Germination of *Persoonia* Species

Australian Flora Foundation
Final Report
March 2019

Kerryn Chia

Kings Park and Botanic Gardens

West Perth, WA 6005

Email: chia@wn.com.au

Tel: 08 9863 1044

This report is provided to the Australian Flora Foundation in fulfilment of the conditions of the grant awarded to the author in 2016. This report presents the results from experiments conduct by Kerryn Chia between 2016 and 2019 on the germination of *Persoonia* species.

The ideas, experimental work, results, analyses and conclusions presented in this report are entirely the authors' own efforts, except where otherwise acknowledged. This work is original.

This work would not have been possible without the support of The Australian Flora Foundation, the Botanic Gardens and Parks Authority and in particular Dr Shane Turner.

Dr Kerryn Chia
15 March 2019

Contents

Cracking A Hard Nut - Germination of *Persoonia* species, a genus with hard woody indehiscent endocarps

K. A. Chia[A,C]*, and S. R. Turner*[A,B]

[A]Kings Park and Botanic Gardens, West Perth, WA 6005, Australia.

[B]School of Plant Biology, Faculty of Science, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia.

[C]Corresponding author. Email: chia@wn.com.au

1.0 Abstract

The mechanisms involved in breaking dormancy in the genus *Persoonia* continue to elude scientists. This study aimed to continue investigations into the use of seasonal simulations in both the nursery and laboratory, including warm and cold stratification and long and short summer cycles, to encourage germination in *P. longifolia*, *P. elliptica*, *P. quinquenervis* and *P. saccata.* This study was unsuccessful in improving germination in *P. longifolia* from previous studies. Germination of *P. quinquenervis* in the nursery, whilst much lower than germination in *P. longifolia*, followed a similar pattern, in that summer rainfall appears to improve germination. Soil stored seed of *P. quinquenervis* remained viable for the duration of the nursery trial. In the laboratory wet/dry trials, germination of 49% was achieved when warm stratification was applied and this was significantly better than all other treatments. *P. elliptica* germinated poorly in all experiments (<10%) and soil stored seed did not remain viable for the duration (2 years) of the nursery trial. *P. saccata* seeds germinated once the endocarp was removed.

2.0 Introduction

Many species in the Australian flora reproduce though a germination unit which includes a seed covered by a hard woody endocarp. The germination of seeds in such species can be controlled by both the hard endocarp and through the embryo itself. The endocarp could provide a mechanical barrier to germination through water impermeability (Li et al., 1999), or by exerting strong mechanical resistance to expansion of the embryo (Nikolaeva, 1969). Additionally, the embryo may have some level of Physiological Dormancy (PD) and warm and/or cold stratification may be required for dormancy break to occur (Baskin et al., 2005, Chien et al., 2002, Persson et al., 2006, Chen et al., 2007, Imani et al., 2011).

The natural sequence of environmental conditions may regulate how germination is broken in such reproductive units through wetting/drying and heating/cooling. These questions were explored through a recent project which investigated dormancy break and germination in *Persoonia longifolia* (Proteaceae, Persoonioideae) (Chia, 2016). Several significant breakthroughs were made including the identification of the drivers of seed dormancy loss which appear to be strongly linked to temperature thresholds, sequence of temperature treatments and the duration of various temperatures experienced by seeds as they reside in the soil seed bank (Chia et al., 2016).

Persoonia longifolia is indicative of many *Persoonia* species, all of which have a hard woody endocarp and all of which are difficult to germinate (Robertson et al., 1996, Stack and Brown, 2003, Norman and Koch, 2008, Norman and Koch, 2006, Preston et al., 2002, Frith and Offord, 2010, Ketelhohn et al., 1998, McIntyre, 1969, Rintoul and McIntyre, 1975, Mullins et al., 2002, Nield et al., 2015, Offord et al., 2015). Removal of the hard woody endocarp improves germination significantly but is time consuming and often results in a high degree of damage to the embryo. The reproductive unit, is similar to many other difficult to germinate species such as *Astroloma, Leucopogon* and *Eremophila* found right across Australia (Turner et al., 2009, Merritt et al., 2007).

This current project aims to extrapolate the results achieved for *P. longifolia* and determine if similar techniques can be used to germinate a number of other *Persoonia* species. Additionally, the research will investigate in more detail the stratification requirements for germination of *P. longifolia* in order to speed up the germination process which currently takes anywhere between 9 months and three years to occur. In particular the project will aim to:

- Determine if similar germination approaches are applicable to other species of *Persoonia* such as *P. elliptica*, *P. quinquenervis,* and *P. angustifolia* which are currently required for mine site restoration and *P. saccata* which is a species of interest in restoration programs undertaken in Bold Park and Kings Park.
- Investigate, in detail, the stratification processes involved in promoting germination of *P. longifolia* through an experiment which builds upon the latest research findings reported by Chia et al. (2016).
- Develop practical and reliable propagation approaches for use by the native plant industry and restoration practitioners for the production of large numbers of plants using novel horticultural approaches.

This proposed work is considered to be essential and logical next step to continue the successes that have been achieved for *P. longifolia* and it is anticipated that the technologies and insights could potentially be transferable to other Australian species with hard woody endocarps such as *Astroloma*, *Leucopogon* and *Eremophila.*

2.1 The Species and Their Habitats

This project is focused on extrapolating the results from the *P. longifolia* studies through additional stratification trials and using similar techniques for three additional *Persoonia* species, *P. elliptica, P. quinquenervis* and *P. saccata,* found in the south west of Western Australia. Distributions maps for these species are shown in Figure 1.

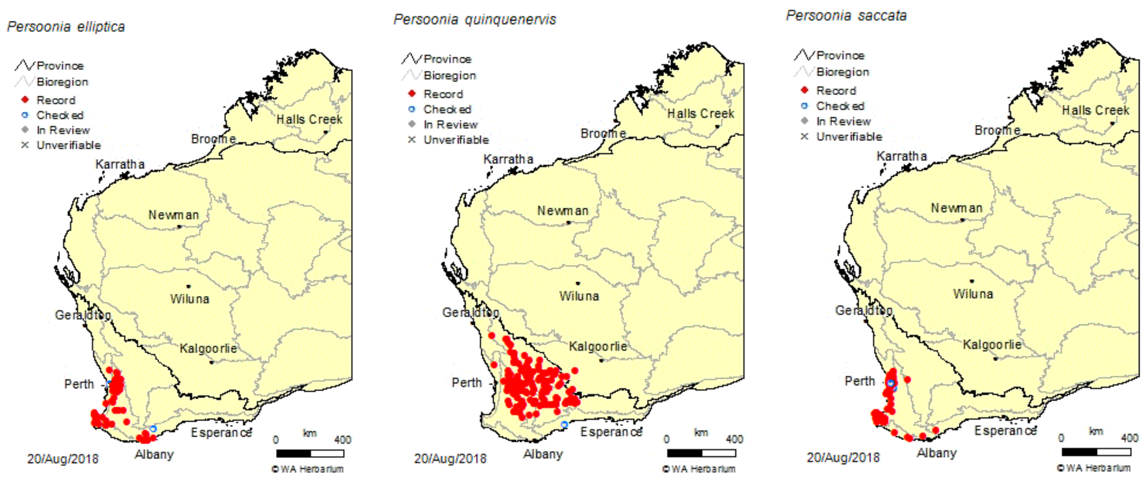

Figure 1: Distribution maps for *P. elliptica*, *P. quinquenervis* and *P. saccata* (Department of Biodiversity Conservation and Attractions, 2018).

Persoonia elliptica is a member of the *Persoonia lanceolata* group (Weston, 2003) and is an erect shrub to tree, 2-8m tall, with corky bark (Figure 2). The leaves are obovate to spathulate, and it flowers between October and February (Weston, 1995).

It occurs within 50km of the coast from Perth to Albany and grows in sclerophyll woodland and forest in sandy soil or laterite. Its common name is spreading snottygobble (Department of Biodiversity Conservation and Attractions, 2018). It is a species that occurs at densities of around 3 plants per hectare around the Jarrahdale area southwest of Perth, WA (Abbott, 1984). Whilst not a major contributor to the tree species in the jarrah forest, the ability to propagate *P. elliptica* is of interest to mining companies for restoration purposes and also to horticulturalists for use in the home garden.

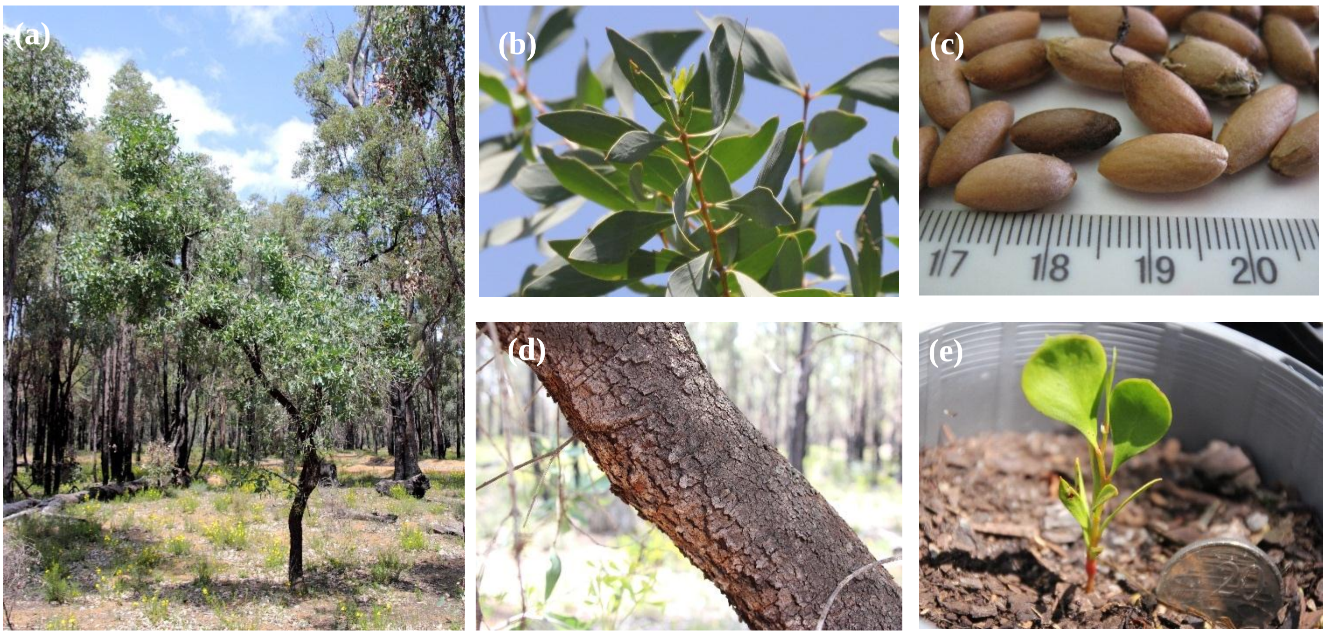

Figure 2: *Persoonia elliptica*. (a) Tree. (b) Leaves (c) Cleaned endocarps. (d) Corky bark on the tree. (e) Young seedling.

Persoonia quinquenervis is an erect spreading shrub which is 2-2.5 m tall (Figure 3). Its leaves are linear to narrowly oblong, to oblanceolate to narrowly spathulate and it is known to exhibit a spectacular variation in leaf morphology. *Persoonia quinquenervis* flowers between November and December and when it germinates it can have between four and eight cotyledons. It grows in the Wheatbelt and Southwestern WA (Figure 1), in heath, mallee heath, *Acacia/Allocasuarina/Eucalyptus* thickets, *Eucalyptus* woodland or dry sclerophyllous forest (Weston, 1995). *Persoonia quinquenervis* is relatively common in the area surrounding South 32 mine site near Boddington and therefore is of interest to those wishing to return this species to restored areas of the mine site.

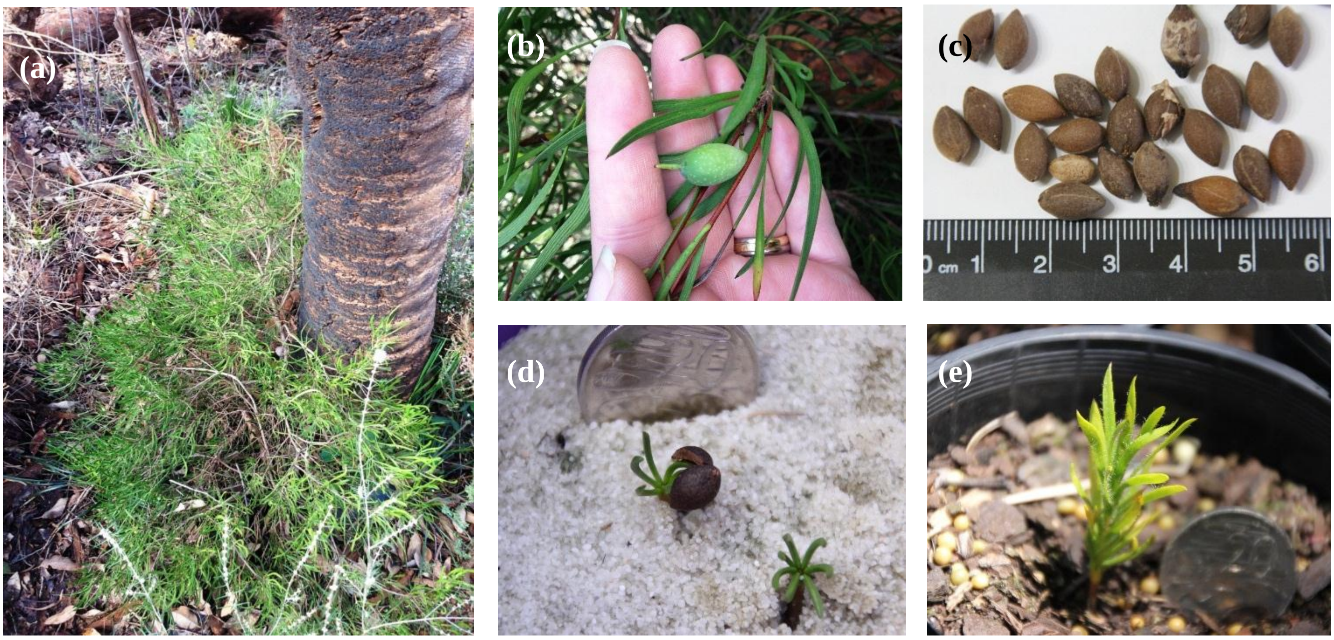

Figure 3: *Persoonia quinquenervis.* (a) *P. quinquenervis* shrub. (b) Drupe and leaves. (c) Cleaned endocarps. (d) Germinants with seven cotyledons. (e) Young seedling.

Persoonia saccata is an erect to spreading shrub 0.2-1.5m tall (Figure 4). It has smooth bark, linear leaves and flowers from July through to January. Germinants have between five and nine cotyledons (Weston, 1995). It occurs north of Perth to Yallingup and Albany in the southwest of WA (Figure 4). After fire, the plant regenerates vigorously and produces large inflorescences the following summer. Each subsequent year following the fire, the size of the new shoots decrease until the plant becomes senescent and does not flower (Weston, 1995). *Persoonia saccata* is common throughout Kings Park and Bold Park.

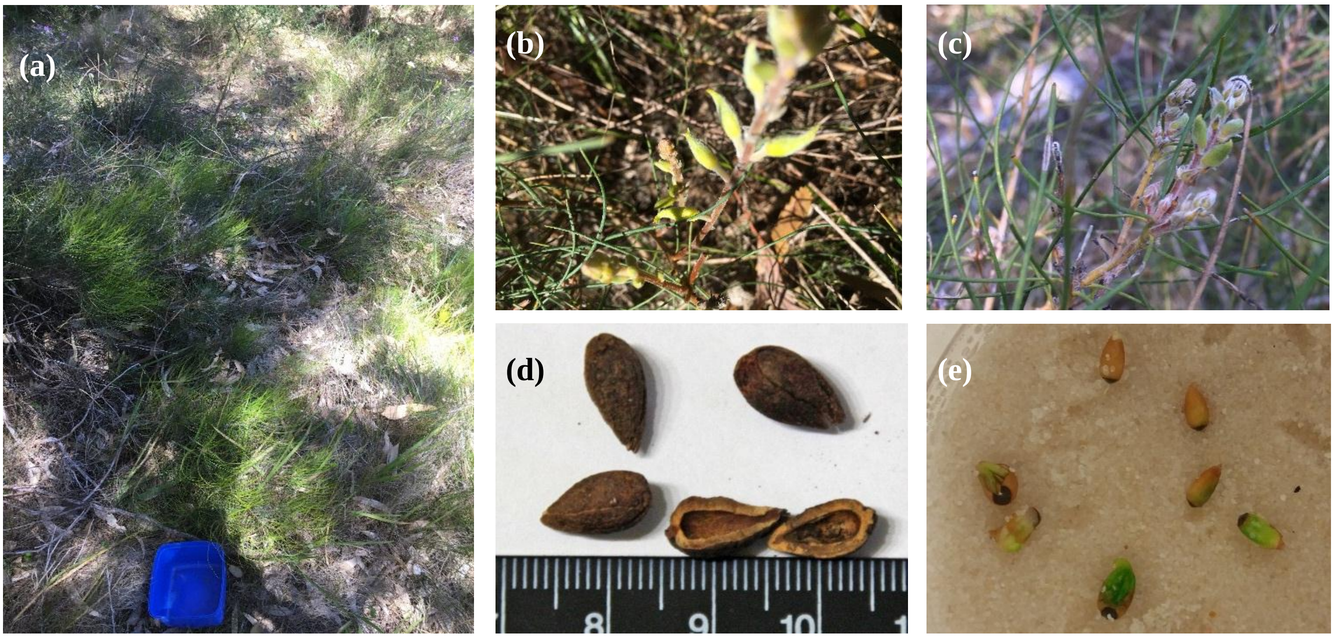

Figure 4: *Persoonia saccata.* (a) *P. saccata* shrub. (b) Flowers. (c) Leaves and buds. (d) Old seeds. (e) Germinants.

2.2 Persoonia longifolia *Stratification Experiment*

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. 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.*

Investigations into germination of *P. longifolia* showed that wet and dry cycles administered over the summer months improved germination significantly but that high germination (>70%) still took three years to occur.

These extensive wet/dry trials led to some preliminary studies into various targeted warm and cold stratification treatments using germination incubators which showed specific warm + cold stratification could significantly reduce the time taken for germination to occur and has been considered a major breakthrough in germination of Australian species with hard woody endocarps (Chia et al., 2016). However there were some limitations of this trial with germination occurring in only one treatment and being limited to only 13% after 30 weeks. Therefore further investigations need to be undertaken to comprehensively determine:

- Optimum length of time for cold stratification (four, six or eight weeks at 5°C).
- Does cold stratification result in better germination if it occurs before or after warm stratification (i.e. determine if the order of stratification is important).
- Do constant wet conditions or wet/dry warm stratification prior to cold stratification results in better germination.

2.3 Germination of Other Persoonia Species

Currently it is unknown if the technologies that have been developed as a result of the recent PhD project are transferable to other species with similarly structured hard woody endocarps. Some small scale trials were undertaken on *P. elliptica* as a part of the PhD project but the results were inconclusive (likely due to poor seed viability). In addition, the bauxite mining company, South 32 has attempted germination of both *P. elliptica* and *P. quinquenervis* in nursery conditions with limited success to date. The reproductive unit of all *Persoonia* species is structurally similar and therefore it is thought that the use of wet/dry cycling may play a role in breaking dormancy in these other important *Persoonia* species.

3.0 Methods

3.1 Endocarp and Seed Characteristics

Various numbers of intact seed-filled endocarps for each species were individually weighed. The seed was then extracted by gently squeezing the endocarp longitudinally in an industrial vice to crack open intact endocarps. The weight of each individual seed was determined 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.

3.1 Persoonia longifolia *Stratification Experiment*

Fruits were collected in September 2016 at Sotico, Boddington, Western Australia. The exocarp and mesocarp layers were removed by fermentation and mechanical means (cleaned using a 0.03 % endozyme solution and agitated for 2-3 hours, adapted from Tieu et al. (2008)). Endocarps were then high pressure cleaned to remove excess mesocarp. Finally the resultant endocarps were dried and stored in the seed drying room at Kings Park until germination tests were established in March 2016.

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). All seeds were X-rayed prior to inclusion in the experiment and empty seeds were removed. A subset of three replicates of 10 seeds were used for TTC tests. The lid was removed using side cutters and 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 which was 100%.

Endocarps were surface sterilized in an acidified 2 % (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. Endocarps were then placed on dry sterilized white silica sand in Petri dishes. Over the following three months endocarps were subjected to different temperature and moisture regimes in alternating 12 hours light and 12 hours dark cabinets. Four replicates of 20 seeds were used for each treatment.

Treatments varied as follows:

- Control treatment (6 weeks cold stratification)
- Cold stratification (either a four, six or eight week period at 5°C) followed by warm stratification (either 12 weeks constant wet at 30°C or 12 weeks at 30°C during which two weeks were wet);
- Warm stratification (either 12 weeks constant wet at 30°C or 12 weeks at 30°C during which two or four weeks were wet) followed by cold stratification (four, six or eight weeks at 5°C)

This resulted in 15 different treatments using the combinations outlined above. At the completion of cold and warm stratification treatments endocarps were moved into an alternating 10/20°C for 12 weeks and scored for germination weekly (i.e. radicle emergence from the endocarp). All treatments were cycled through two summers and two winters, over a period of 2 years.

Additionally, in order to determine the role of warm and cold stratification in dormancy break of *P. longifolia* the following treatments were undertaken:

- Removal of the endocarp lid plus cold stratification (if the mechanical barrier is removed i.e. the stony endocarp lid, will the endocarp germinate if cold stratification is applied to break physiological dormancy);
- Intact seed plus cold stratification
- Removal of the endocarp lid plus warm stratification (if the mechanical barrier is removed i.e. the stony endocarp lid, will the endocarp germinate if warm stratification is applied to break physiological dormancy)
- Intact seed plus warm stratification only

Cold stratification in the above experiment was defined as cold (5°C) with constant moisture. Warm stratification was 12 weeks at 30°C with four weeks throughout the "summer" months whereby the seeds were kept moist (simulating the summer thunderstorms). Following the stratification period, seeds and endocarps were placed in an alternating 10/20°C for 12 weeks to promote germination and scored weekly for germination.

3.2 Germination of Other Persoonia *Species*

3.2.1 *Persoonia saccata* Germination Trial

When completing the germination experiments as part of the PhD project (Chia, 2016), it became quickly apparent that removal of the endocarp resulted in germination. As seed was limited for *P. saccata* it was decided that this simple test would be undertaken with these seeds. Due to seed fruit quality there was insufficient seed to undertaken any more detailed wet/dry germination trials.

Seed was collected in Gnangara and had a seed fill of 75% although only about 39% of the seeds appeared to have plump well developed embryos. TTC testing of these plump embryo filled seeds revealed seed viability of 75%.

Those seeds with plump embryos were removed from the seed batch. Seeds were extracted from the endocarp through gently cracking in a vice and seeds that were undamaged during this process were included in the trial. Germination tests were undertaken with both the endocarp on and endocarp completely removed (naked seed) to determine if the endocarp provides a constraint to germination of *P. saccata* seeds. There were three replicates of 10 seeds or endocarps in each treatment.

3.2.2 Wet/Dry Trial for *P. elliptica* and *P. quinquenervis*

A series of wet/dry trials was undertaken according to the move along experimental technique developed by Baskin and Baskin (2003) with *P. elliptica* and *P. quinquenervis. P. elliptica* fruits were collected on September 2016 at Sotico, Boddington, Western Australia and were cleaned according to the methods outlined in Section 3.1. *Persoonia quinquenervis* seed was also collected from Boddington the following year (August 2017). *Persoonia quinquenervis* seed was cleaned by mechanical means only (i.e. manual removal of exocarp followed by high pressure cleaning of mesocarp). Seed fill and viability testing was also undertaken in accordance with the methods outlined in Section 3.1.

Endocarps were sterilized and plated up according to the methods previously described. Four replicates of 20 seeds were used for each treatment.

The experimental design reflected the limited availability of seed. Over the following three months endocarps were subjected to different summer "rainfall" events (i.e. one week of wet conditions). These summer treatments were:

- 12 weeks constant dry at 30°C with no "rainfall" events
- 12 weeks at 30°C with 4 "rainfall" events

- Extended summer of 20 weeks with 4 "rainfall" events

In addition seeds were treated with a number of winter conditions. These conditions were:
- Constant wet 10/20° for 12 weeks; or
- 6 weeks of cold, wet stratification at 5°C followed by constant wet 10/20°C for 12 weeks.

Due to limited seed availability not all treatments were included for *P. elliptica.*

Additionally, in order to determine if cold stratification played a role in dormancy break of *P. elliptica* the following treatments were undertaken:
- Removal of the endocarp lid plus cold stratification (if the mechanical barrier is removed i.e. the stony endocarp lid, will the endocarp germinate if cold stratification is applied to break physiological dormancy);
- Intact endocarp plus cold stratification

3.4 Nursery Trials

Nursery trials were established for *P. elliptica* and *P. quinquenervis* in November 2016. 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 25 endocarps which were buried 2-3 cm below the soil surface. There were insufficient endocarps to do all four treatments with *P. quinquenervis* so only treatments 1-3 were undertaken. 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.

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. Tubs were put through two summers and two winters with the trial completed in October 2018. At the completion of the experiment the sand was sifted and all intact seeds were removed. Each endocarp was examined and an attempt was made to remove the lid by hand. For those seeds where the lid popped off each seed was examined to determine if the seed inside was still potentially viable (i.e. white and plump).

3.4 Data Analysis and Reporting

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 wet/dry trial for *P. quinquenervis,* treatment name, warm or cold stratification and long or short summer were the factors included in the binomial GLMM. For the nursery trials treatment name, was the only factor included in the binomial GLMM.

The 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 *emmeans* packages (Bates D et al., 2014, Wood, 2011, Lenth et al., 2018).

4.0 Results

4.1 Endocarp and Seed Characteristics

The mean weight of *P. elliptica, P. quinquenervis* and *P. saccata* endocarps is outlined in Table 1.

Table 1: Mean mass of cleaned endocarps and seeds of three *Persoonia* species.

Species	No of seeds weighed	Overall Mass* gm	% by Weight Endocarp	% by Weight Seed
P. elliptica	13	227.0 ± 13.5	92.11 ± 1.01	7.89 ± 1.01
P. quinquenervis	10	115.9 ± 3.8	89.53 ± 2.87	17.45 ± 0.61#
P. saccata	10	158.0 ± 5.8	87.73 ± 1.97	12.26 ± 1.97

* Mass is the mass of the endocarp and seed. Mesocarp was cleaned from the seeds prior to weighing
\# Only 6 seeds were weighed due to difficulty in extraction therefore this is a percentage based on only 6 endocarps + seeds.

The *P. elliptica* seed has two cotyledons and is the biggest of the fruits that were weighed however the ratio of seed to endocarp + seed is the lowest of the three species (Table 1). The structure of the seed and the endocarp are shown in Figure 5. Seed collected for the trial had a seed fill of 85.3 ± 1.9% and viability of 80% based on a tetrazolium test.

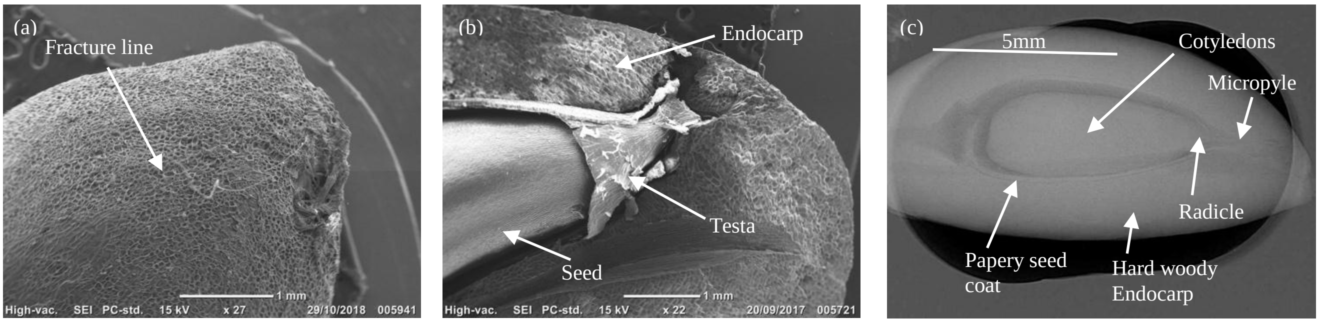

Figure 5: Endocarp and seed structure of *Persoonia elliptica.* (a) Scanning Electron Microscope (SEM) image of hard woody endocarp. (b) SEM image of internal structure of seed and endocarp. (c) X-ray image of seed inside the endocarp.

Persoonia quinquenervis seed is a smaller seed than *P. elliptica* and has a more symmetrical shaped seed which is bulbous on the cotyledon end and tapered on the radicle end. The crack line for the lid is less visible on the outside of the endocarp. Interestingly there are more than 2 cotyledons and the number can vary between four and eight cotyledons (Figure 6).

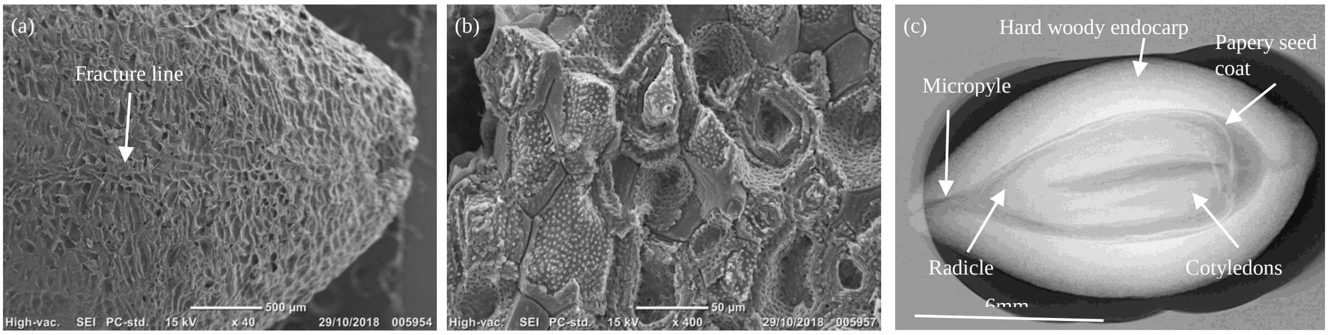

Figure 6: Endocarp and seed structure of *Persoonia quinquenervis.* (a) Scanning Electron Microscope (SEM) image of hard woody endocarp. (b) SEM image endocarp cell structure. (c) X-ray image of seed inside the endocarp.

Persoonia saccata has a similar shaped seed to *P. quinquenervis* with one end being bulbous and the other tapered (Figure 7). Unlike *P. longifolia*, all three endocarps (*P. elliptica, P. saccata* and *P. quinquenervis*) have a straight embryo inside.

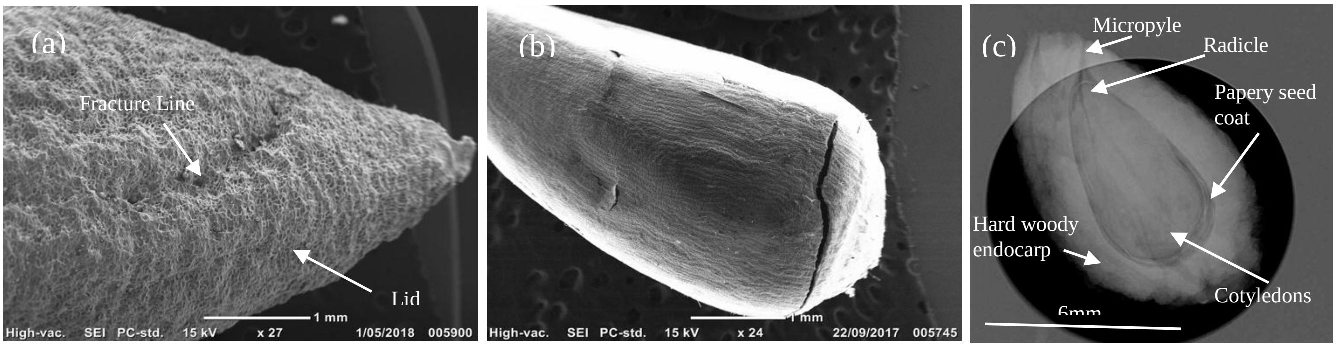

Figure 7: Endocarp and seed structure of *Persoonia saccata.* (a) Scanning Electron Microscope (SEM) image of hard woody endocarp showing the predefined facture line and lid. (b) SEM image of testa. (c) X-ray image of seed inside the endocarp.

4.2 *Persoonia longifolia* Stratification Trial

Germination in the *P. longifolia* stratification trial was very low (<10%) (Figure 8). No germination occurred in a number of treatments however there were no discernible patterns to this lack of germination. Additionally, there was no significant difference in the final germination percentage in any of the variations tested in the stratification trial where germination occurred.

Greatest germination was achieved in the treatment which received eight weeks cold stratification followed by 12 weeks warm stratification including two weeks wet.

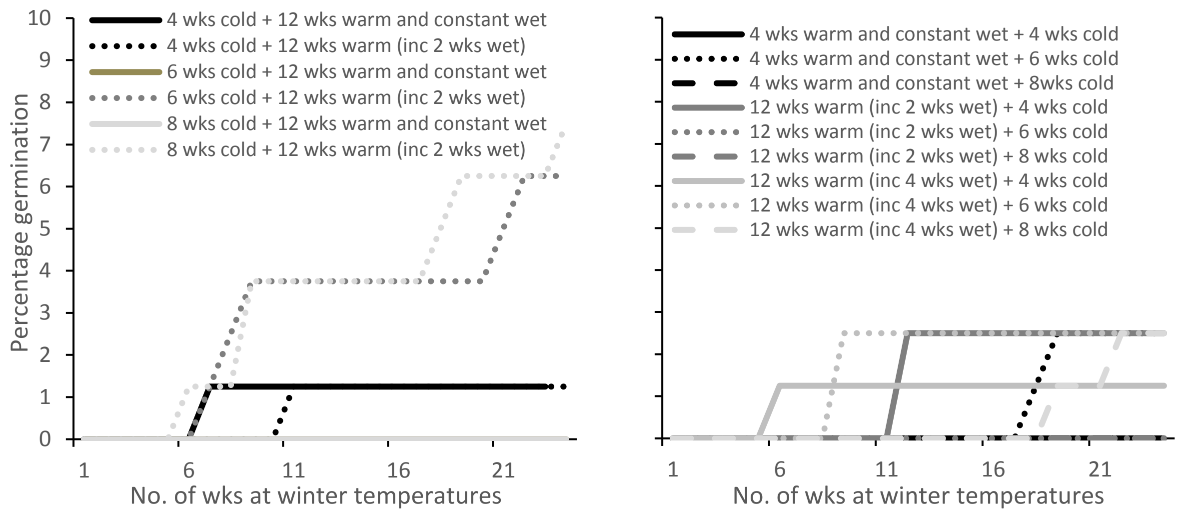

Figure 8: Mean cumulative germination percentages in *P. longifolia* stratification trial. (a) Cold stratification followed by warm stratification (b) warm stratification followed by cold stratification. Final germination percentages in each of the treatments were not significantly different.

In the experiment where the endocarp lid was removed and either warm or cold stratification applied, germination only occurred in those endocarps with the lid removed. It did not appear to matter if warm or cold stratification was applied both resulted in some germination albeit very low (<5%) (Figure 9). Leaving the lid intact resulted in no germination at all. Results between treatments were not significantly different and this is likely to be as a result of the very low germination that was observed in this trial. However leaving the lid on did have a significant negative effect on germination (P=0.02).

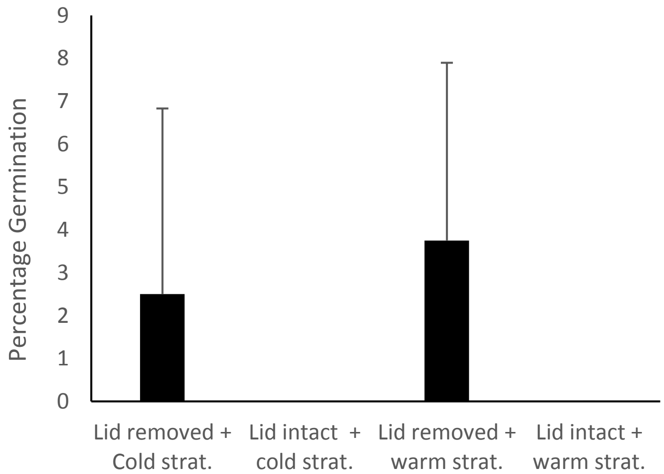

Figure 9: Final germination percentages of endocarps with lids on and off and exposed to warm and cold stratification.

4.3 Germination of Other Persoonia *Species*

4.3.1 *Persoonia saccata*

The only germination that occurred in *P. saccata* was from naked seed (Figure 10) which germinated to 56.7 ± 28.7%. Germination commenced in week 4 and continued through to week 7.

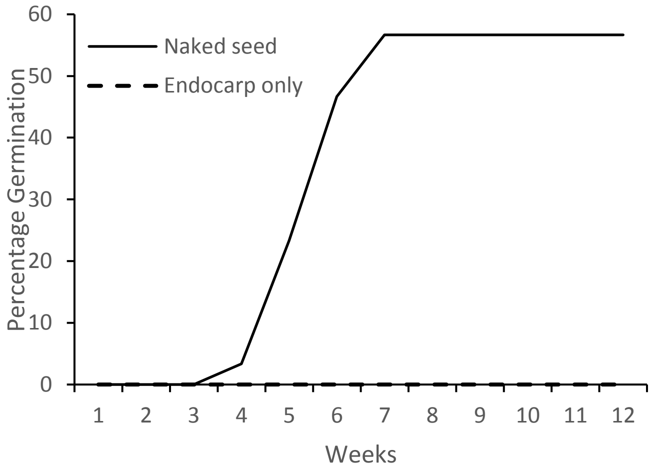

Figure 10: Cumulative germination percentage of *P. saccata* with and without the endocarp.

4.3.2 *Persoonia quinquenervis*

Some germination occurred after the first winter for all treatments however this was very low (<20%) (Figure 11). The treatment that received the long summer plus four weeks of warm stratification germinated the best after the first winter (17.5 ± 6.6%) and this was significantly greater than the control where nothing germinated (P=0.01, Figure 11). However, it was not significantly different from any of the other treatments where germination occurred at the end of the first winter. Additionally, the various factors (warm/cold stratification or long or short summers) had no significant effect on the germination of *P. quinquenervis* after the first winter.

After the completion of the second winter of the trial, germination was significantly different between treatments (P<0.001). Those seeds that received warm stratification only germinated significantly better than all other treatments (Figure 11). The majority of the germination in the warm stratification only treatment occurred during the second winter which is similar to that which was observed for *P. longifolia.*

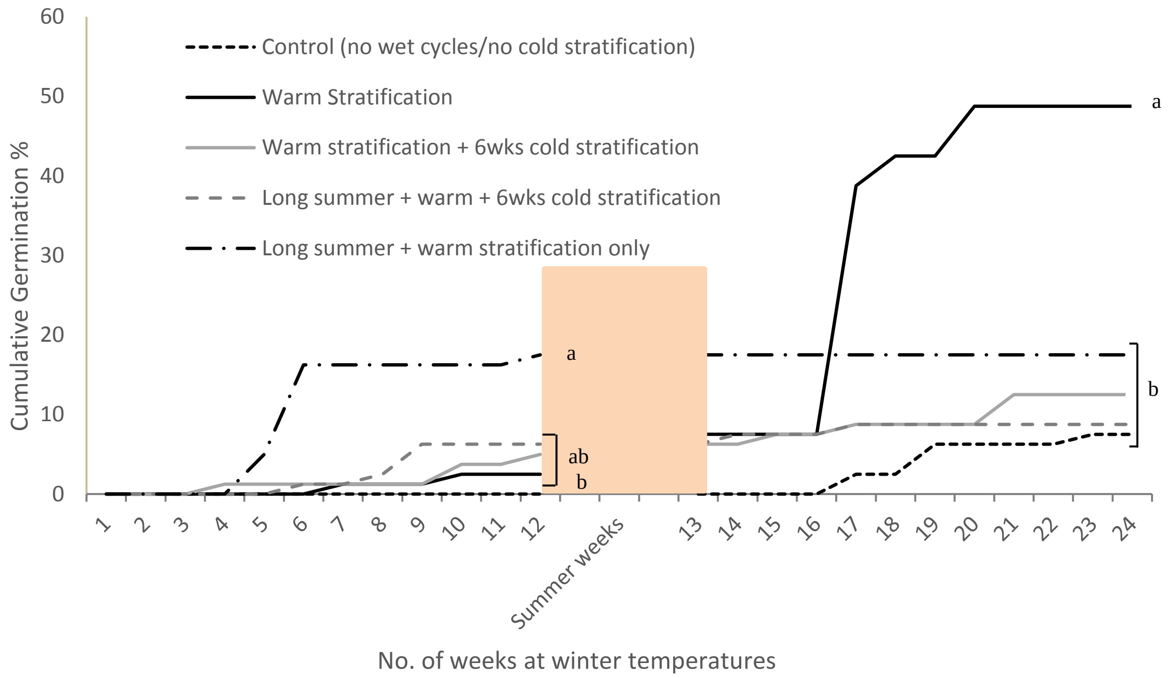

Figure 11: Mean cumulative germination percentage of *Persoonia quinquenervis* with various simulations of summer and winter conditions. Shaded block indicates the second summer with the first summer preceding the data shown. Letters on the graph indicate significant differences between treatments within that season.

When the data was further, analysed a significant difference was seen in the stratification factor (P<0.001), and the length of the summer endured by the seeds (Table 2). Those endocarps exposed to warm stratification only germinated better than those endocarps that received no stratification of warm plus cold stratification. Additionally those endocarps exposed to a shorter summer germinated better than those that were exposed to a long summer period. The interaction between the two factors was not significant.

Table 2: Mean germination percentage of *Persoonia quinquenervis* endocarps with the different simulations of summer wet/dry cycles and with different stratification treatments.

Factor	Treatment	Germination % after 2nd winter	P value
Stratification	None	7.5 ± 5.9	<0.001
	Warm only	33.12 ± 8.2	
	Warm + Cold	10.6 ± 3.8	
Length of summer	12 weeks (short)	22.9 ± 7.0	<0.001
	20 weeks (long)	13.1 ± 3.6	

4.3.3 *Persoonia elliptica*

Like *P. quinquenervis, P. elliptica* endocarps in the treatment with the long summer plus warm and cold stratification were only put through winter temperatures once. At the time of writing this report it was about to start going through the second winter season and therefore the results of that second winter are not included here.

Persoonia elliptica did not germinate well in any of the laboratory trials undertaken. Germination occurred in the treatment with 12 weeks warm stratification only (2.5 ± 4.3%) and this germination occurred in the first winter.

In the experiment where the endocarp lid was removed and cold stratification applied, germination only occurred in those endocarps with the lid removed but was once again very low (3.8 ± 6.5%).

4.4 Nursery Trial

Natural rainfall occurring over the period of this trial is shown in Table 3. Over the summer months, natural rainfall events occurred in December 2016 (54.6 mm), February 2017 (67 mm), December 2017 (25 mm), January 2018 (78 mm), meaning that in each summer there were two natural storm events.

Table 3: Mean monthly rainfall (mm) occurring at Capercup throughout the period of study.

Year	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec	Total
Mean Rainfall*	21.3	15.0	18.1	31.8	62.3	82.1	84.6	69.9	53.6	33.2	24.8	14.2	510.9
2016	192.0	0.6	81.0	40.2	66.8	51.4	57.6	109.0	48.5	30.4	5.4	57.0	739.9
2017	0.0	69.5	13	1.0	38.5	9.0	106.0	91.5	42.0	26.5	9.0	41.0	447.0
2018	78.0	0.0	13.0	10.5	44.5	71.0	77.5	83.75	9.75	30.5	27.5	9.5	455.8

*51 years of data

Seedling emergence for *P. quinquenervis* commenced on the 14th June 2017 with the majority of the germination occurring between weeks three and nine (Figure 12). Very little germination occurred in the second winter for *P. quinquenervis* with emergence occurring after 31st of July 2018. Best emergence was observed in the tubs which received natural rainfall (16 ± 8.5%), followed by those tubs that received four simulated summer rainfall events (11 ± 4.4%). Those tubs that did not receive any rainfall had the lowest emergence of 7 ± 4.4%. However, this difference was not significant.

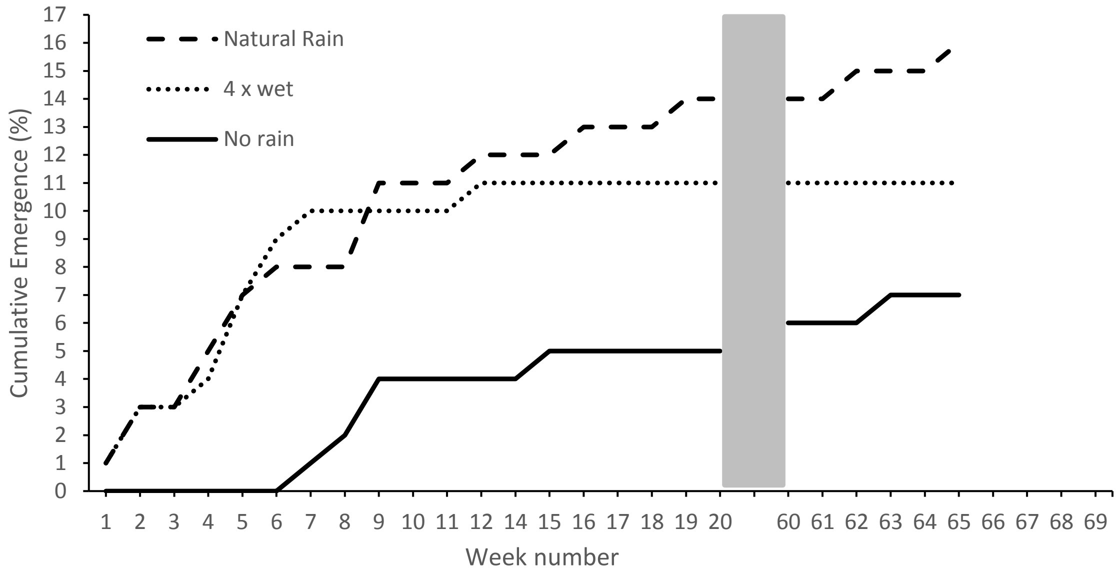

Figure 12: Seedling emergence from *P. quinquenervis* endocarps buried in the nursery for 2 years and treated with different summer watering regimes. The shaded bar indicates summer months.

No *P. elliptica* seedlings emerged during the first winter in which they were buried in the nursery. The second season emergence began to occur in the last week of July 2018. Emergence continued for 11 weeks (Figure 13) but was much lower than that observed for *P. quinquenervis* and *P. longifolia*, with highest mean germination only being 8%.

Figure 13: Seedling emergence from *P. elliptica* endocarps buried in the nursery for 2 years and treated with different summer watering regimes. Only the second winter season is shown as no germination occurred during the first winter.

Table 4: Mean number of seeds where the lids could be removed by hand and the number of seeds that appeared viable at the completion of the experiment. Treatments with the same superscript are not significantly different.

Species	Treatment	% Lids Removed by Hand	P-Value	% of Viable Seeds	P-Value
P. quinquenervis	Natural Rain	93.2 ± 2.1	NS	35.5 ± 2.1^{a}	<0.001
	4 x wet	93.8 ± 3.6		69.6 ± 3.6^{b}	
	No Rain	92.5 ± 3.3		53.9 ± 3.4^{ab}	
P. elliptica	Natural Rain	88.9 ± 2.5	NS	5.4 ± 3.2	NS
	2 x wet	92.9 ± 3.0		1.1 ± 1.1	
	4 x wet	84.9 ± 4.4		1.1 ± 1.1	
	No Rain	88.6 ± 1.8		2.3 ± 1.3	

5.0 Discussion

5.1 Persoonia longifolia

Results from an intensive study of *P. longifolia* found that germination could be achieved after 18 months in the nursery environment or 12 months under more controlled laboratory conditions (Chia, 2016). The breakthroughs made in this research were very encouraging (germination >70 % over a period of three years in some treatments).

A follow up trial looked at the order of stratification and length of time for warm and cold stratification and showed that 12 weeks warm stratification followed by 6 weeks cold stratification resulted in the highest germination. This germination was still low (<15%) but was considered encouraging as it was greater than the germination observed for *P. longifolia* after the first winter in almost all other laboratory trials undertaken.

The stratification trial undertaken in this study aimed to further investigate the impacts of stratification on *P. longifolia* in an attempt to speed up the germination process for propagators. The results however, were not as encouraging, with germination only reaching 7.5% in the treatment where the endocarps were exposed to eight weeks cold stratification followed by 12 weeks warm stratification. There was no significant difference in any of the treatments and this was the reverse of the results found in the previous study.

There was also no clear distinction between germination of those endocarps with the lid removed that were subjected to either warm or cold stratification. However, the lid needed to be removed for any germination to occur.

The results here indicate that whilst warm and cold stratification are required, the mechanisms by which they operate are still not understood and the order in which they are to be applied is still not clear. However, whilst the science behind the germination process of *P. longifolia* is still being teased out it is possible to germinate *P. longifolia* seeds in a nursery environment over a period of two years through intermittent summer watering. Leaving the buried endocarps in a nursery environment without artificial watering will result in germination only if summer thunderstorms occur. Artificial thunderstorms can be used to simulate natural rainfall events if these do not occur and this will result in approximately 30% germination in the first year and a further 30-40% germination in the second year.

Future investigations into germination of this species could look at the impact of the plant hormones such as abscisic acid (ABA) and gibberellins (GAs) and the role they play during the stratification of the endocarps. Such hormones have been found to have an impact on other species with hard woody endocarps such as *Prunus campanulata* (Chen et al., 2007). Initial investigations previously undertaken on *P. longifolia* have shown varying results in relation to the use of GA_3.

5.2 *Persoonia saccata*

Persoonia saccata appears to require the removal of the endocarp in order to germinate and this seems to be a common theme in germination of these species with hard woody endocarps (McIntyre, 1969, Rintoul and McIntyre, 1975, Bauer and Johnston, 1999, Chia, 2016). Like *P. longifolia,* germination of the naked seed of *P. saccata* did not begin until week 4 of the germination trial indicating that there is some block to germination occurring in the embryo itself in addition to the barrier provided by the hard woody endocarp.

Seed for *P. saccata* is hard to come by and expensive to purchase which often limits the trials that can be undertaken. Further experimentation could be undertaken on the use of wet/dry techniques, particularly in nursery environments to encourage germination of this species.

5.3 *Persoonia quinquenervis*

Like *Persoonia longifolia,* it seems that summer rainfall events may be required for germination to occur. Whilst summer rainfall events were not significantly linked to germination in the nursery, the pattern of germination was similar to that observed for *P. longifolia.* Germination with natural rainfall occurred steadily and continuously over the winter months in both the 2017 and 2018 seasons until the maximum germination of 17% occurred. This is much lower than the germination of around 70% observed for *P. longifolia* in previous nursery trials.

Likewise in the laboratory situation, germination for *P. quinquenervis* indicated that some form of warm stratification is required for germination to occur and that most germination occurs after at least two summers and winters. *Persoonia quinquenervis* is found further north and east than *P. longifolia* and it would appear that the warm summer months (warm stratification) are more important for germination than the conditions seeds are exposed to during the winter months.

No experimentation was undertaken looking at variations on winter temperatures and these may have an impact on the germination of the seeds. Winters in the inland areas can be colder than the coastal areas and frosts are common. Therefore there is the possibility that colder stratification temperatures than the 5°C imposed here, may be required to further break dormancy.

Seed viability of *P. quinquenervis* at the completion of the nursery trial was interesting. Lids easily popped of almost all of the seeds remaining in the sand at the completion of the trial indicating that the time buried in the sand had resulted in a breakdown of the endocarp. Examination of the seeds inside those endocarps where the lid had popped off indicated that some of the seeds were still viable (i.e. plump and white). An additional winter and summer may have resulted in further germination and it is possible that this species may require longer burial that *P. longifolia.*

5.4 Persoonia elliptica

Persoonia elliptica has been identified as having a long term regeneration failure that has been attributed to increases in the primary herbivore (western grey kangaroo) in its habitat and low viability of its seed (Nield et al., 2015). Understanding the germination biology of this species is important to ensuring its return to the jarrah forests of the southwest of Western Australia.

Seed testing of batches of seed in this experiment found that whilst seed fill (85%) and viability (80%) were good in freshly collected seed, germination was very low (<10%) in all of the various experiments undertaken despite a range of different treatments being applied. Additionally, at the completion of the nursery trial the majority of the ungerminated seeds could have the lid removed by hand but almost none of these had viable seeds present after being buried for two years indicating that seed viability declines rapidly in soil stored *P. elliptica* endocarps.

This low germination and rapid decline in soil stored seed viability could also contribute to long term regeneration failure of *P. elliptica.* Nield et al. (2015) alludes to the possibility that fire may be required to break dormancy. However, anything more than a very mild fire is more likely to destroy seeds as has been shown to be the case for *P. longifolia* (Chia, 2016). Rather than breaking dormancy, fire could assist germination through the production of a gap in the habitat, allowing the seed to access water and light, which in turn enables germination. Given the continuation of seed production by the parent plants following fire, fresh seeds would be able to take advantage of such gaps in the habitats.

Given the importance of warm and cold stratification on other species of *Persoonia,* and the narrow range of this species habitat, there is also the potential for climatic changes to dramatically impact on germination of *P. elliptica* (and other *Persoonia* species), further reducing regeneration events of this species.

6.0 Conclusion

Climatic conditions for each species of *Persoonia* should be taken into consideration in future experiments. By understanding the climate in which the species grow, conditions could be simulated to produce germination through further move along experiments such as described here and in Baskin and Baskin (2003). It appears that *Persoonia* species have very specific germination requirements and these are likely to be linked to the conditions they experience in their natural habitat.

Critical to any germination success is the removal of the mesocarp. Practitioners should continue to bury the cleaned endocarps in clean washed sand and water over the summer months if summer rainfall events do not occur. Further investigations could be undertaken into watering regimes for different *Persoonia* species depending on the climatic conditions experienced.

7.0 Acknowledgements

This Project would not have been possible without the support of the Australian Flora Foundation and Kings Park and Botanic Gardens. The Australian Flora Foundation contributed funds to undertake the research and Kings Park and Botanic Gardens supplied the laboratory facilities that enabled me to undertake the trials herein.

Special thanks must also go to Dr Shane Turner who assisted with reviewing experimental design and the final report.

8.0 References

ABBOTT, I. 1984. Comparisons of spatial pattern, structure, and tree composition between virgin and cut-over jarrah forest in Western Australia. *Forest Ecology and Management,* 9**,** 101-126.

BASKIN, C. C. & BASKIN, J. M. 2003. When breaking seed dormancy is a problem: Try a move-along experiment. *Native Plants Journal,* 4**,** 17-21.

BASKIN, C. C. & BASKIN, J. M. 2014. *Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination*, 2nd ed., Elsevier Inc.

BASKIN, C. C., BASKIN, J. M., YOSHINAGA, A. & THOMPSON, K. 2005. Germination of drupelets in multi-seeded drupes of the shrub *Leptecophylla tameiameiae* (Ericaceae) from Hawaii: a case for deep physiological dormancy broken by high temperatures. *Seed Science Research,* 15**,** 349-356.

BASKIN, C. C., ZACKRISSON, O. & BASKIN, J. M. 2002. Role of warm stratification in promoting germination of seeds of *Empetrum hermaphroditum* (Empetraceae), a circumboreal species with a stony endocarp. *American Journal of Botany,* 89**,** 486-493.

BATES D, MAECHLER M, B, B. & WALKER S 2014. _lme4: Linear mixed-effects models using Eigen and S4_. R package version 1.1-7.

BAUER, L. M. & JOHNSTON, M. E. 1999. Propagation of *Persoonia virgarta* for the Development of a New Floricultural Export Crop. Gatton College: School of Land and Food The University of Queensland.

CHEN, S.-Y., CHIEN, C.-T., CHUNG, J.-D., YANG, Y.-S. & KUO, S.-R. 2007. Dormancy-break and germination in seeds of *Prunus campanulata* (Rosaceae): role of covering layers and changes in concentration of abscisic acid and gibberellins. *Seed Science Research,* 17**,** 21-32.

CHIA, K. 2016. *Ecology, seed dormancy and germination biology of Persoonia longifolia for use in land restoration and horticulture.* PhD, University of Western Australia.

CHIA, K. A., SADLER, R., TURNER, S. R. & BASKIN, C. C. 2016. 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).*

CHIEN, C. T., CHEN, S. Y. & YANG, J. C. 2002. Effect of stratification and drying on the germination and storage of *Prunus campanulata* seeds. *Taiwan Journal of Forest Science,* 17**,** 413-420.

DEPARTMENT OF BIODIVERSITY CONSERVATION AND ATTRACTIONS. 2018. *Florabase* [Online]. Perth, WA. Available: https://florabase.dpaw.wa.gov.au/browse/profile/2262 [Accessed 2018.

FRITH, A. & OFFORD, C. 2010. Investigation into the germination and propagation of *Persoonia pauciflora* P.H. Weston. *Australian Network for Plant Conservation 8th National Conference.* Perth: Australian Network for Plant Conservation.

IMANI, A., RASOULI, M., TAVAKOLI, R., ZARIFI, E., FATAHI, R., BARBA-ESPÍN, G. & MARTÍNEZ-GÓMEZ, P. 2011. Optimization of seed germination in *Prunus* species combining hydrogen peroxide or gibberellic acid pre-treatment with stratification. *Seed Science and Technology,* 39**,** 204-207.

KETELHOHN, L. M., JOHNSTON, M. E. & WILLIAMS, R. E. 1998. Propagation of *Persoonia virgata* for commercial development. *In:* J.A. CONSIDINE & GIBBS, J. (eds.) *Third International Symposium on New Floricultural Crops.* Perth, Western Australia: International Society for Horticultural Science.

LENTH, R. V., SINGMANN, H., LOVE, J., BUERKNER, P. & HERVE, M. 2018. Estimated Marginal Means, aka Least-Squares Means. *R Package*.

LI, X., BASKIN, J. M. & BASKIN, C. C. 1999. Anatomy of two mechanisms of breaking physical dormancy by experimental treatments in seeds of two north American *Rhus* species (Anacardiaceae). *American Journal of Botany,* 86**,** 1505-1511.

MCINTYRE, D. K. 1969. The Germination of Dormant *Persoonia pinifolia* R.Br. Seeds by the Use of Gibberellic Acid.: Canberra Botanic Gardens, Canberra Australia.

MERRITT, D. J., TURNER, S. R., CLARKE, S. & DIXON, K. W. 2007. Seed dormancy and germination stimulation syndromes for Australian temperate species. *Australian Journal of Botany,* 55**,** 336-344.

MULLINS, R. G., KOCH, J. M. & WARD, S. C. 2002. Practical method of germination for a key jarrah forest species: snottygobble (*Persoonia longifolia*). *Ecological Management & Restoration,* 3**,** 97-103.

NIELD, A. P., MONACO, S., BIRNBAUM, C. & ENRIGHT, N. J. 2015. Regeneration failure threatens persistence of *Persoonia elliptica* (Proteaceae) in Western Australian jarrah forests. *Plant Ecology,* 216**,** 189-198.

NIKOLAEVA, M. G. 1969. *Physiology of Deep Dormancy in Seeds,* Leningrad, Izdatel'stvo "Nauka".

NORMAN, M. A. & KOCH, J. M. 2006. The Investigation of Seed Coat Chipping, Seed Coat Ageing and Warm Temperature Stratificiation for Snottygobble (*Persoonia longifolia*). . Aloca of Australia Pty Ltd., Pinjarra Western Australia.

NORMAN, M. A. & KOCH, J. M. 2008. The effect of *in situ* seed burial on dormancy break in three woody-fruited species (Ericaceae and Proteaceae) endemic to Western Australia. *Australian Journal of Botany,* 56**,** 493-500.

OFFORD, C. A., ROLLASON, A. & FRITH, A. 2015. Tissue culture of *Persoonia* species for horticulture and restoration. *Acta Horticulturae,* 1097**,** 149-154.

PERSSON, L., JENSEN, M., ERIKSEN, E. N. & MORTENSEN, L. C. 2006. The effect of endocarp and endocarp splitting resistance on warm stratification requirement of hawthorn seeds (*Crataegus monogyna*). *Seed Science and Technology,* 34**,** 573-584.

PRESTON, C., ADKINS, S. W., BELLAIRS, S. M., THOMPSON, L., FARLEY, G., GRAVINA, A., DIBBEN, S., ROHDE, T. & BIRT, M. 2002. Dormancy Mechanisms of Australian Native Plant Species.: School of Land and Food Science and Center for Mine Land Rehabilitation, The University of Queensland, Australia.

R CORE TEAM. 2013. *R: A language and environment for statistical computing.* [Online]. Vienna, Austria: R Foundation for Statistical Computing. Available: http://www.R-project.org/.

RINTOUL, I. & MCINTYRE, D. K. 1975. Investigation into Seed Dormancy in *Persoonia pinifolia*. Canberra Botanic Gardens, Canberra Australia.

ROBERTSON, G., MATTHES, M. & SMITH, M. 1996. Conservation research statement and species recovery plan for *Persoonia nutans* R. Br. Endangered Species Project No. 503 ed. Hurtsville, NSW: NSW National Parks and Wildlife Service.

STACK, G. & BROWN, A. 2003. Small-flowered snottygobble (*Persoonia micranthera)* interim recovery plan 2003-2008. *Department of Conservation and Land Management*.

TIEU, A., TURNER, S. & DIXON, K. 2008. The novel use of commercial enzymes to depulp the fruits and seeds of selected Australian native species for seed storage and germination. *Ecological Management & Restoration,* 9**,** 230-232.

TURNER, S., COMMANDER, L. E., BASKIN, J. M., BASKIN, C. C. & DIXON, K. W. 2009. Germination behaviour of *Astroloma xerophyllum* (Ericaceae), a species with woody indehiscent endocarps. *Botanical Journal of the Linnean Society,* 160**,** 299-311.

WESTON, P. H. 1995. Persooniaoideae. *In:* MCCARTHY, P. (ed.) *Flora of Australia: Elaeagnaceae*, *Proteaceae 1*. Melbourne, Australia: CSIRO.

WESTON, P. H. 2003. Proteaceae subfamily Persoonioideae. *Australian Plants,* 22**,** 62-91.

WOOD, S. N. 2011. Fast stable restricted maximum likelihood and marginal likelihood estimation of semiparametric generalized linear models. *Journal of the Royal Statistical Society,* 73**,** 3-36.