address this low level of regeneration investigations have been undertaken over the past 15 years to find a practical and effective means of returning significant numbers of *P. longifolia* to restored areas of the jarrah forest through topsoil return, inclusion in seed broadcasting mixes or as green stock, though with relatively little success to date (Mullins et al., 2002, Norman and Koch, 2005a, Norman and Koch, 2005b, Norman and Koch, 2006, Norman and Koch, 2008, Turner et al., 2010).

Likewise, Worsley has had a similar experience, with *P. longifolia* being almost absent from the restored areas at Boddington despite using the standard restoration approach of direct return of topsoil. Worsley currently include a small amount of *P. longifolia* fruits in its seed mix which is scattered over restoration sites however subsequent monitoring indicates that this current approach is no successful as there is a total absence of *P. longifolia* in restoration sites.

Whilst investigation into the seed germination biology of *P. longifolia* continues, seedlings germinated under nursery conditions (Chapter 3) could be transplanted onto restored mine areas and begin contributing as a future seed source. Newly restored areas are open environments which are very different from a mature jarrah forest in many different ways. There is very little protection from winds and the sun and therefore soil temperatures are likely to be elevated. Seedlings in Mediterranean-type climates must survive long hot dry summers and this is likely to be particularly tough in restored areas where there is no protection from either summer environmental conditions or from grazing (Koch et al., 2004) provided by surrounding plants, leaf litter, logs or other debris. For plants that occur as mid-storey species in the surrounding jarrah forest these conditions are extremely different from those where natural germination and recruitment events typically occur.

The aim of this component of my study was to examine the use and performance of *P. longifolia* in post mining restoration and in particular to evaluate the utility of both seeds and seedlings in restoration through investigations into:

- seedling emergence on restored areas of differing age and attributes;
- seedling survival following planting onto different restored areas; and
- the use of different tree guards to enhance survival and growth of seedlings once planted out.

4.3 Use of seeds in restoration

4.3.1 Introduction

Persoonia longifolia has proven exceptionally difficult to propagate (Mullins et al., 2002, Norman and Koch, 2005a, Norman and Koch, 2006, Norman and Koch, 2008, Turner et al., 2010) and therefore it has long been assumed that seed dormancy is the principal reason that *P. longifolia* fails to germinate and establish on restored areas in the jarrah forest. Both Alcoa and Worsley have indicated that seedling establishment on the restored areas of the mine site is poor and sporadic (J. Koch pers. comm. and B. Stokes pers. comm.). Seeds of *P. longifolia* have been observed to germinate and emerge in the surrounding forest (pers. obs.). Until this study commenced there was no data to indicate how many of the seeds included in the seed mix germinate, if germination is enhanced through burial and the time it takes for germination to commence. Other factors that may affect seedling establishment and survival may include grazing and lack of protection from existing vegetation (i.e. nurse plants).

In the past, *P. longifolia* seed included in seeds mixes utilised by Alcoa and Worsley, has been sown directly onto the soil surface of restored areas as an entire fruit (J. Koch pers. comm.). Burial trials undertaken by researchers at Kings Park and Alcoa have indicated that burial of *P. longifolia* endocarps for a period of 18 months improves germination under laboratory and field conditions (Norman and Koch, 2008, Turner et al., 2010) though the mechanisms for this dormancy loss and improved germination capacity is poorly understood at present. Seed burial has also been shown to improve germination in other Australian species including those with complex seed dormancy (Baker et al., 2005a, Merritt et al., 2007, Roche et al., 1997b, Tieu et al., 2001, Turner, 2013) although this approach to germination enhancement has never been assessed for use in a restoration context.

The effect of burial (rather than scattering onto the soil surface) on germination and emergence of *P. longifolia* seeds has not previously been examined when used for post mining restoration. Likewise, burying seeds under natural conditions (in undisturbed jarrah forest) to initially break dormancy, then retrieving and re-sowing under different conditions has not been examined and may provide a simple cost effective way to cue seeds ready for germination when re-sown into restoration sites.

In order to determine the best methods of using *P. longifolia* seed in restoration this part of the study investigated:

- the amount of seed germination and emergence that occurs when seeds are placed into the post mining restoration environment;
- improving seed germination and emergence by placing endocarps into an environment where more vegetation is available for protection;
- the difference in seed germination between seeds which are buried compared with those that are scattered onto the soil surface in restoration areas; and
- the feasibility of creating and using a synthetic seed bank through burial, retrieval and re-sowing of seeds onto restoration sites to enhance *in situ* germination and emergence in the post mining environment.

4.3.2 Methods

4.3.2.1 Germination on restored areas of different ages

Persoonia longifolia fruit was collected from Dwellingup in August 2010 and was sown onto two different restored areas at Aloca's Huntly mine site on 31st August 2010. The exocarp and mesocarp were retained around the endocarp (and seed within) and the fruits were sown whole (i.e. not cleaned). Fruit numbers for this trial were limited due to the particularly poor seed set during 2010 and this is reflected in the experimental design.

Fruits were sown onto a newly restored pit where earthworks and seed sowing had recently been completed (2010 restoration) and a 3 year old restored pit (restored 2007). Trees, shrubs and ground covers were already well established on the 3 year old restoration at the time the *P. longifolia* fruits were sown (Fig. 4.1).

Figure 4.1. Restored areas of the mine where seeds were buried and scattered. *(a)* Area restored in 2007 with some vegetative cover. *(b)* Area restored in 2010 with no vegetative cover present at the commencement of the experiment.

Four replicates of 20 fruits were established in each selected environment. Fruits were buried to a depth of approximately 2 cm inside wire cages (Fig. 4.1) to protect germinating seedlings from herbivores and 20 fruits were also scattered directly on the soil surface within the cages. Twenty fruits were also scattered on the soil surface adjacent to the cages to investigate the impacts of herbivory. The areas were examined on a monthly basis for the first year (August 2010 to August 2011) to check for germination and emergence. After the first year these areas were examined three times in 2011 (January, April and July 2011). Access to this area then became difficult due to ongoing mining operations and consequently these areas were only re-examined one more time (after the summer months) in April 2013.

4.3.2.2 Synthetic seed bank trial

Fruit was collected in November 2010 from Moodiarrup West Road, near Haddleton Nature Reserve. Fruits were used as whole dispersal units (i.e. not cleaned). Four replicates of 25 fruits were examined by x-ray analysis (MX-20 Digital X-ray, Faxitron, USA) to determine mean seed fill at the commencement of the experiment.

Fruits were initially sown in December 2010 at the Boddington Bauxite Mine site in Western Australia. Fruits were either shallow buried (~2 cm deep) or scattered on the soil surface in natural unburnt bush or in a newly restored pit (2010 restoration). Fruits that were scattered on the soil surface as part of this initial treatment were left in place

for the entire experiment. These fruits were used as a control in this experiment as this treatment is similar to current approaches utilized by Alcoa and Worsley for *P. longifolia* when incorporated into native seed mixes for restoration (i.e. uncleaned fruits scattered on the surface as part of a seed mix). Fruits were also buried and left *in situ* in both bush sites and restored areas. Fruits that were to be retrieved at a later date were buried in mesh bags and wire cages were placed over the bags to prevent disturbance by animals.

At the end of April 2012 a subsample of fruits were retrieved (i.e. after 16 months burial and prior to the onset of the winter season) and x-rayed (as previously described) to ascertain the seed fill and therefore likely viablity. Fruits with no seed were removed from the replicate and the remaining fruits were returned to the bush or restoration within 24 hours of retrieval. Half of the fruits retrieved from the bush environment were returned to the bush (and either buried or scattered on the soil surface). The other half were either scattered or buried on the most recently restored area (2011 restoration) of the mine. Likewise, fruits retrieved from the restored areas of the mine were either returned to new restoration sites on the mine (2011 restoration) or placed in a natural bushland (i.e. jarrah forest) environment (either scattered on the soil surface or buried). This resulted in 11 different treatments, comprising initial burial area (bush vs restoration site), return burial area (bush vs restoration site) and fruit sowing treatment (surface scattered vs soil burial) each with four replicates. These 11 treatments were: endocarps scattered on restoration surface and left in place; buried in bush and left in place; buried in bush, retrieved and reburied in bush; buried in bush retrieved and reburied on restored area; buried in bush retrieved and scattered in bush; buried in bush and scattered on restored area; buried in restored area and left in place; buried in restored area, retrieved and reburied in restored area; buried in restored area, retrieved and reburied in bush; buried in restored area, retrieved and scattered in bush; and buried in restored area, retrieved and scatter on restored area.

Replicates were initially of 20 fruits, however after x-ray analysis the numbers of fruits varied depending on seed fill. Fruits that had been scattered on the soil surface were not retrieved but were left in position and monitored.

After reburial, the trial was assessed for seedling emergence in October 2012 and then quarterly (late December 2012, April 2013, July 2013 and October 2013) for 1 year for emergence. It was then reassessed in April 2014 and again in August 2014.

Some of the results from this trial raised the question that differences in soil temperatures and moisture levels between natural jarrah forest sites and post mining restoration areas were driving some of the disparity in emergence patterns observed. Data loggers (Hobo® Microstation, Onset Computer Corporation, Cape Cod, Massachusetts) with soil moisture probes (Soil Moisture Smart Sensors) and temperature probes (12 –bit Temperature Smart Sensor) were placed in a freshly restored area (restored in 2013) and in an adjacent natural bushland area in October 2013 to quantify soil temperatures and moisture levels at approximately 2 cm below the soil surface, within the two different environments. Measurements were made on an hourly basis and data loggers remained in place for approximately 1 year.

4.3.2.3 Data analysis

Seed fill data was analysed using Analysis of Variance. Germination data was analysed using a binomial GLMM with a logit link function to determine the effects of burial (vs scattering), initial burial area (either restoration or bush), and return burial area (either restoration or bush). The data was examined for zero inflation and models were reduced by omitting all non-significant factors and interactions (5% significance level). Comparisons between the different treatments were made using Tukey's HSD.

Soil temperature and moisture data was analysed with a paired t-test using the mean daily maximum and minimum soil values for the natural bushland and restoration sites.

All analyses were undertaken in the statistical program R (R Core Team, 2013) using the *lme4* package (Bates et al., 2014).

4.3.3 Results

4.3.3.1 Germination on restored areas of different ages

Very low emergence (<1%) was observed from fruits either in the cages or adjacent to the cages on either the 3 year old (2007) or newly established (2010) restored areas during the course of the study. Two germinants were observed in April 2013 and these were recorded from fruits that had been buried inside the cages on the newly established restoration site. It is likely that these seeds germinated in August or September 2012 approximately 24 months after initial burial (this estimate of germination date is based on the size of the seedlings when observed and observations of timing of seedling germination made during the Phenology Study outlined in Chapter 2). However, of the 480 fruits placed out on both the 2010 and 2007 restoration areas, this represents <1.0% emergence after 2 years.

4.3.3.2 Synthetic seed bank trial

Seed fill at the commencement of the study in December 2010 was 83.0 $\pm$ 3.8%. After 16 months *in situ* placement seed fill in fruits retrieved from the restoration area in April 2012 was 76.1 $\pm$ 1.4% in restored areas and 77.5 $\pm$ 3.0% in fruits retrieved from the bush. These values were not significantly different from the initial seed fill or from each other (P=0.424).

Examination of final cumulative germination percentages indicated that zero inflation was present in the data and was associated with the initial burial location. Fruits buried in the restored area were less likely to germinate than those that were initially buried in the natural bushland site (P=0.007).

Modelling indicated that emergence success was significantly affected by all three treatments (i.e. location of initial burial, location of return burial and sowing treatment) (Table 4.1). The best emergence (24.0 $\pm$ 2.7% after 26 months) was observed for fruits that had been initially buried in the natural bushland site, then retrieved and reburied in the restored areas (Fig. 4.2, P<0.001). Once the fruits had been retrieved, burial was the best means of obtaining emergence. Those fruits that were scattered on top of the soil germinated very poorly compared to all the other treatments assessed (Table 4.1, Fig. 4.2).

A total of 64 seedlings was recorded over the period of the study up until August 2014. Eight of these seedlings were recorded in the last assessment and as a result their survival and growth beyond this point is unknown. However, survival of seedlings from previous assessments was generally poor with only 6.4% of seedlings (i.e. three seedlings) observed from previous seasons (2012 and 2013) surviving until August 2014. Two of these seedlings were located in the natural bushland site and one was located on the mine restoration area (Fig. 4.3a). Most seedling deaths occurred as a result of desiccation (Fig. 4.3b) or as a result of heavy insect damage (Fig. 4.3c). Only a few seedlings were observed to have been grazed by herbivores (Fig. 4.3d).

Table 4.1: Cumulative germination percentage (mean ± SE) and results of the binomial analysis for *P. longifolia* fruits within different treatments. Only factors included in the final model are listed in the table. Treatments with the same superscript do not differ significantly from other treatments within the same Factor.

Factor/Interactions	Treatment	Final Cumulative Germination	P-value
Initial burial	Restoration	5.6 ± 2.2^{a}	<0.001
	Bush	9.4 ± 2.0^{b}	
Return burial	Restoration	9.2 ± 2.5^{a}	<0.001
	Bush	3.3 ± 0.9^{b}	
	Remained in position	4.4 ± 1.9^{b}	
Buried/ Scattered	Buried	14.6 ± 3.1^{a}	<0.001
	Scattered	2.7 ± 1.0^{b}	
	Buried (left in position)	6.0 ± 2.4^{b}	
	Scattered (left in position)	0.0	
Initial burial x Buried/scattered			0.001

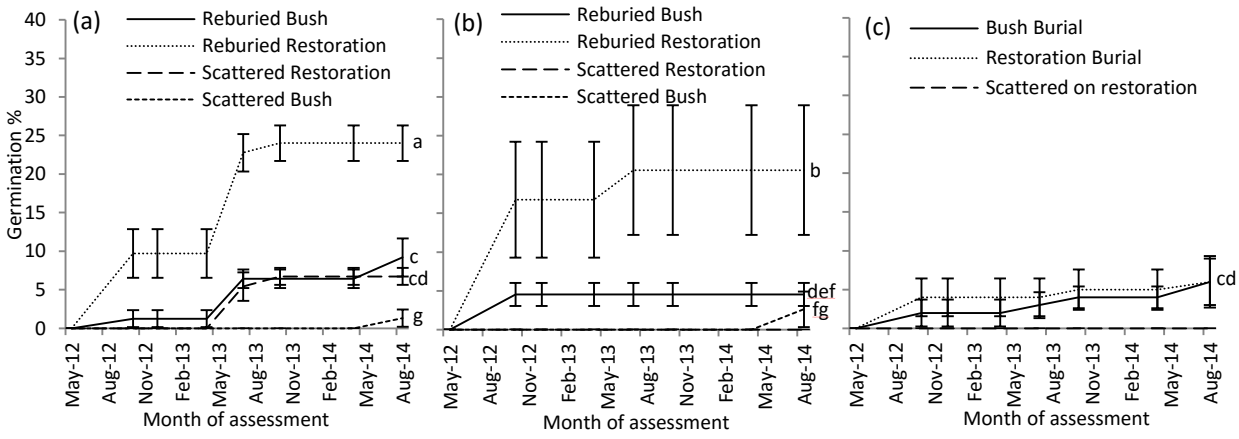

Figure 4.2: Cumulative germination percentage (mean ± SE) recorded in each treatment following retrieval and re-burial/scattering. *(a)* Fruits initially buried in the bush location. *(b)* Fruits initially buried in a restored area. *(c)* Fruits left in place for the duration of the trial. Treatments with the same letters do not differ significantly.

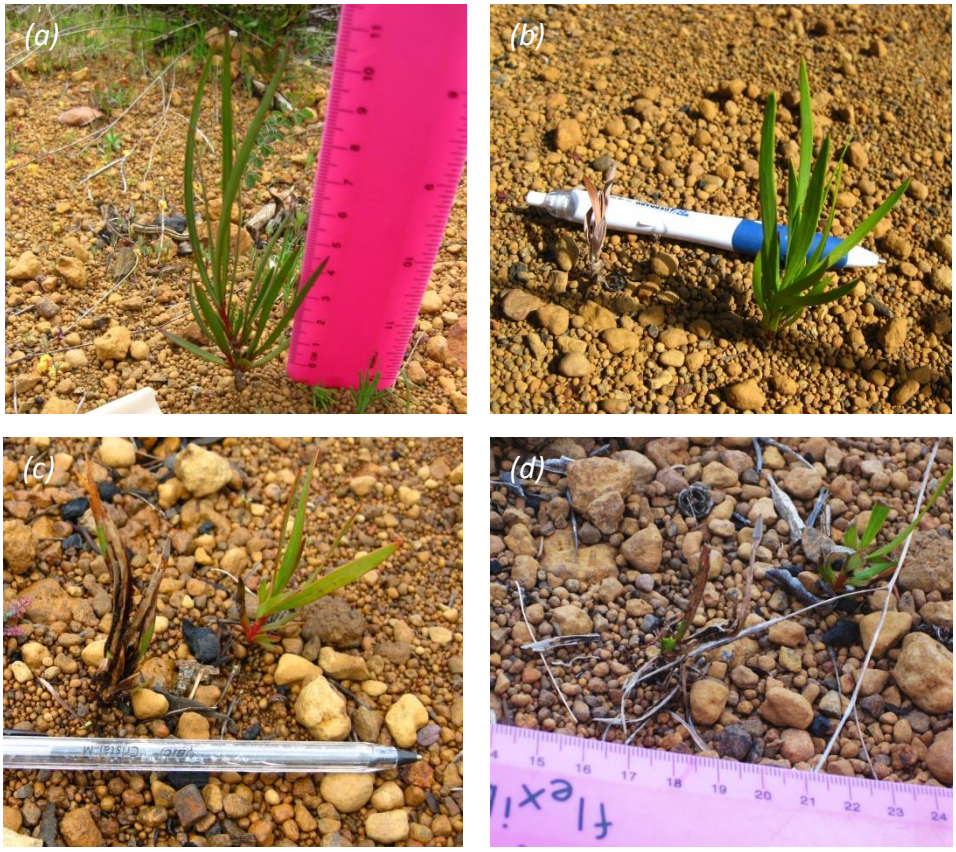

Figure 4.3: Germinants from the synthetic seed bank trial growing in restoration areas**:** *(a)* Healthy seedling that survived to the end of the trial. *(b)* Healthy seedling adjacent to a desiccated seedling. *(c)* Seedling with insect damage adjacent to a healthy seedling *(d)* Seedlings grazed by unknown herbivores.

4.3.3.3 Data logger results

Maximum temperatures recorded at 2 cm below the soil surface were higher on the restored area than in the adjacent natural bushland site (Fig. 4.4, P<0.001). There were several days during the summer months when the soil temperature difference between the two different locations was as high as 13 °C. During the winter months, maximum temperatures on the restored area were generally 5 °C higher than in the adjacent natural bushland site.

While minimum temperatures were more variable, they were still generally a little higher on the restored areas than in the natural bushland sites (Fig. 4.4, P<0.001). However there were occasions during winter when the temperatures recorded on the restored sites were lower than in the natural bushland sites. While the data loggers were in the ground for a full 12 months, due to technical difficulties no records were made from 14 August 2014 to 30 October 2014, during the transition from late winter into spring. Interestingly, both the minimum and maximum soil water contents were generally more extreme in the restored areas compared to the natural bushland sites (Fig. 4.4, P<0.001).

4.3.4 Discussion

Persoonia longifolia does not readily germinate on post mining restoration sites when fruits are included as part of a standard seed mix that is scattered over the soil surface during the restoration process (Mullins et al., 2002, Norman and Koch, 2008). This was confirmed with the low emergence observed in this study (0.4%) in both newly restored areas and 3 year old restoration sites. These results were similar to emergence (0.3%) achieved in other field broadcast trials undertaken by Alcoa (Norman and Koch, 2005b). Therefore, alternative means of returning this species to restored areas of the jarrah forest are required.

The creation of a synthetic seed bank is a new approach to returning recalcitrant species to restored areas which is presented here for the first time. By burying the fruits in their natural environment (the surrounding jarrah forest in this case) it is possible to cue the seed for germination through breaking dormancy within the seed before retrieving the fruits and sowing these at a later time (once dormancy has been alleviated) into areas

requiring restoration. The use of a synthetic seed bank as described in this study is likely to be applicable to other species with similar complex germination requirements and provides a promising new line of research for increasing native plant diversity in restoration programs.

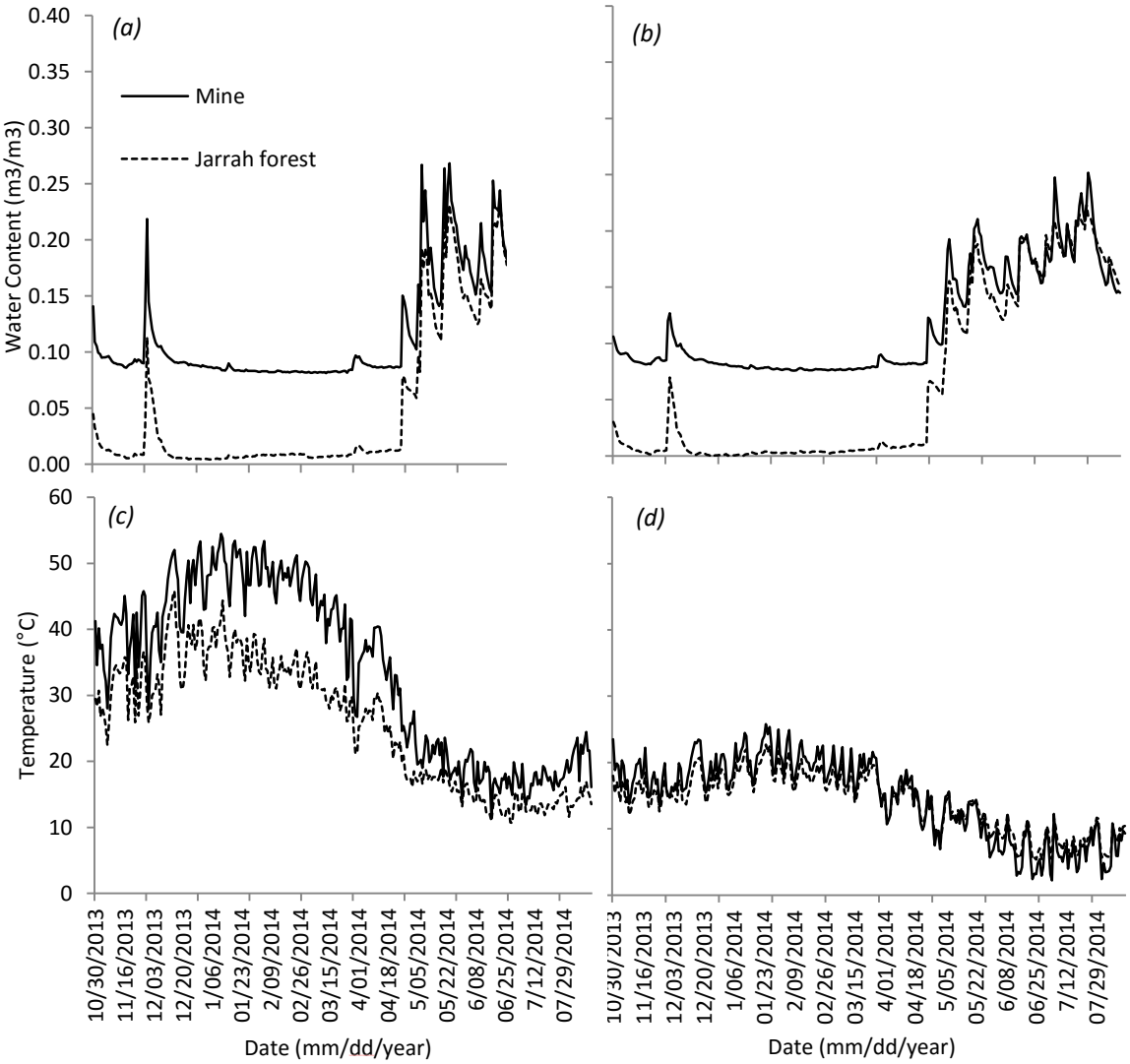

Figure 4.4 Comparison of soil temperatures and moisture levels on a restored pit at Boddington bauxite mine and the adjacent jarrah forest at 2 cm depth. (a) Mean maximum water content (b) Mean minimum water content (c) mean maximum temperature (d) mean minimum temperature.

Results from this trial also indicate that germination is enhanced when fruits or endocarps become buried in the soil. The lack of *P. longifolia* seedlings on post mining

restoration sites is likely to be a result of inappropriate seed broadcasting techniques where intact fruits are scattered onto the soil surface and thus are not incorporated into the topsoil. Inherent dormancy in the embryo rather than herbivory or lack of protection from surrounding vegetation is likely to be the major factor accounting for the low abundance of *P. longifolia* seedlings on restored areas. Whilst herbivory may still play a role in reducing the number of *P. longifolia* seedlings appearing in restoration sites, it is not the principle reason for the general absence of *P. longifolia* seedlings in mine restoration.

Many studies have shown that seed burial up to 5 cm deep improves germination (Blackshaw, 1990, Jurado and Westoby, 1992, Ren et al., 2002). Seed burial for 12 Western Australian jarrah forest species resulted in high level of emergence from 2 cm burial with the optimum germination occurring between 0 and 5 cm for all species (Grant et al., 1996). Grant et al. (1996) also found that emergence from greater than 5 cm was significantly decreased in all but three legume species. Similar results were found for a range of species from a *Banksia* woodland in Western Australia (Rokich et al., 2000) and in this context *P. longifolia* is another species that germinates more readily when buried. Consequently, scattering fruits on the soil surface, results in very low germination and, based on these results, is a highly ineffective way to restore *P. longifolia* following mining and restoration work. Further study is required to determine the optimum depth of burial from which *P. longifolia* seedlings can emerge.

Larger seeds have been generally found to have a greater ability to emerge from depth than smaller seeds due to their greater innate energy reserves (Grant et al., 1996). It is assumed that the weights presented in Grant et al. (1996) are of the reproductive unit that would be sown onto restoration areas so in this context *P. longifolia* may be a little unusual as most of the weight of the dispersal unit is made up of the woody endocarp (>97%) not endosperm, or embryo so their endogenous energy reserves may be much lower than what their endocarp weight would suggest. Taken at face value *P. longifolia* fruits are much heavier than any of the seeds included in Grant et al.'s (1996) study, with a weight of 1019 mg (although the seed component of this is only 17.4 mg) compared with only 87 mg for *Eucalyptus calophylla*, the heaviest seed assessed. To date, the depth from which *P. longifolia* seedlings can emerge is currently unknown. All burial trials included in this thesis involved burial between 2 and 5 cm. Further

study is therefore recommended to determine whether *P. longifolia* seedlings can emerge if endocarps are buried any deeper than 5cm.

In these experiments, the fleshy mesocarp was retained on the endocarp as this would be the least expensive way to sow *in situ P. longifolia* seeds for restoration purposes. However, results from other trials undertaken (see Chapter 3, Section 3.4) show that removal of the mesocarp can greatly improve germination especially when combined with soil burial. If *P. longifolia* is to be directly returned to restoration sites as indehiscent endocarps (rather than planted out as young plants), then it is strongly recommended that the fleshy mesocarp be initially removed (see Chapter 3 for details) leaving behind the hard indehiscent woody endocarp (+ seed) as the reproductive unit to be used for *in situ* sowing.

Burying endocarps in the bushland environment exposes them to the natural conditions that break dormancy and so cue seeds for germination. Burial for up to two summers does not appear to affect seed viability in *P. longifolia*. In order to break dormancy in seeds that can then be returned directly to post mining restoration areas, practitioners could bury clean endocarps in natural bushland for at least one summer (possibly two summers if no summer thunderstorms occur during the first summer – see Chapter 3), retrieve the seeds prior to the onset of winter, then sow these endocarps *in situ* in restored areas making sure these are buried at least 1 cm below the soil surface before the breaking winter rains. Alternatively seeds could be buried under nursery conditions in large tubs of soil or sand where dormancy breaking techniques can be artificially enhanced or imposed through shading, and sporadic supplementary summer watering (See Chapter 3). The seeds could then be easily retrieved and sown *in situ* into restoration sites as required.

Whilst this method, may provide a potential way to increase the numbers of *P. longifolia* emerging *in situ* in restoration sites, the survival of seedlings that manage to germinate is still very poor. In all likelihood, the intensive management of *in situ* seedlings of *P. longifolia* will be difficult and therefore if seedling losses following *in situ* emergence continue to be excessive it may be more practical to germinate seedlings under nursery conditions and then plant these seedlings directly onto restoration sites as is currently done with a range of understorey species (Daws and Koch, 2015). While significantly more expensive than *in situ* seed sowing, the use of nursery grown tube

stock is nevertheless an effective way to restore those species that would otherwise be lost in the post-mining environment.

4.4 Seedling survival on restoration

4.4.1 Introduction

Persoonia longifolia, in its natural environment, grows in relatively shaded areas beneath overstorey trees such as *Eucalyptus marginata* and *Corymbia calophylla*. In contrast, the post mining environment shortly after restoration works is extremely exposed and very different from the surrounding jarrah forest. However, competition is greatly reduced and therefore water and nutrient availability is higher allowing some species to rapidly establish and grow (Daws and Koch, 2015) Once planted on newly established restoration, seedlings are also much more exposed to herbivores who are able to move unobstructed through large open areas searching for what little vegetation may be present (Daws and Koch, 2015, Koch et al., 2004). Grazing has been found to be the major factor reducing seedling survival of other jarrah forest species in the post mining environment on restored bauxite mines (Daws and Koch, 2015, Koch et al., 2004, Nield et al., 2015, Stanton-Clements et al., 2013) and it is likely that grazing could impact on the survival of *P. longifolia* seedlings planted in similar situations. For plants that occur as mid-storey species in the surrounding jarrah forest these conditions are extremely different from those where natural germination and recruitment events occur. Thus the survival and growth of young *P. longifolia* plants could potentially be affected by the age of the restoration they are planted into (microclimate effects), grazing from highly mobile herbivores (such as kangaroos), and the age (size) of the seedling at the time of planting.

A study into survival of jarrah forest understorey species found that despite the initial prediction that older restoration may provide better protection for seedlings, survival and growth were both actually higher on newly established restoration or 10 year old restoration. In comparison, survival and growth were significantly lower on 1 and 4 year old restoration (Daws and Koch, 2015). This was attributed to the 1 and 4 year old areas having a rapidly increasing understorey and therefore were viewed as highly competitive environments. To date no investigation into *P. longifolia* seedling survival on restored areas following bauxite mining has been conducted.

Investigations into the impacts of shading on survival and growth of *Xanthorrhoea gracilis* and *X. pressii* on a restored bauxite mine in the jarrah forest found that shading did not affect survival but rather grazing by kangaroos was the major factor reducing seedling survival and growth in the post mining environment (Koch et al., 2004). Stanton-Clements et al. (2013) also found that guarding increased survival of *Tetraria capillaris* (another jarrah forest species) as a result of reduced grazing pressure even up to 6 ½ years after planting out.

Seedlings of *P. elliptica*, another jarrah forest species closely related to *P. longifolia* have been found to be highly palatable to vertebrates during their first year of establishment (Abbott and van Heurck, 1988). However, during the second summer, seedlings were no longer grazed by herbivores and seedling survival and health significantly increased after this point. A more recent study has found that the main herbivores were the western brush wallaby (*Macropus irma*) and the western grey kangaroo (*Macropus fuliginosus*) and that herbivory is not strictly limited to seedlings but appears to occur to all life stages of *P. elliptica* (Nield et al., 2015). Given that grazing has been linked to survival of many other jarrah forest species (Daws and Koch, 2015, Koch et al., 2004, Nield et al., 2015) it is highly likely that grazing could also impact the survival of *P. longifolia* seedlings planted on restored mine areas and the use of some form of grazing protection may therefore increase seedling survival, growth and health.

Whilst plastic tree guards are suitable for use in temperate or tropical environs (Lai and Wong, 2005), they have been found to have mixed results for plant establishment in Mediterranean type environments (Close et al., 2009, Stanton-Clements et al., 2013) at the height of summer, plastic tree guards (with no holes) create a miniature greenhouse, increasing the average temperature inside the guards by up to 6.7 °C during summer when plants are under the most stress. This increase in temperature may result in seedling being more prone to desiccation and death and thus may be counter-productive. The use of plastic guards with holes punched in them to allow airflow resulted in no negative impact on survival of seedlings in the jarrah forest of Western Australia (Stanton-Clements et al., 2013). Shade cloth tree guards by comparison, may provide significant benefits by minimising extreme temperature effects, reducing wind speeds and retaining soil moisture through reducing evaporation and transpiration as well as

provide protection against browser damage leading to a decrease in mortality (Close et al., 2009).

Variations in the time of year that seedlings are planted may also affect survival and this does not appear to be well researched for restoration programs implemented in the southwest area of Western Australia. Water stress is considered to be the key factor in seedling transplant stress and can cause mortality or limit growth (Burdett, 1990). Under natural conditions, *P. longifolia* seedlings germinate in late July/early August (Chapter 2, Fig. 2.4) and if planted directly onto restored areas following germination, have only 3 months to establish themselves and develop a sufficient root system before the onset of summer drought conditions (December – February). In summer, soil moisture is likely to be the primary factor influencing growth and small plants with shallow root systems are likely to be exposed to very high levels of water stress (Donovan and Ehleringer, 1991). In addition, seedlings planted out in August will be much younger and therefore much more susceptible to transplant shock and moisture stress than older more established plants (6-12 months old) which are more commonly used in restoration programs at present.

Alternatively, seedlings could be maintained over the summer period (after germinating the previous winter) under nursery conditions and then planted around the time of the first rains of the winter season (generally around mid- May). This would provide the seedlings with a full wet season (~late April to October) to develop their roots system in the restored areas before exposure to the long hot dry summers typically experienced in South Western Australia. Previous research indicates that *P. longifolia* develops an extensive root system (Crowhurst, 2006)and anecdotal evidence suggests that root disturbance negatively impacts seedling growth and survival. Effective nursery maintenance over the summer period is imperative to survival of seedlings once planted *in situ* to help foster good root system development and healthy stem and leaf growth.

Given that no previous studies have been undertaken looking at the performance of *P. longifolia* plants when placed into restoration sites, the ability of *P. longifolia* seedlings to survive and grow once planted out requires investigation in order to maximise survival. In addition, an understanding of the influence of seedling age when planted out, and the use of different tree guards to assist with survival and growth through

shading and protection from grazing, also needs to be assessed to identify the most effective means of returning young *P. longifolia* plants to the post mining environment. The aims of this part of the restoration study were to:

- investigate plant survival once placed onto restoration areas and compare with survival of similar aged plants placed into the surrounding jarrah forest,
- compare the survival of plants when placed into older (3 years old) restored areas against those planted in newly established restoration,
- assess the practicalities of maintaining plants under nursery conditions over summer and planting these out into restoration sites once larger and more established; and
- contrast the performance of different types of tree guards on survival and growth of young *P. longifolia* plants.

4.4.2 Methods

Forty seedlings were available during 2010 and given the small numbers it was decided to use these seedlings in a preliminary study of seedling survival at several different sites. Seedlings germinated in early August 2010 and were pricked out into biodegradable pots filled with Native Potting Mix (Baileys Fertilizer, Kwinana, Western Australia) shortly afterwards. They were left to recover from the initial transplant shock in the Alcoa Marrinup Nursery, Dwellingup for 2 weeks before planting out into different sites in late August 2010. The seedlings were planted onto the various areas in the biodegradable pots so no additional disturbance to the roots occurred during the planting process. Ten seedlings were planted into each of the following areas (Figure 4.5):

- newly established restoration with no vegetation (restored 2010);
- 3 year old restoration with some overstorey vegetation (restored 2007);
- an area in natural bush (jarrah forest) burnt in spring 2009 (i.e. no leaf litter but surrounding trees still present) and;
- unburnt natural bush (jarrah forest) with an extensive layer of leaf litter on the soil surface and surrounding trees.

Seedlings planted into the fresh restoration site were planted on the side of the rip line mid-slope. Low areas within the pit were avoided. In the older restored area, the

seedlings were also planted on the eastern side of a large tree or shrub to provide afternoon shade. All seedlings were caged and the cages were staked to prevent movement and grazing.

Figure 4.5: Seedlings planted in different environments. Photographs taken at the time of planting. *(a)* 2010 restoration. *(b)* 2007 restoratoion. *(c)* Burnt area of natural bush. *(d)* Natural bush.

Seedling survival and height (from ground level to the apical meristem) were measured on a monthly basis from September 2010 to April 2011 and then quarterly until April 2012 (a period which covered two summers).

A second trial was established to investigate the impacts of using guards to protect seedlings from herbivores and to determine whether plants of different ages perform differently when planted into fresh restoration. Seedlings for this trial were germinated late July/early August 2011 and were pricked into biodegradable pots as previously described. After potting up, seedlings were drenched with "Seasol" to assist with transplant shock but were not watered again but rather left to natural rainfall before planting out onto restoration sites. Half of the potted seedlings were planted directly onto the restored area in late August 2011 (several weeks after emergence). The seedlings were retained in the biodegradable pots to minimise any transplant shock and so experienced minimal root disturbance during the planting process. Seedlings were planted mid-slope in the middle of the rip line as previously described.

At the time of planting, seedlings were either planted without a tree guard (control plants), or either with an "onion bag" tree guard (Bug-it-off netting, Buono Net Australia Pty Ltd., Homebush NSW) or a purpose built shade cloth (50% shade cloth) tree guard. Both types of guards were held in place with three bamboo poles to pull

them tort. Altogether, 40 seedlings were allocated to each treatment and each treatment was replicated in four different pits (i.e. 10 seedlings per treatment per pit).

Onion bag tree guards (32 cm tall with sides of approximate 15 cm) provide protection from grazing and given their loose weave would allow natural air movement (Fig. 4.6) Eight months after installation they had begun to deteriorate and disintergrate due to their UV sensitivity.

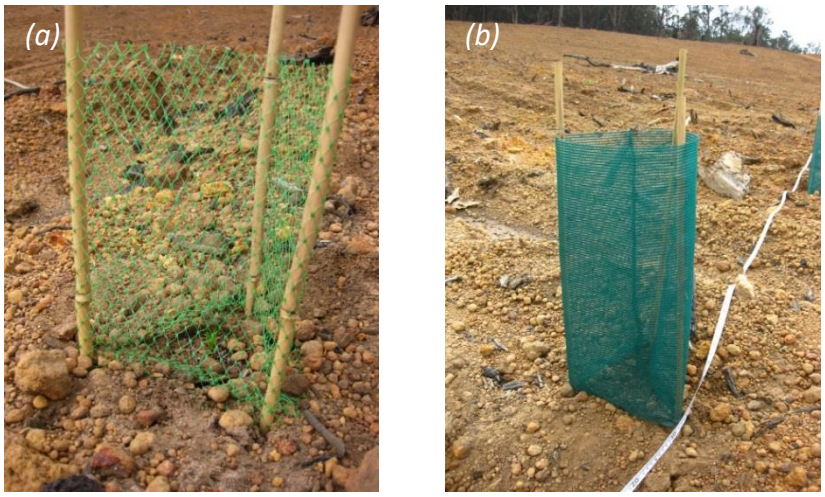

Figure 4.6: Tree guards used to protect seedlings during trials on restored areas of the mine. *(a)* Onion bag tree guard which disintegrates over time. *(b)* Shade cloth tree guard which remained intact for the duration of the trial.

The shade cloth tree guards were made up to a similar size to commercially available plastic tree guards. Shade cloth guards were 46 cm tall and stretched to a triangle with sides of approximately 23 cm.

The second half of the seedlings was retained in the Marrinup nursery for *in situ* planting in August 2012. Unfortunately these seedlings did not survive and another batch of seedlings was obtained that had germinated at a similar time (July/August 2011) as the seedlings planted in August 2011. This second batch of plants was not available in biodegradable pots, but were instead potted up into small tree tubes (5 cm x 5 cm x 12 cm). They were planted into freshly restored areas of the jarrah forest (i.e. 2012 rehabilitation), in August 2012, and were therefore 12 months old at the time of planting. Given the limited numbers there were only sufficient plants for installing two treatments. Due to the results obtained from the previous seasons planting it was

decided to plant these with no protection (control) and with onion bag guards only (which were considered the most economical of the two different guards treatments assessed in terms of price and availability). Fourteen seedlings were allocated to each treatment and each treatment was replicated in 3 different pits.

4.4.2.1 Data Analysis

Survival data was analysed using a binomial GLM with a logit link function. In the guarding trial the model included the age of the seedling at planting time, tree guarding type (either no guard, onion bag or shade cloth), and months after planting. The data was examined for zero inflation and models were reduced by omitting all non-significant factors and interactions (5% significance level). Comparisons between the different treatments were made using Tukey's HSD.

Height data for both trials was arcsine transformed and analysed using a standard linear model. All data analyses were undertaken in the statistical program R (R Core Team, 2013) using the *lme4* package (Bates et al., 2014).

4.4.3 Results

4.4.3.1 Effects of age of restoration and bush planting

The majority of deaths occurred during the first summer (November to March) following planting. All deaths within the newly established restoration occurred within the first 2 months (August to October) of planting (Fig. 4.7). Initially, survival of seedlings in the 3 year old restoration was similar to that in the newly established restoration but as the summer progressed and moisture became scarcer seedling survival in the 3 year old restoration dropped to 50%. After the second summer this then was reduced to only 40% survival.

Those seedlings planted in the burnt bush showed similar survival to those seedlings planted in the restored areas of the mines site with 70% surviving the first summer and two more deaths resulting in a final survival of 50%. No seedlings in the natural unburnt bush survived past the first summer (Fig. 4.7).

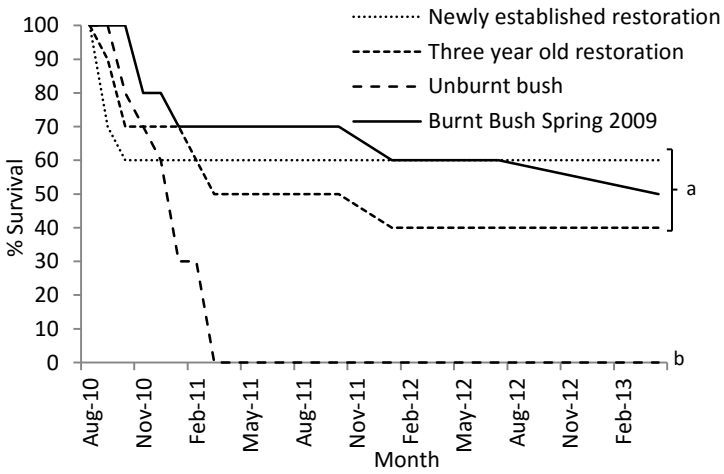

Figure 4.7: Cumulative percentage survival of seedlings after 30 months following planting onto different post mining restoration and bush with different fire histories around the McCoy bauxite mine operated by Alcoa near Dwellingup. Treatments with the same letter do not differ significantly.

A major outcome of this preliminary trial was the rapid and pronounced increase in height of those seedlings planted into the newly established restoration site when compared with all other sites assessed (Fig. 4.8, $P<0.001$). This sizeable height difference was evident from monitoring undertaken in January 2012 which was the second summer after the seedlings were planted (17 months after planting) with seedlings growing rapidly between October 2011 and January 2012 (Fig. 4.7).

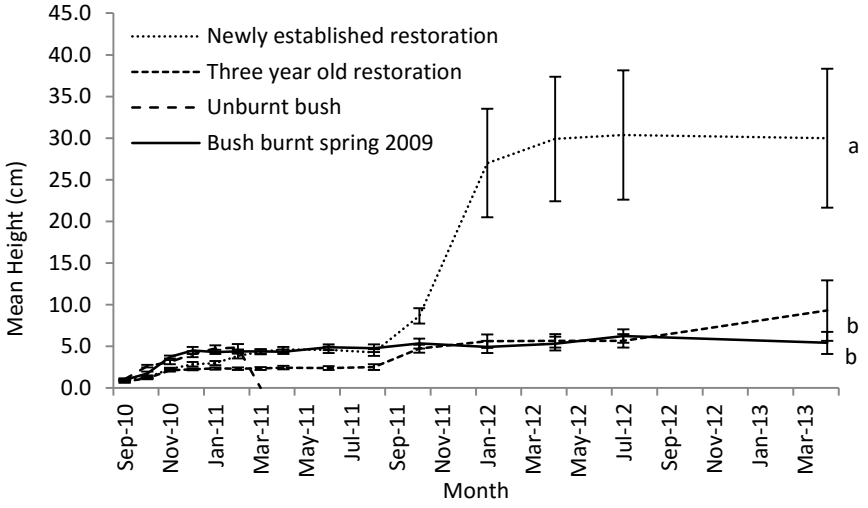

Figure 4.8: Mean height (cm ± SE) of surviving *Persoonia longifolia* plants when placed into different environments around Dwellingup. Treatments with the same letter do not differ significantly.

4.4.2.2 Planting time and tree guard trial

Seedlings kept under nursery conditions for the late planting all died, highlighting the challenges of maintaining *P. longifolia* plants under *ex situ* nursery conditions. Seedlings appear to have very specific watering requirements when maintained in a nursery environment which are still being determined. They are also susceptible to fungal disease (K Chia pers. obs.) both of which may mean micro-managing seedlings which is not always possible in a commercial nursery environment. Due to the loss of all these plants a second batch of seedlings, of a similar age to the first batch, was obtained and used in the later planting.

Results from the binomial analysis initially suggested that planting larger more mature plants 12 months after germination, rather than when recently emerged (i.e. only several weeks old), resulted in greater survival (Table 4.2). This could have been a result of either plant age or climatic variation at the time of planting.

When comparisons were made between the two cohorts of seedlings using the length of time seedlings had been planted, the differences were much less obvious. Three months after planting, survival of the seedlings planted at 12 months of age was greater for both guarding treatments assessed, compared with those seedlings planted at 2-4 weeks of age (Fig. 4.9a).

However, 8 months after planting the seedlings, these differences were no longer significant. Similarly, 20 months after planting it did not matter if the seedlings had been planted at 2-4 weeks of age or 12 months of age as there was no significant difference in survival between the five treatments (Fig. 4.9a).

When comparing the various guarding treatments, survival was greatest in those seedlings planted in the onion bag tree guards (Figure 4.9, Table 4.2). However, whilst use of the shade cloth tree guards did not improve survival of the seedlings, it did result in a significant increase in height when compared with those seedlings planted in either the onion bag tree guards or not guarded in any way (Figure 4.9, Table 4.2).

When comparing the height of the seedlings in each of the guarding treatments at different ages, the difference between those seedlings planted at 2-4 weeks of age and

those planted at 12 months of age was clearly evident once the plants had attained 14 months of age. This was only 3 months after the late plant seedlings were planted onto the restoration. Despite being the same age, those seedlings kept in the nursery and planted out at 12 months of age were significantly smaller than those that were planted out when 2 weeks old. Seedlings that were kept in the nursery for 12 months were similar in size to 3 month old seedlings planted out early (there was no significant difference between late plant 12 months old seedlings and the early plant 3 month old seedlings), indicating that the seedlings grew relatively little under nursery conditions.

Following 20 months *in situ* growth, those seedlings planted when 2 weeks old within the shade cloth tree guards were significantly taller than all other seedlings by the time the seedlings were 20 months of age (P<0.001,). This trend was still evident following 32 months *in situ* growth (Fig. 4.10).

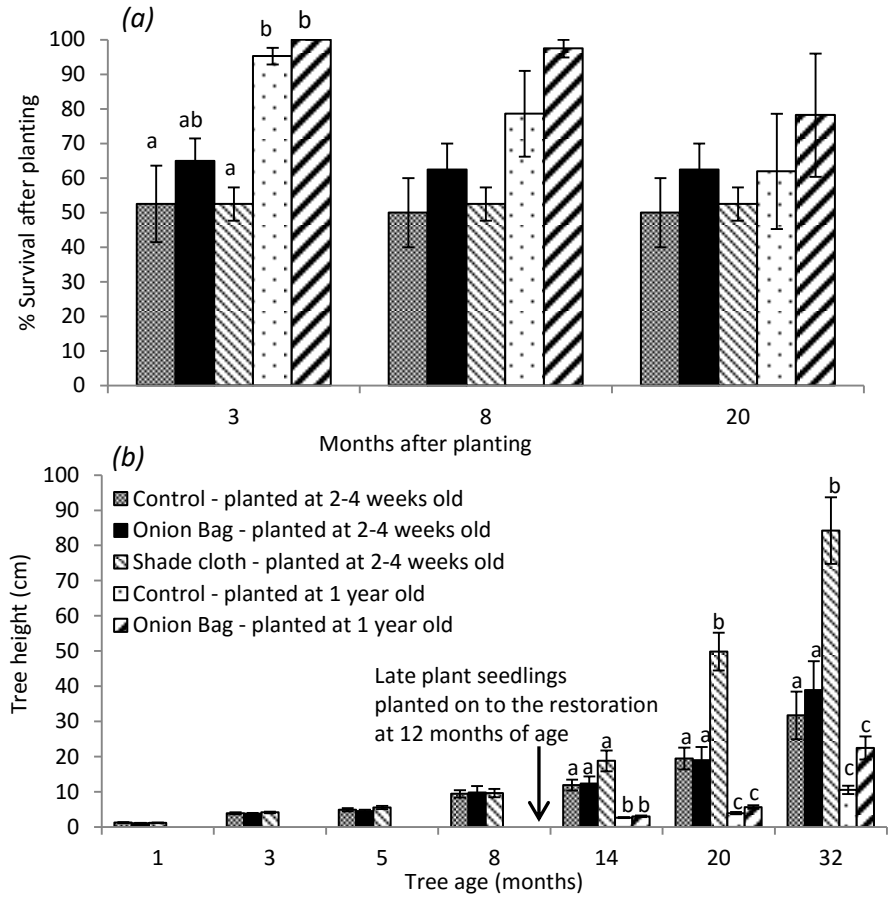

Figure 4.9: Mean survival and height (% + SE) of seedlings planted into several post mining restoration sites around Dwellingup. *(a)* Survival in relation to the months after the seedlings were planted. Treatments within the 3 month assessment with the same letters do not differ significantly (P=0.005). None of the treatments assessed at eight and 20 months after planting were significantly different from one another. *(b)* Height

in relation to tree age at the time of assessment. Treatments within each age group with the same letters do not differ significantly (P=0.005).

Records of insect/herbivore damage show that initially (the first 14 months after planting) there was very little insect damage evident on any of the *P. longifolia* plants. However by the time the seedlings were 20 months old insect herbivory was clearly evident. Only one seedling was noted as having been grazed by a larger herbivore during this experiment. Seedlings in shade cloth guards experienced less insect damage compared with those seedlings left unguarded or those protected by onion bag tree guards, although this was not statistically significant (P>0.05).

Table 4.2: Mean survival (% ± SE) and height (cm ± SE) for *P. longifolia* plants placed into a restoration area following bauxite mining and after the application of different treatments. NS = not significant. NI = not included in the final model.

Treatment	Levels	P-value Survival	Mean Survival (% ± SE)	P-value Height	Mean Height (cm ± SE)
Planting time	Early plant	<0.001	55.3 ± 1.9[a]	<0.001	14.1 ± 1.1[a]
	Late plant		85.2 ± 5.1[b]		5.9 ± 0.4[b]
Shading/guarding	No guard	0.002	59.1 ± 4.4[a]	<0.001	7.2 ± 0.6[a]
	Onion bag		72.0 ± 3.7[b]		9.2 ± 0.9[a]
	Shade Cloth		52.5 ± 1.9[a]		22.1 ± 2.6[b]
Months after Planting	3 months	NS	70.3 ± 5.6	NI	
	8 months		66.0 ± 5.2		
	20 months		60.0 ± 4.9		
Planting time + Shading Treatment		NS		NI	
Planting time + Months after Planting		<0.001		NI	
Shading Treatment + Months after Planting		NS		NI	
Planting time + Shading Treatment + Months after Planting		0.005	Means shown in Figure 4.8	NI	

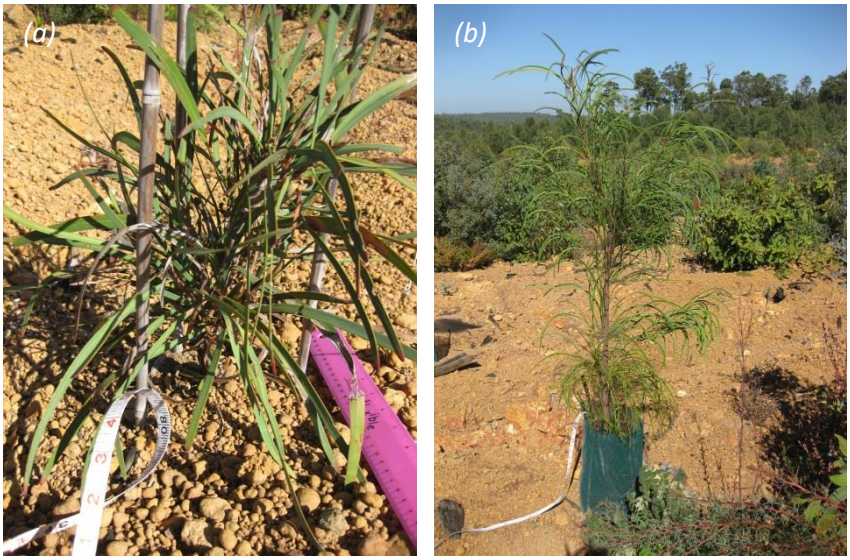

Figure 4.10: Thirty two month old seedlings surviving on the restored areas of a bauxite mine at Dwellingup**.** These two seedlings were adjacent to each other on the restoration site. (*a)* Seedling protected by onion bag guard (onion bag had disintegrated at the time photo was taken). *(b)* Seedling protected by shade cloth guard.

4.4.4 Discussion

Re-establishment of native vegetation with a high botanical diversity is a major priority of mining companies once they have completed all their mining works (Ward et al., 1996). *Persoonia longifolia* is a common, and indeed integral, component of the jarrah forest ecosystem where Alcoa and Worsley currently undertake bauxite mining. It would appear, based on the results outlined in this chapter and from anecdotal evidence, that seed dormancy and specific dormancy alleviating requirements is the primary reason for the lack of *P. longifolia* seedlings on post mining restoration sites. Development of methods to effectively break seed dormancy, prior to seeding in areas undergoing restoration work, while promising, is still in its infancy. Therefore, the current best practice of returning *P. longifolia* plants in the short term to restoration sites, is to plant out seedlings that have been germinated under nursery conditions. Planting out seedlings has, in the past, not been done regularly with *P. longifolia* by mining companies due to lack of seedling availability, hence the importance of the results encapsulated in this study.

Persoonia longifolia is a long lived, mid-storey tree and as such, is likely to germinate in a shaded and protected environment. The initial working hypothesis for this trial was

that areas of the mine requiring restoration are so fundamentally different from the surrounding jarrah forest environment (in terms of temperature, moisture, wind, relative humidity and herbivory), that *P. longifolia* cannot easily germinate, and seedling survival for any that do is difficult, at best. However, this did not prove to be the case with survival of seedlings on the newly established restored sites being greater than in the bush or even on the 3 year old restoration. This is likely to be related to the significant difference in soil temperature and moisture identified between the newly restored areas and the surrounding bush. In fact the increased availability of water in newly restored areas compared with the surrounding natural bush appear to be advantageous for *P. longifolia* and it is likely that this is related to lower levels of competition from surrounding vegetation on the newly restored sites. Reducing competition has been found to improve survival and growth of other jarrah forest species during early growth and development (Abbott, 1984a, Daws and Koch, 2015).

Seedling deaths were greatest in the first few months following planting in both seedling trials, indicating that most deaths are likely to occur as a result of the profound change in conditions from the nursery environment. Transplant shock or seedling damage is less likely to have been an issue for the younger plants as seedlings were placed into the ground in biodegradable pots with minimal root disturbance whilst the later planting did involve the removal of plants from forestry tubes prior to planting which may have unduly impacted or disturbed their root system. Rainfall during September and October of 2010 was well below average, however, despite this very low rainfall, seedling survival in the first trial was still 60 % after 3 months which is similar to survival experienced with other jarrah forest species planted into restored areas (Daws and Koch, 2015). This level of survival was comparable with the second seedling trial which was undertaken during a much more typical rainfall season where survival ranged from 50-80 % after 20 months *in situ* growth.

Seedling survival when planted in the surrounding jarrah forest was variable. Large amounts of deep leaf litter present in unburnt bush may have inhibited water penetration even during heavy rainfall, and this may explain the lack of seedling survival in these areas. In the burnt bush, where leaf litter and competition from surrounding vegetation is greatly reduced, survival was similar to that on the newly established restoration.

Seedlings naturally germinate and emerge *in situ* in July and August at the height of the wet season (Chapter 2). This is a challenging time for restoration practitioners because most of the planting of greenstock is completed prior to this time (June to July) and another pass over the restored areas to plant additional seedlings will result in significant additional costs and may disturb other recently germinated native species. However, results from the tree guarding trial indicated that growth is enhanced when seedlings are planted out earlier. Consequently, a key question for restoration practitioners to now address is whether seedlings need to be planted out in the season that they germinate or alternatively to reduce restoration costs should they be held back in the nursery over the summer months and planted out the following season? Further work is required to clearly establish whether this alternative approach is both beneficial and economical.

Whilst survival is definitely reduced if seedlings are planted out at 2 weeks old (55 % compared with 85 % for seedlings planted out when 12 months old), deaths under nursery conditions were also very common (pers. obs.). To date, no data is available on seedling survival and growth under nursery conditions and how sensitive *P. longifolia* plants are to various pest and diseases commonly encountered in horticulture. If seedlings are to be kept in the nursery until the following planting season then some additional insight is required to determine the best conditions for keeping seedlings alive and growing under nursery conditions over the summer months. Seedlings in the nursery have been observed to die on occasion as a result of fungal attack (K. Chia, personal observations). Watering regimes required for *P. longifolia* seedlings also need to be examined in order to increase survival in the nursery.

Greater survival at 3 months after planting of the older seedlings is an indication that transplant shock is less of an issue in the older seedlings. Variations in rainfall in the months following planting could have also resulted in the differences observed in survival. However, by the time the seedlings had been growing *in situ* for 20 months there was no difference in survival between those *P. longifolia* planted early (i.e. only a few weeks old) and those planted 12 months later (but were, at that point, 32 months old). Maintaining seedlings under nursery conditions for 12 months makes this a more expensive approach compared to planting out newly emerged seedlings almost immediately following germination. This may also be the case for other species that germinate in July/August. To reduce costs and to streamline the restoration approach

further investigations could be undertaken to determine if survival is similar or improved when an autumn planting is completed at the same time as other restoration activities are implemented.

Anecdotal evidence indicates that *P. longifolia* seedlings are known to be palatable to herbivores and planting seedlings with some form of tree guard is likely to improve seedling survival. Onion bag tree guards resulted in the best survival of the seedlings, although it was not always possible to determine the cause of death in any of the treatments as often there were no remnants of the seedlings. However, if a seedling does survive the first summer in the shade cloth tree guards, they were likely to grow significantly bigger in the following seasons. The exact reasons for this rapid burst of growth are currently unknown but could be due to the reduction in insect damage to the plants or variations in microclimate (reduced light intensity, elevated humidity or reduced wind speed) within the guards.

Microclimatic variation was not measured as part of this study, however Close et al. (2009) found that shade cloth tree guards have beneficial effects by preventing browsing, minimising temperature effects on seedlings (temperatures in the Mediterranean environment were 1.4 °C lower in shade cloth tree guards compared with the control) and creating a microenvironment that significantly elevates the levels of photosynthesis in spring which, in turn results in greater growth of *Eucalyptus gomphocephala* and *Banksia attenuata* seedlings

Whilst evidence for herbivory by large animals was lacking, damage to *P. longifolia* seedlings from insects, particularly in those seedlings with no tree guard or with the onion bag tree guard was clearly evident. Further work is required to investigate methods of reducing insect damage as a large number of plants in the seedling trial and in the synthetic seed bank trial experienced extensive damage and in some cases death from insect attack. Plath et al. (2011) found that the use of insecticides resulted in greater growth and reduced leaf damage to seedlings of three native Central American timber tree species, and survival was not directly affected by the use of insecticides.

Based on the results of this study, ideally *P. longifolia* seedlings would be germinated during the winter wet season then once emerged, potted up in July and August and maintained under nursery conditions for 2-3 weeks then planted onto restoration sites in

late August. Nevertheless, this timing is not ideal as most of the *in situ* restoration planting of greenstock is generally complete before this time in June and July. Consequently, further investigation should be undertaken to quantify and enhance survival of young *P. longifolia* plants under nursery conditions and determine if holding the seedlings over summer in the nursery environment and planting at the same time as other seedlings results in similar or greater survival. Planting seedlings in an onion bag guard enhances survival though not growth but the use of a shade cloth guard to increase growth is likely to be less important to practitioners (M. Daws and P. Bullock pers. comm.).

Chapter Five - Conclusions and Recommendations for Practitioners

This six-year study has significantly expanded our knowledge of *Persoonia longifolia* through firstly defining what is currently known about this species, its environment and the present knowledge gaps (Chapter One), then through documenting it's phenology and responses to fire through detailed *in situ* investigations (Chapter Two) and finally thorough describing and understanding its seed biology through detailed *in situ* and *ex situ* germination studies (Chapter Three). Combined with the assessment of various restoration approaches used for reassembling jarrah forest plant communities following bauxite extraction in south west Western Australia (Chapter Four), the findings and outcomes detailed in this thesis make a significant contribution to the restoration ecology of *P. longifolia.* It has also provided some information on how to better manage and utilise *P. longifolia* in undisturbed jarrah forest ecosystems and the likely outcomes stemming from modified fire regimes.

Chapter Five outlines and synthesises the major conclusions of the preceding chapters, provides a prescriptive approach for germination and restoration for practitioners and outlines directions for further studies for research scientists.

5.1 Phenology

An understanding of the phenology of *P. longifolia* resulted in germination studies becoming more targeted. Vegetative growth occurs during the summer months with flowering and fruiting occurring at the same time. Fruits mature from July to September at which time it is dropped to the forest floor. This makes it an ideal time to collect fruits for propagation purposes. Germination occurs in late winter/early spring from fruit that is at least 1 year old.

Following fire, *P. longifolia* plants will resprout prolifically in the next growing season and there is very little fruit production in the first year after a fire. Fruit is not produced until at least the second year, and then seed requires at least 1 year on the ground before germination can occur (3 years post fire). Modelling indicated that maximum fruit

production occurs between 19 and 21 years post fire. Therefore the best areas for collection would be those that have not been burnt for some time.

5.2 Germination biology

As indicated above, seeds germinate in late July/early August. Germination is improved when the mesocarp and the endocarp are removed leaving behind only the naked seed. This is however, very time consuming and is not easy to do without damaging the seed during the extraction process. Removal of the mesocarp, and then burial of indehiscent endocarps provides a more practical method of large-scale propagation through breaking seed dormancy and allowing germination. Burial can occur either in the bush environment or in the nursery environment.

Germination in *P. longifolia* is not straight-forward and a complex sequence of environmental conditions is required in order to break seed dormancy and allow germination to occur. It appears that the conditions that the seeds are exposed to over the summer months are critical to germination success.

Stratification trials indicated that a period of warm incubation (12 weeks, including two weeks wet, at 30 °C) followed by a period of cold stratification (6 weeks constantly wet at 8 °C) prior to incubation under winter-like conditions of 10/20 °C will break dormancy in 13 % of the seeds in the first winter. This is the first time that the sequence of warm + cold stratification has been clearly identified as being important for this species and whilst germination was still low it was greater than germination in the first winter in most other laboratory trial previously undertaken during the course of this study.

If no wet periods are experienced by the endocarps during the summer months germination can still be induced through the use of dry heat pulses (4 weeks at 50 °C).

A longer summer period will also result in greater germination than a shorter summer (20 weeks compared with 12 weeks). The stratification trial undertaken as part of this study only investigated warm incubation over a period of 12 weeks. Further

investigations could be undertaken to determine the optimum length of time for warm incubation followed by cold stratification to maximise germination.

In the nursery environment, manipulation of the watering regimes appears to simulate the warm + cold stratification required for germination. Watering the buried seeds between two and four times with the equivalent of 25 mm rainfall during the summer months resulted in approximately 35 % germination after the first winter and an additional 30 % germination by the second winter. The third winter resulted in only an additional 5 % germination with many of the remaining seeds losing viability. Seeds commenced germinating approximately 4-5 weeks after being constantly wet when exposed to winter temperatures. They also germinate in a staggered manner for about 8 weeks after they commence germination.

The germination sequence in *P. longfolia* is unusual in that hypocotyl and the cotyledons extend and turn green pushing the radicle through the endocarp before any radicle extension occurs indicating that there is a greater degree of dormancy within the radicle than the cotyledons.

5.3 Use of Persoonia longifolia *on restoration*

Seeds need to have their dormancy broken and then be buried (not scattered on soil surface) to germinate in restored areas. Dormancy can be broken through the use of a synthetic seed bank, where seeds are buried and retrieved before sowing on to restoration sites. However, seeds need to be buried onto restoration sites after retrieval in order for germination to occur. If seeds cannot be buried onsite during restoration practices then germination in the nursery and planting out as seedlings may be a more practical approach. Further work needs to be undertaken to determine if burial for dormancy break without the mesocarp results in better germination once sown onto the restored areas. Preliminary evidence strongly indicates that this is likely to be the case.

If *P. longifolia* is to be planted in restored sites as seedlings, these should be planted with some form of tree guard to maximise survival. Currently survival of seedlings can be expected to be between 50-80 % after 1-2 years. Results from tree guard trials showed that best survival was achieved with the use of biodegradable onion bag guards

and best growth was achieved with shade cloth guards. Further investigation into protection from insect damage is required to further improve seedling health and survival.

5.4 Recommendations for practitioners

In summary, the following methodology should be employed to germinate seedlings or prepare seeds for use on restoration sites in the jarrah forest of Western Australia:

1. Collect seeds late July/early August. Seed is considered to be ripe when the mesocarp has turned a yellowish colour and the fruits readily drop to the ground. Fruit can be collected directly from the ground with the yellowing mesocarp. Fruits with a black, dry mesocarp are likely to be at least 1 year old.
2. Remove the fleshy mesocarp layer as soon as possible. The best method to date, of removing the mesocarp involves rupturing the exocarp (the skin) through the use of a coffee depulping machine, and agitating in either a cement mixer or with a paint stirrer in a pectinase solution (10 ml Novozymes VinoClear Classic to 10 L of water). Any remaining flesh can be removed by placing the seeds on a screen and blasting with a high pressure cleaner. Approximately 200 g of clean endocarps will result in approximately 1000 cleaned endocarps.
3. Bury endocarps in clean washed sand under nursery conditions or in mesh bags in the forest for up to 2 years (September/October).

Germination of seeds can be undertaken in the following manner:

1. Bury seeds at least 2 cm below the surface in clean washed sand in tubs in a shaded nursery environment or at least 2 cm below the surface, without leaf litter under natural bush conditions.
2. Water endocarps buried in the nursery four times over the summer months with the equivalent of 25 mm of water and on each occasion between watering events, sand plus endocarps should be allowed to completely dry out.
3. Expose tubs to natural rainfall during winter months (commencing in April) or retrieve endocarps from the bush burial and bury in clean washed sand in a shaded nursery environment.
4. Check weekly for germinants throughout winter (germination is likely to occur approximately 5 weeks after the soil is continuously wet) and prick out into biodegradable pots as required.

5. Allow seedlings to recover from transplant shock for 2-3 weeks.
6. Plant seedlings onto restored areas of the mine in late August (when approximately 2-3 weeks old) and protect with a biodegradable onion bag. Although this does not fit with current restoration practices it will ensure that seedlings do not need to be maintained in the nursery environment over the summer months.

Endocarps buried in the bush environment should be retrieved in April prior to the winter wet season and then if to be directly sown as seed, buried in newly restored areas as soon as practical. Seeds cannot be scattered directly onto the soil surface as they must be buried (>1 cm) for germination to occur.

5.5 Further study

Additional studies are recommended to further increase our understanding of this iconic Western Australian species. These studies could include:

- Further investigation into stratification processes involved in breaking seed dormancy and promoting germination including:
    - The optimum length of time required for warm stratification prior to cold stratification.
    - Determining if constant warm wet stratification followed by cold stratification is effective in breaking seed dormancy or if the alternating wet/dry periods during the warm stratification then followed by cold stratification result in better germination.
    - The optimum length of time required for cold stratification following exposure to warm incubation/stratification/wet and dry cycles.
- Examining the role of warm stratification in terms of dormancy break and endocarp weakening. Does warm or cold stratification weaken the suture line on the endocarp? Does warm or cold stratification alleviate dormancy within the embryo itself?
- Further investigations into the use of *P. longifolia* endocarps and seedlings in the restoration process including:
    - Using clean endocarps in a synthetic seed bank trial (instead of using the entire fruit with the mesocarp intact) to improve germination *in situ.*

- Investigating the possibility of planting seedlings in autumn (which will still require holding seedlings in the nursery environment over the summer months) rather than late-August (early spring) as undertaken in this study.
- Investigating means of controlling insect damage on seedlings planted into restored areas. Insect damage appears to be one of the main issues resulting in seedling death once seedlings have been planted *in situ*.
- Investigating the possibility of using other types of tree guards (such as biodegradable plastic guards) rather than onion bags to further improve growth of seedlings on restored areas of the mine site.

These results outline for the first time how dormancy and germination are regulated in a species with a hard woody endocarp, insights which will significantly improve our understanding of other species with similar reproductive features. The results from this study are likely to be applicable to germinating other species of *Persoonia* from around Australia. Similarly, the techniques described here could potentially be used for breaking seed dormancy in other species with hard woody endocarps both within Australia (such as *Astroloma, Leucopogon* and *Eremophila*) and further afield.

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Appendix 1: Phenology Paper published in the Australian Journal of Botany

CSIRO PUBLISHING

Australian Journal of Botany, 2015, **63**, 415–425
http://dx.doi.org/10.1071/BT14315

Developmental phenology of *Persoonia longifolia* (Proteaceae) and the impact of fire on these events

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Abstract. *Persoonia longifolia* R.Br. is a common understorey tree that is difficult to re-establish following bauxite extraction and land restoration in parts of the jarrah forest of south-western Western Australia. To improve restoration outcomes for *P. longifolia*, understanding its phenology is vital for developing methods for returning this plant to rehabilitated areas. The present study investigated in detail different aspects of the phenology of *P. longifolia* over a 3-year-period. Most vegetative growth occurred during the summer months and flowering and fruiting occurred concurrently. Fruit matured from July through to September, at which time these dropped to the forest floor. Germination occurred in late winter–early spring from fruit that was at least 1-year old, with poor seedling survival in natural bush (<10%) during the first 12 months. Following fire, *P. longifolia* plants resprouted prolifically in the next growing season, although there was very little fruit production in the first year following fire. Fruit was not produced until at least the second year following a fire, and when dispersed, required at least another year in the soil seed bank before germination commenced (i.e. 3 years post-fire). Results from the present study will improve restoration outcomes for this species, by providing guidance on better seed-collection strategies and baseline information concerning growth rates under natural conditions that can then be used to assess performance of this species in restored environments.

Additional keywords: fire, mining, restoration, seed ecology.

Received 25 November 2014, accepted 3 March 2015, published online 11 May 2015

Introduction

Persoonia longifolia R.Br. is an attractive small tree from the Proteaceae family found in jarrah forests in the south-west of Western Australia, an area that is currently subject to mining for the production of bauxite (Koch 2007*a*). It is a common and very distinctive understorey species and the abundance of *P. longifolia* (or snottygobble, as it is commonly known) in the pre-mining environment results in it being considered a priority taxon for return to restored areas (Koch 2007*b*). However, this has proven to be difficult (Mullins *et al.* 2002; Norman and Koch 2005) and, consequently, it is defined as a recalcitrant species, i.e. one that is common in the forest but absent or found only in low densities in restored mine sites (Norman and Koch 2005; Grant and Koch 2007).

In addition to being required for minesite restoration, *P. longifolia*, like other species of *Persoonia* (e.g. *P. virgata* and *P. saccata*) (Ketelhohn *et al.* 1998; Bauer *et al.* 1999; Bauer and Johnston 1999) is also utilised by the cut-flower trade as a filler species in floral arrangements (Weston 2003). Even though the flower is not significant, the foliage is highly prized for its striking weeping appearance and durability and, as a result of these attributes, is exported overseas in significant quantities (B. Long, pers. comm.). Currently, the majority of foliage used in the floriculture industry is wild-sourced from natural populations, because propagation of this species has proven exceptionally difficult either from cuttings or seeds (Mullins *et al.* 2002; Norman and Koch 2005; Crowhurst 2006).

The shape and small size of the tree ensures that there is also potential for this species to be used in the native horticultural industry, as either a feature tree in domestic situations or in amenity plantings in civic parks, gardens or roadside reserves (Crowhurst 2006). Aesthetically, it is very appealing with its graceful symmetry, highly textured flaky bark, lime green weeping leaves and relatively small compact stature (Fig. 1*a*). Interestingly, it was first introduced into cultivation in 1850 in the United Kingdom, but is rarely seen in Australian gardens today (Wrigley and Fagg 1989). If reliable propagation techniques can be developed for this species, it is likely to be

Appendix 2: Germination Paper published on line in the Annals of Botany

Annals of Botany Page 1 of 16
doi:10.1093/aob/mcw100, available online at www.aob.oxfordjournals.org

ANNALS OF BOTANY
Founded 1887

Identification of the seasonal conditions required for dormancy break of *Persoonia longifolia* (Proteaceae), a species with a woody indehiscent endocarp

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Received: 20 January 2016 Returned for revision: 24 February 2016 Accepted: 29 March 2016

• **Background and Aims** The mechanisms involved in breaking seed dormancy in species with woody endocarps are poorly understood. In a landmark study examining the role of endocarps in regulating germination, our aim was to investigate the effects of the natural sequence of environmental conditions on dormancy break of a species with a woody endocarp (*Persoonia longifolia*).
• **Methods** The role of the endocarp in germination was investigated through imbibition and endocarp removal germination tests. The use of burial to break dormancy was examined and results from these experiments were used to guide laboratory investigations into the use of wet/dry cycling and stratification to break dormancy.
• **Key Results** Endocarps were water-permeable. Germination increased from 0 to 92·5 % when endocarps were removed. During burial in the field and nursery, 41·6 and 63·7 % of the endocarps germinated, respectively, after 36 months. *Ex situ* post-burial germination was cyclical and highest after 30 months of burial (45·4 % nursery and 31·8 % field). Highest germination occurred in wet/dry trials when the dry summer was long (20 weeks), had fluctuating temperatures (30/50 °C) and two long (7 d) wet cycles and was followed by moist winters at 10/20 °C. A stratification trial found that highest germination occurred following incubation for 12 weeks at 30 °C (including 2 weeks moist) + 6 weeks moist at 8 °C then placement at 20/10 °C for germination.
• **Conclusions** Summer conditions break physiological dormancy of the embryo and promote opening of the endocarp, allowing seeds to germinate during winter conditions. By closely monitoring the environment that endocarps are exposed to in nature, dormancy breaking mechanisms can be identified and used to improve germination. These results outline for the first time how dormancy and germination are regulated in a species with a hard woody endocarp, insights which will significantly improve our understanding of other species with similar reproductive features.

Key words: *Persoonia longifolia*, woody endocarp, dormancy break, burial, physiological dormancy, stratification, wet/dry cycles, seed germination.

INTRODUCTION

The germination unit of species in various plant families is a seed enclosed by a hard woody indehiscent endocarp (see table 3·10 in Baskin and Baskin, 2014), and species with woody endocarps can be found in vegetation zones from the tropics to the boreal/subalpine. The challenge in understanding the germination ecology of seeds covered by a hard endocarp is that characteristics of both the endocarp and the embryo may be involved in delaying germination. That is, the endocarp could be water-impermeable (Li *et al.*, 1999), or it could be water-permeable but exert strong mechanical resistance to expansion of the embryo (Nikolaeva, 1969). Also, the embryo may have some level of physiological dormancy (PD) and warm and/or cold stratification may be required for dormancy break to occur (Chien *et al.*, 2002; Baskin *et al.*, 2005; Persson *et al.*, 2006; Chen *et al.*, 2007; Imani *et al.*, 2011). However, after PD is broken, it is still uncertain whether the embryo has enough growth potential to overcome the mechanical resistance of the water-permeable endocarp (Nikolaeva, 1969) as the covering structure may require some form of weakening. In some species the endocarp splits into two parts during seed germination, but in others the endocarp opens via a lid-like structure (Hill, 1933, 1937).

For most species with woody indehiscent endocarps, little is known about how the timing of germination is controlled in the natural habitat. Also, the effect that the sequence of environmental conditions between the time of dispersal and germination has on the embryo and the endocarp is largely unknown. To explore these questions, we investigated dormancy break and germination of *Persoonia longifolia* (Proteaceae, Persoonioideae) in its Western Australian Mediterranean habitat.

Persoonia longifolia is one of 98 species of *Persoonia*, all of which are Australian endemics with a long Gondwanan heritage stretching back to the Cretaceous (Dettmann and Jarzen, 1998). It is commonly found in the jarrah forests (*Eucalyptus marginata*) of south-western Western Australia, an area that

Appendix 3: Restoration Paper published online in Ecological Research

Ecol Res
DOI 10.1007/s11284-016-1370-y

ORIGINAL ARTICLE

Kerryn A. Chia · John M. Koch · Rohan Sadler
Shane R. Turner

Re-establishing the mid-storey tree *Persoonia longifolia* (Proteaceae) in restored forest following bauxite mining in southern Western Australia

Received: 8 November 2015 / Accepted: 20 May 2016

Abstract *Persoonia longifolia* is a common mid-storey species that is difficult to return to post-mining environments. This study aimed to quantify in situ emergence of *P. longifolia* seeds on restored areas, investigate seed cueing prior to use in restoration and assess different tree guards for increasing seedling survival and health. Initial investigations found that < 1 % of seeds buried or scattered on restored areas produced seedlings. However, if seeds were cued through burial in surrounding forest, retrieved and sown on restored areas, seedling emergence increased to 24 %. Significantly more seeds emerged as seedlings when buried (14.6 %) compared to those scattered on the soil surface (2.7 %). There was no significant difference in survival between seedlings planted at 2-3 weeks of age compared with those planted at 12 months of age after 20 months in situ growth. Additionally, those seedlings planted when younger were significantly taller (29.0 ± 2.9 cm) than those that were planted at 12 months of age (4.7 ± 0.3 cm). Use of "onion bag" guards improved survival from 58.1 ± 4.0 % (no guard) to 70.8 ± 3.4 % with an onion bag guard. The use of shade cloth guards did not significantly improve survival, however plant height did increase substantially after 32 months growth (22 cm compared with 7.2 cm for no guard). These data demonstrate that consideration needs to be given to specific species requirements to improve seedling emergence and survival when attempting to return difficult to germinate species to the post-mining environment.

Keywords Restoration · Rehabilitation · Seed burial · Tree guards · Mining

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Published online: 07 June 2016

Introduction

Restoration of mine sites requires the return of a diverse range of species commonly found in the pre-mining environment in order to return it to a self-sustaining and resilient ecosystem (Ward et al. 1996; Norman et al. 2006; SERA Standards Reference Group 2016). Germination, growth and return of many of these indigenous species to restored areas can be problematic but is often a requirement of various government authorities in different parts of the world (Bielecka and Król-Korczak 2010). Consequently mining companies are now turning their attention to developing successful restoration techniques for returning plant communities rather than individual species to disturbed areas following mining, though different species may require contrasting reintroduction approaches (Koch 2007b; Todd et al. 2009).

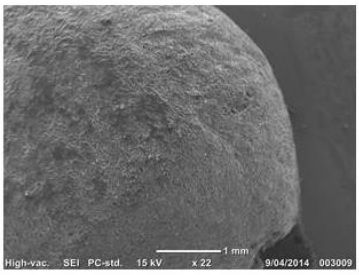
The jarrah forest of southern Western Australia is part of a biodiversity hotspot of international significance (Myers et al. 2000) and is subjected to localised mining for the production of bauxite. Many species found within this area are difficult to germinate, propagate and return to restored areas of these mines and considerable effort is being made to understand the ecology of these species in order to return the forest to a self-sustaining ecosystem reflective of its original composition and function.

Persoonia longifolia, a small, mid-storey resprouter tree found in sandy or lateritic soils in the jarrah forest

Appendix 4: Scanning Electron Microscope images of annual changes in the endocarp of *Persoonia longifolia* when buried for 3 years.

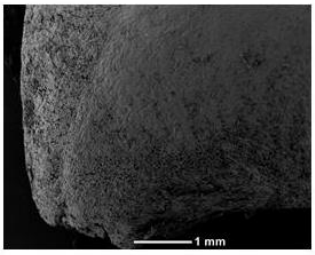
Change in Lid over time

Time 0

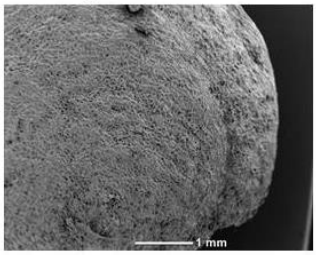


Time 12 months

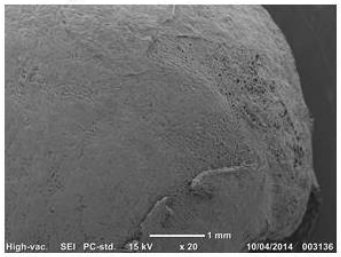
Bush



Nursery

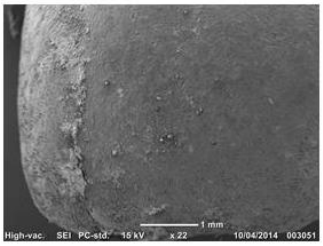


Kings Park

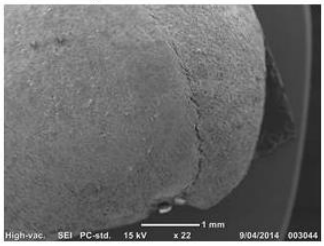


Time 24 months

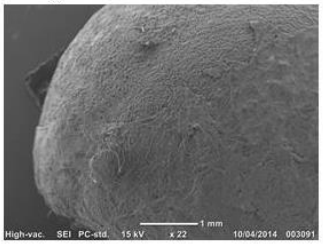
Bush



Nursery

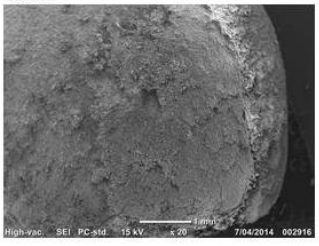


Kings Park

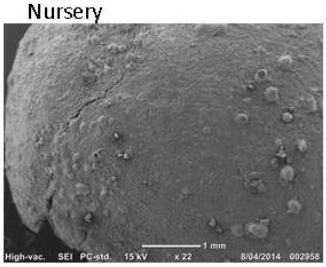


Time 36 months

Bush



Nursery



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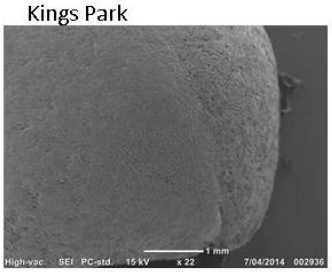
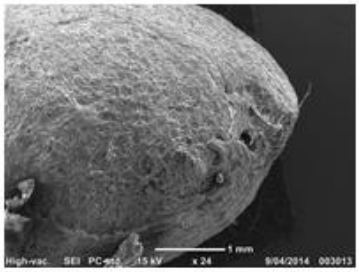


Appendix 4 cont.

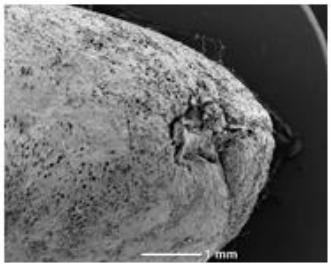
Change in Micropyle over time

Time 0

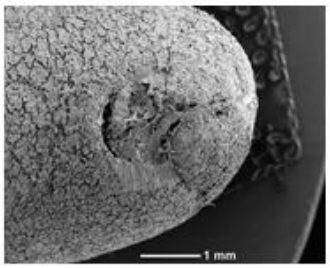


Time 12 months

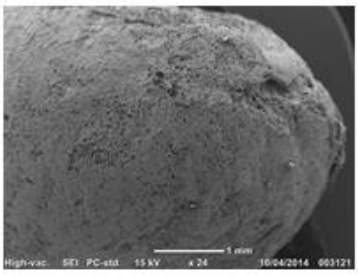
Field



Nursery

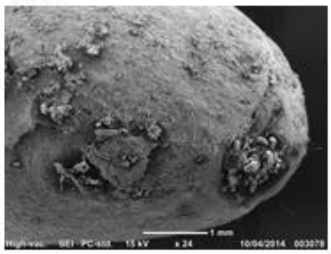


Kings Park

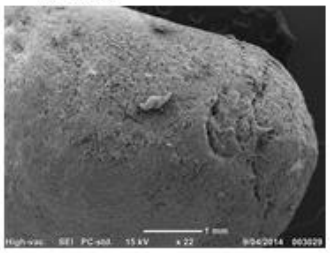


Time 24 months

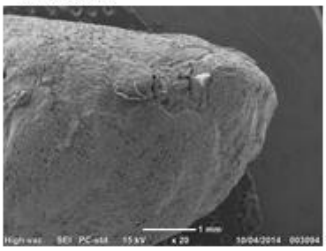
Field



Nursery

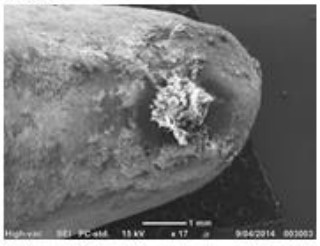


Kings Park



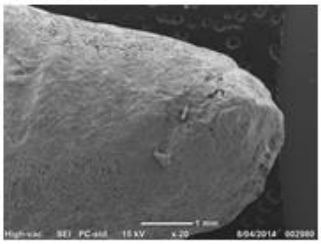
Time 36 months

Field



Nursery

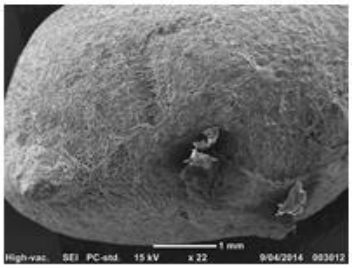
Kings Park



Appendix 4 cont.

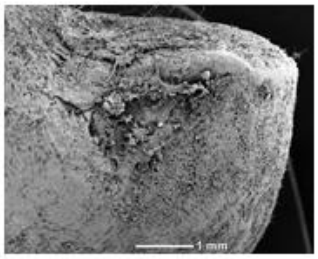
Change in Hilum over time

Time 0



Time 12 months

Field



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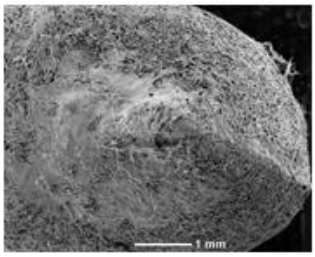

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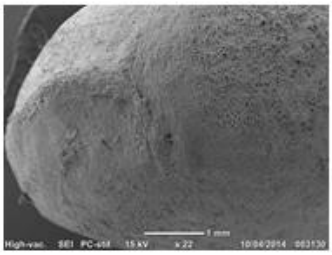
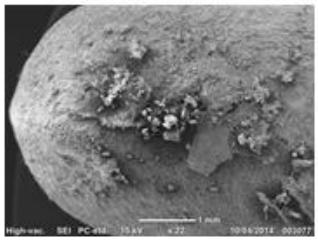


Time 24 months

Field



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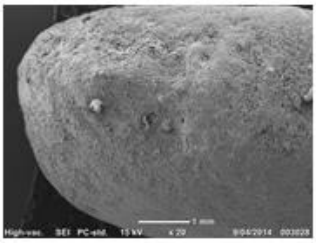
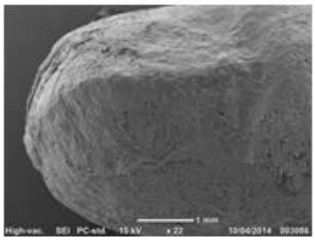
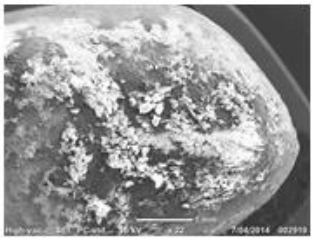


Kings Park



Time 36 months

Field



Nursery

Kings Park